

Molecular and cellular effects of gestational diet on the developing bovine reproductive and immune systems.

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Table of abbreviations

Abbreviation	Description	Abbreviation	Description
µl	Microlitre	LN	Lymph node
µm	Micrometer	LPeri	Low dietary crude protein peri - conception
AI	Artificial insemination	LPost	Low dietary crude protein post - conception
AMH	Antimullerian hormone	M	Male
AR	Androgen receptor	MF	Molecular function
BP	Biological process	Min	Minute
CC	Cellular component	MIS-C20	Mullerian inhibitory substance
CP	Crude protein	MJME	megajoules of metabolisable energy
cTEC	Cortical thymic epithelial cells	ml	Mililitre
DAB	3,3'-diaminobenzidine	mTEC	Medullary thymic epithelial cells
DC	Dendritic cells	MUFA	Monounsaturated fatty acids
DN	Double negative	ng	nanograms
DP	Double positive	PAT	Perinodal adipose tissue
dpc	days post - conception	PBS	Phosphate buffered saline
DPX	Dystirene plasticizer xylene	PGCs	Primordial germ cells
EtoH	Ethanol	PS	Picrosirius
F	Female	PUFA	Polyunsaturated fatty acids
FAME	Fatty acid methyl esther	qPCR	Quantitative polymerase chain reaction
FC	Fold change	RNA	Ribonucleic acid
FCS	Fetal calf serum	Rcf	Relative centrifugal force
FSH	Follicle stimulating hormone	Rpm	Revolution per minute
G	Grams	Sec	Second
GD	Gestation day	SFA	Saturated fatty acids
H	High dietary crude protein	SP	Single positive
Hperi	High dietary crude protein peri - conception	SRY	Sex-determining region Y
HPost	High dietary crude protein post - conception	T3	Triiodothyronine
IF	Immunofluorescence	T4	Thyroxine
IG	Immunoglobulin	TEC	Thymic epithelial cells
IGF	Insulin growth factor	VEGF	Vascular endothelial growth factor
IHC	Immunohistochemistry	VEGFR2	Vascular endothelial growth factor receptor 2
Kg	Kilograms	VS	Versus
L	Low dietary crude protein	WAT	Further white adipose tissue

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Abstract

Protein deficient diets are common in the range beef production system employed in northern Australia. This reflects variation in pasture quality due to local climatic conditions, notably the annual rainy and dry seasons. It is well documented that changes in maternal nutrition during pregnancy can affect the fetal developmental program. The reproductive and immune systems have been demonstrated to be influenced by maternal nutrition; however, much remains unknown. Therefore, the aims of this project were to study the impact of maternal dietary protein intake, mimicking the rainy (14% crude protein dietary content) and the dry (7% crude protein) Australian seasons on the development of the (i) reproductive tract and (ii) immune system of 98 day old bovine fetuses, and on the development of 20 month old bulls. To this end, cows were fed isocalorific high (14%) and low (7%) protein diets during the peri - conception period (60 days prior artificial insemination to 23 days post - conception (dpc)) and then the groups were split into two further groups, half of which remained with the same peri – conception diet, whereas the other half was switched to the opposite diet until 98 dpc (end of the first trimester). This two factorial experimental design provided us with four different dietary groups (low-low, LL; low-high, LH; high-low, HL; and high-high, HH, where low is defined as 7% protein and high as 14% protein), as well as with two different diets per period (LPeri, HPeri, LPost and HPost). The tissues analysed to assess the reproductive tract were the testes in 98 dpc fetuses and the testes and the epididymis in the 20 month old animals. Data pertaining to the fetal testicular cells number, seminiferous tubule abundance and size and blood vessel

quantity and size from fetal and adult testis and adult epididymis were collected using a range of histological techniques and statistical analyses. In addition, proteomic analysis of the epididymal fluid extracted from the 20 month old bulls was performed.

Testicular cell proportions within the d98 testis were affected by the different diets; thus in 98 dpc LL fetuses a decrease in the percentage of Leydig cell and an increase in the percentage of germ cells and in the number of seminiferous tubules compared with the other diet regimes was observed. In addition, the proportion of seminiferous tubules was reduced in the 98 dpc fetuses from the HL group compared to the other diet regimes and tubule size was smaller in 98 dpc LPost fetuses compared with HPost. No significant differences were found in the number, percentage and size of seminiferous tubules, epididymal tubule and blood vessels in 20 month old bulls testis or epididymis, except for a decrease in the percentage of the seminiferous tubules in the adult testes in LPeri compared with HPeri and the number of testicular blood vessels in the LPost compared with the HPost. However, the protein profile of the epididymis fluid from adult bulls showed alterations in proteins that are involved in male fertility related with low protein diets, affecting maturation, capacitation, motility and sperm – zona pellucida binding.

Regarding the assessment of the immune system, thymus and thyroid tissues were collected from 98 dpc male and female fetuses and prescapular lymph node and surrounded adipose tissues were retrieved from the 20 month old bulls. Thymus gene expression was determined using RNA sequencing and

qPCR analysis. Changes in genes potentially involved in thymus development and function were observed between diets and/or sexes. In addition, fatty acids were extracted from the pre-scapular lymph node (LN), perinodal adipose tissue (PAT) and further adipose tissue (WAT) surrounding the pre - scapular lymph node. Fatty acid profiles showed significant differences in the percentage of C14:0, C14:1, C18:0 and C18:1n9c present in LN and the percentage of C16:0 present in WAT during the peri - conception period. A decrease in C18:0 was also observed in LL compared with HL and HH groups in the LN and an increase in C16:0 in LH compared to HL and HH groups in WAT. In conclusion, manipulation of maternal protein intake during early stages of pregnancy altered the reproductive and the immune system tissues development. Whereas histological changes were compensated for later in life, molecular changes were observed in adulthood.

Chapter 1 Literature review

1.1 Introduction

In some regions of Australia, beef cattle production is managed across a diverse collection of rangeland vegetation and pastures and represents the main form of land utilization (Burns et al., 2010). Extended periods of low-rainfall with associated nutritional deficiencies frequently occur in these regions. These nutritional challenges are managed using strategic supplementation (Burns et al., 2010, Bortolussi et al., 2005). In Northern Australia, protein rather than energy is the major limiting nutrient (Copping et al., 2018, Norman, 1963). In extensive beef rangeland systems, instability in quantity and quality of pastures restricts the energy intake, increases the grazing costs and reduces the reproductive performance of the cattle (Astessiano et al., 2012). Usually, the intensive beef production strategy employed in this region is a continuous mating with peak calving in October–December and irregular handling and husbandry of the herds, normally twice annually in April–July and August–September (Bortolussi et al., 2005). Given the economic challenges of beef production globally, infertility and subfertility in ruminants are important problems. In cattle, interruption of the pregnancy between fertilization and term range from 40% to 56%, with the majority occurring in the first 3 weeks of gestation (Geary et al., 2016). Poor reproductive performance is also the most common cause for culling beef cows (Thundathil et al., 2016). Of the environmental factors that affect beef cattle reproduction, nutritional status, can be controlled (in part) by farmers. An optimal diet may improve efficiency

in the management of beef cattle production by enhancing the reproductive performance. On the other hand, reproductive diseases also cause important economic losses for the beef cattle industry (Bellows et al., 2002, Hess et al., 2005). In Northern Australia, endemic viral diseases, such as bovine viral diarrhoea/pestivirus and bovine ephemeral fever, represent one of the most important causes of fertilization failure in beef cattle (Burns et al., 2010).

1.2 Fetal programming

Any alteration in maternal diet before or during pregnancy or during early postnatal life can alter reproductive performance in the progeny (Rhind, 2004). However, although this has been known for at least a century and applied to the rearing of domestic animals to maximize production (Rhind, 2004), it is only in recent decades that the molecular basis of programming of reproductive function appears to have been recognized (Gunn et al., 1972, Gunn, 1977, Allden, 1979). In the beginning, efforts to manipulate the diet in order to enhance the reproductive performance was focused on the days and weeks before mating in sheep and on the postpartum/premating in cattle (Rhind, 1992). It was believed that variations in maternal diet within the early stages of gestation would not cause important changes in fetal development because the nutrient requirements for the growth and maintenance in that period are considerably lower (Rhind et al., 2001). This belief has been reversed by more recent studies including those where ewes were underfed to day 47 and 65 of gestation which showed delays in fetal ovarian structural development (Borwick et al., 1996) and changes in associated patterns of testicular

steroidogenesis (Rae et al., 2002). Any change in the maternal diet during the process of tissue differentiation, gonad development or the association of the enzyme systems can potentially have an effect on the function of these organs, including the reproductive tract (Rhind et al., 2001). Therefore these periods must be considered as pivotal for the development of the organs (Rhind et al., 2001). Although most of the published changes occurred when the maternal diet was modified in the latest stages of gestation and could be explained in terms of effects on gonadotropin profiles, these mechanisms do not interfere in the first month of pregnancy since the gonadotropic-gonadal axis is not in place at this period (Rhind et al., 2001). Hence, to examine the possible effects within the early stages of gestation, the histology and steroidogenic capacity of fetal gonads must be assessed.

1.3 Dietary protein in fetal programming

Protein is an important component in the maternal nutritional regime (Funston et al., 2010). Heifers fed high protein diets during the first and second trimesters of pregnancy showed a higher placental and fetal birth weight than those fed low protein diets (Sullivan et al., 2009b). In addition, heifers fed high protein diets have lower serum concentrations of estrone sulphate, which has been suggested as a useful indicator of placental function (Sullivan et al., 2009a). Increased dietary protein in the first trimester is associated with a reduction in milk production, but conversely increased the protein concentration in colostrum (Sullivan et al., 2009c). Micke et al. (2011b) demonstrated the long-term effects of maternal nutrient intake during the first

two trimesters of gestation on expression of insulin growth factor (IGF) and leptin genes in progeny adipose tissue that are depot-sex-specific. Other factors influenced by the protein intake during gestation are IGF1, IGF1R, IGF2, IGF2R and live weight, height and carcass traits of the progeny (Perry et al., 2002, Micke et al., 2010, Micke et al., 2011a).

1.4 Nutrition in male reproductive tract development

Numerous studies have shown that maternal insults during pregnancy can have negative effects in the development of the reproductive organs in the progeny. Several studies have also focused on males. In small ruminants both Bielli et al. (2002) and Kotsampasi et al. (2009) found a reduction in the number of Sertoli cells in lambs undernourished *in utero* from week 10 to week 17 in the former and from day 31 to day 100 in the latter. Moreover, offspring from rats fed with restricted diets throughout the whole pregnancy and lactation and culled at 100 days post-partum showed a reduction in the number of Sertoli cells (Genovese et al., 2010). The same output occurred when diet was restricted in protein from mating through to weaning in male rats at day 14 post-partum (Rodríguez-González et al., 2012). However Da Silva et al. (2003) observed that maternal over nutrition from embryo transfer until the second third of gestation in sheep did not show effects in the number of Sertoli cells, testicular weight or number of seminiferous cords in the male fetal sheep and Andrade et al. (2013) reported no alteration in the number of Sertoli cells in 110 days male fetus from sheep fed with the 50% of the maintenance ratio since mating period. On the other hand, both maternal protein restricted diets in rats

(Rodríguez-González et al., 2012, Melo et al., 2014) and maternal over nutrition diets in rats (Rodríguez-González et al., 2015) and cattle (Weller et al., 2016) showed smaller seminiferous tubules when compared with control diets. In addition, Rodríguez-González et al. (2012) found a reduction in the number of spermatocytes, a persistence of gonocytes, slower differentiation of the spermatids and a higher number of immature germ cells in male rats fed with restricted protein diets *in utero* and/or lactation compared with the control groups. Similar results in the number of spermatogonias, spermatocytes, spermatids and Leydig cells were observed by Melo et al. (2014) in a study where rats were restricted in protein from mating to weaning and offspring were fed a normal or restricted protein diet from weaning to 70 days of age. However, the alterations suffered by the spermatocytes, spermatids and Leydig cells were recovered in the adult animals. Interestingly, it has been observed that plasma testosterone concentration was greater in 50 day old fetal sheep undernourished *in utero* from day 31 of gestation, although a recovery was showed in 65 and 110 days old fetuses (Rae et al., 2002). Yet over nutrition caused a decreased plasma testosterone concentration in 37 week old rabbits from dams fed hypercholesteraemic diets compared with controls (Dupont et al., 2014). In contrast, androgen receptor (AR) expression was reduced in male rats from mothers fed a low protein diet during gestation and/or lactation (Rodríguez-González et al., 2012). It has also been found that long term effects occurred in rat males overnourished *in utero* and during lactation, where sperm viability, motility and concentration were reduced in 450 days of age males. Also the fertility rate was lower and decreased with time in overfed offspring,

while in normal fed males the fertility rate remained stable (Rodríguez-González et al., 2015). There are a very limited number of studies in bovines into the effects of prenatal diet on the reproductive development of the male offspring. Sullivan et al. (2010b) showed that decreased maternal prenatal protein during first and second trimester reduced levels of prepubertal gonadotropins in male calves and decreased testis volume. However Weller et al. (2016) observed that the expression of FSHB and LHB was not altered by a maternal high energy diet fed from day 60 of gestation to term in the male offspring, nevertheless in females the expression of FSHB was increased in the offspring fed high energy diets.

1.5 Embryonic fetal gonads development

The development of the fetal gonads, independent of whether male or female, initiates in bovine embryos at 27-31 dpc, when the gonadal ridge first arises (Wrobel and Süß, 1998). The coelomic epithelium on the ventromedial surface of the urogenital ridge proliferates and moves into the coelomic cavity as the genital ridge, which elongates longitudinally and medial to the mesonephric duct (Russe and Sinowatz, 1991, George and Wilson, 1994).

Apart from primordial germ cells (PGCs), three different linages of cells exist in the gonad (Capel, 2000); the supporting, the steroidogenic and the connective cell lineages. The supporting cell linage forms Sertoli cells in the testis and follicle cells in the ovary. These supporting cells surround the germ cells and ensure an adequate growth environment (Capel, 2000). The steroidogenic cell lineage differentiates into the Leydig cells in the testis and theca cells in the

ovary. The Leydig and theca cells produce androgens, the sexual hormones that participate in the development of secondary sexual characteristics of the embryo (Capel, 2000). The third cell lineage is the connective cell linage that contributes to the formation of the organ (female and male) as a whole, containing peritubular myoid and stroma cells (Capel, 2000). Figure 1.1 shows the different lineage of cells and the female and male cells they differentiate into.

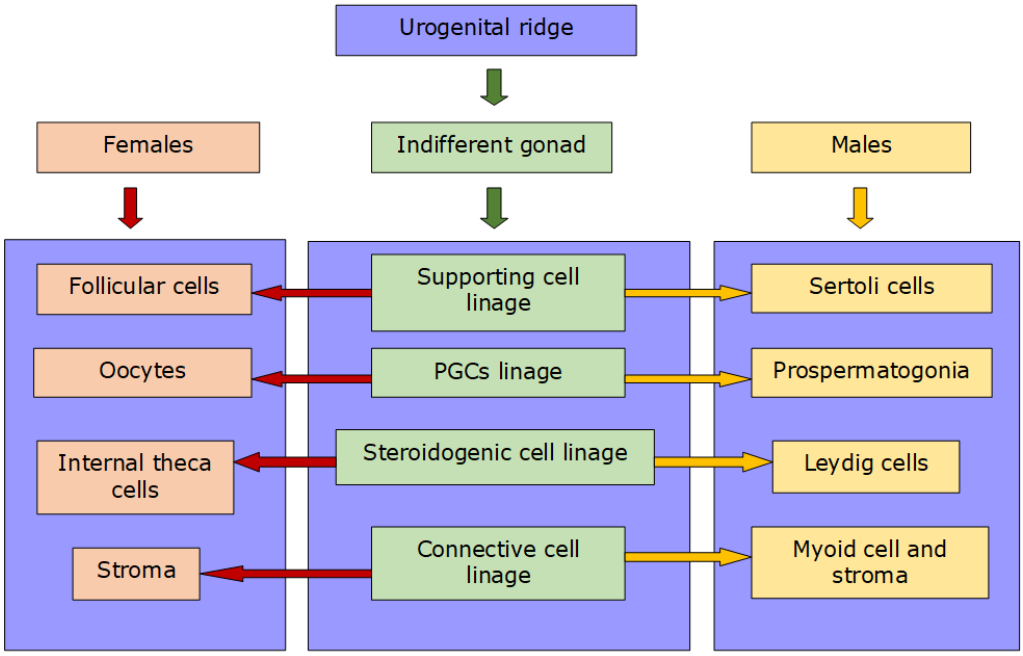


Figure 1.1 Gonadal cell lineages and the cells which they differentiate into. Adapted from Abd-Elmaksoud Ahmed, 2005.

1.6 Fetal testis development

Male reproductive tract differentiation is controlled by the presence of the sex-determining region Y (SRY) gene situated on the Y chromosome (Sinclair et al., 1990, O'Shaughnessy and Fowler, 2011). In mammals, one of the first events in testicular differentiation is the differentiation of fetal Sertoli cells or Pre-Sertoli

cells influenced by SRY gene expression (Magre and Jost, 1979, Sekido et al., 2004). Pre-Sertoli cells play a crucial role in the development of the testis since they are responsible for seminiferous cord formation, stopping meiosis of germ cells, secretion of anti-Müllerian hormone (AMH), which is involved in the regression of the Müllerian ducts in the male fetus, and also secretion of Desert hedgehog, which regulates the differentiation of Leydig cells from mesenchymal like stem cells (Griswold, 1998, Josso et al., 2001, Bitgood et al., 1996, O'Shaughnessy and Fowler, 2011). In cattle, as well as in many other species, proliferation of Sertoli cells starts in fetal life and finishes prior to puberty (Sharpe et al., 2003, Orth, 1993, Bardin et al., 1988, Sinowatz and Amselgruber, 1986). That means that the number of Sertoli cells in the adult animal is determined within this period and therefore disturbances during this time can decrease the amount of these cells, which will reduce testicular size and sperm production (Sharpe et al., 2003, Bardin et al., 1988).

Proliferation of primordial germ cells starts during their migration from the hindgut to the genital ridge, where they form clusters and continue dividing until they are embedded, by Sertoli cells, within the forming testicular cord (Wilhelm et al., 2007, Bendel–Stenzel et al., 1998). In bovine, male germ cells are in transition from PGCs to pre-spermatogonia from 50dpc to 80dpc, during which a high proliferation rate is detected (Wrobel, 2000). After this phase, this proliferation rate declines steadily and ceases at 200dpc and remains inactive until the fourth postnatal week (Wrobel, 2000).

The presence of Leydig cells in the interstitium of the fetal testis has been documented to occur following the development of the testicular cords in almost all species (Orth, 1993, Merchant-Larios et al., 2002, Byskov, 1986). In the bovine testis, testicular cord development happens at 46 dpc (Abd-Elmaksoud, 2005). Fetal Leydig cells differentiate from mesenchymal-like cells present in the interstitium of the testis, which are believed to also be the origin of peritubular cells (Byskov, 1986). Not all of the mesenchymal cells differentiate into Leydig or other interstitial cells; some of them remain as undifferentiated cells and will be the precursors of adult Leydig cells in the postnatal testis (Habert et al., 2001, Mendis-Handagama and Ariyaratne, 2001). In bovine, fetal Leydig cells reach their peak at approximately 88dpc and start to dedifferentiate into mesenchymal cells within the final stage of pregnancy (Abd-Elmaksoud, 2005). The main function of the fetal Leydig cells is the secretion of androgen and more importantly testosterone. Testosterone induces the differentiation of the Wolffian duct and it is 5α-reduced into dihydrotestosterone which promotes development and maintenance of the male external genitalia (Siiteri and Wilson, 1974, Habert et al., 2001, O'Shaughnessy and Fowler, 2011, Tremblay, 2015). Interestingly, the fetal Leydig cells are not the precursors of the adult Leydig cells but adult Leydig cells differentiate post-natally from mesenchymal cells present in the insterstitum of the testis (Mendis-Handagama and Ariyaratne, 2001).

1.7 Blood vessel development

The vascular system is the first system to develop in the vertebrate embryo (Karelina et al., 1995, Breier, 2000), providing oxygen and nutrients to the growing embryo in anticipation of its demands (Breier, 2000). For the development of blood vessels, two consecutive processes are triggered: vasculogenesis and angiogenesis. Vasculogenesis is the formation of blood vessels from stem cells, whereas during angiogenesis the formation of blood vessels occurs from existing blood vessels (Demir et al., 2007). An essential protein in the development of the vascular system is vascular endothelial cell growth factor A (VEGFA), which is involved in endothelial growth and maintenance of the normal vasculature (Cai et al., 2006). This glycoprotein and its receptor, vascular endothelial cell growth factor receptor 2 (VEGFR2), have been observed to be influenced by maternal undernutrition. A significant reduction in VEGF and VEGFR2 was shown in the ovaries of adult rats undernourished *in utero* (Chan et al., 2015) and Liu et al. (2014) observed a downregulation in the expression of VEGF in the placenta and lung of the developing fetus from rats fed a diet restricted in protein during gestation. Another two important proteins in the development of the vascular system are angiopoietin-1 and vascular endothelial (VE)-Cadherin, which play important roles in determining endothelial cells differentiation (Hildbrand et al., 2004) and formation of non-leaky vessels (Jeansson et al., 2011) and in the junction stability of endothelial cells and lumen formation (Rutland et al., 2007), respectively. The effect of protein restriction during murine pregnancy in the

expression of VE-Cadherin in blood vessels was investigated by Rutland et al. (2007), showing a reduction in the intensity of the immunofluorescence of this protein in the casein protein restricted group compared with the control, however in this study the total protein expression levels of VE-Cadherin were not affected. With all this information, it is clear that maternal protein restriction may have a negative impact on the development of the blood vessels present in the male reproductive tract. A proper blood supply is essential for appropriate development and function of any internal organ (Polguj et al., 2015). In the testes the vascular cone also keeps the temperature of the testes 4-5^{o}C below body temperature, which is essential for normal spermatogenesis (Brito et al., 2012). Considering all this, it was essential to investigate whether the shortage of gestational maternal protein investigated in this study altered the development of the testicular blood vessels of their progeny. In addition, another component of the testes, which has an impact in spermatogenesis, is collagen.

1.8 Collagen development

Collagen is a major vertebrate structural protein. Twenty-five different types of collagen have been described to date (Sato et al., 2002, Myllyharju and Kivirikko, 2001, Fitzgerald and Bateman, 2001, Gordon et al., 2000, Gordon et al., 2002, Hashimoto et al., 2002). It can be classified into: fibril-forming collagens, which includes types I, II and III (Kadler et al., 1996); fibril-associated collagens with interrupted triple helices, which includes types IX, XII, XIV and XIX (Koch et al., 2001, Olsen, 1997, Prockop and Kivirikko, 1995) and non-

fibrillar collagens, which include types XIII, XVII and XXV (Hashimoto et al., 2002, Rehn and Pihlajaniemi, 1995, Snellman et al., 2000). Although differences exist between all the types of collagen, all share in common a triple helical structure composed of three polypeptides (Myllyharju and Kivirikko, 2001, Fitzgerald and Bateman, 2001, Gordon et al., 2000, Gordon et al., 2002, Hashimoto et al., 2002, Kadler et al., 1996). Collagen is a major component of the extracellular matrix, which consists of specialized fibrils and networks around cells and in the interstitial spaces between the cells (Sato et al., 2002). During mammalian development this protein is temporally regulated (Sato et al., 2002) and it is a necessary structural component of organs and tissues (He et al., 2005). In the testes, collagen represents more than half of the proteins present in this tissue (Rodriguez et al., 1991). Quantitative changes of collagen in the adult testes have been correlated with percentage of abnormal sperm and sensitivity to testicular degeneration, with a higher amount of this protein linked to a higher presence of the two parameters (Shayakhmetova et al., 2013, Bondarenko et al., 2012, Hoflack et al., 2008). In addition, collagen may have a role in spermatogenesis contributing to the attachment of the immature spermatogonia to the base membrane and detachment of spermatocytes and spermatids towards the lumen during spermatogenesis (He et al., 2005, Bondarenko et al., 2012, Shayakhmetova et al., 2013). An involvement of the type IV collagen in the assembly of inter-Sertoli cell tight-junction has also been observed (Siu et al., 2003). For all these reasons, it seems only reasonable to assess whether changes in maternal nutrition affect the production and/or

distribution of collagen within the testes and epididymis under our nutritional regime.

1.9 Adult testis

The testes are the reproductive organs in the male responsible for producing both spermatozoa and testosterone. The testicular capsule is composed of the visceral vaginal tunic and the tunica albuginea, the inner part of this capsule is a loose connective tissue layer that contains blood vessels (Ross and Pawlina, 2006, Wrobel and Bergmann, 1998). Each testis is divided into approximately 250 lobules by incomplete connective tissue septa that project from the capsule (Ross and Pawlina, 2006, Wrobel and Bergmann, 1998, Bloom and Fawcett, 1975). Each lobule of the testis consists of highly convoluted seminiferous tubules, where sperm are produced, and connective tissue stroma, in which Leydig cells are held (Ross and Pawlina, 2006, Wrobel and Bergmann, 1998, Bloom and Fawcett, 1975). The ends of the lobules take a straight form named tubulus rectus, which becomes continuous with the rete testis, an anastomosing channel system within the mediastinum (Ross and Pawlina, 2006, Wrobel and Bergmann, 1998, Bloom and Fawcett, 1975). The seminiferous tubules are formed by a seminiferous epithelium surrounded by a tunica propria. This epithelium comprises of two cell populations: Sertoli and spermatogenic cells (Ross and Pawlina, 2006, Wrobel and Bergmann, 1998). Adult Sertoli cells are columnar cells that extend from the basal lamina to the lumen of the seminiferous tubules (Trainer, 1987, Russell, 1980). Their cytoplasm contains granular eosinophilic material to phagocytose the

degenerated germ cells and the residual bodies of the spermatids (Trainer, 1987, Kerr, 1992). Sertoli cells also contain androgen receptors in their cytoplasm and secrete inhibin, the hormone regulating the production of Follicle Stimulating Hormone (FSH) (Trainer, 1987). It is thought that Sertoli cells regulate spermatogenesis by carrying out different actions. Firstly, they form tight junctions between Sertoli-Sertoli cells and create the blood-testes barrier, which immunologically protect the auto-antigenic germ cells (Kaur et al., 2014). The creation of this barrier divides the seminiferous tubule into two compartments, the basal and the adluminal compartment. In addition, Sertoli cells are responsible for sperm cell movement from the basal lamina to the lumen of the seminiferous tubules (Russell, 1980, Trainer, 1987, Kerr, 1992, Griswold, 1995). Another important fact that supports the importance of Sertoli cells in spermatogenesis is that each Sertoli cells is able to support a limited number of germ cells (Griswold, 1995, Berndtson et al., 1987). This number varies between species but it is constant within a species (Russell et al., 1990, Griswold, 1998, Orth et al., 1988). Testicular weight and daily sperm production are related to the number of Sertoli cells (Sharpe et al., 2003, Orth et al., 1988, Griswold, 1995). Maturation of germ cells occurs in an orderly and non-random manner (Trainer, 1987, Griswold, 1995). Spermatogonia and young spermatocytes are located in the basal compartment of the seminiferous tubules. They obtain their nutrients from the lymphatic and vascular system and do not show an attachment to the basal lamina of the seminiferous tubules, so are free to move toward the lumen (Russell, 1980, Kaya and Harrison, 1976). Mature spermatocytes and spermatids are situated passing the Blood-Testis

barrier so, as mention previously, it is the Sertoli cells which provide them with nutrients (Russell, 1980, Kaya and Harrison, 1976). As a final step the late spermatid is release into the lumen of the seminiferous tubule as spermatozoon (Johnson et al., 2008, Russell, 1980). Adult Leydig cells are derived from undifferentiated precursor cells present in the interstitium of the testes (Habert et al., 2001, O'Shaughnessy et al., 2009). Adult Leydig cells produce a higher amount of testosterone than the fetal Leydig cells, and this is regulated by the luteinizing hormone (LH) (Habert et al., 2001, Wang et al., 2009). At puberty and into adulthood, testosterone is crucial in order to develop male secondary sex characteristics and initiate and maintain the spermatogenesis cycle (Tremblay, 2015, Wang et al., 2009).

1.10 Epididymis development and anatomy

The epididymis arises from the Wolffian duct (Mesonephric duct) (Capel, 2000, Josso, 1970), the differentiation of which is triggered by the testosterone produced by the Leydig cells in the testis (Behringer et al., 1994, Jost, 1953, Jost, 1947). In bovine the epididymis starts to develop at 60dpc and begins to coil at 110dpc (Alkafafy, 2005). The epididymis is attached to the caudomedial border of the testis and the ductus epididymidis runs along its full length (Dyce et al., 1996). The ductus epididymidis collects the sperm from the last portion of the ductulus efferens testis, which is connected to the rete testis within the testis (Bielli et al., 2007). The mature ductus epididymidis is a long, coiled tube, the size of which varies among species: bovine 40-50 meters (Alkafafy, 2005), rat 3.4m, ovine 50m and porcine 50-100m (França et al., 2005). Anatomically, the

epididymis is divided into three distinct regions called the caput, corpus and cauda (França et al., 2005, Glover and Nicander, 1971, Bielli et al., 2007) however based on its function and structure the epididymis is further subdivided into a number of segments that vary between species (Glover and Nicander, 1971, França et al., 2005, Alkafafy, 2005). Thus while in bulls, rams and stallions there are 6 segments (Nicander, 1958, Glover and Nicander, 1971, Alkafafy, 2005) in pigs (França et al., 2005), rabbits (Nicander, 1956) and guinea pigs (Hoffer and Greenberg, 1978) the epididymis is divided in 9, 12 and 7 segments, respectively. It is in the caput and corpus of the epididymis that the early and late maturation of spermatozoids takes place, respectively, whereas the cauda portion works as a storage area for mature sperm (Glover and Nicander, 1971, Browne et al., 2016, De Miguel et al., 1997). The cellular composition of the epididymis epithelium consists of principal and basal cells throughout its whole length and apical, narrow, clear and halo cells (namely macrophages and T lymphocytes) that are based in different segments of the epididymis depending on their function (França et al., 2005, Browne et al., 2016, De Miguel et al., 1997, Hermo and Robaire, 2002).

1.11 Sperm maturation

Spermatozoids are transported through the ductus epididymidis by peristaltic activity of smooth muscle cells (Axnér, 2006). When spermatozoa leave the testis they are almost immotile and unable to recognize or fertilize an ovum (Gatti et al., 2004b, Ecroyd et al., 2004, Joseph et al., 2009). They acquire these abilities during their transit through the epididymis (Ecroyd et al., 2004, Bassols

et al., 2005, Browne et al., 2016, Joseph et al., 2009). Sperm maturation is a complex process where many modifications such as alteration of the sperm membrane lipids, chromatin condensation, modification in the pattern of movement, migration of the cytoplasmic droplet and changes in the acrosome occurs in a location specific manner (Axnér, 2006, Alkafafy, 2005, Bassols et al., 2005). In bovine, as in other mammals like rabbits, ovine and porcine, the cytoplasmic droplet migrates from the proximal to the distal end of the midpiece (Axnér, 2006, Thundathil et al., 2001). In a study carried out by Thundathil et al. (2001) with bull sperm where migration of the cytoplasmic droplet fails during the transit of the spermatozoa through the epididymis showed impaired motility and a deficient bond of the spermatozoa to the zona pellucida. Another important role of the epididymis is as a quality control organ, which functions by removing spermatozoa with abnormal heads, acrosomal defects and midpiece abnormalities found in transit from the ductuli efferentes testis to the cauda epididymis (Ramos-Ibeas et al., 2013, Axnér, 2006). The mechanism used to carry out this clearance is still unknown, however two hypotheses have been suggested which are phagocytosis by epithelial cells or macrophages present in the epididymis, and/or ubiquitination where ubiquin binds to the defective spermatozoa as a marker for proteolytic degradation by epididymal cells (Axnér, 2006, Ramos-Ibeas et al., 2013). Once the sperm leave the epididymis they go through the capacitation process and only after this are they able to recognize and bind to the zona pellucida, undergo the acrosome reaction and initiate fusion with the oocyte (Ecroyd et al., 2004).

1.12 Epididymis proteomics

In cattle production artificial insemination is a common breeding technique used for transmitting key genes rapidly (Park et al., 2013). Yet, the successful rate of these inseminations is under 50% (Park et al., 2013, Peddinti et al., 2008). Half of the failures can be associated to male subfertility or infertility (Park et al., 2013), which leads to an important necessity to develop different techniques to predict and diagnose male fertility more accurately to avoid important economic losses in livestock breeding.

Male fertility can be described as the success by the mature spermatozoa to fuse with the oocyte and continuance of embryonic and fetal development until birth (Peddinti et al., 2008, Eid et al., 1994). A spermatozoon must be capacitated once it reaches the egg in order for effective fertilization to occur (Peddinti et al., 2008). This process is expedited by sperm surface proteins and leads to specific binding of the sperm surface–active component with the zona pellucida of the egg and, eventually, sperm-egg fusion (Bhattacharyya and Kanjilal, 2003). The wide diversity of proteins from cells surrounding the sperm has been suggested to be an indicator of male fertility (Moura et al., 2006, McCauley et al., 1999, Peddinti et al., 2008).

Proteins secreted into seminal plasma are crucial for sperm capacitation and fertilization (Aslam et al., 2014) since membrane and nuclear proteins are fundamental for inter and intra cellular signalling and are thus key for mediating cell-cell interactions (Bhattacharyya and Kanjilal, 2003). During epididymal transit, sperm absorb new proteins and proteins already present in the

membrane are modified (Moura et al., 2006). Epididymosomes, membranous vesicles secreted by the epididymal epithelium, has been recognized as one of the mechanisms by which sperm are modified during its transit through the epididymis (Sullivan et al., 2005). This epididymosomes are thought to interact and transfer proteins to sperm during epididymal transit (Sullivan et al., 2007). It has been shown that epididymosomes collected from the epididymal lumen are composed of many proteins also identified in sperm (Skerget et al., 2015).

The composition of the epididymal fluid differs in the different portions of the epididymis due to the two opposite activities of its epithelium: secretion and absorption of proteins (Dacheux et al., 2009). Although most of the proteins secreted in the testis are reabsorbed in the efferent ducts, the epididymal fluid composition in the first part of the epididymis is a mixture of testicular and epididymal proteins (Clulow et al., 1994, Dacheux et al., 2009). Regarding protein secretion and luminal protein concentration the caput is the most dynamic part of the organ where a considerable proportion of sperm surface protein from the testis is lost, which is contemplated as the first notable sperm modification (Dacheux et al., 2016). After completing its passage through the epididymal tract, sperm dwell quiescent in the cauda region until ejaculation (Gatti et al., 2004a, Moura et al., 2006). Furthermore, the cauda epididymis milieu also function to impede early capacitation (Roberts et al., 2003), maintain metabolic repose (Hinton and Palladino, 1995) and protect sperm against oxidative/peroxidative reactions, immune attacks and damage caused by substance released by dying cells (Hinton et al., 1996). At ejaculation, it is thought that active interaction between proteins of the cauda epididymal fluid

and accessory sex gland secretions occurs (Moura et al., 2006). To provide the spermatozoon protection against the acidic environment of the vagina, seminal fluid has buffering properties (pH 7.35-7.5) (Herwig et al., 2013).

1.13 Proteins involved in fertility

Numerous researchers have focussed on the study of epididymal and seminal fluid proteins looking for a marker of male fertility. Several proteins have been identified to be differentially expressed depending on the fertility rate. Epididymal angiotensin-converting enzyme (ACE) has been reported to mediate the translocation of ADAM3 in the sperm membrane (Yamaguchi et al., 2006) and to minimize sperm motility during epididymal storage (Köhn et al., 1998). In addition, knockout animals for the ACE gene were sub fertile (Hagaman et al., 1998) suggesting that this enzyme is required for normal fertility. In a study performed by D'Amours et al. (2010), where bulls were categorized as high or low fertile (fertility range based on the number of cows that do not return to service 56 days after the first insemination) the proteins abundant in high fertility bulls were adenylate kinase isoenzyme 1 (AK1) and phosphatidylethanolamine-binding protein 1 (PEBP1), while the proteins abundant in low fertility were T-complex protein 1 subunits ε and θ (CCT5 and CCT8), epididymal sperm-binding protein E12 (ELSPBP1), proteasome subunit α type-6 (PSMA6) and binder of sperm 1 (BSP1). In the same experiment the proteins from motile and immotile sperm subpopulations were extracted and compared and it was found that while AK1 did not change between the 2 subpopulations, PEBP1 was higher expressed in the motile subpopulation.

Moreover, ELSPBP1, BSP1 and PSMA6 were more highly expressed in the immotile subpopulation (D'Amours et al., 2010). It is important to take into consideration that in this study the sperm samples were provided by an artificial insemination company and so the sperm was processed, diluted, frozen and thawed. Therefore it is not showing the inherent fertility of the bulls but is important as artificial insemination techniques are so widely utilised. In another study by Peddinti et al. (2008) it was observed that in bovine spermatozoa, epidermal growth factor (EGF), which promotes proliferation, survival and differentiation of a wide variety of mammalian cells (Dikic, 2003), platelet derived growth factor (PDGF), peroxisome proliferated activator receptor (PPAR), interleukin (IL)-4, NF-kβ, chemokine and insulin growth factor (IGF)-1 were identified only in high fertility spermatozoa. In addition, the ATP synthase, H+ transporting, mitochondrial F1 complex (ATP5B), cytochrome c oxidase III (COX3) and protein kinase (CK2) were highly expressed in this group (Peddinti et al., 2008).

1.14 Immune system

Considering that in Northern Australia, endemic viral diseases represent one of the most important causes of fertilization failure in beef cattle (Burns et al., 2010), the immune system of the animals undernourished *in utero* were investigated.

Different types of immune cells collaborate to sustain a balance in the immune system, which maintains tolerance to self - antigens whilst aiming to eliminate external pathogens (Chaplin, 2010). Once the immune system recognizes the

presence of a non-commensal foreign organism into the body it starts to organize an effective response against it. Two different immune responses exist: innate immunity and adaptive immunity. The innate immunity is the first line of defence. It is an immediate and non-specific response, which retains no immunological memory. Innate immunity is composed of a physical and mucosal barrier (including skin and lung mucosa), phagocytic cells (for example neutrophils, macrophages and natural killer cells) and small molecules (including the complement proteins and cytokines) (Chaplin, 2010, Tomar and De, 2014). The adaptive immune response is a specific and late response activated by the innate immunity. It retains immunological memory, which allows it to act quicker and more efficiently against the same antigen in future infections (Chaplin, 2010, Tomar and De, 2014). This immune response is mainly based on the antigen – specific receptors present on the surface of T and B lymphocytes (Chaplin, 2010, Tomar and De, 2014). There are two types of adaptive immunity: humoral, which involves the creation of antibodies by plasma cells, which in turn are cells formed by the differentiation of B lymphocytes; and cell mediated, which depends on the activation of cytotoxic T lymphocytes, phagocytes and cytokines (Chaplin, 2010, Tomar and De, 2014). Both B and T lymphocytes originate from niches within the bone marrow, however T cells migrate to the thymus, where they undergo positive and negative selection (den Haan et al., 2014). As the thymus gland was the main organ of study in this experiment, the T cell mediated immune response is the primary focus of this chapter.

1.15 Thymus organogenesis

The epithelium of the thymus originates from the endoderm of the pharyngeal pouches (Vaidya et al., 2016, Rodewald, 2008). Other important organs including the thyroid and parathyroid glands develop from the same pharyngeal pouches involved in thymus development. In fact, in the earliest phases of organogenesis the parathyroid and the thymus keep in contact until gestation day (GD) 13.5 in mice and 16.5 GD in rats, at which point they become in two physically distinct organs (Rezzani et al., 2008, Vaidya et al., 2016).

Thymus tissue, primary located in both lateral sides of the neck, descends later in fetal life toward the mediastinum and fuses to form the two lobed cervical thymus (Kuper et al., 2016). This procedure is also followed in human, horses, dogs and rodents, however in ruminants and pigs part of the neck thymus persists in the neck and represents the majority of the tissue (Gasisova et al., 2016, Kuper et al., 2016). During the first year of life an isthmus connects the neck and cervical portion of the thymus in calves, later on this isthmus involves and the connection between these two parts of the organ disappears (Gasisova et al., 2016). In cattle the thymus has a lobed structure in which each lobe comprising of medulla and cortex, and it stops development at the age of 6-9 years (Gasisova et al., 2016). Histologically the thymus is composed of cortex, medulla, a capsule, Hassall's corpuscles, thymocytes, nerves and blood and lymph vessels (Gasisova et al., 2016). The development of the thymus starts at early embryogenesis, reaching maturity in the perinatal period, however it begins to involute as the individual ages (Zdrojewicz et al., 2016, Ribatti et al.,

2006). This involution represents one of the most important factors involved in the decrease of the immune response with the age, since the size of the thymus is reduced and the thymic tissue is replaced by adipose tissue (Ribatti et al., 2006, Zdrojewicz et al., 2016). In bovine the involution of the thymus begins at the age of 10-14 years and it is caused by shrinkage of the cervical branches (Gasisova et al., 2016).

1.16 T- cell development

The thymus is a pivotal organ within the adaptive immune system since in its absence T-lymphocyte differentiation and repertoire selection are not possible (Vaidya et al., 2016, Miller, 1961). In the earliest stage of thymus development the lymphoid progenitors migrates to the rudiment organ through the mesenchyme, however when the thymic vasculature develops they start to enter though the bloodstream (Boehm and Bleul, 2006, Zdrojewicz et al., 2016, Moore and Owen, 1967). The thymic epithelium is divided into cortical and medullary epithelium (Vaidya et al., 2016, Boehm, 2008). The thymic epithelial cells (TECs) of these two compartments have different functions and mediate different aspects during T-cell development (Vaidya et al., 2016, Boehm, 2008, Rossi et al., 2006). In an experiment carried out by Rossi et al. (2006) it was shown that the TECs are bipotent, which means that they are able to differentiate into the full range of epithelial cells present in a functional thymic microenvironment, and not unipotent as previously thought. T-lineage development begins with the migration of hematopoietic progenitors from the bone marrow into the thymus through blood vessels located within the cortical

medullary junction (Gottimukkala et al., 2016, Ma et al., 2013, Vaidya et al., 2016). T- cell development in the thymus can be divided into three progressive stages: Double negative (DN), double positive (DP) and single positive (SP) (Ma et al., 2013). In the DN stage, the thymocyte differentiates successively in DN1 ($CD44^+CD25^-$), DN2 ($CD44^+CD25^+$), DN3 ($CD44^-CD25^+$) and DN4 ($CD44^-CD25^-$) whilst moving from the cortex to the medulla. Before they reach the medulla the DN3 undergoes a rearrangement of the T cell receptor β (TCRβ) chain and forms the pre-TCR complex (Barbarulo et al., 2016). The thymocytes where the rearrangement is successful develop into DP cells and the ones that fail will go through the DN4 stage and then apoptosis (Barbarulo et al., 2016, Ma et al., 2013). Only the new generated DP cells that do not interact with the self-peptide-major histocompatibility complex I/II presented by cortical TEC or dentritic cells (DCs) will survive and move into the medulla (Ma et al., 2013). In the same way only the DP cells that do not interact with the self-antigen presented by the medullary TECs (mTECs) or DCs will pass the negative selection and become single positives ($CD4^+$ and $CD8^+$). These single positive cells will become mature T cells and move out of the thymus (Ma et al., 2013, Barbarulo et al., 2016, Shah and Zúñiga-Pflücker, 2014).

1.17 Thyroid gland

The thyroid gland shares a common origin with the thymus, the endodermal epithelium of the pharyngeal region (Kameda et al., 2013, Varga et al., 2008). In bovine, the differentiation of the thyroid gland occurs between the day 75 and 90 of gestation, and then the glandular proliferation begins (Micke et al.,

2015, Koneff et al., 1949). Although growth and function of the thyroid is regulated by the thyroid-stimulating hormone, thyroid organogenesis does not depend on pituitary control (Nilsson and Fagman, 2017, Postiglione et al., 2002, Fagman and Nilsson, 2010). This endocrine gland is formed by two lobules joined by an isthmus and lies anterior to the cricoid cartilage and trachea (Ritchie and Balasubramanian, 2014). The lobules contain follicles filled with colloid, which are responsible for production of the thyroid hormones and parafollicular cells, which synthesize and secrete calcitonin (Ritchie and Balasubramanian, 2014). The synthesis and secretion of the thyroid hormones by the thyroid gland it is regulated by the release of the thyrotropin-releasing hormone from the hypothalamus to stimulate the secretion of thyrotropin by the anterior pituitary (Duarte-Guterman et al., 2014, Yen, 2001). The production of thyrotropin-releasing hormone and thyrotropin is negatively regulated by thyroid hormones (Yen, 2001). Whereas both of the thyroid hormones thyroxine (T4) and triiodothyronine (T3) are synthesized in the thyroid gland, T4 is the major form of thyroid hormone secreted. The majority of T3, the active form of the thyroid hormones, is produced mainly in target tissues from the 5'- deiodination of the outer ring of T4 by deiodinases (Yen, 2001, De Vito et al., 2012, Duarte-Guterman et al., 2014). There are three types of deoidinases: type 1 (DIO1), type 2 (DIO2) and type 3 (DIO3), which deiodinase the T4 hormone in different manner to obtain T3 and reverse T3 (rT3; inactive); T3; and rT3 and diiodothyronine (T2), respectively (Duarte-Guterman et al., 2014, Yen, 2001). Expression of thyroid hormones in target tissues, like brain,

heart or bone, affects their development, growth and metabolism (Yen, 2001, De Vito et al., 2012).

1.18 Thyroid hormones and Immune system

There are several studies suggesting important interactions between thyroid hormones and the immune system. Both hyperthyroidism and hypothyroidism affect the immune system producing opposite effects. While hyperthyroidism increases the immune response, lymphocyte proliferation, cell migration and antibody production, hypothyroidism decreases all these parameters (De Vito et al., 2012, Klecha et al., 2006, Klecha et al., 2000). Additionally hyperthyroidism decreases the pro - inflammatory markers (De Vito et al., 2012). One of the first scientists to investigate the interaction between thyroid gland and immune system was Fabris (1973), who observed that mice with perinatal thyroidectomy showed lower thymus weights, smaller numbers of peripheral white blood cells, impaired plaque-forming capacity and a reduction in phytohaemagglutinin reaction when compared with control animals. Furthermore, when the animals were treated with a daily injection of thyroxine after thyroid gland ablation all these effects were nullified. In addition, in an experiment carried out by Pierpaoli et al. (1969) using dwarf mice (immunodeficient mice due to a defect in the anterior pituitary gland), a recovery of the thymus and lymphatic tissue structure was observed when the animals were given somatotropic and thyrotropic hormone. In the same experiment, control mice given an anti - thyrotropic hormone sera showed suppression of the immune system (Pierpaoli et al., 1969). Moreover, other

studies found that hyperthyroidism inhibits the differentiation of monocytes into macrophages, and so delayed the inflammatory response, and reduces monocyte migration into the peritoneal cavity (Rosa et al., 1995, Perrotta et al., 2014) whereas hypothyroidism increased the phagocytic activity (Rosa et al., 1995).

1.19 Maternal diet and offspring's immune system development

Maternal malnutrition during pregnancy has been shown to have drastic impacts on the development of the fetal immune system. Calder and Yaqoob (2000) observed that rats fed a restricted protein diet during pregnancy gave birth to pups that presented with reduced thymocyte and lymphocyte proliferation as a response to concavalin A stimulation in addition to decreased natural killer cell activity of spleen lymphocytes. Moreover a reduction in the percentage of singles CD4 ($CD4^+$ $CD8^-$) and CD8 ($CD4^-$ $CD8^+$) cells was found in rat pups which underwent energy restricted during gestation, although the population of double positive ($CD4^+$ $CD8^+$) was increased in these animals (Badr and Mohany, 2011). It has been also found that a reduction in cytokine expression occurs in malnourished animals *in utero*. A decrease in IL-2 and IL-7 cytokine levels in plasma were observed in the offspring of rats undernourished from day 0 of gestation to weaning (Badr and Mohany, 2011) and also in the IL-2, IL-6 and IL-10 expression in the jejunum of neonatal goat kids from protein restricted goats in the last trimester of gestation (He et al., 2014). A drop in component complement 4, immunoglobulin G (IgG) and IgM was also present

in the plasma of the kid goats from the latter study (He et al., 2014). Several studies have focused in the thymus as a possible mediator of the immunological effects of undernutrition, since this gland is a primary lymphoid organ crucial for normal T-lymphocyte development and function, and for the secretion of different thymic hormones which regulates peripheral immune response (McDade et al., 2001). It has been demonstrated that maternal diets lacking protein during gestation produced thymic atrophy and low birth weights in their offspring, related to a higher risk of infection and therefore mortality (Moore et al., 2009, Chen et al., 2010, Odiere et al., 2013, Collinson et al., 2003, Ferreira and Veiga-Fernandes, 2014). It is therefore hypothesized that protein restriction during the peri – conception and/or first trimester of gestation will impact thymus.

1.20 Fatty acids role in the immune system

Fatty acids are major components of triacylglycerols, phospholipids, and other complex lipids (Calder, 2015). They are hydrocarbon chains with terminal methyl and carboxyl groups (Woodside and Kromhout, 2005). The nomenclature indicates the number of carbon atoms in the chain, the number of double bonds in the chain and the position of the first double bond from the methyl terminus of the chain (Calder, 2001). Fatty acids are classified into saturated (SFAs) and unsaturated, which in turn are divided into monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs). SFAs and most MUFAs are considered non-essential fatty acids, since they can be produced in mammalian tissues from non-fat precursors like amino acids or

glucose. Yet PUFAs, because they can only be obtained from dietary intake, are considered essential fatty acids (Bystrická and Ďuračková, 2016, Calder, 2001). The PUFAs omega-6 and omega-3 have been investigated most frequently for their influence on the immune system. Whereas omega-6 is converted by a desaturase into arachidonic acid, which is a precursor of eicosanoids (such as prostaglandins, leukotrienes and thromboxane) that have pro-inflammatory roles, omega-3 is converted into eicosapentaenoic acid, which is the precursor of anti-inflammatory eicosanoids, and docosahexaenoic acid, a precursor of protectins (Calder, 2001, Calder, 2015, Bystrická and Ďuračková, 2016). A diagram of these fatty acids conversion is shown in Figure 1.2. Competition exists between omega-3 and omega-6 fatty acids due to the participation of the same enzymes in both conversions (Bystrická and Ďuračková, 2016, Calder, 2001, Calder, 2015).

Mammalian lymph nodes are surrounded by adipose tissue which, depending on its distance in relation to the lymph node, can be classified as perinodal adipose tissue (PAT) within 2mm to the node, middle adipose tissue between 2 and 5mm from the node and remote adipose tissue which is classified as further than 10mm from the node (Pond, 2005). However this classification is only relative when considering the differences in size and weight between and within animals. It has been observed that PAT has a similar fatty acid composition than the lipids of lymph node and tissue-derived lymphoid cells and that it interacts with adjacent lymphoid tissues (Mattacks et al., 2004, Westcott et al., 2006, Mattacks et al., 2002). During an immune response, stimulated immune cells from the lymph node penetrate in the adjacent

adipose tissue and selectively absorbed fatty acids present within (Acedo et al., 2011, Mattacks et al., 2004, Pond and Mattacks, 2003). Long-chain fatty acids are used as fuels in metabolic processes, and they are also components of cell membranes and precursors of signal molecules secreted by immune cells (Mattacks et al., 2002). Those fatty acids integrated into the membrane can participate in cell signaling, tolerance for oxidative stress and cell mobility, hence the amount and composition of fatty acids for the proliferating lymphoid cells are essential (Pond and Mattacks, 2003). Most of the fatty acids present in the adipose tissue surrounded a lymph node are integrated into the phospholipids of the membranes of the newly formed lymphocytes, albeit some are converted into eicosanoids or oxidised (Pond and Mattacks, 2003, Calder et al., 1994). In PAT the proportion of PUFAs is greater than in the adipose tissues located further from the lymph nodes (Mattacks and Pond, 1997, Westcott et al., 2006). It is well established that adipocytes produce and react to many cytokines, for example tumour necrosis factor-a (TNFα), some of this cytokines stimulate the adipocyte to release fatty acids and/or glutamine (Pond and Mattacks, 1998, Coppack, 2001, Mattacks et al., 2002).

Many studies have shown that protein deficiency affects the synthesis of the enzymes that participate in lipid metabolism, mainly the desaturases involved in PUFA conversions (Narce et al., 1988, Bouziane et al., 1994). However, to our knowledge, this is the first time the effect of maternal low protein diet in the fatty acid profile of the progeny has been investigated.

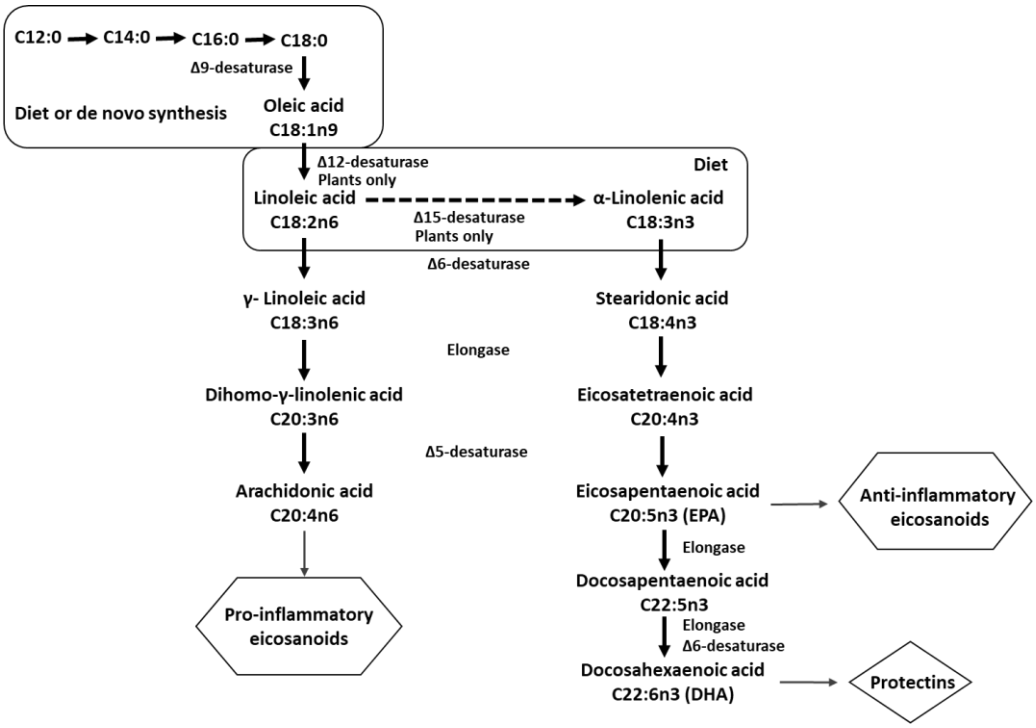


Figure 1.2 Diagram of fatty acid conversion adapted from Calder (2001) and Bystrická and Ďuračková (2016).

1.21 Aims and hypothesis

It has been established that dietary protein deficiency in the diet of pregnant mammals causes negative effects in the development of the future offspring. Considering that dietary protein is deficient in pastures during the dry season in northern Australia, we hypothesized that the progeny from pregnant heifers during the dry season, whose diet is not supplemented with protein, would compromise development resulting in sub-optimal fertility and immunity. In this study, the dietary protein levels used in the present study mimic pasture conditions in Australian rangelands without (low, 7%) and with (high, 14%) protein supplement. To enable comparisons the low protein diet was designated as the control group as it represents a diet based only on grazing and is therefore not influenced by any external factor. There was a 1.9- to 2.1-

fold difference in crude protein (CP) content and a 1.1-fold difference in energy content between the high and low diets. The ration was as isocaloric as possible as ruminants were fed the forage component of the diet under group housing. Dietary fat content was similar and although starch content differed, levels in both low and high diets were moderately low. Protein intake was restricted during both the peri - conception period (60 days prior conception to 23 dpc) and first trimester (24dpc to 98 dpc) in the low group whilst both groups received similar energy intake. As the variation in CP content between the high and low diets was much greater than differences in the energy content of the feeds, we consider that the differences observed in the present study are likely attributable to the effects of protein rather than energy intake. To test our hypothesis, histologically, immunohistochemistry, stereology, next generation sequencing, proteomics and fatty acid analyses were used to analyse the testes, epididymis, thymus, thyroid and pre-scapular lymph node adipose tissue from the offspring of cows restricted in protein intake during the peri – and/or first trimester of gestation.

Chapter 2 General materials and methods

2.1 Animal management

The animal feed trials consisted of 360 maiden 12 month old Santa Gertrudis (Bos Taurus x Bos indicus) heifers, selected based upon age and weight. The animals were acclimatized to the diet, held in covering pens and fed individually before the beginning of the experiment.

Sixty days before artificial insemination (AI) the heifers were split into two equally sized groups determined and randomized by weight. The animals in one of the groups were fed with a low protein diet (7% crude protein (CP)) and the heifers in the other group were fed with a high protein diet (14% CP). The standard diet consisted of limited pellets and straw (5% CP) was fed *ad libitum*. Rations were as isocaloric as possible (Table 2.1) (Copping et al., 2014). The dietary protein levels reflected pasture conditions in Australian rangelands without (low), and with (high), protein supplement. In this study the low protein diet was considered as control diet, since this diet is based in natural resources and not extra supplementation.

Heifers were subjected to an 8-days progesterone based oestrous synchronisation program. On day 0, heifers were artificial inseminated with frozen semen from the same bull. At 23 dpc each group of heifers was again divided into two further groups; a 'high 14%' and 'low 7%' protein diet group, resulting in 4 different diet groups: Low protein-Low protein (LL), Low protein-High protein (LH), High protein-High protein (HH) and High protein-Low protein

(HL) (Figure 2.1) and also two different diet groups per period: LPeri (LL-LH) and HPeri (HH-HL) and LPost (LL-HL) and HPost (HH-LH), Table 2.2.

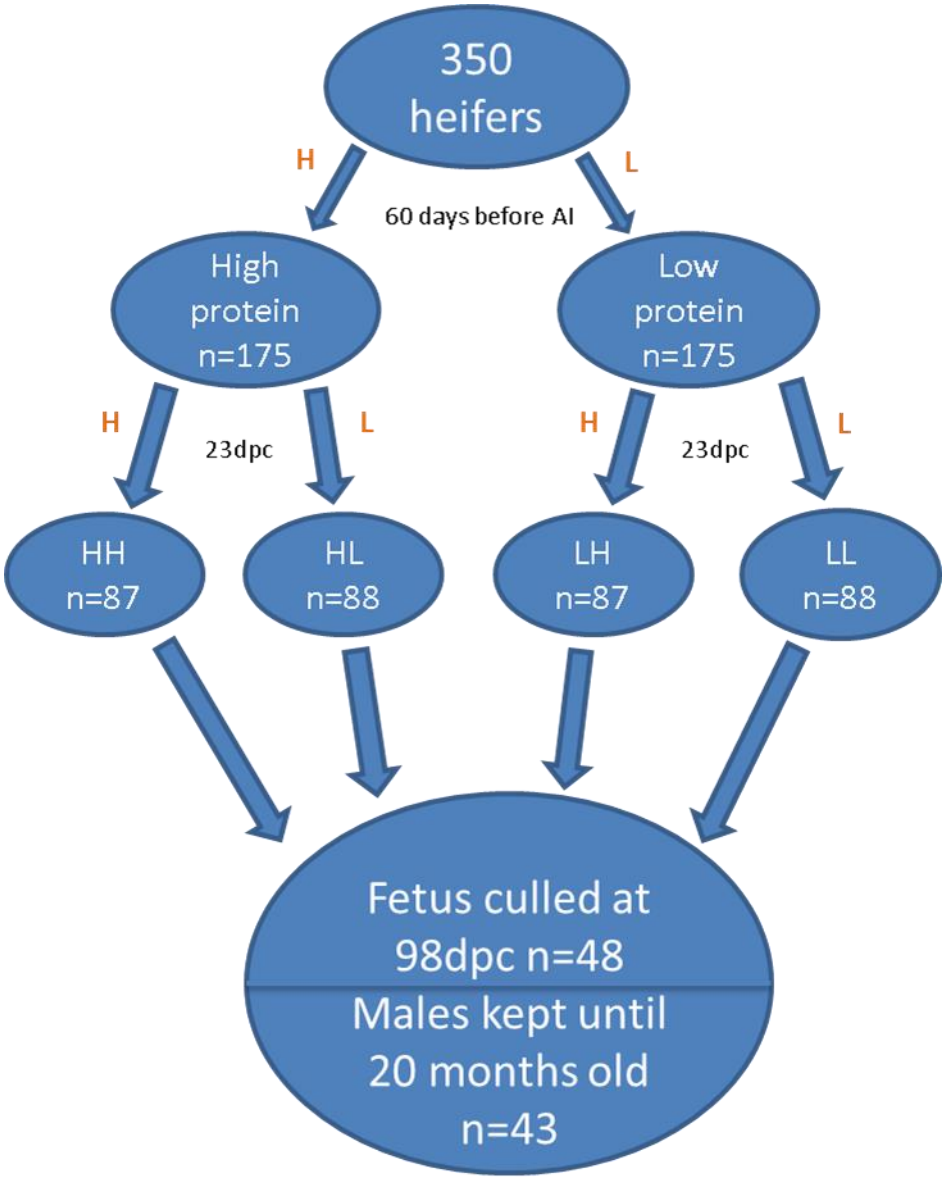


Figure 2.1 Management of the heifers into the different diet groups from which fetal and adult samples were obtained.

At day 36 post conception, pregnancy was diagnosed via transrectal ultrasound in 124 heifers (HH=33; HL=37; LH=30; LL=28), the rest of the heifers were eliminated from the experiment. To enable an equal number of males and

females in each of the four groups, fetuses were sexed at 60 dpc via ultrasonography and 48 heifers selected to be culled at the end of the 98 dpc. At day 98 of pregnancy, 25 male and 23 female fetuses were obtained and 4 fetuses were reabsorbed. Moreover, 2 male twins (4 males) and 1 female twins (2 females), were eliminated from the study. The remaining pregnant heifers were fed with the standard ration until parturition. The male calves obtained from the term parturition of the treated heifers were fed and grown in normal conditions until 20 month old, when the bulls were culled and the tissues of interest dissected. At this stage, 43 bulls were culled, albeit 1 twins (2 males) and a cryptorchid bull were removed from the study. The distribution of the diet groups is shown in Table 2.3.

Table 2.1 Ingredients and nutrient content of heifer rations for induction period.

Ration as fed	Induction	PERI conception		POST conception		2nd & 3rd Trimester
		High	Low	High	Low	
Wheat (kg)	0.66	0.48	1.81	0.56	2.12	0.60
Canola meal (kg)	2.23	-	-	-	-	0.89
Soybean meal (kg)		1.83	0.48	2.14	0.56	0.44
Barley Straw (kg)	7[1]	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 (kg)	9.1[2]	8.3	7.2	12.3	11.8	9.6
Total energy (MJ ME)		71	63	102	98	79
% of energy requirements[3]		96	85	142	136	125
Total crude protein (kg)		1.18	0.62	1.49	0.88	0.92
% of protein requirements[3]		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[3]		130	110	190	185	132
Total phosphorus (g)		17	17	21	21	20
% of phosphorus requirements[3]		130	130	160	160	125

PERI (-60 dpc to 23 dpc) and POST-conception period (24 dpc to 98 dpc) and 2nd & 3rd trimester of gestation (Copping et al., 2014).
[1]assumed value
[2]predicted value
[3]Dietary requirements were calculated using Nutrient Requirements of Domesticated Ruminants (CSIRO, 2007).

Table 2.2 Diet groups obtained per time period.

Groups separated by periods	Final groups
LPeri	LL + LH
HPeri	HH + HL
LPost	LL + HL
HPost	HH + LH

Table 2.3 Number of fetal and adult animals involved in the study following removal of twins and a cryptorchid adult.

	LL	LH	HL	HH
FETUSES				
MALE	4	5	7	5
FEMALE	5	5	5	6
ADULT				
MALE	8	8	14	10

Ethical approval was given by University of South Australia in accordance with institutional and Government guidelines and local ethical approval from the University of Nottingham was obtained to work on the samples (ethical approval number 1117 140320). Samples were transported in accordance to UK Home Office, University of Nottingham and Australian Government guidelines, using appropriate packing, health and safety. This trial and sample collections were carried out by Dr Vivienne Perry.

2.2 Sample collection

2.2.1 98 dpc testis samples

The selected heifers were culled in a commercial slaughterhouse at 98 dpc and their meat used for human consumption. The fetuses were excised from the uterus and the left testes, thyroid and thymus were removed, processed and blocked in paraffin wax. The right testis and a piece of the chest thymus were frozen at -80°C.

Fixation and processing for blocking consisted of the tissues being fixed overnight in 4% paraformaldehyde diluted in 0.1 M phosphate buffered saline (PBS) in a ratio of 1:5 (tissue volume: fixative solution volume). Samples were then washed 3 times for 24 hours each in PBS (0.14 M NaCl, 0.03 M NaH_2PO_4, 0.05 M Na_2HPO_4). Following these three days, the tissue was processed on an automated tissue processor in the following solutions, for 30mins per solution: 70% ethanol (EtOH), 80% EtOH, 95% EtOH, 3X in 100% EtOH, 2X in 100% xylene and 2X in paraffin wax at 60°C under a vacuumed environment. Following processing, the samples were orientated and embedded into paraffin wax. This process was carried out by Dr Vivienne Perry and her colleagues in Australia (Copping et al., 2014).

2.2.2 Adult testis samples

Forty adult males were selected and the left testes and epididymis were removed, dissected and fixed in paraffin wax. Epididymal fluid and pre-scapular lymph node and its surrounding adipose tissue were also collected and stored at -80°C.

The blocked samples were fixed overnight in 4% paraformaldehyde diluted in 0.1 M PBS in a ratio of 1:5 (tissue volume: fixative solution volume). Samples were then washed 3 times in PBS for 24 hours each (0.14 M NaCl, 0.03 M NaH_2PO_4, and 0.05 M Na_2HPO_4). Following these three days, the tissue was processed in an automated tissue processor in the following solutions, for 1 hour per solution: 70% EtOH, 80% EtOH, 95% EtOH, 3X 100% EtOH, 2X 100% xylene and 2X in paraffin wax at 60°C under vacuum conditions, prior to embedding in paraffin. This process was also carried out in Australia by Dr Vivienne Perry and her team.

2.3 Epididymal fluid extraction

The epididymis were excised from the testis and flushed of blood using PBS. A transection was performed in the cauda epididymis before starting the flush and the vas deferens was cut 6 inches distal to the cauda epididymis. A blunted 21 g needle on a 10 ml syringe was inserted into the vas deferens and 5 ml of PBS were pushed retrograde through the sectioned portion of the cauda epididymis. The flushed caudal fluid was collected into a small petri dish, transferred to a falcon tube and centrifuged at 2,850 g (relative centrifugal force) for 10 mins. The sperm formed a good pellet clearly differentiated from the fluid which was collected avoiding the closest area to the sperm pellet to ensure no contamination occurred. Samples were stored at -80°C until posterior analyses.

2.4 Histological techniques

All the histological techniques were performed by the author unless it is specified the contrary. In order to carry out this tissue/cell identification, immunohistochemistry (IHC) processes were used using 3,3'-diaminobenzidine (DAB) staining (Section 2.4.1) and also several different histological techniques described in the following Sections (Figure 2.2).

Fetal testis, adult epididymis and fetal thymus were sectioned at 10 µm, 8 µm and 4 µm, respectively Aziza Alibhai. Adult testes were sectioned at a thickness of 10 µm per section by the author and fetal thyroid were cut at 8 µm by Alan Lasslett. All samples were cut using a microtome (Leica 5M 2255, UK). The sections were dried onto the polysilinated slides (Thermo Scientific, Germany) on a hot plate at 42ºC for one hour and left at room temperature for 24 hours prior to histological staining. A minimum of 30 slides were obtained per testis to ensure systematic random sampling throughout the tissue.

2.4.1 Immunohistochemistry using DAB

Müllerian Inhibitory Substance (MIS-C20) was utilised to identify the interior of the seminiferous tubules and highlight the differences between the Sertoli and germ cells (Section 2.4.1.1; Figure 2.2A). Antibodies were used to stain for collagen (Section 2.4.1.2). The slides were selected at least 5 slides apart throughout the entire sample. This ensured that double counting of cells/structures was avoided. A negative control sample was included per stain, per sample.

2.4.1.1 MIS-C20: Müllerian inhibitory substance staining protocol (Sertoli and germ cell identification)

The Müllerian Inhibitory Substance is secreted by immature Sertoli cells during the development of the reproductive tract (Behringer et al., 1994). This protein is responsible for enabling the regression of the Müllerian duct, which in turn inhibits development of female reproductive organs (Ross and Pawlina, 2006). Marking the Sertoli cells with an antibody against Anti-Müllerian Hormone (AMH; MIS C-20 antibody, Santa Cruz Biotechnology, sc-6886, LOT#K2706) and DAB (Novolink DAB substrate buffer Ref: RE7143 and Novocastra™ DAB chromogen ref: RE7105, Leica Biosystems, UK) ensured that within the morphology of the fetal seminiferous tubules, both the Sertoli cells and by morphological determinants, the germ cells, were both distinct and could be manually identified.

Sections from testis (10 µm thick) were deparaffinised and rehydrated [5 mins xylene, 5 mins 100% EtOH, 5 mins 90% EtOH, 5 mins 70% EtOH and 5 mins in distilled water (dH_2O)], followed by the antigen retrieval step using sodium citrate buffer (sodium citrate 1:10 dH_2O). The slides were submerged in the buffer and placed in the microwave for 1 min on full power and 4 mins on low/medium power. Then, the samples were left to cool for 20 mins and washed with dH_2O.

A Novolink Leica immunostaining kit (Leica Microsystems, UK, Novolink Polymer Detection Kit, REF# RE7150-k, LOT#6012496) was used to immunostain the samples, in accordance to manufacturer's protocols but optimised for the tissue. Firstly, 3 drops of a Novolink Peroxidase blocking

solution were placed on each slide and incubated under parafilm for 15 mins. After that the samples were washed with PBS (2X 5 mins). Non-specific reactivity was blocked using the Novolink protein block solution, 3 drops per slide, incubated under parafilm for 30 mins. 200 µl of a 1:1000 µl dilution of the primary antibody MIS-C20 (Santa Cruz Biotechnology, USA, sc-6886, LOT#K2706) diluted in fetal calf serum (FCS), as previously described by Paul et al. (2005), and incubated at room temperature for 1 hour. Samples were then washed with PBS (2X 5 mins). 200 µl of an unconjugated rabbit anti-goat antibody (Abcam, UK, Unconjugated Rb anti goat IgG, ab6697), instead of the Novolink secondary antibody, was used at a dilution of 1:200 in PBS and incubated for 30 mins. The samples were washed with PBS (2X 5 mins), then covered with 3 drops per slide of a Novolink polymer and incubated for 30 mins and washed with PBS (2X 5 mins). The final immunoreaction step involved adding a Novolink DAB working solution at a dilution of 2 ml of DAB substrate: 25 µl DAB chromogen.

Immunostained sections were counterstained with Harris' haematoxylin solution (Sigma-Aldrich, USA), dehydrated through an ethanol series (70%, 90% and 100% for 5 mins) and then dehydrated in xylene for 2X 5 mins and coverslips mounted with distyrene plasticizer xylene (DPX) mounting solution.

2.4.1.2 Collagen I and III antibody

Prior to starting the immunohistochemistry process the paraffin sections were incubated at 60°C for 20 mins to increase tissue adherence to the microscope slide. Thereafter tissues were deparaffinised and rehydrated in xylene followed by decreasing concentrations of EtoH and dH_2O for 5 mins per step (2X xylene,

100% EtOH, 90% EtOH, 70% EtOH and dH_2O). An antigen retrieval step was performed which consisted of submerging tissue in sodium citrate buffer (concentration of 1:10 in dH_2O) and heated in the microwave 5 mins at medium-high power and 5 mins at medium-low power, always avoiding boiling point. The samples were left for 20 mins to cool and then submerged in dH_2O for 5 mins to remove the residual sodium citrate buffer.

A Novolink Leica immunostaining kit (Leica Microsystems, UK, Novolink Polymer Detection Kit, REF# RE7150-k) was used to immunostain the samples, in accordance with the manufacturer's protocols but optimised for the tissue and primary antibody. Firstly, 3 drops of a Novolink Peroxidase blocking solution were placed on each slide and incubated under parafilm for 15 mins, then washed with PBS (2X 5 mins). Non-specific reactivity was blocked using the Novolink protein block solution, 3 drops per slide, incubated under parafilm for 30 mins. 200 ul of a 1:10 µl dilution of the primary antibody (Bio-rad 2150-2305, USA) diluted in FCS and incubated at room temperature for 1.5 hours. Samples were then washed with PBS (2X 5 mins). As part of the kit 3 drops of a secondary antibody was poured in each slide and covered with parafilm for 30 mins, followed by a wash in PBS 2X 5 mins. 3 drops of a Novolink polymer was then added to the samples and left incubate for 30 mins covered by parafilm and after that washed with PBS 2X 5 mins. The final immunoreaction step was adding 200 µl of DAB working solution at a dilution of 2 ml of DAB substrate: 25 µl DAB chromogen.

Immunostained sections were counterstained with Harris' hematoxylin solution (Sigma-Aldrich, USA), dehydrated through an ethanol series (70%, 90%

and 100% for 5 mins each), dehydrated in xylene for 2X 5 mins and coverslips were mounted with DPX mounting solution.

2.4.2 Masson's trichrome staining of collagen

The Masson's trichrome was a technique employed to visualise the different components of the tissues. In this staining, the collagen fibres appeared green in colour, the cellular nuclei showed as a dark blue/purple and the rest of the tissue (cytoplasm, muscle fibres, erythrocytes) stained red. The main purpose for using this technique was to quantify the amount of collagen in the fetal and adult testes and in the epididymis. The general protocol was optimized by a PhD student in the group, Jennifer Edwards, in the bovine placenta and was adapted by the author for both the testicular and epididymal tissue. The steps of this protocol were:

Samples were deparaffinised for 4 mins in xylene and rehydrated by submerging the samples in a series of EtOH (100%, 90% and 70%) for 5 mins each. The tissue was washed twice with 1X PBS for 5 mins. The sections were mordant in Bouins solution (Polysciences Inc., Lot 646691. USA) and incubated in an oven at 60ºC. After 1 hour the samples were placed under tap water for 5mins and rinsed in dH_2O for another 5 mins prior to hematoxylin staining. The working solution was obtained by mixing Hematoxylin A (Lot 646691, Polysciences Inc., USA) with Haematoxylin B (Lot 664890, Polysciences Inc., USA) in a ratio 1:1. 200 µl of this mix was placed on each slide for 2 mins and then washed under tap water for 5 mins and rinsed with dH_2O for 5 mins. Following this the samples were stained with Biechrich scarlet acid-Fuchsin (BSA) (Lot 664889, Polysciences Inc., USA) diluted at 25% in dH_2O. 200 µl of this

solution per slide was incubated for 30 secs and then samples were rinsed in dH_2O. Following the washes, 200 µl of Phoshotungstic/phoshomolybdic acid (Polysciences Inc., USA) at 5% was placed on each slide and after 10 mins, 200 µl of light green counterstain stock (Lot 664311, Polysciences Inc., USA) was placed onto each slide and left it for 15 mins and then rinsed in dH_2O. As last step, 200 µl of 1% acetic acid (Lot 663682, Polysciences Inc., USA) were placed on each slide for 1 min and then rinsed with dH_2O. The stained samples were dehydrated through an EtOH series (70%, 90% for 5 mins and 100% 2X 5 mins) and then dehydrated in xylene for 2X 5 mins and coverslips were mounted with DPX mounting solution.

2.4.3 Picrosirius staining of collagen

Picrosirius (PS) staining was used to stain collagen in fetal testis and adult testis and epididymis tissue. It differentiates between collagen I, which stained in yellow and collagen III which stained in green. The rest of the collagen stained a red colour. This staining was used to compare the differences between Masson's staining as a secondary technique (as collagen staining has not been reported in this tissue in the literature previously) and also to facilitate the measurement of blood vessels in the fetal and adult testis (allowing better morphological visualization of the vessels). The protocol followed in this technique was the following:

The tissues were deparaffinised first as per Section 2.4.1.1, by placing in xylene for 10 mins and then in a series of EtOH (100, 90 and 70% for 5 mins each). After that the tissue was submerged in dH_2O water for 5 mins. To commence the staining process, 300 µl of Phosphomolybdic Acid (Polysciences, Inc., USA) was

placed on each slide and left for 2 mins, then the slides were rinsed in dH_2O for 5 mins. Following this the tissue was covered by 300 µl of Picrosirius Red F3BA stain (Polysciences Inc., USA) for 60 mins, immediately after that 300 µl of 0.01 N Hydrochloride acid (Polysciences Inc., USA) was placed on each slide for 2 mins and thereafter the tissue was covered with 70% EtoH for 45 secs. Then the stained samples were submerged in dH_2O for 5 mins, dehydrated in EtOH (70, 90 and 100% for 5 mins each), placed in xylene for 10 mins and coverslips mounted with DPX mounting solution.

2.4.4 Van Gienson staining of epididymis collagen

Van Gienson's staining was used to stain the collagen present in the epididymis. This technique was used to corroborate which of the two staining techniques used previously (Massons and PS) was appropriate as differing results had been achieved using the two methods. The following protocol was used:

Tissue was deparaffinised and hydrated as per Section 2.4.1.1. A total of 300 ul of Weigert's Working Haematoxylin, prepared from mixing Weigert's iron haematoxylin A and Weigert's iron haematoxylin B (Merk KGaA, Germany) in equal parts, was added to each slide for 10 mins, and then the tissue washed for 5 mins in running tap water and after that 5 mins in distilled water. Following this, the Van Gienson's solution (Electron Microscopy Sciences, USA) was poured onto the samples and left for 3 mins to react with the tissue. Samples were then dehydrated in 95% EtOH 2X 5 mins, 100% EtOH 2X 5 mins and xylene for 2X 5 mins. Coverslips were mounted using DPX mounting solution.

2.4.5 Haematoxylin and Eosin staining

Haematoxylin and eosin staining was used to differentiate the thyroid follicles within the developing thyroid. This technique was carried out by Alan Lasslett a technician from the School of Veterinary Medicine and Science, Nottingham University. The protocol followed is explained below:

Samples were deparaffinised and hydrated in xylene 2X 5 mins and in a descending concentration of ethanol, 100%, 95% and 70%, for 2 mins in each solution. Then the tissue was washed in running tap water. After that, the slides were submerged in Harris' haematoxylin for 3 mins, washed in tap water and dipped for 2 to 4 secs in 1% acidified IMS. Following this, the sections were washed in tap water and placed in ammoniated water (1% ammonia solution) for 2 to 4 secs. At this stage it was checked that the samples were stained properly with the haematoxylin. Then the tissue was dipped in eosin (1% eosin in 0.02% acetic acid) for 5 mins and washed in running water until excess staining solution was removed. Stained samples were then dehydrated in an ascending concentration of ethanol, 70%, 95% and 100% for 2 mins each and 2X 5 mins in xylene. Coverslips were mounting using DPX.

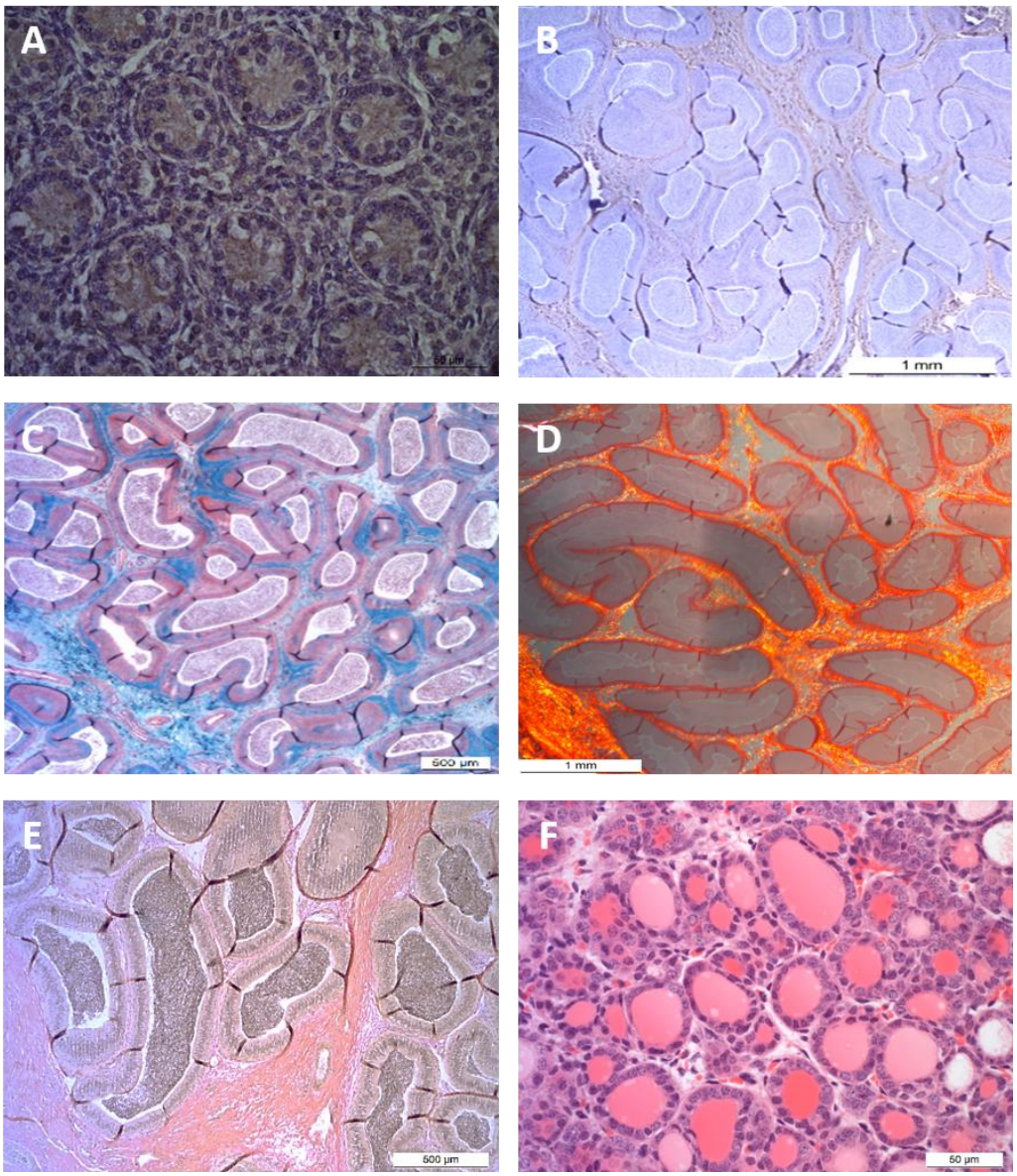


Figure 2.2 Representative examples of the different staining techniques used for the identification of the structures in the fetal and adult testes. A) Immunohistochemistry using MIS-C20 in the fetal testis to highlight the seminiferous tubules, Sertoli cells and germ cells; B) Immunohistochemistry using antibodies for detection of collagen type I and III in adult epididymis; C) Masson's trichrome staining in adult epididymis to detect collagen; D) Picrosirius staining in adult epididymis to detect collagen; E) Van Gienson staining in adult epididymis to detect collagen and F) Hematoxylin and Eosin staining in fetal thyroid to show all features.

2.5 Systematic random sampling and microscopy

Systematic random sampling was carried out by the author. Photomicrographs of the stained samples were taken using a Leica DM5000 microscope, with Leica CTR5000 light box and Leica DFC420 colour capture camera (all from Leica, Germany). One section per slide was selected and systematic random sampling as previously described (Mayhew, 2011) was used to take the photomicrographs throughout the whole section (See Figure 2.3). Different amounts of slides and photomicrographs were taken on the different tissues depending on the minimum requirement for each tissue/structure/cell type to be able to represent the whole tissue, which is explained in more detail in each chapter. Photomicrographs did not overlap to ensure that double counting could not occur. Different magnifications (from 5X to 63X) were used depending on the tissue and structure to be measured – each is explained where appropriate.

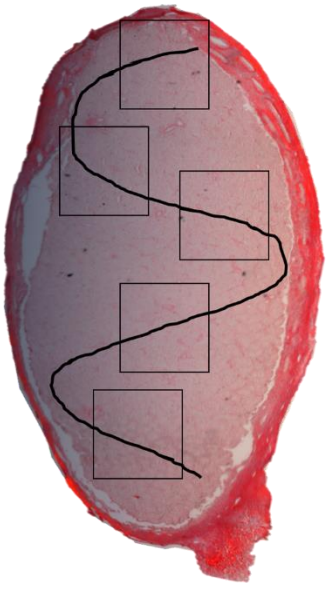


Figure 2.3 A diagram showing how the systematic random sampling was performed.

2.6 Image Analysis

Image analysis was performed by the author. Manual identification and counting of fetal testicular cells (Sertoli, germ and interstitial cells) was carried out. Moreover, in order to measure blood vessels, seminiferous tubules, epididymal tubules and thyroid follicles an imaging analyses software (image-pro plus (version 6.3), Media Cybernetics, USA) was used. The different structures were manually drawn around and this software provided the area and number of the structure measured which were used, following calibration of the imaging software, for the calculation of the average area, number and proportion of the selected structure within the tissue measured. Snapshots of the different structures after their measurement using image-pro are shown in Figure 2.4.

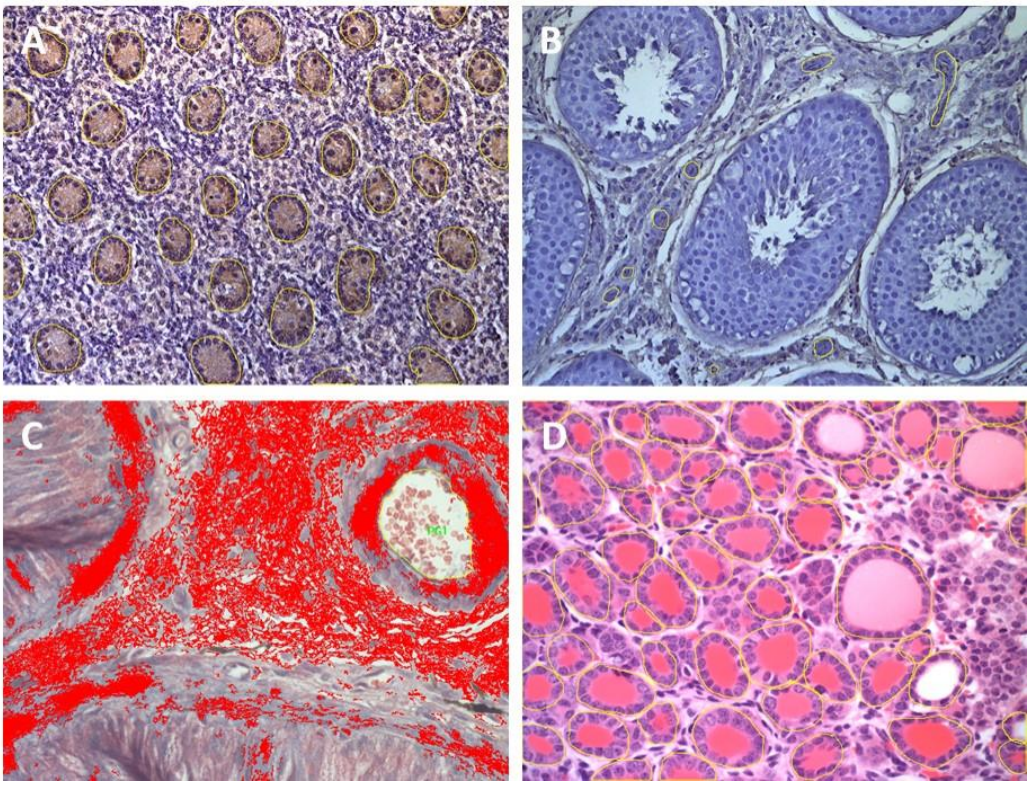


Figure 2.4 Structures manually drawn around in order to obtain measurements using the image analyzing software Image-pro. A) Fetal seminiferous tubules; B) Adult blood vessels; C) Epididymal collagen; D) Thyroid follicles.

2.7 Epididymal fluid Proteomics

2.7.1 2D SDS/PAGE electrophoresis

Proteomics of the epididymal fluid was performed in the INRA institution, France, by Valerie Labas and Lucie Combes-Soia. After separation of proteins by acrylamide SDS-PAGE and Coomassie Blue staining, each lane was cut into 3 slices that were washed in dH_2O, acetonitrile (1:1) for 5 mins followed by a second wash in acetonitrile for 10 mins. Cystein reduction and alkylation were performed by successive incubations in solutions of 10 mM dithiothreitol, 50 mM NH_4HCO_3 for 30 mins at 56°C and 55 mM iodoacetamide, 50 mM NH_4HCO_3 for 20 mins at room temperature in the dark, respectively. Gel slices were

washed by an incubation in 0.1% formic acid, acetonitrile (1:1) for 10 mins followed by an incubation in acetonitrile for 15 mins. Proteins were digested overnight in 25 mM NH_4HCO_3 with 12.5 ng/µl trypsin (Sequencing Grade, Roche, Paris). The resulting peptides were extracted from the gel using an incubation in 0.1% formic acid, acetonitrile (1:1) for 10 mins followed by an incubation in acetonitrile. The two collected extractions were pooled with the initial digestion supernatant, dried in a Speed-Vac, reconstituted with 30 µL of 0.1% formic acid, 2% acetonitrile, and sonicated for 10 mins.

2.7.2 Ms/Ms spectrophotometer

Peptide mixtures were analyzed by on-line nanoflow liquid chromatography tandem mass spectrometry (nanoLC-MS/MS). All experiments were performed on a dual linear ion trap Fourier Transform Mass Spectrometer (FT-MS) LTQ Orbitrap Velos (Thermo Fisher Scientific, Bremen, Germany) coupled to an Ultimate® 3000 RSLC Ultra High Pressure Liquid Chromatographer (Dionex, Amsterdam, The Netherlands) controlled by Chromeleon Software (version 6.8 SR11 ; Dionex, Amsterdam, The Netherlands). 5 µl of each sample were injected using µL-pickup mode and loaded on an LCPackings trap column (Acclaim PepMap 100 C18, 100 µm inner diameter x 2 cm long, 3 µm particles, 100Å pores). Mobile phases consisted of (A) 0.1% formic acid, 97.9% dH_2O, 2% acetonitrile (v/v/v) and (B) 0.1% formic acid, 15.9% water, 84% acetonitrile (v/v/v). Peptides were automatically desalted and pre-concentrated for 10 mins at 5 µL/min with 4% solvent B. The peptide separation was conducted using a LCPackings nano-column (Acclaim PepMap C18, 75 µm inner diameter x 15 cm long, 3 µm particles, 100Å pores). The gradient consisted of 4-55% B for 90

mins, 55 to 99% B for 1 min, constant 99% B 10 mins and return to 4% B in 1 min. The column was re-equilibrated for 25 mins at 4% B between runs. The nanoflow rate was set to 300 nl/min. The eluate was nanoelectrosprayed through a Thermo Finnigan Nanospray Ion Source 1 with a SilicaTip emitter of 15 µm inner diameter (New Objective, Woburn, MA, USA). Standard mass spectrometric conditions for all experiments were spray voltage 1.2 kV, no sheath and auxiliary gas flow; heated capillary temperature, 250°C; predictive automatic gain control (AGC) enabled, and an S-lens RF level of 60%.

Data were acquired using Xcalibur software (version 2.1; Thermo Fisher Scientific, San Jose, CA). The LTQ Orbitrap Velos instrument was operated in positive mode in data-dependent mode to automatically switch between high resolution full scan MS spectra (in the Orbitrap analyzer) collected in centroïd mode and low-resolution CID-MS/MS (in the dual linear ion trap analyzer) in centroïd mode. Resolution in the Orbitrap was set to R=60,000. In the scan range of m/z 400-1800, the 20 most intense peptide ions with charge states ≥2 were sequentially isolated (isolation width, 2 m/z; 1 microscan) and fragmented in the high-pressure linear ion trap by low-energy CID with normalized collision energy of 35% and wideband-activation enabled. Ion selection threshold was 500 counts for MS/MS, and the maximum allowed ion accumulation times were 500 ms for full scans and 25 ms for CID-MS/MS measurements in the LTQ. Target ion quantity for FT full MS was 1×106 and for MS2 was 1×104. The resulting fragment ions were scanned out in the low-pressure ion trap at the “normal scan rate”. An activation q=0.25 and activation time of 10 ms were used. Dynamic exclusion was actived during 30secs with a repeat count of 1.

The lock mass was enabled for accurate mass measurements. Polydimethylcyclosiloxane (m/z, 445.1200025) ions were used for internal calibration.

2.7.3 Protein data analysis

Raw data files were converted to MSF files with Proteome Discoverer software (version 1.3; Thermo Fischer Scientific, San Jose, USA). Precursor mass range of 350–5000Da and signal to noise ratio of 1.5 were the criteria used for generation of peak lists. In order to identify the proteins, the peptide and fragment masses obtained were matched automatically against the Mammalia section of a locally maintained copy of nr NCBI (download July 2015). MS/MS ion searches were performed using MASCOT search engine (version 2.3; Matrix Science, London, UK). The parameters used for database searches include trypsin as a protease with allowed two missed cleavage, carbamidomethylcysteine (+57 Da), oxidation of methionine (+16) and N-terminal acetylation (+42) of peptides as variable modifications. The tolerance of the ions was set to 5ppm for parent and 0.8Da for fragment ion matches and the instrument setting was specified as “ESI-FITCR”.

Mascot results obtained from the target and decoy databases searches were subjected to Scaffold software (version 4.4, Proteome Software, Portland, USA). Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm. Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least two identified peptide. Peptides were considered distinct if they differed in sequence. Protein probabilities were

assigned by the Protein Prophet algorithm. Peptides were considered distinct if they differed in sequence.

2.7.4 Protein quantification

Bottom-up quantitative proteomic analyses were performed from 36 samples that were loaded on SDS-PAGE without fractionation (3 slices per sample). Three nanoLC-MS/MS analyses were performed for each slice. Quantifications were based on a label-free approach using two different quantitative methods: Spectral Counting (SC) and eXtracted Ion Chromatogram peptide pattern (XIC). We used Scaffold Q+ software (version 4.4, Proteome Software, Portland, USA) using the SC or the XIC quantitative module. All proteins with more than two peptides identified were considered for protein quantification. To eliminate quantitative ambiguity in protein groups, we ignored all the spectra matching any peptide shared between proteins. Quantifications performed with normalized data were therefore carried out on distinct proteins. T-test were performed to differentiate proteins that were significantly different between the different conditions. Statistically significant differences were considered for $p<0.05$ with fold change > 2.

2.8 Pre-scapular lymph node and surrounding adipose tissue fatty acid extraction

The fatty acids were extracted from the pre-scapular lymph node (LN), perinodal adipose tissue (PAT) and white adipose tissue (WAT) of 20 month old bulls in each of the four feed groups. In order to analyse the lipids, gas

chromatography was used which required two processing stages: fatty acid extraction from the tissues and methylation.

2.8.1 Fatty acid extraction and methylation

Fatty acid extraction from the lymph node and adipose tissue was carried out by the author. To perform fatty acid extraction 0.25 g of tissue from each sample were cut and homogenized in 2 ml of cold chloroform/methanol (2:1) in a homogenizer. The resulting solution was shaken at 120 revolutions per minute (rpm) for 5 mins, filtered and 3 ml more of cold chloroform/methanol (2:1) was added. After that 1.5 ml of distilled water was added to each sample and vortexed for 5 secs. Following that the samples were centrifuged at 2,400 rpm for 10 mins at room temperature and the upper phase removed and discarded. Then the remaining solution was vortexed and centrifuged again at 2,400 rpm for 5 mins to allow the solution which contained the fatty acids to stablise at the bottom of the tube. After that approximately 1 ml of the lipids were collected from the bottom of the tubes with a 1,000 µl pipette, placed in an Eppendorf tube and stored at -20°C until nitrogen gas evaporation was carried out.

In order to dry the samples, a nitrogen stream was used to remove the chloroform. The samples were placed in a thermostated aluminium block heated at 60°C with a needle that blows out nitrogen into the tubes for 25-30 mins depending on the amount of liquid.

Before starting the methylation of the samples, 2 reagents were prepared in advance: The termination reagent was made by mixing 0.2 g oxalic acid and 6ml of diethyl ether; and the methylation reagent made by mixing 0.9 ml 30%

sodium methoxide and 4.1 ml of methanol. 2 ml of hexane was added into each of the dried samples and vortexed for 30 secs. Next 40 µl of both the methyl acetate and the methylation reagents were added to the samples and vortex for 1 min. After that the samples were left to react for 10 mins. The following step was add 60 µl of the termination reagent and vortex for 30 secs. Then approximate 200 mg of granulated calcium chloride was added to the solution, vortex for 5 secs and left to stand for 1 hour. Samples were then centrifuged at 3,400 rpm at 15°C for 5 mins and the top layer was transferred to a vial used to carry out the GC analysis.

2.8.2 Gas chromatography analysis

The methylated samples were injected into a gas chromatograph (6890 Gas Chromatograph, Agilent, USA), using hydrogen as carrier gas, by a split injector – this part of the protocol was carried out by Ms Dongfang Li, a technician in School of Biosciences, University of Nottingham. The column used to separate the fatty acids was a Varian CP-Sil 88 capillary (Crawford Scientific Ltd, UK). The procedure of separation involved an increase of the temperature of the column, which is located inside an oven, until it reached 240°C. This stage must be done gradually, thus primarily the oven temperature goes from 59°C to 100°C increasing 8°C per minute, then from this temperature to 170°C increasing 6°C per minute and stopped there for 10 mins. Upon reaching 240°C the temperature was increased by 3°C per minute and left at that temperature for 10 mins. The temperature of the injector and detector were set at different temperature to the column at 255°C and 250°C respectively. The identification of the fatty acids was carried out by comparing the retention times of the fatty

acids present in the samples with a fatty acid methyl ester (FAME) standard. In this particular case C19:0 was used as the FAME standard.

The fatty acids found in the samples and analysed included: saturated fatty acids (C6:0, C8:0, C10:0, C11:0, C12:0, C13:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C21:0, C22:0, C23:0, C24:0); monounsaturated fatty acids (C14:1, C15:1, C16:1, C17:1, C20:1, C24:1, C20:2, C22:2) and polyunsaturated fatty acids (C18:3n3, C20:3n3, C20:5n3, C22:6n3, C22:5, C18:2n6t, C18:2n6c, C18:3n6, C20:3n6, C20:4n6, C18:1n9t, C18:1n9c, C22:1n9, C18:1n11t).

For analysis of the samples, the raw results were transformed in percentages and to observe the differences between the different groups a heat map was created using the web-application arraymining.net. This analysis was possible thanks to the help of Dr Alison Mostyn, who taught the dissertation author how to prepared the data and analyse them using these tools. However the analysis was carried out by the author.

2.9 Fetal thymus RNA extraction

2.9.1 RNA extraction

The RNA extraction of the thymus was carried out by the author. To extract RNA, an RNeasy® mini kit (QIAGEN, Germany) was used and the manufacturer's protocol was followed. Briefly, tissue was homogenised using TriZol (lot 11846302, Life Technology, USA). TriZol (1 mL) was added into a GentleMacs M tube (Miltenyi Biotec, Germany) together with the tissue and a GentleMACS dissociator (Miltenyi Biotec GmbH, Germany) at 1000 rpm for 1 min was used to macerate the sample. 600 µl of this solution was pipetted and left at room

temperature for 5mins in a 1.5 ml Eppendorf tubes. After that 200 µl of chloroform was added to the homogenate and vigorously shaken for 15 secs, incubated for 2-3 mins at room temperature and centrifuged at 12,000 xg for 15 mins at 4ºC. The upper, aqueous phase of the solution was transferred to a new 1.5 ml tube and mixed with 600 µl 70% EtoH. After vortexing for 3 secs, the RNA containing solution was transferred to an RNeasy Mini spin column, placed in a 2 ml collection tube and centrifuged for 15 secs at 8,000 xg at room temperature. In order to digest any genomic DNA present in the sample, the next steps were included: 350 µl of buffer RW1 was added to the RNeasy spin column and centrifuged for 15 secs at 8,000 xg. 80 µl DNase (10 µl of DNase l stock and 70 µl of Buffer RDD) was added and incubated at room temperature for 15 mins. 350 µl of Buffer RW1 was applied to the RNeasy spin column and centrifuge at 8,000 xg for 15 secs. Following DNA digestion, 500 µl of buffer RPE was poured into the RNeasy spin column and centrifuged for 15 secs at 8,000 xg. Then another 500 µl of Buffer RPE was added to the RNeasy spin column, but this time the samples were centrifuged for 2 mins at 8,000 xg. After that the collection tubes were replaced, and the samples centrifuged again at full speed for 1 min. To elute the RNA from the column, the column was placed in the final collection tube and 33 µl of RNase-free water was added directly to the membrane and samples centrifuged for 1 min at 8,000 xg at room temperature. The elute liquid was recovered from the last step and a second pass through the column was performed to obtain a higher concentration of RNA.

Once the RNA was extracted, the concentration of RNA (ng/µl) was quantified by absorbance with NanoDrop 8000 spectrophotometer (Thermo scientific, Germany) as well as its purity assessed. The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of RNA. A ratio ~2 is considered as pure in RNA (Thermo scientific, nanodrop instructions), so only the samples that reached the appropriate ratio were sent to be analyzed using an Agilent BioAnalyzer. All the samples were kept at -80ºC for later analysis.

2.9.2 Bioanalyses and Sequence

The samples were sent to the School of Biosciences, University of Nottingham to be analysed with a Bioanalyzer 2100 (Agilent technologies, USA) which provided the concentration and integrity of each RNA sample. In order to know the integrity of the RNA, the RNA Integrity Number (RIN) algorithm, which is the parameter supply by the Bioanalizer must be ≤7, as 1 represents completely degraded and 10 fully intact. After selecting the samples with a low percentage of RNA degradation, diluting to a concentration of 100 ng/µl and the ones that were of interest in order to compare between the different diets and genders (MLL, MHL, MHH and FHH), the RNA was sent for RNA next generation sequencing to Edinburgh Genomics, The Roslin Institute and R(D)SVS, University of Edinburgh or the Barts and the London Genomics Center, Queen Mary's, University of London.

2.9.3 cDNA Library preparation

cDNA preparation and transcriptome sequencing was carried out under the direction of Dr Pablo Fuentes-Utrilla (Edinburgh Genomics, Roslin Institute,

University of Edinburgh) and Dr. Charles Mein, (Barts and the London Genomics Center, Queen Mary's University of London). To quantify genomewide RNA transcription, next generation sequencing was performed using either an Illumina HiSeq 2500 platform (Edinburgh Genomics, Roslin Institute, University of Edinburgh) or Illumina NextSeq platforms (Barts and the London Genomics Center, Queen Mary's University of London). Using TruSeq RNA Sample Preparation Kit V2 (Illumina, San Diego, USA), total RNA (1 µg) was prepared for the cDNA library. Purification of the total RNA, where only the mRNA containing the Poly (A) tail remain, was carried out and posterior to this the mRNA was fragmented. A first single strand of cDNA was synthesised from the fragmented mRNA, subsequently the mRNA was removed from this single strand of cDNA to allow the creation of a second strand of cDNA and so, a double strand of cDNA. After fragmentation, overhangs at the 3' and 5' ends were produced and a repair process was needed. This repair process removed the 3' overhangs and filled in the 5' overhangs using exonuclease activity and polymerase activity, respectively. Then a single 'A' nucleotide was added to the 3' ends to prevent them from ligating to one another during the adapter ligation reaction. This step was needed to keep a low rate of chimera formation. The ligation reaction ligates multiple indexing adapters to the ends of the double stranded cDNA, preparing them for hybridization onto a flow cell. 89 DNA fragments were detected to have adapter molecules at both ends, therefore the enrichment of these fragments was carried out using PCR (10 cycles). Quantification of the DNA libraries with qPCR was preformed using Kapa Illumina library quantification kit (Illumina, San Diego, USA). Finally, to prepare DNA templates

for cluster generation, indexed DNA were normalized to 10 nM in the diluted cluster template (DCT) and then pooled in equal volumes in a pooled DCT plate (PDP).

2.9.4 Quality control and alignment of transcript and differential gene expression

Sequence quality control and alignments were performed by Dr. Nigel Mongan, University of Nottingham. Adapter sequences added to the mRNA during the library preparation and the low quality reads and bases were removed using the Trimgalore wrapper for FastQC and Cutadapt (Martin, 2011) using a phred score of >30 as minimum quality to be retained. The quality of the sequence and the bases as well as the content of the sequences and occurrences of duplication was provided for each sample. Bowtie2, within Tophat2 (Kim et al., 2013) was used to align the trimmed bases to the bovine reference genome UMB3.1 using the Ensembl gene annotation set to generate BAM alignment files.

2.9.5 Differential gene expression analysis

Differential gene expression calculated by Dr Nigel Mongan, from the resultant BAM alignment files were calculated using Cufflinks and Cuffdiff (Trapnell et al., 2010). The level of significance was marked as a false discovery rate (FDR) of less than 0.05, a fold change (FC) of 1.7 and more than 30 FPKM.

2.9.6 Gene ontology and Pathway analysis

Gene ontology and Pathway analysis was carried out by the author. To obtained a more detailed information of the differentially expressed genes, the Ensembl

gene IDs were converted to human orthologous ensemble IDs using Webgestalt software (Zhang et al., 2005). Gene ontology analysis was performed using the human orthologous with ConsensusPathDB (Kamburov et al., 2009) obtaining a report with the GO term categories biological processes (BP), cellular component (CC) and molecular function (MF) of the uploaded genes. To obtain the pathways in which the differentially expressed genes were involved Cytoscape (Genemania) was used, using human database (Warde-Farley et al., 2010, Shannon et al., 2003). In addition to the pathways, a list of transcription factors and suggested additional genes were also provided.

2.9.7 cDNA synthesis and validation by quantitative reverse transcription PCR (qRTPCR)

cDNA synthesis and validation of the RNA sequencing using qRTPCR was performed by the author. The qScript™ cDNA supermix (Quanta, biosciences. Inc, USA) reverse transcriptase enzyme was used to synthesize cDNA from the mRNA extracted from the fetal thymus. Firstly, each sample must have a similar concentration of mRNA (500 ng/µl). To achieve this, the thymus mRNA was diluted in RNAse-free water in the proportion needed for each sample. Then 8 µl of each sample was place in a 1.5 ml Eppendorf and 2 µl of the reverse transcriptase enzyme was added. After that, the Eppendorfs were placed in a water bath at 42°C for 60 mins. The cDNA obtained with this technique was then diluted with 190 µl of RNAse-free water and stored at -20°C. After that, standard PCR was carried out to confirm the success of the cDNA synthesis.

Quantitative Reverse Transcription PCR (qRTPCR) using TaqMan® Gene Expression Assays (Applied Biosystem, Carlsbad, CA, USA) with gene specific

hydrolysis probe sets (Table 2.4) was used to quantify the relative amount of transcript in the samples. Primarily, a master mix was prepared blending the Taq polymerase, probe and RNAse-free water (See Table 2.5 for proportions). The amount of each reagent was multiplied by the number of the triplicated samples that are going to be tested in the reaction. 14 µl of the master mix was then placed in each well and 1 µl of the cDNA sample was added.

Table 2.4 Genes selected for validation of the Next generation sequencing results from the fetal thymus

Gene symbol	***Assay ID***
ACTB	Bt03279174_g1
ARID4A	HS00243140_m1
ARID4B	Bt04289721_m1
ROCK1	HS01127699_m1

Table 2.5 Proportion of reagents used for RT-qPCR

Reagent	***Quantity (µl)***
Roche LightCycler 480 Probes Master mix	7.5
Probe set	0.65
RNase-free water	5.85
cDNA template	1

2.10 Statistical analysis

All the statistical analysis was carried out by the author. Data were checked for normality and transformed before analysis if required. For the histological data

obtained from the fetal testis and adult testis and epididymis the statistical analysis was done using two-way ANOVA (Analysis of Variance) (IBM SPSS statistics v24, USA) to determine the effects of maternal diet during PERI and POST periods and their interaction term. Significant interactions were further explored with Tukey post hoc test as required. Thus, for clarity, the results have been presented as the main effects of PERI and POST maternal diet. When it was not possible to normalise the data, Mann Whitney U test was used. For the statistical analysis of the thyroid tissue and pre – scapular lymph node fatty acids multifactorial ANOVA (IBM SPSS statistic 24, USA) was used to determine the effects of maternal nutrition during peri – conception and first trimester, progeny sex and their interaction terms. One tailed T-test was selected for the statistical analysis of the thymus RT-qPCR results (Graphpad prism 7 software, USA). Statistical significance was reported at $P<0.05$.

Chapter 3 The effect of maternal dietary protein level on the development of the bovine testis at 98 dpc and 20 months of age.

3.1 Introduction

Reproduction efficiency has decreased in the last decades in beef cattle and this poses a great economic lost for the sector (Bellows et al., 2002). The Northern Australian range beef cattle industry depends on the pastures of the area, whose quality, especially in relation to protein, are conditioned by the rainy or dry season (Bortolussi et al., 2005). Because other studies have demonstrated that restriction in maternal dietary protein during pregnancy affects the reproduction and production traits in the progeny (Sullivan et al., 2009c, Sullivan et al., 2010a, Micke et al., 2010, Copping et al., 2018), this experiment focused on the impact of the protein restricted diets during the peri – conception (60 days prior to artificial insemination to 23 dpc) and/or during the first trimester of gestation (24 dpc to 98 dpc) on the testicular development of 98 dpc fetuses and 20 month old bulls.

3.1.1 Hypothesis

We hypothesised that lack of maternal dietary protein during the peri – conception and early gestation will influence the development of the testes and that these effects may last until adulthood, influencing the reproductive efficiency of the male progeny.

3.2 Methods

General methodology and ethical approvals used for this experiment are explained in Chapter 2. Particular methodology used in this experiment are explained below.

3.2.1 Image Analysis

3.2.1.1 Testicular cell counts

Samples were processed, sectioned and immunostained using Mis-C20 prior to systematic random photomicrographs being taken with a light microscope (Chapter 2 Sections 2.2.1, 2.4 and 2.4.1.1, respectively). Following this, cell counts were undertaken. In order to perform counts of the Sertoli, germ and interstitial Leydig cells, a single tissue section from each slide (several systematic slides per animal) was selected and 5 photomicrographs were systematically randomly taken (Mayhew, 2011) at a magnification that allowed identification using morphology and immunostaining (63X). In total 400 photomicrographs were taken from the fetal tissue to count the number of cells, n=4LL, 5LH, 7HL and 5HH. Manual identification and counting of all Sertoli, Germ and Interstitial cells (Leydig cells) was carried out. 80 slides were analysed and 81660 cells counted in total. The numbers of the different cells were quantified and calculated as percentages of the total cell count per area measured.

3.2.1.2 Fetal seminiferous tubules measurements

Samples were processed, sectioned and immunostained using Mis-C20 prior to systematic random samples being taken with a light microscope (Chapter 2

Sections 2.2.1, 2.4 and 2.4.1.1, respectively). After that seminiferous tubules were measured. In order to measure the seminiferous tubules, 5 photomicrographs were systematically randomly (Mayhew, 2011) taken per slide at a magnification of 20X from 3 slides per sample. In total 315 photomicrographs were taken of the fetal tissue to count the number of cells, n=4LL, 5LH, 7HL and 5HH. The areas of the seminiferous tubules in the fetal tissue were measured using an image analysis program (image-pro plus (version 6.3), Media Cybernetics, USA). Tubules were manually drawn around and following calibration of the imaging software, the average number of seminiferous tubules, and their areas were calculated per photomicrograph, an example is shown in Figure 2.3. In total 7308 seminiferous tubules were measured.

3.2.1.3 Fetal vasculature measurements

Samples were processed, sectioned and stained using PS staining prior to photograph being taken with a light microscope (Chapter 2 Sections 2.2.1, 2.4 and 2.4.3, respectively). In order to measure the blood vessels, two different magnifications were needed due to the difference in the sizes of the vessels positioned in the outer capsule and the ones in the testicular parenchyma, using 10X magnification for the former and 40X magnification for the latter. The whole section was photomicrographed at 5X magnification and the pictures were stitched together to show a complete image of the entire section, which was used to calculate the proportion of blood vessels within the section. In total 3 whole sections per animal were created from throughout the tissue. Then a photomicrograph of every blood vessel both in the capsule and in the

parenchyma in each whole section was taken. The number of samples per diet was 4LL, 5LH, 7HL and 5HH. In total, over 2000 pictures were taken for the purpose of measuring the blood vessels. To measure the blood vessels within the fetal testes, Image-pro plus, version 6.3 (Media Cybernetics, USA) was used and vessels manually drawn around. The parameters measured with this program were: average blood vessel area, average blood vessel count and percentage of tissue occupied by vessels. The areas of the blood vessels were calculated and given as a proportion of the analysed area of the testis overall picture. In total 1535 blood vessels were measured.

3.2.1.4 Adult testicular seminiferous tubules measurement

In order to measure adult seminiferous tubule parameters, sections were processed, sectioned and stained with PS and IHC with Mis-C20 antibody (see Chapter 2 Sections 2.2.2, 2.4, 2.4.3 and 2.4.1.1, respectively, for methodology). Five photomicrographs were taken (Mayhew, 2011) per slide at a magnification of 10X from 2 slides per sample. Systematic random sampling was used to take the photomicrographs. In total, 400 photomicrographs were analysed from the adult tissues, n= 8LL, 8LH, 14HL and 10HH. The areas of the seminiferous tubules in the adult tissue were measured using the same image analysis program (image-pro plus (version 6.3), Media Cybernetics, USA) as the one used for measuring fetal structures. Tubules were manually identified and drawn around and following calibration of the imaging software, the average area, number of seminiferous tubules and total area occupied within the picture were calculated per photomicrograph. In total 6120 adult seminiferous tubules were measured.

3.2.1.5 Adult testicular vasculature measurement

In order to visualize vasculature in the adult testis, sections were processed, sectioned and stained with PS and IHC using Mis C-20 antibody (see Chapter 2 Sections 2.2.2, 2.4, 2.4.3 and 2.4.1.1, respectively, for methods) were used in order to make vessels easily identifiable. At a microscopic magnification of 20X, 10 photomicrographs were taken from each section using systematic random sampling and stereological techniques (Mayhew, 2011). Two slides were selected per animal with 80 slides and 800 photomicrographs used in total for measuring the blood vessels in the adult tissue, n =8LL, 8LH, 14HL and 10HH. The areas of the blood vessels in the adult tissues were manually identified and measured using the same image analysis program (image-pro plus (version 6.3), Media Cybernetics, USA) as the fetal samples. In total 1904 adult blood vessels were measured manually. Following calibration of the imaging software, the average area and number of blood vessels were calculated per photomicrograph. In addition the total area of vasculature within each tissue section was calculated. Results were statistically analysed (Section 3.2.2).

3.2.2 Statistics analysis

Data were checked for normality and transformed before analysis if required. The statistical analysis was done using two-way ANOVA (IBM SPSS statistics 24) to determine the effects of maternal diet during PERI and POST periods and their interaction term. Significant interactions were explored with Tukey post hoc test as required. Thus, for clarity, the results have been presented as the

main effects of PERI and POST maternal diet. Statistical significance was reported at $P<0.05$

3.3 Results

3.3.1 Fetal testis analysis following dietary treatments

3.3.1.1 Sertoli, germ and interstitial cell proportions within the fetal testis

In order to compare whether the different diets had an effect on the cell population within the developing testis, the percentages of Sertoli, germ and interstitial cells were calculated based on the numbers obtained from the manual identification and counting. In this stage of testis development, these three types of cells are the only ones that exist within the testes, therefore the percentage of cells was calculated based on total cell number. A photomicrographs showing the different cells counted in the fetal testes is showed in Figure 3.1.

3.3.1.2 Sertoli cell count

Following two-way ANOVA statistical analysis of Sertoli cell counts totaling 29800, no significant differences were found in the percentage of Sertoli cells when peri - and post - conception periods were compared: LH+LL (LPeri) versus HH+HL (HPeri; low and high protein diet in the peri - conception period) and also LL+HL (LPost) versus HH+LH (HPost; low and high protein diet in the post - conception periods; Figures 3.2 B and C, respectively; $P>0.05$). Neither was there an observed interaction between the two periods.

3.3.1.3 Germ cell count

Following two-way ANOVA statistical analysis of 3940 germ cells, significant differences were found in the percentage of germ cells between the low and high protein diets within the peri – conception period (Figure 3.3 A, P=0.014). However, a significant interaction between the peri and post conception period diets was observed (P=0.009), which takes priority in the interpretation of the results, and a post hoc analysis was used to explore it. In the multiple comparison significant differences between the LL versus the HL group was observed (Figure 3.3 C, P=0.002). In addition, no significant differences were shown when low and high protein diets were compared in the post - conception period as main effects (Figure 3.3 B, P>0.05).

3.3.1.4 Interstitial cell count

Following two-way ANOVA statistical analysis of 47920 counted interstitial cells, no significant differences were found in the percentage of interstitial cells when peri - and post - conception periods were compared: LPeri versus HPeri (low and high protein diet in the peri - conception periods) and also LPost versus HPost (low and high protein diet in the post - conception periods) as main effects (Figure 3.4 A and B respectively; P>0.05). Nevertheless, a significant interaction was observed in the diets between the two different periods (Peri*Post; P=0.020). Following multiple comparison, a significant difference was shown between LL versus HL group (Figure 3.4 C, P=0.042).

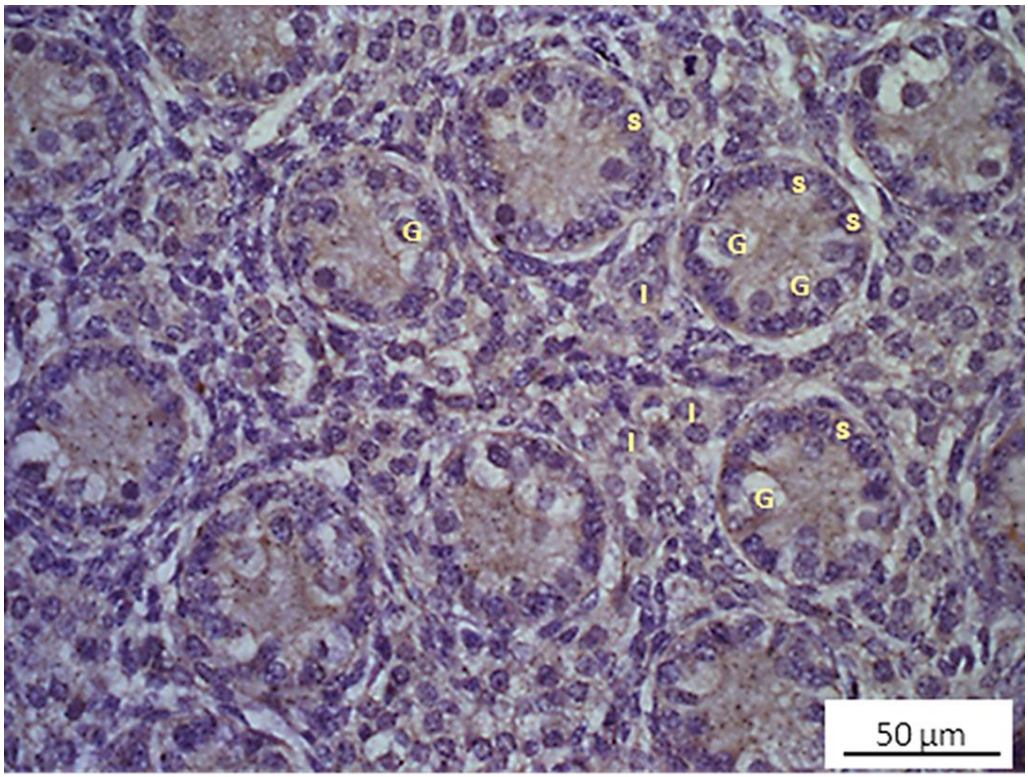


Figure 3.1 Cells present in the testicular lobules of 98 dpc bovine fetus (63X magnification). Photomicrograph was taken by the author using fetal testis samples used in the study. S= Sertoli cells; G= Germ cells; I= Interstitial cells.

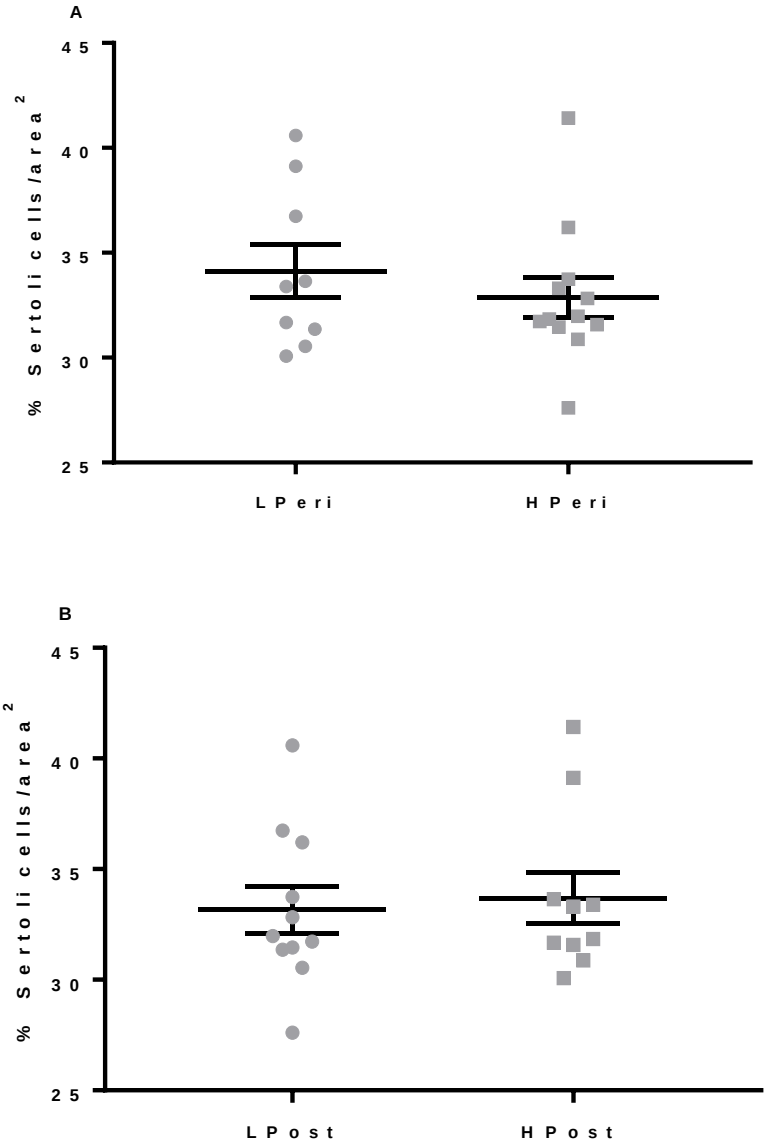


Figure 3.2 Percentage of Sertoli cells following dietary intervention in 98 dpc fetus A) In high and low protein diets in the peri - conception period B) In high and low protein diet in the post-conception period. The data were analysed with two-way ANOVA, no interaction was observed between peri - and post – conception groups. Bars indicate mean±SEM. P<0.05 were considered significant, no significant differences were found.

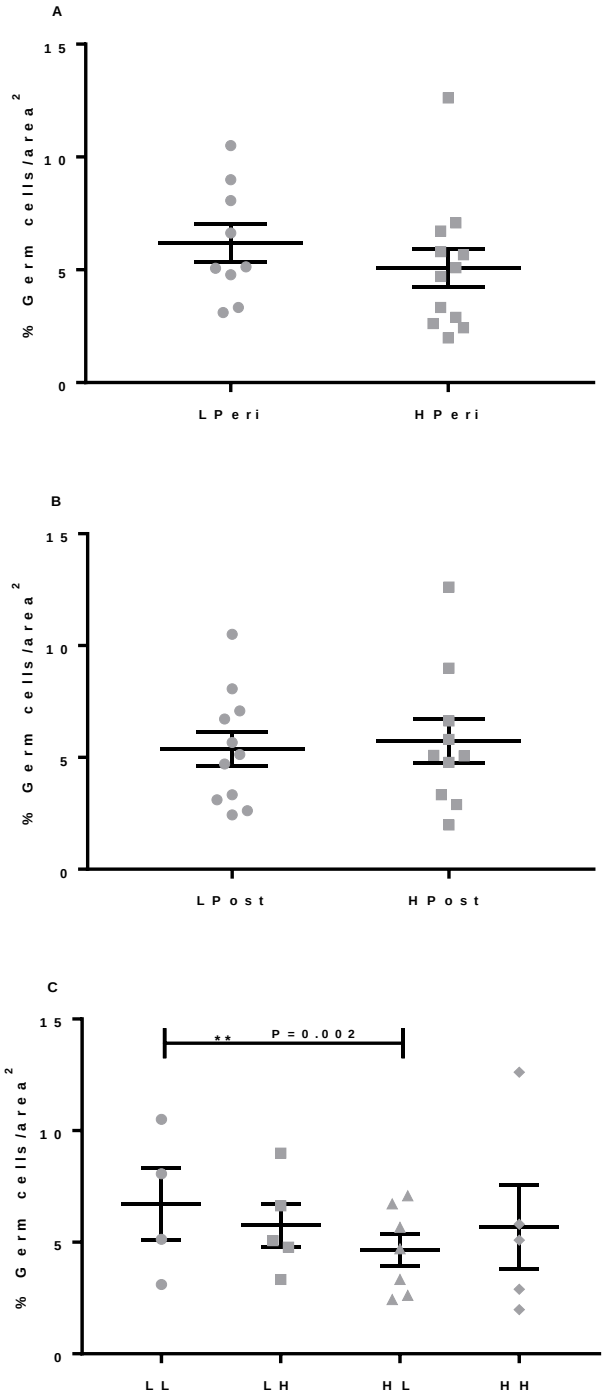


Figure 3.3 Percentage of germ cells following diet trial in 98 dpc fetus A) In high and low protein diet in the peri - conception period B) In high and low protein diet in the post - conception period C) In the four different diets. The data were analysed with two-way ANOVA and a post hoc test was run after a significant interaction between peri – and post – conception groups existed (P=0.009). Bars indicate mean±SEM. P<0.05 were considered significant. Significant differences were found in the fetus fed low protein diet in the peri – and post – conception periods (LL) compared with the fetus fed high protein diets in the peri – conception period and low protein diet in the post – conception period (HL; Figure 3.3 C; P=0.002).

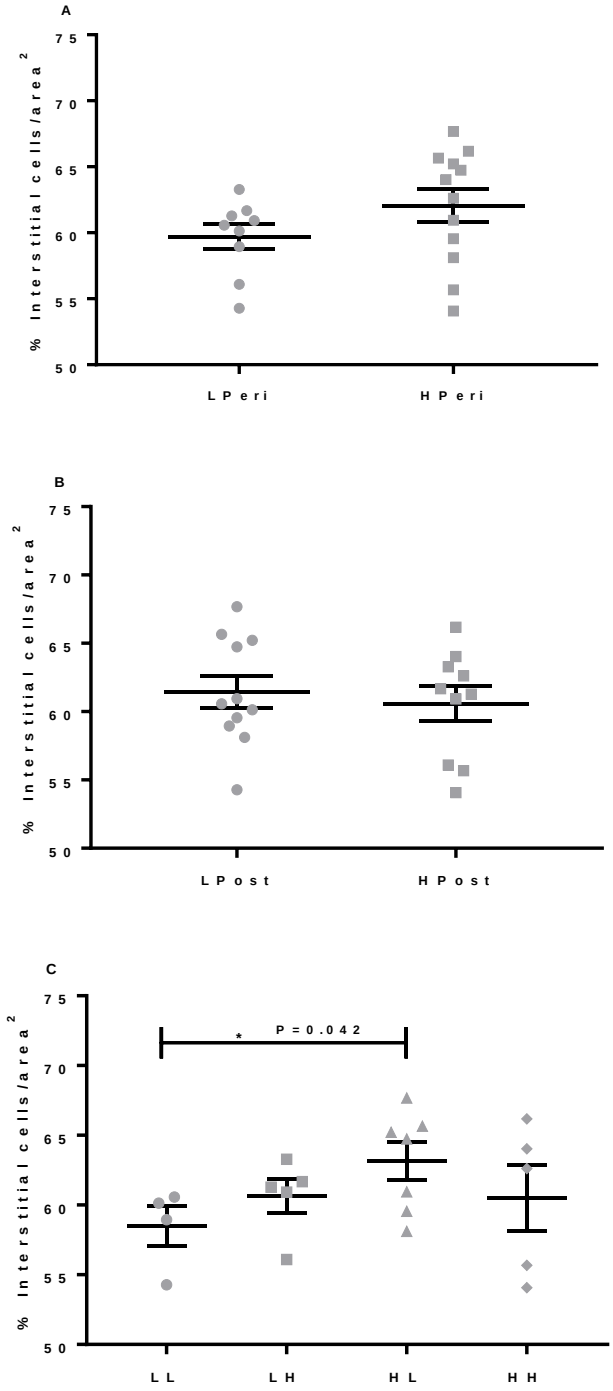


Figure 3.4 Percentage of interstitial cells following the experimental diets in 98 dpc fetus A) In high and low protein diet in the peri-conception period B) In high and low protein diet in the post-conception period C) In the four different diets. The data were analysed with two-way ANOVA and a post hoc test was run after a significant interaction between peri – and post – conception periods existed (P=0.020). Bars indicate mean±SEM. P<0.05 were considered significant. Significant differences were observed in the fetus fed low protein diet in the peri – and post – conception periods (LL) compared with the fetus fed high protein diets in the peri – conception period and low protein diet in the post – conception period (HL; Figure 3.4 C; P=0.042).

3.3.2 Seminiferous tubule measurements within the fetal testis

The data pertaining to the seminiferous tubules in the testis of 98 dpc fetus was obtained by manually drawing the tubules on photomicrographs using Image-pro plus software. 7308 seminiferous tubules were measured to acquire these results. A photomicrographs showing the measurement of the seminiferous tubules it is showed in Figure 3.5.

The percentage tissue coverage, average size and count of seminiferous tubules per photomicrograph for each testis were calculated. Significant differences were observed when the percentage of the seminiferous tubules from low and high protein diets were compared in the peri – conception group (Figure 3.6 A, P=0.003) as main effects. However a significant interaction in the low and high protein diets between the Peri - and Post – conception periods was observed (P=0.002). After the multiple comparison, significant differences were shown between the HL group and the other three different diets (LL, P=0.001; LH, P=0.046; and HH, P=0.042; Figure 3.6 C). The analysis of the percentage of the seminiferous tubules in the post - conception period showed no significant differences (Figure 3.6 B; P>0.05).

Number of seminiferous tubules showed significant differences in both Peri – and Post – conception periods when low and high protein diets were compared (P=0.040 and P=0.041, respectively; Figure 3.7 A and B), a significant interaction was also observed in the two periods (Peri*Post), which prevailed over the simple main effects. To explore this interaction a post hoc test was performed

and it showed significant differences between the LL group and the other 3 group treatments (LH, P=0.002; HL, P=0.001; and HH, P=0.037; Figure 3.7 C).

The average of the areas of the seminiferous tubules coverage were calculated for each testis. No significant differences were observed when the averages were compared between the two diets in the peri - conception period (Figure 3.8 A; P>0.05). Nevertheless, significantly smaller seminiferous tubules were present in the fetus fed a low protein diet during the post – conception period compared with the fetus fed a high protein diet (Figure 3.8 B; P=0.001).

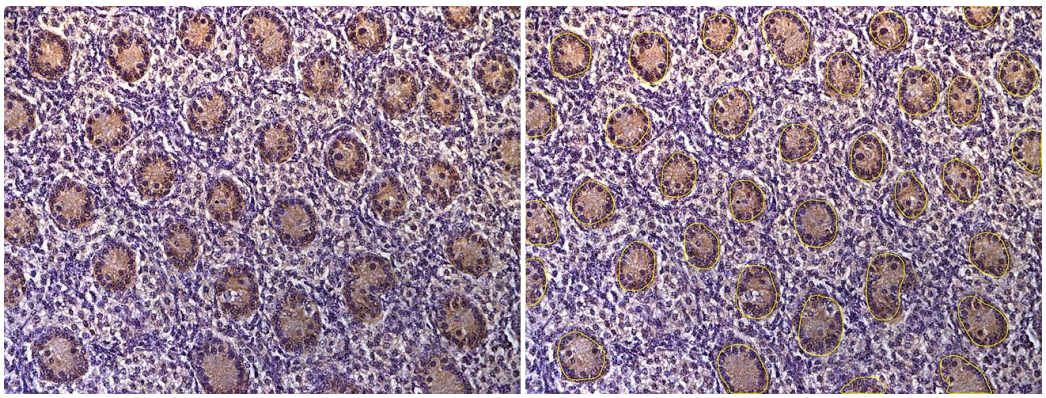


Figure 3.5 Photomicrographs of 98 dpc fetal testes to show how the measurement of the fetal seminiferous tubules was performed using Image-Pro software.

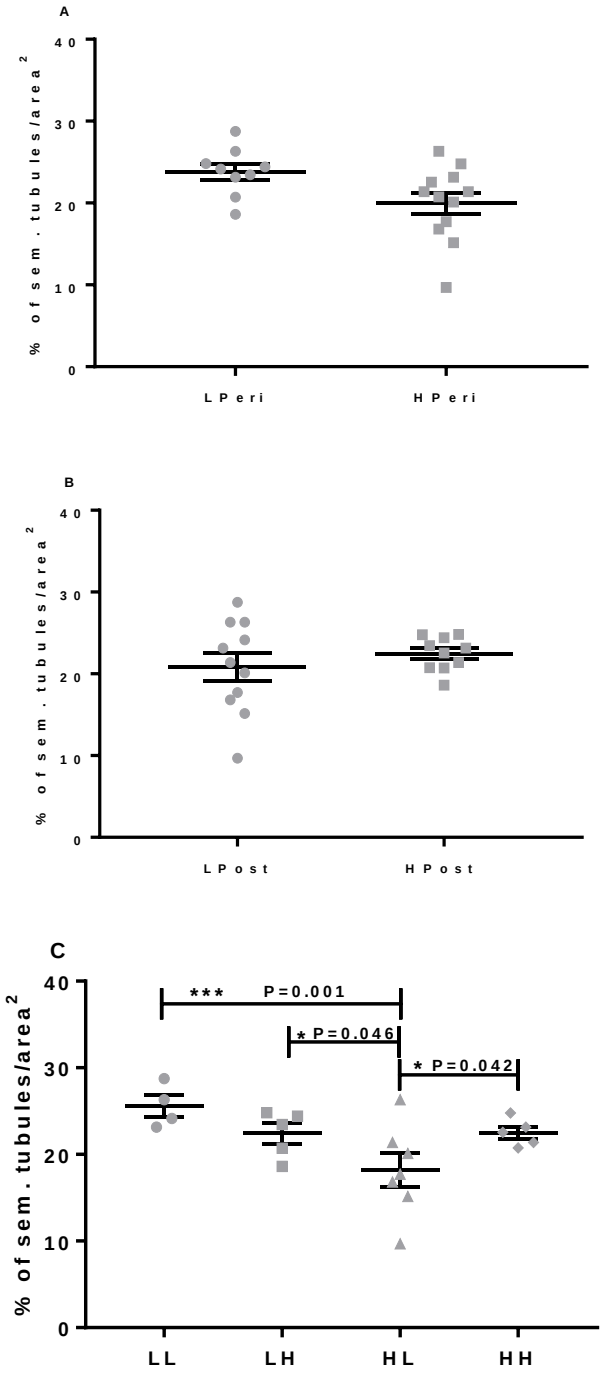


Figure 3.6 Percentage of seminiferous tubules in 98 dpc fetus A) In the four different diets B) When high and low protein diets were compared in the peri-conception period C) In high and low protein diets in the post-conception period. The data were analysed with two-way ANOVA and a post hoc test was run after a significant interaction between peri – and post – conception periods existed (P=0.002). Bars indicate mean±SEM. P<0.05 were considered significant. Significant differences were observed in the fetus fed high protein diet in the peri – conception period and low protein diet in the post conception period (HL) compared with the fetus fed the other three possible diets: low protein diet in both the peri – and post – conception periods (LL, P=0.000) low protein diet in the peri – conception period and high protein diet in the post – conception period (LH; P=0.046) and high protein diet in both peri – and post – conception periods (HH; P=0.042; Figure 3.6 C).

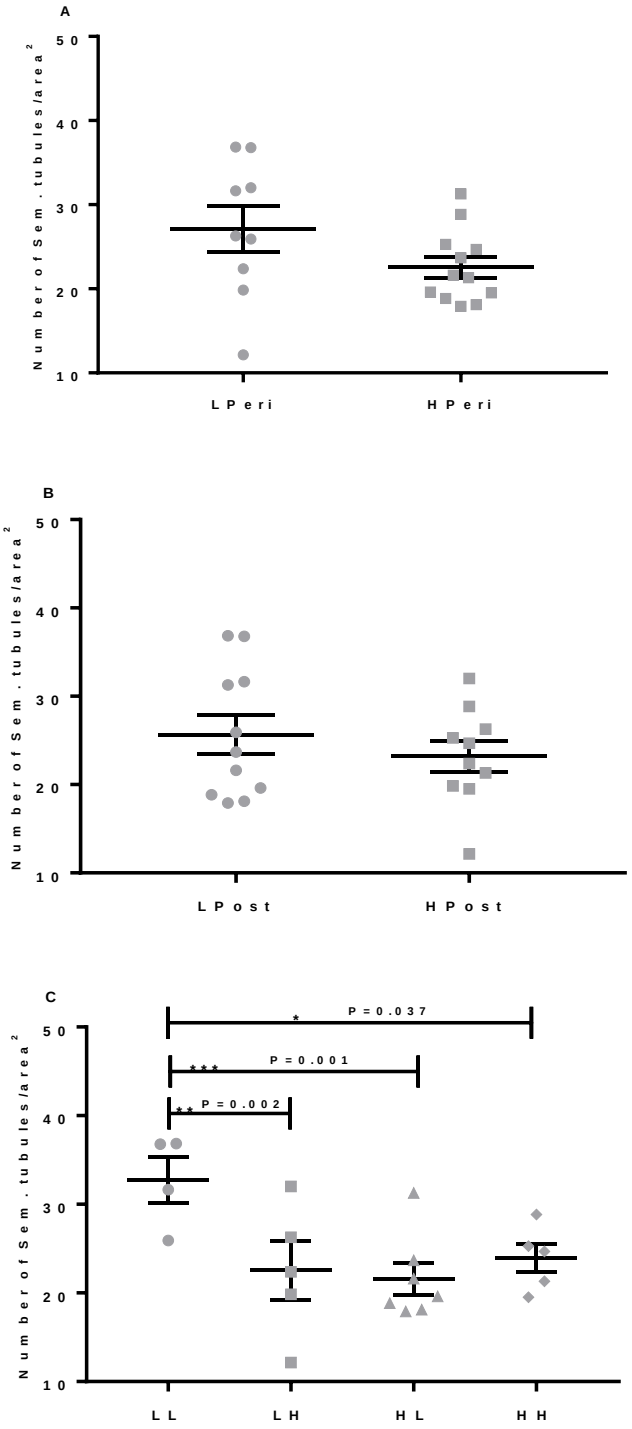


Figure 3.7 Number of seminiferous tubules in the different diets in 98 dpc fetus A) In the peri - conception period B) In the post - conception period C) In the four different diets. The data were analysed with two-way ANOVA and a post hoc test was run after a significant interaction between peri – and post – conception periods existed (P=0.001). Bars indicate mean±SEM. P<0.05 were considered significant. Significant differences were observed in the fetus fed low protein diet in both the peri – and post – conception periods (LL) compared with the fetus fed the other three possible diets: low protein diet in the peri – conception period and high protein diet in the post – conception period (LH; P=0.002), high protein diet in the peri – conception period and low protein diet in the post conception period (HL, P=0.001) and high protein diet in both peri – and post – conception periods (HH; P=0.037; Figure 3.7 C).

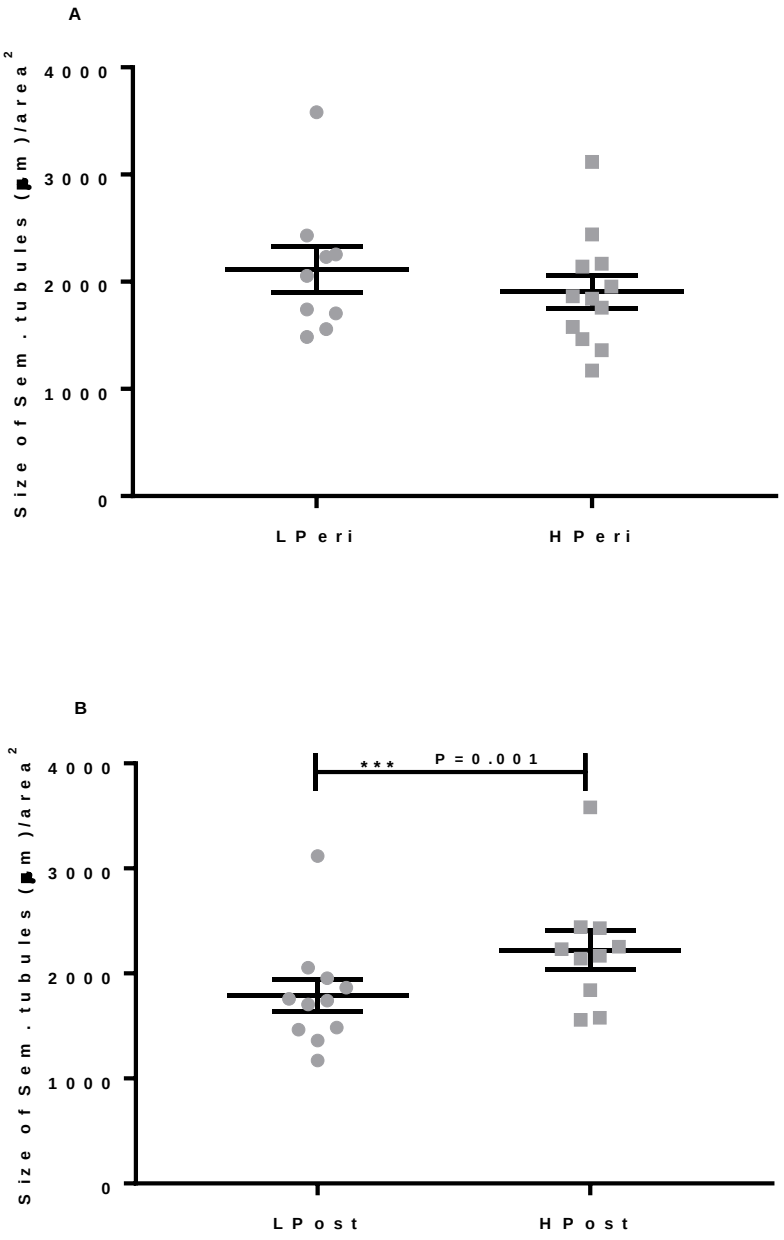


Figure 3.8 Average area of the seminiferous tubules in the diet interventions in 98 dpc fetus A) In the peri - conception period B) In the post-conception period. The data were analysed with two-way ANOVA. No interaction between the peri – and post – conception periods were observed. Bars indicate mean±SEM. P<0.05 were considered significant. Significant differences were present in the fetus from mothers fed a low protein diet during the post – conception period (LPost) compared with fetus from mothers fed a high protein diet during the post – conception period (HPost; Figure 3.8 B; P=0.001).

3.3.3 Testicular vasculature in 98 dpc fetus

Multiple data were collected from the blood vessels: average blood vessel area, average blood vessel count/number and percentage of tissue occupied by blood vessels. The data were obtained from the combination of the separated measurements of the parenchymal and the capsular blood vessels. These data were compared between the high and low protein diets in the peri - and post - conception periods and its interaction.

The percentage of blood vessels within the testis did not show significant differences between low and high protein diet in the peri - and post - conception periods, neither was a significant interaction observed (Figure 3.9 A and B; $P>0.05$). In contrast, significant differences were observed in the number of blood vessels when compared high and low protein diets in both the peri - and post - conception period ($P=0.028$ and $P=0.002$, respectively; Figure 3.10 A and B). While the low protein diet showed a significantly higher number of blood vessels in the peri – conception period (Figure 3.10 A; $P=0.028$), in the post – conception period it was the high protein diet which showed the bigger amount of blood vessels (Figure 3.10 B; $P=0.002$).

In addition, changes were not significantly different in the average blood vessel area when comparing the high and low protein diets in the post - conception period (Figure 3.11 B; $P>0.05$). However, in the peri - conception period, the average of the blood vessel areas were significantly smaller in the low protein diet groups when compared with the high protein diet group (Figure 3.11 A; $P=0.008$).

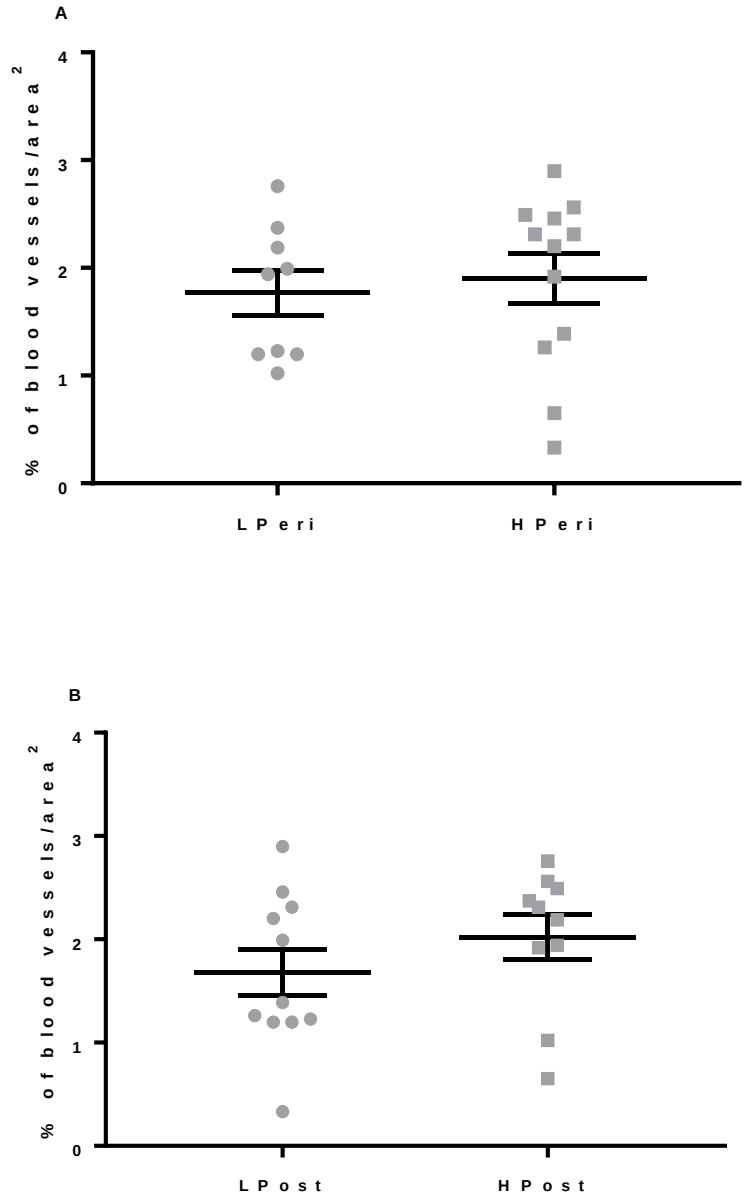


Figure 3.9 Percentage of blood vessels in 98 dpc fetus A) In the peri-conception period B) In the post-conception period. The data were analysed with two-way ANOVA. No significant interaction between the peri – and post – conception periods was observed. Bars indicate mean±SEM. P<0.05 were considered significant, no significant differences were shown.

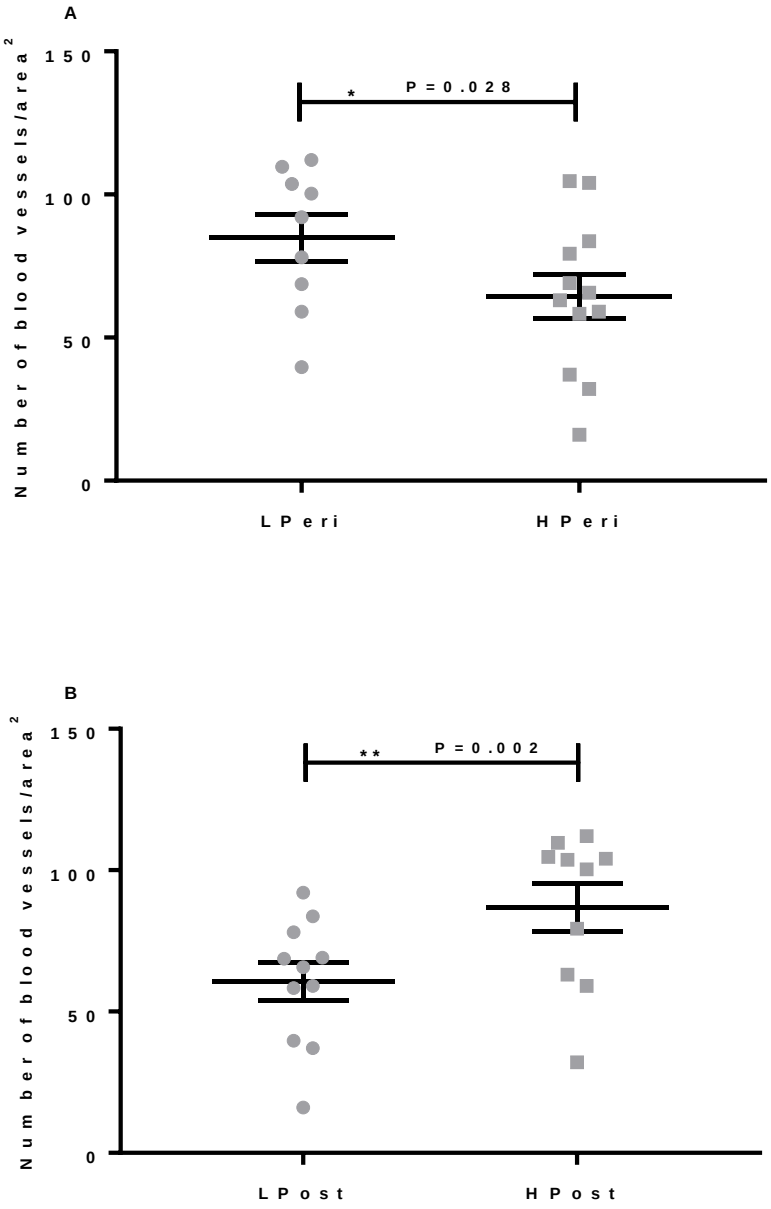


Figure 3.10 Blood vessels quantification in 98 dpc fetus. A) In the peri - conception period B) In the post - conception period. The data were analysed with two-way ANOVA. No interaction between the peri – and post – conception periods were observed. Bars indicate mean±SEM. P<0.05 were considered significant. Significant differences were present in the fetus fed low protein diet *in utero* during the peri – conception period (LPeri) compared with the fetus fed high protein diet (HPeri; P=0.028; Figure 3.10 A). Moreover, significant differences were present in the fetus from mothers fed a low protein diet during the post – conception period (LPost) compared with fetus from mothers fed a high protein diet during the post – conception period (Figure 3.10 B; P=0.002).

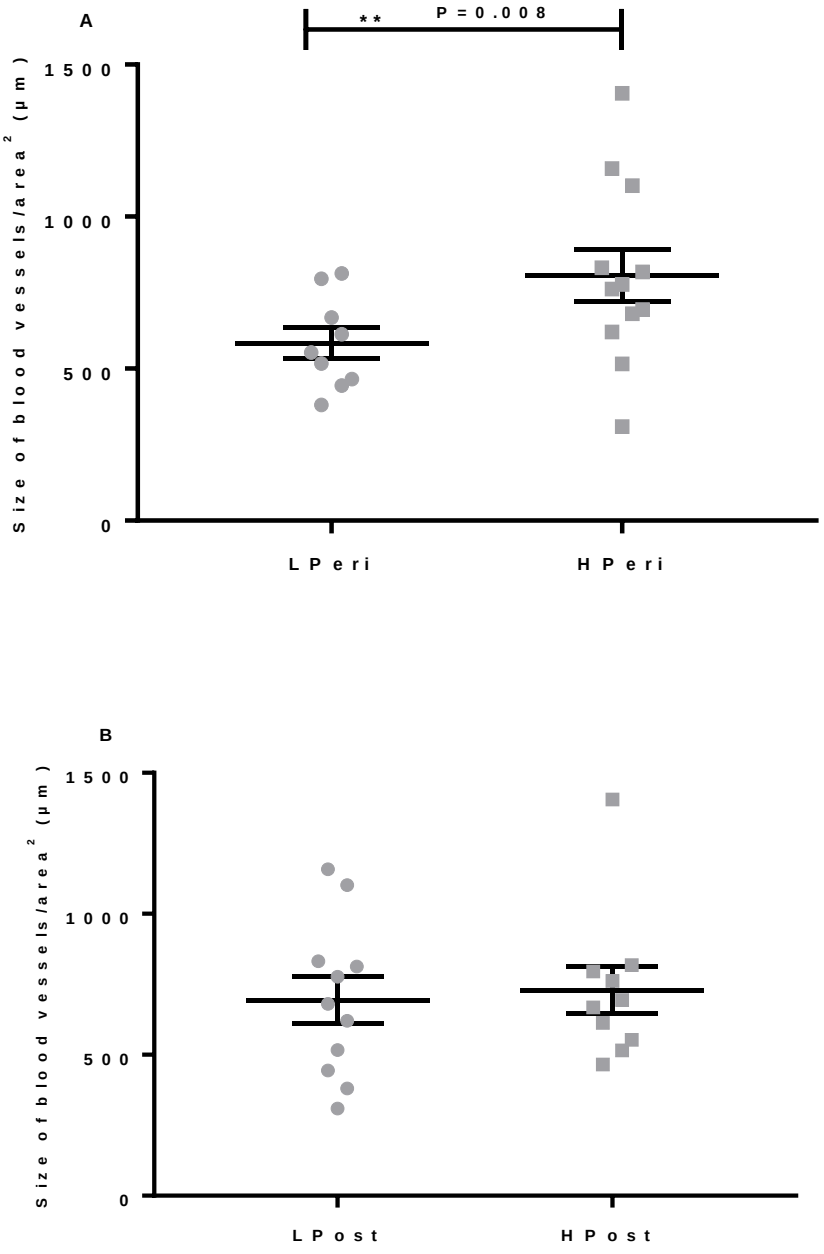


Figure 3.11 Blood vessels area in 98 dpc fetus A) In the peri-conception period B) In the post-conception period. The data were analysed with two-way ANOVA. No interaction between the peri – and post – conception periods were observed. Bars indicate mean±SEM. P<0.05 were considered significant. Significant differences were present in the fetus fed low protein diet *in utero* during the peri – conception period (LPeri) compared with the fetus fed high protein diet (HPeri; P=0.008; Figure 3.11 A).

3.3.3.1 Parenchymal blood vessels

Due to the obvious observable differences in size and amount of blood vessels between the capsule and parenchyma in the testis of 98 dpc fetus, percentage, area and number of capsular and parenchymal blood vessels were calculated separately and analysed. A photomicrographs showing how the parenchymal and the capsular blood vessels were measured using the Image-Pro software are showed in Figure 3.12 and 3.16, respectively.

No significant differences were obtained when comparing the percentage of parenchymal blood vessels between the low and high protein diet in the peri – and post - conception periods (Figure 3.13 A and B; $P>0.05$). In addition, changes in the number of blood vessels were also not significant when compared low and high protein diets in the peri - and post - conception periods (Figure 3.14 A and B; $P>0.05$). No interaction between the two conception periods was observed either in the percentage nor the number of parenchymal blood vessels.

Nevertheless, low protein diet samples showed significantly smaller blood vessels than the high protein diet samples in the peri - conception period (Figure 3.15 A; $P=0.002$). However no significant differences were found when comparing the two diets in the post - conception period neither had an interaction between the two conception periods (Figure 3.13 A and C; $P>0.05$).

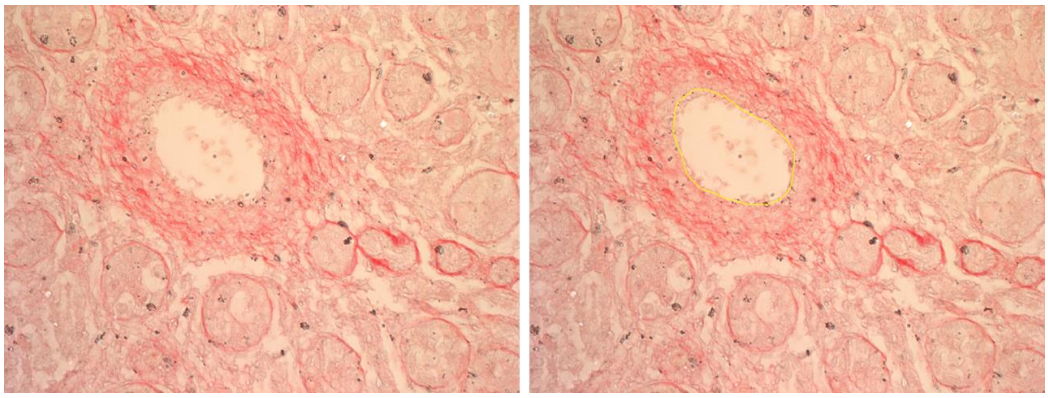


Figure 3.12 Photomicrographs of 98 dpc fetal testes to show how the measurement of the fetal parenchymal blood vessel was performed using Image-Pro software.

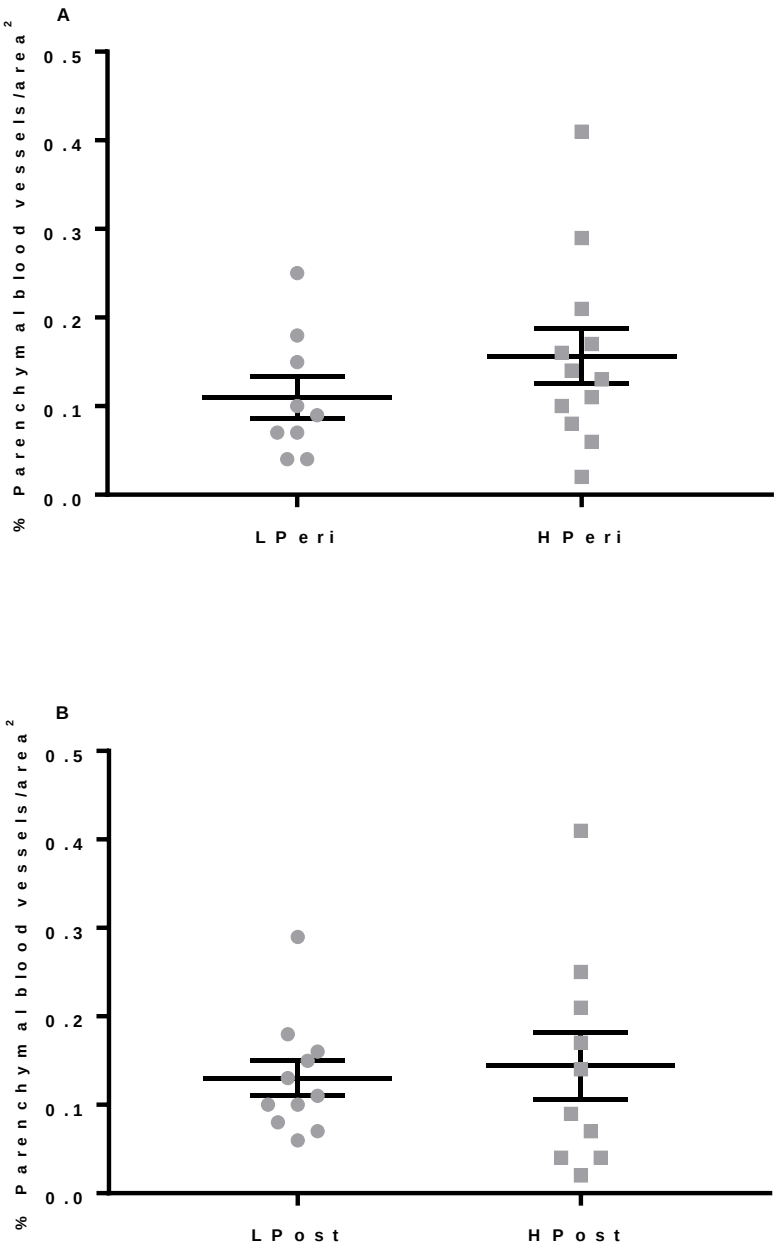


Figure 3.13 Percentage of parenchymal blood vessels in 98 dpc fetus A) In the peri-conception period B) In the post-conception period. The data were analysed with two-way ANOVA. No significant interaction between the peri – and post – conception periods was observed. Bars indicate mean±SEM. P<0.05 were considered significant, no significant differences were shown.

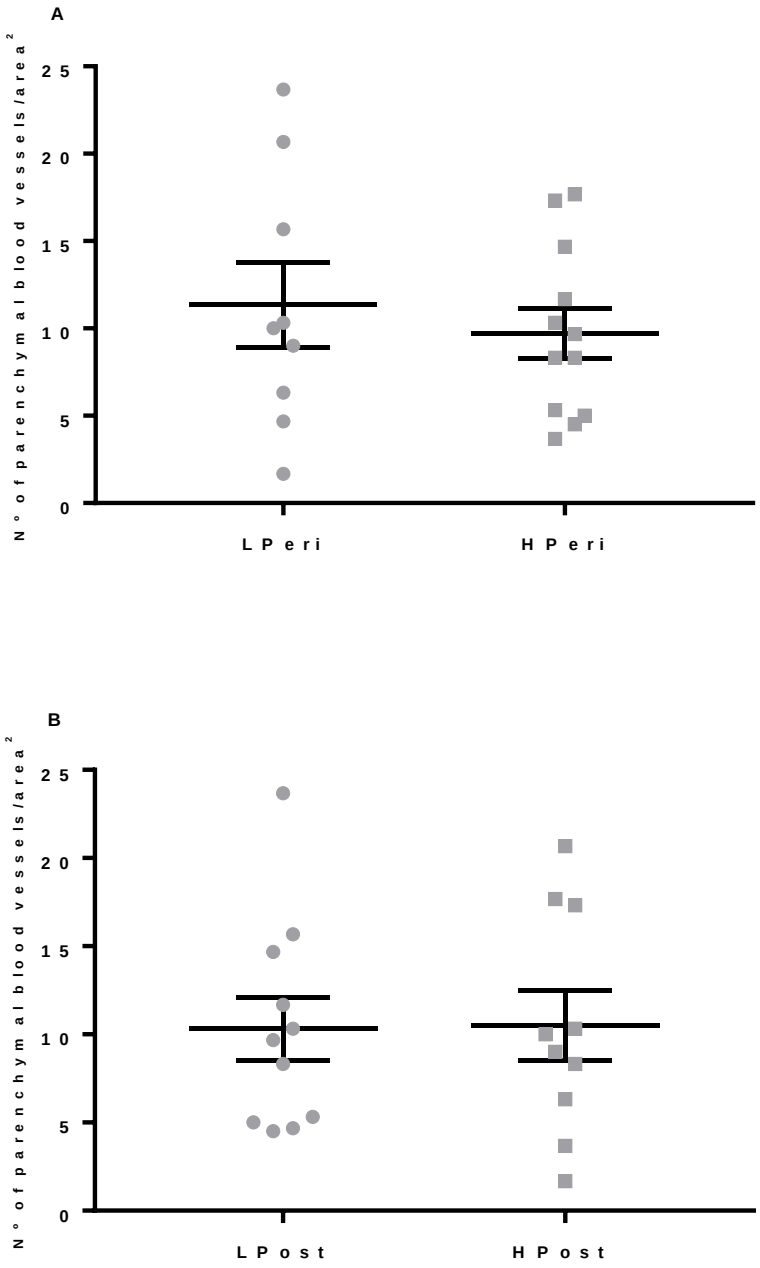


Figure 3.14 Parenchymal blood vessels quantification in 98 dpc fetus A) In the peri-conception period B) In the post-conception period. The data were analysed with two-way ANOVA. No significant interaction between the peri – and post – conception periods was observed. Bars indicate mean±SEM. P<0.05 were considered significant, no significant differences were shown.

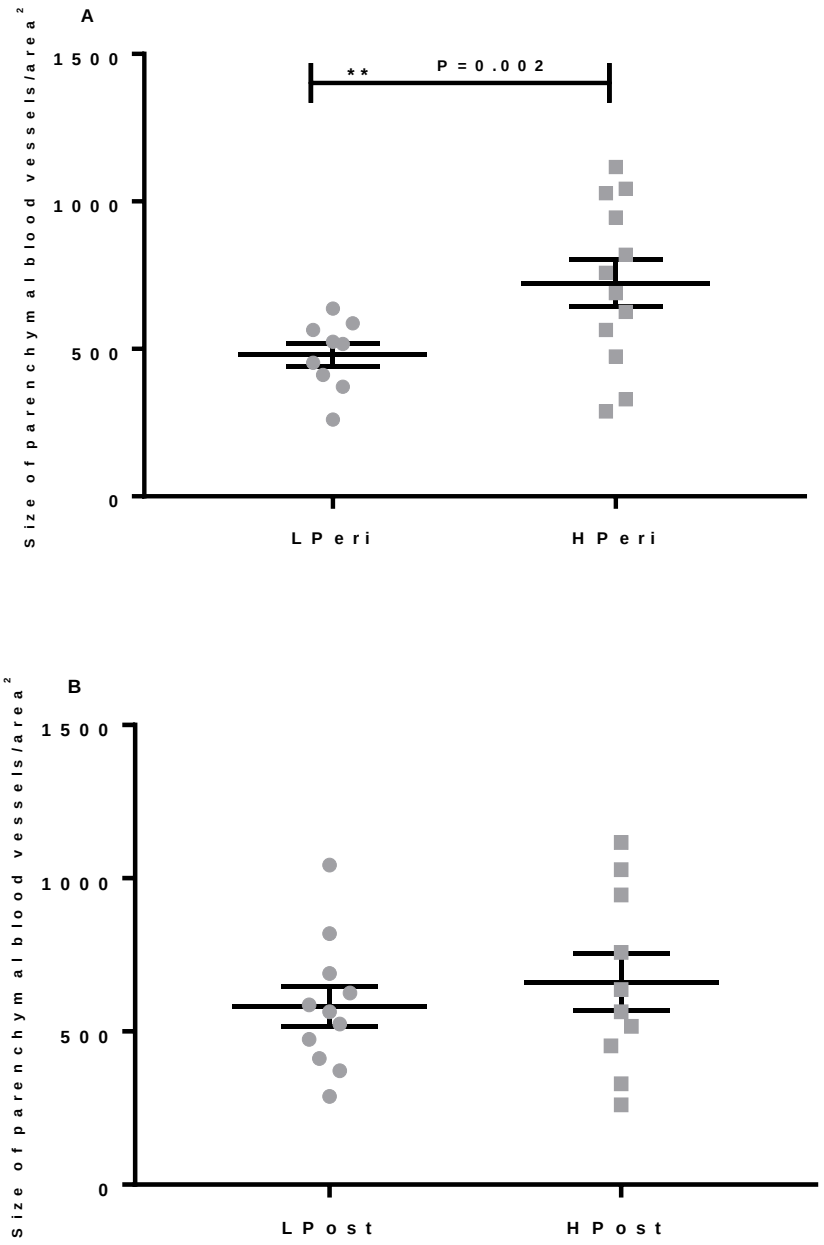


Figure 3.15 Parenchymal blood vessels area in 98 dpc fetus A) In the peri - conception period B) In the post-conception period. The data were analysed with two-way ANOVA. No interaction between the peri – and post – conception periods was observed. Bars indicate mean±SEM. P<0.05 were considered significant. Significant differences were present in the fetus from mothers fed a low protein diet during the peri – conception period (LPeri) compared with fetus from mothers fed a high protein diet during the peri – conception period (HPeri; P=0.002; Figure 3.15 A).

3.3.3.2 Capsular blood vessels

Percentage, size and number of capsular blood vessels were calculated using the data obtained after carrying out the measurements with the image-pro software. No significant differences were found in the percentage of capsular blood vessels when comparing low and high protein diets in the peri - and post - conception periods (Figure 3.17 A and B; P>0.05). On the other hand, the amount of capsular blood vessels showed to be significantly larger in the low protein diet compared with high protein diet in the peri - conception period (Figure 3.18 A; P=0.032). In contrast, the number of capsular blood vessels was significantly smaller in the low protein diets compared with high protein diets in the post - conception period (Figure 3.18 B; P=0.001). Moreover the size of the capsular blood vessels did not show significant differences between the low and high protein diets in the peri - and post - conception periods (Figure 3.19 A and B; P>0.05), neither was there an interaction between peri – and post – conception period.

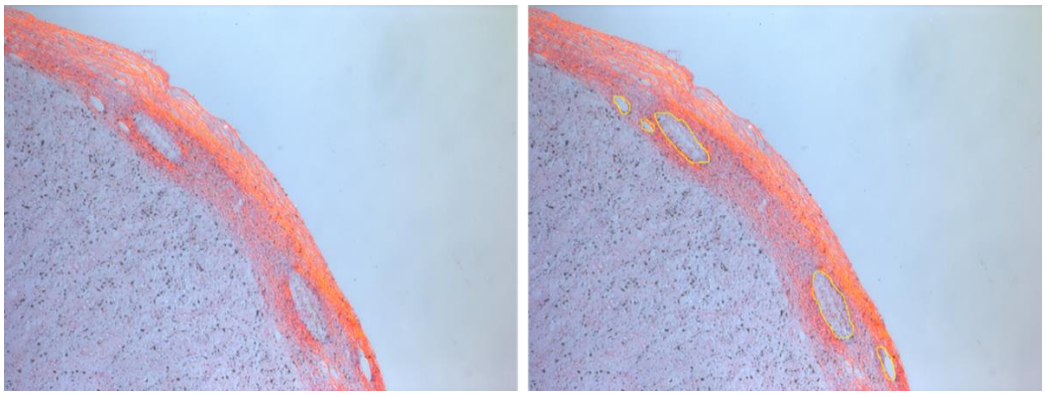


Figure 3.16 Photomicrographs of 98 dpc fetal testes to show how the measurement of the fetal capsular blood vessel was performed using Image-Pro software.

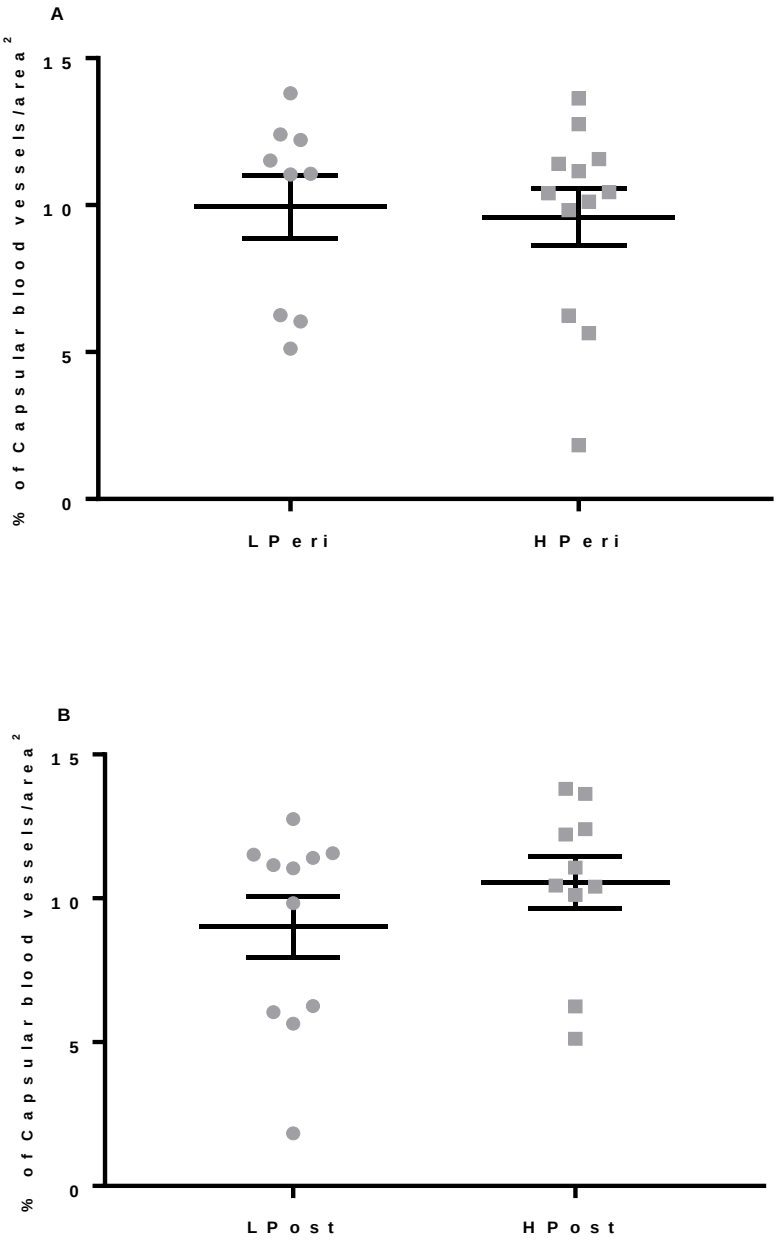


Figure 3.17 Percentage of capsular blood vessels in 98 dpc fetus A) In the peri-conception period B) In the post-conception period. The data were analysed with two-way ANOVA. No significant interaction between the peri – and post – conception periods was observed. Bars indicate mean±SEM. P<0.05 were considered significant, no significant differences

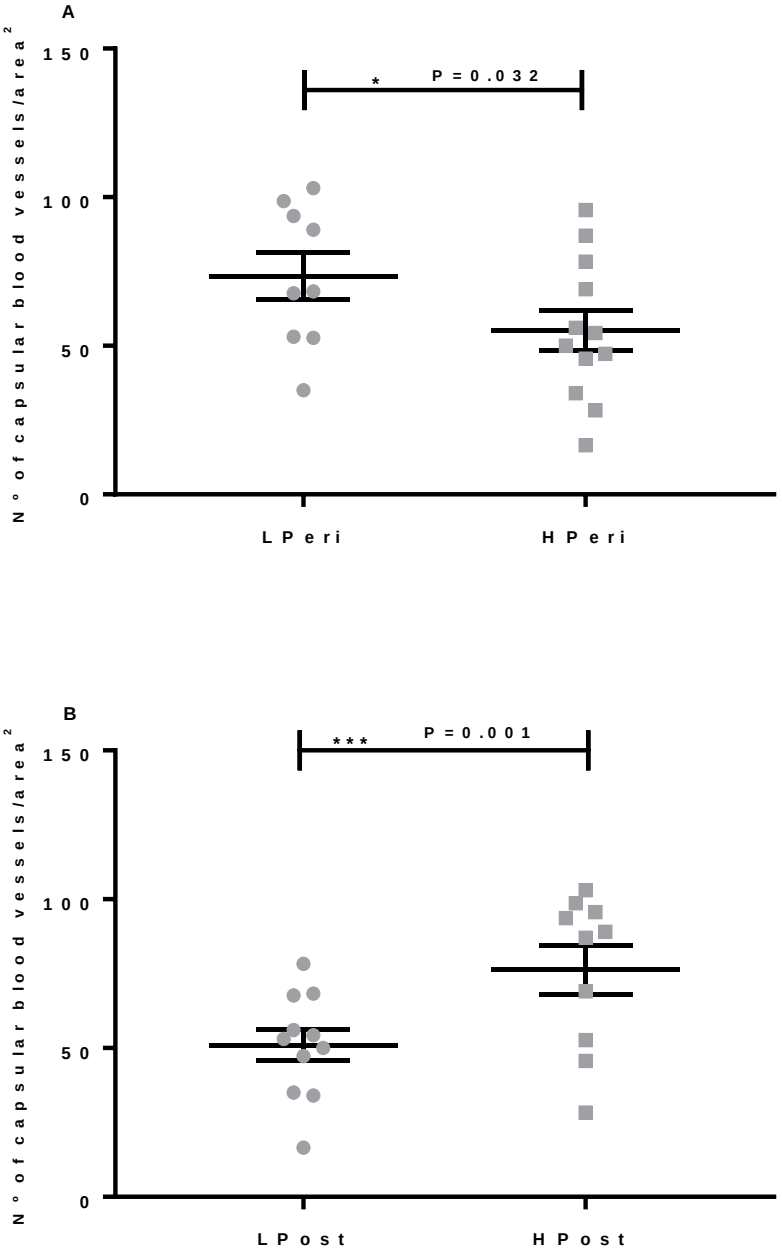


Figure 3.18 Capsular blood vessels quantification in 98 dpc fetus A) In the peri - conception period B) In the post - conception period. The data were analysed with two-way ANOVA. No interaction between the peri – and post – conception periods were observed. Bars indicate mean±SEM. P<0.05 were considered significant. Significant differences were present in the fetus fed low protein diet *in utero* during the peri – conception period (LPeri) compared with the fetus fed high protein diet (HPeri; P=0.032; Figure 3.18 A). In addition, significant differences were present in the fetus from mothers fed a low protein diet during the post – conception period (LPost) compared with fetus from mothers fed a high protein diet during the post – conception period (Figure 3.18 B; P=0.001).

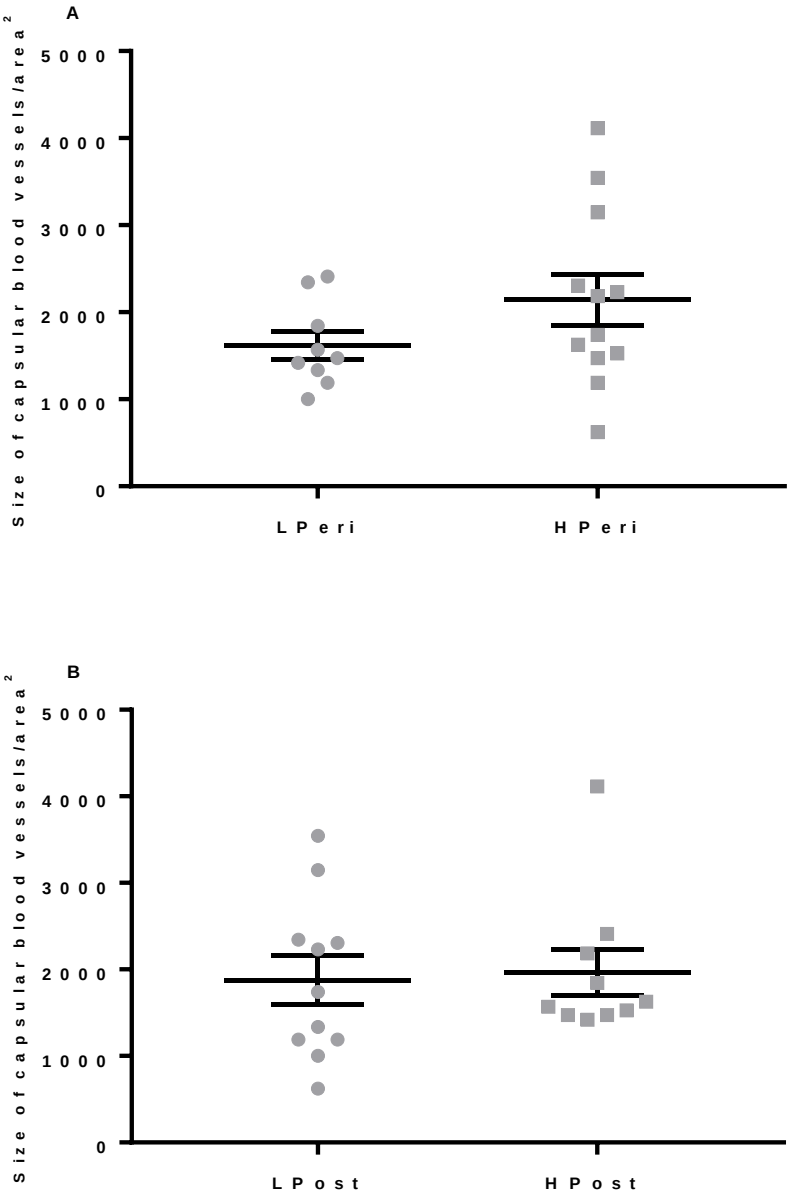


Figure 3.19 Capsular blood vessels area in 98 dpc fetus A) In the peri-conception period B) In the post-conception period. The data were analysed with two-way ANOVA. No significant interaction between the peri – and post – conception periods was observed. Bars indicate mean±SEM. P<0.05 were considered significant, no significant differences were shown.

3.3.4 Seminiferous tubules measurement within the adult testis

In the adult animals, like in the fetus, the average area, number and proportion of seminiferous tubules within the tissue were calculated with the data obtained from the image analysing software. In total 6120 seminiferous tubules were measured in the adult testes. A photomicrographs showing how the measurement of the adult seminiferous tubules was performed using Image-Pro software is showed in Figure 3.20.

No significant differences were found in the number and area of seminiferous tubules when comparing high and low protein diets between the two different time periods (Figures 3.21 A-B and 3.22 A-B; P>0.05). Moreover, no significant differences were observed in the percentage of seminiferous tubules when low and high protein diets were compared in post – conception period (Figure 3.20 B; P>0.05). However, the percentage of seminiferous tubules was significantly smaller in the low protein diet compared with high protein diet in the peri - conception period (Figure 3.23 A; P=0.005).

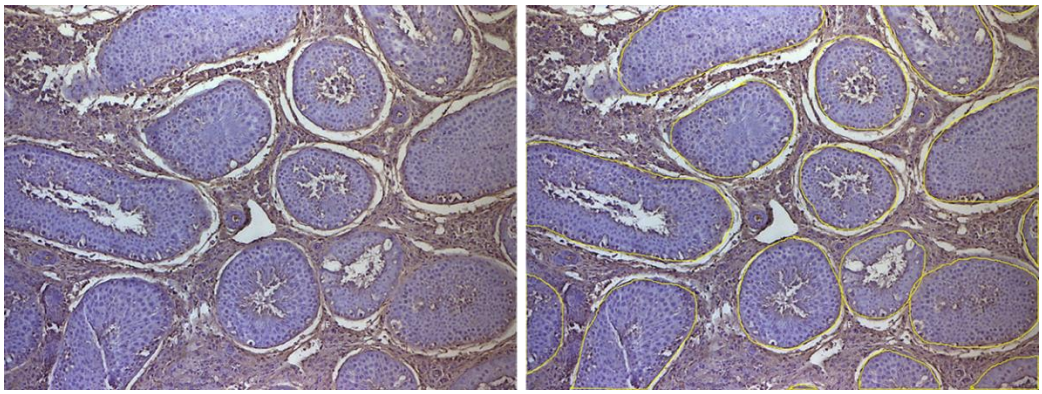


Figure 3.20 Photomicrographs of the testis of 20 month old bulls to show how the measurement of the adult seminiferous tubules was performed using Image-Pro software.

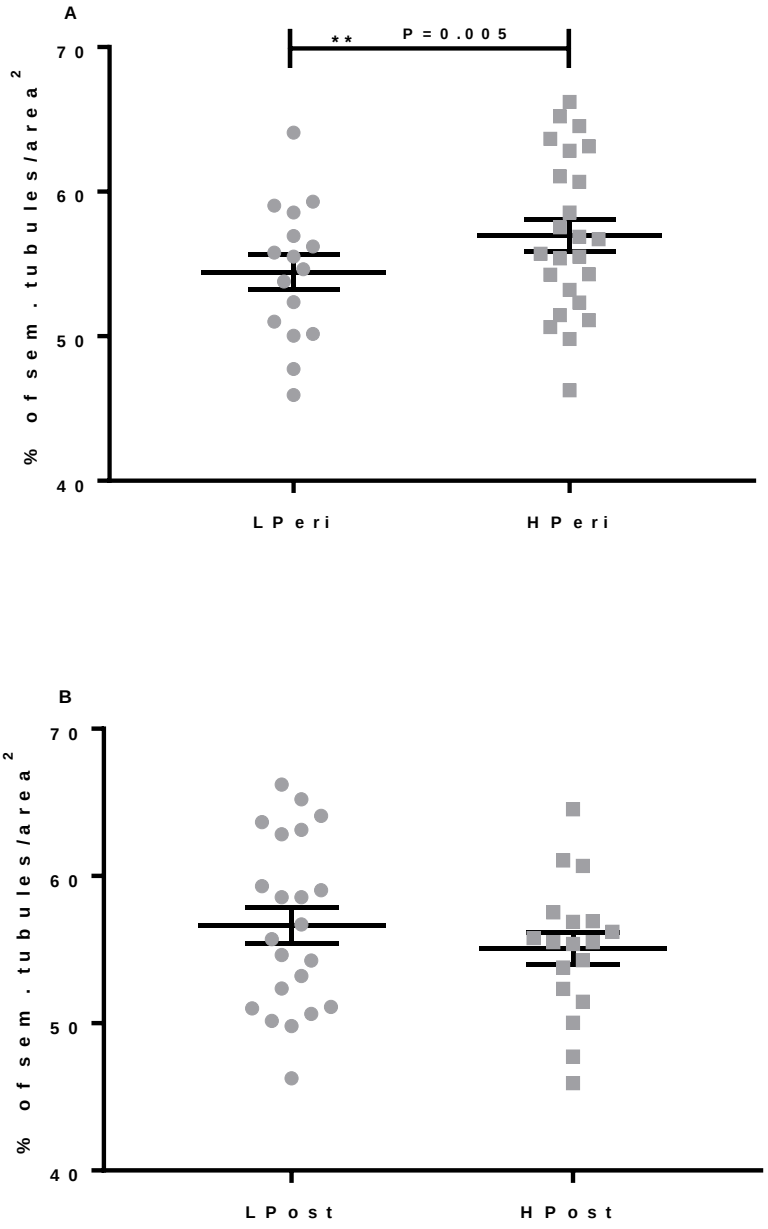


Figure 3.21 Percentage of seminiferous tubules in 20 month old bulls A) In the peri - conception period B) In the post-conception period. The data were analysed with two-way ANOVA. No interaction between the peri – and post – conception periods was observed. Bars indicate mean±SEM. P<0.05 were considered significant. Significant differences were present in adult bulls from mothers fed a low protein diet during the peri – conception period (LPeri) compared with bulls from mothers fed a high protein diet during the peri – conception period (HPeri; P=0.005; Figure 3.18 A).

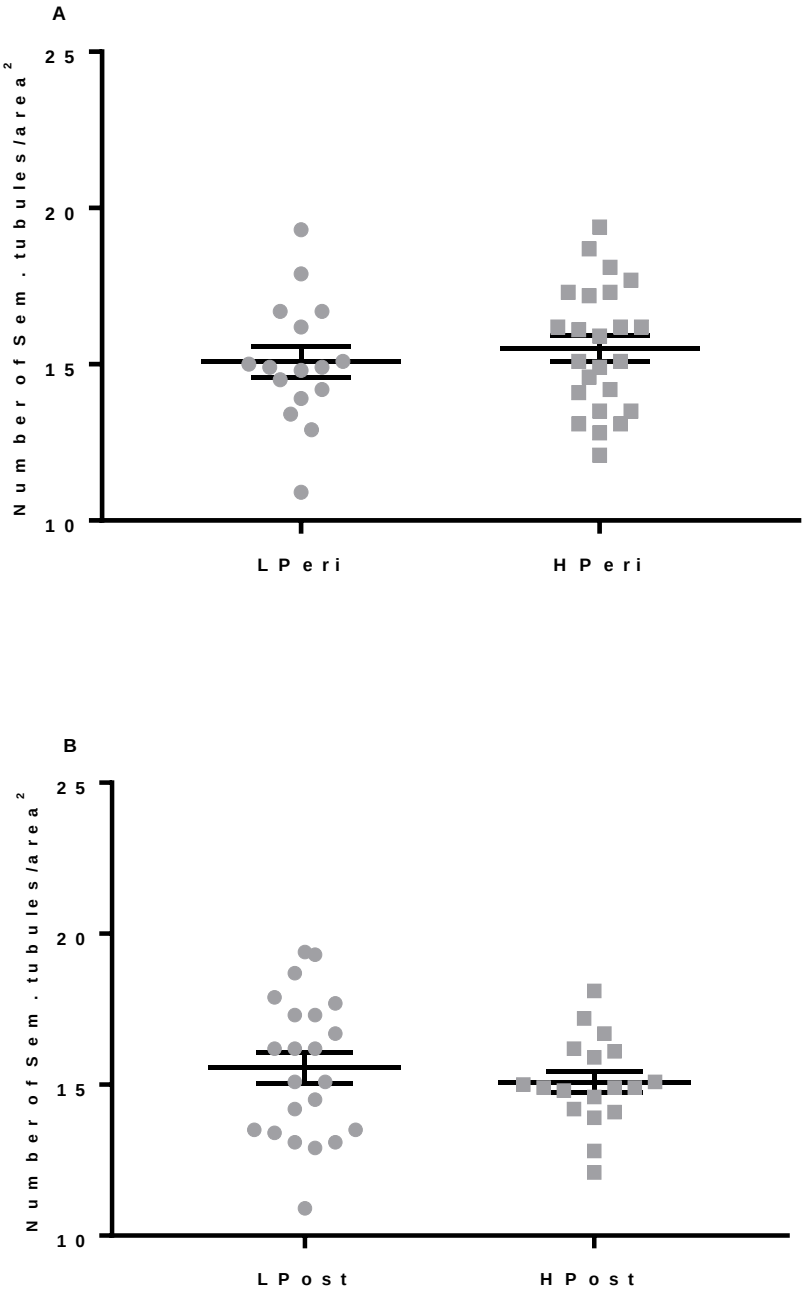


Figure 3.22 Seminiferous tubules quantification in 20 month old bulls A) In the peri-conception period B) In the post-conception period. The data were analysed with two-way ANOVA. No significant interaction between the peri – and post – conception periods was observed. Bars indicate mean±SEM. P<0.05 were considered significant, no significant differences were shown.

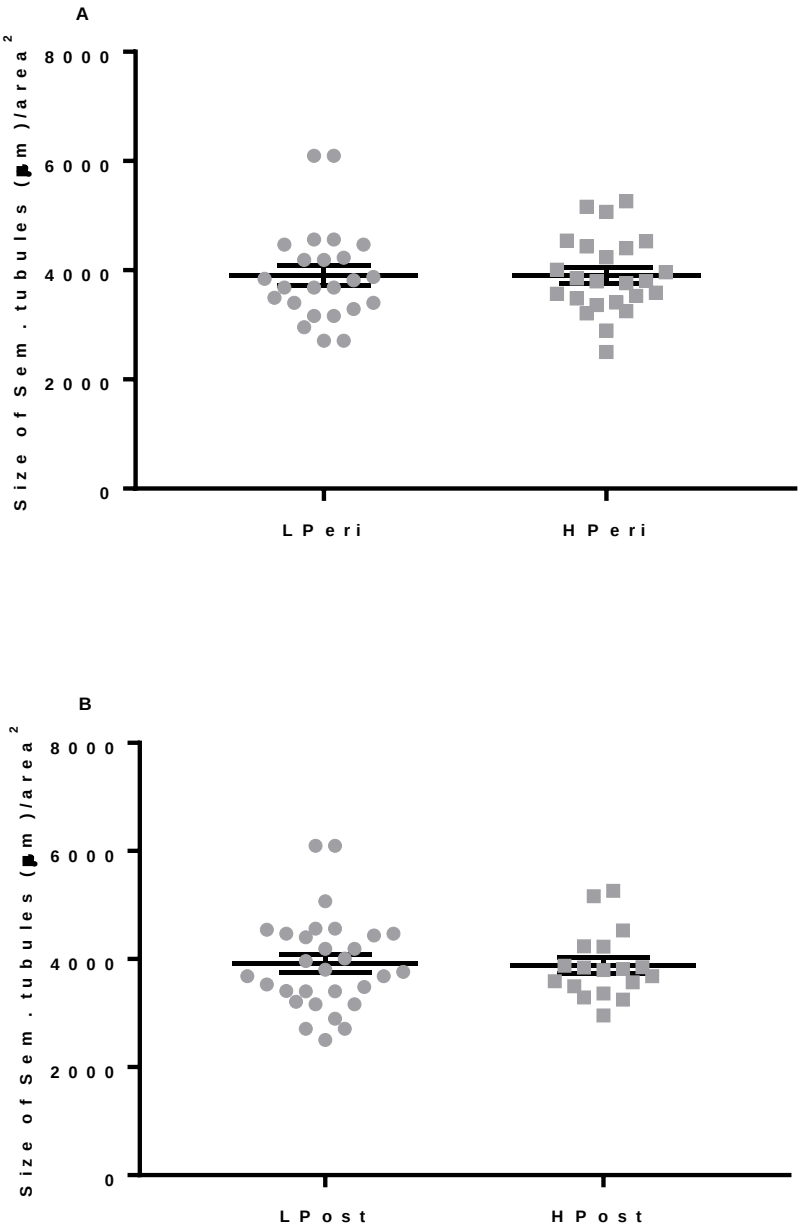


Figure 3.23 Seminiferous tubules area in 20 month old bulls A) In the peri-conception period B) In the post-conception period. The data were analysed with two-way ANOVA. No significant interaction between the peri – and post – conception periods was observed. Bars indicate mean±SEM. P<0.05 were considered significant, no significant differences were shown.

3.3.5 Testicular vasculature in the 20 month old bulls

Following the same criteria as shown in fetal vasculature measurement, in the adult testes the average area, number and proportion of blood vessels occupied within the testes were calculated. With the exception that, in adults, only parenchymal blood vessels were measured. In total 1904 adult blood vessels were measured. A photomicrographs showing how the measurement of the adult blood vessels was performed using Image-Pro software is showed in Figure 3.24. On this occasion, due to the non-normal distribution of the data, Mann-Whitney U test was used to statistically analyse the data.

No significant differences were found in the percentage or in the area of adult blood vessels when comparing the low and high protein diets in the peri - and post - conception periods (Figure 3.25 A-B and 3.26 A-B; P>0.05). However, the number of adult blood vessels were significantly increased in the low protein diets when compared with the high protein diets in the post - conception period (Figure 3.27 B; P=0.000). In contrast, the number of adult blood vessels did not show significant differences between the two different diets in the peri - conception period when compared (Figure 3.27 A; P>0.05).

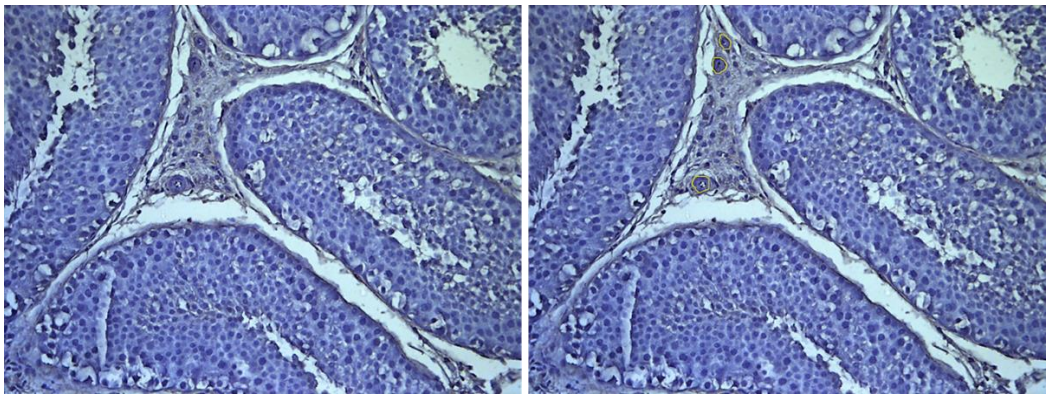


Figure 3.24 Photomicrographs of the testis of 20 month old bulls to show how the measurement of the adult blood vessels was performed using Image-Pro software.

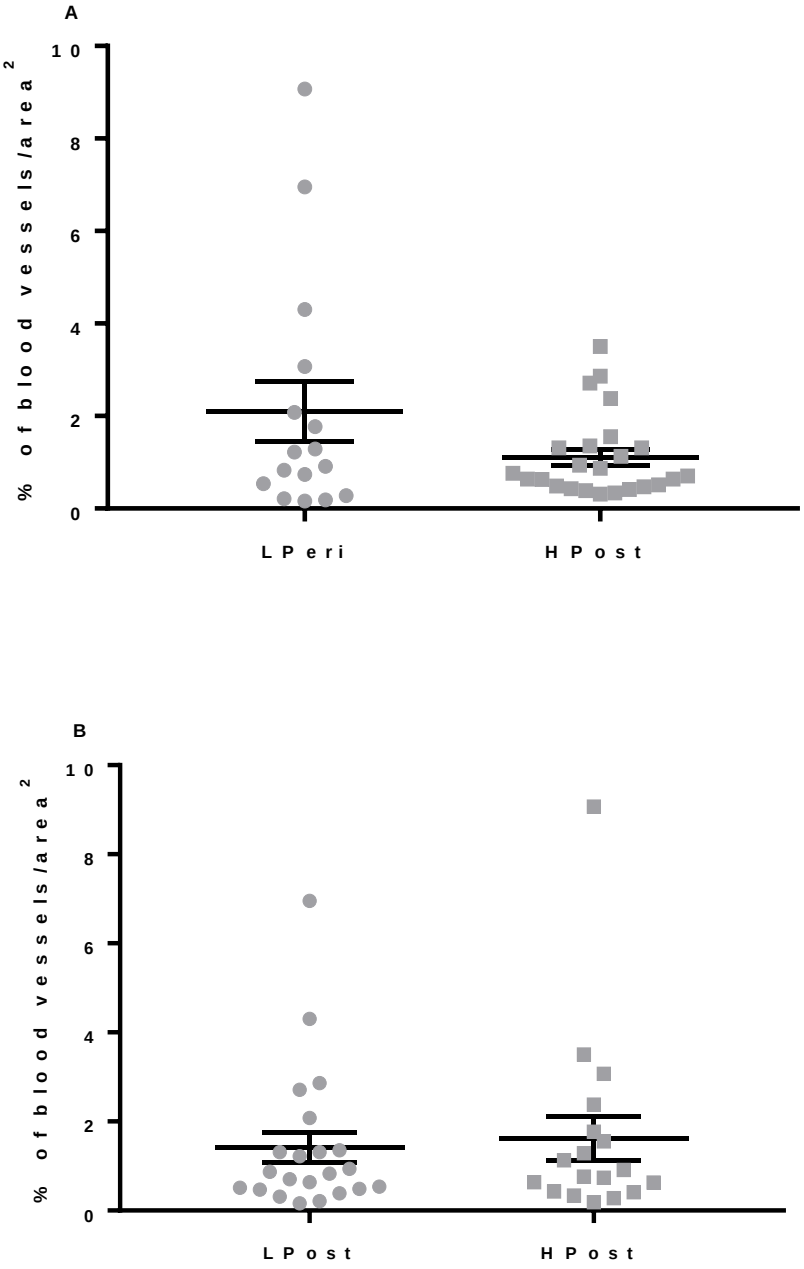


Figure 3.25 Percentage of blood vessels in 20 month old bulls A) In the peri-conception period B) In the post-conception period. The data were analysed with two-way ANOVA. No significant interaction between the peri – and post – conception periods was observed. Bars indicate mean±SEM. P<0.05 were considered significant, no significant differences were shown.

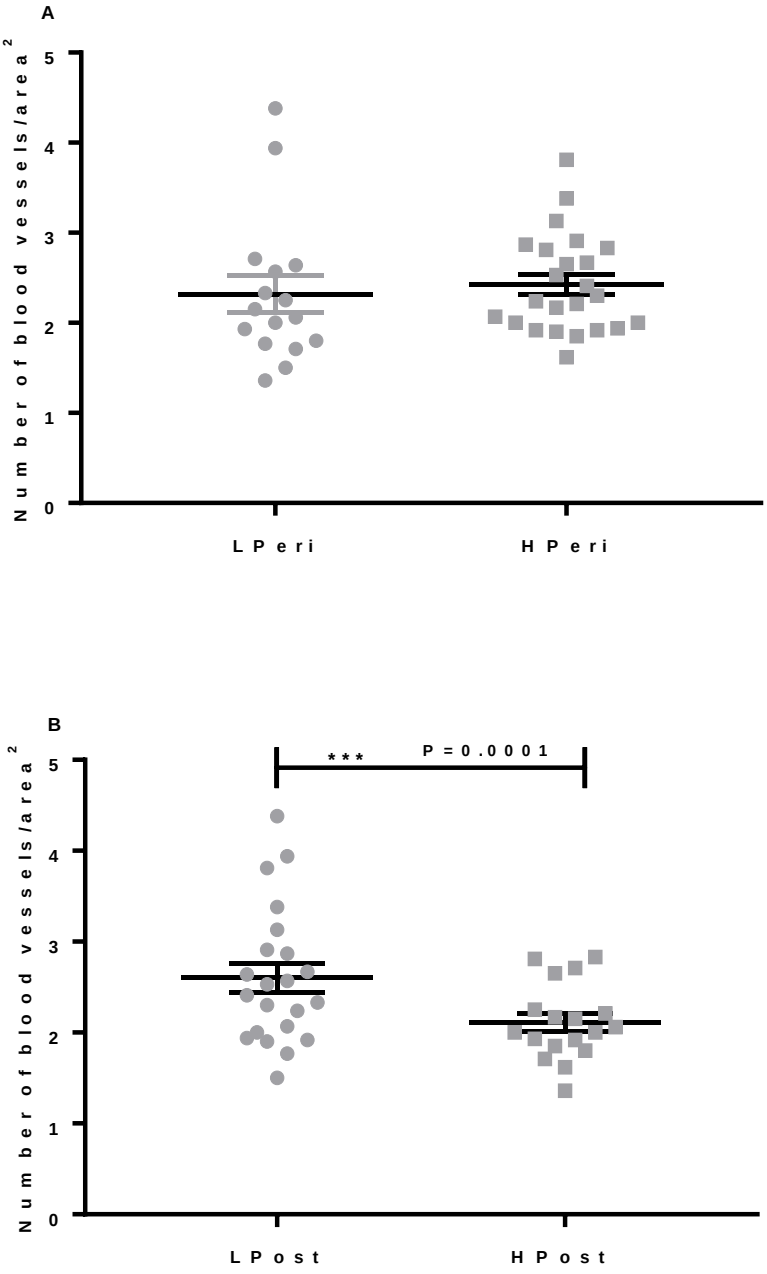


Figure 3.26 Blood vessels quantification in 20 month old bulls A) In the peri - conception period B) In the post-conception period. The data were analysed with two-way ANOVA. No interaction between the peri – and post – conception periods was observed. Bars indicate mean±SEM. P<0.05 were considered significant. Significant differences were observed in adult bulls from mothers fed a low protein diet during the post – conception period (LPost) compared with bulls from mothers fed a high protein diet during the post – conception period (HPost; P=0.0001; Figure 3.26 B).

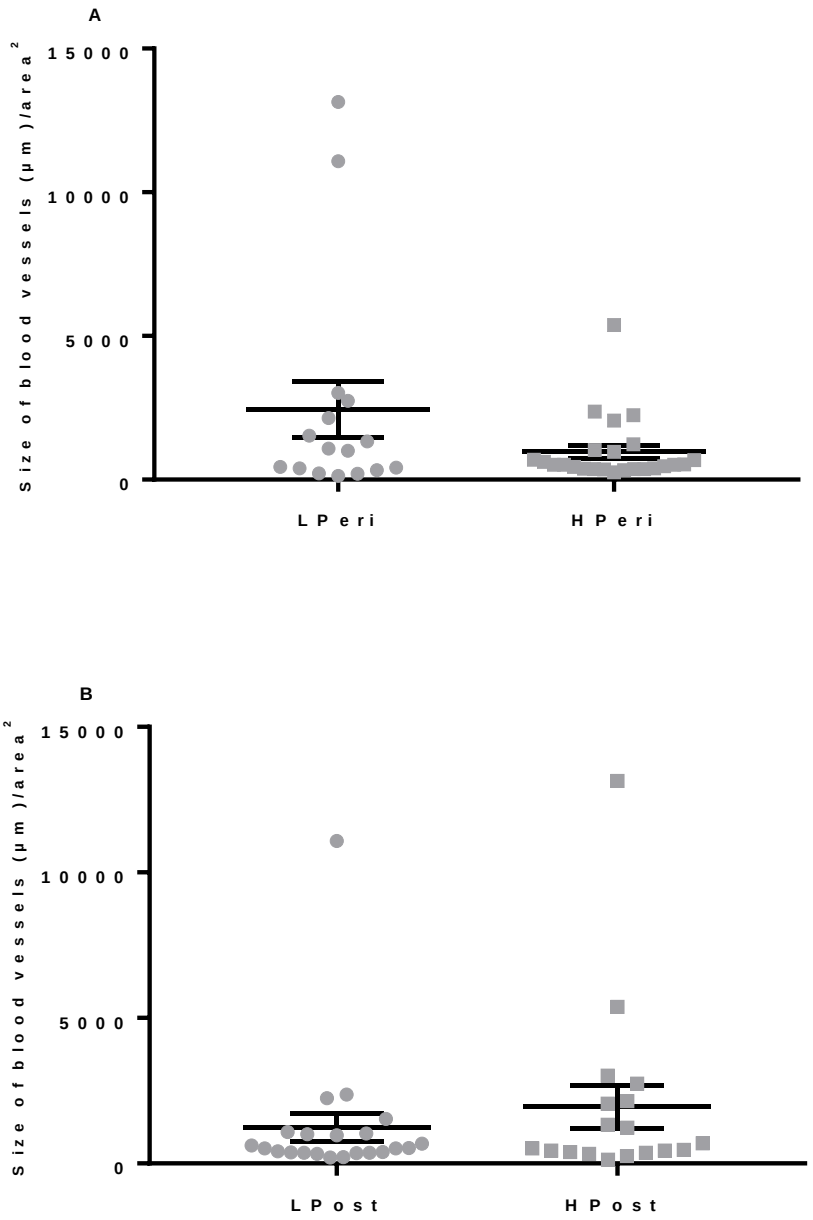


Figure 3.27 Blood vessels area in 20 month old bulls A) In the peri-conception period B) In the post-conception period. The data were analysed with two-way ANOVA. No significant interaction between the peri – and post – conception periods was observed. Bars indicate mean±SEM. P<0.05 were considered significant, no significant differences were present.

3.4 Discussion

Improved nutrition has been one of the primary interventions employed to obtain high productivity in both dairy and beef cattle during the last few decades. However, these changes have led to nutritional imbalances like hypocalcaemia or displaced abomasa (CHAWG, 2014). Fertility, one of the most important concerns in both beef and dairy cattle production, is greatly influenced by nutrition. In this experiment, the effects of two different maternal diets (simulating the real protein content in pastures during the dry (7%) and the wet (14%) seasons in Australia) in the testicular development of bulls were studied. For that purpose the testicular cells, seminiferous tubules and blood vessels within the testes were assessed at two points: 1- just at the end of the nutritional restriction at 98 dpc; 2- 20 months after birth. Using this method not only was the protein restriction effect in situ in the fetus assessed, but also the possible long term effects in adult animals.

3.4.1 Dietary effects on testicular development

From the embryo to the adult animal testicular cells play an essential role in the development of a fertile male individual. Fetal Sertoli cells are especially important during the first stages of testicular formation since they control the differentiation of Leydig cells and stop the meiosis of germ cells, the other two testicular cells assessed in this study. Fetal Sertoli cells also secrete anti-Müllerian hormone and are responsible for the formation of the seminiferous cords (Griswold, 1998, Josso et al., 2001, Bitgood et al., 1996, O'Shaughnessy and Fowler, 2011). In adult animals, Sertoli cells are also crucial because they

create the Blood-Testis barrier (Kaur et al., 2014) and help the sperm cells in their movement from the basal compartment of the seminiferous tubules towards the lumen (Russell, 1980, Trainer, 1987, Kerr, 1992, Griswold, 1995). Other important qualities of the Sertoli cells is the secretion of inhibin and the presence of androgen receptors in their cytoplasm (Trainer, 1987). Because the number of germ cells that each Sertoli cell is able to support is limited (Berndtson et al., 1987, Griswold, 1995), for an adequate daily sperm production it is essential that nothing alters the proliferation and differentiation of Sertoli cells.

To assess how the deprivation of protein during peri – conception and first trimester of gestation might affect the reproductive tract development of the male progeny, the percentage of the different type of cells (Sertoli, germ and interstitial cells) present in the fetal testes was calculated using manual identification and counting. Sertoli cells did not show changes in their percentages when low and high protein diets were compared in the peri – or post – conception periods. These findings concur with the investigation reported by Da Silva et al. (2003), where the fetus from ewes subjected to a moderate and high protein diets from the AI day to 103dpc did not show significant differences in the number of Sertoli cells. Likewise Andrade et al. (2013) discovered that fetus undernourished *in utero* from mating to 110 days of gestation presented no significant differences in the number of Sertoli cells when compared with a control group. In contrast, other studies indicated a reduction in the number of Sertoli cells in lambs when the ewes were underfed in energy during pregnancy (Kotsampasi et al., 2009, Bielli et al., 2002).

However in these two experiments the diet was restricted to the second trimester of gestation and the testis were collected from born animals while in our study the percentage of protein was manipulated just during peri - conception and the first trimester and the tissues analysed were from the 98 dpc fetus. Interestingly, the percentage of germ cells did show significant differences in the interaction between diets and periods. Thus, in 98 dpc fetuses fed low protein diet in the peri – and post – conception periods (LL) showed an increase in the percentage of germ cells compared with the 98 dpc fetus fed high protein diet in the peri – conception period and low protein diet in the post – conception period (HL). In the same comparison, LL versus HL, the percentage of Leydig cells was significantly decreased in the LL group. As Sertoli cells exert a control over germ cells mitosis and Leydig cells differentiation, a diminution in the function of Sertoli cells would have explained the changes in the percentage of these cells, however the only feature assessed in this study was the number of Sertoli cells but not their size, which might have been altered. That would also explain the smaller size of seminiferous tubules showed in the 98 dpc fetus fed low protein diets in the post – conception period, and also the greater number of seminiferous tubules in 98 dpc from the LL groups compared with the other three dietary treatments (LH, HL and HH) and the smaller percentage of seminiferous tubules in the 98 dpc fetus of HL groups compared with the LL, LH and HH groups, since all received protein restricted diets in the post – conception period. Several studies have investigated the effect of maternal nutrition in male fertility. Rodríguez-González et al. (2012) observed a reduction in AR expression and in the number

of spermatocytes, slower differentiation of the spermatids and a higher number of immature germ cells in male rats fed with restricted protein diets *in utero* and/or lactation compared with the control groups. Similar results in the number of spermatogonias, spermatocytes, spermatids and Leydig cells were observed by Melo et al. (2014) in a study where rats were restricted in protein from mating to weaning and offspring were fed a normal or restricted protein diet from weaning to 70 days of age. However, the alterations observed in the spermatocytes, spermatids and Leydig cells were recovered in the adult animals. These results concur with our study, where a reduction in the percentage of Leydig cells in fetal animals was observed and it might agree with the increase in the percentage of germ cells in that if there is an increment in the number of immature germ cells in fetal life but the number of Sertoli cells remains the same and the Leydig cells are decreased, it might not be possible for these two cells to support and trigger the differentiation of that many germ cells. It also support our speculation of smaller Sertoli cells which would agree with a decrease in AR expression. Interestingly, it has been observed that plasma testosterone concentration was greater in 50 day old fetal sheep undernourished in energy *in utero* from day 31 of gestation, although a recover was showed at later age (Rae et al., 2002). These results contrasts with our results, since it could be expected that a decrease in the percentage of Leydig cells would also reduce the production of testosterone. Yet in another study, overnutrition caused a decreased plasma testosterone concentration in 37 week old rabbits from dams fed hypercholesteraemic diets compared with control (Dupont et al., 2014). It has been also observed that a reduction in the

levels of prepubertal gonadotropins and a decrease in testis volume was present in male calves restricted in protein during the first and second trimesters (Sullivan et al., 2010b). These findings are consistent with our results and are further evidence that a restriction in protein during fetal development alters the reproductive system at a cellular, hormonal and pituitary level. In contrast, a high energy diet fed from day 60 of gestation to term showed no alteration in the expression of FSHB and LHB in the males offspring (Weller et al., 2016).

Regarding the effect of maternal diet in the development of the seminiferous tubules, numerous studies has explored the effect of under – and over - nutrition *in utero* in the development of this testicular structure. Some studies found smaller diameters in seminiferous tubules in male lambs undernourished *in utero* (Kotsampasi et al., 2009) and also in rats fed restricted protein diet *in utero* (Rodríguez-González et al., 2012, Melo et al., 2014). These results are consistent with the smaller seminiferous tubules found in the fetus fed with low protein diet during the first trimester of gestation in our study. Besides, our results showing that the size of the seminiferous tubules was not significantly affected in LL and HL groups, the size of seminiferous tubules did show a non-significant tendency to be smaller in both groups, which might explain why the number of seminiferous tubules was increased in the LL fetus and also why the percentage of seminiferous tubules was decreased in the HL fetus. On the other hand, the effect of overnutrition of the fetus during gestation is somewhat less clear. Thus, Weller et al. (2016) found a decrease in the size of the seminiferous tubules in fetal bovine overnourished *in utero.* These results concur with the

study by Rodríguez-González et al. (2015) where male rats from obese mothers fed with high energy diet during pregnancy and weaning showed smaller seminiferous tubule area at 650 postnatal day and Sullivan et al. (2010a) who observed a tendency in bull calves over supplemented *in utero* to show a smaller diameter in the seminiferous tubules than those on reduced protein levels. However, new born lambs overnourished *in utero* showed an increase in the volume of seminiferous tubules (Bielli et al., 2002) and no significant differences were observed in 103dpc fetus lambs overnourished *in utero* when compared with a moderate diet (Da Silva et al., 2003). Clearly, a big controversy exists regarding the effects of the maternal diet on the development of the seminiferous tubules both in the fetus and as long term effects in adult animals. These differences could be species specific and also due to the different applied diets and periods of gestation chosen for their administration in each experiment. Another circumstance to take into consideration is whether the pregnancy was terminated at the end of the experimental period or whether the pregnancy was left until term and also whether the offspring received a normal, restricted or overenergised diet after parturition. Interestingly, although in our study the main effects in the fetal testicular cells and seminiferous tubules was caused by a reduction in protein intake during the first trimester of gestation, in the adult animals (20 months old) was in the peri – conception periods (60 days before AI to 23 dpc) where a reduction in the percentage of seminiferous tubules was observed. It has been found that long term effects occurred in rat males overnourished *in utero* and during lactation, where sperm viability, motility and concentration were reduced in 450 day old

males. Also the fertility rate was lower and decreased with time in overfed offspring's, while in normal fed males the fertility rate remained stable (Rodríguez-González et al., 2015). Nevertheless, in our experiment, it might be possible that, in the case of the LPost animals, a compensation occurred in the adult animals during/after puberty, where modifications in size, morphology and number of the testicular cells take place in order to be set for reproduction.

The last structure assessed in our study were the blood vessels. Blood vessels in the early stages of the pregnancy are very important since blood supply is essential to provide the requirements of cells to proliferate (Tilmann and Capel, 1999, Brennan et al., 2002). Moreover, for an adequate sustenance of microenvironments in seminiferous tubular and interstitial compartments, the testicular microcirculation is essential (Ergün et al., 1997). Dietary protein seemed to significantly affect the number and size of blood vessels in the testes of 98 dpc fetuses. Significant differences were found in both peri – and post - conception periods. In the former, low protein diets showed a smaller blood vessels size and an increase in the number of blood vessels in the testes of 98 dpc fetuses. In addition, when the blood vessels were categorized between parenchymal and capsular it was observed that parenchymal blood vessels were significantly smaller in the LPeri diet compare with HPeri and it was also found that there was a decreased number of capsular blood vessels in the LPeri when compared with HPeri. The number of blood vessels was the only parameter affected by low protein diet in the post – conception period, showing a smaller number of total blood vessels and capsular blood vessels in LPost versus HPost. Interestingly, in the 20 month old bulls the number of

blood vessels was statistically bigger in the LPost diet compare with the HPost. Some studies focused on low maternal diet in fetal development and found that a restricted diet during pregnancy caused a decrease in microvascular density in ovary (in rats), kidney and placenta (both in sheep) (Chan et al., 2015, Dunford et al., 2014, Luther et al., 2007) and also a reduction in the percentage of blood vessels in murine placenta (Rutland et al., 2007). Although these represent different species and tissues, it is clear that a restricted diet during gestation has an impact in the development of the vasculature, as it was observed in our study. VEGFA is essential for angiogenesis in embryo development and also in postnatal growth (Reddy et al., 2012, Nalbandian et al., 2003). Besides, this protein has also a major role in male fertility, orchestrating testicular cord and vasculature formations during embryo development (Dores and Dobrinski, 2014, Bott et al., 2006) and regulating postnatal spermatogenesis (Reddy et al., 2012, Nalbandian et al., 2003, Schön et al., 2010, Caires et al., 2009). VEGF has been found to be expressed in Sertoli and Leydig cells and its receptors in germ cells and testicular blood vessels (Seval-Celik et al., 2014, Reddy et al., 2012). It has been observed that an inhibition of VEGFA caused loss of undifferentiated spermatogonia and reduced number of sperm in the corpus epididymis in mice (Lu et al., 2013) and inhibition of seminiferous cord formation in rats (Bott et al., 2006). Reductions in the expression of VEGFA and its receptor, VEGFR2, have been observed in the ovaries of adult rats undernourished *in utero* (Chan et al., 2015) and in the placenta and lung of developing fetuses from rats fed a diet restricted in protein during gestation (Liu et al., 2014). Considering this information, it might be

possible that the alterations caused in the seminiferous tubules and testicular vasculature in the 98 dpc fetus in our experiments are caused by a reduction of VEGFA due to a limitation of protein in the maternal diet, which in the case of the animals restricted in the peri – conception period persisted until adulthood with a decrease in the percentage of seminiferous tubules.

In summary, restricted maternal protein diets in both peri – and post – conception periods affected the development of the testes, although in the peri – conception period was the vasculature the main structure affected whereas in the post – conception period vasculature, seminiferous tubules and testicular cells were affected.

Chapter 4 Long term effects of low maternal dietary protein on the epididymis of adult bulls

4.1 Introduction

This chapter explores the effect of maternal diet on offspring epididymis development and epididymal fluid protein profile. Few studies have focused on the maternal nutritional impact on the male reproductive tract development at a histological level despite the importance of the epididymis in sperm maturation. Recently there have been several studies to investigate the proteins present in the epididymis and their roles in sperm maturation. There are no studies in the role that maternal diet could play in the epididymal protein profile. Therefore, in this chapter an anatomical and molecular examination of epididymal development in 20 month bulls undernourished *in utero* during the peri – conception period and first trimester of gestation is presented.

4.1.1 Hypothesis

We hypothesised that the lack of protein in the maternal diet during the peri – conception and first trimester of gestation will impact the development of the epididymis and the protein profile of the epididymal fluid of 20 month old bulls. Due to the interaction of the sperm with the proteins present in the epididymis during sperm maturation, it would be possible that these changes influence sperm quality and reproductive efficiency.

4.2 Methods

4.2.1 Sample acquisition at 20 months

4.2.1.1 Epididymal tissue

4.2.1.1.1 Fixation and processing

To assess the development of the epididymis a 1cm^3 piece from the tail of the epididymis was dissected. The fixation and processing was as described in Chapter 2 Section 2.2.2 of the materials and methods chapter. Several adaptations to adjust the techniques performed (Chapter 2 Section 2.4.2, 2.4.3 and 2.4.4) to the requirement of the epididymis tissue were followed as explained in the Sections below.

4.2.1.1.1.1 Masson´s trichrome staining

Several optimisations were carried out in the staining protocols of the epididymis:

- BSA time reduced from 30 to 25secs.
- Light green time increased from 15 to 30mins.

A comparison of antigen retrieval methods was also undertaken between sodium citrate buffer treatment and no treatment. Following the first xylene step, samples were placed in sodium citrate buffer at a ratio of 1:10 in dH_2O and heated at 1min high power and 4mins low-medium power in the microwave.

4.2.2 Image analysis

4.2.2.1 Adult epididymal tubules measurement

Tissue sections stained with PS and Masson's trichrome were used for this technique. For the measurement of the epididymal tubules 6 photomicrographs at 5X magnification were obtained using a systematic random sampling method (Mayhew, 2011), the photomicrographs were then electronically stitched together to obtain a larger image ensuring that most of the tubules were complete, only complete tubules were measured (as is always required by this technique). In total, 432 photomicrographs were taken and 1080 tubules measured, n=8HH, 12HL, 8LH and 8LL.

Image-pro plus, version 6.3 (Media Cybernetics, USA) was used to manually measure the area, number and the percentage of the tubule vs other epididymal tissue within each picture. In addition, the lumens of each tubule were separately measured using the same technique. The tubules and lumens were manually identified and drawn around, the total area of each picture was also measured. Statistical analysis was carried out as described in Chapter 2 Section 2.10.

4.2.2.2 Adult epididymal vasculature measurement

Tissue sections stained with Masson's trichrome were used for the measurement of the blood vessels as they were clearly visible using this technique. Twelve photomicrographs at 20X were taken using a systematic random sampling criteria (Mayhew, 2011) to measure the blood vessels in the epididymis of the bulls. From these 12 photomicrographs 7 were used to

measure the area and count the blood vessels. In total 864 photomicrographs were taken and 4183 blood vessels were measured and counted, n=8HH, 12HL, 8LH and 8LL.

The area, number and percentage of blood vessel within the epididymis was measure using Image-pro plus, version 6.3 (Media Cybernetics, USA). Blood vessels were visually identified and measured manually drawing around. Statistical analysis was carried out as described in Chapter 2 Section 2.10.

4.2.2.3 Adult epididymal collagen measurement

To measure the collagen within the epididymis 1 slide per sample was stained using Masson's trichrome and PS. Six photomicrographs were taken at 40X magnification using a systematic random sampling (Mayhew, 2011) method. Five of these photomicrographs were used to measure the amount of collagen type I, III and other types of collagen (PS) and total collagen (Masson's) present in the epididymis. In the tissue stained with PS the collagen I and III stained yellow and green, respectively and the rest of collagen was indicated by a red colour. The tissue stained with Masson's showed all collagen in green. In total 432 photomicrographs were taken and collagen was measured, n=8HH, 12HL, 8LH and 8LL.

The amount of collagen was measured using Image-pro plus, version 6.3 (Media Cybernetics, USA). The different types of collagen were individually identified and measured manually using colour identification software. The percentage of each type of collagen as well as the total collagen within the epididymis was

calculated and used for the statistical analysis following Section 2.10 in Chapter 2.

The distribution and amount of the stained collagen with the Masson's trichrome and the PS staining showed significant differences, therefore one more histological stain was performed (Van Gieson) and immunohistochemistry undertaken (antibody for collagen I and III) to prove which staining was more accurate. Comparing the results obtained from the different methods, PS was determined to be the most accurate method.

4.3 Results

4.3.1 The effect of diet on adult epididymal tubules

Using the data obtained with the imaging software, the size, number and proportion of epididymal tubules within the epididymal area were measured in the adult tissue (See Figure 4.1). In total, 1080 tubules were measured. No significant differences were observed in any of the measured parameters when comparing either the four treatments or peri- and post- conception periods (Figure 4.2 – 4.3; P>0.05).

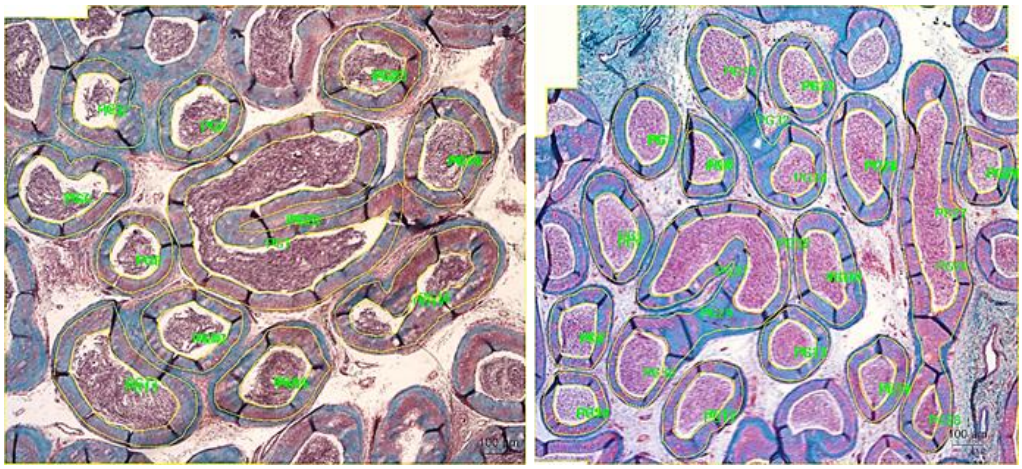


Figure 4.1 Photomicrographs of the epididymal tubules after measurement using Image-Pro software

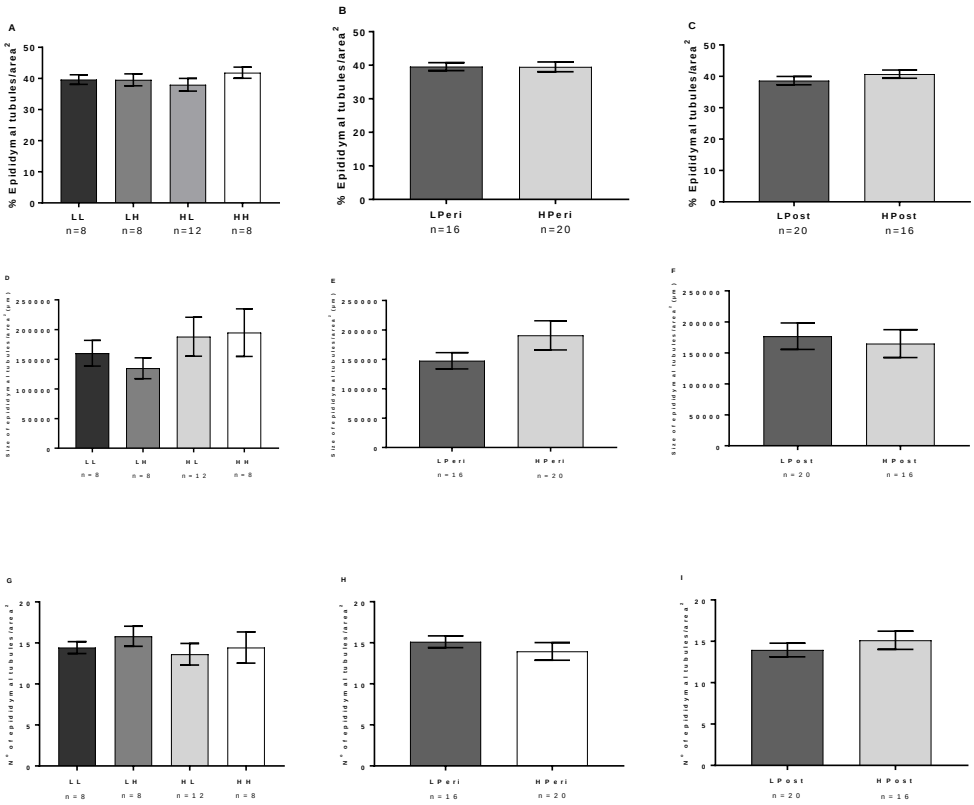


Figure 4.2 Percentage of epididymal tubules in 20 month old bulls A) In the four different diets B) When low and high protein diets were compared in the peri-conception period C) In low and high protein diets during the post-conception period; Size of epididymal tubules in 20 month old bulls D) In the four different diets E) When low and high protein diets were compared in the peri-conception period F) In low high protein diets during the post-conception period; Number of epididymal tubules in 20 month old bulls G) Within the four different diets H) In low and high protein diets in the peri-conception period I) When low and high protein diets were compared in the post-conception period. The data were analysed with two-way ANOVA. Graphs indicate mean±SEM. P<0.05 were considered significant, no significant differences were observed.

4.3.2 The effect of diet on adult epididymal blood vessels

Following the same criteria as in the tubules, the size, number and percentage of blood vessels per area within the epididymis were measured (Figure 4.3). In total, 4183 blood vessels were measured and the data obtained was compared between the 4 different treatments as well as the two time periods (Peri- and Post-conception). No significant differences were found in the size, number or proportion of tissue occupied by blood vessels within the epididymis, when comparing between the four groups or the two different periods (Figure 4.4; P>0.05).

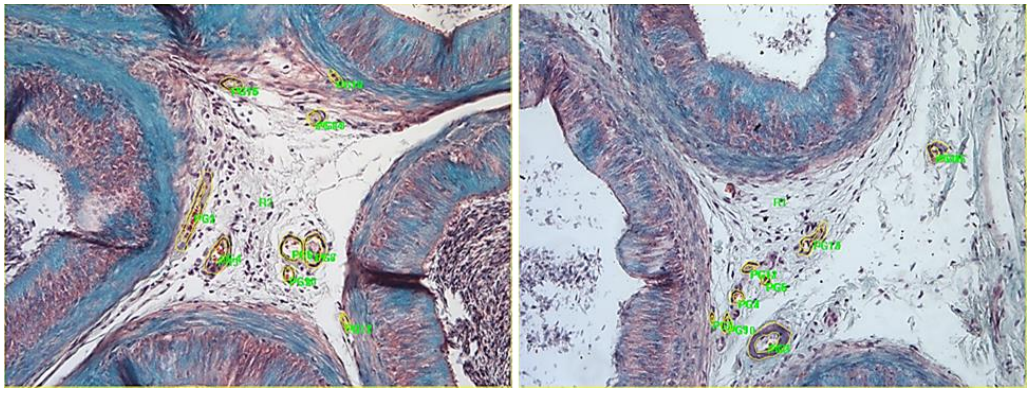


Figure 4.3 Photomicrographs of epididymal blood vessels after measurement using Image-Pro software

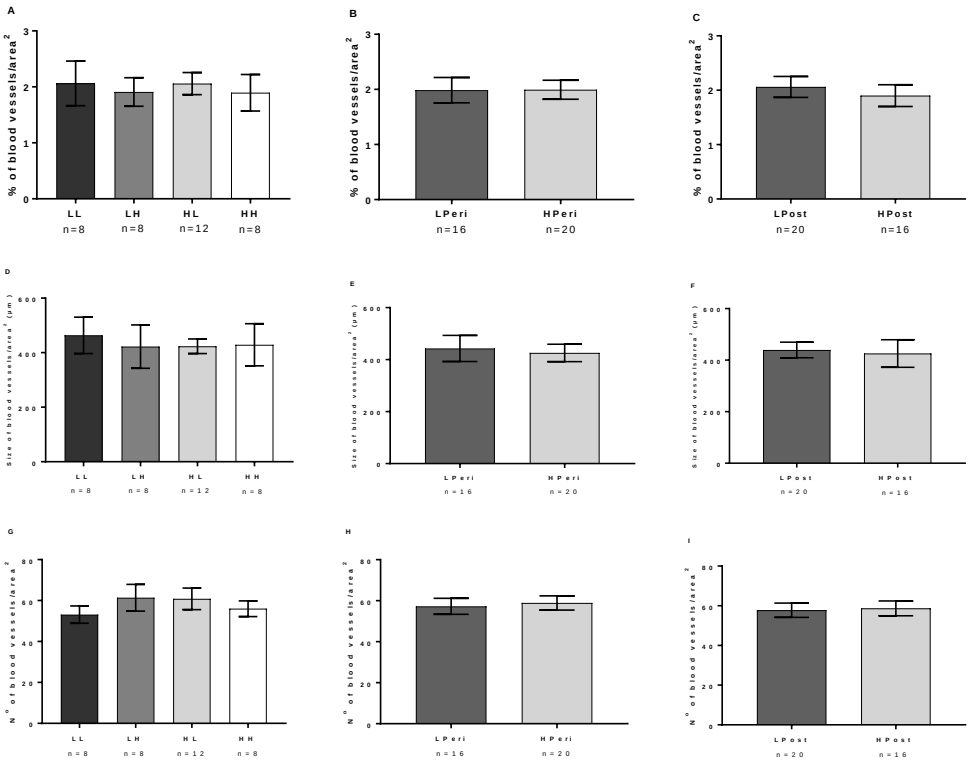


Figure 4.4 Percentage of epididymal blood vessels in 20 month old bulls A) In the four different diets B) When low and high protein diets were compared in the peri-conception period C) In low and high protein diets during the post-conception period; Size of epididymal blood vessels in 20 month old bulls D) In the four different diets E) When low and high protein diets were compared in the peri-conception period F) In low and high protein diets in the post-conception period; Number of epididymal blood vessels in 20 month old bulls G) In the four different diets H) When low and high protein diets were compared in the peri-conception period I) In low and high protein diets during the post-conception period. The data were analysed with two-way ANOVA. Graphs indicate mean±SEM. P<0.05 were considered significant, no significant differences were observed.

4.3.3 The effect of diet on adult epididymal collagen

The collagen in the epididymis was measured in order to analyse the histological structure of the organ. The percentage of the collagen was obtained from two different staining: PS, where it was possible to differentiate between collagen types I, III and all remaining collagens, and Masson's Trichrome staining where all collagen types stained a green colour. An example of both stainings it is showed in Figure 4.5. For this purpose 432 photomicrographs were measured.

The structure and distribution of the collagen in the epididymis was similar in all the samples when comparing the same staining procedure. Strangely, although both techniques are used for staining the collagen, statistical differences were found between the data obtained in tissue stained with PS and the one from the same tissue stained with Masson's. No statistical differences were present in the percentage of collagen between the 4 treatments or the two different periods within the same staining techniques (Figure 4.6.; $P>0.05$).

In order to identify which technique (PS or Masson's) was more accurate in measuring the collagen present in the epididymal tissue, an IHC using antibody specific for collagen I and III (Biorad 2150-2305, USA) and Van Gienson staining to stain the total collagen were carried out. It was observed that the staining obtained with the IHC and the Van Gienson technique matched with the staining showed in the epididymis stained with PS.

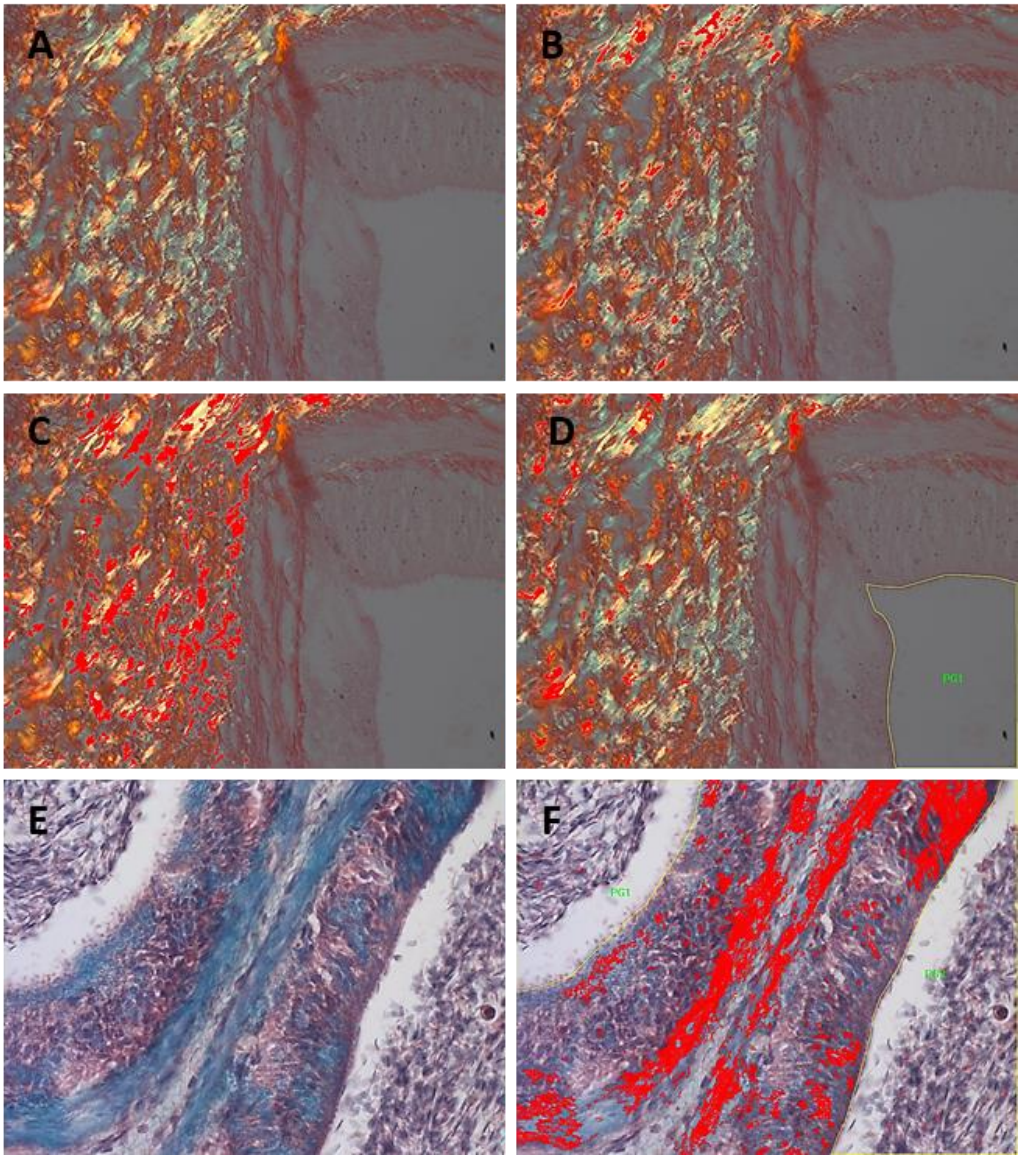


Figure 4.5 Photomicrographs of the collagen in the epididymis tissue after measurement using Image-Pro software A) Photomicrograph of the epididymis stained with PS staining B) Measurement of the collagen type I C) Measurement of the collagen type III D) Measurement of all the collagen except type I and III E) Photomicrograph of the epididymis stained with Masson's trichrome F) Measurement of the total collagen

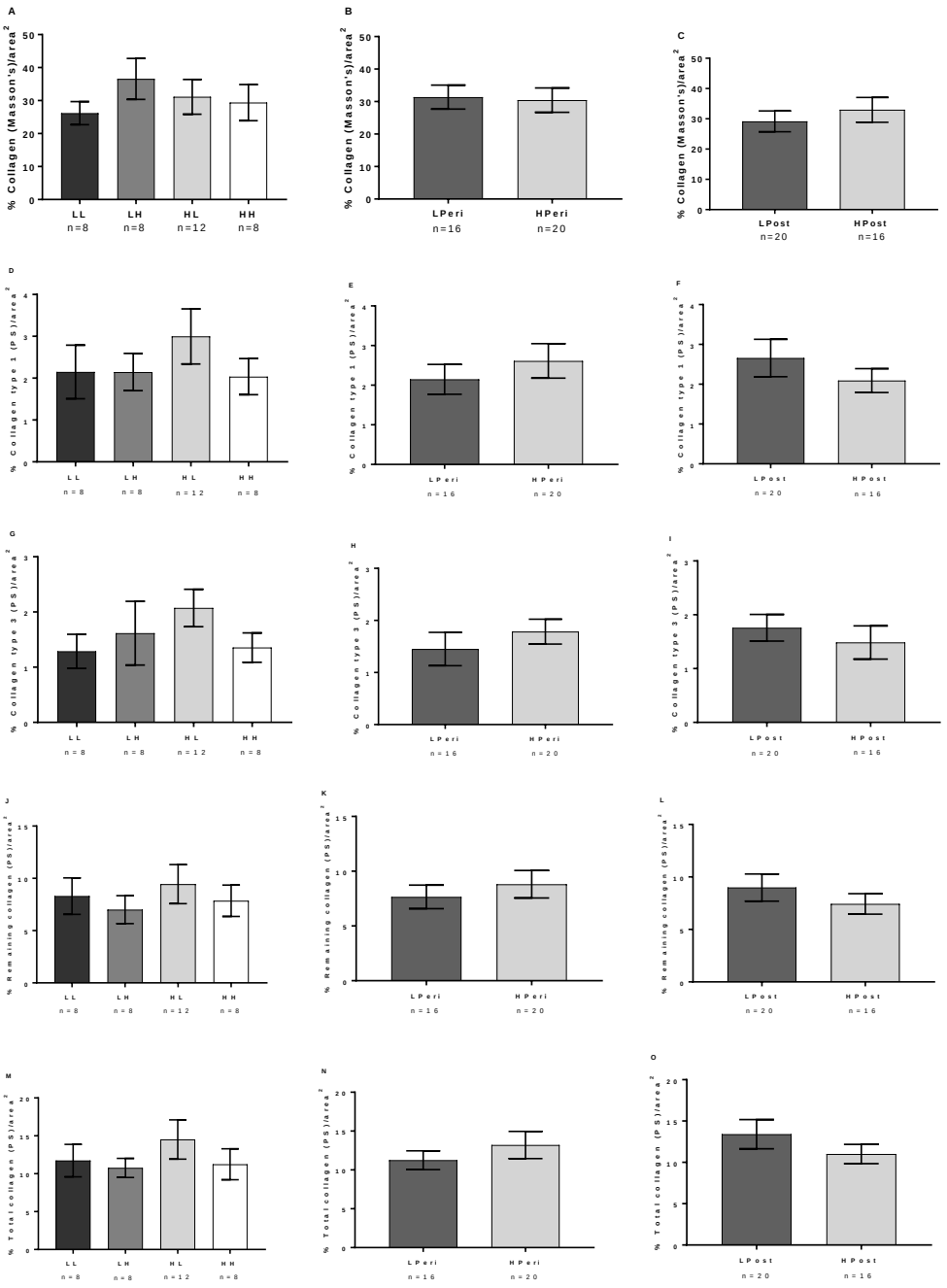


Figure 4.6 Percentage of collagen stained with Masson's within the epididymal tissue A) In the four different diets B) When low and high protein diets were compared in the peri-conception period C) In low and high protein diets during the post-conception period; Percentage of collagen type I stained with PS staining within the epididymis D) In the four different diets E) When low and high protein diets were compared in the peri-conception period F) In low and high protein diets during the post-conception period; Percentage of collagen type III stained with PS staining within the epididymis G) In the four different diets H) When low and high protein diets were compared in the peri-conception period I) In low and high protein diets during the post-conception period; Percentage of remaining collagen stained with PS staining within the epididymis J) In the four different diets K) When low and high protein diets were compared in the peri-conception period L) In low and high protein diets during the post-conception period; Percentage of total collagen stained with PS staining within the epididymis M) In the four different diets N) When low and high protein diets were compared in the peri-conception period O) In low and high protein diets during the post-conception period. The data were analysed with two-way ANOVA. Graphs indicate mean±SEM. P<0.05 were considered significant, no significant differences were observed.

4.3.4 The effect of diet on protein profile of adult epididymis

Frozen epididymal fluid samples were sent for analysis to the INRA institution in France. The 36 samples (n=8LL, 8LH, 12HL, and 8HH) were pooled by groups (LL, LH, HL, HH, LPeri, HPeri, LPost and HPost). The following methods were conduced by Valerie Labas and Lucie Combes-Soia. Samples were separated in 1D SDS Page gels, passed through LC MS/MS mass spectrometry for measurement and then the spectral counting data obtained was analysed. Significant differences in the expression of proteins were found between the different diet groups and also between the 2 different time periods. The analysis and interpretation of the data was done by the author.

Comparisons of the low versus high protein diets between the two different periods (Peri- and Post-conception) were performed. The 4 different diets were grouped depending on the diet they received in the different time periods. Therefore, LL and LH represented the LPeri group and HL and HH formed the HPeri group, whereas to assess the effect of the low protein diet in the first trimester of gestation the four different treatments were grouped as follow: LL and HL (LPost) and LH and HH (HPost).

4.3.4.1 Protein expression in LPeri vs HPeri

Differentially expressed proteins between LPeri and HPeri were observed (Table 4.1). The over-expressed proteins with the highest Log2FC in this comparison were filamin B (FLNB), casein kinase 2 alpha 1 (CSNK2A1) and crystallin zeta (CRYZ). On the other hand, chaperonin containing TCP1 subunit 6A (CCT6A), sperm acrosome associated 3 (SPACA3), nitrilase 1 (NIT1) and glyoxalase domain containing 4 (GLOD4) showed the highest Log2FC within the under-expressed proteins.

The gene ontologies (GO) with the highest significance in each category of the differentially expressed proteins between LPeri and HPeri showed enriched categories in: GO:0009056 catabolic process; GO:0043230 extracellular organelle and GO:0016787 hydrolase activity (Table 4.2).

Pathway analysis (for the human orthologs of the identified genes) completed using the Cytoscape Genemania plugin (Montojo et al., 2014) are shown in the Table 4.3. Using the differentially expressed proteins to form a network, this program inferred new proteins related to the search proteins of interest and therefore added a better understanding of their function (see results), transcription factors and pathways. Looking at this table it can be observed that the main pathways altered when the proteins were reduced during the peri - conception period, involved the CCT complex. Transcription factors inferred include those known to play key roles in male fertility and development including NRF2 (nuclear factor, erythroid 2 like 2) and SRY (sex determining region Y).

Table 4.1 Differentially expressed protein in LPeri vs HPeri

GI Number	Gene name	Log2FC	P value
300794998	*FLNB*	3.8	0.00096
1094400	*CSNK2A1*	3.7	0.038
114326274	*CRYZ*	3.3	0.019
149758330	*GNB1*	2.1	0.017
115497818	*XPNPEP1*	2.1	0.018
114051361	*SELENBP1*	2.0	0.0023
296475407	*MPI*	1.9	0.048
296484714	*TLN1*	1.8	0.014
528976936	*APOA4*	1.7	0.027
75812934	*CTSS*	1.6	0.043
146345491	*PRDX5*	1.5	0.0023
195539525	*SAO*	1.3	0.026
529001778	*ACY1*	1.1	0.049
311266740	*PRPSAP1*	1.1	0.04
156120547	*AKR7A2*	1.1	0.045
301760045	*ALAD*	1.0	0.032
488556002	*EIF4A1*	1.0	0.048
331284213	*ACE*	-1.0	0.0025
115496448	*QDPR*	-1.0	0.00023
27806789	*TTR*	-1.0	0.00018
1699167	*IGCGAMMA*	-1.3	0.0004
528999364	*MAN2C1*	-1.3	0.023
148681465	*Efhd2*	-1.3	0.0014
274325364	*CCT5*	-1.7	0.00029
27806617	*GDI1*	-1.7	0.049
440912826	*PSMA1*	-1.7	0.028
156120555	*GLOD4*	-2.3	0.0001
126723227	*CCT6A*	-2.3	0.0001
157279923	*SPACA3*	-2.3	0.022
296489845	*NIT1*	-2.3	0.012

Table 4.2 Gene ontologies (GO) categories for differentially expressed proteins in LPeri vs HPeri.

GO term		category	set size	candidates contained	q-value
GO:0009056	catabolic process	BP 2	1920	12 (0.6%)	0.000366
GO:0044710	single-organism metabolic process	BP 2	4126	15 (0.4%)	0.00518
GO:0006457	protein folding	BP 2	227	4 (1.8%)	0.00518
GO:0043230	extracellular organelle	CC 2	2827	21 (0.7%)	5.20E-11
GO:0043227	membrane-bounded organelle	CC 2	12089	26 (0.2%)	0.00715
GO:0016787	hydrolase activity	MF 2	2567	11 (0.4%)	0.00783

BP: biological process; CC: Cellular component; MF: molecular function
Showing the GO terms, GO categories, set size, number of proteins present in the set and q value.

Table 4.3 Cytoscape (Genemania) analysis of the LPeri vs HPeri differentially expressed proteins, showing the pathways, suggested extra genes (result) and suggested transcription factors.

Pathway	Results	Transcription factors
Visual phototransduction	APOC2	SP1
Retinoid metabolism and transport	PROS1	USF
Regulation of Insulin Secretion by Glucagon-like Peptide-1	CCT2	CP2
Protein folding	CCT3	GATA1
Prefoldin mediated transfer of substrate to CCT TriC	CCT8	ELF1
Peroxisome	TCF7L2	GABP
Nectin adhesion pathway	DYNLT1	PEA3
Metabolism of proteins	ANXA7	SREBP1
Metabolism of Angiotensinogen to Angiotensins	TJAP1	HNF6
Metabolism	CCT7	NRF2
Lissencephaly gene (LIS1) in neuronal migration and development	LDLR	FOX
Lipoprotein metabolism	TCP1	SRY
Folding of actin by CCT TriC	CCT4	E4F1
Formation of tubulin folding intermediates by CCT TriC	CYTIP	ATF6
Diseases associated with visual transduction	APOC3	PIT1
Cooperation of Prefoldin and TriC CCT in actin and tubulin folding	SUV420H1	
Chylomicron-mediated lipid transport	LPXN	
Chaperonin-mediated protein folding	CBX5	
Association of TriC CCT with target proteins during biosynthesis	ACTB	
	HIVEP2	

4.3.4.2 Protein expression in LPost vs HPost

Differentially expressed proteins found in this comparison are shown in Table 4.4. Within the over-expressed proteins several have been identified to be involved in male fertility. These are: ribosomal protein SA (RPSA), serpin family C member 1 (SERPINC1), ceruloplasmin (CP) and alpha-1-B glycoprotein (A1BG).

The under-expressed proteins that have been identify to play a role in male reproduction were: proteasome subunit α type-6 (PSMA6), gelsolin (GSN),

alpha-L-fucosidase 1 (FUCA1), epididymal sperm binding protein 1 (ELSPBP1), cathepsin A (CTSA), cyclase associated actin cytoskeleton regulatory protein 1 (CAP1), cysteine rich secretory protein 3 (CRISP3), cysteine rich secretory protein 1 (CRISP1), lipocalin-type prostaglandin D synthase (PTGDS), ribonuclease A family 10 (RNASE10), Sperm Adhesion Molecule 1 (SPAM1), cathepsin C (CSTC), ADAM metallopeptidase domain 7 (ADAM7) and prosaposin (PSAP).

The gene ontologies (GO) with the highest significance in each category of the differentially expressed proteins between LPost and HPost showed enriched categories in: GO:0072376 protein activation cascade; GO:0043230 extracellular organelle and GO:0030234 enzyme regulator activity (Table 4.5).

The main pathways, results genes and transcription factors obtained using the Cytoscape Genemania plugin are shown in the Table 4.6. The main pathways altered by the reduction in maternal protein diet during the first trimester of gestation are involved in the vascular system. PSAP, ACTN4 (actinin alpha 4), CAP1, ALB (albumin), A2M (alpha-2-macroglobulin), FGA (fibrinogen alpha chain), F2 (coagulation factor II, thrombin) and SERPINC1 (serpin family C member 1) were the main differentially expressed proteins that participated in these pathways. An important suggested transcription factor was androgen receptor (AR).

Table 4.4 Differentially expressed protein in LPost vs HPost

Accession Number	Gene Symbol	Log2FC	P value
119584991	*RPSA*	3.6	0.001
555957525	*SERPINC1*	3.2	0.0031
296491101	*CP*	2.8	0.0094
114589077	*COPG1*	2.6	0.0021
12845853	*Hba-a2*	2.5	0.063
114051379	*LRG1*	1.9	0.0045
115545495	*IGK*	1.6	0.032
27806751	*AHSG*	1.6	0.0093
135806	*F2*	1.4	0.0035
114051586	*TAGLN*	1.4	0.03
160332365	*SERPINA3-1*	1.2	0.048
157954061	*A2M*	1.0	0.0001
741932316	*C3*	1.0	0.0045
148745450	*FGA*	1.0	0.0001
24496448	*IGHM*	1.0	0.0017
30794280	*ALB*	0.7	0.0001
74267962	*ALB*	0.6	0.0001
114053019	*A1BG*	0.5	0.028
296483195	*PPIB*	-0.7	0.033
148704781	*Psma6*	-0.7	0.0038
296473586	*PRSS22*	-0.7	0.017
164452943	*GSN*	-1.0	0.014
114051279	*FUCA1*	-1.0	0.0084
300796502	*ELSPBP1*	-1.0	0.00038
117935057	*TKFC*	-1.0	0.0067
440904596	*WFDC8*	-1.0	0.022
440912546	*LOC102286148*	-1.0	0.0024
114051401	*PLBD2*	-1.0	0.032
149676188	*CTSA*	-1.0	0.0068
119577215	*ACTN4*	-1.0	0.016
78042488	*CAP1*	-1.0	0.013
78042470	*TPPP2*	-1.0	0.0044
528970160	*C11H9orf9*	-1.0	0.033
296477761	*FCGBP*	-1.3	0.0018
149642641	*DDT*	-1.3	0.012
118601862	*CRISP3*	-1.3	0.0001

440904602	*SPINT4*	-1.3	0.018
329664664	*CRISP1*	-1.3	0.012
46405151	*IFI30*	-1.3	0.0012
126010821	*SCIN*	-1.3	0.0009
1888363	*ACR*	-1.3	0.049
118150406	*SCGB1D*	-1.7	0.0056
27807521	*PTGDS*	-1.7	0.00065
115495283	*CREG1*	-1.7	0.00036
3914346	*PIGR*	-1.7	0.0077
440913247	*LCN6*	-1.7	0.00054
75812934	*CTSS*	-1.7	0.016
194670534	*RNASE10*	-1.7	0.001
328887941	*SPAM1*	-1.7	0.0077
14249144	*Rab11b*	-1.7	0.0036
426257071	*RBM3*	-1.7	0.0022
27806675	*CST3*	-2.3	0.0003
300797860	*NAALAD2*	-2.3	0.0001
155372309	*SCGB2A2*	-2.3	0.0046
30038325	*CTSC*	-2.3	0.00019
156120497	*TCAF2*	-2.3	0.048
149643069	*ACPP*	-2.3	0.0058
555974191	*ADAM7*	-3.3	0.032
120419464	*PSAP*	-3.8	0.029
296472216	*RET*	-3.8	0.0016

Table 4.5 Gene ontologies (GO) categories for differentially expressed proteins in LPost vs HPost.

GO term		category	set size	candidates contained	q-value
GO:0043230	extracellular organelle	CC 2	2827	33 (1.2%)	1.60E-13
GO:0072562	blood microparticle	CC 2	158	11 (7.0%)	8.49E-12
GO:0005615	extracellular space	CC 2	1416	23 (1.6%)	8.49E-12
GO:0072376	protein activation cascade	BP 2	151	6 (4.0%)	0.000246
GO:0030234	enzyme regulator activity	MF 2	960	10 (1.0%)	0.00638

BP: biological process; CC: Cellular component; MF: molecular function

Showing the GO terms, GO categories, set size, number of proteins present in the set and q value.

Table 4.6 Cytoscape (Genemania) analysis of the LPost vs HPost differentially expressed proteins, showing the pathways, suggested extra genes (result) and suggested transcription factors.

Consolidated pathway	Results	Transcription factor
Complement and coagulation cascades	FGG	HNF3
Platelet activation, signaling and aggregation	FGB	CEBPB
Platelet degranulation	F13A1	HNF1
Hemostasis	F8	ERR1
Response to elevated platelet cytosolic Ca2+	PROS1	ALPHACP1
Platelet Aggregation (Plug Formation)	VWF	PAX4
BIOCARTA_AMI_PATHWAY	F5	IRF2
BIOCARTA_INTRINSIC_PATHWAY	SERPING1	ICSBP
Lysosome	KNG1	ETS2
Glycosphingolipid metabolism	PLG	LEF1
Metabolism	SERPINF2	PEA3
MHC class II antigen presentation	CFD	CEBP
Uptake of Carbon Dioxide and Release of Oxygen by Erythrocytes	SERPINE1	AR
Scavenging of Heme from Plasma	ITGA2B	GABP
Uptake of Oxygen and Release of Carbon Dioxide by Erythrocytes	VCL	STAT5B
Binding and Uptake of Ligands by Scavenger Receptors	CFB	E2F
O2 CO2 exchange in erythrocytes	ITGB3	
pentose phosphate pathway	APOA1	
	F2R	
	SERPINA1	

4.4 Discussion

4.4.1 Effect of low protein diet on epididymal development

The luminal environment of the epididymis is necessary for maintaining normal sperm structure, therefore defects in the epididymal epithelium and tubules structure may cause changes in the composition of the luminal milieu and affect the sperm maturation and function (Ecroyd et al., 2004, Joseph et al., 2009, Choi et al., 2015a). Blood vessel development is also key for optimal function of the epididymis since blood supply is essential for cell proliferation and function (Tilmann and Capel, 1999, Brennan et al., 2002). In this project the long-term effects of protein restricted diets during pregnancy on male offspring fertility were studied. Histological examinations of the epididymis of 20 month old bulls undernourished *in utero* were carried out. Size, number and proportion of epididymal tubules and blood vessels, as well as the proportion of collagen in this tissue were measured, analysed and compared between the different diets and periods. Deficiencies in maternal protein diet during the peri- and/or post-conception periods did not show significant differences in any of the parameters measured. In Chapter 3, these effects were investigated in the testis of fetuses and of adult animals who received the same maternal dietary regime. These same adults were also used for the epididymis evaluation in this Chapter. In the adult testis it was observed that a compensation in the alterations which had been seen during fetal development had occurred. It was hypothesised that such compensation in the testis development might have happened after the end of the restriction period (first trimester), during

lactation or pre-pubertal period, this could also have happened in the epididymal tissue. Our results coincide with Kotsampasi et al. (2009) who found no significant differences in the number of Sertoli cells or seminiferous tubules diameter in 10 month old male sheep undernourished *in utero* during early pregnancy. However these two parameters were reduced when the diet was restricted during the second trimester of gestation. A reduction in the number of Sertoli cells and seminiferous tubules absolute volume was also observed in adult rats undernourished *in utero*, although the maternal diet of these animals was restricted throughout gestation and lactation (Genovese et al., 2010). One possible explanation of why animals are able to compensate for the delays in development produced by restricted maternal diets during early days of gestation and no in later stages of pregnancy might be that at such early stages cell differentiation has not been completely finished and so it can be continued until the deficiency in cells are solved. Therefore it is likely that compensatory mechanisms are being activated in order to maintain the size, number and proportion of epididymal tubules and blood vessels, and the amount of collagen within the bovine epididymal tissue following the differing maternal dietary protein levels given in this study. To our knowledge no studies have focus in the histological development of the epididymis in adult animals undernourished *in utero.*

4.4.2 Effect of low protein diet in epididymal fluid protein profile

Investigating the proteins present in the reproductive tract to find target biomarkers of male infertility has been an area of increasing interest in the last

decade. Several studies have compared epididymal fluid and/or seminal plasma proteins from fertile animals with infertile/subfertile animals to better understand which proteins might be involved in male fertility. However, to our knowledge this is the first study to focus on the possible changes in epididymal fluid protein profile in adult animals undernourished *in utero*.

In this experiment comparisons between low and high protein diets in two different periods (LPeri vs HPeri and LPost vs HPost) were perfomed. Numerous proteins were differentially expressed when comparing the low protein diet with the supplemented diet. Some of these proteins have previously been investigated in other models/animals for their possible implications on male fertility, and most have been implicated in sperm capacitation and binding capabilities. These proteins included SPAM1, PTGDS, GSN, PRDX5, ACE, CCT5, CCT6A, AHSG, PSMA6, ELSPBP1, CTSA, CRISP1, CRISP3, RNASE10, ADAM7 and PSAP. The majority of these proteins were under-expressed in the groups fed low protein diet, which may indicate subfertility; however some were over-expressed. The different diets and the proteins of interest are discussed in Sections 4.4.2.1-2.

4.4.2.1 LPeri vs HPeri

In the LPeri vs HPeri comparison there were 17 common differentially over-expressed proteins and 13 common differentially under-expressed proteins. From these proteins the following were prioritised for their possible role in male reproduction: PRDX5, ACE, CCT5, CCT6A and PSMA1.

PRDX5 is a thiol-dependent peroxidase, that is present in the cauda of the epididymis and is thought to protect the sperm from reactive oxygen species (ROS)(Nagdas et al., 2014). High levels of ROS leads to the lipid peroxidation of the sperm membrane changing its fluidity, which will affect the sperm capacity of fertilize the ova (Hsieh et al., 2006, Park et al., 2012, Nagdas et al., 2014). However a small concentration of ROS is essential for initiation of capacitation and acrosome reaction (Nagdas et al., 2014, Park et al., 2012). An over-expression of this protein in LPeri animals may indicate the necessity of a compensation for a higher concentration of ROS in the epididymal fluid, however per se it does not seem to have a negative impact in the sperm but the opposite. Although the number of over-expressed proteins was greater than the under-expressed proteins in the LPeri group vs the HPeri group, it was within the under-expressed proteins that more proteins with a known role in fertility were observed, for example ACE. ACE apart from being involved in the regulation of blood pressure, also plays a role in male fertility (Köhn et al., 1998, Deguchi et al., 2007, Fujihara et al., 2013, Kondoh et al., 2008). However, it has been argued that ACE does not have a direct function in sperm - zona pellucida binding but it works as a catalytic enzyme for the glycosylphosphatidylinositol (GPI) anchored proteins (Fujihara et al., 2013, Kondoh et al., 2005), a process that seems to be essential for sperm capacitation (Boerke et al., 2014). In addition it has been also considered that its dipeptidase activity has a function in fertilization (Fuchs et al., 2005). Since LPeri animals showed a reduction in the expression of ACE, an impairment in the removal of the GPI anchored proteins from the membrane surface of the sperm and so the no exposition of

proteins needed for capacitation could be expected to occur in these animals. CCT5 and CCT6A were also found to be under-expressed in LPeri animals. These proteins are part of the CCT complex. Because of its function to assist in the folding of other proteins (Valpuesta et al., 2002), it has been suggested that this complex has an important function in spermatogenesis by being involved in the cytodifferentiation of spermatids (Souès et al., 2003, Dun et al., 2011). Moreover in mature spermatozoa this complex has been observed to play an intermediary role presenting adhesion molecules to the zona pellucidae proteins to facilitate the sperm-oocyte binding (Redgrove et al., 2011, Dun et al., 2011). Surprisingly, no studies have focused in the possible role of CCT in sperm maturation in spite of the fact that these proteins have been found to reside in the epididysomes, which are involved in sperm maturation (Dun et al., 2012). It might be possible that a lower expression of these proteins in the epididymal fluid of the LPeri animals could affect the composition of the CCT complex and hence alter sperm maturation, since the changes in protein structure that take place during the pass of the sperm through the epididymis are essential for a correct maturation. Interestingly, PSMA1, a protein part of the UPS, was under-expressed in LPeri animals which it might indicate two things: 1) the number of aberrant sperm is lower in this group, 2) the abnormal sperm have not been detected by the proteins present in the epididymis.

4.4.2.2 LPost vs HPost

Eighteen common differentially over-expressed proteins and 42 common differentially under-expressed proteins were observed in the comparison

between LPost vs HPost. Which represents the comparison with the highest number of differentially expressed proteins. Only under-expressed proteins were prioritised in this comparison, since are the ones that have been investigated for its role in male fertility. They were: PSMA6, GSN, PTGDS, SPAM1, ELSPBP1, CSTA, CRISP1, CRISP3, RNASE10, ADAM7 and PSAP.

The function of ELSPBP1 is not yet well known, however its similarity with the binder of sperm proteins (BSPs) suggest that it is implicated in lipid binding and sperm plasma membrane interaction and therefore they have different roles in sperm physiology (D'Amours et al., 2012b). This protein is secreted by epithelial cells and it is transferred by epididysomes to dead sperm (D'Amours et al., 2012b, D'Amours et al., 2012a). In addition this protein has been found to be over-expressed in subfertile animals (D'Amours et al., 2010). The fact that this protein is under-expressed in LPost animals may indicate that the number of dead spermatozoa in the epididymis is lower than in the HPost group or the dead sperm are not being recognized. In addition PSMA6, a protein part of the UPS, was also found to be under-expressed in this group. This suggests that the number of defective sperm was lower, or that dead sperm recognition is not appropriate, which supports the above argument. Several of the under-expressed proteins were related to sperm motility, including CTSA, RNASE10, ADAM7, PSAP, GSN and PTGDS. CTSA is a lysosomal enzyme that associates with β-D-galactosidase and N acetyl-α-neuraminidase to create a fully functional and stable enzyme complex (Zhou et al., 1996) and it is present in the epididymis in a specific region manner (Luedtke et al., 2000). CTSA Knockout mice showed structural alterations in epididymis cells and also a

reduction in sperm population and motility (Hermo et al., 2007). RNASE10 is secreted and reabsorbed in the epididymal caput and does not have ribonucleolytic activity (Castella et al., 2004). RNASE10 knock out mice showed a sperm impairment in ascending the female reproductive tract and also in binding to the zona pellucida (Krutskikh et al., 2012). Interestingly this protein does not have direct interaction with the sperm (Castella et al., 2004, Krutskikh et al., 2012). ADAM7 is a transmembranose protein expressed by epididymis and male and female anterior pituitary (Cornwall and Hsia, 1997). The protein, expressed in the epididymis, is transferred to the spermatozoa surface (Oh et al., 2005, Choi et al., 2015a). ADAM7 knock out mice showed a reduction in the ability of fertilize eggs and sperm tail deformations *in vivo* (Choi et al., 2015a). It was also observed a reduction in motility, alteration of tail morphology and lower levels of tyrosine phosphorylation, an essential protein in capacitation, under *in vitro* conditions (Choi et al., 2015a). PSAP is secreted by Sertoli cells and epididymal principal cells and it has been described to increase binding capabilities of frozen sperm in its presence (Hammerstedt et al., 2001). The lack of this protein in mice treated with polychlorinated biphenyls, a chemical that has been associated with a reduction of this protein in caput epididymis, showed a reduction of sperm concentration and motility (Cai et al., 2015). PTGDS is a lipocalin secreted mainly by Sertoli cells but also by Leydig cells, germ cells and epididymis cells (Gerena et al., 2000, Leone et al., 2002). In addition it has been observed that this protein is abundant in compartment beyond blood-tissue barriers, such as seminal fluids (Heshmat et al., 2008, Melegos et al., 1996). It has been discovered that a greater concentration of

PTGDS in seminal plasma is beneficial for sperm motility, morphology and density (Diamandis et al., 1999, Heshmat et al., 2008), in contrast a reduction of the expression of this protein was found in azoospermic and vasectomized patients (Diamandis et al., 1999). Despite its functions in male fertility is not yet clear many investigators think, due to its affinity for lipophilic ligands, that it may be related with its capability of transfer retinoic acids, thyroid hormones and essential fatty acids beyond the testis barrier, which would be essential for the developing of germ cells in the seminiferous tubules and the maturation of spermatozoa in the epididymis (Heshmat et al., 2008, Diamandis et al., 1999, Leone et al., 2002). As we extracted this protein from epididymal fluid, it is likely this protein in these animals could be linked to sperm maturation and hence the under-expression of PGTDS in LPost animals will impair this process. Finally, GSN is an actin-binding protein responsible of polymerize and depolymerize F-actin. It is activated by Ca^{2+} and inhibited by Phosphatidylinositol 4,5-bisphosphate (PIP_2) (Finkelstein et al., 2010, Finkelstein et al., 2013, Breitbart and Finkelstein, 2015). It has been discovered that F-actin is polymerized in the tail of the spermatozoa during capacitation where it plays a role in sperm hyperactive motility acquisition, however it must be depolymerized in the head of the spermatozoa to allow the acrosome reaction to take place. Due to its control over F-actin gelsolin is key in these two processes (Finkelstein et al., 2010, Finkelstein et al., 2013, Breitbart and Finkelstein, 2015). In our study gelsolin was under-expressed in LPost animals, which due to the closeness to the capacitation process it may negatively influence the capability of the sperm to acquire hyperactive motility. Taking all of this evidence together it can be

predicted that males undernourished *in utero* during the first trimester of gestation may present with a reduction in sperm motility. Moreover, two proteins from the CRISP family were found to be under-expressed in LPost. CRISP1 is involved in several steps of male fertility. This protein is secreted to the lumen by the epididymis epithelia and added to the sperm during maturation (Kohane et al., 1980). CRISP1 is expressed in two forms D and E, and while the former binds in a reversible manner to the sperm and is almost completely lost once the sperm leave the epididymis, the latter is tightly bound to the sperm and it is found to be implicated in sperm – egg fusion (Roberts et al., 2008, Kohane et al., 1980, Roberts et al., 2006, Busso et al., 2007). The D form it is thought to inhibit sperm capacitation in the epididymis (Kohane et al., 1980, Roberts et al., 2008). CRISP3 has been suggested to supress the binding of polymorphonuclear neutrophils to spermatozoa and phagocytosis which is important in the transport of the sperm to the oviduct (Doty et al., 2011). Hence, a lack of these two proteins in the epididymal fluid of LPost animals could lead to an early capacitation of the sperm and less protection against female immune cells, which will kill the sperm on their way to the oocyte. In addition, under-expression of a protein involved in sperm-ZP binding was also found in LPost animals (SPAM1). SPAM1 is a Glycosyl phosphatidylinositol (GPI) linked form of hyaluronidase, which are enzymes that catalyse the hyaluronic acid present in the extracellular matrix (ECM) (Baba et al., 2002, Modelski et al., 2014). SPAM1 is expressed in sperm but also in the epididymis where they are transferred to sperm surface during maturation (Deng et al., 2000, Modelski et al., 2014, Zhang et al., 2004b). Because the zona pellucida is an abundant supply

of ECM surrounding the mammalian oocyte, it is thought that SPAM1 is involved in sperm-ZP binding by catalysing the hyaluronic acid and allowing the sperm to pass through the cumulus matrix of the oocyte (Baba et al., 2002, Morin et al., 2010, Kimura et al., 2009, Zhang et al., 2004b, Primakoff and Myles, 2002, Primakoff et al., 1985). With all this it is likely that sperm from LPost animals may have difficulties in fertilizing the ova.

In this experiment we found that a low protein diet in both peri- and post-conception periods could result in a negative impact on male fertility, however the effects were more pronounced when the animals were undernourished during the first trimester of gestation. Sperm maturation and capacitation were the steps most likely to be affected in LPeri animals when the functions of the altered proteins are considered. In the LPost group sperm motility could be the most affected process with 6 proteins that are thought to be involved in this process under-expressed in these animals. Proteins related with capacitation, immune protection and sperm – ZP binding were also under-expressed in LPost animals. Surprisingly, proteins related with marking and destruction of abnormal sperm were under-expressed in both LPeri and LPost groups.

In conclusion, although the different dietary treatments applied in this study did not showed histological alterations in the epididymis structures of 20 month old bulls, at a molecular level changes in the expression of proteins from the epididymal fluid linked to male infertility/ subfertility were observed in the bulls from cows fed a low protein diet from day 60 before AI to 23 dpc and from 24

dpc to day 98 of gestation. These changes were more pronounced when the restriction was imposed during the first trimester of pregnancy.

Chapter 5 The effect of maternal dietary protein on bovine thyroid development and thymus gene expression on 98 dpc animals

5.1 Introduction

In the Australian cattle industry, bacterial and parasite infections and viral diseases represent a huge economical loss affecting both the health of the animals (Sackett et al., 2006) and also the reproductive performance (Bellows et al., 2002) . A strong immune system is essential to protect against external pathogens and it has been proven that maternal environmental changes, including malnutrition, will have an impact in the development of the offspring's immunity (Calder and Yaqoob, 2000, Badr and Mohany, 2011, He et al., 2014). It is also well known that endocrine hormones play an important role in the function of the immune system (Pierpaoli et al., 1969, Fabris, 1973, De Vito et al., 2012). In this chapter the effects of low dietary protein during the peri – conception and first trimester of gestation on the histology and global gene expression in thyroid and thymus glands, respectively, of 98 dpc bovine fetus were evaluated. In this chapter male and female fetal tissues were assessed, since they both have the same organs implicated on the function of the immune system.

5.1.1 Hypothesis

We hypothesised that the deprivation of protein in maternal diets during the peri – conception and first trimester of gestation will impact the development of crucial organs implicated in the establishment of a strong immune system. In this experiment we focused in the thymus, as it is an essential organ in the

development of the immune system and also the thyroid for the implication of the thyroid hormones in the function of the immune system.

5.2 Methods

5.2.1 Animals and feeding regime

The experiment design and ethics and sample collection were as described in Chapter 2 Section 2.2.1.

5.2.2 Sample acquisition and processing

Thymus and thyroid tissues were dissected at 98 dpc. The thymus glands were dissected and stored at -80°C immediately after fetal dissection. RNA sequencing and RT-qPCR was performed in this tissue as was described in Chapter 2 Section 2.9. The thyroid gland was processed and stained as described in Chapter 2 Section 2.2.1 and 2.4.5, respectively. This tissue was stained and sectioned at 8µm by Alan Lanslett from the Department of Pathology, University of Nottingham.

5.2.3 Image analysis

Stained samples were photomicrographed using a Leica DM5000 microscope, with Leica CTR5000 light box and Leica DFC420 colour capture camera (all from Leica, Germany), following systematic random sampling and stereology (Mayhew, 2011) methodology. Photomicrographs were taken avoiding overlapping to ensure that double counting could not occur.

In order to measure the size, number and proportion of the thyroid follicles within the tissue, 5 photomicrographs were taken at 40X magnification. In total 220 photomicrographs were taken, n= 5FLL, 5FLH, 5FHL, 6FHH, 4MLL, 5MLH, 8MHL and 6MHH.

Using an image analysing program (Image-pro plus (version 6.3), Media Cybernetics, USA) thyroid follicles were manually drawn around and the area of the follicles was calculated using the software. Following calibration of the imaging software, the average size, number and percentage of the thyroid follicles within the tissue was calculated per photomicrograph.

5.2.4 Statistical analysis

For the statistical analysis of the thyroid tissue multifactorial ANOVA was used to determine the effects of maternal nutrition during peri – conception and first trimester, progeny sex and their interaction terms. One tailed T-test was selected for the statistical analysis of the RT-qPCR results. ANOVA was performed using SPSS statistic software (IBM SPSS statistic 24) whereas for T-test analysis Graph pad prism 7 software was used.

5.3 Results

5.3.1 Effect of low protein diet on thyroid development

Using image-pro plus software the area of the follicles were measured. With the data obtained the average size and number of follicles and also the percentage of follicles within the thyroid was calculated. In total 1514 follicles were measured. In this chapter both sexes, male and female, were analysed so in addition to investigating dietary differences, the two sexes were also analysed. No significant differences in follicular measurement were observed between the comparisons of the 4 different diets or peri- or post- conception periods, or between the male and females in any of the parameters measured (Figure 5.1; $P>0.05$).

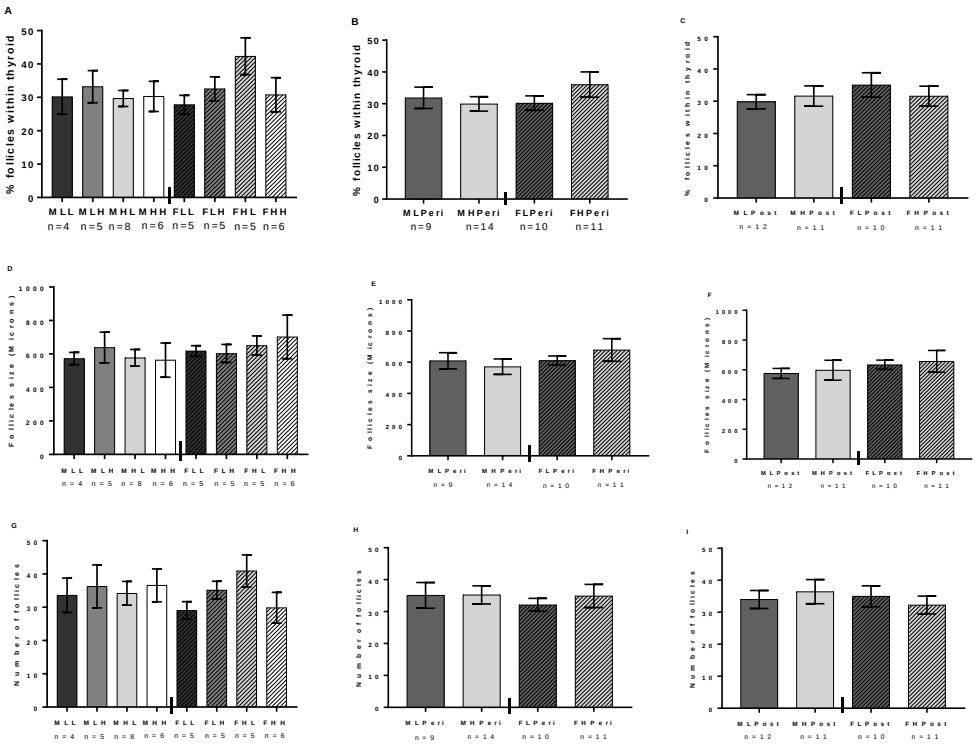


Figure 5.1 Percentage of follicles within fetal thyroid A) In the 4 different diets B) In high and low protein diet comparison in the peri - conception period C) In high and low protein diet comparison in the post - conception period; Area of follicles within fetal thyroid D) In the 4 different diets E) In high and low protein diet comparison in the peri - conception period F) In high and low protein diet comparison in the post - conception period; Quantification of fetal thyroid follicles G) In the 4 different diets H) In low and high protein diet comparison in the peri - conception period I) In low and high protein diet comparison in the post-conception period. Data were analysed using two-way ANOVA. Graphs indicate mean±SEM. P<0.05 were considered significant, no significant differences observed.

5.3.2 Effect of low protein diet on thymus gene expression

5.3.2.1 Differentially expressed genes

The thymus tissues were collected from the 98 dpc male and female fetuses. Eight samples were sent for RNA sequencing (2 MLL, 2 MHL, 2 MHH and 2 FHH). With these 4 groups, 6 comparisons were made (Table 5.1). Most of the differentially expressed genes identified were down regulated in the low protein diets compared with control diets, however sex differences were less pronounced.

Table 5.1 Number of up and down regulated genes in each comparison

Comparison	*Up regulated*	*Down regulated*
MHL vs MLL	14	2
MHH vs MLL	16	2
MHH vs MHL	1	0
FHH vs MLL	21	5
FHH vs MHL	6	6
FHH vs MHH	2	9

MLL= Males fed low protein diet in both peri – and post – conception periods.
MHL= Males fed high protein diet in the peri – conception period and low protein diet in the post – conception period.
MHH= Males fed high protein diet in both peri – and post – conception period.
FHH= Females fed high protein diet in both peri – and post – conception period.

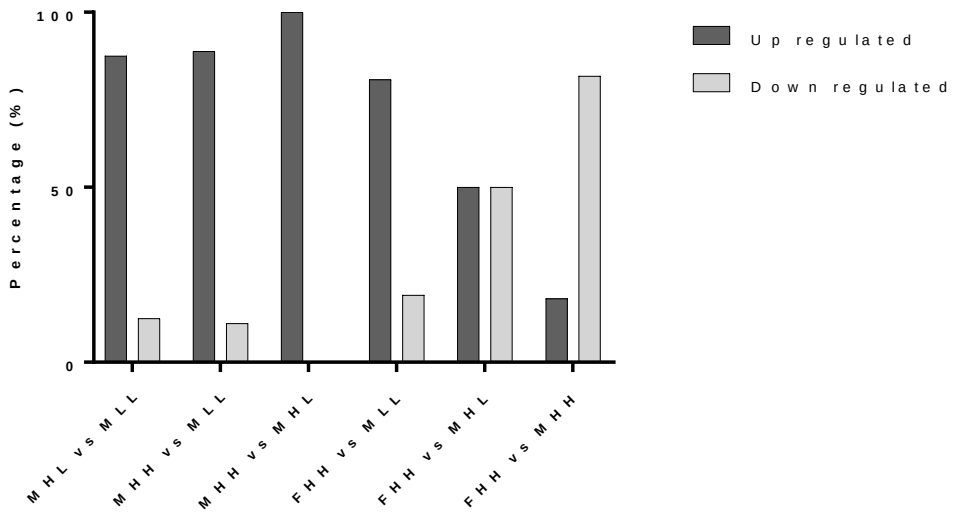


Figure 5.2 Percentage of up and down regulated genes within each gene comparison. The number of differentially expressed genes within each comparison was transformed to show percentages.

5.3.2.2 Differentially gene expression in MLL compared with MHL and MHH

The number of differentially expressed genes in the two comparisons (MHH vs MLL and MHL vs MLL) was similar, with 18 and 16 differentially expressed genes respectively. The two comparisons shared the majority of differentially expressed genes and all were up regulated. Other genes were only expressed in one or the other comparison, for example Rho associated coiled-coil containing protein kinase 1 (ROCK1), which was differentially expressed only in the MLL vs MHL comparison and bromodomain adjacent to zinc finger domain 2B (BAZ2B), remodelling and spacing factor 1 (RSF1) and coiled-coil domain containing 18 (CCDC18), which were present exclusively in the comparison of MLL vs MHH. These results suggest that the biggest impact in the development of the thymus was caused by deprivation of protein during the peri - conception

period (-60 to 23 dpc). Table 5.2 shows the log2FC of the common differentially expressed genes, excluding those with 0 FPKM expression in one of the conditions, in the comparison of MLL with MHL and MHH. In both comparisons the genes with the highest log2FC were alpha thalassemia/mental retardation syndrome X-linked (ATRX; a chromatin remodeler), eukaryotic translation elongation factor 1 alpha 1 (EEF1A1) and ankyrin repeat domain 12 (ANKRD12). Another over-expressed gene in the supplemented diets that plays a crucial role in immune system was the bovine major histocompatibility complex, class II, DQ beta (Bola – DQB).

The gene ontologies (GO) of the common differentially expressed genes in MHL and MHH compared with LL showed enriched categories in: GO:0032259 methylation (Table 5.3). The main pathways, results genes and transcription factors obtained using cytoscape (genemania) are shown in Table 5.4. The pathways altered in these comparisons were thymic stromal lymphopoietin (TSLP) and Epidermal Growth Factor Receptor 1 (EGFR1). From the results the transcription factor LEF1 (Lymphoid Enhancer Binding Factor 1) is notable as it is required for early stages of thymocyte maturation (Choi et al., 2015b, Xing et al., 2016).

Table 5.2 Common differentially up regulated genes in the comparisons of MHH and MHL vs MLL (excluding those with 0 FPKM in one of the samples).

		Log2FC	
Ensembl ID	**Gene symbol**	**MHH vs MLL**	**MHL vs MLL**
ENSBTAG00000038434	*ATRX*	5.34924	5.34522
ENSBTAG00000034185	*EEF1A1*	3.93504	3.91843
ENSBTAG00000002755	*ANKRD12*	3.80665	3.80994
ENSBTAG00000017469	*CCDC88A*	3.27779	3.26971
ENSBTAG00000000222	*ARID4B*	3.20711	3.19139
ENSBTAG00000024688	*PHIP*	3.14299	3.11311
ENSBTAG00000001821	*PRRC2C*	2.94688	2.93365
ENSBTAG00000019588	*BOLA-DQB*	2.6895	2.01877
ENSBTAG00000018898	*ARID4A*	2.65542	2.61518
ENSBTAG00000014609	*PPP1R12A*	2.53786	2.52497
ENSBTAG00000006869	*PHF20L1*	2.49025	2.50491

Table 5.3 Gene ontologies (GO) categories for common up regulated genes in the comparison between MHL and MHH vs MLL.

Gene ontology term	**Category**	**Set size**	**Candidates contained**	**q-value**
GO:0032259 methylation	BP 2	340	3 (0.9%)	0.0204

Including GO terms, GO categories, set size, number of genes present in the set and q value.

Table 5.4 Cytoscape (Genemania) analysis of the common up-regulated genes in the MHL and MHH vs MLL comparisons, showing the pathways, suggested extra genes (result) and inferred transcription factors.

Pathway	Results	Transcription factor
TSLP	*SUDS3*	NFY
EGFR1	*KAT8*	LEF1
	TADA3	MAZ
	ING2	CREB
	H3F3B	SRF
	ZBED1	FREAC2
	HINFP	E2F1
	SAP130	E2F
	HOOK3	E2F1DP1RB
	HOOK2	YY1
	HOOK1	NRF1
	BRMS1	FREAC3
	STMN4	
	ELAVL4	
	HLA-DQA1	
	HLA-DQA2	
	PRRC2B	
	PRRC2A	
	CCDC88B	
	CCDC88C	

5.3.2.3 FHH vs MLL differential gene expression

In the comparisons between FHH and MLL, where two different variables existed: sex and diet; 26 differentially expressed genes were found (Table 5.5). As found in the males fed with supplemented protein diet (MHH) compared with MLL, *ATRX, EEF1A1* and *ANKRD12* were some of the highest differentially expressed genes, and so this adds evidence to the theory that it is the diet

which is affecting these genes. Other genes differentially expressed which were also common to the comparison between MHH and MLL were *CCDC88A, ARID4B, PRRC2C, PHIP, ARID4A, PPP1R12A, BAZ2B, ROCK1, RSF1* and *PHF20L1*. The remaining genes were not differentially expressed in the comparisons of males fed different diets and are therefore more likely to be influenced by the sex. These were: HMG-box containing (*BBX*), early endosome antigen 1 (*EEA1*), chromodomain helicase DNA binding protein 9 (*CHD9*), A-kinase anchoring protein 9 (*AKAP9*), structural maintenance of chromosomes 2 (*SMC2*), splicing regulatory glutamic acid and lysine rich protein 1 (*SREK1*) and DExD-box helicase 21 (*DDX21*). Interestingly the only under-expressed gene in the comparison between FHH vs MLL was a pseudogene of *EEF1A1*, one of the highest up regulated genes in the same comparison.

The gene ontologies (GO) of the differentially expressed genes in FHH and MLL showed enriched categories in: GO: 0043233 organelle lumen (Table 5.6). In Table 5.7 the main pathways are shown, in addition to the results of genes and transcription factors obtained using the Cytoscape Genemania plugin in the differentially expressed genes present in this FHH vs MLL comparison. Two important pathways in the development of the immune system were RhoA signaling pathway and TSLP. LEF1 was highlighted in the suggested transcription factors for its function in the maturation of thymocytes (Choi et al., 2015b, Xing et al., 2016).

Table 5.5 Up and down regulated genes in the comparison between FHH vs MLL (excluding those with 0 FPKM in one of the samples).

Ensembl ID	Gene symbol	Log2FC
ENSBTAG00000038434	*ATRX*	5.31048
ENSBTAG00000006893	*BBX*	4.26337
ENSBTAG00000034185	*EEF1A1*	3.92408
ENSBTAG00000002755	*ANKRD12*	3.81111
ENSBTAG00000024688	*PHIP*	3.3211
ENSBTAG00000000421	*EEA1*	3.22243
ENSBTAG00000017469	*CCDC88A*	3.10372
ENSBTAG00000000222	*ARID4B*	2.98958
ENSBTAG00000001821	*PRRC2C*	2.80301
ENSBTAG00000014609	*PPP1R12A*	2.66536
ENSBTAG00000018898	*ARID4A*	2.60932
ENSBTAG00000020654	*BAZ2B*	2.44009
ENSBTAG00000002287	*CHD9*	2.38955
ENSBTAG00000006869	*PHF20L1*	2.33895
ENSBTAG00000020268	*RSF1*	2.22942
ENSBTAG00000007442	*AKAP9*	2.1962
ENSBTAG00000008403	*ROCK1*	2.15689
ENSBTAG00000008772	*SMC2*	2.13794
ENSBTAG00000007321	*SREK1*	2.06343
ENSBTAG00000014558	*DDX21*	1.87854
ENSBTAG00000022796	*EEF1A1 Pseudogene*	-5.42536

Table 5.6 Gene ontologies (GO) categories for up and down regulated genes in the comparison between FHH vs MLL.

Gene ontology term	Category	Set size	Candidates contained	q-value
GO:0043233 organelle lumen	CC 2	4421	12 (0.3%)	0.00911

Showing the GO terms, GO categories, set size, number of genes present in the set and q value.

Table 5.7 Cytoscape (Genemania) analysis of the FHH vs MLL up and down regulated genes, showing the pathways, suggested extra genes (result) and suggested transcription factors.

Pathway	Results	Transcription factor
Cell Cycle	*NR1I3*	LEF1
Cell Cycle, Mitotic	*SUDS3*	IRF1
RhoA signaling pathway	*HINFP*	E2F
Vascular smooth muscle contraction	*ZBED1*	PAX4
TSLP	*TADA3*	FOXO3
Mitotic G2-G2 M phases	*ING2*	E2F1
G2 M Transition	*SRPK3*	DR3
	KAT8	E2F
	TARBP1	IRF2
	NCAPG2	CART1
	CENPA	IRF
	SAP130	SRF
	NCAPH2	
	PXK	
	APTX	
	NCAPD2	
	STMN4	
	ELAVL4	
	SAMD9L	
	SAMD9	

5.3.2.4 FHH vs MHL differential gene expression

The comparison between FHH and MHL showed 12 differentially expressed genes. Only 3 of these genes were expressed in both groups (not 0 FPKM; Table 5.8). One of these genes was over-expressed in the FHH group compared to the MHL group, the predicted multidrug resistance-associated protein 4-like gene. From the two under-expressed genes, the gene with the highest Log2FC was a pseudogene of the *EEF1A1* gene and the other was *BOLA – DQB*.

The gene ontologies (GO) of the differentially expressed genes in FHH and MHL showed no enriched categories. In Cytoscape (genemania), all the pathways showed for these genes were involved in a correct functionality of the immune system (Table 5.9).

Table 5.8 Up and down regulated genes in the comparison between FHH vs MHL (excluding those with 0 FPKM in one of the samples).

Ensembl ID	Gene symbol	Log2FC
ENSBTAG00000039714	Predicted: multidrug resistance-associated protein 4-like	3.17902
ENSBTAG00000019588	*BOLA-DQB*	-2.11444
ENSBTAG00000022796	*EEF1A1 Pseudogene*	-5.19739

Table 5.9 Cytoscape (Genemania) analysis of the FHH vs MHL up and downs regulated genes, showing the pathways, suggested extra genes (result) and suggested transcription factors.

Pathway	Results
Type I diabetes mellitus	*HLA-DMA*
Interferon gamma signalling	*HLA-DRB1*
Autoimmune thyroid disease	*HLA-DMB*
Rheumatoid arthritis	*HLA-DRA*
Downstream TCR signalling	*CD3E*
MHC class II antigen presentation	*CD3G*
Leishmaniasis	*PIK3R2*
Costimulation by the CD28 family	*HLA-DQA1*
TCR signalling	*HLA-DRB5*
Antigen processing and presentation	*CD4*
Viral myocarditis	*HLA-DQA2*
	CD3D
	HLA-DOA
	HLA-DOB
	HLA-DPB1
	HLA-DPA1
	PDPK1
	HLA-DRB4
	HLA-DQB2
	HLA-DRB3

5.3.2.5 FHH vs MHH differential gene expression

In the comparison between FHH and MHH 11 genes were differentially expressed. Only 3 of these genes were expressed in both groups (not 0 FPKM; Table 5.10) and they were under-expressed in FHH compared with MHH. These genes were, from the lowest to the highest Log2FC, *EEF1A1 Pseudogene, BOLA*

– *DQB* and *BOLA DQA1* (Bovine major histocompatibility complex, class II, DQ alpha, type 1).

The gene ontologies (GO) of the differentially expressed genes in FHH and MHH showed no enriched categories. The pathways provided by cytoscape (genemania) for these genes were involved in the immune system function (Table 5.11).

Table 5.10 Down regulated genes in the comparison between FHH vs MHH (excluding those with 0 FPKM in one of the samples).

Ensembl ID	Gene symbol	Log2FC
ENSBTAG00000037605	*BOLA-DQA1*	-1.72401
ENSBTAG00000019588	*BOLA-DQB*	-2.78517
ENSBTAG00000022796	*EEF1A1 Pseudogene*	-5.10078

Table 5.11 Cytoscape (Genemania) analysis of the FHH vs MHH down regulated genes, showing the pathways, suggested extra genes (result) and suggested transcription factors.

Pathway	Results
Viral myocarditis	*HLA-DMA*
Rheumatoid arthritis	*HLA-DRB1*
Type I diabetes mellitus	*HLA-DMB*
Interferon gamma signalling	*HLA-DRA*
Costimulation by the CD28 family	*CD3E*
TCR signalling	*CD3G*
MHC class II antigen presentation	*LCK*
Leishmaniasis	*CD4*
Antigen processing and presentation	*PIK3R2*
Autoimmune thyroid disease	*HLA-DRB5*
Downstream TCR signalling	*HLA-DQA2*
	CD3D
	HLA-DOA
	HLA-DOB
	HLA-DPB1
	HLA-DPA1
	PDPK1
	HLA-DRB4
	HLA-DQB2
	HLA-DRB3

5.3.2.6 RT-qPCR validation and specific gene targets

Validation of the RNA sequencing results was carried out using RT-qPCR. The differentially expressed genes commonly identified in most of the comparisons and with the highest differential count and Log2FC (not less than 2) were selected for validation. These genes were *ARID4A, ARID4B* and *ROCK1*. Other genes, *Bola DQB* and *Bola DQA1*, showed a high differential count and Log2FC in the different comparison, however due to the high levels of polymorphism

of these particular genes between animals it was not possible to undertake validation of the results obtained in the RNA sequencing using Taqman assay based RT-qPCR analysis. Given time constraints of the project it was not possible to develop and optimize SYBR green based assays for these genes.

5.3.2.6.1 ARID4A (AT-rich interaction domain 4A)

ARID4A showed a lower Fragments Per Kilobase of transcript per Million (FPKM) mapped reads in the MLL animals compared with MHL, MHH and FHH in the RNA sequence (Figure 5.3, A). However only in the comparison between MLL and MHH was a significantly smaller relative expression of this gene observed when using RT-qPCR (Figure 5.3, B-D).

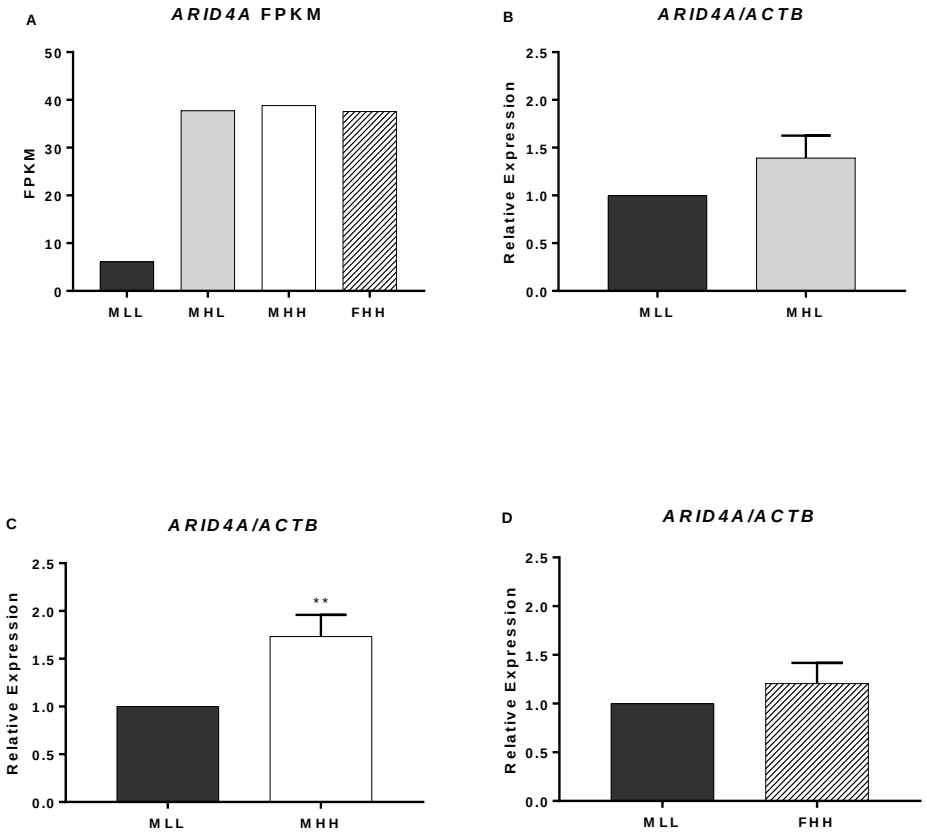


Figure 5.3 Quantification of *ARID4A* expression showing A) the RNAseq FPKM, RT-qPCR quantification normalized to expression of ACTB in B) MLL vs MHL, C) MLL vs MHH and D) MLL vs FHH in bovine 98 dpc thymus. M=male, F=Female, LL=Low/Low, HL=High/Low, HH=High/High. T-test, ** represents P<0.01.

5.3.2.6.2 ARID4B (AT-rich interaction domain 4B)

Results from RNA sequencing showed a lower FPKM expression of *ARID4B* in the MLL group compared with the MHL, MHH and FHH (Figure 5.4, A). When these results were compared using RT-qPCR the relative expression of ARID4B was downregulated in MLL compared with MHL, MHH and FHH (Figure 5.4, B-D).

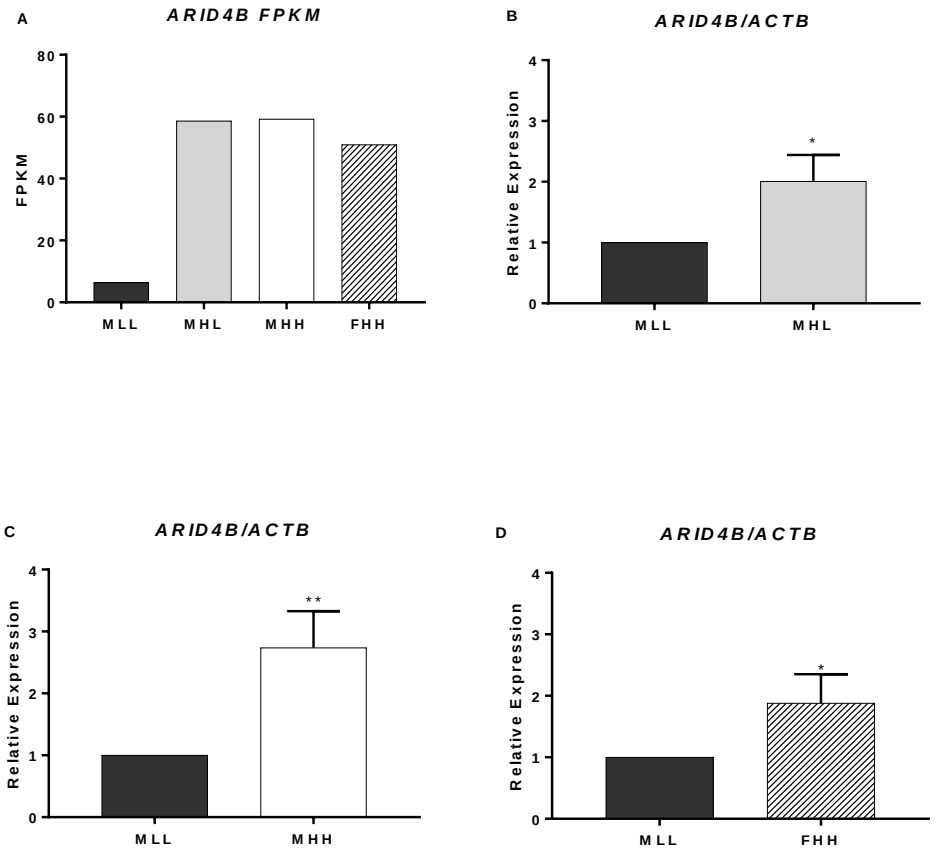


Figure 5.4 Quantification of *ARID4B* expression showing A) the RNAseq FPKM, RT-qPCR quantification normalized to expression of ACTB in B) MLL vs MHL, C) MLL vs MHH and D) MLL vs FHH in bovine 98 dpc thymus. M=male, F=Female, LL=Low/Low, HL=High/Low, HH=High/High. T-test, * represents P<0.05, ** represents P<0.01.

5.3.2.6.3 ROCK1 (Rho associated coiled-coil containing protein kinase 1)

ROCK1 FPKM expression was reduced in the MLL group compared with MHL and FHH as is shown in Figure 5.5 A. These results were validated with RT-qPCR, where both comparisons showed a downregulation in the relative expression of this gene in MLL (Figure 5.5 B-C).

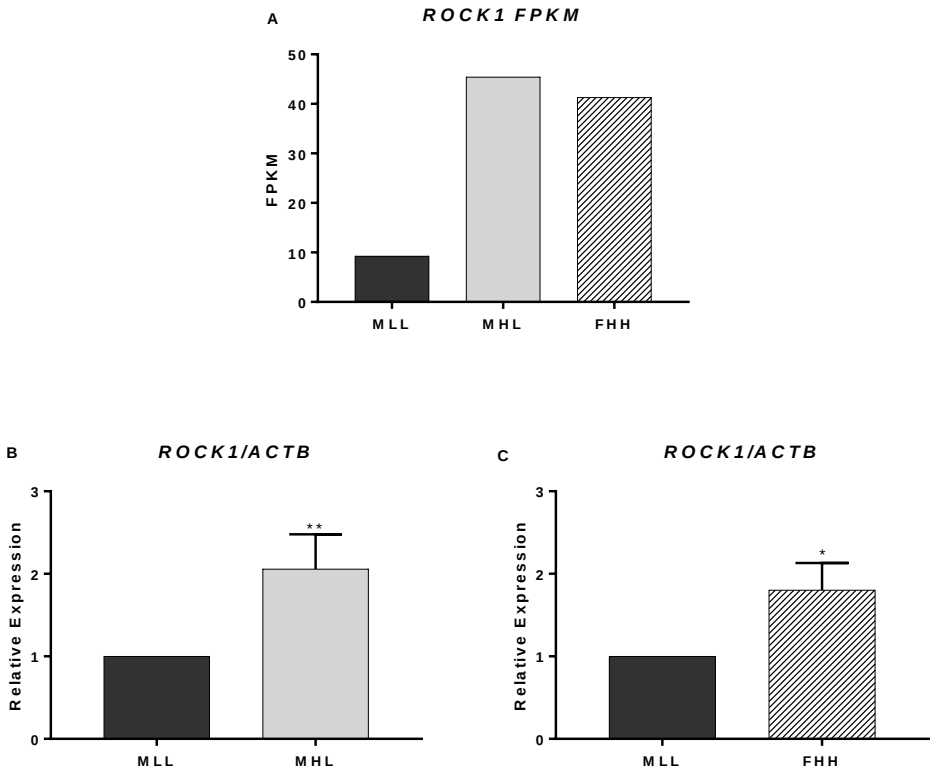


Figure 5.5 Quantification of *ROCK1* expression showing A) the RNAseq FPKM, RT-qPCR quantification normalized to expression of ACTB in B) MLL vs MHL, C) MLL vs FHH in bovine 98 dpc thymus. M=male, F=Female, LL=Low/Low, HL=High/Low, HH=High/High. T-test, * represents P<0.05.

5.4 Discussion

5.4.1 Protein deprivation effects on thyroid development

It has been well documented that thyroid hormones play a role in the correct function of the immune system. Various studies have focused on the role of these hormones in the onset of the immune response against an infection showing that in hyperthyroidism the immune response is enhanced whereas in hypothyroidism the response is diminished (Klecha et al., 2000, Klecha et al., 2006, De Vito et al., 2012). Furthermore, it has been observed that there is a positive interaction between thyroid hormones and thymus development and function (Pierpaoli et al., 1969, Fabris, 1973). Fetal programming is a well-accepted term for the process where maternal environmental changes affect the development of the fetus. Within these environmental changes, diet has been highlighted as a key factor with protein intake being one of the main components affecting fetal development. Despite of this knowledge only few experiments have focused in the study of protein restriction during pregnancy in relation to the concentration of thyroid hormones in the offspring and, as far as we know, none in the histological development of the thyroid.

In this study the number, size and percentage of follicles within the thyroids of fetal bovine from mothers fed a supplemented and unsupplemented diet during the peri - conception and/or first trimester of gestation were calculated. No significant differences were found between all of the comparisons including differences in treatment, time period or sex. These results were consistent with a study performed by Heasman et al. (2000), where 144 fetus from sheep fed

with a restricted protein diet from day 28 to mid-gestation showed no significant differences in plasma concentrations of T3 and T4. Moreover in a study carried out in rats fed restricted protein diets during gestation and lactation it was also observed that the 65 day old offspring showed no significant differences in their plasma levels of T3 and T4 (Qasem et al., 2012). In contrast, our results differ from the only study performed in beef in which the progeny of beef cattle fed a restricted protein diet from conception to the end of the second trimester showed significant differences in plasma concentrations of T3 and T4, in relation to both diet and sex, during the first three months of life. However by day 191 no significant differences were found between any of the groups (Micke et al., 2015). The fact that our results do not concur with the latter study might be related with the protein restriction time frame used in both experiments. While in the Micke et al. (2015) study the protein was restricted until the end of the second trimester in our study the diet was restricted only until the end of the first trimester. It is also important to take into consideration that our samples were taken from the fetus and not from neonates. All this data suggests that genetic changes in the fetal thymus are not highly linked to alterations in thyroid development.

5.4.2 Protein deprivation effects on thymic gene expression

In this experiment it was observed that both diet and sex have an impact in the development of the immune system. Several genes were differentially expressed only in the female HH group compared to male LL and others were shared between all of the supplemented animals, regardless of the sex. The

genes differentially expressed exclusively in the FHH vs MLL comparison were mostly involved in RNA replication and cell differentiation, including HMG-box containing (*BBX*), a transcription factor which has been found to be involved in stem cells and progenitors differentiation (Dixon et al., 2013, Choi et al., 2014, Wang et al., 2016); early endosome antigen 1 (*EEA1*), a coil – coiled protein present in the cytoplasm face of early endosomes and the cytosol. This protein has an important role in endocytosis by promoting endosome vesicle fusion (Mu et al., 1995, Waite et al., 1998, Selak et al., 2003); chromodomain helicase DNA binding protein 9 (*CHD9*), an ATP-dependent chromatin remodelling enzyme which has been found to be expressed during the differentiation of osteoprogenitors (Salomon-Kent et al., 2015, Shur et al., 2006); structural maintenance of chromosomes 2 (*SMC2*), that belongs to the structural maintenance of chromosomes protein family and plays a key role in chromatin segregation and condensation (Strunnikov et al., 1995, Stursberg et al., 1999); splicing regulatory glutamic acid and lysine rich protein 1 (*SREK1*), the only member of the SR protein family which contains a glutamic acid – lysine domain between the serine-arginine (SR)-rich domains. While SR proteins are key regulators of RNA splicing, it seems that the function of *SREK1* is the regulation of the SR proteins, both positively and negatively (Li et al., 2002, Li et al., 2003). Moreover, there were two differentially expressed genes in this comparison which have been studied for their functions in the immune response. These genes were A-kinase anchoring protein 9 (*AKAP9*), found to be a regulator of microtubules, structures that are involved in polarization and migration of T cells (Herter et al., 2015) and DExD-box helicase 21 (*DDX21*), an RNA helicase

involved in the restriction of virus replication (Dong et al., 2016). This latter protein also plays a role in the activation of type I interferon (IFN) and pro - inflammatory cytokine responses in myeloid dendritic cells by forming a complex with *DDX36* and TRIF and binding to the Poly I:C, which is a synthetic form of RNA which mimics double-stranded viral RNA (Zhang et al., 2011). In addition, in macrophages, *DDX21*-TRIF complex seems to activate the production of S100A9 protein, which is a protein that induces pro - inflammatory responses and regulates apoptosis in virus infections (Tsai et al., 2014). Therefore, with the known function of these genes it may be possible that the replication and movement of the thymocytes and also the response against viruses would be reduced in FHH compared with MLL.

In the comparisons where the animals were fed with supplemented protein diets (FHH, MHH and MHL) versus the unsupplemented males the common differentially expressed genes with the highest Log2FC were *ATRX, EEF1A1* and *ANKRD12*. *ATRX* it is key for heterochromatin formation (Sadic et al., 2015) and also it has been found to play an important role in the regulation of telomere chromatin in embryonic stem cells through interaction with H3.3 (universal histone) and *CBX5* (chromobox 5) (Wong et al., 2010). *EEF1A1* is a GTP binding protein, which transfers the aminoacyl-tRNA to an A site of the ribosome during elongation in translation (Koiwai et al., 2008). It is also involved in apoptosis and degradation of damaged proteins through the Ubiquitin Proteasome System (UPS) (Chuang et al., 2005, Koiwai et al., 2008). *ANKRD12* has both repression and activation domains and can inhibit the transcriptional activity of nuclear receptors through the recruitment of histone deacetylases (HDACs)

(Zhang et al., 2004a, Bai et al., 2013). With these functions in mind, it is possible that the replication and elimination of defective thymocytes may be adversively affected in males fed low protein diet in both peri - and post – conception periods.

ARID4A and *ARID4B* were also up regulated in the comparisons between MHH, MHL and FHH vs MLL and were selected for the validation of the RNA sequencing data using RT-qPCR. However, when the RT-qPCR results were analysed it was found that there were not significant differences in the expression of *ARID4A* in the MHL and FHH versus MLL, although an increase in the expression of this gene in MHL and FHH versus MLL is clear ($P<0.05$). This output differs from the RNAseq results and an explanation for these differences might be the number of animals compared in each technique, which were higher in RT-qPCR and so added more individual variability to these results. *ARID4A* and *ARID4B* are part of the AT- rich interaction domain gene family and they play a role in the development of the immune system through interaction with other genes. For example, *ARID4A* and *ARID4B* participate in the regulation of forkhead box P3 (Foxp3) (Wu et al., 2008). Foxp3 functions as a transcriptional regulator by assembling chromatin remodelling complexes involved in histone modification and it is implicated in the development and function of natural regulatory T cells (Zheng and Rudensky, 2007, Wu et al., 2008). Foxp3 also interact physically with runt related transcription factor 1 (RUNX1/ AML1), a gene which is essentially required for normal haematopoiesis including thymic T-cell development, to control regulatory T cell function (Ono et al., 2007, Wu et al., 2008). In a study performed by Wu et

al. (2008) it was observed that *ARID4A* $^{-/-}$ and *ARID4B* $^{+/-}$ mice showed a reduced expression of Foxp3 while the expression of AML1 was unaffected. With all of this evidence, it would be reasonable to think that in our study a greater expression of *ARID4A* and *ARID4B* in the animals fed with supplemented protein diets might lead to better thymic T cell development and function. It has also been observed that both *ARID4A* and *ARID4B* interact with retinoblastoma protein (RB), a protein which plays a role in many cellular processes, and so they act as repressors of the E2F transcription factor (Wilsker et al., 2005, Wu et al., 2008). RB is widely known for its function as a tumour suppressor due to its interaction with E2F (Lai et al., 1999, Classon and Harlow, 2002, Henley and Dick, 2012, Morales et al., 2014). However it has been found that RB protein also plays a role in immune function by interacting with the major histocompatibility complex (*MHC*). Thus RB-null cells showed a reduction in the expression of *MHC* class I and II proteins, a reduction that was restored with the introduction of RB (Zhu et al., 1999, Eason et al., 2001, Markey et al., 2007). Moreover, Osborne et al. (1997) observed that RB promoted the induction of interferon gamma (IFN-γ) by *MHC* class II, since it facilitated the transcriptional activation of the *MHC* class II gene. In the same study the reconstitution of RB in RB-null cell lines led to a chromatin remodelling at the *MHC* class II DRA promoter. In addition, as a suppressor of E2F, RB plays a crucial cell-autonomous role in thymic epithelial cells (TECs) to control maximal thymic size and aging (Garfin et al., 2013). This is because the E2F transcription factor binds to and regulates the transcription of Foxn1 promoter, which is involved in differentiation and proliferation of TECs, and RB inhibits this

activation (Garfin et al., 2013). The finding of a lower expression of *ARID4A* and *ARID4B* in the LL animals might implicate a reduction in the *MHC* expression, which is consistent with our results. Another two up regulated genes from our results that interact with RB proteins were protein phosphatase 1 regulatory subunit 12A (*PPP1R12A*) and PHD finger protein 20 like 1 (*PHF20L1*). *PPP1R12A* is part of the myosin phosphatase targeting protein family. This gene regulates the catalytic activity of the protein phosphatase 1, which is involved in smooth muscle contraction, cell migration and adhesion and cell cycle regulation (Zhang et al., 2015). Furthermore, Cho et al. (2010) demonstrated that the expression of *PPP1R12A* is a key factor in the regulation of the phosphorylation status of RB1 proteins and hence in the activation of E2F transcription factors. *PHF20L1* is a component of the MOF complex, a complex which participates in transcriptional regulation and has been implicated in several stages of the DNA damage response. The interaction of *PHF20L1* with RB proteins allows the recruitment of the MOF acetyltransferase complex to E2F target genes. Its presence is required for the methylation of RB proteins by MOF acetyltransferase complex, and so with transcriptional control (Carr et al., 2017).

ROCK1, another of the genes used for validation of the RNA sequencing results using RT-qPCR, was up regulated in the comparison of FHH and MHL versus MLL. Interestingly, this gene showed no significant differences between MLL and MHH. *ROCK1* has been widely study for its implication in the immune system. This gene encodes a protein serine/threonine kinase that is activated when bound to the GTP-bound form of Rho (Liu et al., 2016c). It plays a role in

actin reorganization, which is a crucial step for a proper immune function of the macrophages, since all immune functions of macrophages depend on proper organization of their actin cytoskeleton (Liu et al., 2016b, Liu et al., 2016c). It has been observed that inhibition of *ROCK1* obliterates macrophages polarity, motility and phagocytosis (Liu et al., 2016c) and also decreases the recruitment of leukocytes (Noma et al., 2008). Other key roles of *ROCK1* are negative regulation of macrophage and neutrophil function by binding to PTEN and regulating its activation (Vemula et al., 2010) and suppression of the protrusions at the rear of migrating monocytes to allow productive migration (Worthylake and Burridge, 2003). Apart from its role in antigen presenting cells, *ROCK1* is involved in the development and function of T lymphocytes. In a study by Henning et al. (1997) it was observed that Rho had an important role in T lymphocytes intracellular signalling, which makes it essential for normal thymic development. These signals were critical for thymocyte proliferation and cell survival but had not involvement in thymocyte differentiation. Furthermore, other studies demonstrated a function of *ROCK1* in promoting T cell activation and proliferation, with the inhibition of *ROCK1* causing the suppression of cytokine expression, such as interleukin (IL)-2, IFN and IL-13 (Woodside et al., 1998, Tharaux et al., 2003). It is known that activation of cytokine gene expression programs is a key component of T cell activation and proliferation (Tharaux et al., 2003). With all the above implications of *ROCK1* in the appropriate function of the immune system, it is an unexpected finding that animals from FHH and MHL groups but not MHH group compared with MLL showed a significantly higher expression of this gene. One possible explanation

might be related with the mechanism of disposing of aberrant T cells in the thymus. It is known that T cells that do not pass the positive or negative selection in the thymus go through apoptosis and are digested by macrophages. It is possible that the amount of defective T cells were increased in FHH and MHL and so *ROCK1* was necessary for a proper migration and phagocytosis by macrophages. In addition, another upregulated gene in the supplemented diets compared with the MLL was coiled-coil domain containing 88A (*CCDC88A*). *CCDC88A* is a gene that encodes Girdin, an actin binding protein involves in cell migration, actin organization and cell motility and also in regulation of early endosome maturation (Bhandari et al., 2015, Nahorski et al., 2016). Although the function of this protein in the immune system has not been investigated yet, this protein might also be involved in macrophage motility, since it is implicated in actin organization.

Interestingly, *Bola–DQB* (*MHC* class II) was upregulated in MHH and MHL versus MLL and also in the comparison between MHH and MHL versus FHH. It was also found that there was an increase in the expression of *Bola-DQA1* in the comparison between MHH and FHH. These results suggest that this gene expression was influenced by both diet and sex. *MHC* class II plays a crucial role in the immune adaptive response, since its function is to present antigens to $CD4^+$ T lymphocytes (Norimine and Brown, 2005, Wang et al., 2015a, Liu et al., 2016a). *MHC* class II alleles are redundant and greatly polymorphic, magnifying the repertoire of epitopes that an individual can recognize (Norimine and Brown, 2005). In normal conditions, once foreign pathogens enter the host, antigen presenting cells such as macrophage engulf them, degrade the antigen

present in the pathogen into small peptides and load them upon specific *MHC* class II molecules to form a *MHC* class II-peptides complex. This complex is then placed on the surface of the antigen presenting cells where it binds to the T cell receptor (TCR) of $CD4^+$ T cells and triggers the adaptive immune response (Wang et al., 2015a, Strawbridge and Blum, 2007, Blum et al., 2013). Furthermore, it has been observed that *MHC* class II is essential for an appropriate development and differentiation of immature thymocytes (Kim et al., 2003, Lev et al., 2013). In the thymus, immature T lymphocytes go through a positive (cortex) and a negative (medulla) selection to discard self- reactive T cells. For that, cortical thymic epithelial cells in the cortex and the dendritic cells (DCs) and medullary thymic epithelial cells in the medulla, test the affinity of the TCR from T cells to the *MHC* present in their surface (Kyewski and Klein, 2006, Klein et al., 2009, Liu et al., 2016a). It is expected then that a decrease in *Bola-DQB* and *Bola-DQA1* expression would deeply affect the capacity of the immune system to respond to infection.

Taking into consideration these results it can be concluded that feeding pregnant animals with just pasture and not adding protein supplements to their food might produce future animals with a weaker immune system, and so they could be more vulnerable to infection and diseases. Also an interesting finding is the upregulation of the *Bola-DQB* and *Bola-DQA1* genes between males and females fed the same supplemented diet, suggesting a sex difference in the function of the immune system. It might be an explanation as to why females could present a narrower variability of *MHC* class II than males to avoid the attack of T cells to the sperm.

Chapter 6 The effect of maternal dietary protein level on the fatty acid profile of adult bovine pre-scapular lymph node and surrounded adipose tissue

6.1 Introduction

Although it was previously believed that the adipose tissue surrounding the lymph nodes only functioned as fuel for the body, it is now well established that the fatty acid composition of the perinodal adipose tissue interacts with the stimulated immune cells of the lymph nodes (Mattacks et al., 2002, Mattacks et al., 2004). These fatty acids produce eicosanoids that promote or reduce inflammation and alter the fluidity of the immune cells membrane (Calder, 2008, Calder, 2001). Many studies have investigated the effect of different compositions of fatty acids, mainly polyunsaturated fatty acids, in diets but only a few have focused in the effect of protein intake. To our knowledge, this is the first study to focus on the long-term effects of maternal protein restricted diets on the fatty acid composition of lymph node and surrounded adipose tissue in bovine offspring.

6.1.1 Hypothesis

We hypothesised that restricted maternal protein diets during peri – conception and early pregnancy will influence the fatty acid profile of the perinodal adipose tissue reducing the amount of polyunsaturated fatty acid available for the stimulated immune cells and so this might impact the effective function of these immune cells.

6.2 Methods

6.2.1 Animals and feeding regime

Animal and feeding regimes, sample collection and ethics were as described in Chapter 2.

6.2.2 Sample acquisition

To extract the fatty acid profile of the lymph node and surrounding adipose tissue, the pre-scapular lymph node, PAT and remote adipose tissue were dissected from the 20 month old bulls. Tissue was classified as previously described (Pond, 2005). PAT was located less than 5mm from the pre-scapular lymph node and remote adipose tissue was situated more than 1cm away from the node. Samples were collected, transported in dry ice (in accordance to UK Home Office, University of Nottingham and Australian Government guidelines, using appropriate packing, health and safety) and stored at -80°C until the fatty acid extraction and gas chromatography was carried out (see Chapter 2 Section 2.8.1 and 2.8.2 for methodology).

6.2.3 Statistical analyses

Statistical comparison of the percentage of the different fatty acids detected in each tissue was carried out using multifactorial ANOVA (IBM SPSS statistic 24, USA) to analyse the effects of maternal nutrition during peri – conception and first trimester, in the percentage of fatty acids in the same tissue and the interaction between the different tissues. Statistical significance was reported at $P<0.05$.

6.3 Results

6.3.1 The effect of maternal low protein diet on the fatty acid profile of the pre-scapular lymph node

When comparing the fatty acids percentages between the different diets and dietary periods (Table 6.1) it was found that the majority of the fatty acids showed no significant differences, however in the lymph node tissue a statistical difference ($P<0.05$) was observed in the stearic acid (C18:0) between LL and HL ($P=0.033$) and between the LL and HH ($P=0.003$) groups. The amount of stearic acid was higher in the LL group compared with the other two groups (Figure 6.1 A). Significant differences were also found in the amounts of stearic acid ($P=0.017$), myristic acid (C14:0; $P=0.025$), myristoleic acid (C14:1; $P=0.015$) and oleic acid (C18:1n9c; $P=0.029$) between the LPeri and HPeri groups (See Figure 6.1 B-E). All of these fatty acids showed a greater amount present in the HPeri group except for stearic acid which was higher in the LPeri. No significant variations were found between the post-conception groups.

Table 6.1 Percentage of fatty acid within the scapular lymph node of 20 month old bulls

Fatty acid	Mean± SEM (%)			
	LL (n=8)	LH (n=8)	HL (n=14)	HH (n=9)
C10:0	0.35±0.23 (0.05)	0.61±0.41 (0.05)	0.58±0.24 (0.04)	0.00±0.00 (0.00)
C12:0	0.10±0.10 (0.01)	0.00±0.00 (0.00)	0.19±0.13 (0.01)	0.00±0.00 (0.00)
C14:0	0.31±0.21 (0.04)	0.58±0.38 (0.04)	11.65±8.17 (0.89)	7.87±3.84 (1.44)
C14:1	0.00±0.00 (0.00)	0.00±0.00 (0.00)	1.66±1.12 (0.13)	1.62±0.77 (0.30)
C15:0	0.17±0.17 (0.02)	0.17±0.17 (0.01)	1.66±1.02 (0.13)	1.34±0.54 (0.25)
C15:1	0.21±0.21 (0.03)	0.00±0.00 (0.00)	0.79±0.60 (0.06)	0.23±0.23 (0.04)
C16:0	0.18±0.18 (0.02)	8.03±8.03 (0.61)	82.77±60.04 (6.32)	70.30±32.39 (12.85)
C16:1	0.26±0.26 (0.03)	156.93±156.42 (11.96)	97.44±85.84 6.3.2	9.20±4.18 (1.68)
C17:0	4.84±0.98 (0.65)	7.03±2.19 (0.54)	10.50±3.00 (0.80)	5.06±1.25 (0.92)
C17:1	2.40±0.54 (0.32)	3.27±0.75 (0.25)	4.52±1.73 (0.35)	3.42±0.99 (0.63)
C18:0	257.84±62.47 (34.55)	356.86±146.17 (27.20)	315.47±67.57 (24.09)	119.03±13.06 (21.76)
C18:1n11t	313.09±128.48 (41.95)	587.90±285.11 (44.81)	438.08±156.05 (33.45)	71.95±36.48 (13.15)
C18:1n9c	4.87±1.28 (0.65)	10.59±5.55 (0.81)	133.77±95.33 (10.22)	94.93±44.58 (17.35)
C18:1n9t	78.02±50.59 (10.45)	88.88±34.65 (6.77)	111.52±22.04 (8.52)	63.49±23.01 (11.61)
C18:2n6c	1.95±0.51 (0.26)	7.69±4.81 (0.59)	10.32±5.90 (0.79)	12.89±5.40 (2.36)
C18:2n6t	2.32±0.37 (0.31)	3.68±0.77 (0.28)	5.88±3.19 (0.45)	7.47±3.70 (1.37)
C18:3n3	1.48±0.37 (0.20)	1.25±0.70 (0.10)	3.00±1.13 (0.23)	1.95±0.67 (0.36)
C18:3n6	2.90±0.63 (0.39)	2.24±0.67 (0.17)	2.84±0.69 (0.22)	1.43±0.51 (0.26)
C20:0	3.18±1.01 (0.43)	3.27±1.17 (0.25)	4.09±1.04 (0.31)	2.06±0.73 (0.38)
C20:1	2.16±0.24 (0.29)	2.24±0.48 (0.17)	2.25±0.18 (0.17)	1.97±0.36 (0.36)

C20:2	44.93±3.50 (6.02)	37.18±5.64 (2.83)	37.40±5.47 (2.86)	24.39±7.07 (4.46)
C20:3n3	0.00±0.00 (0.00)	0.00±0.00 (0.00)	0.38±0.38 (0.03)	0.29±0.29 (0.05)
C20:3n6	0.00±0.00 (0.00)	0.82±0.82 (0.06)	1.36±0.67 (0.10)	2.07±0.97 (0.38)
C20:4n6	0.00±0.00 (0.00)	6.16±6.16 (0.47)	6.36±4.46 (0.49)	13.81±7.11 (2.52)
C20:5n3	0.00±0.00 (0.00)	0.26±0.26 (0.02)	0.16±0.16 (0.01)	0.41±0.21 (0.07)
C21:0	2.65±1.03 (0.35)	2.56±0.84 (0.19)	1.88±0.42 (0.14)	2.00±0.90 (0.37)
C22:0	0.00±0.00 (0.00)	0.00±0.00 (0.00)	0.27±0.18 (0.02)	1.15±0.81 (0.21)
C22:1n9	1.64±0.29 (0.22)	1.19±0.20 (0.09)	1.42±0.23 (0.11)	0.84±0.28 (0.15)
C22:2	5.61±0.90 (0.75)	5.65±1.04 (0.43)	5.89±0.93 (0.45)	3.56±1.14 (0.65)
C22:5 DPA	1.38±0.90 (0.18)	0.00±0.00 (0.00)	0.00±0.00 (0.00)	1.69±1.69 (0.31)
C22:6n3	0.00±0.00 (0.00)	0.22±0.22 (0.02)	0.18±0.18 (0.01)	0.28±0.19 (0.05)
C23:0	0.00±0.00 (0.00)	6.16±6.1 (0.47)	4.01±4.01 (0.31)	13.37±7.20 (2.44)
C24:0	0.00±0.00 (0.00)	0.00±0.00 (0.00)	0.13±0.09 (0.01)	0.12±0.12 (0.02)
C24:1	13.50±0.97 (1.81)	10.47±1.63 (0.80)	11.06±1.62 (0.84)	6.83±2.23 (1.25)
SUM	746.33 (100.00)	1311.88 (100.00)	1309.51 (100.00)	547.01 (100.00)

LL= animals fed low protein diet in both peri and post-conception periods

LH= animals fed low protein diet in the peri-conception period and high protein diet in the post-conception period

HL= animals fed high protein diet in the peri-conception period and low protein diet in the post conception period

HH= animals fed high protein diet in both peri and post-conception period

Numbers indicate mean±SEM of fatty acids

Brackets indicate percentage of fatty acids

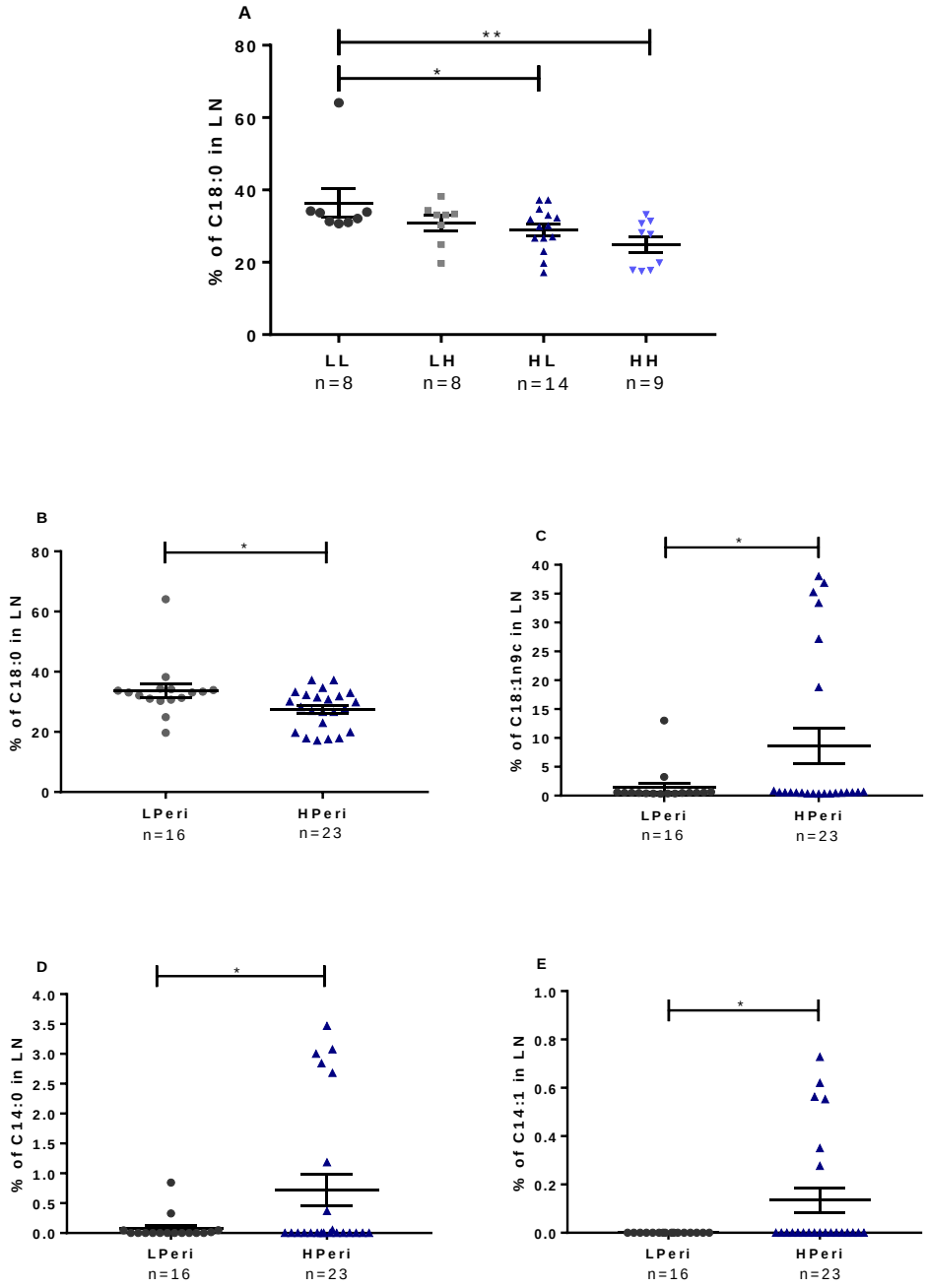


Figure 6.1 Percentage of the fatty acids showing significant differences between the lymph nodes from differing dietary regimes. A) Of C18:0 in the scapular lymph node in the four different dietary treatments B) Of C18:0 in the lymph node in the peri – conception comparison C) Of C18:1n9c in low and high protein diets in the peri – conception period D) Of C14:0 in the comparison of protein diets in the peri – conception period E) Of C14:1 in low and high protein diets in the peri – conception period. The data were analysed with linear models univariance. Graphs indicate mean±SEM. P<0.05 were considered significant. * represents P<0.05; ** represents P<0.01.

6.3.3 The effect of maternal low protein diet on fatty acid profile of the perinodal adipose tissue (PAT)

No significant differences were found in the comparisons between the percentage of fatty acids within PAT between the different diets or peri- and post-conception periods ($P>0.05$; Table 6.2).

Table 6.2 Percentage of fatty acids within the perinodal tissue surrounding the pre-scapular lymph node

Fatty acids	Mean±SEM (%)			
	LL (n=8)	LH (n=8)	HL (n=14)	HH (n=10)
C10:0	0.29±0.19 (0.02)	0.53±0.15 (0.04)	0.19±0.10 (0.02)	0.12±0.12 (0.01)
C12:0	0.67±0.27 (0.05)	0.81±0.19 (0.06)	0.46±0.14 (0.04)	0.55±0.18 (0.05)
C14:0	47.10±8.99 (3.69)	47.88±4.71 (3.67)	39.12±4.17 (3.64)	36.14±4.86 (3.45)
C14:1	8.52±2.0 (0.67)	8.38±1.02 (0.64)	6.31±0.50 (0.59)	8.00±1.50 (0.76)
C15:0	6.52±1.11 (0.51)	6.23±0.60 (0.48)	5.32±0.53 (0.49)	5.06±0.63 (0.48)
C16:0	304.15±51.83 (23.85)	300.68±25.93 (23.05)	259.30±24.88 (24.11)	246.95±26.96 (23.58)
C16:1	44.18±8.19 (3.46)	42.11±3.58 (3.23)	36.89±3.61 (3.43)	35.95±4.58 (3.43)
C17:0	17.33±2.47 (1.36)	17.95±1.86 (1.38)	14.96±1.56 (1.39)	14.15±1.56 (1.35)
C17:1	10.47±1.63 (0.82)	10.94±0.86 (0.84)	8.82±0.88 (0.82)	8.74±1.05 (0.83)
C18:0	247.64±42.82 (19.42)	256.51±26.20 (19.66)	213.67±26.05 (19.87)	194.48±20.81 (18.57)
C18:1n9c	469.88±14.87 (36.84)	484.59±11.89 (37.14)	394.32±7.99 (36.66)	401.87±9.32 (38.37)
C18:1n9t	82.45±78.7 (6.46)	89.31±51.62 (6.85)	65.89±41.97 (6.13)	63.91±45.26 (6.10)
C18:2n6c	19.01±0.51 (1.49)	20.55±0.35 (1.58)	15.93±0.29 (1.48)	16.29±0.37 (1.56)
C18:2n6t	3.14±3.29 (0.25)	3.14±2.93 (0.24)	2.47±1.81 (0.23)	2.54±2.18 (0.24)
C18:3n3	3.17±0.33 (0.25)	3.49±0.22 (0.27)	2.62±0.21 (0.24)	2.95±0.16 (0.28)
C18:3n6	0.00±0.00 (0.00)	0.00±0.00 (0.00)	0.05±0.05 (0.01)	0.16±0.11 (0.02)
C20:0	1.92±0.43 (0.15)	1.90±0.54 (0.15)	1.56±0.35 (0.14)	1.44±0.45 (0.14)
C20:1	3.21±0.55 (0.25)	3.54±0.75 (0.27)	2.77±0.30 (0.26)	3.13±0.53 (0.30)
C20:3n6	0.11±0.30 (0.01)	0.00±0.33 (0.00)	0.00±0.16 (0.00)	0.07±0.22 (0.01)

C20:4n6	0.20±0.27 (0.02)	0.37±0.24 (0.03	0.37±0.06 (0.03)	0.00±0.07 (0.00)
C21:0	1.09±0.11 (0.09)	1.00±0.00 (0.08)	0.72±0.00 (0.07)	0.91±0.07 (0.09)
C22:0	0.35±0.13 (0.03)	0.24±0.26 (0.02)	0.06±0.19 (0.01)	0.07±0.00 (0.01)
C24:0	0.00±0.00 (0.00)	0.16±0.16 (0.01)	0.00±0.00 (0.00)	0.00±0.00 (0.00)
SUM	1271.40 (99.69)	1300.31 (99.66)	1071.78 (99.65)	1043.47 (99.62)

LL= animals fed low protein diet in both peri and post-conception periods

LH= animals fed low protein diet in the peri-conception period and high protein diet in the post-conception period

HL= animals fed high protein diet in the peri-conception period and low protein diet in the post conception period

HH= animals fed high protein diet in both peri and post-conception period

Numbers indicate mean±SEM of fatty acids

Brackets indicate percentage of fatty acids

6.3.4 The effect of maternal low protein diet on the fatty acid profile of the remote adipose tissue

Only the palmitic acid (C16:0) showed significant differences when comparing the percentage of fatty acids in the remote adipose tissue (Table 6.3) between the 4 different treatments and also between the two different periods. Significant differences were observed between LH and HL (P=0.026) and the LH and HH groups (P=0.006), where the percentage of palmitic acid was greater in the LH group (Figure 6.2 A). Statistical differences in the percentage of palmitic acid between the LPeri and Hperi were also observed with a higher percentage of palmitic acid in the LPeri group (P=0.005; Figure 6.2 B). No significant differences were found in fatty acid composition between the LPost and HPost groups.

Table 6.3 Percentage of fatty acids within the lymph node surrounding further adipose tissue

Fatty acids	Mean±SEM (%)			
	LL (n=7)	LH (n=8)	HL (n=13)	HH (n=10)
C10:0	0.55±0.20 (0.03)	0.93±0.15 (0.05)	0.75±0.13 (0.04)	0.86±0.17 (0.04)
C12:0	1.25±0.15 (0.07)	1.44±0.16 (0.07)	1.18±0.07 (0.07)	1.35±0.16 (0.07)
C14:0	62.62±6.65 (3.58)	72.73±6.67 (3.69)	62.62±2.32 (3.46)	65.97±4.90 (3.34)
C14:1	10.94±1.05 (0.63)	14.26±2.27 (0.72)	11.17±0.75 (0.62)	13.06±1.62 (0.66)
C15:0	9.16±0.83 (0.52)	9.77±0.86 (0.50)	8.72±0.43 (0.48)	9.35±0.73 (0.47)
C16:0	399.83±33.10 (22.86)	466.95±37.73 (23.69)	396.74±13.35 (21.89)	425.12±28.33 (21.51)
C16:1	61.95±4.99 (3.54)	71.19±7.18 (3.61)	62.84±3.21 (3.47)	63.80±4.61 (3.23)
C17:0	23.99±1.83 (1.37)	25.60±2.14 (1.30)	25.02±1.32 (1.38)	28.34±1.98 (1.43)
C17:1	15.30±1.50 (0.87)	15.53±1.34 (0.79)	15.82±0.69 (0.87)	16.62±0.98 (0.84)
C18:0	327.04±35.24 (18.70)	367.55±31.98 (18.65)	343.19±22.41 (18.94)	400.34±35.44 (20.26)
C18:1n9t	115.54±15.10 (6.61)	134.90±15.83 (6.84)	122.31±7.05 (6.75)	128.46±10.01 (6.50)
C18:1n9c	668.16±51.82 (38.20)	729.61±54.54 (37.02)	709.10±30.81 (39.13)	762.70±42.30 (38.60)
C18:2n6t	4.85±0.65 (0.28)	4.98±0.44 (0.25)	4.51±0.28 (0.25)	5.04±0.32 (0.26)
C18:2n6c	27.02±2.66 (1.54)	33.21±2.96 (1.68)	28.66±1.69 (1.58)	31.11±1.56 (1.57)
C20:0	2.62±0.31 (0.15)	2.73±0.21 (0.14)	2.52±0.21 (0.14)	2.91±0.30 (0.15)
C20:1	5.06±0.71 (0.29)	5.46±0.55 (0.28)	5.40±0.45 (0.30)	6.11±0.49 (0.31)
C18:3n3	5.41±0.77 (0.31)	6.07±0.73 (0.31)	4.67±0.39 (0.26)	5.67±0.57 (0.29)
C21:0	1.92±0.45 (0.11)	1.84±0.20 (0.09)	1.57±0.19 (0.09)	2.02±0.30 (0.10)
C20:2	0.17±0.17 (0.01)	0.11±0.11 (0.01)	0.00±0.00 (0.00)	0.00±0.00 (0.00)
C22:0	0.22±0.14 (0.01)	0.10±0.10 (0.01)	0.06±0.06 (0.00)	0.17±0.11 (0.01)

C20:3n6	0.00±0.00 (0.00)	0.00±0.00 (0.00)	0.00±0.00 (0.00)	0.27±0.19 (0.01)
C20:4n6	0.14±0.14 (0.01)	0.33±0.16 (0.02)	0.00±0.00 (0.00)	0.08±0.08 (0.00)
C23:0	0.24±0.15 (0.01)	0.11±0.11 (0.01)	0.22±0.12 (0.01)	0.47±0.20 (0.02)
SUM	1743.98 (99.70)	1965.38 (99.73)	1807.08 (99.73)	1969.81 (99.69)

LL= animals fed low protein diet in both peri and post-conception periods

LH= animals fed low protein diet in the peri-conception period and high protein diet in the post-conception period

HL= animals fed high protein diet in the peri-conception period and low protein diet in the post conception period

HH= animals fed high protein diet in both peri and post-conception period

Numbers indicate mean±SEM of fatty acids

Brackets indicate percentage of fatty acids

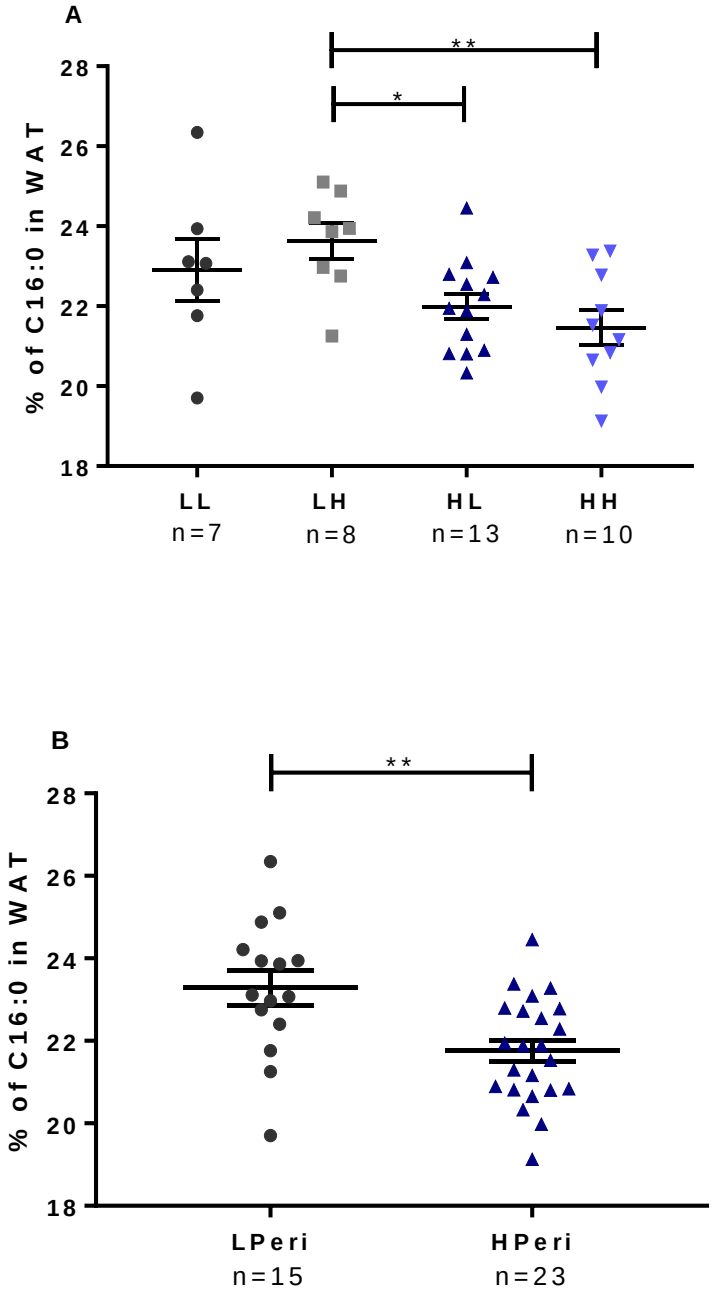


Figure 6.2 Percentage of C16:0 present in the further adipose tissue A) In the four different dietary treatments B) In the comparison between low and high protein diets in the peri-conception period. The data were analysed with linear model univariance. Graphs indicate mean±SEM. P<0.05 were considered significant. * represents P<0.05; ** represents P<0.01.

6.4 Discussion

Fatty acids play an important role in the development of the immune response, with unsaturated fatty acids reportedly exerting a greater influence in several immune cells. Two highlighted fatty acids from this group are the essential PUFAs, omega-6 and omega-3. Omega-6 is the precursor of arachidonic acid, which is known for being the precursor of pro-inflammatory eicosanoids whereas omega-3 is converted into EPA, which is the precursor of anti-inflammatory eicosanoids (Calder, 2001, Calder, 2015). The role of MUFAs, mainly oleic acid, in the activation of different cells from the immune system have also been investigated, however their effects are less pronounced than the ones shown by PUFAs (Yaqoob, 2002). Apart from the production of eicosanoids, PUFAS are part of the membrane of immune cells and changes in the composition of fatty acids alter membrane fluidity (Calder et al., 1990, Calder, 2008). Previous *in vitro* studies have proved that addition of unsaturated fatty acids into the macrophage membrane enhanced macrophage phagocytosis of different bacteria (Adolph et al., 2012, Fuhrmann et al., 2007, Lecchi et al., 2011), probably due to the changes in membrane fluidity. Exogenous supplementation of mast cell cultures with omega-3 showed suppressed degranulation, cytokine production and lipid-derived mediator release from activated mast cells (Wang et al., 2015b). In addition, it has been observed that omega-3 PUFA caused a suppression in $CD4^+$ T-cell proliferation by promoting the formation of lipid rafts (Kim et al., 2008, Hou et al., 2016), since modifications in the lipid-protein composition of lipid rafts are necessary

for initiation of signalling cascades, protein trafficking, cell-cell communication and endocytosis in T cells (Kim et al., 2008). Furthermore, antigen presenting proteins major histocompatibility complex I (MHCI) and MHCII expression was reduced on *in vitro* cells supplemented with arachidonic acid and docosahexaenoic acid (Shaikh and Edidin, 2007) and in rats fed with supplemented fish oil (rich in omega-3) (Sanderson et al., 1997), respectively.

The composition of fatty acid profiles in lymph nodes is similar to that of the PAT, what indicates that a movement of fatty acids from the PAT to the lymph node when the immune cells are stimulated occurred (Pond and Mattacks, 2003, Westcott et al., 2006).

In the present study, the percentage of PUFAs within the total percentage of fatty acids in the different tissues (lymph node, PAT and remote adipose tissue) was around 2% in all the tissues analysed and in the different diets (LL, LH, HL), except in the HH group within the lymph node where it was greater (over 7.7%). Nevertheless, the comparison between the different PUFAs present in the 3 tissues were not significantly different between the four diets. This means that no differences in the effects caused by the composition of PUFAs in the immune cells should be seen between the different dietary treatments. Interestingly, the percentage of oleic acid was lower in the lymph node than in both adipose tissue deposits. However, in both adipose tissue deposits the percentage of this monounsaturated fatty acid was similar, suggesting that this fatty acid is stored and only transferred to the immune cells when it is needed. A similar pattern was observed in the palmitic and myristic acids, which were very limited in the lymph node compared with the percentage of these two fatty acids present in

the PAT and remote adipose tissue. However, the percentage of stearic acid was increased in the lymph node when compared with the two adipose tissue deposits. Moreover, vaccenic acid (C18:1n11t) was absent in the adipose tissue surrounding the lymph node despite being present in one of the highest percentages in the lymph node. In ruminants, despite ingesting a higher ratio of PUFAs over SFA (2:1) within the diet, the majority of the PUFAs are biohydrogenated in the rumen by rumen bacteria into stearic acid, via a number of intermediates (Nam and Garnsworthy, 2007). The main intermediate of linoleic and α-linolenic biohydrogenation is vaccenic acid (Nam and Garnsworthy, 2007). Once the fatty acids have been absorbed in the duodenum, this vaccenic acid is then converted into rumenic acid (C18:2nc9, t11), which is an isomer of conjugated linoleic acid, by the enzyme Δ9-desaturase (Nam and Garnsworthy, 2007, Strillacci et al., 2014). Surprisingly, no rumenic acid was detected neither in the lymph node nor in the adipose tissues analysed.

6.4.1 Fatty acid profile in the pre-scapular lymph node

In this experiment the composition of fatty acids in the pre-scapular lymph nodes from bulls undernourished *in utero* was studied. In this tissue the percentage of MUFAs was the highest within the total percentage of fatty acids present, followed by SFAs and finally PUFAs. Several fatty acids were affected by the restriction of maternal protein diet during the peri-conception period (60 days before artificial insemination to 23 days after conception). These altered fatty acids were myristic, myristoleic, stearic and oleic acids. All of them were higher in HPeri compared with LPeri except the stearic acid which was

greater in LPeri vs HPeri. Moreover, significant differences were also observed in stearic acid when the 4 different treatments were compared, with an increase of this SFA in LL group compared with HL and HH. The conversion of myristic and stearic acids into myristoleic and oleic acids, respectively, is regulated by the enzyme Δ9-desaturase. Several studies have related the activity of Δ9-desaturase with protein intake. In a study by de Tomás et al. (1980) it was observed that 31 days old pup rats from dams restricted in protein during gestation and lactation showed a reduced activity of liver Δ9-desaturase. Moreover, fetal and maternal liver Δ9-desaturase activity was lower in pregnant rats fed low protein diet during gestation (Mercuri et al., 1979). Furthermore, in adult rats fed low protein diet for 26 days a reduction in Δ9-desaturase was also observed (Narce et al., 1992). Taking all together, our results suggest that maternal low protein diet during the peri-conception period may reduce Δ9-desaturase activity in their male progeny and so impair the conversion of myristic acid into myristoleic acid and stearic acid into oleic acid. This argument is clearer in the case of stearic acid and oleic acid, since these two fatty acids showed different patterns in the peri-conception period. While stearic acid was increased in LPeri oleic acid was reduced. This may indicate that due to the lack of Δ9-desaturase activity in LPeri the SFA stearic acid could not be converted into oleic acid and so the percentage of this saturated fatty acid was risen in this group compared with HPeri. In the case of myristic and myristoleic acids, since both of the fatty acids were increased in the HPeri compared with LPeri, the greater percentage of MUFA myristoleic acid in HPeri group could be because of the reduced activity of Δ9-desaturase

in LPeri but also because of the percentage of its precursor, myristic acid, was increased in the HPeri group compared with LPeri.

It has been observed that oleic acid influences the onset of the immune response. In a study carried out by Yaqoob et al. (1994) rats fed with different diets supplemented with oleic acid, omega-6 or omega-3 for 10 weeks showed lower thymidine stimulation index values in lymph node lymphocytes, and so a reduction in lymphocyte proliferation, than the rats fed no supplemented diets. In addition, other studies observed a stimulation in neutrophil activation when oleic acid was added to isolated neutrophils from human blood and peritoneal fluid in rats (Hatanaka et al., 2006, Carrillo et al., 2011). Both studies showed an increase in reactive oxygen species, which is associated with phagocyte defense, in the neutrophils supplemented with oleic acid. These results contrast with a study reported by Vázquez et al. (2004), where the control group compared with rats supplemented with oleic acid diets from 8 weeks did not show an increase in reactive oxygen species, although an increase in neutrophils activation due to the higher release of lysozyme, β-glucoronidase and myeloperoxidase (lysosomal enzymes) was observed. In this last study a decrease in the production of prostaglandin E_2 was also observed in the animals fed a diet supplemented with oleic acid (Vázquez et al., 2004). These differences between studies might be caused by the differences in the methodology followed. Suppression of natural killer cells activity in rats fed diets rich in oleic acid compared with low-fat diets has been also demonstrated (Yaqoob et al., 1994, Jeffery et al., 1996, Yaqoob, 2002). Therefore a reduction in the percentage of oleic acid in the lymph node might undermine an immune

response against non-commensal microorganisms, and so the bulls from dams underfed during the peri-conception period could be more sensitive to infections. On the other hand, excess saturated fatty acids alter cell growth and cytotoxicity and can promote apoptosis (Harvey et al., 2010), which can be prevented with the addition of mono/polyunsaturated fatty acids (Listenberger et al., 2003, Maedler et al., 2001). Myristic acid also seems to be key for regulating protein-protein and/or protein-membrane interaction (Johnson et al., 1994) and it is also used as a substrate for some desaturases (Rioux et al., 2011). Taken altogether, our results indicate that low maternal protein diet during the peri-conception might lead to a lower production of the enzyme Δ9-desaturase in the male progeny which can unbalance the ratio of saturated:monounsaturated fatty acids (specifically stearic and oleic acids) in the lymph node. Whether the cells are able to tolerate these differences, or if it could have a negative impact in the onset of the immune response, is presently unknown. Moreover, the very low levels of myristic acid in LPeri animals might reduce the interactions between proteins needed for a good communication between the different immune cells, albeit why the restriction of proteins *in utero* is affecting the percentage of this fatty acid is not yet understood.

6.4.2 Fatty acid profile in adipose tissue surrounding the pre-scapular lymph node

The adipose tissue surrounding the pre-scapular lymph node showed fewer difference in fatty acid profiles between the differing maternal diets, with only the remote adipose tissue showing significant differences in the percentage of

the fatty acids. In this tissue a significant increase in the percentage of palmitic acid in the animals fed low protein diets *in utero* compared with the animal fed supplemented diets was observed. As explained above, an overabundance of saturated fatty acids is related with cell apoptosis (Harvey et al., 2010), however as this is adipose tissue, and its main function is as storage, it is not clear if the increase in palmitic fatty acids could have a negative impact in the development of an immune response.

In summary, maternal low protein intake from day 60 before artificial insemination to day 23 post-conception altered the fatty acid profiles of the pre-scapular lymph node and remote adipose tissue in male progeny. These differences may be caused by the reduction of the enzyme Δ9-desaturase, which is involved in the conversion of SFAs into MUFAs, and might exert a negative impact in the development of an immune response against an external microorganism.

Chapter 7 General discussion

This final discussion chapter summarizes the study and highlights some of the main findings and limitations of the study and identifies possible future directions. The primary limitation to the current project is that no functional studies were performed to support either the histological or the molecular changes observed in the different tissues. Another limitation is that it relates to the unique conditions found in Australian beef production systems and its relevance to beef production in more temperate climates in the UK and northern Europe, is unclear. However, analysis of the dietary conditions in Australian herds permits us to examine the physiological, histological and molecular changes induced by variation in maternal dietary protein in the peri- and post- conception periods.

Bacterial infection and diseases that affect the reproductive performance together with reproductive conditions are a challenge to the economic viability of the beef cattle industry (Bellows et al., 2002). In cattle, around half of the of the cows that become pregnant suffer an interruption in their gestation in the period between fertilization and term (Geary et al., 2016). In North Australia subfertility of bulls is a major issue where 10 – 20 % of the bulls fail a routine bull breeding soundness evaluation (Fordyce et al., 2006). From all the environmental factors that affect reproductive performance, nutrition has been found to clearly impact reproduction efficiency and is amenable to cost-effective on-farm interventions (Hess et al., 2005). In Northern Australia there is a seasonal variability in quality of pastures, due to the existence of a dry and a wet season. Although feed energy levels do not appear to be a limiting factor

under these situations, dietary protein is often deficient during the dry season for pregnant beef cattle and therefore protein supplementation is necessary (Copping et al., 2014, Sullivan et al., 2009b, Bortolussi et al., 2005). Numerous studies have shown that maternal disturbances during pregnancy may affect post-natal reproduction and production traits in progeny (Sullivan et al., 2009c, Sullivan et al., 2009b, Sullivan et al., 2009a, Micke et al., 2010, Micke et al., 2011a, Micke et al., 2011b). Further studies have focused on testicular development of animals restricted in nutrients during gestation. These have shown a reduction in the number of Sertoli and Leydig cells, smaller seminiferous tubules and an increase in immature germ cells (Bielli et al., 2002, Kotsampasi et al., 2009, Rodríguez-González et al., 2012, Genovese et al., 2010, Melo et al., 2014). Considering this, investigating how protein deficiency during pregnancy during the dry season may affect the development of the progeny seems to be urgent and it is why the experiments in this dissertation were completed.

In summary, in this two factorial experimental design, heifers were fed either a low protein (7%) or a high protein (14%) diet, mimicking the real amount of protein present in the Australian pastures during the dry and the wet seasons, respectively, during the peri – conception and/or the post – conception period. Following this design, 4 dietary regimes in total (LL, LH, HL and HH) and two different diets per period (LPeri, HPeri, LPost and HPost) were obtained. Different tissues were analysed, testes, epididymis, thymus, thyroid and perinodal adipose tissue. Testes and epididymis were studied for their direct

involvement in male reproduction. The thymus, thyroid and perinodal adipose tissue were investigated for their role in the immune system.

Table 7.1 Summary of the significant differences observed in the fetal and adult testes

		Dietary treatments							
		Comparison				Comparison		Comparison	
Parameters measured		LL	LH	HL	HH	LPeri	HPeri	LPost	HPost
Fetus	% germ cells	⇧	--	⇩	--	--	--	--	--
	% Leydig cells	⇩	--	⇧	--	--	--	--	--
	% Seminiferous tubules	⇧	⇧	⇩	⇧	--	--	--	--
	N° Seminiferous tubules	⇧	⇩	⇩	⇩	--	--	--	--
	Area Seminiferous tubules	--	--	--	--	⇩	⇧	--	--
	N° blood vessels	--	--	--	--	⇧	⇩	⇩	⇧
	Area blood vessels	--	--	--	--	⇩	⇧	--	--
Adults	% Seminiferous tubules	--	--	--	--	⇩	⇧	--	--
	N° blood vessels	--	--	--	--	--	--	⇧	⇩

In the first experiment, it was observed that low protein diets during the peri – conception and first trimester of gestation altered testicular development in cattle. A summary of the significant changes found in the fetal and adult testes when low and high protein diets were compared is showed in Table 7.1. It was found that 98 dpc LL fetuses showed a reduction in the percentage of Leydig cell and an increase in the percentage of germ cells and in the number of seminiferous tubules compared with the rest of the diet regimes. Furthermore the proportion of seminiferous tubules was decreased in the 98 dpc HL fetuses and the size was smaller in 98 dpc LPost fetuses. Interestingly, the percentage of Sertoli cells, one of the most crucial testicular cells for an adequate function of the reproductive tract which is involved in differentiation of Leydig cells,

meiosis of germ cells and formation of seminiferous tubules (Griswold, 1998, Josso et al., 2001, Bitgood et al., 1996, O'Shaughnessy and Fowler, 2011) was not affected by protein restriction, which could have explained our results. However, the size and performance of the Sertoli cells was not measured in our experiments and the presence of smaller Sertoli cells in the 98 dpc fetuses fed low protein diet during the first trimester of pregnancy and the reduction in the performance of the Sertoli cells in the 98 dpc fetuses fed low protein diet during the peri – conception period, is consistent with our results. Further studies in this area are therefore warranted. In addition, the development of the testicular vasculature was also affected by dietary protein restriction. LPeri 98 dpc fetuses showed smaller total blood vessels due to the smaller size of the parenchymal blood vessels and a larger amount of total blood vessel because of the increase number of capsular blood vessels compared with HPeri 98 dpc fetuses. Furthermore, the number of total testicular blood vessels in LPost 98 dpc fetuses was reduced compared with HPost 98 dpc fetuses due to a decrease in the number of capsular blood vessels. All these alterations in the testicles of 98 dpc fetuses fed a diet restricted in protein might be explained by a reduction in the production of the vascular endothelial cell growth factor A, a protein that not only has been proven essential for angiogenesis (Reddy et al., 2012, Nalbandian et al., 2003) but has also been observed to be involved in the formation of seminiferous cords and spermatogenesis (Dores and Dobrinski, 2014, Bott et al., 2006, Caires et al., 2009, Schön et al., 2010). Vascular endothelial cell growth factor A is expressed in Sertoli and Leydig cells, and a

reduction in VEGFA expression has been observed in animals undernourished *in utero* (Chan et al., 2015, Liu et al., 2014).

Regarding the reproductive organs which were histologically analysed in the adult bulls, the percentage of seminiferous tubules was decreased in LPeri animals compared with HPeri and a greater number of blood vessels was observed in the LPost group compared with HPost. On the other hand, none of the structures analysed from the epididymis of the adult bulls were shown to be altered by maternal protein restriction. These findings suggest that the histological changes observed at 98 dpc in bovines fed restricted protein diet *in utero* during the peri – conception period and first trimester of pregnancy tends to be compensated later in life.

In addition, in order to assess how the deprivation of protein during fetal development may affect the reproductive performance of the adult bulls, the protein profile of the epididymal fluid from the adult bulls was also analysed. Proteomics has become popular in the last decade for its use in the search of new markers of male fertility. Interactions between the proteins secreted by the epididymosomes of the epididymis and the proteins present in the surface of the sperm seem to be crucial for sperm maturation, capacitation and fertilization (Moura et al., 2006, Sullivan et al., 2005, Sullivan et al., 2007, Skerget et al., 2015). In our experiment, alterations in the expression of proteins which may influence the reproductive performance in males was observed in the animals fed low protein diet. The changes in the protein profile in LPeri bulls might lead to problems in sperm maturation and capacitation and

it could be expected that motility, capacitation, immune protection and sperm – zona pellucida binding were compromised in LPost animals. In a recent study published by our team (Copping et al., 2018) using the same adult animals from the present study, it was observed that the bulls fed low protein diet during the peri – conception period showed the greatest changes in sperm analyses. Thus, LPeri bulls achieved puberty later and showed a reduction in the sperm concentration and motility and an increase of sperm abnormalities compared with HPeri. On the other hand, LPost did not seem to affect motility or concentration compared with HPost. However, in the study by Copping et al. (2018) only a visual assessment of sperm quality was completed. Future functional studies are required to determine whether the different expression of protein between low and high protein diets would have an impact on the fertility of the bulls.

Infectious diseases which negatively impact on reproductive efficiency are important in the cattle industry (Bellows et al., 2002). For this reason we considered it important to investigate the impact of maternal diet restricted in protein on the development of the thymus, a key organ in the maturation of T-lymphocytes (Vaidya et al., 2016). In this experiment, both female (F) and male (M) thymuses from 98 dpc fetuses were assessed. RNA sequencing analysis of the RNA extracted from FHH, MLL, MHL and MHH were performed. When the different diets and sexes were compared it was observed that the expression of genes that might be involved in development of the thymus and its function were altered when the 98 dpc fetuses from dams fed low protein diets were compared with 98 dpc fetuses from supplemented dams. Of special interest

was the up regulation of Bola-DQB and Bola-DQA1, two genes involved in the presentation of antigens to T-lymphocytes (Norimine and Brown, 2005, Wang et al., 2015a, Liu et al., 2016a), linked to sexes and not to diet. In addition, because of the developmental relation between the thymus and the thyroid gland and the positive interaction between thyroid hormones and thymus development and function (Pierpaoli et al., 1969, Fabris, 1973, Varga et al., 2008, Kameda et al., 2013), the development of the thyroid gland was also assessed in this experiment. However, no significant findings were identified between the diets and any of the thyroid parameters measured. Furthermore, the fatty acids profile of the adipose tissue surrounding the pre-scapular lymphocyte were also assessed. During an immune response, stimulated immune cells from the lymph node penetrate in the adjacent adipose tissue and selectively absorbed fatty acids present within, which are mainly long-chain fatty acids (Acedo et al., 2011, Pond and Mattacks, 2003, Mattacks et al., 2004). As protein deficient nutrition affect the conversion of saturated fatty acids into long-chain fatty acids (Narce et al., 1988, Bouziane et al., 1994), we investigated if maternal protein restriction was affecting this conversion. It was observed that animals restricted in protein during the peri – conception period might show problems to convert saturated fatty acids into polyunsaturated fatty acids, which may be caused by a reduction of the enzyme Δ9-desaturase. This might lead to a decrease of long – chain fatty acids available for the lymphocytes during an immune response and so reduce the efficacy of the response.

To conclude, these findings indicate that maternal dietary intake restricted in protein during the peri – conception and first trimester of pregnancy impact the reproductive output and the immune response of their progeny. Whereas histologically these alterations appear to be compensated for later in life, our data indicate that the molecular changes exist in adulthood. Considering these findings, cattle farmers should supplement with protein their animal diets from the very beginning of gestation and in the days prior to artificial insemination. Further investigations to develop an optimal diet that improves bovine male reproductive performance should be encouraged. Moreover, an improved system to measure the reproductive performance of bulls must be develop using molecular markers instead of measuring the scrotal circumference of the bulls since it has been observed in this and other studies that the biggest circumferences not always correlate with better sperm quality.

Publications

COPPING, K. J.*, **RUIZ-DIAZ, M. D.*,** RUTLAND, C. S., MONGAN, N. P., CALLAGHAN, M. J., MCMILLEN, I. C., RODGERS, R. J. & PERRY, V. E. A. 2017. Peri-conception and first trimester diet modifies reproductive development in bulls. Reproduction, Fertility and Development. *Joint First Authors

Maria Dolores Ruiz-Diaz, Hannah Partridge, Francesca Davidson, Nigel P Mongan, David S. Gardner, Catrin Sian Rutland. 2017. Parental low protein diet programmes offspring growth and vasculature to increase risk of cardiovascular, pancreatic and metabolic disease. Review in the European Medical Journal.

Conference abstract list

Maria Dolores Ruiz-Diaz, Nigel Mongan, Katrina Copping, Aziza Alibhai, Matthew Keane, Imogen Purvis, Viv E. A. Perry, Catrin S Rutland. Effect of maternal protein intake in the development of the bovine epididymis. European Society for Domestic Animal Reproduction (ESDAR) 21st annual conference, 2017.

Maria Dolores Ruiz-Diaz, Nigel Mongan, Katrina Copping, Aziza Alibhai, Matthew Keane, Imogen Purvis, Viv E. A. Perry, Catrin S Rutland. The effects of gestational nutrition on the male reproductive system. European Association of Veterinary Anatomists conference, 2017.

Appendix 1. Additional work

Appendix 1.1 Extra histology work

Immunofluorescence

The objective of using immunofluorescence in this project was to carry out a double immunofluorescence with antibodies against MIS-C20 (to stain AMH observed in immature Sertoli cells) and Wilms tumour 1 (WT1; to stain both mature and immature Sertoli cells) to observe whether cells remained immature in the adult in any of the feed groups. Several published studies have previously used AMH as an identification of infertility in males due to the presence of immature Sertoli cells, which were hypothesised to cause a limitation in the differentiation and proliferation of spermatids during the spermatogenesis (Richard M Sharper, et al 2003). To ensure a specific staining with MIS-C20 in the adult tissue, since the AMH is also secreted outside of these cells, the Sertoli cells were also stained with an antibody against WT1 to locate these cells within the seminiferous tubules and looked for possible surrounded staining using MIS-C20. A second objective was to try and stain for CD3, CD4 and/or CD8 immuno positive cells in the thymus.

MIS-C20 immunofluorescence protocol

MIS-C20 (Santa Cruz Biotechnology, USA, sc-6886, LOT#K2706) was used as a primary antibody to identify immature Sertoli cells in the immunofluorescence technique. Fetal testicular tissue was used as a positive control as the cells are expected to be immature at that stage. Examples of positive staining in 98 dpc fetal tissue and negative staining in adult tissue using MIS-C20 is shown in the Figure 2.2C and D, respectively. To stain the samples the following protocol was used:

Sections from adult testis (10µm) were deparaffinised and rehydrated [5mins xylene, 5mins 100% EtoH, 5mins 90% EtoH, 5mins 70% EtoH and 5mins in dH2O], followed by 3X 5mins washes in PBS buffer. After that, as antigen retrieval, the samples were placed in sodium citrate buffer in a ratio of 1:10 and heated in the microwave for 18mins and then the slides were left to cool for

20mins. Once the slides were cooled they were submerged in dH2O for 5mins and washed with PBS 3X 5mins. A blocking solution 200µl per slide of fetal calf serum (FCS) for 1 hour was used. After that, the slides were washed with PBS (3X 5mins). Successive, 200µl of the antibody (MIS C-20, Santa Cruz Biotechnology, sc-6886, LOT#K2706) was placed on each slide in concentrations of either 1:50; 1:100; 1:300 and 1:1000 (for the positive control we only used a ratio of 1:1000, since it works in the immunohistochemistry) per slides for 1 hour, leaving the negative control slide submerged in FCS. Following to this step, the tissue was washed with PBS (3X 5mins) and then 200µl per slides of the secondary antibody (Alexa fluor 488 rabbit anti-goat, Life technology, Lot 1613914. USA) in a ratio of 1:300 was added to the samples and left for 30mins. As finals steps, the samples were washed with PBS (3X 5mins) and mounted with 2 drops per slide of DAPI (which stains all nuclei blue UV; Sigma Aldrich, USA. Lot SLBL5097V) and a glass coverslip.

Wilms tumour 1 immunoflourescence protocol

WT1 antibody was used to stain the Sertoli cells in adult tissue. This antibody stains both immature and mature Sertoli cells, this is important for applicability in this experiment, considering that the objective of using this technique was to search for the presence of immature Sertoli cells in adult tissue. The protocol for this technique was the following:

The samples were deparaffinised and rehydrated [5mins xylene, 5mins 100% EtoH, 5mins 90% EtoH, 5mins 70% EtoH and 5mins in dH2O]. After that, triton at 0.3% in PBS was used as antigen retrieval. 200µl of this solution was placed for 30mins in each slide and then washed 3X for 5mins with PBS. Samples were blocked in FCS for 30mins and subsequently 200µl of the WT1 primary antibody (ab64681, Lot GR144290-1, Abcam, UK) in a ratio of 1:100 in FCS was added to the slides and incubated at room temperature for 1 hour. The negative control tissue was kept submerged in FCS during this period. Following this step, the tissue was washed with PBS (3X 5mins) and then 200µl per slides of the secondary antibody (Alexa fluor 594 goat anti-rabbit, Lot 1420898, Life technology, USA) in a ratio of 1:300 was added to the samples and left for

30mins. Finally the samples were washed with PBS (3X 5mins) and mounting with 2 drops per slides of DAPI and a glass coverslip added. After 24 hours the slides where observed under the microscope and no staining was observed in the adult testes tissue. The IF trial was stopped in order to observed the results provided by the PCR in the adult tissue, which could not be carried out in the end due to the presence of epididymis in the frozen tissues.

Frozen adult testes in OCT and cryosection

Frozen adult testes kept at -80°C were embedded in OCT and cryosected at 10µm. The tissue was then stained with haematoxylin and eosin following the protocol explained in Chapter 2 Section 2.4.5. After analysing under the microscope, it was decided that the tissue was very damage to count the testicular cells. Another finding was the presence of epididymal tissue, which impeded us to performed PCR in these testes.

Fetal femur, dissection, processing, staining and microscopy

The objective was counting the common lymphoid, myeloid and erythroid progenitors cells and looking for variations between the different diets. In order to do that, dissection of the frozen femur from the 98 dpc fetuses was carried out. These femurs were processed, embedded in paraffin, cut at 8µm and stained with H&E as described in Chaper 2 Section 2.4.5. The counting was not possible due to the difficulty in differentiate the types of cells present in the bone.

Lymph node brown adipose tissue in adults

To try to differentiate the adipose tissue surrounding the pre – scapular lymph node between brown or white adipose tissue, IHC using UCP1 was performed. Visually, the tissue looked like white adipose tissue and it did not stained using UCP1, which is specific for staining brown adipose tissue. However, the positive control did stained using the same untibody. After that all the adipose tissue surrounding the lymph node was considered as white adipose tissue.

CD3, CD4 and CD8 immunostaining in 98 dpc fetal thymus

Thymus was a tissue we had not worked with prior to this project, therefore optimisation of protocols were required. The aim was to stain the CD3, CD4 and CD8 antibody positive cells present in the fetal thymus. The first optimisations used the IHC protocol and kit used for the IHC in the testis (Chapter2 Section 2.4.1).

CD3, CD4 and CD8 immunoflourescence protocol in fetal thymus

Due to the failure of the DAB IHC method for detecting CD3, CD4 and CD8 thymus tissue, IF optimisation was trialled in the fetal thymus tissue. As it was done in the IHC, same protocol used for staining the testis with a few modifications was followed:

Sections (3µm thick) on glass microcope slides were heated in an oven for 20mins at 60°C, deparaffinised and rehydrated [5mins xylene, 5mins 100% EtoH, 5mins 90% EtoH, 5mins 70% EtoH and 5mins in dH2O], followed by 3X 5mins washes in PBS buffer. Using a heat-induced epitope retrieval (HIER) method for retrieving the antigen, the samples were submerged in sodium citrate at a ratio of 1:10 in dH2O and heated in a microwave for 18mins at a medium-low power. Then they were left to cool for 20mins at room temperature. After that the samples were washed with PBS and Triton (0.3% in PBS) was added to the slides, covered with parafilm and kept for 30mins. Then the samples were washed 3X 5mins with PBS and 200µl of 5% FCS were added to the tissue as a blocking solution for 30mins. Then the primary antibodies CD3, CD4 and CD8 (Sigma Aldrich C7930, USA; GeneTex CC58, USA; Biorad MCA1653GA, USA, respectively) at a dilution of 1:100 for 1.5 hours, leaving the negative control tissue in 5% FCS. Samples were then washed in PBS 3X 5mins and the secondary antibodies with fluorochrome (Alexa fluor 594® and alexa fluor 488®, Thermo fisher, USA) was added to the slides at a dliution of 1:100 for 30 mins, covered with parafilm and kept it in a dark container to avoid loss of fluorescence. Finally the samples were washed 3X 5mins with PBS and

mounting with DAPI in glycerol (3 drops/slide, Sigma Aldrich, USA. Lot SLBL5097V).

Samples were covered with foil at 4°C and analysed microscopically within 24 hours.

The protocol above was further systemically optimised using the alterations below:

- Antigen retrieval:
    - Instead of a HIER method, a proteolytic-induced epitope retrieval (PIER) was trialled by adding trypsin 0.1% at 37°C for 10mins.
- Permeabilitation:
    - Achieved by refreshing the triton solution every 5 mins.
- Primary antibody:
    - Increased dilution from 1:100 to 1:50 and 1:10.
    - Increased the time of incubation from 1.5 hours to overnight, leaving the samples in the fridge until the next day.
    - Used many different brands and types of antibodies for CD4 and CD8 that were reported to work for the staining of these cells.
- Washing:
    - Used Tris-buffered saline (TBS) instead of PBS, since some published literature used this buffer.

New design protocols for immunostaining CD4 and CD8 cells

Due to the constant failure for immunostaining of the CD cells using the above modified protocol, new protocols were design by the author based in a search of the available literature. Both IHC and IF protocols were designed. Unfortunately, these protocols were never applied.

Protocols 1 IHC

Day 1

- Place samples in the oven at 60ºC for 20’

- Dewaxed samples in xylene 3X 10’
- Rehydrate samples going through alcohol 100, 96 and 70% 5’ each
- Place in PBS 2X 5’
- Antigen retrieval: Coplin jar filled with 0.1M formic acid in 2% gelatin (Merck) heated in the microwave at high power until the boiling point is reached and then reduced to 150W for 1’. This operation must be carry out in a fume hood. Cover the jar with the caps.
- Stop the treatment rinsing the slides several times in 5mM TBS (Tris buffer saline).
- Biotin and Avidin blocking kit (DAKO, Glostrup, Denmark). according manufacture instruction
- Wash with TBS 3X 5’
- Peroxidase blocking (0.05% H_2O_2) 30’
- Wash with TBS
- Non-specific protein binding blocking: FCS for 30’
- Wash with TBS 3X 5’
- Primary antibody (CD4 or CD8) diluted X in TBS. Incubated overnight at 4ºC.

Day 2

- Leave the slides for 1 hour at room temperature
- Wash with TBS
- Secondary antibody: Biotinylated goat anti-mouse. (Jackson ImmunoResearch Laboratories (JIRL), West Grove, PA, USA). 1:500 in TBS/3% non-fat milk, pH7.4, containing 10% FCS for 1 hour at room temperature
- Wash in TBS 3X 5’
- Incubation with peroxidase labelled streptavidin (JIRL) diluted 1:100 for 30’
- Wash with TBS 3X 5’
- Incubate with biotinylated-tyramide (Renaissance TSA-Direct kit) for 3-10’ according with the manufacturer instructions (NEN, Boston, USA) or

own preparation 100mg NHS-LC biotin (Pierce, Bender and Hobein; Munich, Germany) and 32 mg tyramine HCL (Sigma) added to 50ml boric acid buffer, pH 8 (Sigma) as stock solution. This stock was used as a dilution 1:50 in 50mM TBS, 0.05% H_2O_2, pH 8.

- Wash with TBS 3X 5'
- Avidin-biotin complex (ABC) reagent from the Vectorstain ABC elite kit prepared according to the instructions given by the manufacturer (Vector Laboratories, Peterborough, Canada). 30'
- DAB staining.
- Counterstain with haematoxylin 1'
- Wash in running tap water for 5'
- Place in DH_2O 5'
- Dehydrate going through alcohol 70%, 90 and 100%
- Clear in Xylene 2X 5'
- Mounting with DPX

Protocol 2 IHC

Day 1

- Place samples in the oven at 60ºC for 20'
- Dewaxed samples in xylene 3X 10'
- Rehydrate samples going through alcohol 100, 96 and 70% 5' each
- Place in PBS 2X 5'
- Antigen retrieval: Incubate with Protease K (25µg dissolved in 1ml TBS) at 37 ºC 5'
- Stop the treatment rinsing the slides several times in 5mM TBS (Tris buffer saline).
- Biotin and Avidin blocking kit (DAKO, Glostrup, Denmark) according manufacture instruction
- Wash with TBS 3X 5'
- Peroxidase blocking (0.05% H_2O_2) 30'
- Wash with TBS

- Non-specific protein binding blocking: FCS for 30’
- Wash with TBS 3X 5’
- Primary antibody (CD4 or CD8) diluted X in TBS. Incubated overnight at 4ºC.

Day 2

- Leave the slides for 1 hour at room temperature
- Wash with TBS
- Secondary antibody: Biotinylated goat anti-mouse. (Jackson ImmunoResearch Laboratories (JIRL), West Grove, PA, USA). 1:500 in TBS/3% non-fat milk, pH7.4, containing 10% FCS for 1 hour at room temperature
- Wash in TBS 3X 5’
- Incubation with peroxidase labelled streptavidin (JIRL) diluted 1:100 for 30’
- Wash with TBS 3X 5’
- Incubate with biotinylated-tyramide (Renaissance TSA-Direct kit) for 3-10’ according with the manufacturer instructions (NEN, Boston, USA) or own preparation 100mg NHS-LC biotin (Pierce, Bender and Hobein; Munich, Germany) and 32 mg tyramine HCL (Sigma) added to 50ml boric acid buffer, pH 8 (Sigma) as stock solution. This stock was used as a dilution 1:50 in 50mM TBS, 0.05% H_2O_2, pH 8.
- Wash with TBS 3X 5’
- Avidin-biotin complex (ABC) reagent from the Vectorstain ABC elite kit prepared according to the instructions given by the manufacturer (Vector Laboratories, Peterborough, Canada). 30’
- DAB staining.
- Counterstain with haematoxylin 1’
- Wash in running tap water for 5’
- Place in DH_2O 5’
- Dehydrate going through alcohol 70%, 90 and 100%
- Clear in Xylene 2X 5’

- Mounting with DPX

Protocol 1 IF

Day 1

- Place samples in the oven at 60ºC for 20'
- Dewaxed samples in xylene 3X 10'
- Rehydrate samples going through alcohol 100, 96 and 70% 5' each
- Place in PBS 2X 5'
- Antigen retrieval: Coplin jar filled with 0.1M formic acid in 2% gelatin (Merck) heated in the microwave at high power until the boiling point is reached and then reduced to 150W for 1'. This operation must be carry out in a fume hood. Cover the jar with the caps.
- Stop the treatment rinsing the slides several times in 5mM TBS (Tris buffer saline).
- Biotin and Avidin blocking kit (DAKO, Glostrup, Denmark). according manufacture instruction
- Wash with TBS 3X 5'
- Peroxidase blocking (0.05% H_2O_2) 30'
- Wash with TBS
- Non-specific protein binding blocking: FCS for 30'
- Wash with TBS 3X 5'
- Primary antibody (CD4 or CD8) diluted X in TBS. Incubated overnight at 4ºC.

Day 2

- Leave the slides for 1 hour at room temperature
- Wash with TBS
- Secondary antibody: Biotinylated goat anti-mouse. (Jackson ImmunoResearch Laboratories (JIRL), West Grove, PA, USA). 1:500 in TBS/3% non-fat milk, pH7.4, containing 10% FCS for 1 hour at room temperature

- Wash in TBS 3X 5’
- Incubation with peroxidase labelled streptavidin (JIRL) diluted 1:100 for 30’
- Wash with TBS 3X 5’
- Incubate with biotinylated-tyramide (Renaissance TSA-Direct kit) for 3-10’ according with the manufacturer instructions (NEN, Boston, USA) or own preparation 100mg NHS-LC biotin (Pierce, Bender and Hobein; Munich, Germany) and 32 mg tyramine HCL (Sigma) added to 50ml boric acid buffer, pH 8 (Sigma) as stock solution. This stock was used as a dilution 1:50 in 50mM TBS, 0.05% H_2O_2, pH 8.
- Wash with TBS 3X 5’
- Incubate with FITC labelled streptavidin (JIRL) diluted 1:100 for 30’
- Mounting with DAPI

Protocol 2 IF

Day 1

- Place samples in the oven at 60ºC for 20’
- Dewaxed samples in xylene 3X 10’
- Rehydrate samples going through alcohol 100, 96 and 70% 5’ each
- Place in PBS 2X 5’
- Antigen retrieval: Incubate with Protease K (25µg dissolved in 1ml TBS) at 37 ºC 5’
- Stop the treatment rinsing the slides several times in 5mM TBS (Tris buffer saline).
- Biotin and Avidin blocking kit (DAKO, Glostrup, Denmark). according manufacture instruction
- Wash with TBS 3X 5’
- Peroxidase blocking (0.05% H_2O_2) 30’
- Wash with TBS
- Non-specific protein binding blocking: FCS for 30’

- Wash with TBS 3X 5’
- Primary antibody (CD4 or CD8) diluted X in TBS. Incubated overnight at 4ºC.

Day 2

- Leave the slides for 1 hour at room temperature
- Wash with TBS
- Secondary antibody: Biotinylated goat anti-mouse. (Jackson ImmunoResearch Laboratories (JIRL), West Grove, PA, USA). 1:500 in TBS/3% non-fat milk, pH7.4, containing 10% FCS for 1 hour at room temperature
- Wash in TBS 3X 5’
- Incubation with peroxidase labelled streptavidin (JIRL) diluted 1:100 for 30’
- Wash with TBS 3X 5’
- Incubate with biotinylated-tyramide (Renaissance TSA-Direct kit) for 3-10’ according with the manufacturer instructions (NEN, Boston, USA) or own preparation 100mg NHS-LC biotin (Pierce, Bender and Hobein; Munich, Germany) and 32 mg tyramine HCL (Sigma) added to 50ml boric acid buffer, pH 8 (Sigma) as stock solution. This stock was used as a dilution 1:50 in 50mM TBS, 0.05% H_2O_2, pH 8.
- Wash with TBS 3X 5’
- Incubate with FITC labelled streptavidin (JIRL) diluted 1:100 for 30’
- Mounting with DAPI

Protocol 3 IF

Day 1

- Place samples in the oven at 60ºC for 20’
- Dewaxed samples in xylene 3X 10’
- Rehydrate samples going through alcohol 100, 96 and 70% 5’ each
- Place in PBS 2X 5’

- Antigen retrieval: Coplin jar filled with 0.1M formic acid in 2% gelatin (Merck) heated in the microwave at high power until the boiling point is reached and then reduced to 150W for 1'. This operation must be carry out in a fume hood. Cover the jar with the caps.
- Stop the treatment rinsing the slides several times in 5mM TBS (Tris buffer saline).
- Biotin and Avidin blocking kit (DAKO, Glostrup, Denmark). according manufacture instruction
- Wash with TBS 3X 5'
- Peroxidase blocking (0.05% H_2O_2) 30'
- Wash with TBS
- Non-specific protein binding blocking: FCS for 30'
- Wash with TBS 3X 5'
- Primary antibody (CD4 or CD8) diluted X in TBS. Incubated overnight at 4ºC.

Day 2

- Leave the slides for 1 hour at room temperature
- Wash with TBS 3X 5'
- Secondary antibody: Alexa fluor 594 goat anti mouse. Diluted in 1:100 in TBS for 1 hour.
- Wash with TBS 3X 5'
- Mounting with DAPI

Protocol 4 IF

Day 1

- Place samples in the oven at 60ºC for 20'
- Dewaxed samples in xylene 3X 10'
- Rehydrate samples going through alcohol 100, 96 and 70% 5' each
- Place in PBS 2X 5'

- Antigen retrieval: Incubate with Protease K (25µg dissolved in 1ml TBS) at 37 ºC 5’
- Stop the treatment rinsing the slides several times in 5mM TBS (Tris buffer saline).
- Biotin and Avidin blocking kit (DAKO, Glostrup, Denmark). according manufacture instruction
- Wash with TBS 3X 5’
- Peroxidase blocking (0.05% H_2O_2) 30’
- Wash with TBS
- Primary antibody
- Non-specific protein binding blocking: FCS for 30’
- Wash with TBS 3X 5’
- Primary antibody (CD4 or CD8) diluted X in TBS. Incubated overnight at 4ºC.

Day 2

- Leave the slides for 1 hour at room temperature
- Wash with TBS
- Secondary antibody: Alexa fluor 594 goat anti mouse. Diluted in 1:100 in TBS for 1 hour
- Wash with TBS 3X 5’
- Mounting with DAPI

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