The effect of ewe nutrition during pregnancy on the reproductive system of the offspring

A thesis presented in partial fulfilment of the requirements
for the degree of Doctor of Philosophy
in Animal Science at Massey University,
Palmerston North, New Zealand

Asmad Kari

2014

Abstract

Human and domestic animal epidemiological studies have shown that the early life intrauterine environment can play a critical role in determining the development of various organs and systems at the cellular level, and the lifetime health status and productive performance of an individual. However, only sparse information exists for sheep, regarding the effects of maternal feeding during pregnancy under New Zealand grazing conditions on offspring growth and reproductive performance.

In this thesis, two paradigms were examined; (i) Dam size (heavy *vs* light; selected based on extreme live weights from a commercial flock) and dam nutrition for a prolonged period in pregnancy (*ad libitum vs* maintenance; P21-140), and (ii) Dam nutrition during early P21-50 (*ad libitum vs* maintenance *vs* sub maintenance) and mid-to-late pregnancy P50-139 (*ad libitum vs* maintenance) which are to the range of nutritional treatments used by New Zealand farmers. *Ad libitum* was used to provide unrestricted access to pasture forage, maintenance was to ensure total live weight gain equivalent to the expected conceptus mass and sub maintenance was to achieve a loss in total ewe live weight 0.1 kg/day. The growth and reproductive performance of the offspring during both the pre-natal and post-natal periods were examined.

The results from this thesis indicated that it was possible to alter ovarian cell development of the female offspring during fetal and adult life by varying dam size at the time of conception. In male offspring, only minor effects of dam size on fetal testicular cell development were observed. Maternal nutrition during pregnancy altered female offspring fetal ovarian cell development but there was no effect on reproductive performance as an adult. In fetal male offspring, maternal nutrition did not alter testicular cell development, however, minor effects were observed on adult reproductive performance. Overall, there was little effect of both paradigms on male and female offspring lifetime performance.

Combined, the results suggest that farmers using similar grazing conditions to the present studies do not need to take into account nutrition of the dam when selecting male or female replacements. Future studies may consider more extreme underfeeding, but this may not be relevant on sheep farming in New Zealand. Further studies are required to further investigate the possible effects of maternal size on lifetime performance of the offspring.

Acknowledgements

First and foremost, with an overwhelming sense of gratitude I thank the Almighty God for giving me strength and perseverance to achieve the successful completion of this work.

I would like to express my sincere thanks and gratitude to my supervisors Professor Paul Kenyon, Professor Hugh Blair, Dr. Sarah Pain and Professor Tim Parkinson for their guidance, advice and encouragement throughout this study. Their great supervision, sustained encouragement, help, cooperation, inspiration, support, constructive criticism, quick feedback and friendliness made it truly a privilege to work under all of them. Working under their supervision was a priceless experience and they will always be my role models throughout my future career.

My special thanks to Assoc. Prof. Nicolas Lopez Villalobos and Dr. Shinichi Nakagawa for their help in statistical analysis. Their help, cooperation, guidance, advices and friendliness is much appreciated.

The financial assistance provided by Ministry of Higher Education Malaysia Doctoral scholarship, Gravida, National Centre for Growth and Development and Institute of Veterinary, Animal and Biomedical Sciences (IVABS), Massey University, Palmerston North, for funding the research is duly acknowledged.

My sincere thanks are to Debbie Hill and Kristen Story for guiding and assisting me regarding the necessary administrative work. I also extend thanks to International Student Support Office, Massey University, especially Dianne Reilly and Natalia Benquet for their help and support. I am also thankful to all the staff members of Keeble Farm and the technical staff of IVABS, Massey University who helped me in this research, especially Dean Burham, Geoff Purchas, Natalia Martin, Evelyn Lupton and Saritha Gills for their help and support in conducting the practical field work and laboratory work.

My special thanks are conveyed to Stefan Smith and Lisanne Fermin for their help in the semen collection trial. I am thankful to Dr. Matthew Perrot and Dr. Kalyani Perera for their help in the histology and microscopy work; Professor Mike Dragunow and Dr.

Pritika Narayan for their help in image analysis and Assoc. Prof. Mark Hampton and his laboratory members for their help in the oxidative stress study.

I also extend thanks to my co-workers, office mates and friends Sara Azarpeykan, Sharini Sinhadipathige, Tessy George, Shirli Notcovich, Emily Vallee, Deepa Patel, Lisanne Fermin, Amy Paten, Maria Loureiro, Lydia Cave, Gabriella Grongvist, Javier Roca, Emi Sakata, Nick Sneddon, Doris Adeyinka, Jose Solis Ramirez, Kandarp Patel, Daniela Tapia, Felipe Lembeye, Ali Karkaba, Ali Alhajouj, Mazidah Abdul Rahman, Suryani Eka Wijaya, Haslinda Hassan, Amissa Arifin, Khairul Farihan Kassim and many others for their support, encouragement and friendliness.

My special thanks are also conveyed to my parents (Kari b Chik and Nik bt Abdullah), sisters (Rodziah, Fatimah, Wahidah, Amirah, Nurul Farhana and Aisyah), brothers (Mohd Ridzuan, Mohd Hanafi and Muhammad Hafizuddin), brothers in law (Bakhtiar Adha and Fadli), nephews (Basyirun and Syafiy), family and friends in Malaysia for their concern, prayers, support and encouragement. I really appreciate all of you. I also extend my warm thanks to my teachers Dr. Kassim Hamid and Dr. Aziz Taib for their encouragement and unflinching support, all of which are gratefully acknowledged.

Table of Content

List of Tables

List of Figures

Appendices

Chapter 1

Introduction

1.1. Introduction

The early life intrauterine environment plays a critical role in determining the lifetime health status of an individual. Epidemiological studies in humans helped develop the 'Barker hypothesis'; which postulates that impeded fetal growth is associated with a number of chronic health conditions later in life (Godfrey and Barker, 2000). This idea has been the genesis for further work in many species, including examining the effects of the intrauterine environment in sheep on the growth and reproductive development of the fetus and their subsequent lifetime outcomes (Harding and Johnston, 1995; Bell and Ehrhardt, 2002; Redmer *et al*., 2004; Rhind *et al*., 2001; Reynolds *et al*., 2005; Kenyon, 2008; Gardner *et al*., 2009; Blair *et al*., 2010).

A common practice of New Zealand farmers is to restrictively feed ewes during early pregnancy in order to conserve pasture for later stages of pregnancy when fetal growth is greatest (Kenyon and Webby, 2007). However, nutrition during early pregnancy is also critical for fetal growth, due to high embryonic metabolic activity and rapid developmental changes (Ehrhardt and Bell, 1995; Redmer *et al*., 2004). There are some evidences to show that restriction of maternal nutrition can negatively affect fetal growth (Bielli *et al*., 2001), fetal mammary gland development (Jenkinson, 2003), oxidative stress (Bernal *et al*., 2010) and the reproductive performance of male (Bielli *et al*., 2001; Rae *et al*., 2002; Gardner *et al*., 2009) and female (Rae *et al*., 2001; Borwick *et al*., 1997) offspring. Therefore, the feeding practice of New Zealand's farmers could inadvertently affect the growth and reproductive development of ewes' offspring in their later life. However, the long-term effects of maternal nutrition are not well understood especially in regards to critical levels of nutrient restriction and critical time windows of restriction upon the regulation of cell development and subsequent post-natal growth and reproductive development of offspring. In addition to nutrition,

maternal size can also affect the offspring development by limiting the mother's capability to supply nutrients to the fetus(es) through the placenta (Gluckman and Hanson, 2004). This notion is supported by previous findings which show that maternal size can influence gestational length (Bloomfield *et al*., 2003), placental weight (MacLaughlin *et al*., 2005) and birth and weaning weight of the offspring (Kenyon *et al*., 2009; Aliyari *et al*., 2012).

The objectives of this thesis are to examine the effects of pregnancy nutritional regimen under New Zealand grazing conditions and maternal size on:

- Testicular cell development of the offspring at Day 140 of gestation (Chapter 5).
- Growth (live weight) and reproductive performance (scrotal circumference, semen characteristics and testosterone levels) of two-year-old male offspring (Chapter 6).
- Ovarian cell development of offspring during pre-natal (Day 65, Day 100 and Day 140 of gestation) and post-natal life (six-year-old) (Chapter 7).
- Lifetime performance (growth, pregnancy diagnosis, ovulation rate, lambs born, lambs weaned, lambs weaned weight and ewe efficiency) of female offspring from two to six years of age (Chapter 8).
- Oxidative stress levels of fetal ovaries at Day 140 of gestation (Chapter 9).

Two different meta-analyses focusing on males and females respectively, of existing literature examining the effect of maternal nutrition on the growth and reproductive performance of offspring, were conducted to determine which factors appear to be most critical to the growth and reproductive development of offspring (Chapter 3 and 4).

Therefore the hypothesis of this study that differential maternal size and/or nutrition (maintenance or moderately restricted feeding) during pregnancy can lead to the alteration of the growth and reproductive development of offspring.

1.2. References

Aliyari, D., Moeini, M. M., Shahir, M. H., & Sirjani, M. A. (2012). Effect of body condition score, live weight and age on reproductive performance of Afshari ewes. *Asian Journal of Animal and Veterinary Advances, 7*(9), 904-909.

Bell, A. W., & Ehrhardt, R. A. (2002). Regulation of placental nutrient transport and implications for fetal growth. *Nutrition Research Reviews, 15*(2), 211-230.

Bernal, A. B., Vickers, M. H., Hampton, M. B., Poynton, R. A., & Sloboda, D. M. (2010). Maternal undernutrition significantly impacts ovarian follicle number and increases ovarian oxidative stress in adult rat offspring. *PLoS ONE, 5*(12), e15558.

Bielli, A., Katz, H., Pedrana, G., Gastel, M. T., Morana, A., Castrillejo, A., Lundeheim, N., Forsberg, M., & Rodriguez-Martinez, H. (2001). Nutritional management during fetal and post-natal life, and the influence on testicular stereology and Sertoli cell numbers in Corriedale ram lambs. *Small Ruminant Research, 40*(1), 63-71.

Blair, H. T., Jenkinson, C. M. C., Peterson, S. W., Kenyon, P. R., van der Linden, D. S., Davenport, L. C., Mackenzie, D. D. S., Morris, S. T., & Firth, E. C. (2010). Dam and granddam feeding during pregnancy in sheep affects milk supply in offspring and reproductive performance in grand-offspring. *Journal of Animal Science, 88*, E40-E50.

Bloomfield, F. H., Oliver, M. H., Giannoulias, C. D., Gluckman, P. D., Harding, J. E., & Challis, J. R. G. (2003). Brief undernutrition in late-gestation sheep programs the hypothalamic-pituitary-adrenal axis in adult offspring. *Endocrinology, 144*(7), 2933-2940.

Borwick, S. C., Rhind, S. M., McMillen, S. R., & Racey, P. A. (1997). Effect of undernutrition of ewes from the time of mating on fetal ovarian development in mid gestation. *Reproduction Fertility and Development, 9*(7), 711-715.

Ehrhardt, R. A., & Bell, A. W. (1995). Growth and metabolism of the ovine placenta during mid-gestation. *Placenta, 16*(8), 727-741.

Gardner, D. S., Ozanne, S. E., & Sinclair, K. D. (2009). Effect of the early-life nutritional environment on fecundity and fertility of mammals. *Philosophical Transactions of the Royal Society B-Biological Sciences, 364*(1534), 3419-3427.

Gluckman, P. D., & Hanson, M. A. (2004). Living with the past: Evolution, development, and patterns of disease. *Science, 305*(5691), 1733-1736.

Godfrey, K. M., & Barker, D. J. (2000). Fetal nutrition and adult disease. *The American Journal of Clinical Nutrition, 71*(5), 1344S-1352S.

Harding, J. E., & Johnston, B. M. (1995). Nutrition and fetal growth. *Reproduction Fertility and Development, 7*(3), 539-547.

Jenkinson, C. M. C. (2003). The pattern and regulation of mammary gland development during fetal life in the sheep. PhD Thesis. Massey University, Palmerston North, New Zealand.

Kenyon, P. R. (2008) A review of *in-utero* environmental effects on sheep production. *Proceedings of the New Zealand Society of Animal Production,* 68, 142–155.

Kenyon, P. R., & Webby, R. W. (2007). Pastures and supplements in sheep production systems. In P. V. Rattray, I. M. Brookes & A. M. Nicol (Eds.), *Pasture and Supplements for Grazing Animals* (pp. 255-273): New Zealand Society of Animal Production Occassional Publication.

Kenyon, P. R., Blair, H. T., Jenkinson, C. M. C., Morris, S. T., Mackenzie, D. D. S., Peterson, S. W., Firth, E. C., & Johnston, P. L. (2009). The effect of ewe size and nutritional regimen beginning in early pregnancy on ewe and lamb performance to weaning. *New Zealand Journal of Agricultural Research, 52*(2), 203-212.

MacLaughlin, S. M., Walker, S. K., Roberts, C. T., Kleemann, D. O., & McMillen, I. C. (2005). Periconceptional nutrition and the relationship between maternal body weight changes in the periconceptional period and feto-placental growth in the sheep. *The Journal of Physiology, 565*(1), 111-124.

Rae, M. T., Palassio, S., Kyle, C. E., Brooks, A. N., Lea, R. G., Miller, D. W., & Rhind, S. M. (2001). Effect of maternal undernutrition during pregnancy on early ovarian development and subsequent follicular development in sheep fetuses. *Reproduction, 122*(6), 915-922.

Rae, M. T., Rhind, S. M., Fowler, P. A., Miller, D. W., Kyle, C. E., & Brooks, A. N. (2002). Effect of maternal undernutrition on fetal testicular steroidogenesis during the CNS androgen-responsive period in male sheep fetuses. *Reproduction, 124*(1), 33-39.

Redmer, D. A., Wallace, J. M., & Reynolds, L. P. (2004). Effect of nutrient intake during pregnancy on fetal and placental growth and vascular development. *Domestic Animal Endocrinology, 27*(3), 199-217.

Reynolds, L. P., Borowicz, P. P., Vonnahme, K. A., Johnson, M. L., Grazul-Bilska, A. T., Redmer, D. A., & Caton, J. S. (2005). Placental angiogenesis in sheep models of compromised pregnancy. *Journal of Physiology-London, 565*(1), 43-58.

Rhind, S. M., Rae, M. T., & Brooks, A. N. (2001). Effects of nutrition and environmental factors on the fetal programming of the reproductive axis. *Reproduction, 122*(2), 205-214.

Chapter 2

Literature Review

2.1 Overview of literature review

The uterine environment in which early embryonic and fetal development takes place is essential in determining pregnancy success, as well as the health and performance of offspring in later life (Barker and Osmond, 1986; Barker *et al*., 1993; Godfrey and Barker, 2000). Intrauterine development can be influenced by maternal diet, endocrine milieu, physical condition and other factors that act during critical stages of embryonic and fetal life and, by interacting with the genome, can induce long-term changes in not only the fetal phenotype and but also its functional genotype (Gardner *et al*., 2009). This process is known as fetal programming. The term 'fetal programming' was originally used by David Barker to describe his findings from epidemiological studies that correlated health problems (e.g: coronary heart disease, stroke, diabetes and hypertension) to low birth weight in adult humans. The 'Barker' hypothesis states that undesirable effects during early embryonic/fetal life can result in permanent changes in physiology and metabolism in adult life (Armitage *et al*., 2004; De Boo and Harding, 2006).

Maternal nutrition is arguably the most critical factor that can influence the development of offspring since nutritional supply is a key regulator of fetal development (Harding and Johnson, 1995). The effects of maternal nutrition upon the fetus are also dependent on the effectiveness of the placenta; the most important organ for exchange of respiratory gases, nutrients and wastes between the maternal and fetal system (Redmer *et al*., 2004).

The importance of maternal nutrition in the programming of growth and reproductive function of the offspring has been known for some time (Gunn *et al*., 1972; Gunn, 1977; Allden, 1979; Rhind *et al*., 2001). Maternal nutrition can alter the size and nutrient

transfer capacity of the placenta, especially in the early- to mid-pregnancy period as this is when the majority of placental growth occurs, thus ultimately determining pre-natal growth trajectory of the fetus (Redmer *et al*., 2004) (Figure 2.1.). Studies in sheep have shown that maternal undernutrition during pregnancy can result in a reduction in the lifetime reproductive capacity of both male and female offspring (Gunn *et al*., 1995; Rhind *et al*., 2001; Kotsampasi *et al*., 2009a). Recent evidence has also shown that maternal nutrition can cause oxidative stress, DNA damage and alteration of gonadal tissues, potentially persisting into later life (Bernal *et al*., 2010; Igwebuike, 2010).

This review will examine normal reproductive organ development during pre-natal and early post-natal life; the potential constraining effects of maternal size and nutrition, critical windows of nutrition restriction and nutritional manipulation during pregnancy on fetal growth and reproductive development. The perspective and hypotheses of this thesis will also be described.

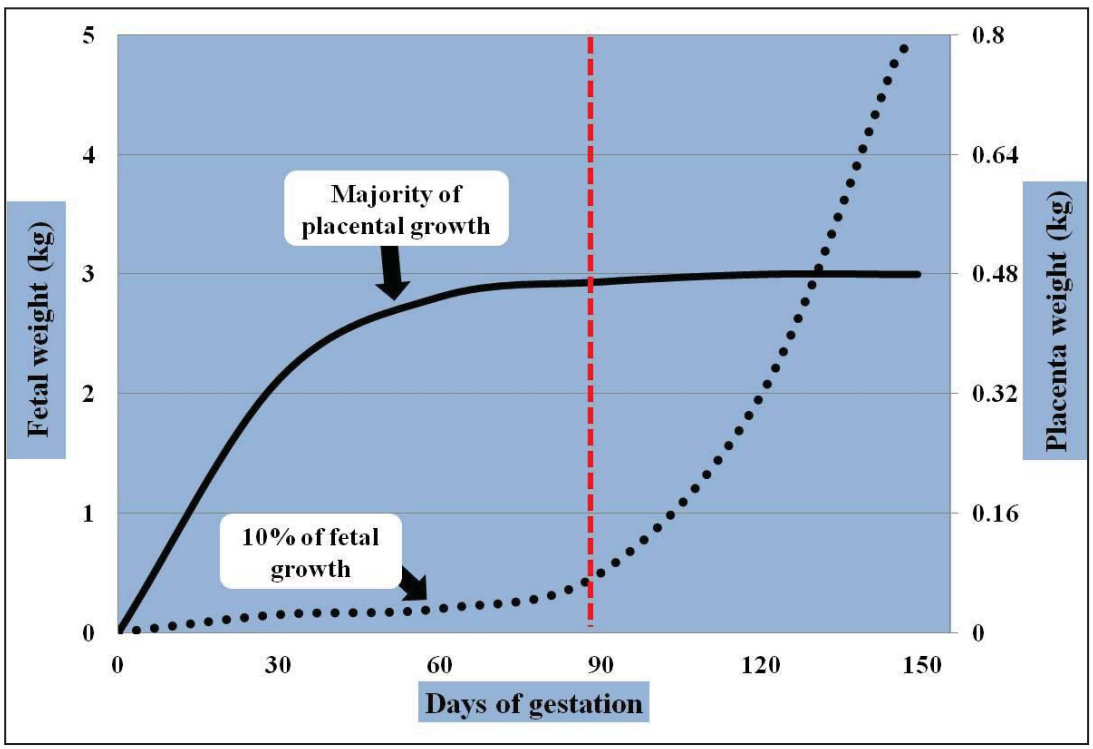


Figure 2.1. Normal growth trajectories of the ovine fetus and placenta from conception to end of gestation period (Adapted and modified from Barcroft, 1946).

2.2 Gonadal development of male and female sheep during the pre-natal and post-natal period

There are a number of fetal development processes that occur during all stages of pregnancy with specific effects on gonadal development (Rhind, 2004). Male and female gonads develop during both pre-natal and post-natal life before they are reproductively functional. The stages of ovine gonadal development; sexual differentiation, proliferation, meiosis (in the female), primordial cell development and follicular formation and growth mostly occur during fetal life. Each stage of development involves regulation by hormones and growth factors, as well as interactions among differing cell types.

The male and female reproductive tracts are derived from the same embryonic tissue. The gonads develop from the mesothelium (coelomic epithelium) lining the posterior abdominal wall, the underlying mesenchyme (intermediate mesoderm), and the primordial germ cells (McGeady *et al*., 2006; Schoenwolf *et al*, 2009). The indifferent gonad consists of a medulla and cortex. In males (XY) embryo, the medulla will develop into the testes and the cortex will regress, whilst in females (XX) ovary will develop from the cortex and medulla will decline (Schoenwolf *et al*, 2009).

The gonads, internal and external genetalia begin as bipotential tissues (Wilhelm *et al*., 2007; Schoenwolf *et al*, 2009). There are two genital duct systems (Müllerian and Wolffian ducts) which are present at the bipotential stage (Wilhelm *et al*., 2007). In males, the Müllerian duct degenerate under the influence of AMH secreted by the testicular Sertoli cells which also stimulates the differentiation of mesenchymal cells to Leydig cells (testosterone secreting), whereas the Wolffian duct differentiate into epididymides, vasa differentia and seminal vesicle under the control of androgens

produced by Leydig cells (Wilhelm *et al*., 2007). The differentiation of male gonad is dependent on expression of SRY (sex reversal Y) which is expressed in the Sertoli cells results in a cascade of events leading to the development of seminiferous tubules (Wilhelm *et al*., 2007). In females, in the absence of testosterone the Wolffian duct degenerate and Müllerian duct (in the absence of AMH) differentiates into oviduct, uterus and upper vagina (Wilhelm *et al*., 2007). The following section outlines the remainder of normal development of the testis and ovary during pre-natal and post-natal life.

2.2.1 Male

Pre-natal

Testicular differentiation in the sheep occurs around Day 27 of gestation followed by steroidogenesis and activation of associated enzyme systems (Rhind, 2004). The main testicular structures are gonocytes, Sertoli cells, seminiferous tubules and Leydig cells (Figure 2.2.). During the pre-natal stage, Sertoli cells are present at around Day 34 of gestation, seminiferous cords by about Day 35 to 40 (Sweeney *et al*, 1997; Rhind *et al*., 2001), and Leydig cells from Day 42 (Hochereau-de Reviers *et al*., 1995). Sertoli cells surround the gonocytes and occupy around 80% of the sex cord volume (Hochereau-de Reviers *et al*., 1995). Gonocytes appear as large cells, about 12 µm in diameter, which are centrally located in the sex cords (Hochereau-de Reviers *et al*., 1995). Leydig cells appear as large, round cells mainly in groups of two or more near small blood vessels (Hochereau-de Reviers *et al*., 1995).

The relative volume in the intertubular tissues of the testis decrease from 20% at Day 42 to 9% at Days 139-150 of gestation and, by Day 70, the rete testis is organized in the

centre of the testis (Sweeney *et al*, 1997). Meanwhile, between Days 35 and 85 of gestation, the gonadotrophin releasing hormone (GnRH) neuronal system develops in the hypothalamus (Caldani, *et al*., 1995). A functional hypothalamic-pituitary-gonadal (HPG) axis is present in the sheep fetus around Day 70 of gestation. Luteinising hormone (LH) and follicle stimulating hormone (FSH) gonadotrophs are present in the anterior pituitary gland by Day 70 and Day 100 of gestation, respectively (Thomas *et al*., 1993). Studies of sexual differentiation in ruminants have shown steroidal influences as early as Day 50 to 80 of gestation can affect gonadotrophin secretion and therefore, by birth, the male reproductive system development is almost complete (Ford and Klindt, 1989; Rae *et al*., 2002b).

Post-natal

Sertoli cells continue to develop and divide after birth, with their maximal numbers being set before puberty at around 40-80 days of post-natal age (Hochereau-de Reviers *et al*., 1995). However, the basic structure of testis (seminiferous tubules/Leydig cells) remains unchanged from gonadal sex differentiation at 37 days of post-natal age (Eckery *et al*., 1996) until the onset of puberty (Hochereau-de Reviers *et al*., 1995).

At puberty, gonocytes move to the periphery of the tubules and start to differentiate into spermatogonia which are supported by Sertoli cells. These changes occur with the prepubertal rise in gonadotrophin concentrations. Sertoli cells remain present during the animal's entire sexual life and influence sperm production. At puberty; the activity of the gonadotrophs increase and the testis becomes capable of simultaneously producing hormones (steroidogenesis) and sperm (gametogenesis) for fertilisation indicating that the testicular cells are able of releasing gametes (Amann and Schanbacher, 1983).

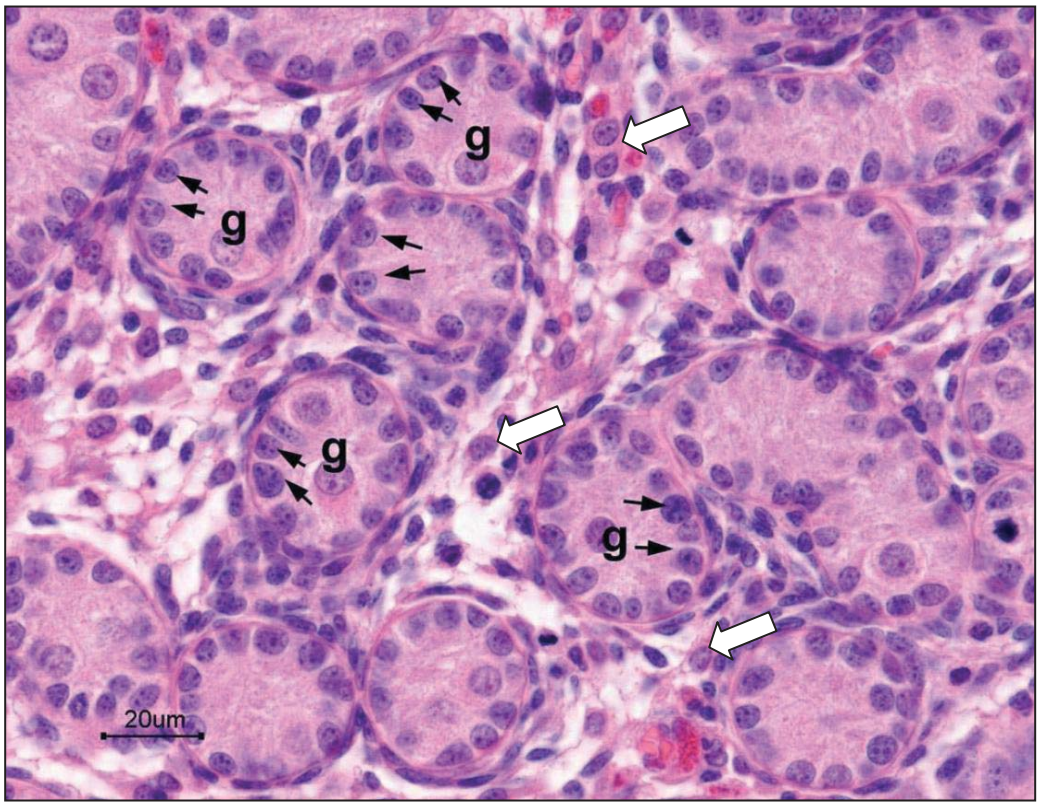


Figure 2.2. Morphology of the fetal ovine testis on Day 103 of gestation, comprising seminiferous cords with one or two gonocytes (g) centrally located in the middle of the cord, Sertoli cells (black arrows) located at the periphery and Leydig cells (white arrows) near the basement membrane of the cord (Adapted from Da Silva *et al*., 2003).

2.2.2 Female

Pre-natal

The very early structure of the ovary is similar to the structure of testis. Sex cords structures, formed by somatic and germ cells, basically remain unchanged, and are present at the beginning of ovarian differentiation (Eckery *et al*., 1996). Oocytes (germ cells) within interstitial tissues are wrapped by a few somatic cells to form primordial structures. The mesonephros and coelomic epithelium cells in the ovary contribute to gonadal formation (Wartenberg, 1981). This formation, into a functional excretory organ, is determined by the thickening of cells along the medial aspect of the

mesonephros (Grinsted, 1981). Primordial cells start to populate the gonad following migration from extragonadal sites once the gonadal ridge is formed (Figure 2.3.).

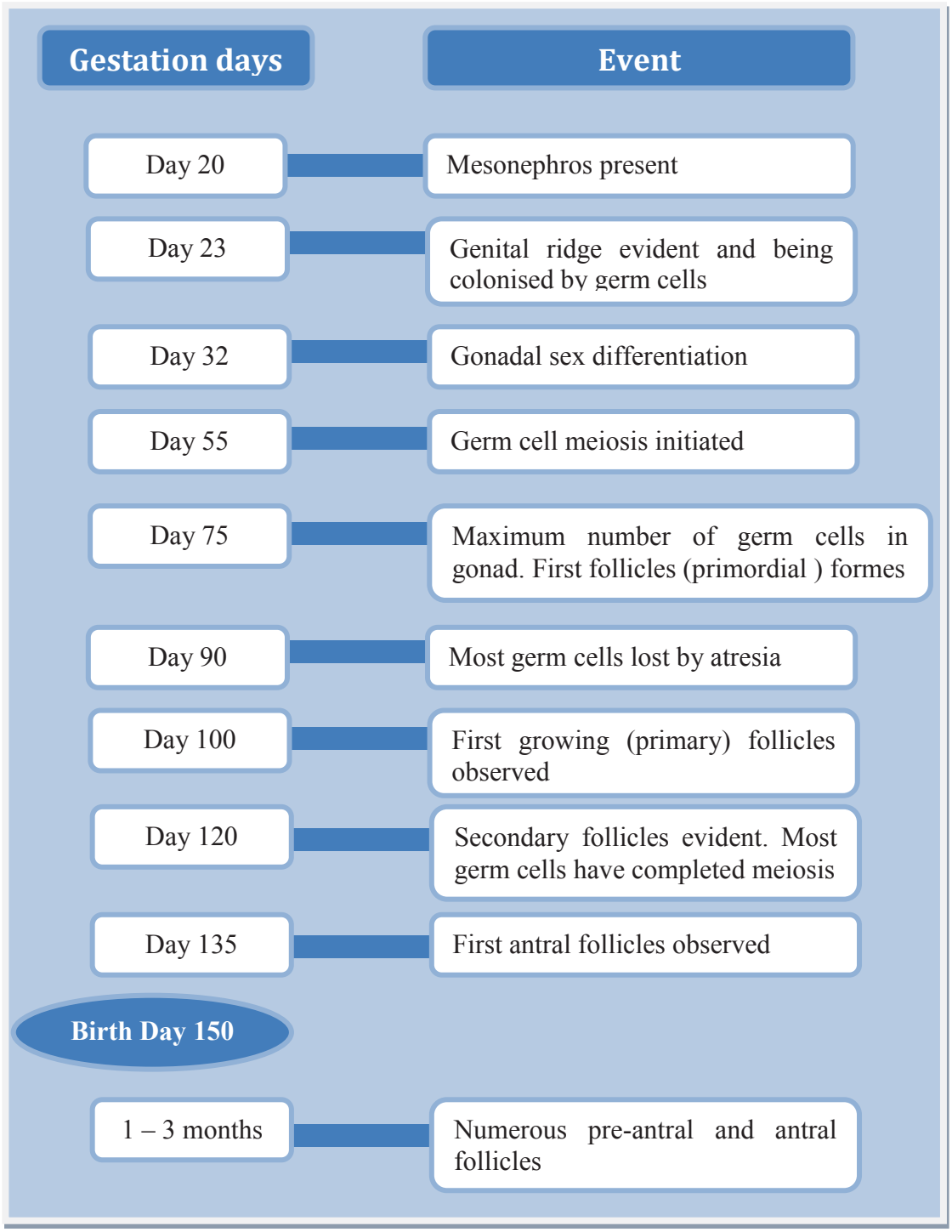


Figure 2.3. Sequences of ovarian formation and key events occurring during gestation and after birth in the sheep (Eckery *et al*., 1996).

Sexual differentiation of the female sheep starts at around Day 32 of gestation (Eckery *et al*., 1996). The population of germ cells in the ovary rapidly increases by mitosis. This process continues until a maximum number of primordial follicle cells is reached, usually by about Day 75 of gestation. The rapid increase of germ cells is followed by a massive decline/atresia (80%) of the same cells by Day 90 of gestation (Eckery *et al*., 1996), however, mitotic processes are still observed in germ cells until Day 120.

At around Day 55 of gestation, germ cell meiosis is initiated. The first germ cells to enter meiosis are those at the innermost region of the ovarian cortex (Eckery *et al*., 1996). Meiosis persists until about Day 120 of gestation. The ovarian medulla and cortex are clearly defined when meiosis starts, with nearly all germ cells being localised in the cortex region. Primordial follicles form in the cortex region at Day 75 of gestation, while germ cells enter meiosis in the immediately adjacent region and oogonia undergo mitosis in the outermost region (Eckery *et al*., 1996). The development of primordial to primary follicles in the female sheep fetus begins at about Day 90 of gestation (McNatty *et al*., 2000). Primary follicles start to grow at around Day 100 of gestation, secondary follicles at Day 120 and the first antral follicles are present at Day 135 of gestation (Eckery *et al*., 1996; Rhind, 2004).

Post-natal

At birth, the sheep ovary contains many preantral and antral follicles (Figure 2.4.). Three months after birth, some follicles have reached preovulatory size and many are steroidogenically active (McNatty *et al*., 1987). Follicular growth and maturation in the ovary follow a series of sequential sub-cellular and molecular transformations of

various follicular components (the oocyte, granulosa and theca cells). These transformations are controlled by intraovarian and intrafollicular factors and hormonal signals that lead the secretion of androgens and oestrogens (Hafez and Hafez, 2000). Complete follicular development and ovulation occur only when the concentrations and secretion patterns of FSH or LH reach adult values (Hafez and Hafez, 2000). At puberty, the ovary is able to release ova and balance the ratio of steroid hormones required to maintain the development of reproductive organs and facilitate fertilisation (Foster *et al*., 1985).

In addition, there are a number of growth factors from the transforming growth factor β (TGFβ) that play impotant roles in the regulation of animals during fetal and adult age. These growth factors can stimulate or inhibit differentiation and other critical processes in cell function (Sporn *et al*., 1986). The GDF9 and AMH are among the growth factors from the transforming growth factor β (TGFβ) family in the ovary that play an important role for normal follicular development (Dong *et al*., 1996; Visser *et al*., 2006). GDF9 gene is associated with infertility due to follicular growth arrest at the primary stage (Dong *et al*., 1996), whilst AMH plays an important role in both ovarian primordial follicle recruitment and selection of the dominant follicle, and is established as a marker for ovarian reserve (Weenen *et al*., 2004; Anderson, 2012).

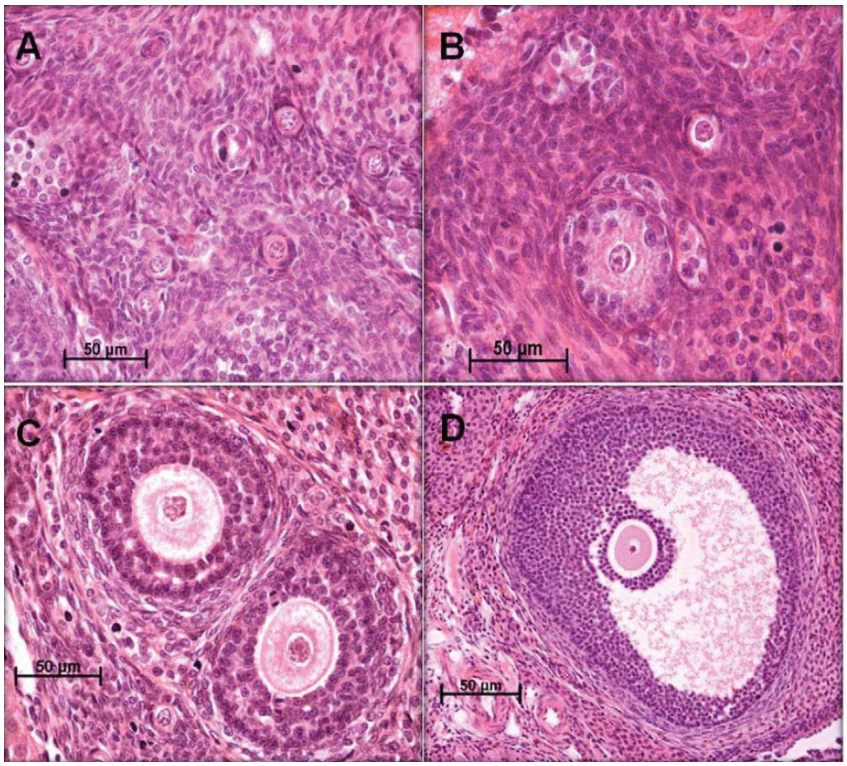


Figure 2.4. Morphological classification of ovarian follicles. A) primordial - germ cells surrounded by flattened follicular cells; B) primary - enlarge oocyte completely surrounded by one or two layers of cubodial follicular cells; C) secondary - enlarged oocyte surrounded by two or more concentric layers of cubodial cells, and; D) antral follicles - an oocyte surrounded by multiple layers of cuboidal granulose cells and containing antral spaces, cumulus oophorus and theca layer (Bernal *et al*., 2010).

2.3 Effects of maternal constraints on offspring growth and reproductive development

There are several stressors that may contribute to maternal constraints during gestation which, in turn, can affect fetal development (Harding and Johnston, 1995; Redmer *et al*., 2004; Rhind *et al*., 2001; Rhind, 2004; Gootwine *et al*, 2007, Wu *et al*., 2006; Kenyon, 2008; Gardner *et al*., 2009). In this thesis, the focus is on maternal size and nutritional stressors during pregnancy which may affect fetal growth and reproductive development.

Maternal size (live weight and body condition) can influence offspring development by limiting the mother's capability to supply nutrients to the fetus(es) via the placenta (Gluckman and Hanson, 2004). Lighter mothers can have smaller placentas which influence the capacity or efficiency of maternal nutrient and oxygen transfer to the fetus, especially in dams carrying multiple fetuses (Gootwine *et al*., 2007), resulting in a higher probability of intrauterine growth retardation (IUGR). Therefore, having a moderately heavier mother may reduce the risk of IUGR. Maternal size can influence gestation length (Bloomfield *et al*., 2003), placental weight (MacLaughlin *et al*., 2005), birth weight, and weaning weight of the offspring (Russel *et al*., 1981; Kenyon *et al*., 2009; Aliyari *et al*., 2012). Maternal size can also influence the fertility and fecundity of female offspring by altering their ovulation rate (reviewed by Michels *et al*., 2000). In addition, a genetic correlation between ewe weight and fecundity has been reported (Baker *et al*., 1979; Fogarty *et al*., 1994), and Safari *et al*. (2005) showed that reproductive performance of the mother was positively influenced by their size and that this trait can be inherited by the offspring.

Fetal growth is a complex biological process that is influenced by genetic, epigenetic and environmental factors. These factors regulate placental transfer of nutrients and oxygen from mother to fetus, and they also determine the endocrine environment in which the fetus develops (Harding and Johnston, 1995; Bell and Ehrhardt, 2002; Redmer *et al*., 2004; Wu *et al*., 2006). Thus, changes in maternal nutrition and endocrine status can potentially alter the structure, physiology and metabolism status of the fetus (Wu *et al*., 2006).

Epigenetic regulation of gene expression during fetal development (Jammes, *et al*., 2011) has the potential to affect later life performance (Whorwood *et al*., 2001; Arrighi *et al*., 2010). Fetal gene expression can be mediated nutritionally or endocrinologically

by DNA methylation (Rees, 2002). Epigenetic modification of gene expression can be altered not only in the offspring themselves, but these modifications can also be inherited by subsequent generations (Lumey, 1992). Liang *et al.* (2007) reported that food restriction during pregnancy in female rat-like hamsters had negative effects on body growth of first generation (F1) offspring, and on the physical and neurodevelopment of both F1 and F2 offspring. Other studies have shown that food restriction in female rat-like hamsters decrease the size of reproductive organs and the concentration of reproductive hormones in F1 (male and female) and F2 (male) offspring (Liang and Zhang, 2006). Likewise, studies in rats have shown that growth trajectories of F2 generations derived from malnourished dams were even longer and slower than the growth trajectories of the F1 generation (Lobe *et al*., 2006).

Oxidative stress due to maternal undernutrition can alter the fertility of male and female offspring by disrupting genome sequences (DNA damage and DNA fragmentation), modifying structural lipids (lipid peroxidation) and proteins (alteration of enzymes and formation of protein carbonyls), thus impacting on later physiological function (Bernal *et al*., 2010; Tarry-Adkins *et al*., 2010). At present there are a number of studies in rodents (Bernal *et al*., 2010; Igosheva *et al*., 2010; Tarry-Adkins *et al*., 2010) examining the effects of oxidative stress due to pre-natal and early post-natal nutrition on the offspring development. To date there is limited data available on the effects of oxidative stress due to maternal nutrition on offspring development in the ruminant.

2.4 Critical time windows when maternal nutrition can affect offsprings' development

Gestation is normally classified into three different stages; early, mid and late. In sheep, these stages correspond to Days 0 to 50, 51 to 100 and 101 to 145, respectively. Early studies examining the effects of maternal nutrition on fetal development have typically focused on the later stages of pregnancy and the early neonatal period (e.g. Gunn *et al*., 1995). However, there is a growing body of evidence that nutrition during all stages of pregnancy can affect fetal development (Redmer *et al*., 2004; Rhind *et al*., 2001; Rhind, 2004; Gootwine *et al*, 2007, Wu *et al*., 2006) (Figure 2.5.). During pregnancy there are a number of developmental events that occur not only during early, mid or late pregnancy, but throughout the whole period of pregnancy (Figure 2.6.). As fetal development processes are associated with a wide range of mechanisms, it is unsurprising that the effects of maternal over- and under-nutrition can be exerted at all stages of pregnancy (Figure 2.5.). However, the understanding of the mechanisms by which maternal nutrition at different stages of pregnancy affects the growth and future reproduction of ruminant offspring remains limited.

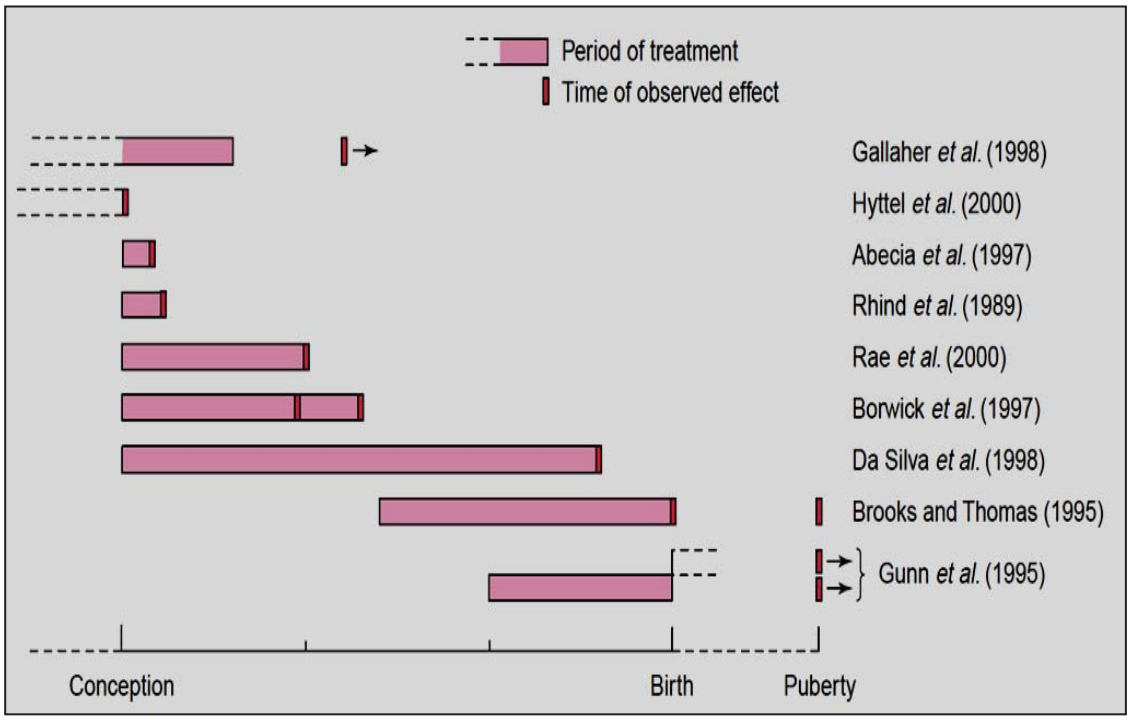


Figure 2.5. Periods of development in sheep, cattle and pigs during which maternal nutritional manipulations have been applied (light pink colour) and the stages which effects on structure or function of the reproductive system have been identified (dark red colour) (Rhind *et al*., 2001).

2.4.1 Early pregnancy

Early pregnancy is associated with high fetal metabolic activity per unit of weight, although the total energy requirement for fetal growth is relatively small (Robinson *et al*., 1997). During early pregnancy, embryonic and trophoblast development are potentially influenced by the concentration of nutrients (metabolisableable energy and protein) because these are highly metabolically active tissues and the placenta achieves its maximum weight during the first two-thirds of pregnancy (Barker *et al*., 1993; Redmer *et al*., 2004). Therefore, nutritional restriction during this early pregnancy implantation period can retard growth and reproductive development of the fetus (Tables 2.1,, 2.2., 2.3. and 2.4.).

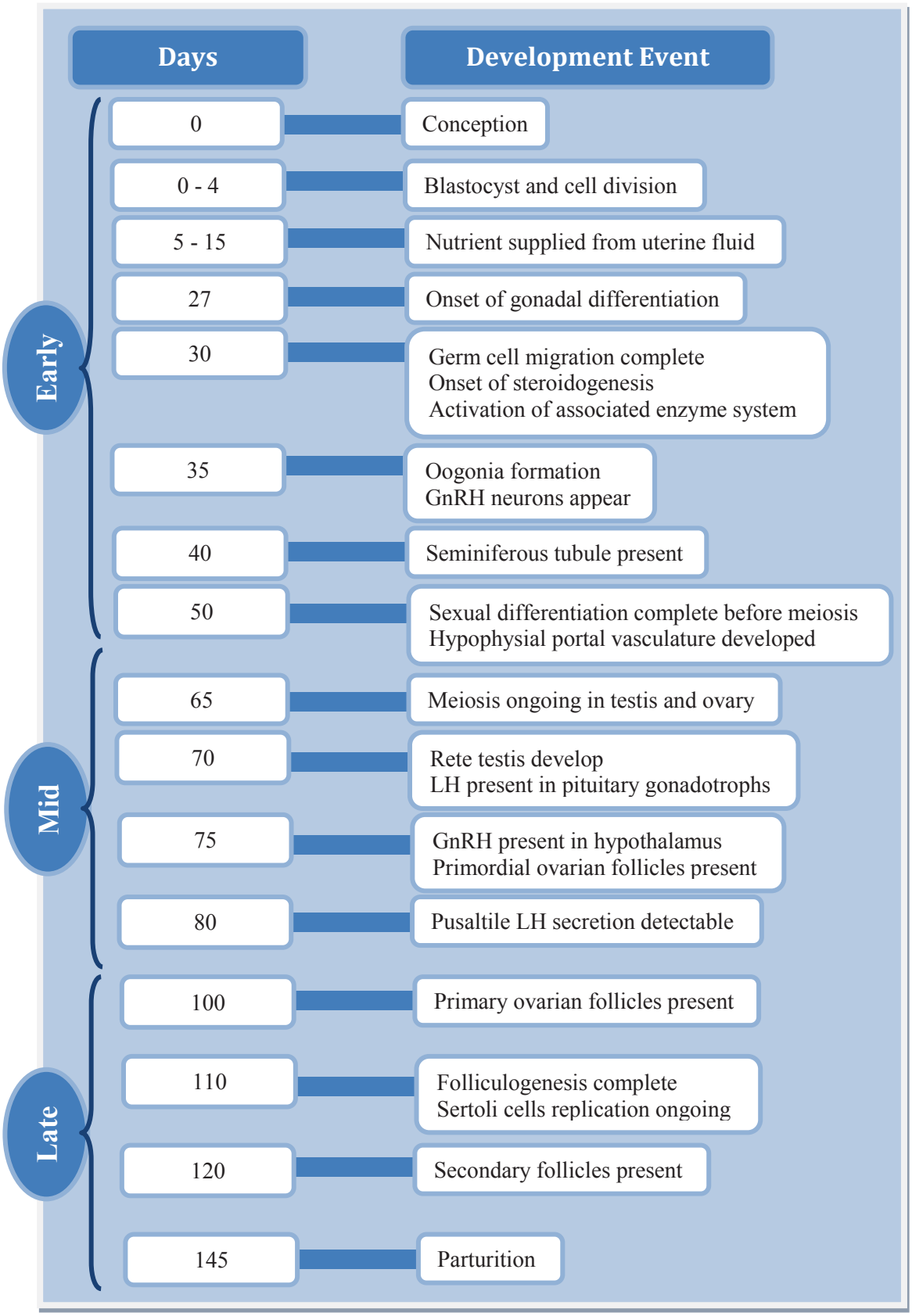


Figure 2.6. Approximate timing of reproductive development events in the sheep which may be sensitive to early life nutritional influences, expressed as days of gestation and percentage of gestation (Adapted from Rhind *et al*., 2001 and Rhind, 2004)

2.4.2 Mid pregnancy

In mid pregnancy, placental growth is rapid compared to fetal growth (Amoroso, 1959; Ehrhardt and Bell, 1995; Redmer *et al*., 2004). Restriction of nutrition during this period might influence the development of the placenta and thus affect its ability to perform transplacental exchange interactions between the fetus and the mother (Bell and Ehrhardt, 2002; Redmer *et al*., 2004) (Table 2.1., 2.2., 2.3. and 2.4.).

2.4.3 Late pregnancy

Late pregnancy is associated with maximum fetal growth and fetal nutritional demand (Foote *et al*., 1959; Robinson *et al*., 1997; Redmer *et al*., 2004). Almost 75% of ovine fetal growth occurs during the last 50 days of gestation (Robinson *et al*., 1997). Consequently, this is the time at which a deficiency in nutrient supply to the fetus would be expected to be damaging. Restriction of nutrition during this period can also delay growth and reproductive development of the fetus (Barker *et al*., 1993; Redmer *et al*., 2004; Rhind *et al*., 2001; Rhind, 2004) (Tables 2.1., 2.2., 2.3. and 2.4.).

2.5 Effects of maternal nutritional manipulation during pregnancy on the fetal growth

The following section reviews studies investigating the effects of manipulation of maternal nutrition during pregnancy on the growth of male and female offspring.

2.5.1 Male

A number of studies have investigated the impact of maternal nutrient restriction on the growth of male offspring (Table 2.1.). Several studies in sheep (Bielli *et al*., 2001; Da Silva *et al*., 2001), rats (Léonhardt *et al*., 2003; Genovese *et al*., 2010), and pigs (Kind *et al*., 2002, 2003) have shown that maternal nurient restriction during pregnancy reduced fetal weight, birth weight and subsequent body weight of male offspring (Table 2.1.). In contrast, other studies in sheep (Rae *et al*., 2002a, b, c; Da Silva *et al*., 2003; Kenyon *et al*., 2009, 2011; Simitzis *et al*., 2009; Smith *et al*., 2010), rats (Zambrano *et al*., 2005), pigs (Bauer *et al*., 2009) and cattle (Long *et al*., 2010) reported no effect of maternal nutrient restriction on fetal weight, birth weight, or body weight of male offspring. The inconsistencies between studies are likely due species, breed, climate and the level, time and duration of the nutritional manipulation imposed, suggesting that additional studies addressing these issues are warranted (see Literature Review Section 2.5.3).

Table 2.1. The effect of maternal nutrition regimen and timing of nutritional regimen on the growth of male offspring.

Species	Dietary manipulation	Time of dietary manipulation (Day)	Effect on restricted group	Reference
Sheep	Improve pasture + grain supplement *vs* Native pasture	1-99	Reduced birth weight and live weight at 99 days of age	Bielli *et al*., 2001
Sheep	*ad libitum vs* Moderate (to allow liveweight gain of approximately 75 g/day)	1-145	Increased fetal weight	Da Silva *et al*., 2001
Pig	*ad libitum vs* 85% *ad libitum*	1-115	Reduced birth weight and live weight at 80 days of age	Kind *et al*., 2002

Sheep	100% ME *vs* 50% ME	1-145	No differences in birth weight and live weight at 20 months of age	Rae *et al*., 2002a
Sheep	High (100% ME) or Low (50% ME)	1-30, 31-50, 1-50, 31-65, 1-65, d65-110, 1-110,	No differences in fetal weight	Rae *et al*., 2002b
Sheep	100% protein intake *vs* 50% protein intake	1-119	No differences in fetal weight	Rae *et al*., 2002c
Sheep	*ad libitum vs* Moderate (to allow liveweight gain of approximately 75 g/day)	1-103	No differences in male fetal weight	Da Silva *et al*., 2003
Pig	*ad libitum vs* 85% *ad libitum vs* 70% *ad libitum*	1-115	Reduced birth weight and live weight at 80 days of age	Kind *et al*., 2003
Rat	100% ME *vs* 50% ME	14-21	Reduced body weight at weaning	Léonhardt *et al*., 2003
Rat	100% protein intake *vs* 50% protein intake	1-22	No differences in fetal weight	Zambrano *et al*., 2005
Sheep	*ad libitum vs* Maintenance (to ensure no change in total live weight).	21-140	No differences in fetal weight and live weight	Kenyon *et al*., 2009
Sheep	100% ME *vs* 50% ME	1-30, 31-100	No differences in live weight	Kotsampasi *et al*., 2009a
Sheep	100% ME *vs* 50% ME	1-30, 31-100	No differences in birth weight and live weight	Simitzis *et al*., 2009
Pig	23% CP *vs* 14% CP	1-115	No differences in birth weight	Bauer *et al*., 2009
Sheep	110% ME *vs* 70% ME	110-145	No differences in birth weight	Smith *et al*., 2010
Rat	*ad libitum* vs 33.5% *ad libitum*	1-22	Reduced birth weight	Genovese *et al*., 2010
Bull	100% NRC *vs* 55% NRC	32-115	No differences in birth weight, weaning weight and live weight at 5 months of	Long *et al*., 2010

			age.	
Sheep	*ad libitum vs* Maintenance (to ensure no change in total live weight) *vs* Sub-Maintenance (to achieve a loss in total live weight of 100g/d)	21-50, 50-140	No differences in fetal weight and live weight	Kenyon *et al*., 2011

*CP (crude protein); ME (metabolisable energy); NRC (National Research Council requirement (1985))

2.5.2 Female

Many studies have examined the impact of maternal nutritional manipulation on the growth of female offspring (Table 2.2.). Evidence from sheep, cattle, rats and pigs (Gunn *et al*., 1995; Kind *et al*., 2002; Borwick *et al*., 2003; Léonhardt *et al*., 2003; Daniel *et al*., 2007; Martin *et al*., 2007; van der Linden *et al*., 2007; van der Linden *et al*., 2009; Neville *et al*., 2010) have all shown a reduction of fetal weight, birth weight and/or live weight due to maternal nutrient restriction during pregnancy. Interestingly, some studies (Da Silva *et al*., 2001; van der Linden *et al*., 2010a; Paten *et al*., 2011) have demonstrated that maternal nutrition restriction during pregnancy can actually increase fetal weight, birth weight and/or live weight of female offspring, whilst other studies in sheep, cattle, mice/rats and pigs (Gunn *et al*., 1995; Borwick *et al*., 1997; Da Silva *et al*., 2001; Meikle and Westberg., 2001; Rae *et al*., 2001, 2002b, c; Kind *et al*., 2002; Borwick *et al*., 2003; Chadio *et al*., 2007; Daniel *et al*., 2007; Martin *et al*., 2007; Chernoff *et al*., 2009; Kotsampasi *et al*., 2009b; Munoz *et al*., 2009; van der Linden *et al*., 2007; van der Linden *et al*., 2009; Neville *et al*., 2010; Smith *et al*., 2010; van der Linden *et al*., 2010a; Vonnahme *et al*., 2010; Paten *et al*., 2011) demonstrated that there was no effect of maternal nutrient restriction on either female offspring fetal weight, birth weight, and/or live weight. The results show inconsistencies between studies but, more importantly, most of the effects of maternal undernutrition upon offspring live

weight disappear with age (see 2.5.3), suggesting that any such effects are more likely to be associated with *in utero* development rather than imparting permanent effects upon the offspring.

Table 2.2. The effect of maternal nutrition regimen and timing of nutritional regimen on the growth of female offspring.

Species	Dietary manipulation	Time of dietary manipulation (Day)	Effect on restricted group	Reference
Sheep	Upland grazing + 525 g commercial pellet/day/animal *vs* Upland grazing *vs* Hill grazing.	50-145	Reduced birth weight, no difference in live weight at weaning and mating, no difference in live weight and body condition score at 18, 30 and 42 months of post-natal age	Gunn *et al*., 1995
Sheep	150% ME *vs* 50% ME.	mating to 47	No difference in fetal weight at d47 or d62	Borwick *et al*., 1997
Sheep	*ad libitum vs* Moderate (to allow 75 g/day liveweight gain).	0-145	Increased birth weight, no difference in live weight	Da Silva *et al*., 2001
Mice	*ad libitum vs* below *ad libitum*	1-21	No differences in birth and weaning weight	Meikle and Westberg., 2001
Sheep	100% ME *vs* 50% ME.	0-30, 31-50, 0-50, 31-65, 0-65, 65-110, 0-110	No difference in fetal weight at d50	Rae *et al*., 2001
Pig	*ad libitum vs* 85% *ad libitum*	1-115	No differences in birth weight	Kind *et al*., 2002
Sheep	100% ME *vs* 50% ME.	1 to 95	No difference in birth weight or live weight at 6 weeks/20 months of age,	Rae *et al*., 2002a
Sheep	100% ME *vs* 50% ME.	1 to 119	No difference in fetal weight	Rae *et al*., 2002c

Sheep	100% ME *vs* 70% ME.	100-parturition	Lower birth weight and live weight at 14 weeks of age, no difference in live weight at 26 weeks of age.	Borwick *et al*., 2003
Sheep	*ad libitum vs* Moderate (to allow live weight gain 75 g/day).	0-103	No difference in fetal weight	Da Silva *et al*., 2003
Pig	*ad libitum vs* 85% *ad libitum vs* 70% *ad libitum*	1-115	Reduced birth weight, no differences in growth rate from birth to weaning	Kind *et al*., 2003
Rat	*ad libitum vs* Low 50% *ad libitum*	14-21	Retardation of body growth	Léonhardt *et al*., 2003
Sheep	100% ME *vs* 50% ME.	0-30, 31-100	No differences in birth weight and live weight at 2, 5.5 and 10 months of age	Chadio *et al*., 2007
Cow	0.45 kg/d of 42% CP supplement *vs* no CP supplement	168-252	No differences in birth weight, lighter at weaning, pre-weaning, prebreeding, first pregnancy diagnosis and before second breeding season	Martin *et al*., 2007
Sheep	100% ME *vs* 50% ME.	30-70, 30-85	No differences in birth weight and live weight at 24 weeks, however restricted group at d30-70 had lower live weight at 22 weeks of age.	Daniel *et al*., 2007
Sheep	*ad libitum vs* Medium (Maintenance; to allow live weight gain 75 g/day).	21-140	No differences in birth weight, lighter weight at 46, 80 and 100 days of age, lower growth rates during the first 22 days of life.	van der Linden *et al*., 2007
Rat	*ad libitum vs* 50% *ad libitum*	1-22	No differences in body weight	Chernoff *et al*., 2009
Sheep	100% ME *vs* 50% ME.	0-30, 31-100	No differences in birth weight and live weight	Kotsampasi *et al*., 2009b

Sheep	EP=200% ME, and MP=140% ME *vs* EP=100% ME, and MP=80% ME *vs* 60% ME.	1-39, 40-90	No differences in offspring live weight and body condition score dam restricted during early or mid late pregnancy.	Munoz *et al*., 2009
Sheep	*ad libitum vs* Medium (Maintenance; to allow live weight gain 75 g/day).	21-140	No differences in fetal weight, lighter live weight during the first 49 days of lactation	van der Linden *et al*., 2009
Sheep	110% ME *vs* 50% ME.	0-7	No differences in birth weight and placenta weight	Smith *et al*., 2010
Sheep	140% NRC *vs* 100% NRC *vs* 60% NRC.	50-145	Lighter birth weight, no differences in live weight at d78, d162, d120 and d180 of age	Neville *et al*., 2010
Sheep	*ad libitum vs* Medium (Maintenance; to allow live weight gain 75 g/day).	21-140	No differences in birth weight, growth rates to weaning, post weaning and post pubertal. But higher growth during pre pubertal	van der Linden *et al*., 2010a
Sheep	*ad libitum vs* Medium (Maintenance; to allow live weight gain 75 g/day).	21-140	No differences in live weight and body condition score during pregnancy	van der Linden *et al*., 2010b
Sheep	140% NRC *vs* 100% NRC *vs* 60% NRC.	50-145	No differences in birth weight, live weight and average daily gain	Vonnahme *et al*., 2010
Sheep	*ad libitum vs* Medium (Maintenance; to allow live weight gain 75 g/day) *vs* Sub-maintenance	21-50, 50-140	Increased birth weight (mid late pregnancy), no difference in live weight	Paten *et al*., 2011

*CP (crude protein); ME (metabolisable energy); NRC (National Research Council requirement (1985))

2.5.3 Summary of maternal nutritional effect on offspring growth

Many studies have investigated the effect of maternal nutrition during pregnancy on the growth of male (Table 2.1.) and female (Table 2.2.) offspring and reported an alteration of the offspring's growth. However, the results are variable and inconsistent. Although there were variations between studies, most of the results show that the differences in offspring live weight due maternal nutrition manipulation have not persisted after weaning. This scenario is potentially related to the management of feeding and health in post-natal life which is well recognised as having effects on subsequent growth performance of the livestock animals. In addition, the alteration of either physical (phenotype) or cellular (genotype) development during fetal life may not have been significant enough to change their productive performance in adult life.

2.6 Effects of maternal nutritional manipulation during pregnancy on the fetal reproductive development

The following section reviews studies upon the effects of manipulating of nutrition during pregnancy on the reproductive development of male and female offspring.

2.6.1 Male

Maternal nutrition during pregnancy has the potential to influence reproductive development of male offspring (Table 2.3.). Structural differences in testicular development could result from direct effects of nutrient supply to the testis, through indirect effects could be mediated via hypothalamic–pituitary function or altered

gonadotrophin and other pituitary hormone profiles, or via direct effects of other hormones on the gonads (Deliogeorgis *et al*., 1996; Rae *et al*., 2002c).

The effects of maternal nutrition on testicular development of male offspring vary between studies. Studies by Deligeorgis *et al*. (1996), Bielli *et al*. (2002), Rae *et al*. (2002a, b, c), Osgerby *et al*. (2002), Da Silva *et al*. (2003), and Sullivan *et al*. (2010) have all demonstrated that testis weight of male offspring was not affected by maternal nutrition. However, Bielli *et al*. (2001), Liang and Zhang (2006) and Léonhardt *et al*. (2003), found a reduction in testis weight of male offspring from dams exposed to nutritional restriction during pregnancy. Bielli *et al*. (2002) and Sullivan *et al*. (2010) found that there was no difference in seminiferous tubule diameter, whilst Bielli *et al*. (2001) and Kotsampasi *et al*. (2009a) showed a decreased in seminiferous tubule diameter in male offspring from undernourished mothers. Decreased numbers of Leydig cells (Bielli *et al*. 2001) and Sertoli cells (Kotsampasi *et al*., 2009a; Bielli *et al*., 2002) have been reported in male offspring from undernourished mothers. Conversely, Bielli *et al*. (2001) and Da Silva *et al*. (2003) found no effect on Sertoli cell count. Fetal plasma testosterone concentrations were increased in fetuses from undernourished mothers compared with controls in the study of Rae *et al*. (2000). In contrast, Rae *et al*. (2002b) and Liang and Zhang (2006) found that maternal undernutrition can cause a reduction in offspring testosterone concentrations.

In conclusion, the majority of the previous studies of maternal nutrition on male offspring found no or minor effects on testis weight, testosterone concentration, number of testicular cells and diameter of seminiferous tubules. However results do not appear to be consistent and most of the studies were focussing on the development at certain periods of age and did not continuously follow the animals and examine their lifetime reproductive performance. Therefore, there are questions; what are the causes of these

inconsistencies and do the differences that appear in early life affect the subsequent development or productive performance of male offspring or not? (see Literature Review Section 2.6.3).

Table 2.3. The effect of maternal nutrition regimen and timing of nutritional regimen on the reproductive development of male offspring.

Species	Dietary manipulation	Time of dietary manipulation (day)	Effect on restricted group	Reference
Sheep	110% ME *vs* 90% ME	30-146	No differences in testis weight	Deligeorgis *et al*., 1996
Sheep	Improve pasture + grain supplement *vs* Native pasture	1-99	Reduced left and right testis weight, diameter of seminiferous tubule, Leydig cells and no differences in number of Sertoli cells.	Bielli *et al*., 2001
Sheep	*ad libitum vs* Moderate (to allow liveweight gain of approximately 75 g/day)	1-145	Reduced testosterone concentration	Da Silva *et al*., 2001
Sheep	110% ME *vs* 70% ME	70-145	Increased number of Sertoli cells, and no differences in testicular weight (left, right or paired), and semineferous tubules diameter.	Bielli *et al*., 2002
Sheep	100% ME *vs* 70% ME	22-d90, 22-135	No differences in testis weight at day 90 and day 135 of gestation	Osgerby *et al*., 2002
Sheep	100% ME *vs* 50% ME	1-95	No differences in scrotal circumference and number of spermatozoa	Rae *et al*., 2002a
Sheep	100% ME *vs* 50% ME	1-30, 31-50, 1-50, 31-65, 1-65, d65-110, 1-110,	Increased and decreased in testosterone concentration and no differences in fetal	Rae *et al*., 2002b

			testicular weight	
Sheep	100% protein intake *vs* 50% protein intake	1-119	No differences in testis weight	Rae *et al*., 2002c
Sheep	*ad libitum vs* Moderate (to allow liveweight gain of approximately 75 g/day)	1-103	No significant differences in fetal testicular weight, number of semineferous cords and Sertoli cell count	Da Silva *et al*., 2003
Rat	*ad libitum vs* Low 50% *ad libitum*	14-21	Reduce area and intratubular lumen of seminiferous tubules, delayed onset of puberty	Léonhardt *et al*., 2003
Rat	*ad libitum vs* 70% *ad libitum*	1-22	Reduced in testis weight and testosterone concentration	Liang and Zhang, 2006
Sheep	*ad libitum vs* Maintenance (to ensure no change in total live weight)	21-140	No differences in testis weight	Kenyon *et al*., 2009
Sheep	100% ME *vs* 50% ME	1-30, 31-100	Reduced number of Sertoli cells, seminiferous diameter, no differences in testosterone levels and testis weight	Kotsampasi *et al*., 2009a
Bull	250% CP and 243% ME) *vs* 75% CP and 199% ME (1-93 days) and 229% CP and 228% ME *vs* 63% CP and 176% ME (94-180 days)	1-93, 93-180, 1-180	No differences in paired testicular weight and semineferous tubules diameter	Sullivan *et al*., 2010
Sheep	*ad libitum vs* Maintenance (to ensure no change in total live weight) *vs* Sub-Maintenance (to achieve a loss in	21-50, 50-140	No differences in testis weight	Kenyon *et al*., 2011

	total live weight of 100g/d)			
Sheep	*ad libitum vs* Maintenance (to ensure no change in total live weight) *vs* Sub-Maintenance (to achieve a loss in total live weight of 100g/d)	21-50, 50-140	No differences in testis weight	Martín, 2012

*CP (crude protein); ME (metabolisable energy)

2.6.2 Female

Nutrition during pregnancy can influence the reproductive development and performance of female offspring during pre-natal and post-natal life respectively (Figure 2.7.). Maternal nutrient restriction during pregnancy alters germ cell meiosis, follicular development and reduces ovarian weight (Borwick *et al*. 1997; Rae *et al*., 2001; Da Siva *et al*., 2002; Robinson *et al*., 2002; Da Silva *et al*., 2003; Grazul-Bilska *et al*., 2009; Léonhardt *et al*., 2003; Bernal *et al*., 2010). Restricted maternal nutrition increased or had no effect on the ovarian weight (Deligeorgis *et al*., 1996; Borwick *et al*., 1997; Da Siva *et al*., 2002; Orgesby *et al*., 2002; Rae *et al*., 2002c; Murdoch *et al*., 2003) of fetal and adult offspring (Table 2.4.). Other studies showed that maternal undernutrition during pre-natal life caused lowered or had no effect on subsequent ovulation rate, and delayed or had no effect on the onset of puberty (Corah *et al*., 1975; Gunn *et al*., 1995; Da Siva *et al*., 2001; Rae *et al*., 2002a; Léonhardt *et al*., 2003; Robinson *et al*., 2006; Martin *et al*., 2007; Kotsampasi *et al*., 2009b).

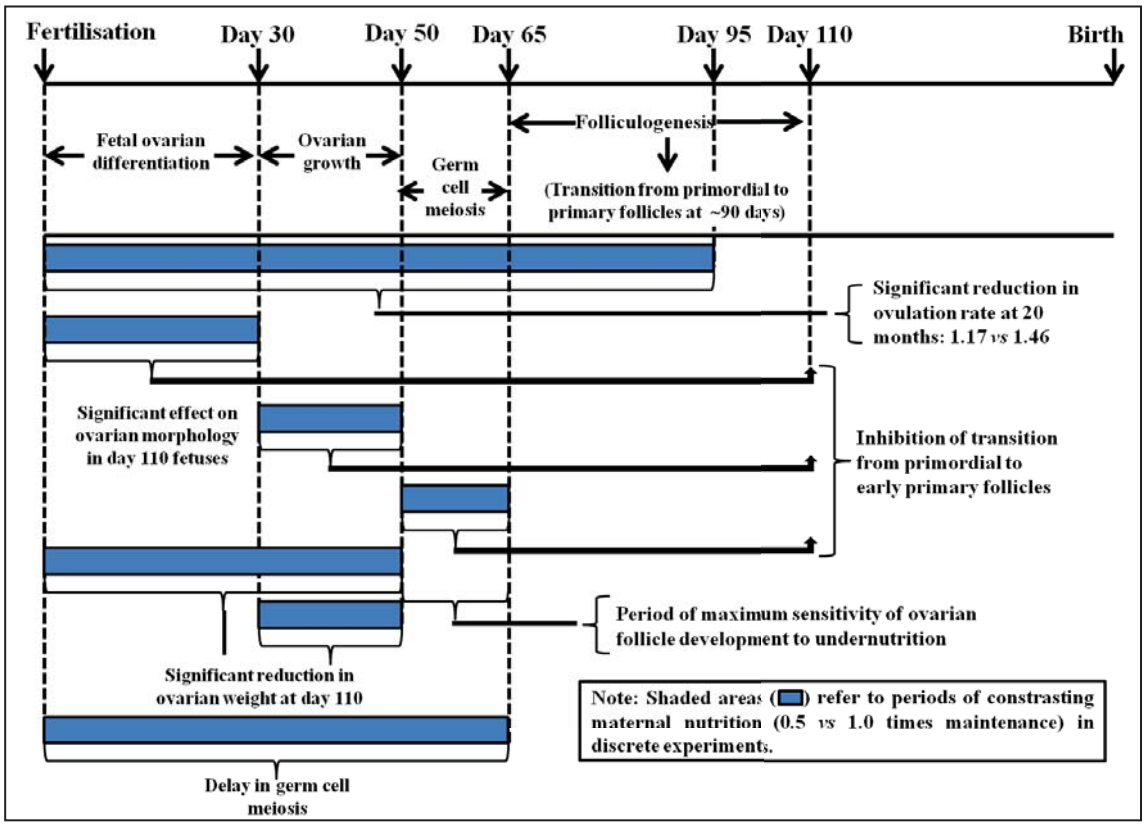


Figure 2.7. Critical periods during gestation in sheep for effects of maternal nutrition (0.5 x maintenance *vs* 1.0 x maintenance) on fetal ovarian development (Robinson *et al*., 2006).

Previous studies mostly investigated the effect of maternal undernutrition during pregnancy on offspring development in pre-natal life. There have been few studies undertaken that continuously follow the animal's reproductive development from fetal life through until adulthood, making it difficult to draw any sensible conclusion as to how effect that appeared during fetal life influences performance as an adult. However, based on previous studies that examined reproductive development during fetal and/or adult life; the differences that appeared during pre-natal life in the ovary are unlikely to affect reproductive performance in the adult animal (see Literature Review Section 2.6.3).

Table 2.4. The effect of maternal nutrition regimen and timing of nutritional regimen on the reproductive development of female offspring.

Species	Dietary manipulation	Time of dietary manipulation (day)	Effect on restricted group	Reference
Cow	100% NRC *vs* 65% NRC	152-252	No differences in the age of puberty	Corah *et al*., 1975
Sheep	Upland grazing + 525 g commercial pellet/day/animal *vs* Upland grazing *vs* Hill grazing.	50-145	No difference in ovulation rate	Gunn *et al*., 1995
Sheep	110% ME *vs* 90% ME.	30-145	No difference in fetal ovary weight (d55)	Deligeorgis *et al*., 1996
Sheep	150% ME *vs* 50% ME.	mating to 47	No difference in fetal ovarian weight at d47 or d62, no difference in oestradiol production, increased concentration of germ cells on d47 and d62, higher % of pigmented oocytes, no different in germ cell diameter	Borwick *et al*., 1997
Mice	*ad libitum vs* below *ad libitum*	1-21	Lower reproductive success	Meikle and Westberg., 2001
Sheep	*ad libitum vs* Moderate (to allow 75 g/day liveweight gain).	0-145	Higher plasma LH concentrations at weeks 2, 10, 15, 17 and 20 of age, no difference in age ovulation began, duration of first ovarian cycle, no of ovulatory cycle per first season or duration of first breeding season	Da Silva *et al*., 2001
Sheep	100% ME *vs* 50% ME.	0-30, 31-50, 0-50, 31-65, 0-65, 65-110, 0-110	Lower fetal ovarian weight at d50, fewer germ cells at d65	Rae *et al*., 2001
Sheep	*ad libitum vs* Moderate (10.2 MJME/kg dry matter).	0-131	Lower LHβ and FHβ mRNA expression, no different in ovarian weight, higher primordial follicles and total number of follicles,	Da Silva *et al*., 2002

			no different in primary or secondary follicles.	
Sheep	100% ME *vs*70% ME.	22-145	Heavier fetal ovarian weight at d90, no different in fetal ovarian weight at d135	Osgerby *et al*., 2002
Sheep	100% ME *vs* 50% ME.	mating to 95	Reduced in ovulation rate, no different in FSH profiles, basal LH profiles or response to GnRH.	Rae *et al*., 2002a
Sheep	100% ME *vs* Low 50% ME.	mating to 119	No difference in fetal ovarian weight, no difference in mean basal LH concentration in response to an exogenous GnRH challenge	Rae *et al*., 2002c
Sheep	100% ME *vs* 70% ME.	100-parturition	No difference either before or after ovariectomy, higher pituitary GnRh receptor binding and ERα at 31 weeks of age, no difference in the abundance of mRNA for LHβ, FSHβ or GnRH receptor binding or ERα mRNA at 31 weeks or 18 months of age	Borwick *et al*., 2003
Sheep	*ad libitum vs* Moderate (to allow live weight gain 75 g/day).	d0-d103	No difference in LHβ and FSHβ mRNA expression, higher primordial follicles and total number of follicles, no difference in isolated, primary and secondary follicles	Da Silva *et al*., 2003
Rat	*ad libitum vs* 50% *ad libitum*	14-21	Retardation of ovarian growth, delayed onset of puberty	Léonhardt *et al*., 2003
Sheep	100% NRC *vs* 50% NRC	d21-d78	No differences in fetal ovarian weight and germ cells concentration, increase oxidative base lesions within DNA of mid-gestational fetal oogonia	Murdoch *et al*., 2003

Sheep	100% ME *vs* 50% ME	d0-30, d31-50, d0-50, d31-65, d0-65, d65-110, d0-110	No differences at d50 in Ki67, predominant in germ cells or Bax and Mcl-1, predominant in the oocytes.	Lea *et al*., 2006
Cow	0.45 kg/d of 42% CP supplement *vs* no CP supplement	168-252	No differences on age of puberty	Martin *et al*., 2007
Rat	*ad libitum vs* 50% *ad libitum*	1-22	No differences in the age of vaginal opening	Chernoff *et al*., 2009
Sheep	EP=200% ME, and MP=140% ME *vs* EP=100% ME, and MP=80% ME *vs* 60% ME.	1-39, 40-90	No differences in conception rate	Munoz *et al*., 2009
Sheep	100% ME *vs* 60% ME	50-135	Lighter fetal ovarian weight, smaller proportion of proliferating primordial follicles/area and labeling index in primordial follicles in ovine fetal ovaries.	Grazul-Bilska *et al*., 2009
Sheep	100% ME *vs* 50% ME.	0-30, 31-100	No difference in onset of puberty, LH and FSH challenge at 5.5 months, at 10 months of age FSH higher in restricted group	Kotsampasi *et al*., 2009b
Sheep	*ad libitum vs* Medium (Maintenance; to allow live weight gain 75 g/day).	21-140	No differences in PD, lambs born, weaned, and ewe efficiency. But, had higher weight of lambs weaned.	van der Linden *et al*., 2010b
Sheep	*ad libitum vs* Maintenance (to allow live weight gain 75 g/day) *vs* Sub-maintenance.	21-50, 50-140	No differences in fetal ovarian weight	Martín, 2012

*CP (crude protein); ME (metabolisable energy); NRC (National Research Council requirement (1985))

2.6.3 Summary of maternal nutritional effects on offspring reproductive development

Evidence from previous studies suggest that maternal nutrition during pregnancy can influence reproductive development of offspring during fetal life but that these effects are unlikely to influence their reproductive performance as adults. In males, the majority of studies found little or no differences in testis weight, testosterone concentration and scrotal circumference, whilst some studies found minor differences in number of Sertoli cells and diameter of seminiferous tubules during either pre- or post-natal life (although limited studies have been conducted in later adulthood). In females, the effects observed in early life were mostly associated with ovarian follicular cell development which either reduced or increased in the number of follicles due to maternal nutrition. In adult female life, there were little effects apparent on ovulation rate, puberty onset or hormones that related to reproduction. Ovarian weight of the offspring did not appear to be greatly affected by maternal nutrition either during fetal or adult life.

There were a limited number of studies that investigated the long term effects of maternal nutrition on reproductive performance or gonadal functionality in adult life. Although there were some studies that investigated the effects of maternal nutrition on the age at which puberty was attained and subsequent ovulation rate. Most of the studies investigated the effects of maternal nutrition on gonadal development during fetal and early post-natal life, suggesting that additional studies addressing the longer term impacts of maternal nutrition are warranted.

The results from the literature showed variation of maternal nutritional effects on male and female offspring's reproductive development which could potentially be associated with variability between the methods used (e.g. animal management during and after

pregnancy), a potential species effect (difference in physiology of the body which potentially influence the adaptation processes to the unnourished environment during pregnancy), levels and duration of nutrition and nutritional treatments (i.e. longer periods and higher levels of nutritional restriction might have more severe effects on offspring and periods of maternal nutrition restriction may have different effects on the development of tissues or organs of the offspring). The current literature is a confusing collection of loosely relates studies with disparate results making a traditional review of literature difficult and not particularly informative. Meta-analysis is a potential tool/approach that may provide a means to quantitatively and qualitatively organize and summarize the findings of a large number of previous studies in order to identify and focus future research on key outcome areas.

2.7 Application of meta-analysis in science area

The growth of this research area has led to a large body of related research studies, however, it is common for there to be inconsistencies between studies. For example, in the present review there was considerable variation between the magnitude and direction of differences between the offspring of nutrient-restricted *versus* maintenance-fed *versus ad-libitum* dams. Moreover, there are differences in the design of trials, including variations of species, breed, live weight, body condition, environment, climate, and level, timing and duration of maternal nutrition treatment. Under such circumstances, it becomes important to agglomerate the data from individual studies in a way which organizes and summarizes findings in order to identify and focus future research on key outcome areas (Garvey and Griffith, 1971). This problem has led to the development of a systematic method for quantitative synthesis of disparate research

findings (Cooper and Hedges, 1994), known as meta-analysis. Meta-analysis is a quantitative method that combines results from different studies on the same topic in order to quantitatively construct overall conclusions from the literature or general context of systematic reviews (Lipsey and Wilson, 2001). Since its introduction in 1976 by a psychologist, Gene Glass, meta-analysis has evolved into an essential and established tool for literature review and research synthesis in the social and medical sciences (Arnqvist and Wooster, 1995). Meta-analysis relies on evaluating effect size using a scale free indicator of the intervention effect to quantitatively construct overall conclusions from the literature (Cohen, 1998; Nakagawa and Cuthill, 2007). Thus, the integration of findings from different studies with different units and different methodologies are possible (Cohen, 1998; Lipsey and Wilson, 2001; Nakagawa and Cuthill, 2007).

There are meta-analysis studies related to fetal programming currently in the literature, reporting the effects of maternal nutrition on the fetal origins of hypertension (Van Abeelen *et al*., 2012), the effect of energy and micronutrient intakes on offspring health (Blumfield *et al*., 2012), smoking during pregnancy and offspring obesity (Oken *et al*., 2007; Ino, 2010) and pre-natal care and maternal health on the incidence of low birth weight offspring (Scholl *et al*., 1994). As there are a number of studies investigating the effects of maternal nutrition on offspring development; this literature is precisely the type of data that are amenable to meta-analysis. However, to date meta-analysis examining the effects of maternal nutrition of male and female offspring reproductive performance has not been undertaken.

2.8 Objectives and hypothesis

The evidence from previous findings demonstrated that maternal nutritional regimen during pregnancy could have importance consequences for the offspring and farmers. Therefore, it is important to know how pregnancy nutrition can affect the growth and reproductive performance of the offspring. However, to date the majority of studies have been confined to the primarily to the effects of maternal undernutrition, often very severe. Furthermore, although the effects of size (i.e. body condition/weight within a breed) have been widely studied in relation to their impact on the reproductive performance of the affected animals, very little attention has been given to whether the body size or condition of the dam can affect the reproductive potential of its offspring. Of the studies that have examined the effects of maternal nutrition, and of the few studies that have examined the effects of maternal size and body condition, on the performance and reproduction of the progeny, very few have attempted to elucidate the effects at the cellular level.

Therefore, the objective of this thesis was to examine the effects of *in utero* environment on the development of reproductive system in male and female offspring, and their subsequent performance as an adult. Two differing maternal paradigms were used;

1) Dam size (heavy *vs* light) and dam nutrition for a prolonged period in pregnancy (*ad libitum vs* maintenance; P21-140)
2) Dam nutrition during early (*ad libitum vs* maintenance *vs* sub maintenance; P21-50) and mid-to-late pregnancy (*ad libitum vs* maintenance; P50-139).

The duration of nutritional treatments studied represent the feeding practices that are commonly applied by New Zealand sheep farmers. In the first paradigm (the dam size

and pregnancy nutrition study) the duration of nutrition treatment was relatively long (P21 – P140) making it difficult to define the exact periods of pregnancy that resulted in the observed effects. This was improved by the second paradigm (early and mid-to-late pregnancy dam nutrition study) which had divided the timing of the maternal nutritional treatments into two different windows; early (P21 – P50) and mid-to-late pregnancy (P50 – P139).

2.9 Background information of dams

This thesis utilized male and female progeny from 2 large studies that were undertaken to examine the effects of maternal nutrition under pastoral conditions on offspring growth, lactation and reproductive performance. Previous research by the Massey University group showed that maternal feeding could affect fetal mammary gland development (Jenkinson, 2003; Blair *et al*., 2010) however functionality had not been examined.

This section describes the treatments of the dams [(i) maternal size and nutrition trial (Chapter 5, 7, and 8), (ii) maternal nutrition during early and mid-to late pregnancy (Chapter 6 and 9)], to which the offspring studied in this thesis were born in more detail (the methodology is from Kenyon *et al*., 2009 and Kenyon *et al*., 2011). Only a brief description of the maternal treatments is given in later chapters.

2.9.1 Dam treatments for maternal size and nutrition trial (offspring in chapters 5, 7 and 8)

The heaviest (heavy (H), n = 450, live weight 60.8 kg ± S.E. 0.18 and body condition score (CS; scale 1-5 (Jefferies, 1961)) 3.02 ± 0.03) and lightest (light (L), n = 450, live

weight 42.5 kg ± 0.17, and body condition score 1.97 ± 0.03) multiparous Romney ewes (3-5 years of age) were selected from a commercial flock of 2900 ewes at 69 days (D69) before artificial insemination and managed under commercial grazing conditions as one group (Kenyon *et al*., 2009).

At day 14 (D14), ewes had progesterone controlled internal drug release devices (CIDR, 0.3 g progesterone, Pharmacia & UpJohn, Auckland, New Zealand) inserted vaginally. On day 2 (D2) half of the dams, including individuals from each of the dam size groups had their CIDRs removed. The following morning (D1), the remainder of the dams had their CIDRs removed. On D0 those dams which had their CIDRs removed on D2 were artificially inseminated, via intra-uterine laproscopy using semen from one of four Suffolk rams, randomly allocated to each dam. On D1 the remaining dams underwent the same procedure and then both cohorts of dam were merged. At D4, eight crayon-harnessed entire Suffolk rams were introduced to the dams and dams were managed under commercial conditions (Kenyon *et al*., 2009).

At day 21 (P21) the Suffolk rams were removed as were any dams displaying harness marks indicating returns to service. The remaining dams (n = 612), were randomly allocated to one of two nutitional regimens until day 140 (P140) (maintenance (M) *vs ad libitum* (A)) under pastoral grazing conditions which is applicable by New Zealand farmers. The average pre- and post-grazing herbage masses during the period P21 to P140 were described in Table 2.5.

The aim of the maintenance nutritional regimen was to ensure that total dam live weight increased in pregnancy at a level similar to that of the expected conceptus mass (Rattray *et al*., 1974). The aim of the *ad libitum* nutritional regimen was to provide unrestricted access to pasture forage. Within each regimen singleton- and twin-bearing dams were not separated.

Therefore, from P21 until P140 the treatment groups included; heavy-*ad libitum* (n = 151), heavy-maintenance (n = 153), light-*ad libitum* (n = 155) and light-maintenance (n = 153) including singleton- and twin-bearing dams. To achieve these feeding regimens dams were rotationally grazed. The live weight and body condition of the dams prior and during pregnancy are shown in Table 2.6 and Table 2.7.

At P141 all dams were set stocked for lambing and offered a minimum cover of 1200 kg DM/ha. During the period from ten days after the mid-point of lambing (L10) until L100 dams were managed in three groups under commercial grazing conditions with a minimum cover of 1200 kg DM/ha, with each group containing individuals from each treatment.

Post weaning all progeny were managed under commercial conditions. Only twin born offspring were maintain for the studies in chapters 5, 7 and 8 because under current NZ farming conditions twin born offspring are the common animal used of interest for farmers.

Table 2.5. Mean (±s.e.) pre- and post- grazing herbage masses (kg DM/ha) for ewe nutritional treatments; maintenance (M_1) *vs ad libitum* (A_1) applied Day 21-140 (P21-140) of pregnancy (adapted from Kenyon *et al*., 2009).

Parameter	Herbage mass (kg DM/ha)	
	Pre-grazing	Post-grazing
Maintenance	1330 ± 140.0	804.0 ± 133.4
Ad libitum	2304.0 ± 156.8	1723.3 ± 149.7

Table 2.6. The effect of pregnancy rank, ewe size and nutritional regimen on ewe live weight (kg). Means (±SE) within pregnancy rank, ewe size and nutritional regimens and columns with letters in common or without superscripts are not significantly different (P<0.05) (adapted from Kenyon *et al*., 2011).

		Ewe liveweight			
	n	P-69*	P1	P53	P140
Pregnancy rank					
1	230	$51.15^{a} \pm 0.24$	$54.81^{a} \pm 0.27$	$60.56^{a} \pm 0.28$	$70.23^{a} \pm 0.37$
2	280	$52.12^{b} \pm 0.22$	$56.08^{b} \pm 0.25$	$61.99^{b} \pm 0.25$	$73.14^{b} \pm 0.36$
Ewe size					
Light (L)	255	$42.56^{a} \pm 0.22$	$46.80^{a} \pm 0.26$	$53.12^{a} \pm 0.26$	$64.49^{a} \pm 0.37$
Heavy (H)	255	$60.72^{b} \pm 0.23$	$64.08^{b} \pm 0.26$	$69.43^{b} \pm 0.27$	$78.88^{b} \pm 0.36$
Ewe nutrition					
Maintenance (M)	268	51.37 ± 0.22	55.21 ± 0.25	$56.11^{a} \pm 0.26$	$65.01^{a} \pm 0.35$
Ad libitum (A)	242	51.91 ± 0.23	55.68 ± 0.26	$66.45^{b} \pm 0.27$	$78.36^{b} \pm 0.37$

*P, Days before or after insemination

Table 2.7. The effect of pregnancy rank, ewe size and nutritional regimen on ewe condition score. Means (±SE) within pregnancy rank, ewe size and nutritional regimens and columns with letters in common or without superscripts are not significantly different (P<0.05). Condition score scale 0-5 in 0.5 units (Jefferies, 1961) (adapted from Kenyon *et al*., 2009).

		Ewe condition score		
	n	P-69*	P1	P140
Pregnancy rank				
1	230	2.49 ± 0.04	$2.36^{a} \pm 0.04$	$2.61^{a} \pm 0.03$
2	280	2.51 ± 0.03	$2.49^{b} \pm 0.04$	$2.47^{b} \pm 0.03$
Ewe size				
Light (L)	255	$1.98^{a} \pm 0.03$	$1.91^{a} \pm 0.04$	$2.32^{a} \pm 0.03$
Heavy (H)	255	$3.02^{b} \pm 0.03$	$2.93^{b} \pm 0.04$	$2.76^{b} \pm 0.03$
Ewe nutrition				
Maintenance (M)	268	$2.46 \pm 0.0.3$	2.41 ± 0.04	$1.78^{a} \pm 0.03$
Ad libitum (A)	242	2.54 ± 0.03	2.44 ± 0.04	$3.30^{b} \pm 0.03$

*P, Days before or after insemination

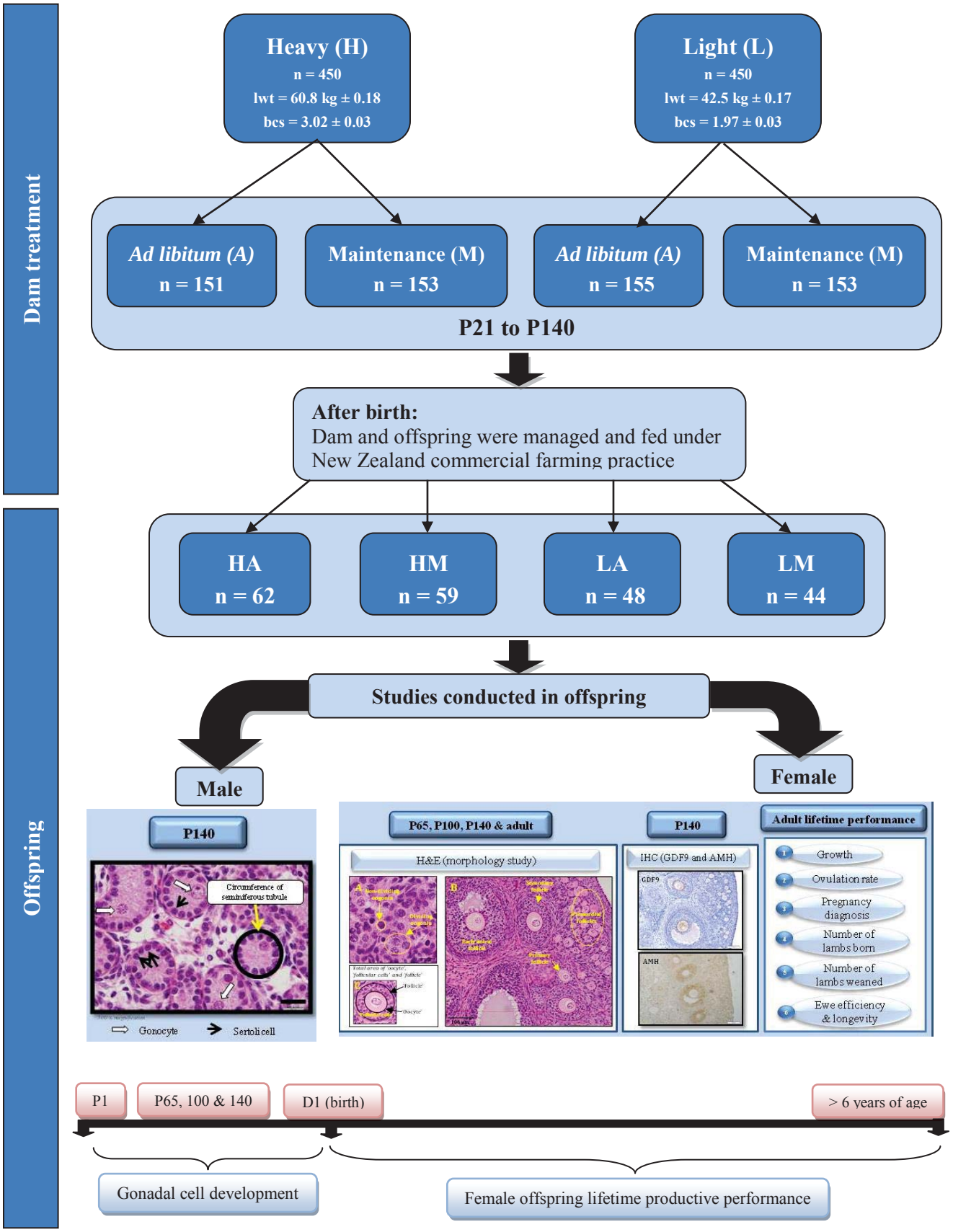


Figure 2.8. Study design and overview of maternal size and nutrition studies conducted in the offspring.

2.9.2 Dam treatments for maternal nutrition during early and mid-to-late pregnancy trial (offspring in chapters 6 and 9)

A second study was undertaken in 2009. This study built on the first study which showed feeding of the dam can affect milk production in offspring (van de linden *et al*., 200?). The new study aimed to examine specifically the early pregnancy period, a period in which the fetal mammary gland is developing in.

In total, 1169 romney ewes (average live weight 66.3 kg ± 0.18, condition score 2.96 ± 0.02) from commercial flock were treated vaginally with progesterone-controlled internal drug-release devices (CIDR, 0.3 g progesterone, Pharmacia & UpJohn, Auckland, New Zealand) 14 days before artificial insemination (P14) (Kenyon *et al*., 2011). On P-2, CIDRs were removed from half of the ewes, with the remainder removed the following morning (P1). On P0, those ewes that had their CIDRs removed on P-2 were artificially inseminated via intra-uterine laproscopy, using fresh semen randomly allocated from one of five Romney rams. On P1, the remaining ewes underwent the same procedure and then both cohorts of ewes were merged. At P5, 12 crayon-harnessed entire Romney rams were introduced and the ewes and rams were managed under commercial conditions (Kenyon *et al*., 2011).

Ewes were offered herbage with a minimum post-grazing herbage mass of 1200 kg DM/ha until P21. At P21, any ewes displaying harness marks on their rumps (indicating returns to service) were removed. The remaining ewes (n 879), were randomly allocated to one of three nutritional treatments (Kenyon *et al*., 2011) until P50 [Sub maintenance$_{P21\text{-}50}$ ($Sm_{P21\text{-}50}$) *vs* Maintenance$_{P21\text{-}50}$ ($M_{P21\text{-}50}$) vs Ad libitum ($A_{P21\text{-}50}$)]. The aim of $Sm_{P21\text{-}50}$ treatment was to achieve a loss in total ewe live weight of 0.1 kg/day, whereas the aim of $M_{P21\text{-}50}$ treatment was to ensure no change in total ewe live weight. Target pre- and post- grazing herbage masses for $Sm_{P21\text{-}50}$, $M_{P21\text{-}50\ or\ P50\text{-}140}$ and $A_{P21\text{-}50\ or}$

$_{P50\text{-}140}$ were as Table 2.8. Morris and Kenyon (2004) have previously shown that ewe intakes do not differ above a minimum herbage cover of ~1200 kg DM/ha, therefore, $A_{P21\text{-}50 \text{ or } P50\text{-}140}$ were not offered herbage masses below this level.

Table 2.8. Mean (±s.e.) pre- and post- grazing herbage masses (kg DM/ha) for ewe nutritional treatments; sub maintenance (Sm_1) *vs* maintenance (M_1) *vs ad libitum* (A_1) applied Day 21-50 (P21-50) of pregnancy; and maintenance (M_2) *vs ad libitum* (A_2) applied Day 50-139 of pregnancy (adapted from Kenyon *et al*., 2011).

Parameter	Herbage mass (kg DM/ha)			
	P21-50		P50-139	
	Pre-grazing	Post-grazing	Pre-grazing	Post-grazing
sub maintenance	$996^{a} \pm 89.3$	$814^{a} \pm 54.2$		
maintenance	$1479^{b} \pm 107.7$	$1112^{b} \pm 59.4$	$1450^{a} \pm 83.9$	$1011^{a} \pm 32.8$
Ad libitum	$2331^{c} \pm 82.0$	$1649^{c} \pm 54.2$	$1828^{b} \pm 76.0$	$1301^{b} \pm 37.8$

Mean within columns followed by different letters are significantly different ($P<0.05$)

At P48, all ewes were pregnancy scanned via transabdominal ultrasonography. Non-pregnant, single-, triplet- and quadruplet-bearing ewes were removed at this stage (n = 33, 228, 110 and 1, respectively). A further of 10 ewes were removed because of incomplete data (Kenyon *et al*., 2011).

At P50, the remaining 497 twin-bearing ewes were randomly allocated to one of further nutritional treatments for the period P50-139 ($M_{P50\text{-}139}$ vs $A_{P50\text{-}139}$). Each of the two nutritional treatments included animals from the three P21-50 treatments. The aim of the MP50-139 nutritional regimen was to achieve a total ewe live weight increase similar to that of expected conceptus mass (Rattray *et al*., 1974; Rattray, 1986). The length of the grazing period within each paddock for each nutritional treatment, for both periods, was dependent on herbage-mass and liveweight changes observed (Table 2.8 and Table 2.9). The length of the grazing period within each paddock for each nutritional treatment, for both periods, was dependent on herbage-mass and live weight

changes observed. Therefore, from P50 there was a 3 x 2 factorial nutrition design [$Sm_{P21-50} - M_{P50-139}$, $Sm_{P21-50} - A_{P50-139}$, $M_{P21-50} - M_{P50-139}$, $M_{P21-50} - A_{P50-139}$, $A_{P21-50} - M_{P50-139}$, $A_{P21-50} - A_{P50-139}$].

At P139, the nutritional treatments were merged and all ewes were placed in paddocks for lambing at a rate of 12.1 ewes/ha, with an average herbage mass of 1558 ± 73 kg DM/ha. Ewes were randomly allocated to lambing paddocks.

Ewes remained in their paddocks until 23 days after the mid-point of the lambing period (L3), at which time they were managed in three groups, under commercial grazing conditions with a minimum herbage mass of 1200 kg DM/ha, each group containing individuals from each nutritional treatment. Post weaning all progeny managed under commercial grazing conditions.

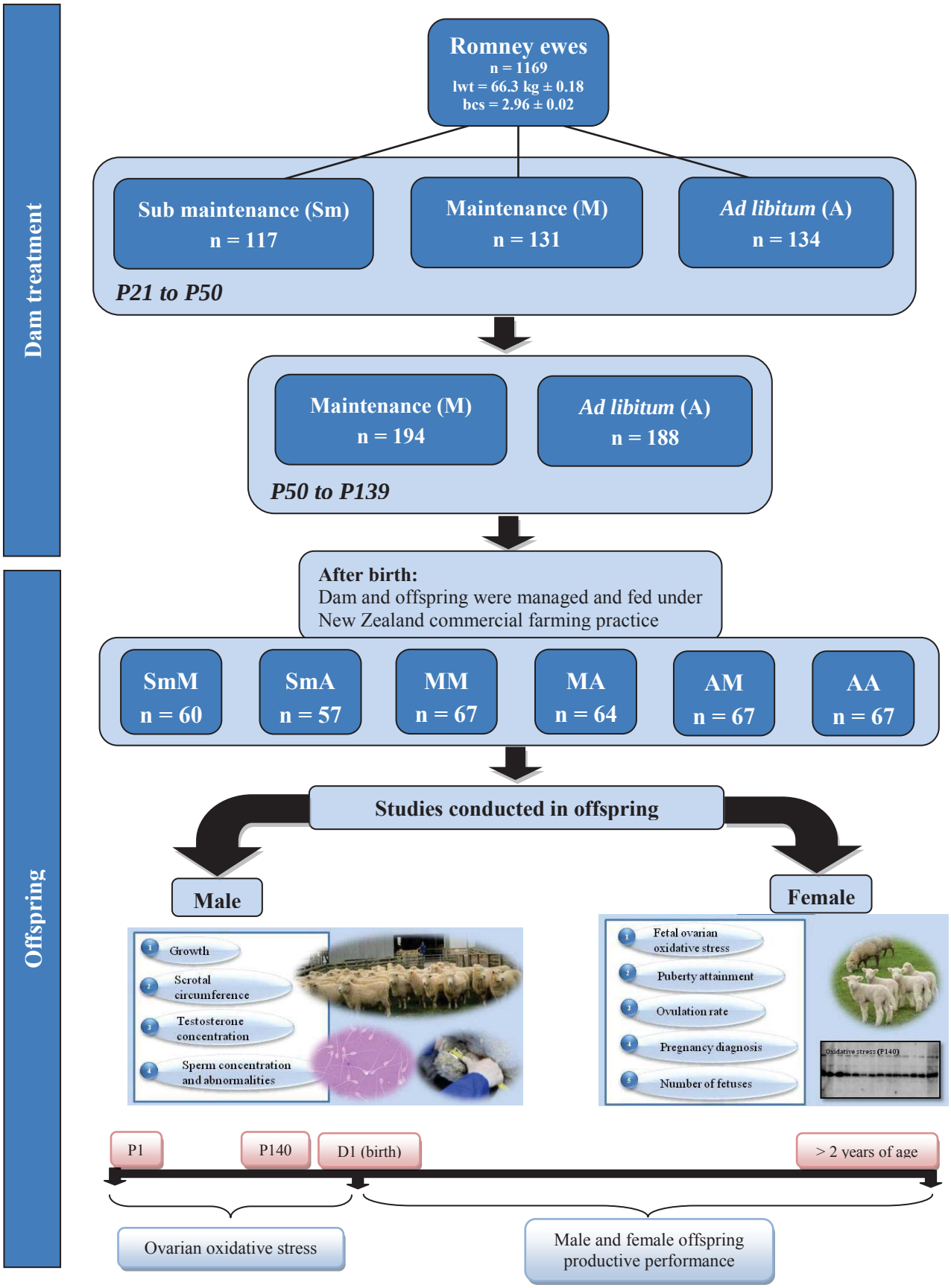


Figure 2.9. Study design and overview of maternal nutrition during early and mid-to-late pregnancy studies conducted in the offspring.

Table 2.9. Effect of ewe nutritional treatments during P21-50 [Sub maintenance (Sm) *vs* Maintenance (M) *vs Ad libitum* (A)] and P50-139 [Maintenance (M) vs Ad libitum (A)] on mean (±s.e.) ewe live weight (kg) during pregnancy (Adapted from Kenyon *et al*., 2011).

Parameter	*n*	Ewe live weight (kg)					
		P21	P30	P50	P69	P113	P137
P21 – 50							
Sm	*117*	66.0 ± 0.58	63.8^{a} ± 0.65	62.2^{a} ± 0.56	70.0^{a} ± 0.60	74.5^{a} ± 0.60	84.3 ± 0.68
M	*131*	65.6 ± 0.55	66.8^{b} ± 0.61	65.1^{b} ± 0.53	72.6^{b} ± 0.57	76.2^{b} ± 0.57	85.5 ± 0.67
A	*134*	66.2 ± 0.55	66.6^{b} ± 0.61	69.5^{c} ± 0.53	74.5^{b} ± 0.57	77.7^{b} ± 0.57	86.4 ± 0.67
P50 – 139							
M	*194*				69.4^{a} ± 0.47	71.8^{a} ± 0.48	82.6^{a} ± 0.53
A	*188*				75.4^{b} ± 0.48	80.5^{b} ± 0.48	88.2^{b} ± 0.53
P21 – 50 X P50 – 139							
SmM	*60*				66.5^{a} ± 0.85	70.0^{a} ± 0.87	81.0^{a} ± 0.95
SmA	*57*				73.6^{bc} ± 0.87	79.1^{b} ± 0.85	87.7^{b} ± 0.97
MM	*67*				70.5^{b} ± 0.80	72.5^{a} ± 0.80	83.3^{a} ± 0.90
MA	*64*				74.8^{cd} ± 0.83	79.9^{bc} ± 0.82	87.7^{b} ± 0.92
AM	*67*				71.3^{b} ± 0.82	72.9^{a} ± 0.81	83.6^{a} ± 0.92
AA	*67*				77.7^{d} ± 0.81	82.5^{c} ± 0.81	89.1^{b} ± 0.90

Means within columns and main effects or interactions followed by different letters are significantly different ($P<0.05$).

2.10 References

Aliyari, D., Moeini, M. M., Shahir, M. H., & Sirjani, M. A. (2012). Effect of body condition score, live weight and age on reproductive performance of Afshari ewes. *Asian Journal of Animal and Veterinary Advances, 7*(9), 904-909.

Allden, W. G. (1979). Undernutrition of the Merino sheep and its sequelae .5. the influence of severe growth restriction during early post-natal life on reproduction and growth in later life. *Australian Journal of Agricultural Research, 30*(5), 939-948.

Amann, R. P., & Schanbacher, B. D. (1983). Physiology of male reproduction. *Journal of Animal Science, 57*(2), 380-403.

Amoroso, E. C. (1959). Comparative anatomy of the placenta. *Annals of the New York Academy of Sciences, 75*(2), 855-872.

Armitage, J. A., Khan, I. Y., Taylor, P. D., Nathanielsz, P. W., & Poston, L. (2004). Developmental programming of the metabolic syndrome by maternal nutritional imbalance: how strong is the evidence from experimental models in mammals? *The Journal of Physiology, 561*(2), 355-377.

Arnqvist, G., & Wooster, D. (1995). Meta-analysis: synthesizing research findings in ecology and evolution. *Trends in Ecology andamp; Evolution, 10*(6), 236-240.

Arrighi, S., Aralla, M., Genovese, P., Picabea, N., & Bielli, A. (2010). Undernutrition during foetal to prepubertal life affects aquaporin 9 but not aquaporins 1 and 2 expression in the male genital tract of adult rats. *Theriogenology, 74*(9), 1661-1669.

Baker, R. L., Clarke, J. N., Carter, A. H., & Diprose, G. D. (1979). Genetic and phenotypic parameters in New-Zealand Romney sheep; body weights, fleece weights, and estrous activity. *New Zealand Journal of Agricultural Research, 22*(1), 9-21.

Barcroft, J. (1946). Researches on pre-natal life: Oxford: Blackwell Scientific Publications.

Barker, D. J., Gluckman, P. D., Godfrey, K. M., Harding, J. E., Owens, J. A., & Robinson, J. S. (1993). Fetal nutrition and cardiovascular disease in adult life. *Lancet, 341*(8850), 938-941.

Barker, D. J., Osmond, C. (1986). Infant mortality, childhood nutrition, and ischaemic heart disease in England and Wales. *Lancet*; 1, 1077–1081.

Bauer, B., Dittami, J., & Huber, S. (2009). Effects of nutritional quality during early development on body weight and reproductive maturation of guinea pigs (*Cavia aperea f. porcellus*). *General and Comparative Endocrinology, 161*(3), 384-389.

Bell, A. W., & Ehrhardt, R. A. (2002). Regulation of placental nutrient transport and implications for fetal growth. *Nutrition Research Reviews, 15*(2), 211-230.

Bernal, A. B., Vickers, M. H., Hampton, M. B., Poynton, R. A., & Sloboda, D. M. (2010). Maternal undernutrition significantly impacts ovarian follicle number and increases ovarian oxidative stress in adult rat offspring. *PLoS ONE, 5*(12), e15558.

Bielli, A., Katz, H., Pedrana, G., Gastel, M. T., Morana, A., Castrillejo, A., Lundeheim, N., Forsberg, M., & Rodriguez-Martinez, H. (2001). Nutritional management during fetal and post-natal life, and the influence on testicular stereology and Sertoli cell numbers in Corriedale ram lambs. *Small Ruminant Research, 40*(1), 63-71.

Bielli, A., Perez, R., Pedrana, G., Milton, J. T. B., Lopez, A., Blackberry, M. A., Duncombe, G., Rodriguez-Martinez, H., & Martin, G. B. (2002). Low maternal

nutrition during pregnancy reduces the number of Sertoli cells in the newborn lamb. *Reproduction Fertility and Development, 14*(6), 333-337.

Blair, H. T., Jenkinson, C. M. C., Peterson, S. W., Kenyon, P. R., van der Linden, D. S., Davenport, L. C., *et al*. (2010). Dam and granddam feeding during pregnancy in sheep affects milk supply in offspring and reproductive performance in grand-offspring. *Journal of Animal Science, 88* (13 electronic suppl), E40-E50.

Bloomfield, F. H., Oliver, M. H., Giannoulias, C. D., Gluckman, P. D., Harding, J. E., & Challis, J. R. G. (2003). Brief undernutrition in late-gestation sheep programs the hypothalamic-pituitary-adrenal axis in adult offspring. *Endocrinology, 144*(7), 2933-2940.

Blumfield, M. L., Hure, A. J., Macdonald-Wicks, L., Smith, R., & Collins, C. E. (2012). Systematic review and meta-analysis of energy and macronutrient intakes during pregnancy in developed countries. *Nutrition Reviews, 70*(6), 322-336.

Borwick, S. C., Rae, M. T., Brooks, J., McNeilly, A. S., Racey, P. A., & Rhind, S. M. (2003). Undernutrition of ewe lambs *in utero* and in early post-natal life does not affect hypothalamic-pituitary function in adulthood. *Animal Reproduction Science, 77*(1-2), 61-70.

Borwick, S. C., Rhind, S. M., McMillen, S. R., & Racey, P. A. (1997). Effect of undernutrition of ewes from the time of mating on fetal ovarian development in mid gestation. *Reproduction Fertility and Development, 9*(7), 711-715.

Caldani, M., Antoine, M., Batailler, M. & Duittoz, A. (1995). Ontogeny of GnRH systems. *Journal of Reproduction and Fertility Supplement, 49*, 147–162.

Chadio, S. E., Kotsampasi, B., Papadomichelakis, G., Deligeorgis, S., Kalogiannis, D., Menegatos, I., & Zervas, G. (2007). Impact of maternal undernutrition on the hypothalamic–pituitary–adrenal axis responsiveness in sheep at different ages post-natal. *Journal of Endocrinology, 192*(3), 495-503.

Chernoff, N., Gage, M. I., Stoker, T. E., Cooper, R. L., Gilbert, M. E., & Rogers, E. H. (2009). Reproductive effects of maternal and pre-weaning undernutrition in rat offspring: Age at puberty, onset of female reproductive senescence and intergenerational pup growth and viability. *Reproductive Toxicology, 28*(4), 489-494.

Cohen, J. (1988). Statistical Power Analysis for the Behavioral Sciences. Lawrence Erlbaum Associates, Hillsdale, New Jersey.

Cooper, H.M. & Hedges, L.V. (Eds.) (1994). The handbook of research synthesis. New York: The Russell Sage Foundation.

Corah, L. R., Dunn, T. G., & Kaltenbach, C. C. (1975). Influence of prepartum nutrition on the reproductive performance of beef females and the performance of their progeny. *Journal of Animal Science, 41*(3), 819-824.

Da Silva, P., Aitken, R. P., Rhind, S. M., Racey, P. A., & Wallace, J. M. (2001). Influence of placentally mediated fetal growth restriction on the onset of puberty in male and female lambs. *Reproduction, 122*(3), 375-383.

Da Silva, P., Aitken, R. P., Rhind, S. M., Racey, P. A., & Wallace, J. M. (2002). Impact of maternal nutrition during pregnancy on pituitary gonadotrophin gene expression and ovarian development in growth-restricted and normally grown late gestation sheep fetuses. *Reproduction, 123*(6), 769-777.

Da Silva, P., Aitken, R. P., Rhind, S. M., Racey, P. A., & Wallace, J. M. (2003). Effect of maternal overnutrition during pregnancy on pituitary gonadotrophin gene expression and gonadal morphology in female and male foetal sheep at day 103 of gestation. *Placenta, 24*(2-3), 248-257.

Daniel, Z., Brameld, J. M., Craigon, J., Scollan, N. D., & Buttery, P. J. (2007). Effect of maternal dietary restriction during pregnancy on lamb carcass characteristics and muscle fiber composition. *Journal of Animal Science, 85*(6), 1565-1576.

De Boo, H. A., & Harding, J. E. (2006). The developmental origins of adult disease (Barker) hypothesis. *Australian and New Zealand Journal of Obstetrics and Gynaecology, 46*(1), 4-14.

Deligeorgis, S. G., Chadio, S., & Menegatos, J. (1996). Pituitary responsiveness to GnRH in lambs undernourished during fetal life. *Animal Reproduction Science, 43*(2-3), 113-121.

Eckery, D. C., Tisdall, D. J., Heath, D. A., & McNatty, K. P. (1996). Morphology and function of the ovary during fetal and early neonatal life: A comparison between the sheep and brushtail possum (*Trichosurus vulpecula*). *Animal Reproduction Science, 42*(1-4), 551-561.

Ehrhardt, R. A., & Bell, A. W. (1995). Growth and metabolism of the ovine placenta during mid-gestation. *Placenta, 16*(8), 727-741.

Fogarty, N., Brash, L., & Gilmour, A. (1994). Genetic parameters for reproduction and lamb production and their components and liveweight, fat depth and wool production in Hyfer sheep. *Australian Journal of Agricultural Research, 45*(2), 443-457.

Foote, W. C., Pope, A. L., Chapman, A. B., & Casida, L. E. (1959). Reproduction in the yearling ewe as affected by breed and sequence of feeding levels. II. Effects on fetal development. *Journal of Animal Science, 18*(1), 463-474.

Ford, J. J., & D'Occhio, M. J. (1989). Differentiation of sexual behavior in cattle, sheep and swine. *Journal of Animal Science, 67*(7), 1816-1823.

Ford, J. T., & Klindt, J. (1989). Sexual differentiation and growth process. In D. R. Campion, G. J. Hausman R. J. Martin (Eds.), *Animal Growth Regulation* Plenum, New York.

Foster, D. L., Yellon, S. M., & Olster, D. H. (1985). Internal and external determinants of the timing of puberty in the female. *Journal of Reproduction and Fertility, 75*(1), 327-344.

Gardner, D. S., Ozanne, S. E., & Sinclair, K. D. (2009). Effect of the early-life nutritional environment on fecundity and fertility of mammals. *Philosophical Transactions of the Royal Society B-Biological Sciences, 364*(1534), 3419-3427.

Garvey, W. D., & Griffith, B. C. (1971). Scientific communication: Its role in the conduct of research and creation of knowledge. *American Psychologist, 26*(4), 349-362.

Genovese, P., Núñez, M. E., Pombo, C., & Bielli, A. (2010). Undernutrition during foetal and post-natal life affects testicular structure and reduces the number of Sertoli cells in the adult rat. *Reproduction in Domestic Animals, 45*(2), 233-236.

Gluckman, P. D., & Hanson, M. A. (2004). Living with the past: Evolution, development, and patterns of disease. *Science, 305*(5691), 1733-1736.

Godfrey, K. M., & Barker, D. J. (2000). Fetal nutrition and adult disease. *The American Journal of Clinical Nutrition, 71*(5), 1344S-1352S.

Gootwine, E., Spencer, T. E., & Bazer, F. W. (2007). Litter-size-dependent intrauterine growth restriction in sheep. *Animal, 1*(04), 547-564.

Grazul-Bilska, A. T., Caton, J. S., Arndt, W., Burchill, K., Thorson, C., Borowczyk, E., Bilski, J. J., Redmer, D. A., Reynolds, L. P., & Vonnahme, K. A. (2009). Cellular proliferation and vascularization in ovine fetal ovaries: effects of undernutrition and selenium in maternal diet. *Reproduction, 137*(4), 699-707.

Grinsted, J. (1981). The relation between mesonephric kidney function and onset of meiosis in the mammalian ovary. In: A.G. Byskov & H. Peters (Eds.), *Development and Function of Reproductive Organs*, Excerpta Medica, Amsterdam, pp. 13-17.

Gunn R. G. (1977). The effects of two nutritional environments from 6 weeks prepartum to 12 months of age on lifetime performance and reproductive potential of Scottish Blackface ewes in two adult environments. *Animal Production*, *25*, 155–164.

Gunn, R. G., Doney, J. M., & Russel, A. J. F. (1972) Embryo mortality in Scottish Blackface ewes as influenced by body condition at mating and by post-mating nutrition. *Journal of Agricultural Science, Cambridge*, *79*, 19–25

Gunn, R. G., Sim, D. A., & Hunter, E. A. (1995). Effects of nutrition in-utero and in early-life on the subsequent lifetime reproductive-performance of Scottish Blackface ewes in 2 management-systems. *Animal Science, 60*, 223-230.

Hafez, B., & Hafez, E. S. E. (2000). Reproduction in Farm Animals, 7th edition. Lippincott Williams and Wilkins, USA.

Harding, J. E., & Johnston, B. M. (1995). Nutrition and fetal growth. *Reproduction Fertility and Development, 7*(3), 539-547.

Hochereau-de Reviers, M. T., de Reviers, M. M., Monet-Kuntz, C., Perreau, C., Fontaine, I., & Viguier-Martinez, M. C. (1987). Testicular growth and hormonal parameters in the male Snell dwarf mouse. *Acta Endocrinologica, 115*(3), 399-405.

Hochereau-de Reviers, M. T., Perreau, C., Pisselet, C., Locatelli, A., & Bosc, M. (1995). Ontogenesis of somatic and germ cells in sheep fetal testis. *Journal of Reproduction and Fertility, 103*(1), 41-46.

Igosheva, N., Abramov, A. Y., Poston, L., Eckert, J. J., Fleming, T. P., Duchen, M. R., & McConnell, J. (2010). Maternal diet-induced obesity alters mitochondrial activity and redox status in mouse oocytes and zygotes. *PLoS ONE, 5*(4), e10074.

Igwebuike, U. M. (2010). Impact of maternal nutrition on ovine foetoplacental development: A review of the role of insulin-like growth factors. *Animal Reproduction Science, 121*(3-4), 189-196.

Ino, T. (2010). Maternal smoking during pregnancy and offspring obesity: Meta-analysis. *Pediatrics International, 52*(1), 94-99.

Jammes, H., Junien, C., & Chavatte-Palmer, P. (2011). Epigenetic control of development and expression of quantitative traits. *Reproduction, Fertility and Development, 23*, 64-74.

Jefferies, B. C. (1961). Body condition scoring and its use in management. *Tasmanian Journal of Agriculture, 32*, 19-21.

Jenkinson, C. M. C. (2003). The pattern and regulation of mammary gland development during fetal life in the sheep. PhD Thesis. Massey University, Palmerston North, New Zealand.

Kenyon, P. R. (2008) A review of *in-utero* environmental effects on sheep production. *Proceedings of the New Zealand Society of Animal Production, 68*, 142–155.

Kenyon, P. R., Blair, H. T., Jenkinson, C. M. C., Morris, S. T., Mackenzie, D. D. S., Peterson, S. W., Firth, E. C., & Johnston, P. L. (2009). The effect of ewe size and nutritional regimen beginning in early pregnancy on ewe and lamb performance to weaning. *New Zealand Journal of Agricultural Research, 52*(2), 203-212.

Kenyon, P. R., Pain, S. J., Hutton, P. G., Jenkinson, C. M. C., Morris, S. T., Peterson, S. W., & Blair, H. T. (2011). Effects of twin-bearing ewe nutritional treatments on ewe and lamb performance to weaning. *Animal Production Science, 51*(5), 406-415.

Kind, K. L., Clifton, P. M., Grant, P. A., Owens, P. C., Sohlstrom, A., Roberts, C. T., Robinson, J. S., & Owens, J. A. (2003). Effect of maternal feed restriction during pregnancy on glucose tolerance in the adult guinea pig. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology, 284*(1), R140-R152.

Kind, K. L., Simonetta, G., Clifton, P. M., Robinson, J. S., & Owens, J. A. (2002). Effect of maternal feed restriction on blood pressure in the adult guinea pig. *Experimental Physiology, 87*(04), 469-477.

Kotsampasi, B., Balaskas, C., Papadomichelakis, G., & Chadio, S. E. (2009a). Reduced Sertoli cell number and altered pituitary responsiveness in male lambs undernourished *in utero*. *Animal Reproduction Science, 114*(1-3), 135-147.

Kotsampasi, B., Chadio, S., Papadomichelakis, G., Deligeorgis, S., Kalogiannis, D., Menegatos, I., & Zervas, G. (2009b). Effects of maternal undernutrition on the hypothalamic-pituitary-gonadal axis function in female sheep offspring. *Reproduction in Domestic Animals, 44*(4), 677-684.

Lea, R. G., Andrade, L. P., Rae, M. T., Hannah, L. T., Kyle, C. E., Murray, J. F., Rhind, F. M., & Miller, D. W. (2006). Effects of maternal undernutrition during early pregnancy on apoptosis regulators in the ovine fetal ovary. *Reproduction, 131*(1), 113-124.

Léonhardt, M., Lesage, J., Croix, D., Dutriez-Casteloot, I., Beauvillain, J. C., & Dupouy, J. P. (2003). Effects of perinatal maternal food restriction on pituitary-gonadal axis and plasma leptin level in rat pup at birth and weaning and on timing of puberty. *Biology of Reproduction, 68*(2), 390-400.

Liang, H., & Zhang, Z. B. (2006). Food restriction affects reproduction and survival of F1 and F2 offspring of rat-like hamster (*Cricetulus triton*). *Physiology and Behavior, 87*(3), 607-613.

Liang, H., Xiong, W. H., & Zhang, Z. B. (2007). Effect of maternal food restriction during gestation on early development of F1 and F2 offspring in the rat-like hamster (*Cricetulus triton*). *Zoology, 110*(2), 118-126.

Lipsey, M. W., & Wilson, D. B. (2001). *Practical meta-analysis / Mark W. Lipsey, David B. Wilson*: Thousand Oaks, Calif: Sage Publications.

Lobe, S. L., Bernstein, M. C., & German, R. Z. (2006). Life-long protein malnutrition in the rat (*Rattus norvegicus*) results in altered patterns of craniofacial growth and smaller individuals. *Journal of Anatomy, 208*(6), 795-812.

Long, N. M., Nijland, M. J., Nathanielsz, P. W., & Ford, S. P. (2010). The effect of early to mid-gestational nutrient restriction on female offspring fertility and hypothalamic-pituitary-adrenal axis response to stress. *Journal of Animal Science, 88*(6), 2029-2037.

Lumey, L. H. (1992). Decreased birthweights in infants after maternal *in utero* exposure to the Dutch famine of 1944–1945. *Paediatric and Perinatal Epidemiology***,** *6*, 240–253.

MacLaughlin, S. M., Walker, S. K., Roberts, C. T., Kleemann, D. O., & McMillen, I. C. (2005). Periconceptional nutrition and the relationship between maternal body weight changes in the periconceptional period and feto-placental growth in the sheep. *The Journal of Physiology, 565*(1), 111-124.

Martin, J. L., Vonnahme, K. A., Adams, D. C., Lardy, G. P., & Funston, R. N. (2007). Effects of dam nutrition on growth and reproductive performance of heifer calves. *Journal of Animal Science, 85*(3), 841-847.

Martín, N. P. (2012). *Ewe nutrition during pregnancy: effects on the development of twin fetuses* Master of AgriScience, Massey University, Palmerston North, New Zealand.

McGeady, T. A., Quinn, P. J., FitzPatrick, E. S., & Ryan, M. T. (2006). Male and female reproductive system. *Veterinary embryology* (pp. 244-267): Oxford, UK: Blackwell

McNatty, K. P., & Henderson, K. M. (1987). Gonadotrophins, fecundity genes and ovarian follicular function. *Journal of Steroid Biochemistry, 27*(1-3), 365-373.

McNatty, K. P., Fidler, A. E., Juengel, J. L., Quirke, L. D., Smith, P. R., Heath, D. A., Lundy, T., & O'Connell, A. (2000). Growth and paracrine factors regulating follicular formation and cellular function. *Molecular and Cellular Endocrinology, 163*(1-2), 11-20.

Meikle, D., & Westberg, M. (2001). Maternal nutrition and reproduction of daughters in wild house mice (*Mus musculus*). *Reproduction, 122*(3), 437-442.

Michels, H., Decuypere, E., & Onagbesan, O. (2000). Litter size, ovulation rate and pre-natal survival in relation to ewe body weight: genetics review. *Small Ruminant Research, 38*(3), 199-209.

Munoz, C., Carson, A. F., McCoy, M. A., Dawson, L. E. R., Wylie, A. R. G., & Gordon, A. W. (2009). Effects of plane of nutrition of ewes in early and mid-pregnancy on performance of the offspring: Female reproduction and male carcass characteristics. *Journal of Animal Science, 87*(11), 3647-3655.

Murdoch, J. W., Edward, A. V. K., Vonnahme, K. A., & Stephen, P. F. (2003). Ovarian responses to undernutrition in pregnant ewes, USA. *Reproductive Biology and Endocrinology, 1*(6), 1-8.

Nakagawa, S., & Cuthill, I. C. (2007). Effect size, confidence interval and statistical significance: a practical guide for biologists. *Biological Reviews, 82*(4), 591-605.

National Research Council (1985). Nutrient Requirements of Sheep, 6th edition. National Academy of Sciences.

Neville, T. L., Caton, J. S., Hammer, C. J., Reed, J. J., Luther, J. S., Taylor, J. B., Redmer, J. B., Reynolds, L. P., & Vonnahme, K. A. (2010). Ovine offspring growth and diet digestibility are influenced by maternal selenium supplementation and nutritional intake during pregnancy despite a common post-natal diet. *Journal of Animal Science, 88*(11), 3645-3656.

Oken, E., Levitan, E. B., & Gillman, M. W. (2007). Maternal smoking during pregnancy and child overweight: systematic review and meta-analysis. *International Journal of Obesity, 32*(2), 201-210.

Osgerby, J., Wathes, D., Howard, D., & Gadd, T. (2002). The effect of maternal undernutrition on ovine fetal growth. *Journal of Endocrinology, 173*(1), 131-141.

Paten, A. M., Asmad, K., Loureiro, M. F. P., Kenyon, P. R., Pain, S. J., Peterson, S. W., Pomroy, W. E., Scott, I., & Blair, H. T. (2011). The effects of dam nutrition during pregnancy on the post-natal growth and puberty attainment of ewe progeny. *Proceedings of the New Zealand Society of Animal Production, 71,* 56-58.

Rae, M. T., Kyle, C. E., Miller, D. W., Hammond, A. J., Brooks, A. N. & Rhind, S. M. (2002a). The effects of undernutrition, *in utero*, on reproductive function in adult male and female sheep. *Animal Reproduction Science, 72,* 63–71.

Rae, M. T., Rhind, S. M., Fowler, P. A., Miller, D. W., & Brooks, A. N. (2002b). Effect of maternal undernutrition on fetal testicular steroidogenesis during the CNS androgen-responsive period in male sheep fetuses. *Reproduction, 124*, 33–39.

Rae, M. T., Rhind, S. M., Kyle, C. E., Miller, D. W., & Brooks, A. N. (2002c). Maternal undernutrition alters triiodothyronine concentrations and pituitary response to GnRH in fetal sheep. *Journal of Endocrinology, 173*, 449–455.

Rae, M. T., Rhind, S. M., Kyle, C. K., Fowler, P. A., Miller, D. W. & Brooks, A. N. (2000). Maternal undernutrition during early pregnancy up-regulates fetal testicular steroidogenesis in sheep *Journal of Reproduction and Fertility*, Abstract Series 25.

Rae, M., Palassio, S., Kyle, C., Brooks, A., Lea, R., Miller, D., & Rhind, S. M. (2001). Effect of maternal undernutrition during pregnancy on early ovarian development and subsequent follicular development in sheep fetuses. *Reproduction, 122*(6), 915-922.

Redmer, D. A., Wallace, J. M., & Reynolds, L. P. (2004). Effect of nutrient intake during pregnancy on fetal and placental growth and vascular development. *Domestic Animal Endocrinology, 27*(3), 199-217.

Rees, W. (2002). Manipulating the sufur amino acid content of the early diet and its implications for long-term health. *Proceedings of the Nutrition Society, 61*, 71–77.

Rhind, S. M. (2004). Effects of maternal nutrition on fetal and neonatal reproductive development and function. *Animal Reproduction Science, 82-3*, 169-181.

Rhind, S. M., Rae, M. T., & Brooks, A. N. (2001). Effects of nutrition and environmental factors on the fetal programming of the reproductive axis. *Reproduction, 122*(2), 205-214.

Robinson, J. J., Ashworth, C. J., Rooke, J. A., Mitchell, L. M., & McEvoy, T. G. (2006). Nutrition and fertility in ruminant livestock. *Animal Feed Science and Technology, 126*(3-4), 259-276.

Robinson, J. J., Rooke, J. A., & McEvoy, T. G., (2002). *Nutrition for conception and pregnancy*. In: Freer, M., & Dove, H. (Eds.), Sheep Nutrition. CAB International, Wallingford, UK, pp. 189–211.

Robinson, J. S., Hartwich, K. M., Walker, S. K., Erwich, J., & Owens, J. A. (1997). Early influences on embryonic and placental growth. *Acta Paediatrica, 86*, 159-163.

Russel, A. J. F., Foot, J. Z., White, I. R., & Davies, G. J. (1981). The effect of weight at mating and of nutrition during mid-pregnancy on the birth weight of lambs from primiparous ewes. *The Journal of Agricultural Science, 97*(03), 723-729.

Safari, E., Fogarty, N. M., & Gilmour, A. R. (2005). A review of genetic parameter estimates for wool, growth, meat and reproduction traits in sheep. *Livestock Production Science, 92*(3), 271-289.

Schoenwolf, G. C., Bleyl, S., B., Brauer, P. R., & Francis-West, P. H. (2009). *Larsen's Human Embryology* (4th ed.). Philadelphia, PA Churchill Livingstone.

Scholl, T. O., Hediger, M. L., & Belsky, D. H. (1994). Pre-natal care and maternal health during adolescent pregnancy: A review and meta-analysis. *Journal of Adolescent Health, 15*(6), 444-456.

Simitzis, P. E., Charismiadou, M. A., Kotsampasi, B., Papadomichelakis, G., Christopoulou, E. P., Papavlasopoulou, E. K., & Deliogeorgis, S. G. (2009). Influence of maternal undernutrition on the behaviour of juvenile lambs. *Applied Animal Behaviour Science, 116*(2-4), 191-197.

Smith, N. A., McAuliffe, F. M., Quinn, K., Lonergan, P., & Evans, A. C. O. (2010). The negative effects of a short period of maternal undernutrition at conception on the glucose–insulin system of offspring in sheep. *Animal Reproduction Science, 121*(1-2), 94-100.

Sporn, M., Roberts, A., Wakefield, L., & Assoian, R. (1986). Transforming growth factor-beta: biological function and chemical structure. *Science, 233*(4763), 532-534.

Sullivan, T. M., Micke, G. C., Greer, R. M., & Perry, V. E. A. (2010). Dietary manipulation of Bos indicus x heifers during gestation affects the prepubertal reproductive development of their bull calves. *Animal Reproduction Science, 118*(2-4), 131-139.

Sweeney, T., Saunders, P. T. K., Millar, M. R., & Brooks, A. N. (1997). Ontogeny of anti-Müllerian hormone, 3β-hydroxysteroid dehydrogenase and androgen receptor expression during ovine fetal gonadal development. *Journal of Endocrinology, 153*(1), 27-32.

Tarry-Adkins, J. L., Chen, J.-H., Jones, R. H., Smith, N. H., & Ozanne, S. E. (2010). Poor maternal nutrition leads to alterations in oxidative stress, antioxidant defense capacity, and markers of fibrosis in rat islets: potential underlying mechanisms for development of the diabetic phenotype in later life. *Faseb Journal, 24*(8), 2762-2771.

Thomas, G. B., McNeilly, A. S., & Brooks, A. N. (1993). Development of gonadotrophs and thyrotrophs in the female foetal sheep pituitary: immunocytochemical localization studies. *Journal of Neuroendocrinology, 5*(2), 157-161.

Van Abeelen, A. F. M., Veenendaal, M. V. E., Painter, R. C., De Rooij, S. R., Thangaratinam, S., Van Der Post, J. A. M., Bossuyt, P. M. M., Elias, S. G., Uiterwaal, C. S. P. M., Grobbee, D. E., Saade, G. R., Mol, B. W. J., Khan, K. S., & Roseboom, T. J. (2012). The fetal origins of hypertension: a systematic review and meta-analysis of the evidence from animal experiments of maternal undernutrition. *Journal of Hypertension, 30*(12), 2255-2267.

van der Linden, D. S., Kenyon, P. R., Blair, H. T., Lopez-Villalobos, N., Jenkinson, C. M. C., Peterson, S. W., & Mackenzie, D. D. S. (2009). Effects of ewe size and nutrition on fetal mammary gland development and lactational performance of offspring at their first lactation. *Journal of Animal Science, 87*(12), 3944-3954.

van der Linden, D. S., Kenyon, P. R., Blair, H. T., Lopez-Villalobos, N., Jenkinson, C. M. C., Peterson, S. W., & Mackenzie, D. D. S. (2010a). Relationships between early post-natal growth and metabolic function of 16-month-old female offspring born to ewes exposed to different environments during pregnancy. *Journal of Developmental Origins of Health and Disease, 1*(1), 50-59.

van der Linden, D. S., Kenyon, P. R., Lopez-Villalobos, N., Jenkinson, C. M. C., Peterson, S. W., & Blair, H. T. (2010b). Effects of ewe size and nutrition during

pregnancy on performance of 2-year-old female offspring. *Journal of Agricultural Science, 148*, 465-475.

van der Linden, D.S., Kenyon, P.R., Jenkinson, C.M.C., Peterson, S.W., Lopez-Villalobos, N. & Blair, H.T. (2007). The effects of ewe size and nutrition during pregnancy on growth and onset of puberty in female progeny. *Proceedings of the New Zealand Society of Animal Production 67*, 126-129.

Visser, J. A., de Jong, F. H., Laven, J. S. E., & Themmen, A. P. N. (2006). Anti-Müllerian hormone: a new marker for ovarian function. *Reproduction, 131*(1), 1-9.

Vonnahme, K. A., Luther, J. S., Reynolds, L. P., Hammer, C. J., Carlson, D. B., Redmer, D. A., & Caton, J. S. (2010). Impacts of maternal selenium and nutritional level on growth, adiposity, and glucose tolerance in female offspring in sheep. *Domestic Animal Endocrinology, 39*(4), 240-248.

Wartenberg, H. (1981). The influence of the mesonephric blastema on gonadal development and sexual differentiation. In: A.G. Byskov & H. Peters (Eds), *Development and Function of Reproductive Organs*. Excerpta Medica, Amsterdam, pp. 3- 12.

Weenen, C., Laven, J. S. E., von Bergh, A. R. M., Cranfield, M., Groome, N. P., Visser, J. A., *et al*. (2004). Anti-Müllerian hormone expression pattern in the human ovary: potential implications for initial and cyclic follicle recruitment. *Molecular Human Reproduction, 10*(2), 77-83.

Whorwood, C. B., Firth, K. M., Budge, H., & Symonds, M. E. (2001). Maternal undernutrition during early to midgestation programs tissue-specific alterations in the expression of the glucocorticoid receptor, 11β-hydroxysteroid dehydrogenase isoforms, and type 1 angiotensin ii receptor in neonatal sheep. *Endocrinology, 142*(7), 2854-2864.

Wilhelm, D., Palmer, S., & Koopman, P. (2007). Sex determination and gonadal development in mammals. *Physiological Reviews, 87*(1), 1-28.

Wu, G., Bazer, F. W., Wallace, J. M., & Spencer, T. E. (2006). Intrauterine growth retardation: Implications for the animal sciences. *Journal of Animal Science, 84*(9), 2316-2337.

Zambrano, E., Rodriguez-Gonzalez, G. L., Guzman, C., Garcia-Becerra, R., Boeck, L., Diaz, L., Menjivar, M., Larrea, F., & Nathanielsz, P. W. (2005). A maternal low protein diet during pregnancy and lactation in the rat impairs male reproductive development. *Journal of Physiology-London, 563*(1), 275-284.

Chapter 3

Effects of maternal nutrition during pregnancy on the growth and reproductive development of male sheep: a meta-analysis

Chapter published in part as:

Asmad, K., Nakagawa, S., Lopez-Villalobos, N., Kenyon, P. R., Pain, S. J., Blair, H. T. (2012). Effects of maternal nutrition during pregnancy on the growth and reproductive development of male sheep: a meta-analysis. *Proceedings of the New Zealand Society of Animal Production, 72*, 51-57.

3.1 Abstract

Nutrient supply to the fetus is one of the crucial factors in the regulation of fetal growth and reproductive development. The effect of maternal nutrition can be exerted at all stages of fetal development, from conception until birth. A meta-analysis of maternal nutrition effects on the growth and reproductive development of male sheep offspring was undertaken using sixteen sheep studies. Twelve studies were included for prediction of the growth curves using a logistic growth model, and eleven studies were included for reproductive development and analysed using a mixed model. The meta-analysis suggested that, male sheep offspring undernourished during gestation had significantly slower growth and lower testosterone levels while there were no statistically significant effects on testis weight, seminiferous tubule diameter or Sertoli cell count. The lack of a statistical effect on seminiferous tubule diameter and Sertoli cell count may be due to the limited number of studies examining the effects of maternal nutrition during pregnancy on male sheep offspring reproductive development. The meta-analysis reported here suggests that additional studies are warranted to more conclusively determine whether maternal nutrition during pregnancy effects male sheep growth and reproductive development.

3.2 Introduction

In animal production systems of temperate countries, sheep are pregnant throughout winter where feed is limited in availability. This creates a situation where maternal feeding level may not be optimal. However, pre-natal nutrition is one of the crucial factors regulating fetal growth (Lumey, 1992) and development of the fetal reproductive system (Rhind, 2004). Evidence from previous studies also indicated that maternal

undernutrition altered weight, testicular development and hormonal regulation in male offspring (Bielli *et al*., 2001; Da Silva *et al*., 2001; Rae *et al*., 2002b).

During the pre-natal period, there are a number of critical phases of fetal reproductive development. For example, in sheep, testicular differentiation occurs around Day 27 of gestation followed by steroidogenesis and the activation of associated enzyme systems (Rhind, 2004). Sertoli cells are present at around Day 34 of gestation, seminiferous cords between Day 35 to 40 (Sweeney *et al*, 1997; Rhind *et al*., 2001), and Leydig cells from Day 42 (Hochereau-de Reviers *et al*., 1995). Sertoli cell replication continues throughout fetal life and after birth with maximum numbers reached before 40-80 days of post-natal age (Hochereau-de Reviers *et al*., 1987). By Day 70 of gestation, the rete testis is organised in the centre of testis (Sweeney *et al*, 1997) and between Days 35 and 85 of gestation the GnRH neuronal system develops in the hypothalamus (Caldani *et al*., 1995). Therefore, development of the male's reproductive system is almost complete by birth, except for the Sertoli cells.

Generally, pregnancy is divided into 3 trimesters. Gunn *et al*. (1995) and Rhind *et al*. (1998) showed that nutrition during the first and last trimesters of pregnancy are crucial for fetal development. During the first trimester of pregnancy, the energy requirements for fetal development are relatively small but fetal metabolic activity is high. During the last trimester of pregnancy, fetal growth is maximum and associated with the highest nutritional demand. Ehrhardt and Bell (1995) showed that, in sheep, placental growth is completed before Day 100 of pregnancy when the developing fetus was approximately 25% of its final birth weight (Robinson *et al*., 1977).

There have been a number of studies in sheep examining the effect of maternal nutrition during gestation on growth and reproductive development, but the results have not been

consistent. Meta-analysis relies on effect size using a scale free indicator of the intervention effect to quantitatively construct overall conclusions from the literature (Cohen, 1988) on these effects. It is hypothesised that restricted maternal nutrition during gestation affects the growth and reproductive development of male offspring. To test this hypothesis, sixteen sheep studies were reviewed and meta-analyses undertaken to examine the effect of maternal nutrition restriction on the growth and reproductive development of male sheep offspring.

3.3 Materials and methods

3.3.1 Literature search

A systematic search was undertaken using 'PubMed' and 'Web of Science' databases, reviews, and reference lists of relevant papers. The search strategy employed keywords of maternal nutrition or maternal diet and male growth or male reproduction and trial without language and time limitation. Sixteen sheep studies were identified for this meta-analysis. Twelve studies were included for prediction of growth curves, and eleven studies were included to examine reproductive development.

3.3.2 Study selection

Studies were eligible for inclusion if they included a control or comparison group level of maternal nutrition during pregnancy and measured the growth or reproductive development of sheep male offspring. The details of studies included in the meta-analysis for predicting growth curves are summarised in Table 3.1. and for examining reproductive development in Table 3.2. The maternal nutritional regimen, the specific

periods of treatment and the growth and reproductive development of male sheep offspring are included in the tables.

3.3.3 Data extraction

A total of 16 studies with growth and reproductive development data met the eligibility criteria for meta-analysis. The recorded data included the journal/year of publication, country and author, duration of feeding restriction, number and age of sheep for Control (*ad libitum* intake) and Restricted feeding groups offered less than *ad libitum* intake, mean values related to growth as measured by fetal weight, birth weight and liveweight, and mean values related to reproductive development as measured by individual testis weight, plasma testosterone level, seminiferous tubule diameter and Sertoli cell count of male sheep offspring. The age of the male offspring used in the studies from which the data for meta-analysis were collected ranged from a minimum of 30 days gestation to 301 post-natal days of age.

3.3.4 Statistics and analysis

Statistical analyses were carried out using the the Statistical Analysis System (SAS, 2008; SAS 9.2, SAS Institute, North Carolina, USA) for analysis of variance. Growth data were analysed in two steps. Firstly, growth curves were modeled using the NLMIXED procedure to obtain random regression coefficients for a group of animals in the Control and Restricted treatments in each study using the following logistic growth model:

$$Wt = A/(1 + Be^{-Ct})$$

where W_t is the weight at age t, A is the asymptotic live weight and B and C are model parameters that characterise the shape of the curve.

Secondly, predicted values of liveweight at 50 days intervals after conception were predicted for each treatment and study, and analysed using the MIXED procedure with a linear model that included the fixed of treatment, age in days and the interaction of treatment and age plus the random effect of study. Means and standard errors were obtained for each treatment and age.

Reproductive variables involving testis weight, seminiferous tubule and Sertoli cell count were analysed after $\log_{10}$ transformation using the MIXED procedure with a mixed model including fixed effect of treatment, age as a covariable and random effect of study. Predicted means and their standard errors for each treatment were obtained from the mixed models. Testosterone level was analysed using the Proc GLIMMIX procedure with a model including treatment as a fixed effect, age as a covariate and study as a random effect. The distribution of data to follow a Poison distribution and log transformation was used as a link function. Comparison between means was compared using the Tukey adjustment.

In meta-analytic studies, effect size statistics such as standardized mean difference (Hedges's *g* or Hedges's *d* for small sample sizes) are used to compare effects.

Hedge's d was calculated as:

Hedge's d = $(m_1 - m_2)/\sqrt{(((n_2 - 1)S_2^2 + (n_1 - 1)\ S_1^2/\ (n1 + n2 - 2))}$ (Hedges and Olkin 1985)

Where m = the mean of each treatment group in the trial, n = the number of individuals in each treatment group and s = the standard deviation of the measurements derived from each treatment group.

An unbiased estimate of the difference in standard deviation units suitable for small sample size (Hedge's *d*) was calculated from Hedge's *g* as:

Hedges' d = Hedge's g $(1 - (3/(4(n_1 + n_2 - 2)-1))$ (Hedges and Olkin 1985)

The magnitude of Hedge's d statistics was grouped into small (< 0.2), medium (0.21 to 0.79) and large (> 0.8) ranges for the purpose of discussion (Cohen, 1988).

Overall, 16 studies were included with data from 12 studies for prediction of growth curve, and 11 studies for reproductive development of male sheep offspring.

Table 3.1. Summary of references used in the meta-analysis for determining the effect of maternal nutrition regimen and timing of nutritional regimen on the growth of male sheep offspring.

Nutritional treatment	Days post conception during which nutritional treatment imposed	Effect on Restricted group	Reference
Control group (Improve pasture + grain supplement) or Treated group (Native pasture).	1-99	Reduced birth weight and live weight at 99 days of age (P<0.01)	Bielli *et al*., 2001
High (*ad libitum*) or Moderate (to allow live weight gain of approximately 75 g/day).	1-145	Increased fetal weight (P<0.001)	Da Silva *et al*., 2001
High (100% ME) or Low (50% ME).	1-95	No differences in birth weight and live weight at 20 months of age	Rae *et al*., 2002a
High (100% ME) or Low (50% ME).	1-30, 31-50, 1-50, 1-30, 31-65, 1-65, 1-30, 31-65, 65-110, 1-110	No differences in fetal weight	Rae *et al*., 2002b
Control (100% protein intake) or Restricted (50% protein	1-119	No differences in fetal weight	Rae *et al*., 2002c

intake).			
High (*ad libitum*) or Moderate (to allow live weight gain of approximately 75 g/day).	1-103	No differences in male fetal weight	Da Silva *et al*., 2003
High (*ad libitum*) or Maintenance (to ensure no change in total live weight).	21-140	No differences in fetal weight and live weight	Kenyon *et al*., 2009
Control (100% ME) or Restricted (50% ME).	1-30, 31-100	No differences in live weight	Kotsampasi *et al*., 2009
Control (100% ME) or Restricted (50% ME).	1-30, 31-100	No differences in birth weight and live weight	Simitzis *et al*., 2009
Control (110% ME) or Restricted (70% ME).	110-145	No differences in birth weight	Smith *et al*., 2010
Control (*ad libitum*)or Maintenance (to ensure no change in total live weight) or Sub-Maintenance (to achieve a loss in total live weight of 100g/d)	21-50, 50-140	No differences in fetal weight and live weight	Kenyon *et al*., 2011

ME = Metabolisable Energy

Table 3.2. Summary of references used in the meta-analysis for determining the effect of maternal nutrition regimen and timing of nutritional regimen on the reproductive development of male sheep offspring

Nutritional treatment	Days post conceptio during which nutritional treatment imposed	Effect on Restricted group	Reference
High (110% ME) or Low (90% ME)	30-146	No differences in testis weight	Deligeorgis *et al*., 1996
Control group (improve pasture + grain supplement) or Treated group (native pasture)	1-99	Reduced left and right testis weight (P<0.001), diameter of seminiferous tubule (P<0.01), leydig cells (P<0.001) and no differences in number of Sertoli cells.	Bielli *et al*., 2001
High (*ad libitum*) or Moderate (to allow liveweight gain of approximately 75 g/day)	1-145	Reduced testosterone concentration (P<0.05)	Da Silva *et al*., 2001
High (110% ME) or Low (70% ME)	70-145	Decrease number of Sertoli cells per testis, and	Bielli *et al*., 2002

		no differences in Sertoli cells per cord, testicular weight (left, right or paired), and semineferous tubules diameter.	
High (100% ME) or Low (70% ME)	22-d90, 22-135	No differences in testis weight at day 90 and day 135 of gestation	Osgerby *et al*., 2002
High (100% ME) or Low (50% ME)	1-95	No differences in scrotal circumference and number of spermatozoa	Rae *et al*., 2002a
High (100% ME) or Low (50% ME)	1-30, 31-50, 1-50, 31-65, 1-65, d65-110, 1-110,	Increased and decreased in testosterone concentration ($P<0.05$) and no differences in fetal testicular weight	Rae *et al*., 2002b
Control (100% protein intake) or Restricted (50% protein intake)	1-119	No differences in testis weight	Rae *et al*., 2002c
High (*ad libitum*) or Moderate (to allow liveweight gain of approximately 75 g/day)	1-103	No significant differences in fetal testicular weight, number of semineferous cords and Sertoli cells count	Da Silva *et al*., 2003
High (*ad libitum*)or Maintenance (to ensure no change in total live weight)	21-140	No differences in testis weight	Kenyon *et al*., 2009
Control (100% ME) or Restricted (50% ME)	1-30, 31-100	Reduced number of Sertoli cells ($P<0.01$), seminiferous diameter ($P<0.05$), no differences in testosterone levels and testis weight	Kotsampasi *et al*., 2009
Control (*ad libitum*)or Maintenance (to ensure no change in total live weight) or Sub-Maintenance (to achieve a loss in total live weight of 100g/d)	21-50, 50-140	No differences in testis weight	Kenyon *et al*., 2011
Control (*ad libitum*)or Maintenance (to ensure no change in total live weight) or Sub-Maintenance (to achieve a loss in total live weight of 100g/d)	21-50, 50-140	No differences in testis weight	Martin, 2011.

ME = Metabolisable Energy

3.4 Results

3.4.1 Live weight

Figure 3.1. shows the predicted live weight of male offspring from conception to 800 days of age. The live weight of male offspring from either the Control or Restricted dam feeding during pregnancy groups were significantly different (P = 0.02). The effect was in the expected direction whereby the Restricted group had slower growth rate compared to the Control group. There was a significantly age effect (P<0.001) for overall live weight.

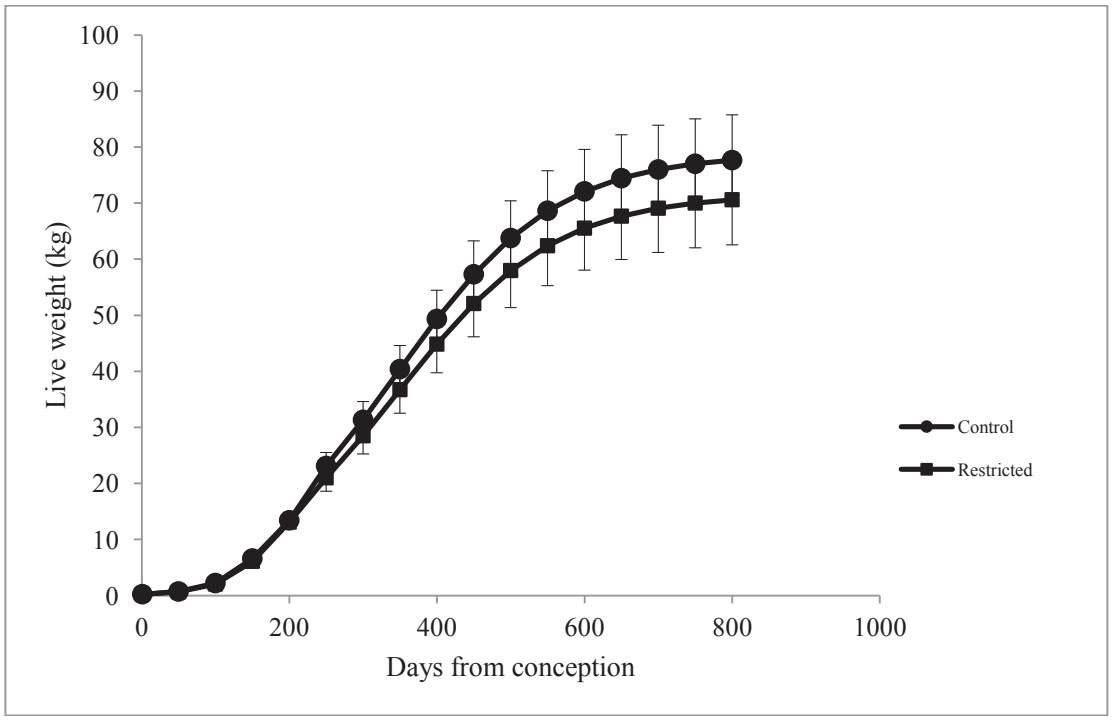


Figure 3.1. Predicted live weights (Mean ± SEM) (using a logistic function) of male offspring from conception to 800 days for Control and Restricted maternal nutrition treatments during pregnancy using twelve sheep studies.

3.4.2 Reproductive development

Table 3.3. shows that, there was no significant difference (P > 0.05) for testis weight, seminiferous tubule diameter or Sertoli cell count of male sheep offspring between the Control and Restricted dam feeding during pregnancy groups. However, the Control group showed a higher testosterone concentration (P=0.004) than the Restricted group. The mean point estimates of the unbiased difference between trial means in standard deviation units (Hedges' *d*) showed a small effect between the Control and Restricted groups of 0.02 for testis weight, 0.18 for testosterone concentration and 0.14 for seminiferous tubule diameter. The effect was larger (0.38) for Sertoli cell count. There was a significant effect of age for testis weight (P < 0.001) and testosterone concentration (P < 0.001), with no significant effect of age for seminiferous tubule diameter (P = 0.36) or Sertoli cell count (P = 0.68).

Table 3.3. Back-transformed mean ± standard error of mean and corresponding Hedges' *d* statistic of testis weight (eleven studies), testosterone level (three studies), seminiferous tubule diameter (three studies) and Sertoli cell count (three studies) of pre-natal ranging from a minimum of 30 days gestation to a maximum of 301 post-natal days of age born to ewes that were controlled and restrictively fed during pregnancy. m = Number of value means used for meta-analysis where the number of sheep used to obtain each ranged from 4 to 14. Bolding of P value indicates significance (P < 0.05).

Measurements	Treatment				P value	Hedge's *d*
	m	Control	m	Restricted		
Testis weight (mg)	17	528.3 ± 3.4	39	464.1 ± 3.4	0.18	0.02
Testosterone level (ng/mL)	59	1.9 ± 0.7[b]	79	1.3 ± 0.5[a]	**0.004**	0.18
Seminiferous tubule diameter (μm)	3	100.0 ± 1.8	4	79.4 ± 1.8	0.26	0.14
Sertoli cell (per tubule)	3	12.6 ± 0.1	4	10.0 ± 1.2	0.38	0.31

[ab] Means between columns within rows with differing superscripts are significantly different (P<0.05)

3.5 Discussion

In this study only sixteen papers were identified for inclusion in the meta-analysis which limits the power of the analysis. Results achieved by using both, the NLMIXED and MIXED procedures estimates of the effects were all in the expected direction where the group of male progeny born following restricted feeding of the dam during pregnancy all had a lower performance than the Control group (Figure 3.1. and Table 3.3.). However, there are many critical limitations in performing and interpreting meta-analysis as it relies only on pre-existing studies rather than any original data collection. Therefore, any flaws that consistently run through the early studies may be magnified in the meta-analysis (Walker *et al*., 2008).

3.5.1 Live weight

The results from comparative meta-analysis demonstrated that maternal nutrition restriction during pregnancy altered the growth of male sheep offspring. This is in agreement with several studies in rats (Léonhardt *et al*. 2003) and pigs (Kind *et al*., 2002) which showed that maternal nutrition restriction during pregnancy reduced fetal weight, birth weight or live weight of male offspring. In contrast though, studies in rats and bulls (Zambrano *et al*., 2005; Long *et al*., 2010), demonstrated that there was no effect of maternal nutrition restriction on male offspring fetal weight, birth weight, or live weight. However, the meta-analysis of sheep growth to account for differences between studies provides quantitative and qualitative evidence that nutrition during pre-natal life can alter the live weight performance of male offspring.

Physiologically, growth that results in enlargement of cells in foetal tissue and development that results in changes of the structure and function of cells in foetal tissue

are complex biological events that are sensitive to maternal nutrition with a potential carry-over effect in later life (Wu *et al*. 2006). On average maternal nutrition restriction during gestation is likely to adversely affect male offspring live weight up to approximately two years of age.

3.5.2 Testis weight

Our meta-analytic results suggest that maternal undernutrition is unlikely to affect the testis weight of male sheep offspring. This was consistent with several studies in rats and bulls (Léonhardt *et al*. 2003; Sullivan *et al*. 2010) which showed that maternal nutrition during pregnancy did not alter testis weight of male offspring. Only two studies in rats by Liang and Zhang (2006) and Genovese *et al.* (2010) showed testis weight of male offspring was reduced from mothers exposed to restricted nutrition during pregnancy. From a physiological perspective undernutrition during pre-natal life in sheep would not be expected to be critical for fetal testis weight as the development and cell differentiation in the testis is almost complete by Day 100 of pregnancy when the energy requirement for fetal development is low relative to high fetal metabolic activity.

3.5.3 Testosterone concentration

The three studies in this analysis all showed a small (d = 0.185) but significant difference of testosterone levels between the Control and Restricted maternal nutrition groups. Other studies have reported variable responses. Zambrano *et al*. (2005) and Liang and Zhang (2006) reported reduced and Sullivan *et al*. (2010) reported no effect

on testosterone concentration in male offspring following a restriction in maternal nutrition during pregnancy. These inconsistent findings may be due to variation among studies due to different species, breeds, level and duration of maternal restriction between treatment and control fetuses. However, the meta-analysis results are consistent with the changes of hypothalamic-pituitary axis secretion due to pre-natal undernutrition (Rhind *et al*. 2001), thereby reducing plasma testosterone concentration (Zambrano *et al*. 2005). More testosterone concentration data are required before conclusive statements regarding the effect of maternal nutrition on offspring testosterone levels can be made.

3.5.4 Seminiferous tubules diameter and Sertoli cells count

The meta-analyses demonstrated that there was no statistically significant differences between the Control and Restricted dam nutrition during pregnancy groups for seminiferous tubule diameter and Sertoli cell count. Data for these two measurements was limited such that it was not possible to draw any robust conclusions.

As with testosterone concentration, reports on the effect of restrictions to maternal nutrition during pregnancy have been associated with no change (Genovese *et al*. 2010), or a decrease in seminiferous tubule lumen diameter (Léonhardt *et al*. 2003) in male offspring. Similarly the restriction to maternal nutrition during pregnancy has been associated with a decrease (Genovese *et al*. 2010) in Sertoli cell count.

In rams, nutrition not only alters the output of GnRH and testis weight but also the efficiency of spermatogenic tissues such as seminiferous tubule diameter and Sertoli cell count (Martin *et al*. 2010). Brooks and Thomas (1995) also showed that a reduction of testis weight at birth was positively related to the number of Sertoli cells, while the

diameter of seminiferous tubules was highly correlated to the number of Sertoli cells (Hochereau-de Reviers *et al.* 1987). Therefore, the lack of a difference in testis weight shown in this study is consistent with there being no differences in either the diameter of seminiferous tubules or the Sertoli cell count.

3.6 Conclusions

Our quantitative and qualitative reviews both indicated that maternal nutrition during gestation altered growth and had minor effects on the reproductive development of male offspring. Although there were only a limited number of studies, meta-analysis results showed similar outcomes for growth and reproductive development in male offspring, with those offspring born to dams experiencing restricted nutrition during pregnancy having lower performance compared to the Control group. However, there still appears to be a need for future studies to determine those critical levels of restriction and windows where restriction is applied that lead to changes in the regulation of cell development. Also, studies are required to investigate further the effect of restricting specific dietary components, such as protein, from the maternal diet during gestation that can affect the pre-natal and subsequent post-natal growth and reproductive development of male offspring.

3.7 Acknowledgements

Thanks goes to the National Research Centre for Growth and Development for funding this research. The first author is funded by Ministry of Higher Education (Malaysia) Doctoral Scholarship.

3.8 References

Bielli, A., Katz, H., Pedrana, G., Gastel, M. T., Morana, A., Castrillejo, A., Lundeheim, N., Forsberg, M., & Rodriguez-Martinez, H. (2001). Nutritional management during fetal and post-natal life, and the influence on testicular stereology and Sertoli cell numbers in Corriedale ram lambs. *Small Ruminant Research, 40*(1), 63-71.

Bielli, A., Perez, R., Pedrana, G., Milton, J. T. B., Lopez, A., Blackberry, M. A., Duncombe, G., Rodriguez-Martinez, H., & Martin, G. B. (2002). Low maternal nutrition during pregnancy reduces the number of Sertoli cells in the newborn lamb. *Reproduction Fertility and Development, 14*(6), 333-337.

Brooks, A. N., & Thomas, G. B. (1995). Ontogeny and function of the pituitary-gonadal axis during fetal development in sheep. *Reproduction in Domestic Animals, 30*(4), 158-162.

Caldani, M., Antoine, M., Batailler, M., & Duittoz, A. (1995). Ontogeny of GnRH systems. *Journal of Reproduction and Fertility Supplement 49*, 147–162.

Cohen, J. (1988). Statistical Power Analysis for the Behavioral Sciences. Lawrence Erlbaum Associates, Hillsdale, New Jersey.

Da Silva, P., Aitken, R. P., Rhind, S. M., Racey, P. A., & Wallace, J. M. (2001). Influence of placentally mediated fetal growth restriction on the onset of puberty in male and female lambs. *Reproduction, 122*(3), 375-383.

Da Silva, P., Aitken, R. P., Rhind, S. M., Racey, P. A., & Wallace, J. M. (2003). Effect of maternal overnutrition during pregnancy on pituitary gonadotrophin gene expression and gonadal morphology in female and male foetal sheep at day 103 of gestation. *Placenta, 24*(2-3), 248-257.

Deligeorgis, S. G., Chadio, S., & Menegatos, J. (1996). Pituitary responsiveness to GnRH in lambs undernourished during fetal life. *Animal Reproduction Science, 43*(2-3), 113-121.

Ehrhardt, R. A., & Bell, A. W. (1995). Growth and metabolism of the ovine placenta during mid-gestation. *Placenta, 16*(8), 727-741.

Genovese, P., Núñez, M. E., Pombo, C., & Bielli, A. (2010). Undernutrition during foetal and post-natal life affects testicular structure and reduces the number of Sertoli cells in the adult rat. *Reproduction in Domestic Animals, 45*(2), 233-236.

Gunn, R. G., Sim, D. A., & Hunter, E. A. (1995). Effects of nutrition in-utero and in early-life on the subsequent lifetime reproductive-performance of Scottish blackface ewes in 2 management-systems. *Animal Science, 60*, 223-230.

Hedges, L., & Olkin, I. (1985). Statistical Methods for Meta-Analysis. Academic Press, New York, NY.

Hochereau-de Reviers, M. T., de Reviers, M. M., Monet-Kuntz, C., Perreau, C., Fontaine, I., & Viguier-Martinez, M. C. (1987). Testicular growth and hormonal

parameters in the male Snell dwarf mouse. *Acta Endocrinologica, 115*(3), 399-405.

Hochereau-de Reviers, M. T., Perreau, C., Pisselet, C., Locatelli, A., & Bosc, M. (1995). Ontogenesis of somatic and germ cells in sheep fetal testis. *Journal of Reproduction and Fertility, 103*(1), 41-46.

Kenyon, P. R., Blair, H. T., Jenkinson, C. M. C., Morris, S. T., Mackenzie, D. D. S., Peterson, S. W., Firth, E. C., & Johnson, P. L. (2009). The effect of ewe size and nutritional regimen beginning in early pregnancy on ewe and lamb performance to weaning. *New Zealand Journal of Agricultural Research, 52*(2), 203-212.

Kenyon, P. R., Pain, S. J., Hutton, P. G., Jenkinson, C. M. C., Morris, S. T., Peterson, S. W., & Blair, H. T. (2011). Effects of twin-bearing ewe nutritional treatments on ewe and lamb performance to weaning. *Animal Production Science, 51*(5), 406-415.

Kind, K. L., Simonetta, G., Clifton, P. M., Robinson, J. S., & Owens, J. A. (2002). Effect of maternal feed restriction on blood pressure in the adult guinea pig. *Experimental Physiology, 87*(04), 469-477.

Kotsampasi, B., Balaskas, C., Papadomichelakis, G., & Chadio, S. E. (2009). Reduced Sertoli cell number and altered pituitary responsiveness in male lambs undernourished *in utero*. *Animal Reproduction Science, 114*(1-3), 135-147.

Léonhardt, M., Lesage, J., Croix, D., Dutriez-Casteloot, I., Beauvillain, J. C., & Dupouy, J. P. (2003). Effects of perinatal maternal food restriction on pituitary-gonadal axis and plasma leptin level in rat pup at birth and weaning and on timing of puberty. *Biology of Reproduction, 68*(2), 390-400.

Liang, H., & Zhang, Z. (2006). Food restriction affects reproduction and survival of F1 and F2 offspring of Rat-like hamster (*Cricetulus triton*). *Physiology & Behavior, 87*(3), 607-613.

Long, N. M., Nijland, M. J., Nathanielsz, P. W., & Ford, S. P. (2010). The effect of early to mid-gestational nutrient restriction on female offspring fertility and hypothalamic-pituitary-adrenal axis response to stress. *Journal of Animal Science, 88*(6), 2029-2037.

Lumey, L. H. (1992). Decreased birthweights in infants after maternal *in utero* exposure to the Dutch famine of 1944–1945. *Paediatric and Perinatal Epidemiology, 6*, 240–253.

Martin, G. B., Blache, D., Miller, D. W., & Vercoe, P. E. (2010). Interactions between nutrition and reproduction in the management of the mature male ruminant. *Animal*, *4*(7), 1214-1226.

Martín, N. P. (2011). Ewe nutrition during pregnancy: effects on the development of twin fetuses: a thesis for the degree of Master of AgriScience in Agriculture at Massey University, Palmerston North, New Zealand.

Osgerby, J., Wathes, D., Howard, D., & Gadd, T. (2002). The effect of maternal undernutrition on ovine fetal growth. *Journal of Endocrinology, 173*(1), 131-141.

Rae, M. T., Kyle, C. E., Miller, D. W., Hammond, A. J., Brooks, A. N., & Rhind, S. M. (2002a). The effects of undernutrition, *in utero*, on reproductive function in adult male and female sheep. *Animal Reproduction Science, 72*(1-2), 63-71.

Rae, M. T., Rhind, S. M., Fowler, P. A., Miller, D. W., Kyle, C. E., & Brooks, A. N. (2002b). Effect of maternal undernutrition on fetal testicular steroidogenesis during the CNS androgen-responsive period in male sheep fetuses. *Reproduction, 124*(1), 33-39.

Rae, M. T., Rhind, S. M., Kyle, C. E., Miller, D. W., & Brooks, A. N. (2002c). Maternal undernutrition alters triiodothyronine concentrations and pituitary response to GnRH in fetal sheep. *Journal of Endocrinology, 173*, 449-455.

Rhind, S. M., Elston, D. A., Jones, J. R., Rees, M. E., McMillen, S. R., & Gunn, R. G. (1998). Effects of restriction of growth and development of Brecon Cheviot ewe lambs on subsequent lifetime reproductive performance. *Small Ruminant Research, 30*(2), 121-126.

Rhind, S. M., Rae, M. T., & Brooks, A. N. (2001). Effects of nutrition and environmental factors on the fetal programming of the reproductive axis. *Reproduction, 122*(2), 205-214.

Rhind, S. M. (2004). Effects of maternal nutrition on fetal and neonatal reproductive development and function. *Animal Reproduction Science, 82*(3), 169-181.

Robinson, J. J. (1977). Influence of maternal nutrition on ovine fetal growth. *Proceedings of the Nutrition Society, 36*(1), 9-16.

SAS. (2008). Statistical Analysis System. SAS Institute Incorporation, Cary, North Carolina, USA.

Simitzis, P. E., Charismiadou, M. A., Kotsampasi, B., Papadomichelakis, G., Christopoulou, E. P., Papavlasopoulou, E. K., & Deligeorgis, S. G. (2009). Influence of maternal undernutrition on the behaviour of juvenile lambs. *Applied Animal Behaviour Science, 116*(2-4), 191-197.

Smith, N. A., McAuliffe, F. M., Quinn, K., Lonergan, P., & Evans, A. C. O. (2010). The negative effects of a short period of maternal undernutrition at conception on the glucose–insulin system of offspring in sheep. *Animal Reproduction Science, 121*, 94-100.

Sullivan, T. M., Micke, G. C., Greer, R. M., & Perry, V. E. A. (2010). Dietary manipulation of Bos indicus x heifers during gestation affects the prepubertal reproductive development of their bull calves. *Animal Reproduction Science, 118*(2-4), 131-139.

Sweeney, T., Saunders, P. T. K., Millar, M. R., & Brooks, A. N. (1997). Ontogeny of anti-Müllerian hormone, 3β-hydroxysteroid dehydrogenase and androgen receptor expression during ovine fetal gonadal development. *Journal of Endocrinology, 153*(1), 27-32.

Walker, E., Hernandez, A. V., & Kattan, M. W. (2008). Meta-analysis: Its strengths and limitations. *Cleveland Clinic Journal of Medicine, 75*(6), 431-439.

Wu, G., Bazer, F. W., Wallace, J. M., & Spencer, T. E. (2006). Intrauterine growth retardation: Implications for the animal sciences. *Journal of Animal Science, 84*(9), 2316-2337.

Zambrano, E., Rodriguez-Gonzalez, G. L., Guzman, C., Garcia-Becerra, R., Boeck, L., Diaz, L., Menjivar, M., Larrea, F., & Nathanielsz, P. W. (2005). A maternal low protein diet during pregnancy and lactation in the rat impairs male reproductive development. *Journal of Physiology-London, 563*(1), 275-284.

Chapter 4

Effects of maternal nutrition during pregnancy on the growth and reproductive development of female sheep offspring: a meta-analysis

4.1 Abstract

Epidemiological evidence has suggested that nutritional status during pre-natal life can influence the growth and reproductive development of offspring at all stages of fetal development, with effects lasting through into post-natal and even adult life. A meta-analysis examining the effects of maternal nutrition on the growth and reproductive development of female sheep offspring was undertaken using twenty five studies. Nineteen studies were included for growth development, of which eighteen (those with growth data from day 145 to 450 after conception) were included for prediction of the post-birth growth curves using an orthogonal polynomial growth model. Thirteen studies were included for assessment of reproductive development and analysed using Hedges' *d* statistic. The data were grouped into studies of either overnutrition or undernutrition during pregnancy. The meta-analysis suggested that female sheep offspring from undernourished dams had significantly slower growth compared to controls, whilst female offspring from overnourished and undernourished dam had lower concentrations of luteinising hormone (LH) compared to controls. There was no effect of dam nutrition treatments on the ovarian weight or follicle number. The meta-analysis therefore indicates that undernutrition of ewes during pregnancy can potentially delay the growth development of their offspring. However, there was no effect of either restricted or superfluous nutrition of dams during pregnancy on the reproductive development of their progeny. These conclusions are based on only a limited number of studies on the effects of pre-natal nutrition on reproductive performance of female sheep offspring, so additional studies are warranted to more conclusively determine the extent and magnitude of those effects.

4.2 Introduction

In recent years, there has been increasing evidence to indicate that maternal nutrition status can have long-term impact on the physiology and cellular development of the offspring (Ashton, 2000; Bernal *et al*., 2010). Studies have indicated that so-called 'fetal programming' effects can lead to an increased chance of diseases such as cardiovascular disease, hypertension and diabetes in humans (Barker *et al*., 1993; Ashton, 2000; Jansson and Powell, 2007). In sheep, poor maternal nutrition can affect growth (Borwick *et al*., 2003; Daniel *et al*., 2007; Kenyon, 2008), reproductive performance (Rhind *et al.*, 2001; Redmer *et al.*, 2004; Rhind, 2004; Dupont *et al*., 2012) and health (Oliver *et al*., 2007) of the offspring. In pastoral sheep production systems in temperate countries, pregnancy occurs during the winter when pasture growth and availability can be low (Moot *et al*., 2007; Kenyon and Webby, 2007), which can create a situation in which maternal nutrition may not be optimal, impairing the supply of nutrients from mother to fetus during the period when the energy demand for fetal growth is at its highest (Robinson, 1977).

Reproductive system development in the female sheep fetus follows a specific timeline. Sexual differentiation of the gonad occurs at around day 32 after conception (Eckery *et al*., 1996). During the period from day 32 the population of germ cells in the ovary increases rapidly by mitosis until it reaches a maximum by day 75 (Eckery *et al*., 1996). At approximately day 55 of gestation, germ cell meiosis is initiated and persists until day 120 (Eckery *et al*., 1996). Primordial follicles form in the cortex region of the ovary from day 75 of gestation (Eckery *et al*., 1996) and develop into primary follicles after about day 90 (McNatty *et al*., 2000).

A number of studies in sheep have investigated the effects of maternal nutrition in gestation on the growth and reproductive development of female offspring, although, to date, the results have been remarkably inconsistent. This inconsistency between studies is likely to be due to differences in the level of maternal nutrition and the stage of gestation at which the nutritional treatment was applied. Meta-analysis is a technique that can be used to organise and summarise findings in a systematic way for quantitative synthesis of these apparently-disparate research findings (Cooper and Hedges, 1994). Meta-analysis relies on effect size, using a scale free indicator of the intervention effect to quantitatively construct overall conclusions from the literature (Cohen, 1988). The previous chapter of this thesis (Chapter 3) used meta-analysis of male offspring to show that undernutrition during gestation delayed growth and reduced testosterone concentrations. It is postulated that overfeeding or underfeeding dam during gestation may also delay the growth and reproductive development of female offspring. To test this hypothesis, twenty five sheep studies were reviewed and meta-analyses undertaken to examine the effects of maternal nutrition restriction on the growth and reproductive development of female offspring.

4.3 Materials and methods

4.3.1 Literature search

A systematic search was undertaken using 'PubMed' and 'Web of Science' databases, reviews, and reference lists of relevant papers. The search strategy used the keywords of 'maternal nutrition' or 'maternal diet' and 'female growth' or 'female reproduction' of 'sheep' and 'trial' without language and time limitation. Twenty five sheep studies were

identified for this meta-analysis. Nineteen studies were included for growth analysis, and thirteen studies for reproductive development analysis.

4.3.2 Study selection

Studies were eligible for inclusion if they (i) included a control or comparison group level of maternal nutrition (either undernutrition or overnutrition) during pregnancy and (ii) measured the growth or reproductive development of female offspring. The details of studies included in the meta-analysis for growth performance are summarized in Table 4.1. and those for reproductive development in Table 4.2.

4.3.3 Data extraction

The recorded data included the journal/year of publication, country and author, level and duration of feeding (control groups: 100% maintenance ME, overnutrition groups: above 100% maintenance ME, and undernutrition groups: below 100% maintenance ME), number and age of sheep, growth characteristics (fetal weight, birth weight, live weight, body condition score and growth rate), and reproductive characteristics (ovarian weight, follicular cell counts and LH concentration) of female offspring. The age of the female offspring used in the studies from which the data for meta-analysis were collected ranged from a minimum of 50 days gestation to 1405 days after conception.

4.3.4 Statistics and analysis

Statistical analyses were carried out using the Statistical Analysis System (SAS, 2008; SAS 9.2, SAS Institute, North Carolina, USA) and R statistical package (v. 2.15.2, R Foundation for Statistical Computing, Vienna, Austria) for analysis of variance.

Growth curves were modeled using mixed linear model to obtain random regression coefficients for a group of animals in the control, undernutrition and/or overnutrition treatments as appropriate in each study using the orthogonal polynomial growth model (Suchocki and Szyda, 2011). However, due to the limited number of studies available examining growth, only data from day ranges 145 (birth) to 450 after conception were included for prediction of a post-natal growth curve. This analysis was undertaken using the MIXED procedure

All meta-analyses were conducted in the R environment (v. 2.15.2; R Development Core Team 2012), using the linear mixed-effect (lme function) in the R package, (Pinheiro *et al*. 2012). The lme can be used to run random-effects meta-analysis and also can account for correlated structures arising from multiple effect sizes from the same studies (Lumley 2002; Nakagawa and Santos 2012). In meta-analytic studies, standardized-mean-difference metrics are used to compare effects between two groups (i.e. control and experimental groups). In this study, Hedges' *d* was used as an effect size statistic (Hedges and Olkin 1985; Cooper *et al*. 2009). The magnitude of Hedges' *d* statistics was grouped into small (< 0.2), medium (0.21 to 0.79) and large (> 0.8) ranges for the purpose of discussion (Cohen, 1988).

Also, for random-effects meta-analysis, heterogeneity (i.e., inconsistence of results among studies) is quantified with the I^2 statistic (Higgins *et al*., 2003); I^2 is the ratio between the between-study variance and the total variance (which is the sum of the

between-study variance and the within-study variance). The I^2 statistic can be interpreted as low = 25%, moderate = 50% and high = 75%, respectively (Higgins *et al*., 2003). The data from day 50 to 1405 after conception were included for examination growth and reproductive development effects.

In this chapter, a different statistical analysis approach (using R and I^2) from Chapter 3 has been used for meta-analysis due to limitation of the data (i.e. age during sample collection, unit measurements (i.e. the whole ovary or certain area of the ovary)) which meant an alternate analysis was required.

Table 4.1. Summary of references used in the meta-analysis for determining the effect of maternal nutrition regimen and timing of nutritional regimen on the growth of female sheep offspring.

Nutritional treatment	Days post conception during which nutritional treatment imposed	Overnutrition	Undernutrition	Reference
Upland grazing + 525 g commercial pellet/day/animal *vs*. Upland grazing *vs*. Hill grazing.	50-145	heavier birth weight, no difference in live weight at weaning and mating, no difference in live weight and body condition score at 18, 30 and 42 months of age.	no difference in birth weight, lighter at 2 months of age,no difference in live weight at weaning and mating, no difference in live weight and body condition score at 18, 30 and 42 months of age.	Gunn *et al*., 1995
High (*ad libitum*), Moderate (10.2 MJME/kg or to allow 75 g/day live weight gain)	1-145	lower birth weight, no difference in live weight.	-	Da Silva *et al*., 2001
High (100% ME) or Low (50% ME)	0-30, 31-50, 0-50, 31-65, d0-65, 65-110, 0-110	-	no difference in fetal weight at day 50.	Rae *et al*., 2001

High (100% ME) or Low (50% ME)	1-95	-	no difference in birth weight or live weight at 6 weeks and 20 months of age.	Rae *et al*., 2002a
High (100% ME) or Low (50% ME)	1-119	-	no difference in fetal weight	Rae *et al*., 2002b
High (100% ME) or Low (70% ME)	100-145	-	lower birth weight and live weight at 14 weeks of age, no difference in live weight at 26 weeks of age.	Borwick *et al*., 2003
High (*ad libitum*) or Moderate (10.2 MJME/kg or to allow 75 g/day live weight gain)	1-103	no difference in fetal weight.	-	Da Silva *et al*., 2003
High (100% ME) or Low (50% ME)	1-30, 31-100	-	no difference in birth weight and live weight at 2, 5.5 and 10 months of age.	Chadio *et al*., 2007
High (100% ME) or Low (50% ME)	30-70, 30-85	-	no difference in birth weight and live weight at 24 weeks, had lower live weight at 22 weeks of age (restricted P30-70).	Daniel *et al*., 2007
High (*ad libitum*) or Medium (maintenance; to allow live weight gain 75 g/day)	21-140	heavier live weight from 22 days of age until weaning.	-	van der Linden *et al*., 2007
High (EP=200% ME, MP=140% ME), Medium (EP=100% ME, MP=80% ME), Low (60% ME)	1-39, 40-90	no difference in offspring live weight and bcs dam restricted during early or mid late pregnancy.	no difference in offspring live weight and bcs dam restricted during early or mid late pregnancy.	Munoz *et al*., 2009

High (100% ME) or Low (50% ME)	1-30, 31-100	-	no difference in birth weight and live weight.	Kotsampasi *et al*., 2009
High (*ad libitum*) or Medium (maintenance; to allow live weight gain 75 g/day)	21-140	lower birth weight, grew slower until weaning compared to maintenance group.	-	van der Linden *et al*., 2009
High (110% ME) or Low (50% ME)	1-7	-	no differences in birth weight and placenta weight.	Smith *et al*., 2010
High (140% NRC) or Medium (100% NRC) Low (60% NRC)	50-145	no difference in birth weight and live weight at 78, 162, 120 and 180 days of age.	lower birth weight, no differences in live weight at 78, 162, 120 and 180 days of age.	Neville *et al*., 2010
High (140% NRC) or Medium (100% NRC) Low (60% NRC)	50-145	no differences in birth weight, live weight and average daily gain	no differences in birth weight, live weight and average daily gain	Vonnahme *et al*., 2010
High (*ad libitum*) or Medium (maintenance; to allow live weight gain 75 g/day)	21-140	no difference in birth weight, growth rates to weaning, post weaning and post pubertal.	-	van der Linden *et al*., 2010a
High (*ad libitum*) or Medium (maintenance; to allow live weight gain 75 g/day)	21-140	no differences in live weight and body condition score during pregnancy, lighter lambs live weight at birth and weaning and lamb growth rates of the grand-offspring.	-	van der Linden *et al*., 2010b

High (*ad libitum*) or Medium (maintenance; to allow live weight gain 75 g/day), Low (sub maintenance)	21-50, 50-140	lower birth weight, no difference in live weight from 90 to 360 days of age.	no difference in live weight from birth to 360 days of age.	Paten *et al*., 2011

NRC = National Research Council requirement (1985); ME = Metabolisable Energy; EP = early pregnancy; MP = mid-pregnancy

Table 4.2. Summary of references used in the meta-analysis for determining the effect of maternal nutrition regimen and timing of nutritional regimen on the reproductive development of female sheep offspring.

Nutritional treatment	Days post conception during which nutritional treatment imposed	Overnutrition	Undernutrition	Reference
High (110% ME) or Low (90% ME)	30-145	-	no difference in fetal ovarian weight at day 55 of gestation.	Deligeorgis *et al*., 1996
High (*ad libitum*), Moderate (10.2 MJME/kg or to allow 75 g/day live weight gain)	0-145	lower plasma LH concentrations at weeks 2, 10, 15, 17 and 20 of age, no difference in age ovulation began, duration of first ovarian cycles, no number of ovulatory cycle per first season or duration of first breeding season.	-	Da Silva *et al*., 2001
High (100% ME) or Low (50% ME)	1-30, 31-50, 0-50, 31-65, 0-65, 65-110, 0-110	-	lower fetal ovarian weight at day 50, fewer germ cells at day 65, delayed ovarian follicular development at day 110.	Rae *et al*., 2001
High (*ad libitum*), Moderate (10.2 MJME/kg or to allow 75 g/day live	0-131	Higher LHβ and FHβ mRNA expression, no difference in ovarian weight, lower number of primordial	-	Da Silva *et al*., 2002

weight gain)		follicles and total number of follicles, no difference in primary or secondary follicles.		
High (100% ME) or Low (70% ME)	22-145	-	heavier fetal ovarian weight at day 90, no difference in fetal ovarian weight at day 135.	Osgerby *et al*., 2002
High (100% ME) or Low (50% ME)	1-95	-	reduced ovulation rate, no difference in FSH profiles, basal LH profiles or response to GnRH.	Rae *et al*., 2002a
High (100% ME) or Low (50% ME)	1-119	-	no difference in fetal ovarian weight, no difference in mean basal LH concentration in response to an exogenous GnRH challenge.	Rae *et al*., 2002b
High (100% ME) or Low (70% ME)	100-145	-	had higher pituitary GnRH receptor binding and ERα at 31 weeks of age, no difference in the abundance of mRNA for LHβ, FSHβ or GnRH receptor binding or ERα mRNA at 31 weeks or 18 months of age.	Borwick *et al*., 2003
High (*ad libitum*) or Moderate (10.2 MJME/kg or to allow 75 g/day live weight gain)	1-103	no difference in LHβ and FSHβ mRNA expression, lower number of primordial follicles and total number of follicles, no difference in isolated, primary	-	Da Silva *et al*., 2003

		and secondary follicles.		
High (100% NRC) or Low (50% NRC)	21-78	-	no difference in fetal ovarian weight and germ cells concentration, increase oxidative base lesions within DNA of mid-gestational fetal oogonia, arrest modulator p53, antiapoptic factor Bcl-2, and base-excision repair polymerase β.	Murdoch *et al*., 2003
High (100% ME) or Low (60% ME)	50-135	-	lighter fetal ovarian weight, smaller proportion of proliferating primordial follicles/area and labeling index in primordial follicles in ovine fetal ovaries. No difference of labeling index in primary and secondary follicles. Bigger proportion of proliferating antral follicles/area.	Grazul-Bilska *et al*., 2009
High (100% ME) or Low (50% ME)	0-30, 31-100	-	no difference in onset of puberty, LH and FSH challenge at 5.5 months, at 10 months of age FSH higher in restricted group.	Kotsampasi *et al*., 2009
High (*ad libitum*) or Medium (maintenance; to allow live weight gain 75 g/day), Low (sub maintenance)	21-50, 50-140	no difference in fetal ovarian weight.	no difference in fetal ovarian weight.	Martín, 2011

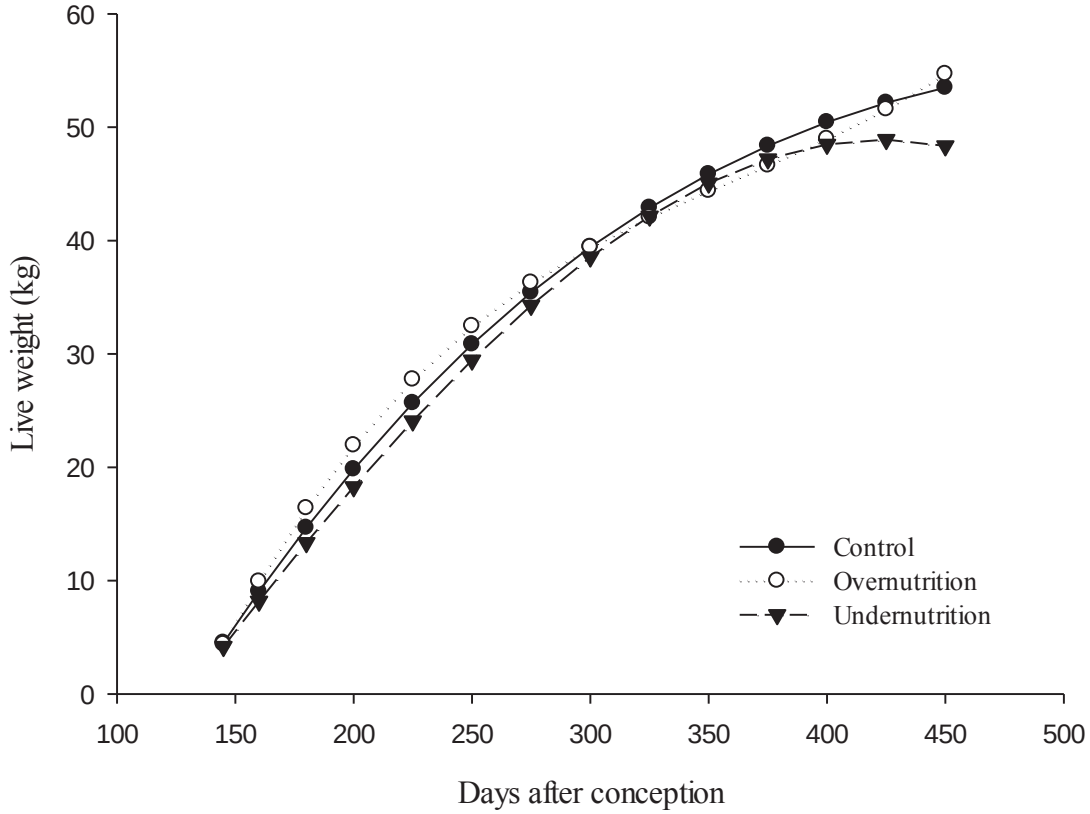
NRC = National Research Council requirement (1985); ME = Metabolisable Energy

4.4 Results

4.4.1 Live weight

There were no differences between either maternal control, overnutrition or undernutrition groups in relation to predicted live weights of female offspring from days 145 (birth) to 450 after conception (Figure 4.1.). Conversly, Hedges' *d* statistic (d = 0.2770 ± 0.0991) for growth data from day 100 to 1405 after conception showed that female offspring born to control fed dams had significantly (P = 0.006) higher growth performance than those born to underfed dams (Table 4.3.). However, the heterogeneity across the studies was moderate (I^2 = 54.5%).

Figure 4.1. Predicted live weights (Mean) (using an orthogonal polynomial) of female offspring from day 145 (birth) to 450 after conception for Control and Restricted maternal nutrition treatments during pregnancy. This data was generated using fourteen sheep studies.



4.4.2 Reproductive development

There was no significant effect of maternal nutrition on ovarian weight and number of follicles (Table 4.3.). However, the concentration of LH was higher in control compared to groups that were overfed (P = 0.0295, d = 0.2508 ± 0.1084) or underfed (P = 0.0295, d = 0.2503 ± 0.1084) during pregnancy.

The heterogeneity among studies was moderate for both ovarian weight for both underfed (I^2 = 42.1%) and overfed groups (I^2 = 32.2%), and was moderate for follicle number in overfed groups (I^2 = 36.8%). Both LH concentrations and numbers of follicles of overfed and underfed groups showed low heterogeneity among studies (I^2 = <0.10 and 20.7%, respectively).

Table 4.3. Hedges' *d* statistic mean ± s.e., 95% confidence interval and heterogeneity (I^2) of growth (19 studies), ovarian weight (9 studies), number of follicles (5 studies) and luteinising hormone (LH) concentrations (7 studies) of female sheep offspring born to ewes either overnutrition or undernutrition during pregnancy. The age of the offspring during data collection is ranging from 50 days of gestation to a maximum of 1405 days after conception. Bolding of P value indicates significance (P<0.05).

Measurements	Maternal nutrition	N study	N observation	Means Hedges' *d* (SE)	P value	95% CI	I^2 (%)
Growth	Overnutrition	12	183	-0.0469 ± 0.0523	0.3713	-0.056 to 0.150	27.8
	Undernutrition	13	136	0.2770 ± 0.0991	**0.0060**	0.081 to 0.473	54.5
Ovarian weight	Overnutrition	3	7	0.0349 ± 0.2623	0.9005	-0.693 to 0.763	42.1
	Undernutrition	7	18	0.2175 ± 0.1541	0.1859	-0.122 to 0.557	32.2

Number of follicles	Overnutrition	2	13	0.3085 ± 0.3889	0.4444	-0.5475 to 1.1646	36.8
	Undernutrition	3	45	0.2546 ± 0.1318	0.0601	-0.0114 to 0.5205	<0.10
LH concentration	Overnutrition	3	28	0.2508 ± 0.1084	**0.0295**	0.0276 to 0.4741	<0.10
	Undernutrition	4	66	0.2503 ± 0.1084	**0.0292**	-0.4800 to 0.1227	20.7

Mean Hedges' *d*; positive values = Control > Treatment (Overnutrition/ Undernutrition).

4.5 Discussion

Twenty five papers were utilised for this meta-analysis study and results indicate that there are some consistent effects of maternal nutrition upon the growth and reproductive development of female offspring. Conceptually, a meta-analysis uses a statistical approach which allows it to generalise results from a large population and inconsistency of results across studies can be quantified and analysed (Walker *et al*., 2008). However, there are many critical limitations in performing and interpreting meta-analysis as it relies only on pre-existing studies rather than any original data collection. Therefore, any flaws that consistently run through the early studies (e.g. biases of the methods used in different studies) may be magnified in the meta-analysis (Walker *et al*., 2008).

The meta-analysis demonstrated that maternal undernutrition during pregnancy reduced the growth of female offspring (Table 4.3.). This is in agreement with several individual studies in rats (Kind *et al*., 2003; Léonhardt *et al*., 2003; Sloboda *et al*., 2009; Zhang *et al*., 2010) and cows (Martin *et al*., 2007), which reported that maternal undernutrition

reduced birth weight, weaning weight and live weight of female offspring. Although there are also some individual studies that have failed to identify any such effect (mice: Meikle and Westberg, 2001, rats: Chernoff *et al*., 2009; Iwasa *et al*., 2010, cows: Martin *et al*., 2007; Evans *et al*., 2012). However, the meta-analysis approach used in this study accounts for differences between studies and provides quantitative and qualitative evidence that maternal undernutrition during gestation can alter the growth of female offspring. The present meta-analysis does not aim to determine the cause of the impaired growth of female offspring born to these underfed dams. It does, however, indicate that there can be long term impacts of undernutrition on growth which may influence the productivity of offspring in later life.

The meta-analysis results also suggest that maternal nutrition does not affect the ovarian weights of the progeny, which is consistent with some studies in cows (Mossa *et al*., 2013) and rats (Bernal *et al*., 2010). However, it is important to note that the apparent lack of an effect on ovarian weight may not necessarily imply a lack of an effect on ovarian functionality. There is some evidence in rats (Léonhardt *et al*. 2003; Liang and Zhang, 2006; Iwasa *et al*., 2010) that there is ovarian growth retardation and delayed onset of puberty of female offspring born to mothers that were underfed during pregnancy.

Likewise, offspring born to ewes which were either overfed or underfed had lower LH concentrations than those born to controls. This result is consistent with studies in rats (Iwasa *et al*., 2010) and rat-like hamsters (Liang and Zhang, 2006) which showed that maternal undernutrition negatively affected LH and oestradiol concentrations in female offspring. However, there were limited studies were investigated the effect maternal overnutrition on LH in female offspring. The studies overnutrition studies that used in the meta-analysis showed lower plasma LH concentrations at weeks 2 to 20 of age,

whilst higher (Da Silva *et al*., 2002) and no difference (Da Silva *et al*., 2003) of LHβ expression in overnourished offspring.Whether this is due to a reduction in secretion of gonadotrophins is unclear, because, in contrast, another study has reported that maternal undernutrition increased serum oestradiol concentrations, and expression of follicle stimulating hormone receptor and luteinizing hormone receptor (da Silva Faria *et al*., 2008). Yet other studies have reported that maternal undernutrition either decreased LH concentrations and follicular development (Romano *et al*., 2007) or had no effect on LH concentrations (Iwasa *et al*., 2010) in the offspring. The present meta-analysis results are consistent with the negative changes of hypothalamic-pituitary axis secretion due to pre-natal undernutrition (Rhind *et al*. 2001), as the level of nutrition can influence the gonadotrophin content of the anterior pituitary gland and thus the release of gonadotrophin from the gland (Landefeld *et al*., 1989; Thomas *et al*., 1990; Cameron and Nosbisch, 1991).

The meta-analyses was also unable to show an effect of maternal nutrition on follicle numbers. It is acknowledged however that data for this parameter was limited which restricts the ability to draw clear conclusions. The inability to show a clear response is, however, consistent with the variability in the literature. Whereby, some studies have shown no effect of maternal undernutrition on the number of primordial (Bernal *et al*., 2010) or primary follicles (da Silva Faria *et al*., 2008; Bernal *et al*., 2010), whilst other studies have found a decrease in primordial (da Silva Faria *et al*., 2008) and antral follicle numbers (Mossa *et al*., 2009; Bernal *et al*., 2010).

4.6 Conclusions

The meta-analysis indicates that maternal undernutrition can negatively affect the growth of the female offspring; and that maternal over and undernutrition reduces the concentration of LH but does not alter ovary weight or follicle numbers of female sheep offspring. Although there was only a limited number of studies, most of the results of the meta-analysis showed similar outcomes, such that those offspring born to dams that were overfed or underfed during pregnancy have lower performance of growth and reproductive parameters measured (although they were not significantly different) compared to the control group. Further studies are warranted to investigate the possible effects of maternal nutrition on offspring endocrine development and potential effects on reproductive performance in adult life.

4.7 Acknowledgements

The authors thank Gravida, National Centre for Growth and Development for providing funding for this project. The first author is funded by Ministry of Higher Education (Malaysia) Doctoral Scholarship.

4.8 References

Ashton, N. (2000). Perinatal development and adult blood pressure. *Brazilian Journal of Medical and Biological Research, 33*(7), 731-740.

Barker, D. J., Gluckman, P. D., Godfrey, K. M., Harding, J. E., Owens, J. A., & Robinson, J. S. (1993). Fetal nutrition and cardiovascular disease in adult life. *Lancet, 341*(8850), 938-941.

Bernal, A. B., Vickers, M. H., Hampton, M. B., Poynton, R. A., & Sloboda, D. M. (2010). Maternal undernutrition significantly impacts ovarian follicle number and

increases ovarian oxidative stress in adult rat offspring. *PLoS ONE, 5*(12), e15558.

Borwick, S. C., Rae, M. T., Brooks, J., McNeilly, A. S., Racey, P. A., & Rhind, S. M. (2003). Undernutrition of ewe lambs *in utero* and in early post-natal life does not affect hypothalamic–pituitary function in adulthood. *Animal Reproduction Science, 77*(1-2), 61-70.

Cameron, J. L., & Nosbisch, C. (1991). Suppression of pulsatile luteinizing hormone and testosterone secretion during short term food restriction in the adult male rhesus monkey (*Macaca mulatta*). *Endocrinology, 128*(3), 1532-1540.

Chadio, S. E., Kotsampasi, B., Papadomichelakis, G., Deligeorgis, S., Kalogiannis, D., Menegatos, I., & Zervas, G. (2007). Impact of maternal undernutrition on the hypothalamic–pituitary–adrenal axis responsiveness in sheep at different ages post-natal. *Journal of Endocrinology, 192*(3), 495-503.

Chernoff, N., Gage, M. I., Stoker, T. E., Cooper, R. L., Gilbert, M. E., & Rogers, E. H. (2009). Reproductive effects of maternal and pre-weaning undernutrition in rat offspring: Age at puberty, onset of female reproductive senescence and intergenerational pup growth and viability. *Reproductive Toxicology, 28*(4), 489-494.

Cohen, J. (1988). Statistical power analysis for the behavioral sciences. Lawrence Erlbaum Associates, Hillsdale, New Jersey.

Cooper, H. M., & Hedges, L. V. (1994). The Handbook of research synthesis. New York: Russell Sage Foundation.

Cooper, H. M., Hedges, L. V., & Valentine, J. C. (2009). The handbook of research synthesis and meta-analysis. New York: Russell Sage Foundation.

da Silva Faria, T., de Bittencourt Brasil, F., Sampaio, F. J. B., & da Fonte Ramos, C. (2008). Maternal malnutrition during lactation alters the folliculogenesis and gonadotropins and estrogen isoforms ovarian receptors in the offspring at puberty. *Journal of Endocrinology, 198*(3), 625-634.

Da Silva, P., Aitken, R. P., Rhind, S. M., Racey, P. A., & Wallace, J. M. (2001). Influence of placentally mediated fetal growth restriction on the onset of puberty in male and female lambs. *Reproduction, 122*(3), 375-383.

Da Silva, P., Aitken, R. P., Rhind, S. M., Racey, P. A., & Wallace, J. M. (2002). Impact of maternal nutrition during pregnancy on pituitary gonadotrophin gene expression and ovarian development in growth-restricted and normally grown late gestation sheep fetuses. *Reproduction, 123*(6), 769-777.

Da Silva, P., Aitken, R. P., Rhind, S. M., Racey, P. A., & Wallace, J. M. (2003). Effect of maternal overnutrition during pregnancy on pituitary gonadotrophin gene expression and gonadal morphology in female and male foetal sheep at day 103 of gestation. *Placenta, 24*(2-3), 248-257.

Daniel, Z., Brameld, J. M., Craigon, J., Scollan, N. D., & Buttery, P. J. (2007). Effect of maternal dietary restriction during pregnancy on lamb carcass characteristics and muscle fiber composition. *Journal of Animal Science, 85*(6), 1565-1576.

Deligeorgis, S. G., Chadio, S., & Menegatos, J. (1996). Pituitary responsiveness to GnRH in lambs undernourished during fetal life. *Animal Reproduction Science, 43*(2-3), 113-121.

Dupont, C., Cordier, A. G., Junien, C., Mandon-Pépin, B., Levy, R., & Chavatte-Palmer, P. (2012). Maternal environment and the reproductive function of the offspring. *Theriogenology, 78*(7), 1405-1414.

Eckery, D. C., Tisdall, D. J., Heath, D. A., & McNatty, K. P. (1996). Morphology and function of the ovary during fetal and early neonatal life: A comparison between the sheep and brushtail possum (*Trichosurus vulpecula*). *Animal Reproduction Science, 42*(1-4), 551-561.

Evans, A. C. O., Mossa, F., Walsh, S. W., Scheetz, D., Jimenez-Krassel, F., Ireland, J. L. H., Smith, G. W., & Ireland, J. J. (2012). Effects of maternal environment during gestation on ovarian folliculogenesis and consequences for fertility in bovine offspring. *Reproduction in Domestic Animals, 47*(Supplement S4), 31-37.

Grazul-Bilska, A. T., Caton, J. S., Arndt, W., Burchill, K., Thorson, C., Borowczyk, E., Bilski, J. J., Redmer, D. A., Reynolds, L. P., & Vonnahme, K. A. (2009). Cellular proliferation and vascularization in ovine fetal ovaries: effects of undernutrition and selenium in maternal diet. *Reproduction, 137*(4), 699-707.

Gunn, R. G., Sim, D. A., & Hunter, E. A. (1995). Effects of nutrition *in-utero* and in early-life on the subsequent lifetime reproductive-performance of Scottish blackface ewes in two management-systems. *Animal Science, 60*, 223-230.

Hedges, L., & Olkin, I. (1985). Statistical Methods for Meta-Analysis. Academic Press, New York, NY.

Higgins, J. P. T., Thompson, S. G., Deeks, J. J., & Altman, D. G. (2003). Measuring Inconsistency in Meta-Analyses. *British Medical Journal, 327*(7414), 557-560.

Iwasa, T., Matsuzaki, T., Murakami, M., Fujisawa, S., Kinouchi, R., Gereltsetseg, G., Kuwahara, A., Yasui, T., & Irahara, M. (2010). Effects of intrauterine undernutrition on hypothalamic Kiss1 expression and the timing of puberty in female rats. *The Journal of Physiology, 588*(5), 821-829.

Jansson, T., & Powell, T. L. (2007). Role of the placenta in fetal programming: underlying mechanisms and potential interventional approaches. *Clinical Science, 113*(1), 1-13.

Kenyon, P. R. (2008). A review of *in utero* environmental effects on sheep production. *Proceedings of the New Zealand Society of Animal Production, 68*, 142-155.

Kenyon, P. R., & Webby, R. W. (2007). Pastures and supplements in sheep production systems. In P. V. Rattray, I. M. Brookes & A. M. Nicol (Eds.), *Pasture and Supplements for Grazing Animals* (pp. 255-273): New Zealand Society of Animal Production Occasional Publication.

Kind, K. L., Clifton, P. M., Grant, P. A., Owens, P. C., Sohlstrom, A., Roberts, C. T., Robinson, J. S., & Owens, J. A. (2003). Effect of maternal feed restriction during pregnancy on glucose tolerance in the adult guinea pig. *American Journal of*

Physiology - Regulatory, Integrative and Comparative Physiology, 284(1), R140-R152.

Kotsampasi, B., Chadio, S., Papadomichelakis, G., Deligeorgis, S., Kalogiannis, D., Menegatos, I., & Zervas, G. (2009). Effects of maternal undernutrition on the hypothalamic–pituitary–gonadal axis function in female sheep offspring. *Reproduction in Domestic Animals, 44*(4), 677-684.

Landefeld, T. D., Ebling, F. J. P., Suttie, J. M., Vannerson, L. A., Padmanabhan, V., Beitins, I. Z., & Foster, D. L. (1989). Metabolic interfaces between growth and reproduction. ii. characterization of changes in messenger ribonucleic acid concentrations of gonadotropin subunits, growth hormone, and prolactin in nutritionally growth-limited lambs and the differential effects of increased nutrition. *Endocrinology, 125*(1), 351-356.

Léonhardt, M., Lesage, J., Croix, D., Dutriez-Casteloot, I., Beauvillain, J. C., & Dupouy, J. P. (2003). Effects of perinatal maternal food restriction on pituitary-gonadal axis and plasma leptin level in rat pup at birth and weaning and on timing of puberty. *Biology of Reproduction, 68*(2), 390-400.

Liang, H., & Zhang, Z. B. (2006). Food restriction affects reproduction and survival of F1 and F2 offspring of rat-like hamster (*Cricetulus triton*). *Physiology & Behavior, 87*(3), 607-613.

Lumley, T. (2002). Network meta-analysis for indirect treatment comparisons. *Statistics in Medicine, 21*(16), 2313-2324.

Martín Natalia de la Paz. (2011). Ewe nutrition during pregnancy: effects on the development of twin fetuses: a thesis for the degree of Master of AgriScience in Agriculture at Massey University, Palmerston North, New Zealand.

Martin, J. L., Vonnahme, K. A., Adams, D. C., Lardy, G. P., & Funston, R. N. (2007). Effects of dam nutrition on growth and reproductive performance of heifer calves. *Journal of Animal Science, 85*(3), 841-847.

McNatty, K. P., Fidler, A. E., Juengel, J. L., Quirke, L. D., Smith, P. R., Heath, D. A., Lundy, T., & O'Connell, A. (2000). Growth and paracrine factors regulating follicular formation and cellular function. *Molecular and Cellular Endocrinology, 163*(1-2), 11-20.

Meikle, D., & Westberg, M. (2001). Maternal nutrition and reproduction of daughters in wild house mice (*Mus musculus*). *Reproduction, 122*(3), 437-442.

Moot, D. J., Matthew, C., & Kemp, P. D. (2007). Growth of pasture and supplementary crops. In P. V. Rattray, I. M. Brookes & A. M. Nicol (Eds.), *Pasture and Supplements for Grazing Animals* (pp. 13-22): New Zealand Society of Animal Production Occasional Publication.

Mossa, F., Carter, F., Walsh, S. W., Kenny, D. A., Smith, G. W., Ireland, J. L. H., Hildbrandt, T. B., Lonergan, P., Ireland, J. J., & Evans, A. C. O. (2013). Maternal undernutrition in cows impairs ovarian and cardiovascular systems in their offspring. *Biology of Reproduction, 88*(4), 92-92.

Mossa, F., Kenny, D., Jimenez-Krassel, F., Smith, G. W., Berry, D., Butler, S., Fair, T., Lonergan, P., Ireland, J. J., & Evans, A. C. O. (2009). Undernutrition of heifers during the first trimester of pregnancy diminishes size of the ovarian reserve in female offspring. 42nd Annual Meeting of the Society for Study of Reproduction, Vol. Abstract 135. Pittsburg, PA, P. 77.

Munoz, C., Carson, A. F., McCoy, M. A., Dawson, L. E. R., Wylie, A. R. G., & Gordon, A. W. (2009). Effects of plane of nutrition of ewes in early and mid-pregnancy on performance of the offspring: Female reproduction and male carcass characteristics. *Journal of Animal Science, 87*(11), 3647-3655.

Murdoch, J. W., Edward, A. V. K., Vonnahme, K. A., & Ford S. P. (2003). Ovarian responses to undernutrition in pregnant ewes, USA. *Reproductive Biology and Endocrinology, 1*, 6-8.

Nakagawa, S., & Santos, E. S. A. (2012). Methodological issues and advances in biological meta-analysis. *Evolutionary Ecology, 26*(5), 1253-1274.

National Research Council (1985). Nutrient Requirements of Sheep, 6th edition. National Academy of Sciences.

Neville, T. L., Caton, J. S., Hammer, C. J., Reed, J. J., Luther, J. S., Taylor, J. B., Redmer, D. A., Reynolds, L. P., & Vonnahme, K. A. (2010). Ovine offspring growth and diet digestibility are influenced by maternal selenium supplementation and nutritional intake during pregnancy despite a common post-natal diet. *Journal of Animal Science, 88*(11), 3645-3656.

Oliver, M. H., Jaquiery, A. L., Bloomfield, F. H., & Harding, J. E. (2007). The effects of maternal nutrition around the time of conception on the health of the offspring. *Society of Reproduction and Fertility Supplement, 64*, 397-410.

Osgerby, J., Wathes, D., Howard, D., & Gadd, T. (2002). The effect of maternal undernutrition on ovine fetal growth. *Journal of Endocrinology, 173*(1), 131-141.

Paten, A. M., Asmad, K., Loureiro, M. F. P., Kenyon, P. R., Pain, S. J., Peterson, S. W., Pomroy, W. E., Scott, I., & Blair, H. T. (2011). The effects of dam nutrition during pregnancy on the post-natal growth and puberty attainment of ewe progeny. *Proceedings of the New Zealand Society of Animal Production, 71*, 56-58.

Pinheiro J., Bates D., DebRoy S., & Sarkar S. The R Development Core Team (2012). Linear and Nonlinear Mixed Effects Models. R package version 3.1-104.

R Development Core Team (2012) *R: a language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing.

Rae, M. T., Kyle, C. E., Miller, D. W., Hammond, A. J., Brooks, A. N., & Rhind, S. M. (2002a). The effects of undernutrition, *in utero*, on reproductive function in adult male and female sheep. *Animal Reproduction Science, 72*(1-2), 63-71.

Rae, M. T., Palassio, S., Kyle, C. E., Brooks, A. N., Lea, R. G., Miller, D. W., & Rhind, S. M. (2001). Effect of maternal undernutrition during pregnancy on early ovarian development and subsequent follicular development in sheep fetuses. *Reproduction, 122*(6), 915-922.

Rae, M. T., Rhind, S. M., Kyle, C. E., Miller, D. W., & Brooks, A. N. (2002b). Maternal undernutrition alters triiodothyronine concentrations and pituitary response to GnRH in fetal sheep. *Journal of Endocrinology, 173*, 449-455.

Redmer, D. A., Wallace, J. M., & Reynolds, L. P. (2004). Effect of nutrient intake during pregnancy on fetal and placental growth and vascular development. *Domestic Animal Endocrinology, 27*(3), 199-217.

Rhind, S. M. (2004). Effects of maternal nutrition on fetal and neonatal reproductive development and function. *Animal Reproduction Science, 82-3*, 169-181.

Rhind, S. M., Rae, M. T., & Brooks, A. N. (2001). Effects of nutrition and environmental factors on the fetal programming of the reproductive axis. *Reproduction, 122*(2), 205-214.

Robinson, J. J. (1977). The influence of maternal nutrition on ovine foetal growth. *Proceedings of the Nutrition Society, 36*(1), 9-16.

Romano, M. A., Barnabe, V. H., Kastelic, J. P., De Oliveira, C. A., & Romano, R. M. (2007). Follicular dynamics in heifers during pre-pubertal and pubertal period kept under two levels of dietary energy intake. *Reproduction in Domestic Animals, 42*(6), 616-622.

SAS (2008). Statistical Analysis System. SAS Institute Incorporation, Cary, North Carolina, USA.

Sloboda, D. M., Howie, G. J., Pleasants, A., Gluckman, P. D., & Vickers, M. H. (2009). Pre- and post-natal nutritional histories influence reproductive maturation and ovarian function in the rat. *PLoS ONE, 4*(8), 1-8.

Smith, N. A., McAuliffe, F. M., Quinn, K., Lonergan, P., & Evans, A. C. O. (2010). The negative effects of a short period of maternal undernutrition at conception on the glucose–insulin system of offspring in sheep. *Animal Reproduction Science, 121*, 94-100.

Suchocki, T., & Szyda, J. (2011). Statistical modelling of growth using a mixed model with orthogonal polynomials. *Journal of Applied Genetics, 52*(1), 95-100.

Thomas, G. B., Mercer, J. E., Karalis, T., Rao, A., Cummins, J. T., & Clarke, I. J. (1990). Effect of restricted feeding on the concentrations of growth hormone (GH), gonadotropins, and prolactin (PRL) in plasma, and on the amounts of messenger ribonucleic acid for GH, gonadotropin subunits, and PRL in the pituitary glands of adult ovariectomized ewes. *Endocrinology, 126*(3), 1361-1367.

van der Linden, D. S., Kenyon, P. R., Blair, H. T., Lopez-Villalobos, N., Jenkinson, C. M. C., Peterson, S. W., & Mackenzie, D. D. S. (2009). Effects of ewe size and nutrition on fetal mammary gland development and lactational performance of offspring at their first lactation. *Journal of Animal Science, 87*(12), 3944-3954.

van der Linden, D. S., Kenyon, P. R., Blair, H. T., Lopez-Villalobos, N., Jenkinson, C. M. C., Peterson, S. W., & Mackenzie, D. D. S. (2010a). Relationships between early post-natal growth and metabolic function of 16-month-old female offspring born to ewes exposed to different environments during pregnancy. *Journal of Developmental Origins of Health and Disease, 1*(1), 50-59.

van der Linden, D. S., Kenyon, P. R., Blair, H. T., Lopez-Villalobos, N., Jenkinson, C. M. C., Peterson, S. W., & Mackenzie, D. D. S. (2010b). Effects of ewe size and nutrition during pregnancy on glucose metabolism, fat metabolism and adrenal function of postpubertal female twin offspring. *Animal Production Science, 50*(9), 869-879.

van der Linden, D. S., Kenyon, P. R., Jenkinson, C. M. C., Peterson, S. W., Lopez-Villalobos, N., & Blair, H. T. (2007). The effects of ewe size and nutrition during pregnancy on growth and onset of puberty in female progeny. *Proceedings of the New Zealand Society of Animal Production, 67*, 126-129.

Vonnahme, K. A., Luther, J. S., Reynolds, L. P., Hammer, C. J., Carlson, D. B., Redmer, D. A., & Caton, J. S. (2010). Impacts of maternal selenium and nutritional level on growth, adiposity, and glucose tolerance in female offspring in sheep. *Domestic Animal Endocrinology, 39*(4), 240-248.

Walker, E., Hernandez, A. V., & Kattan, M. W. (2008). Meta-analysis: Its strengths and limitations. *Cleveland Clinic Journal of Medicine, 75*(6), 431-439.

Zhang, Y., Li, N., Yang, J., Zhang, T., & Yang, Z. (2010). Effects of maternal food restriction on physical growth and neurobehavior in newborn Wistar rats. *Brain Research Bulletin, 83*(1–2), 1-8.

Chapter 5

Effects of dam size and nutrition during pregnancy on the fetal testicular development

Chapter published in part as:

Asmad, K., Kenyon, P. R., Parkinson, T. J., Pain, S. J., Lopez-Villalobos, N., Blair, H. T. (2013). Effect of dam size and nutrition during pregnancy on fetal testicular development in sheep. *Proceedings of the New Zealand Society of Animal Production, 73*, 41-44.

5.1 Abstract

Epidemiological studies have shown that the maternal environment may compromise development of offspring and it's subsequent health. The objective of this study was to examine the effect of maternal size and nutrition during pregnancy on the development of fetal testes. Romney ewes of either heavy (H; mean: 60.8 kg ± 0.18, n=450) or light (L; mean: 42.5 kg ± 0.17, n=450) live weight at the time of mating were oestrus-synchronized and artificially inseminated. Ewes were allocated to either *ad libitum* (A) or maintenance (M, total live weight gain equivalent to expected conceptus mass) nutrition from Days 21 to 140 of pregnancy, resulting in four treatment groups: HA (n= 151), HM (n= 153), LA (n= 155) and LM (n= 153). On day 140 of pregnancy (P140), dams were slaughtered (n = 79) and fetal testicles were collected, weighed and placed in Bouin's fixative. Fetal testicles were then sectioned (5 μm) and stained (H&E) for morphological assessment. Total area of seminiferous tubules per field (90977 μm2; 10 fields/animal), and circumference, total number of Sertoli cells and gonocytes per tubule from 50 seminiferous tubules per animal were counted and measured using Image J software. Results showed that dam size affected ($P<0.05$) the number of gonocytes with fetuses from H-ewes having a higher number of testicular gonocytes than fetuses from L-ewes (1.3 ± 0.02 vs 1.2 ± 0.02). However, there was no effect ($P>0.05$) of dam size, nutrition or their interaction on the total area of seminiferous tubules, circumference of seminiferous tubule and total number of Sertoli cells observed. These results indicate that both low dam live weight at the time of conception and a low plane of nutrition during pregnancy had little impact on fetal testicular development. This suggests that for male offspring size of their dam or nutrition of the dam during pregnancy does not affect future reproductive performance.

5.2 Introduction

In male sheep, testicular development is almost complete at birth. Testicular cells start to differentiate as early as day 27 of gestation (P27) followed by steroidogenesis and the activation of associated enzyme systems (Rhind, 2004). Sertoli cells are present at around P34 and replication continues post-natally (Hochereau-de Reviers *et al*., 1987). Seminiferous cords that will develop into tubules appear between P35 to P40 (Sweeney *et al*., 1997; Rhind *et al*., 2001). By P70 the rete testis is organised in the centre of testis (Sweeney *et al*., 1997) and between P35 and P85 the gonadotrophin releasing hormone (GnRH) neuronal system develops in the hypothalamus (Caldani *et al*., 1995). Testicular gonocytes are are typically observed between birth and 25 days of age and progressively differentiate into spermatogonia up to 70 days after birth (Sharpe *et al*., 2003).

Previous studies have demonstrated that maternal undernutrition during pregnancy can impact testicular development of the fetus. Maternal undernutrition decreased seminiferous tubule diameter at P99 (Bielli *et al*., 2001) and at 10 months of age (Kotsampasi *et al*., 2009), decreased the number of Sertoli cells at 2 days of age (Bielli *et al*., 2002) and at 10 months of age (Kotsampasi *et al*., 2009) and increased the number of spermatocytes at 14 days of age (Rodríguez-González *et al*., 2012) in male offspring.

Effects such as these have the potential to affect reproductive capability of the male in later life as testicular cell development is highly correlated with sperm productivity and quality (Martin *et al*., 2010). The present study investigated the effects of dam size and nutrition during pregnancy on the development of fetal testicular cells at P140 to

investigate the effect of maternal nutrition applied in the present study on male offspring testicular development.

5.3 Materials and methods

The study was conducted at the Massey University Keeble Sheep and Beef farm (latitude 41° 10'S longitude 175° 35'E), 5 km south of Palmerston North, New Zealand. All experimental animal procedures were approved by the Massey University Animal Ethics Committee (MUAEC 09/18), Palmerston North, New Zealand.

5.3.1 Dams

Romney ewes of either heavy (H; mean: 60.8 kg ± 0.18, n=450) or light (L; mean: 42.5 kg ± 0.17, n=450) live weight at the time of mating were oestrus-synchronised and artificially inseminated as previously described by Kenyon *et al*., (2009). Ewes were allocated to either *ad libitum* (A) or maintenance (M, total live weight gain equivalent to the expected increase in conceptus mass) nutrition from P21 to P140 (represent the feeding practice that commonly applied by New Zealand sheep farmers during pregnancy), resulting in four treatment groups. The average pre- and post-grazing pasture covers during the period P21 to P140 were 1330 ± SE 140.0 and 804.0 ± SE 133.4 kg DM/ha for the maintenance regimen and 2304.0 ± ± SE 156.8 and 1723.3 ± SE 149.7 kg DM/ha for *ad libitum* regimen (Kenyon *et al*., 2009).

5.3.2 Fetal testicular collection

Seventy nine ewes were euthanised at P140 and fetuses collected (HA n = 21; HM n = 20; LA n = 18; LM n= 20 as previously described by Blair *et al*. 2011). Blair *et al*., (2011) reported fetal weight at P140 (LM-fetus 4.5±0.2 kg; LA-fetus 4.9±0.2 kg; HM-fetus 4.7±0.2 kg; HA-fetus 5.3±0.2 kg). Fetal weight of LM-, LA- and HM-fetuses did not differ from each other, but HA-fetuses were heavier than all other groups (Blair *et al*., 2011). Fetal testes from each group were dissected, weighed and place in Bouin's fixative for 20 hours. Testes were removed and excess fixative was washed out in two changes of 70% ethanol and the testes were stored in 70% ethanol before processing into paraffin wax (Leica Histoembedder, Leica Instruments GmbH, Nussloch, Germany).

5.3.3 Fetal testicular cells measurements

A total of 30 male testes were randomly selected (one per fetus) from 14 single and 16 twin fetuses (HA = 8, HM = 8, LA = 7 and LM = 7). Paraffin embedded testes were weighed and sectioned (5 µm thick), 5 µm apart, five sections per fetus and stained (H&E) for morphological assessment (Fischer *et al*., 2008). Total area of seminiferous tubules (under 250 x magnification; 90977 μm^2/per field) were measured from two fields per section (10 fields per animal) (Figure 5.1.). The circumference of 10 seminiferous tubules (round in shape with apparent gonocytes inside the tubule) was measured per section (equal to 50 tubules per fetus, under 500x magnification). The total number of Sertoli cells and gonocytes were counted from the same seminiferous tubules used to measure the circumference. Measurement of the total area and number of Sertoli cells and gonocytes were conducted by using ImageJ software (Rasband, 1997).

5.3.4 Statistical analysis

Statistical analyses were carried out using the Statistical Analysis System (SAS, 2008; SAS 9.2, SAS Institute, North Carolina, USA). Values for testes weight and mean values for total area and circumference of seminiferous tubules, total number of Sertoli cells and gonocytes from each animal were analysed using the MIXED procedure including fixed effects of fetal rank (singleton *vs* twin), dam size treatment (heavy *vs* light), dam feeding treatment (A *vs* M). All two-way and three-way interactions were included in the initial model. Testis weight was included as a covariate (except for testis weight analysis where fetal rank was included as a covariate). Dam size and nutrition and the interaction between dam size and nutrition remained in all models to allow for testing of the study design.

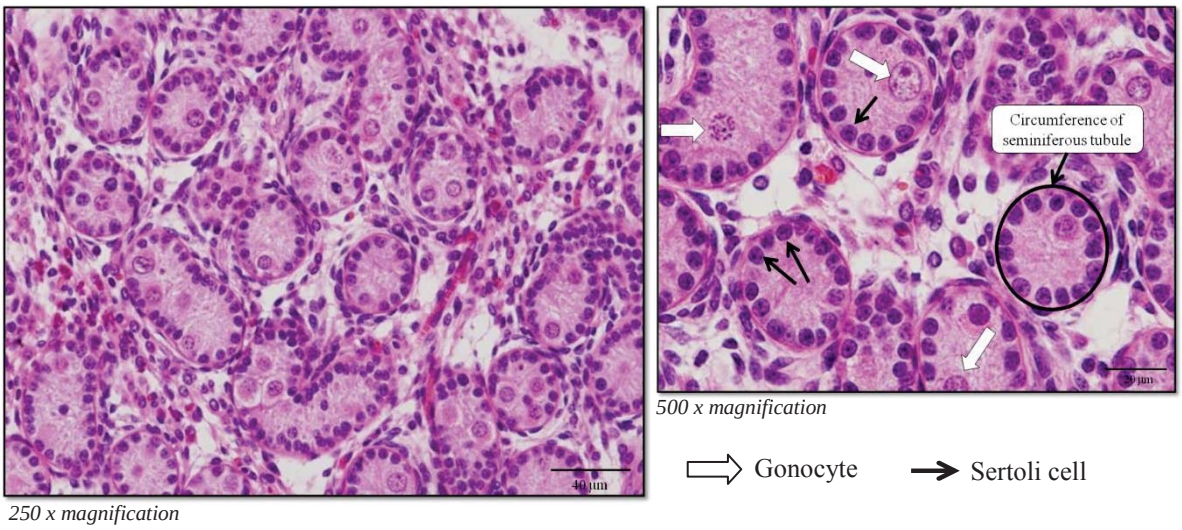


Figure 5.1. Histological slide to visualize the measured variables of seminiferous tubules per field of 90,977 µm^2 in area, circumference of seminiferous tubule, Sertoli cells per seminiferous tubule and number of gonocytes per seminiferous tubule in a sample of fetal testis at Day 140 of gestation.

5.4 Results and Discussion

5.4.1 Testis weight

Results from the present study suggest that maternal size and nutrition did not affect fetal testis weight at P140 (Table 5.1. $P<0.05$). Similarly Kotsampasi *et al*., (2009) showed that maternal nutrition during pregnancy did not alter male lambs testis weight. However, the study by Bielli *et al*., (2001) showed testis weight was reduced when dams were exposed to restricted nutrition during pregnancy. The absent of an effect in the present study is likely due to a lack of a maternal undernutrition treatment.

5.4.2 Total area and circumference of seminiferous tubules

There was no effect ($P>0.05$) of either dam size or nutrition or an interaction between size and nutrition for the total area and circumference of seminiferous tubules per field (Table 5.1.). However, there was an effect of fetal rank, whereby singletons had a greater total area compared to twins (singles: 4.2 ± 0.80 μm^2 *vs* twins: 3.9 ± 0.87 $x10^4$ μm^2; $P=0.02$). Similarly, no effect of maternal nutrition on seminiferous tubule diameter has been reported (Bielli *et al*., 2002). Other studies have reported reduced diameter (Kotsampasi *et al*., 2009) and total area of seminiferous tubules (Sullivan *et al*., 2010) in offspring born to undernourished mothers. As testicular cell development is strongly associated with testicular weight (Jafariahangari *et al*., 2012), the absence of an effect on total area and circumferance of seminiferous tubules is likely due to the fact that there were no differences in testes weight.

5.4.3 Sertoli cell count

Studies typically focus on Sertoli cell development due to its important role in spermatogenesis (Sharpe *et al*., 2003). There was no effect (P>0.05) of either dam size or nutrition or an interaction between size and nutrition treatments or fetal rank on the number Sertoli cells per seminiferous tubule in the present study (Table 5.1.). This has been previously reported (Bielli *et al*., 2001) although both a decrease (Kotsampasi *et al*., 2009) and an increase (Bielli *et al*., 2002) in number of Sertoli cells has also been found. The absence of an effect on Sertoli cell numbers in the present study is perhaps not surprising due to the lack of a difference in seminiferous tubule circumference. The number of Sertoli cells is highly correlated with the size of seminiferous tubules (Hochereau-de Reviers *et al*., 1987).

Table 5.1. Least square mean ± standard error of the mean for testis weight, total area of seminiferous tubules per a field area of 90,977 μm^2, total number of Sertoli cells and number of gonocytes per tubule at Day 140 of gestation for each dam size, dam nutrition and dam size by nutrition group.

Treatment group	N fetus	Testis weight (g)	Total area of semi. tub. ($x10^4$ μm^2)	N semi. tub.	Circumference of semi. tub. (μm)	Sertoli cell (no./tub.)	Gonocyte (no./tub.)
Size							
H	16	3.4 ± 0.11	4.1 ± 0.72	800	124 ± 1.59	12.2 ± 0.19	1.26 ± 0.02^{b}
L	14	3.4 ± 0.12	4.1 ± 0.77	700	127 ± 1.70	12.6 ± 0.21	1.19 ± 0.02^{a}
Nutrition							
A	15	3.6 ± 0.11	4.0 ± 0.75	750	125 ± 1.71	12.5 ± 0.20	1.22 ± 0.02
M	15	3.3 ± 0.11	4.2 ± 0.75	750	126 ± 1.71	12.4 ± 0.20	1.23 ± 0.02
Size x Nutrition							
HA	8	3.5 ± 0.16	3.9 ± 1.02	400	124 ± 2.25	12.5 ± 0.27	1.25 ± 0.03
HM	8	3.4 ± 0.16	4.3 ± 1.02	400	125 ± 2.25	11.9 ± 0.27	1.27 ± 0.03
LA	7	3.6 ± 0.17	4.0 ± 1.10	350	126 ± 2.52	12.4 ± 0.29	1.19 ± 0.03
LM	7	3.2 ± 0.17	4.1 ± 1.09	350	128 ± 2.52	12.8 ± 0.29	1.20 ± 0.03

[ab]Means between rows within a column with differing superscripts are significantly different (P<0.05)
semi. tub. = semineferous tubule

5.4.4 Gonocyte cell count

Male fetuses from H-ewes had a higher number ($P<0.05$) of gonocyte cells in seminiferous tubules at P140 compared to males from L-ewes (Table 5.1.). This result is interesting but somewhat unexpected given the lack of differences in the other parameters measured. Maternal nutrition and fetal rank had no effect on the number of gonocyte cells. To date no other studies have examined the effect of maternal size and nutrition on gonocyte development.

5.5 Conclusions

The majority of studies have investigated the impact of maternal undernutrition on fetal testes development, whilst the present study examined maintenance and *ad libitum* maternal feeding levels. The results reported here demonstrate that provision of maternal nutrition of maintenance or above during pregnancy does not appear to negatively affect testis weight, seminiferous tubule development, Sertoli cells number or gonocyte cell number in the fetal testis relative to feeding the ewes at maintenance. However, given the influence of maternal size on gonocyte cell number, it may be worthwhile to further investigate the effect to maternal size on gonocyte functionality as this is an early indicator of fertility potential in males.

5.6 Acknowledgements

The authors thank Gravida, National Centre for Growth and Development and Massey University for providing funding for this project. The first author is funded by Ministry of Higher Education (Malaysia) Doctoral Scholarship.

5.7 References

Bielli, A., Katz, H., Pedrana, G., Gastel, M. T., Morana, A., Castrillejo, A., Lundeheim, N., Forsberg, M., & Rodriguez-Martinez, H. (2001). Nutritional management during fetal and post-natal life, and the influence on testicular stereology and Sertoli cell numbers in Corriedale ram lambs. *Small Ruminant Research 40*(1), 63-71.

Bielli, A., Perez, R., Pedrana, G., Milton, J. T. B., Lopez, A., Blackberry, M. A., Duncombe, G., Rodriguez-Martinez, H., & Martin, G. B. (2002). Low maternal nutrition during pregnancy reduces the number of Sertoli cells in the newborn lamb. *Reproduction Fertility and Development 14*(6), 333-337.

Blair, H. T., van der Linden, D. S., Jenkinson, C. M. C., Morris, S. T., Mackenzie, D. D. S., Peterson, S. W., Firth, E. C., & Kenyon, P. R. (2011). Do ewe size and nutrition during pregnancy affect foetus and foetal organ weight in twins? *Livestock Science, 142*(1-3), 99-107.

Fischer, A. H., Jacobson, K. A., Rose, J., & Zeller, R. (2008). Hematoxylin and Eosin Staining of Tissue and Cell Sections. *Cold Spring Harbor Protocols, 2008*(5), pdb.prot4986.

Caldani, M., Antoine, M., Batailler, M., & Duittoz, A. (1995). Ontogeny of GnRH systems. *Journal of Reproduction and Fertility Supplement 49*, 147–162.

Hochereau-de Reviers, M. T., de Reviers, M. M., Monet-Kuntz, C., Perreau, C., Fontaine, I., & Viguier-Martinez, M. C. (1987). Testicular growth and hormonal parameters in the male Snell dwarf mouse. *Acta Endocrinologica 115*(3), 399-405.

Jafariahangari, Y., Smith, S., Sharma, R. K., Zerehdaran, S., & Blair, H. T. (2012). The effect of pre-natal maternal environment on live weight, reproductive and semen characteristics in ram lambs. *Small Ruminant Research 103*(2–3), 200-204.

Kenyon, P. R., Blair, H. T., Jenkinson, C. M. C., Morris, S. T., Mackenzie, D. D. S., Peterson, S. W., Firth, E. C., & Johnson, P. L. (2009). The effect of ewe size and nutritional regimen beginning in early pregnancy on ewe and lamb performance to weaning. *New Zealand Journal of Agricultural Research 52*(2), 203-212.

Kotsampasi, B., Balaskas, C., Papadomichelakis, G., & Chadio, S. E. (2009). Reduced Sertoli cell number and altered pituitary responsiveness in male lambs undernourished *in utero*. *Animal Reproduction Science 114*(1-3), 135-147.

Martin, G. B., Blache, D., Miller, D. W., & Vercoe, P. E. (2010). Interactions between nutrition and reproduction in the management of the mature male ruminant. *Animal 4*(7), 1214-1226.

Rasband, W. S. (1997). ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA. (http://imagej.nih.gov/ij/).

Rhind, S. M. (2004). Effects of maternal nutrition on fetal and neonatal reproductive development and function. *Animal Reproduction Science 82*(3), 169-181.

Rhind, S. M., Rae, M. T., & Brooks, A. N. (2001). Effects of nutrition and environmental factors on the fetal programming of the reproductive axis. *Reproduction 122*(2), 205-214.

Rodríguez-González, G. L., Vigueras-Villaseñor, R. M., Millán, S., Moran, N., Trejo, R., Nathanielsz, P. W., Larrea, F., & Zambrano, E. (2012). Maternal protein restriction in pregnancy and/or lactation affects seminiferous tubule organization in male rat offspring. *Journal of Developmental Origins of Health and Disease 3*(5), 321-326.

SAS. (2008). Statistical Analysis System. SAS Institute Incorporation, Cary, North Carolina, USA.

Sharpe, R., McKinnell, C., Kivlin, C., & Fisher, J. (2003). Proliferation and functional maturation of Sertoli cells, and their relevance to disorders of testis function in adulthood. *Reproduction 125*(6), 769-784.

Sullivan, T. M., Micke, G. C., Greer, R. M., & Perry, V. E. A. (2010). Dietary manipulation of Bos indicus x heifers during gestation affects the prepubertal reproductive development of their bull calves. *Animal Reproduction Science 118*(2-4), 131-139.

Sweeney, T., Saunders, P. T. K., Millar, M. R., & Brooks, A. N. (1997). Ontogeny of anti-Müllerian hormone, 3ß-hydroxysteroid dehydrogenase and androgen fetal programming of the reproductive axis 213 receptor expression during ovine fetal gonadal development. *Journal of Endocrinology 153*, 27–32.

Chapter 6

Effects of dam nutrition during pregnancy on growth and reproductive performance of male offspring

Chapter published in part as:

Asmad, K., Paten, A. M., Loureiro, M. F. P., Kenyon, P. R., Pain, S. J., Parkinson, T. J., Pomroy, W. E., Scott, I., Blair, H. T. (2011). Brief communication: The effects of dam nutrition during pregnancy on the growth of male offspring. *Proceedings of the New Zealand Society of Animal Production, 71*, 59-61.

6.1. Abstract

The maternal uterine environment has the potential to affect the growth and reproductive performance of male offspring. The aim of this study was to determine the effect of three pastoral nutritional treatments between days 21 and 50 (P21-50) of pregnancy (sub maintenance (Sm_1) *vs* maintenance (M_1) *vs ad libitum* (A_1)) and two further nutritional treatments between P50 and 139 (M_2 *vs* A_2) on the performance of male offspring (n = 292) to 27 months of age. Male lambs were regularly weighed from birth to 27 months of age, faecal egg counts (FEC: strongyloides eggs/g; n=227) were measured at 163 days and carcass weight and GR (fat depth) were recorded on 184 lambs slaughtered at 252 days of age. Scrotal circumference, semen characteristics and testosterone concentrations were measured in the breeding (March 2011; n = 65; 19 months of age) and non-breeding (November 2011; n = 62; 27 months of age) seasons. The results from this study showed that there was no effect of ewe nutritional treatment during pregnancy on male offspring live weight from birth to 27 months of age. Ewe nutrition during Days P21-50 had no effect (P>0.05) on FEC (2626 ± 354 *vs* 1930 ± 382 *vs* 1417 ± 375 strg/g for Sm_1, M_1 and A_1 respectively) or GR (8.3 ± 0.2 *vs* 8.6 ± 0.2 *vs* 8.5 ± 0.2 mm). Similarly, ewe nutrition during Days P50-139 had no effect (P>0.05) on FEC (2212 ± 306 *vs* 1770 ± 298 strongyloides/g for M_2 and A_2 respectively) or GR (8.2 ± 0.2 *vs* 8.7 ± 0.2 mm). However, carcass weight of lambs born to ewes fed M_2 during Days P50-139 were lighter (P<0.05, 19.1 ± 0.3 kg) than those born to A_2 ewes (20.3 ± 0.3 kg). Testosterone concentration and sperm quality were not affected (P>0.05) by ewe nutrition during Days P21-50 or P50-139. However, there was an interaction between early and mid-to-late pregnancy nutrition where rams born to M_1A_2 fed ewes had greater mean scrotal circumference (P=0.05) than those born to M_1M_2 fed ewes (38.5 ± 0.6 *vs* 36.7 ± 0.7 cm). Reproductive parameters showed significant seasonal

variation. There were significant differences between breeding and non-breeding season for scrotal circumference (40.0 ± 0.6 *vs* 34.7 ± 0.6, respectively), testosterone concentration (3.7 ± 04 *vs* 1.5 ± 0.2 ng/mL, respectively), sperm concentration (8.3 ± 1.36 $x10^9$/mL *vs* 13.0 ± 1.39 $x10^9$/mL, respectively) and sperm abnormalities (total abnormal (10.4 ± 1.94% *vs* 23.4 ± 2.83%, respectively), total major (5.4 ± 1.3% *vs* 11.1 ± 2.0%, respectively) and total minor (4.6 ± 1.5% *vs* 12.5 ± 2.0%, respectively)). These results indicate that maternal nutrition levels applied in this study had little impact on the post-natal growth and reproductive performance of male offspring. As expected, poorer reproductive parameters were observed in the non-breeding season relative to the breeding season.

6.2. Introduction

It has been well documented that nutrition during post-natal life can affect growth and reproductive performance of male animals (e.g. Brito *et al*., 2007; Martin *et al*., 2010). More recently, evidence has emerged that pre-natal nutrition also has a pivotal role in the regulation of fetal growth, and reproductive development of male offspring (see reviews by Rhind *et al.*, 2001; Redmer *et al.*, 2004; Rhind 2004; Dupont *et al*., 2012). Manipulation of fetal development can affect the structure and physiology of adult offspring through the process of fetal programming (Barker *et al*., 1993).This finding is supported by epidemiological studies in sheep, which also indicate that pre-natal nutritional status has an impact on offspring in their post-natal life (McMillen *et al.*, 2001). Such evidence emphasises the importance of maternal nutrition during gestation upon fetal and subsequent post-natal development. This is of significance to sheep husbandry in temperate countries, where environmental changes due to seasonality

influence feed quality and quantity especially during winter when most livestock are pregnant. This creates a situation where maternal feeding level may well be suboptimal and, consequently, this affects nutrient supply to the fetus. Under such circumstances, the pre-natal growth and reproductive development of male offspring could be adversely affected. This is supported by experimental studies in which nutritional restriction during gestation affected fetal, birth and live weight (Bielli *et al*., 2001; Da Silva *et al*., 2001; Chapter 3); carcass quality (Bell, 1992); testis weight (Bielli *et al*., 2001); scrotal circumference (Rae *et al*., 2002a); testosterone concentrations (Da Silva *et al*., 2001; Chapter 3); and semen characteristics (Rae *et al*., 2002a) of male offspring. It is therefore postulated that maternal nutrition restriction during mid-to-late pregnancy would affect post-natal growth and reproductive performance of male offspring. The objective of this study was to examine the effects of maternal nutrition during pregnancy on the growth (from birth to 27 months of age), parasite load (at 12 months of age) and reproductive development (in the breeding and non-breeding seasons) of male offspring.

6.3. Materials and methods

The study was conducted at the Massey University Keeble Sheep and Beef farm (latitude 41° 10'S longitude 175° 35'E), 5 km south of Palmerston North, New Zealand. All experimental animal procedures were approved by the Massey University Animal Ethics Committee (MUAEC 09/18), Palmerston North, New Zealand.

6.3.1. Experimental animal

In total, 1169 romney ewes (average live weight 66.3 kg ± 0.18, condition score 2.96 ± 0.02) from commercial flock, that had conceived to artificial insemination using fresh

semen from one of five Romney rams, were randomly allocated to one of three nutritional treatments from day 21 of pregnancy (P21) until P50: sub maintenance (Sm_1), maintenance (M_1) or *ad libitum* (A_1) (Kenyon *et al.*, 2011). The Sm_1 treatment aimed to achieve a loss in mean ewe live weight of 0.1 kg/d, the M_1 treatment aimed to achieve no change in ewe live weight, whilst the A_1 treatment aimed to provide unrestricted herbage under grazing conditions. On P50, non pregnant and single and triplet bearing ewes were removed for the remainder of the study. The remaining twin bearing ewes were reallocated to one of two further nutritional treatments for the period P50 to P139 (pregnancy maintenance (M_2) *vs ad libitum* (A_2)). The aim of the M_2 treatment was to achieve a mean ewe live weight increase similar to that of the expected gravid uterine mass, whilst A_2 aimed to provide unrestricted herbage intake conditions (Table 6.1.). Therefore, this study had a 3 x 2 factorial design (A_1A_2, A_1M_2, M_1A_2, M_1M_2, Sm_1A_2 and Sm_1M_2). On P139, all groups were merged and managed under commercial conditions from then onwards (Kenyon *et al.*, 2011). The present study only reports on twin-born male lambs (n = 292) from both mixed and same sex sets. This study, conducted at the Massey University Keeble Sheep and Beef farm (winter solstice 21 July), 5 km south of Palmerston North, New Zealand, was approved by the Massey University's Animal Ethics Committee.

Table 6.1. Mean (±s.e.) pre- and post- grazing herbage masses (kg DM/ha) for ewe nutritional treatments; sub maintenance (Sm_1) *vs* maintenance (M_1) *vs ad libitum* (A_1) applied Day 21-50 (P21-50) of pregnancy; and maintenance (M_2) *vs ad libitum* (A_2) applied Day 50-139 of pregnancy (adapted from Kenyon *et al*., 2011).

Parameter	Herbage mass (kg DM/ha)			
	P21-50		P50-139	
	Pre-grazing	Post-grazing	Pre-grazing	Post-grazing
sub maintenance	$996^{a} \pm 89.3$	$814^{a} \pm 54.2$		
maintenance	$1479^{b} \pm 107.7$	$1112^{b} \pm 59.4$	$1450^{a} \pm 83.9$	$1011^{a} \pm 32.8$
ad libitum	$2331^{c} \pm 82.0$	$1649^{c} \pm 54.2$	$1828^{b} \pm 76.0$	$1301^{b} \pm 37.8$

Mean within columns followed by different letters are significantly different ($P<0.05$)

6.3.2. Live weight, carcass measurements and FEC

Lambs were weighed at birth (D1) (lambing dates; from 2 to 13 September 2009) and then approximately once a month until 27 months of age. A faecal sample was collected at D252 for faecal egg count (FEC strongyloides eggs/g; n=227). On D253, 216 of the males were randomly assigned within treatment to be slaughtered (n = 38, 37, 42, 34, 30 and 34, for A_1A_2, A_1M_2, M_1A_2, M_1M_2, Sm_1A_2 and Sm_1M_2 treatments, respectively) in a commercial slaughter plant. Carcass weight, dressing-out percentage (DO%) and GR measurement (fat depth at 12^{th} rib) were determined.

6.3.3. Scrotal circumference

The remaining sixty-five Romney rams (n = 11, 10, 12, 10, 11 and 11, for A_1A_2, A_1M_2, M_1A_2, M_1M_2, Sm_1A_2 and Sm_1M_2 treatments, respectively) were managed under commercial conditions until they had their scrotal circumference measured at D570 and D813. D570 was in March 2011 and D813 was in November 2011. In New Zealand March is within the breeding season while November is within the non-breeding season (McNatty *et al*., 1984). Scrotal measurement was via a scrotal measuring tape with both testes were kept firmly pressed against the wall and base of scrotum by one hand at the neck of the scrotum.

6.3.4. Blood collection

Blood samples were collected at D570 and D813 using 3 mL heparinized vacutainer tubes by jugular venipuncture (Becton Dickinson and Company, NJ, USA), and immediately placed on ice. Plasma was separated by centrifugation (3000xg for 15

minutes). Duplicate samples were stored at -20°C within 4 hours of bleeding until plasma testosterone analyses were performed.

Testosterone assay

Plasma testosterone concentrations were measured by radioimmunoassay, as described by Oliver *et al.* (1992). Samples were measured in duplicate using testosterone^{125} label and a monoclonal antibody. The limit of sensitivity of detection, as determined by twice the standard deviation of blank values (representing the least amount of hormone which could cause a significant displacement of radiolabelled hormone from the antibody), which was 0.1 ng/mL. Aliquot size used was 50 µL per sample. The CV% between duplicate ranged from 0.1% to 30% (most of the samples had less than 10% CV). Those samples with a CV more than 10% were repeated to ensure the assay variation value was less than 10%. The within assay variation was between 5 to 10%.

6.3.5. Semen collection

Semen samples were collected at D570 and D813 by electro-ejaculation. The sample was immediately evaluated for visual density (either creamy, milky or watery), forward motility and wave motion under 100x magnification. Smears were prepared and stained with eosin/nigrosin (Björndahl *et al*., 2003) and air-dried for later morphological examination. Semen samples were then fixed in buffered formal saline for later density assessment using a haemocytometer; all sperm heads within the 5 chambers of 25 square chambers were counted under 200x magnification and the sperm cell concentration per mL (average sperm count x 25 x 10,000 x dilution factor) for each animal was calculated (Evans and Maxwell, 1987). Morphology was examined at 1000x magnification, and defects/abnormalities of sperm cells were classified as major

(associated with impaired fertility) or minor abnormalities (minor consequence to male fertility) (Bloom, 1983; Chenoweth, 2005) (Table 6.2.). Abnormalities were also classified as being in the head, the mid-piece or the tail.

Table 6.2. List of Major and minor abnormalities observed in sperm cells (Chenoweth, 2005).

Major abnormalities	Minor abnormalities
Underdeveloped	Narrow heads
Double forms	Small normal heads
Acrosome defect (knobbed acrosome)	Giant and short broad heads
Decapitated sperm defect (active tail)	Free normal heads
Diadern defect	Detached acrosome membranes
Pear-shaped defect	Abaxial implantation
Narrow at base	Distal droplet
Abnormal contour	Simple bent tail
Small abnormal heads	Terminally coiled tail
Free pathological heads	*Other abnormal cells*
Corkscrew defect	• Epithelial cells
Other midpiece defects (tail stump)	• Erythrocytes
Proximal droplet	• Medusa formation
Pseudodroplet	• Boat cells
Strongly coiled or folded tail ('Dag' defect)	• Round cells
	• Pus cells

6.3.6. Statistical Analysis

Live weight

Statistical analyses were carried out using the Statistical Analysis System (SAS, 2008; SAS 9.2, SAS Institute, North Carolina, USA) for analysis of variance using the MIXED procedure. Post-hoc differences between the groups were detected using least significant differences. Correlations between repeated measures were assumed to have compound symmetric error structure (Littell *et al*., 2000).

Growth curves were modeled using a third order polynomial that included the fixed effect of dam feeding treatment (early: A_1 *vs* M_1 *vs* Sm_1; mid-to-late pregnancy: A_2 *vs* M_2, and the interaction between early and mid-to-late pregnancy), a random effect of ram, and the age as a covariate.

Live weight data for birth, weaning, 187, 394 and 813 days of age were analysed with a linear mixed model that included the main effects of sire, dam feeding treatments (early: A_1 *vs* M_1 *vs* Sm_1; mid-to-late pregnancy: A_2 *vs* M_2) and all two-way and three-way interactions were included in the initial model, but interactions were removed if found to be non-significant ($P<0.05$) and the model re-fitted. The models also included date of birth as a covariate. The nutrition treatments and interaction between early and mid-to-late pregnancy nutrition treatments remained in all models irrespective of whether they were significant, or not, to allow for testing of the study design.

Reproductive variables

Reproductive variables (scrotal circumference, testosterone concentration, sperm concentration and sperm abnormalities) were analysed using linear mixed model that included the fixed effect of dam feeding treatment (early: A_1 *vs* M_1 *vs* Sm_1; mid-to-late pregnancy: A_2 *vs* M_2, and the interaction between early and mid-to-late pregnancy), season (breeding *vs* non-breeding; and the interaction between season with feeding treatment), and a random effect of sire. The model also included live weight of the rams as a covariate. The nutrition treatments and interaction between early and mid-to-late pregnancy nutrition treatments remained in all models irrespective of whether they were significant or not to allow for testing of the study design. This analysis was undertaken using the MIXED procedure.

Carcass measurements and FEC

Lamb carcass weight, DO%, GR and FEC data were subjected to analyses of variance with respect to the fixed effects and interaction between, dam nutritional regimens during P21-50 and P50-139. Unless reported in the text, interactions between P21-50 and P50-139 were not significant (P>0.05). Sire was fitted as a fixed effect and date of birth was fitted as a covariate in models for carcass weight, DO%, GR and FEC. To normalise FEC values they were log10 transformed using the GLM procedure in Minitab (Minitab 2010; Minitab 16 Inc, Pennsylvania, USA).

6.4. Results

6.4.1. Live weight

There was no significant affect (P>0.05) of either nutritional treatment during early (P21-50), mid-to-late late pregnancy *(*P50-139) or interaction between early and mid-to-late pregnancy on male offspring live weight from D1 to D813 of age (Figure 6.1., Table 6.3.).

6.4.2. Carcass measurements and FEC

Ewe nutrition during early, mid-to-late pregnancy or interaction between early and mid-to-late pregnancy had no effect (P>0.05) on carcass DO%, GR or FEC of male offspring (Table 6.4.). However, there was an interaction between P21-50 and P50-139 treatments for carcass weight (P<0.05) such that carcasses of ram lambs born to M_1A_2-fed ewes were heavier (P<0.05) than those of ram lambs born to M_1M_2-fed ewes. Carcass weights

of ram lambs born to A_1A_2, A_1M_2, Sm_1A_2 and Sm_1M_2-fed ewes did not differ ($P>0.05$) from either M_1A_2 or M_1M_2 groups or from each other.

6.4.3. Reproductive performance

There was no effect ($P>0.05$) of nutritional treatment during early and mid-to-late pregnancy or interaction between early and mid-to-late pregnancy on testosterone concentration, sperm concentration or sperm abnormalities (Table 6.5.). However, for scrotal circumference there was an interaction ($P<0.05$) between early and mid-to-late pregnancy nutrition where by rams born to M_1A_2 fed ewes had a larger scrotal circumference ($P=0.05$) than those born to A_1A_2, M_1M_2 and Sm_1M_2-fed ewes. No difference was observed between M_1A_2 and A_1M_2 and Sm_1M_2.

The reproductive performance parameters measured showed significant seasonal changes (Table 6.5.). There were differences ($P<0.05$) between the breeding and non-breeding seasons for scrotal circumference, testosterone concentration, sperm concentration and sperm abnormalities (total abnormal (major and minor), total major and total minor). However, there was no interaction ($P>0.05$) between breeding and non-breeding seasons with early and mid-to-late pregnancy nutrition.

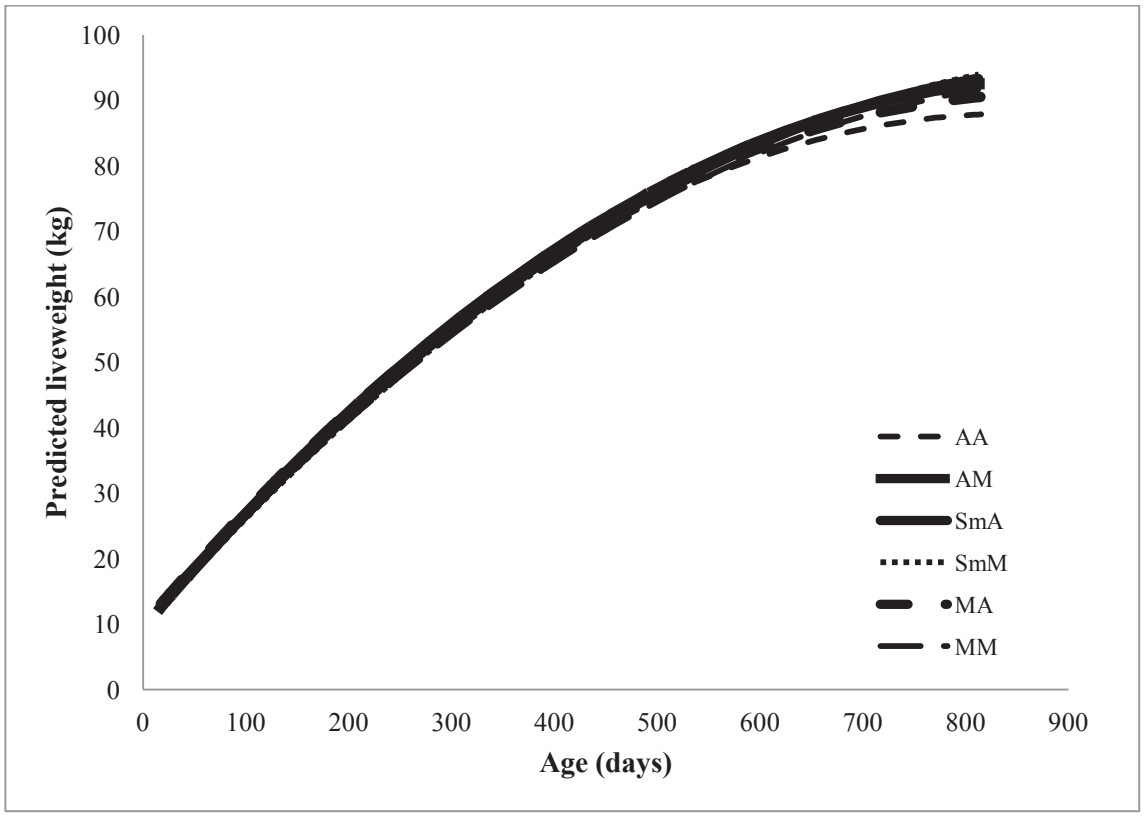


Figure 6.1. Growth curve of male offspring born to ewe nutrition during pregnancy day 21 to day 50 (P21 – 50) (sub maintenance (Sm_1) *vs* maintenance (M_1) *vs ad libitum* (A_1)) and P50 – 139 (maintenance (M_2) *vs ad libitum* (A_2)) (least square mean ± SE, kg). There was no significant difference (P>0.05) between group from birth to 820 days of age.

Table 6.3. Effects of ewe nutrition during pregnancy day 21 to day 50 (P21 – 50) (sub maintenance (Sm_1) *vs* maintenance (M_1) *vs ad libitum* (A_1)) and P50 – 139 (maintenance (M_2) *vs ad libitum* (A_2)) on male offspring birth weight (D1), weaning weight (D92), 6 months of age (D187), 13 months of age (D394) and 27 months of age (D813; non-breeding season).

Treatment group	*n*	Birth weight (kg)	*n*	Weaning weight (kg)	*n*	D187	*n*	D394	*n*	D813
A_1A_2	*51*	5.4 ± 0.11	*51*	26.6 ± 0.48	*47*	38.8 ± 0.66	*11*	69.0 ± 1.92	*11*	96.7 ± 2.83
A_1M_2	*50*	5.5 ± 0.11	*50*	27.2 ± 0.49	*48*	39.8 ± 0.66	*12*	68.8 ± 1.84	*9*	101.1 ± 3.14
M_1A_2	*54*	5.5 ± 0.10	*53*	27.2 ± 0.47	*51*	39.5 ± 0.62	*12*	66.3 ± 1.80	*12*	97.9 ± 2.65
M_1M_2	*45*	5.5 ± 0.12	*45*	26.0 ± 0.52	*44*	38.0 ± 0.69	*11*	67.9 ± 1.88	*10*	101.0 ± 2.90
Sm_1A_2	*43*	5.4 ± 0.12	*42*	26.8 ± 0.53	*41*	38.1 ± 0.70	*12*	67.3 ± 1.75	*10*	102.5 ± 2.84
Sm_1M_2	*49*	5.5 ± 0.11	*48*	26.7 ± 0.50	*49*	37.8 ± 0.64	*12*	67.1 ± 1.83	*11*	101.0 ± 2.90

Table 6.4. Effects of ewe nutrition during pregnancy day 21 to day 50 (P21 – 50) (sub maintenance (Sm_1) *vs* maintenance (M_1) *vs ad libitum* (A_1)) and P50 – 139 (maintenance (M_2) *vs ad libitum* (A_2)) on live weight (kg; D247), carcass weight (least square mean ± se, kg), dressing out % (least square mean ± se, %) GR (least square mean ± se, mm) and faecal egg count (mean ± se, strongyloides/g) of male lamb offspring.

	P21 to 50						P50 to 139			
	n	sub maintenance	*n*	maintenance	*n*	*ad-libitum*	*n*	maintenance	*n*	*ad-libitum*
Carcass measures										
Live weight (kg)	*91*	46.6 ± 0.55	*98*	48.2 ± 0.54	*100*	47.8 ± 0.53	*141*	47.2 ± 0.45	*148*	47.9 ± 0.43
Carcass weight (kg)	*54*	19.66 ± 0.33	*64*	19.70 ± 0.31	*66*	19.72 ± 0.30	*89*	19.13 ± 0.26[b]	*95*	20.25 ± 0.25[a]
Dressing out (%)	*54*	0.413 ± 0.003	*64*	0.408 ± 0.003	*66*	0.413 ± 0.003	*89*	0.408 ± 0.002	*95*	0.415 ± 0.002
GR (fat depth; mm)	*54*	8.3 ± 0.24	*64*	8.6 ± 0.23	*66*	8.5 ± 0.22	*89*	8.2 ± 0.19	*95*	8.7 ± 0.19
Faecal egg count (FEC)										
Log 10 (strg/g)	*81*	2.9 ± 0.11 (2625.7)[1]	*73*	2.8 ± 0.10 (1929.9)[1]	*73*	2.6 ± 0.10 (1417.4)[1]	*116*	2.8 ± 0.08 (2212.4)[1]	*111*	2.7 ± 0.08 (1769.6)[1]

[ab] Means between columns within rows with differing superscripts are significantly different (P <0.05).
[1] Back-Transformed FEC values shown in parenthesis

Table 6.5. Effect of ewe nutrition during pregnancy day 21 to day 50 (P21 – 50) (sub maintenance (Sm_1) *vs* maintenance (M_1) *vs ad libitum* (A_1)) and P50 – 139 (maintenance (M_2) *vs ad libitum* (A_2)) on reproductive performance of male lamb offspring (least square mean ± SE) during breeding and non-breeding season.

Treatment group	*n*	Scrotal circumference (cm)	Testosterone concentration (ng/ml)	Sperm concentration ($x10^8$ sperm/ml)	Sperm abnormalities (%)		
					Total abnormal	Total major	Total minor
Nutrition (P21 – P50)							
A_1	*41*	37.2 ± 0.53	2.5 ± 0.30	9.5 ± 1.21	18.4 ± 2.23	8.8 ± 1.51	9.5 ± 1.62
M_1	*44*	37.6 ± 0.50	2.9 ± 0.29	10.4 ± 1.15	15.0 ± 2.16	7.3 ± 1.46	8.0 ± 1.56
Sm_1	*43*	37.2 ± 0.51	2.4 ± 0.29	12.1 ± 1.18	17.3 ± 2.20	8.7 ± 1.49	8.4 ± 1.60
Nutrition (P50 – P140)							
A_2	*67*	37.4 ± 0.44	2.4 ± 0.26	10.8 ± 1.00	16.6 ± 1.82	8.1 ± 1.24	8.3 ± 1.30
M_2	*61*	37.3 ± 0.46	2.7 ± 0.26	10.5 ± 1.03	17.2 ± 1.85	8.4 ± 1.26	8.9 ± 1.33
Nutrition (P21 – P50) x (P50 – P139)							
A_1A_2	*22*	36.9 ± 0.65^{a}					
A_1M_2	*19*	37.5 ± 0.70^{ab}					
M_1A_2	*24*	38.5 ± 0.62^{b}					
M_1M_2	*20*	36.7 ± 0.66^{a}					
Sm_1A_2	*21*	36.9 ± 0.66^{a}					
Sm_1M_2	*22*	37.6 ± 0.64^{ab}					
Season							
Breeding	*65*	40.0 ± 0.56^{b}	3.7 ± 0.36^{b}	8.3 ± 1.36^{a}	10.4 ± 1.94^{a}	5.4 ± 1.28^{a}	5.0 ± 1.48^{a}
Non-breeding	*63*	34.7 ± 0.56^{a}	1.5 ± 0.19^{a}	13.0 ± 1.39^{b}	23.4 ± 2.83^{b}	11.1 ± 1.97^{b}	12.3 ± 2.00^{b}

[ab]Mean between rows within columns with differing superscripts are significantly different (P<0.05)

6.5. Discussion

6.5.1. Live weight

Results from the present study demonstrate that maternal nutrition (above or below maintenance) during early pregnancy or pregnancy maintenance or above in mid-to-late pregnancy did not alter the growth of male offspring from birth to 2 years of age. This absence of an effect of maternal nutrition is in agreement with several studies in sheep (Rae *et al*., 2002a, b, c; Da Silva *et al*., 2003; Kenyon *et al*., 2009; Simitzis *et al*., 2009; Smith *et al*., 2010), rats (Zambrano *et al*., 2005), pigs (Bauer *et al*., 2009) and cattle (Long *et al*., 2010). Although, two studies in sheep have shown long term effects of maternal nutrition to weaning (Bielli *et al*., 2001) and 25 weeks of age (Da Silva *et al*., 2001), they followed the animals until 99 days and 43 weeks of age, respectively which were much shorter period of time compared to the current study. Similarly, meta-analysis results (Chapter 3) also showed a slower growth in undernourished male sheep offspring until 655 days of age. In contrast, the present study showed no effect of maternal nutrition on birth weight, weaning weight or later live weight. The nutritional treatments used in this study were not sufficiently extreme to affect fetal growth (Martín *et al*., 2012).

6.5.2. Carcass measurements

This study showed that maternal nutrition during pregnancy had no effect on DO% or GR of male offspring. This finding agrees with that of Munõz *et al.* (2009) who reported that maternal nutrition during pregnancy had no effect on DO% and Ford *et al.* (2007) who reported that restricted feeding of ewe from d28 to d78 of gestation did not alter fat depth of lambs at 280 days of age. In contrast, other studies showed that

maternal nutrition restriction during early (Jaquiery *et al*., 2012) and late pregnancy (Gardner *et al*., 2005) increased fat mass in male progeny at maturity and fat levels at one year of age, respectively. Given the absence of effects on carcass weight, DO% and GR, there is unlikely to be any impact of the maternal nutrition treatments examined on the income generated from offspring at slaughtered.

6.5.3. FEC

This study demonstrated that nutrition of the dam during early or mid-to-late pregnancy had no effect on the FEC of male progeny on Day 252. Similarly, Paganoni (2005) reported no effect of maternal nutrition during gestation on the FEC of offspring at 7 to 27 months of age. A study using the female counterparts to the rams in this study showed that ewe lambs born to M_1 dams had more strongyloides eggs at Day 244 of age, but not at Day 277 of age (Paten *et al*., 2011). Rooke *et al.* (2010) found that restricted maternal nutrition (75% metabolisable energy requirement) during early-to-mid pregnancy resulted in greater FEC in Suffolk lambs at weaning but not in Scottish Blackface lambs. The difference between the current study and Rooke *et al.* (2010) might be due to different levels and duration of nutrition restriction imposed during pregnancy. Across studies it appears that maternal nutrition during pregnancy has little impact on the offspring's susceptibility to parasites, although further research is required to confirm this.

6.5.4. Scrotal circumference

An interaction between early and mid-to-late pregnancy nutrition for scrotal circumference of male offspring was found, whereby rams born to M_1A_1 fed ewes had larger circumference than those born to M_1M_2 fed ewes although the difference in magnitude was small. Martín *et al.* (2012), utilising late gestation counterparts to the rams used in this study, reported that ewe nutrition during early and mid-to-late pregnancy had no effect on testes weight of male fetuses at day 140 of gestation. Similarly, Rae *et al*. (2002a) and Kotsampasi *et al.* (2009) showed at 20 and 10 months of age, respectively, that there was no difference in testis size and live weight of male offspring born to ewes that were fed 100% Metabolisable Energy (ME) and 50% ME fed during pregnancy. However, in rats (Liang and Zhang, 2006) showed that testis weights were reduced in male offspring born to mothers exposed to restricted nutrition (70% *ad libitum*) during pregnancy at 60 days of age. From a physiological perspective, scrotal and testis characteristics have been shown to be positively influenced by live weight; where heavier animals have bigger testis and reach puberty earlier (Salhab *et al*, 2001; Jafariahangari *et al*., 2012). The minor difference in scrotal circumference observed in the present study is consistent with the lack of differences in either ram live weight, growth or other reproductive parameters as testis size is positively correlated with live weight (Jafariahangari *et al*., 2012). In addition, there was no interaction ($P>0.05$) between breeding and non-breeding seasons with early and mid-to-late pregnancy nutrition.

6.5.5. Semen quality parameters

Sperm concentration

There was no effect of dam nutrition during either early or mid-to-late pregnancy on sperm concentration in the present study. This is consistent with, Rae *et al*. (2002a) who found no effect of maternal nutrition on sperm concentration in male offspring at 20 months of age. Physiologically, sperm concentration is positively correlated with testis size, because larger testis has a larger mass of spermatogenic tissue (Martin *et al.*, 2010). This result is consistent with the absence of a difference in scrotal circumference. However, method of collection also can have a major effect on the concentration of sperm (Sarsaifi *et al*., 2013).

Sperm abnormalities

The current study showed that ewe nutrition during pregnancy had no effect on either major or minor sperm abnormalities. This is in contrast to a study in rats that showed sperm abnormalities were higher at Day 90 of age in offspring whose mother had been exposed to a lower-than-normal protein diet throughout pregnancy (Toledo *et al*., 2011). Whilst there are no studies on the effect of maternal nutrition (either protein or energy) restriction during pregnancy on sperm abnormalities in male sheep offspring, there are studies in sheep which have investigated the effects of maternal nutritional restriction on male testicular cell development. In these studies, male offspring whose dams were exposed to a 50% of requirement for maintenance from P31 to P100 of gestation showed a significantly lower number of Sertoli cells and smaller diameter of seminiferous tubules at 10 months of age (Kotsampasi *et al*., 2009). Whilst other findings found no effect of moderate nutrition (10.2 MJ metabolisable energy/kg dry

matter (DM)) during early to mid-pregnancy on seminiferous tubules diameter and Sertoli cells count at P103 of gestation (Da Silva *et al*., 2003). Bielli *et al*. (2002) also reported no effect of a 70% nutrition restriction during mid-to-late pregnancy on seminiferous tubules diameter at two days of age. Combined, as reported in Chapter 3, meta-analysis results showed that there was no effect of maternal nutrition restriction on seminiferous tubule diameter and Sertoli cells count of male offspring. Further in Chapter 5, there was no effect of either having dams fed *ad libitum* or maintenance during pregnancy on the total area or circumference of seminiferous tubules and number of Sertoli cells. These findings suggest that nutrition during pregnancy has potential to alter the testicular development if feeding restriction is severe and therefore male fertility. The number of Sertoli cells is positively correlated with testis size and the rate of germ cell production (Sharpe *et al*., 2003); which potentially can influence the quality of the sperm produced (Sharpe, 1994). Jafariahangari *et al.* (2012) showed that lambs with larger testes and higher concentration of sperm had less major sperm defects compared to those with small testes. Therefore, the absence of an effect in the present study on sperm abnormalities is consistent with the absence of an effect of dam nutrition during pregnancy on scrotal circumference and sperm concentration.

6.5.6. Testosterone concentration

This study showed that ewe nutrition during early and mid-to-late pregnancy had no effect on testosterone concentration. Other studies have reported inconsistent responses. Several studies in sheep (Da Silva *et al*., 2003; Kotsampasi *et al*., 2009) and pigs (Bauer *et al*., 2009) have shown that there was no difference in testosterone concentration, whilst other evidence from sheep (Rae *et al*., 2002b) suggested that maternal

undernutrition (50% metabolisable energy requirement) during early pregnancy caused reduced testosterone concentrations in offspring. This difference might be due to different levels and duration of maternal nutrition restriction and only a single sample of testosterone concentration being measured. The absence of an effect on testosterone concentration in the present study is perhaps not surprising given the lack of any effect on scrotal circumference, sperm quality and live weight.

6.5.7. Seasonal effect on reproductive performance

Several reproductive parameters showed significant differences between the breeding and non-breeding seasons. There was however no interaction between season and maternal nutrition treatments. Scrotal circumference and testosterone concentration were greater in the breeding season, whereas sperm concentration was lower and sperm abnormalities were fewer in the breeding season. These results were in agreement with previous studies which showed that scrotal circumference, semen characteristics, efficiency of spermatogenesis and testosterone concentrations of rams are maximal during the breeding season and decrease during the non-breeding season (Lincoln *et al*., 1990; Colas, 1990; Gastel *et al*., 1995; Karagiannidis *et al*., 2000). The present study showed that the proportion of sperm abnormalities was inversely related to scrotal circumference size. This negative correlation between sperm abnormalities and scrotal circumference size is consistent with previous studies which showed rams with larger testis have lesser sperm abnormalities (Jafariahangari *et al*., 2012). Lower sperm concentrations in the breeding relative to the non-breeding season is contrary to much of the literature available (e.g. Hochereau-de Reviers and Lincoln, 1978; Hochereau-de Reviers *et al*., 1985; Karagiannidis *et al*., 2000; Paris *et al*., 2005), but may be related

to sperm depletion due to increased ejaculation activity during the breeding season (Paris *et al*., 2005).

6.6 Conclusions

In conclusion, this study suggests that up to two years of age, nutrition during early and mid-to-late pregnancy has little impact on the growth and reproductive performance of male offspring. Moreover, it did not affect the response of the reproductive system to environmental season. Therefore, under the conditions of the present study farmers do not need to take into consideration dam nutrition during pregnancy on the potential performance of male offspring.

6.7 Acknowledgements

Thanks goes to the Gravida, National Centre for Growth and Development for funding this research. The first author is funded by Ministry of Higher Education (Malaysia) doctoral scholarship. The authors would like to thank to S.L. Smith and L.M. Fermin who assisted with sperm collection, blood collection and scrotal circumference measurement.

6.8 References

Barker, D. J., Gluckman, P. D., Godfrey, K. M., Harding, J. E., Owens, J. A., & Robinson, J. S. (1993). Fetal nutrition and cardiovascular disease in adult life. *Lancet, 341*(8850), 938-941.

Bauer, B., Dittami, J., & Huber, S. (2009). Effects of nutritional quality during early development on body weight and reproductive maturation of guinea pigs (*Cavia aperea f. porcellus*). *General and Comparative Endocrinology 161*(3), 384-389.

Bell, A.W. (1992). Fetal growth and its influence on post-natal growth and development. *In*: The Control of Fat and Lean Deposition. Boormann, K.N.; Buttery, P.J.; & Lindsey, D.B. eds. Butterworth-Heinemann Ltd., Oxford, UK. pp 111-127.

Bielli, A., Katz, H., Pedrana, G., Gastel, M. T., Morana, A., Castrillejo, A., Lundeheim, N., Forsberg, M., & Rodriguez-Martinez, H. (2001). Nutritional management during fetal and post-natal life and the influence on testis stereology and Sertoli cell numbers in Corriedale ram lambs. *Small Ruminant Research, 40*(1), 63-71.

Bielli, A., Perez, R., Pedrana, G., Milton, J. T. B., Lopez, A., Blackberry, M. A., Duncombe, G., Rodriguez-Martinez, H., & Martin, G. B. (2002). Low maternal nutrition during pregnancy reduces the number of Sertoli cells in the newborn lamb. *Reproduction Fertility and Development, 14*(6), 333-337.

Björndahl, L., Söderlund, I., & Kvist, U. (2003). Evaluation of the one step eosin nigrosin staining technique for human sperm vitality assessment. *Human Reproduction, 18*(4), 813-816.

Blom E. (1983). Pathological conditions in genital organs and sperm as a cause for the rejection of breeding bulls for import into and export from Denmark. *Nordisk Veterinaer Medicin*, *45*, 105-130.

Brito, L. F. C., Barth, A. D., Rawlings, N. C., Wilde, R. E., Crews, D. H., Mir, P. S., & Kastelic, J. P. (2007). Effect of nutrition during calfhood and peripubertal period on serum metabolic hormones, gonadotropins and testosterone concentrations, and on sexual development in bulls. *Domestic Animal Endocrinology, 33*(1), 1-18.

Chenoweth, P. J. (2005). Genetic sperm defects. *Theriogenology, 64*(3), 457-468.

Colas, G., Lefebvre, J., & Guerin, J. (1990). Father-male offspring transmission of seasonal variations in testis diameter and percentage of abnormal sperm in the Ile-de-France rams: 1. Male offspring born in February). *Reproduction Nutrition Development*, *30*, 589-603.

Da Silva, P., Aitken, R. P., Rhind, S. M., Racey, P. A., & Wallace, J. M. (2001). Influence of placentally mediated fetal growth restriction on the onset of puberty in male and female lambs. *Reproduction, 122*(3), 375-383.

Da Silva, P., Aitken, R. P., Rhind, S. M., Racey, P. A., & Wallace, J. M. (2003). Effect of maternal overnutrition during pregnancy on pituitary gonadotrophin gene expression and gonadal morphology in female and male foetal sheep at day 103 of gestation. *Placenta, 24*(2-3), 248-257.

Dupont, C., Cordier, A. G., Junien, C., Mandon-Pépin, B., Levy, R., & Chavatte-Palmer, P. (2012). Maternal environment and the reproductive function of the offspring. *Theriogenology, 78*(7), 1405-1414.

Evans G. & Maxwell W. M. C. (1987). Salamon 's Artificial Insemination of Sheep and Goats. Butterworths, Sidney, pp. 85-106.

Ford, S. P., Hess, B. W., Schwope, M. M., Nijland, M. J., Gilbert, J. S., Vonnahme, K. A., Means, W. J., Han, H., & Nathanielsz, P. W. (2007). Maternal undernutrition during early to mid-gestation in the ewe results in altered growth adiposity, and glucose tolerance in male offspring. *Journal of Animal Science, 85*, 1285-1294.

Gastel, T., Bielli, A., Perez, R., Lopez, A., Castrillejo, A., Tagle, R., Franco, J., Laborde, D., Forsberg, M., & Rodriguez-Martinez, H. (1995). Seasonal variations in testicular morphology in Uruguayan Corriedale rams. *Animal Reproduction Science, 40* (1-2), *59-75.*

Gardner, D. S., Tingey, K., Van Bon, B. W. M., Ozanne, S. E., Wilson, V., Dandrea, J., Keisler, D. H., Stephenson, T., & Symonds, M. E. (2005). Programming of glucose-insulin metabolism in adult sheep after maternal undernutrition. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology, 289*(4), R947-R954.

Hochereau-de Reviers, M. T., & Lincoln, G. A. (1978). Seasonal-variation in histology of testis of red deer, cervus-elaphus. *Journal of Reproduction and Fertility, 54*(2), 209-213.

Hochereau-de Reviers, M. T., Perreau, C., & Lincoln, G. A. (1985). Photoperiodic variations of somatic and germ cell populations in the Soay ram testis. *Journal of Reproduction and Fertility, 74*(2), 329-334.

Jafariahangari, Y., Smith, S., Sharma, R. K., Zerehdaran, S., & Blair, H. (2012). The effect of pre-natal maternal environment on live weight, reproductive and semen characteristics in ram lambs. *Small Ruminant Research, 103*(2–3), 200-204.

Jaquiery, A. L., Oliver, M. H., Honeyfield-Ross, M., Harding, J. E., & Bloomfield, F. H. (2012). Periconceptional undernutrition in sheep affects adult phenotype only in males. *Journal of Nutrition and Metabolism,* Article ID 123610.

Karagiannidis, A., Varsakeli, S., Alexopoulos, C., & Amarantidis, I. (2000). Seasonal variation in semen characteristics of Chios and Friesian rams in Greece. *Small Ruminant Research, 37*(1-2), 125-130.

Kenyon, P.R., Blair, H. T. Jenkinson, C. M. C. Morris, S. T. Mackenzie, D. D. S. Peterson, S. W., Firth, E. C., & Johnston, P. L. (2009). The effect of ewe size and nutritional regimen beginning in early pregnancy on ewe and lamb performance to weaning. *New Zealand Journal of Agricultural Research, 52*(2), 203-212.

Kenyon, P. R., Pain, S. J., Hutton, P. G., Jenkinson, C. M. C., Morris, S. T., Peterson, S. W., & Blair, H. T. (2011). Effects of twin-bearing ewe nutritional treatments on ewe and lamb performance to weaning. *Animal Production Science, 51*(5), 406-415.

Kotsampasi, B., Balaskas, C., Papadomichelakis, G., & Chadio, S. E. (2009). Reduced Sertoli cell number and altered pituitary responsiveness in male lambs undernourished *in utero*. *Animal Reproduction Science, 114*(1-3), 135-147.

Liang, H., & Zhang, Z. (2006). Food restriction affects reproduction and survival of F1 and F2 offspring of Rat-like hamster (*Cricetulus triton*). *Physiology and Behavior, 87*(3), 607-613.

Lincoln, G. A., Lincoln, C. E., & McNeilly, A. S. (1990). Seasonal cycles in the blood-plasma concentration of FSH, inhibin and testosterone, and testicular size in rams of wild, feral and domesticated breeds of sheep. *Journal of Reproduction and Fertility, 88*(2), 623-633.

Littell, R. C., Pendergast, J., & Natarajan, R. (2000). Modelling covariance structure in the analysis of repeated measures data. *Statistics in Medicine, 19*(13), 1793-1819.

Long, N. M., Nijland, M. J., Nathanielsz, P. W., & Ford, S. P. (2010). The effect of early to mid-gestational nutrient restriction on female offspring fertility and hypothalamic-pituitary-adrenal axis response to stress. *Journal of Animal Science, 88*(6), 2029-2037.

Martin, G. B., Blache, D., Miller, D. W., & Vercoe, P. E. (2010). Interactions between nutrition and reproduction in the management of the mature male ruminant. *Animal, 4(7),* 1214-1226.

Martín, N. P., Kenyon, P. R., Morel, P. C. H., Pain, S. J., Jenkinson, C. M. C., Hutton, P. G., Morris, S. T., Peterson, S. W., Firth, E. C., & Blair, H. T. (2012). Ewe nutrition in early and mid- to late pregnancy has few effects on fetal development. *Animal Production Science, 52*(7), 533-539.

McMillen, I. C., Adams, M. B., Ross, J. T., Coulter, C. L., Simonetta, G., Owens, J. A., Robinson, J. S., & Edwards, L. J. (2001). Fetal growth restriction: Adaptations and consequences. *Reproduction, 122,* 195-204.

McNatty, K. P., Hudson, N. L., Henderson, K. M., Lun, S., Heath, D. A., Gibb, M., Ball, K., McDiarmid, J. M., & Thurley, D. C. (1984). Changes in gonadotrophin secretion and ovarian antral follicular activity in seasonally breeding sheep throughout the year. *Journal of Reproduction and Fertility, 70*(1), 309-321.

Minitab (2010). Minitab 16 Statistical Software. Minitab, Institute Incorporation, Pennsylvania, USA: (www.minitab.com)

Munõz, C., Carson, A. F., McCoy, M. A., Dawson, L. E. R., Wylie, A. R. G., & Gordon, A. W. (2009). Effects of plane of nutrition of ewes in early and mid-pregnancy on performance of the offspring: Female reproduction and male carcass characteristics. *Journal of Animal Science, 87,* 3647-3655.

Oliver, M. H., Harding, J. E., Breier, B. H., Evans, P. C., & Gluckman, P. D. (1992). The nutritional regulation of circulating placental-lactogen in fetal sheep. *Pediatric Research, 31,* 520-523.

Paganoni, B. L. (2005). Increasing feed-on-offer to Merino ewes during pregnancy and lactation can increase muscle and decrease fat, but does not affect the faecal worm egg count of their progeny. MSc thesis: University of Western Australia.

Paris, D. B. B. P., Taggart, D. A., Shaw, G., Temple-Smith, P. D., & Renfree, M. B. (2005). Changes in semen quality and morphology of the reproductive tract of the male tammar wallaby parallel seasonal breeding activity in the female. *Reproduction, 130*(3), 367-378.

Paten, A. M., Asmad K., Loureiro M. F. P., Kenyon P. R., Pain S. J., Peterson S. W., Pomroy, W. E., Scott, I. & Blair, H. T. (2011). The effects of dam nutrition during

pregnancy on the post-natal growth and puberty attainment of ewe progeny. *Proceedings of the New Zealand Society of Animal Production, 71*, 56-58.

Rae, M. T., Kyle, C. E., Miller, D. W., Hammond, A. J., Brooks, A. N., & Rhind, S. M. (2002a). The effects of undernutrition, *in utero*, on reproductive function in adult male and female sheep. *Animal Reproduction Science, 72,* 63–71.

Rae, M. T., Rhind, S. M., Fowler, P. A., Miller, D. W., & Brooks, A. N. (2002b). Effect of maternal undernutrition on fetal testicular steroidogenesis during the CNS androgen-responsive period in male sheep fetuses. *Reproduction, 124*, 33–39.

Rae, M. T., Rhind, S. M., Kyle, C. E., Miller, D. W., & Brooks, A. N. (2002c). Maternal undernutrition alters triiodothyronine concentrations and pituitary response to GnRH in fetal sheep. *Journal of Endocrinology, 173*, 449–455.

Redmer, D. A., Wallace, J. M., & Reynolds, L. P. (2004). Effect of nutrient intake during pregnancy on fetal and placental growth and vascular development. *Domestic Animal Endocrinology, 27*(3), 199-217.

Rhind, S. M. (2004). Effects of maternal nutrition on fetal and neonatal reproductive development and function. *Animal Reproduction Science, 82*, 169-181.

Rhind, S. M., Rae, M. T., & Brooks, A. N. (2001). Effects of nutrition and environmental factors on the fetal programming of the reproductive axis. *Reproduction, 122*(2), 205-214.

Rooke, J. A., Houdijk, J. G. M., McIlvaney, K., Ashworth, C. J., & Dwyer, C. M. (2010). Differential effects of maternal undernutrition between days 1 and 90 of pregnancy on ewe and lamb performance and lamb parasitism in hill or lowland breeds. *Journal of Animal Science, 88,* 3833-3842.

Sarsaifi, K., Rosnina, Y., Wahid, H., Yimer, N., Ariff, M., Hani, H., Vejayan, J., Win Naing, S. & Abas, M. (2013). Effect of semen collection methods on the quality of pre- and post-thawed bali cattle (*Bos javanicus*) spermatozoa. *Reproduction in Domestic Animals, 48*(6), 1006-1012.

Salhab, S. A., Zarkawi, M., Wardeh, M. F., Al-Masri, M. R., & Kassem, R. (2001). Development of testicular dimensions and size, and their relationship to age, body weight and parental size in growing Awassi ram lambs. *Small Ruminant Research, 40*(2), 187-191.

SAS. (2008). Statistical Analysis System. SAS Institute Incorporation, Cary, North Carolina, USA.

Sharpe, R. M. (1994) Regulation of spermatogenesis. In The Physiology of Reproduction 2nd Edn. pp 1363–1434 Eds E. Knobil and J. D. Neill. Raven Press, New York

Sharpe, R. M., McKinnell, C., Kivlin, C., & Fisher, J. (2003). Proliferation and functional maturation of Sertoli cells, and their relevance to disorders of testis function in adulthood. *Reproduction, 125*(6), 769-784.

Simitzis, P. E., Charismiadou, M. A., Kotsampasi, B., Papadomichelakis, G., Christopoulou, E. P., Papavlasopoulou, E. K., & Deligeorgis, S. G. (2009).

Influence of maternal undernutrition on the behaviour of juvenile lambs. *Applied Animal Behaviour Science, 116*(2), 191-197.

Smith, N. A., McAuliffe, F. M., Quinn, K., Lonergan, P., & Evans, A. C. O. (2010). The negative effects of a short period of maternal undernutrition at conception on the glucose–insulin system of offspring in sheep. *Animal Reproduction Science, 121*(1-2), 94-100.

Toledo, F., Perobelli, J., Pedrosa, F., Anselmo-Franci, J., & Kempinas, W. (2011). *In utero* protein restriction causes growth delay and alters sperm parameters in adult male rats. *Reproductive Biology and Endocrinology, 9*(1), 94.

Zambrano, E., Rodriguez-Gonzalez, G. L., Guzman, C., Garcia-Becerra, R., Boeck, L., Diaz, L., Menjivar, M., Larrea, F., & Nathanielsz, P. W. (2005). A maternal low protein diet during pregnancy and lactation in the rat impairs male reproductive development. *Journal of Physiology-London, 563*(1), 275-284.

Chapter 7

Effects of dam size and nutrition during pregnancy on the ovarian development of their offspring

Chapter published in part as:

Asmad, K., Kenyon, P. R., Pain, S. J., Parkinson, T. J., Blair, H. T. (2012). Effect of dam size and nutrition during pregnancy on fetal ovary development of offspring. *International Congress of Animal Reproduction (ICAR)*, pp. 596 (Abstract no: 3252).

7.1 Abstract

The aim of this study was to examine the effects of maternal size and nutrition during pregnancy on the ovarian development of their offspring. Romney ewes were selected at the time of breeding for heavy (H; mean: 60.8 kg ± 0.18) or light (L; mean: 42.5 kg ± 0.17) live weights, and then allocated to either *ad libitum* (A) or pregnancy maintenance (M) nutrition from days 21 to 140 of pregnancy, resulting in four treatment groups; HA, HM, LA and LM. Ewes were euthanised on days 65 (P65), 100 (P100) and 140 (P140) of pregnancy to collect fetal tissue. At 6 years of age offspring were also euthanised for tissue collection. Ovarian tissues were processed for morphological evaluation (P65, P100, P140 and 6-years of age) and immunohistochemical study of anti Müllerian hormone (AMH) and Growth Differentiation Factor 9 (GDF9) (P140). Fetuses from H dams had a higher number of dividing oogonia at P65 (P=0.05) and total number of antral follicles (P=0.018) and total number of follicles (P=0.05) at six-years of age compared to progeny from L dams. Fetuses of dams fed at maintenance during pregnancy had more (P=0.016) total number of follicles but smaller (P=0.027) antral follicles at P140. However, there was no effect of dam size or nutrition on the size of preantral follicles at P100 and P140. There was an interaction (P=0.018) between dam size and nutrition at P100, whereby LA-fetuses had more pre-antral follicles than HA- and LM-fetuses. At P140 an interaction between dam size and nutrition was found for the expression of AMH and GDF9, whereby the tendency of effect was on dam size; HM-fetuses had more AMH expression than LM-fetuses, whilst HA-fetuses had a higher degree of GDF9 expression than LA-fetuses. These results indicate that light live weight at breeding affects fetal and adult ovary structure. However, nutrition at or above the maintenance requirements for pregnancy is unlikely to affect ovarian development. Combined, these data suggest that dam size at conception may be an important indicator

of fertility potential of female progeny, thus further study is warranted to investigate the effect of dam size on lifetime reproductive performance.

7.2 Introduction

Many of the key physiological processes that determine the structure and function of cells in animals are established during pre-natal life (Eckery *et al*., 1996; Rhind *et al*., 2001; Rhind, 2004). The reproductive system can be affected since most gonadal development occurs during the gestation period (Rhind *et al*., 2001; Rhind, 2004). Therefore pre-natal exposure to adverse environmental factors such as small maternal size and undernutrition might be expected to subsequently affect the reproductive potential of an individual. Under New Zealand's spring lambing pastoral conditions, the gestation period occurs during winter, a time when pasture growth and availability may be inadequate to meet the nutritional demands of the pregnant ewe (Moot *et al*., 2007; Kenyon and Webby, 2007). New Zealand farmers often provide ewes only a maintenance ration during early pregnancy, in order to conserve feed, before increasing nutrition in late pregnancy (Kenyon and Webby, 2007). When conditions occur that result in sub maintenance feeding, the growth of the fetus can be buffered by the ewe by drawing upon her body reserves. Thus, there is a need to investigate whether disparate size of the animals at breeding and feeding levels during pregnancy affects offspring development.

In females the number of oogonia in early fetal age would be a good indicator for potential number of follicles in the ovary and fertility in adult (Sawyer *et al*., 2002). Reproductive functionality of the female depends on ovarian follicular cell development. Ovarian follicular growth is a process involving a complex exchange of

hormonal signals between the hypothalamus, the pituitary gland and the ovary. It also requires a localised exchange of growth factors and hormones within ovarian cells, including the oocyte and its adjacent somatic cells, which are essential for follicle development and ovulation (Montgomery *et al*., 2001; Juengel *et al*., 2002). The growth factors, GDF9 and AMH, are from the transforming growth factor β (TGFβ) family that play an important role for normal follicular development in the ovary (Dong *et al*., 1996; Visser *et al*., 2006). Absence of the GDF9 gene is associated with infertility due to follicular growth arrest at the primary stage (Dong *et al*., 1996), whilst AMH an established marker for ovarian reserve plays an important role in both ovarian primordial follicle recruitment and selection of the dominant follicle (Weenen *et al*., 2004; Anderson, 2012).

A growing body of literature indicates that undernutrition during pregnancy can alter germ cell meiosis (Borwick *et al*., 1997; Rae *et al*., 2001), follicular development (Rae *et al*., 2001; Borwick *et al*., 2003; Da Silva *et al*., 2003; Grazul-Bilska *et al.*, 2009; Léonhardt *et al*., 2003; Bernal *et al.*, 2010) and ovarian weight (Rae *et al*., 2001; Grazul-Bilska *et al*., 2009). Additionally, Mossa *et al*. (2013) showed that maternal restriction caused a reduction in antral follicle count and concentration of anti Mülerian hormone (AMH). Moreover, Bernal *et al*., (2010) reported low ovarian mRNA expression for Growth Differentiation Factor 9 (GDF9) in the offspring of undernourished dams, which could affect early ovarian growth (Vitt *et al*., 2000). Combined these results suggest reduced reproductive productivity of offspring due to poor maternal feeding.

It is therefore postulated that a small dam size and plane of nutrition that only meets maintenance requirements during pregnancy could have a negative effect on ovarian follicular development and concentration of AMH and GDF9 in the ovaries of their

offspring, compared to dams which either received *ad libitum* feeding or were of large size. The objective of the present study was to investigate the effects of dam size and nutrition during pregnancy on (i) the development of ovarian follicles during fetal and adult life and (ii) the expression of AMH and GDF9 in the fetal ovaries off their offspring.

7.3 Materials and methods

The study was conducted at the Massey University Keeble Sheep and Beef farm (latitude 41° 10'S longitude 175° 35'E), 5 km south of Palmerston North, New Zealand. All experimental animal procedures were approved by the Massey University Animal Ethics Committee (MUAEC 09/18), Palmerston North, New Zealand.

7.3.1 Dams

Romney ewes of 3 to 5 years of age were selected at the time of breeding based on live weight: heavy (H; mean: 60.8 kg ± 0.18, n=450; CS; (scale 1-5 (Jefferies, 1961): 3.02 ± 0.03) or light (L; mean: 42.5 kg ± 0.17, n=450; CS 1.97 ± 0.03) from a commercial flock of 2900 ewes, and then underwent estrus-synchronisation and artificial insemination as previously described by Kenyon *et al*. (2009). Ewes were allocated to either *ad libitum* (A) or pregnancy maintenance (M) feeding regimens under New Zealand pastoral grazing conditions between Days 21 and 140 (P21 – P140; represent the whole period of gestation) of pregnancy which are commonly employed by New Zealand sheep farmers. The average pre- and post-grazing pasture covers during the period between P21 and P140 of pregnancy were 1330 ±140.0 and 804 ±133.4 kg

DM/ha for the M regimen, and 2304 ± 156.8 and 1723 ± 149.7 kg DM/ha for the A regimen (Kenyon *et al*., 2009). The aim of the M-nutritional regimen was to ensure that the total ewe live weight during pregnancy increased at a rate similar to that of the predicted conceptus weight, whilst the A-nutritional regimen was designed to offer unrestricted herbage. Live weight change in the period P0 – P140 differed ($P<0.05$) between *ad libitum* (29.5 ± 0.9 kg) and pregnancy maintenance nutrition (12.8 ± 0.9 kg) (Blair, *et al*., 2011). From P140 through to weaning, all dams and their lambs were provided with *ad libitum* pasture (Kenyon *et al.*, 2009). After weaning, all female offspring were managed and fed as one group under commercial conditions for the remainder of their lifetime. Therefore, this study utilised a two-by-two factorial design, including two dam-size treatments (H *vs* L) and two dam-nutrition treatments (M *vs* A); resulting in four offspring treatment groups (HA, HM, LA and LM).

7.3.2 Ovarian tissue collection

Twin-bearing dams were euthanised for collection of ovarian tissue at P65 (HA: n = 4, HM: n= 3, LA: n = 5, LM: n = 2); and at P100 (HA: n = 7, HM: n = 5, LA: n = 9, LM: n = 2).

Single- and twin-bearing dams were euthanised at P140 and fetal ovarian tissue was collected (HA: n = 8, HM: n = 9, LA: n = 12, LM: n = 9). There were low numbers in some of the stage/treatment/gender combinations due to unknown male:female fetal status prior to euthanasia. Fetal testis tissue was also collected for use as a control in later immunohistochemical studies.

Six years later surviving twin female offspring (daughters) were euthanised (HA: n = 11, HM: n= 10, LA: n = 10, LM: n = 10) and their ovaries were collected.

Ovaries from each fetus or adult ewe were dissected, weighed and place in Boiun's fixative for 20 hours. After this time, excess fixative was washed out in two changes of 70% ethanol and the testis and ovary were stored in 70% ethanol before processing into paraffin wax (Leica Histoembedder, Leica Instrumens GmbH, Nussloch, Germany).

The sampling choice was to represent the three main periods of pregnancy (i.e. early (Day 65), mid (100) and late pregnancy (140)). These three sampling times were designed to examine the maternal nutrition affects at different stages of pregnancy and whether any effects (if observed) persisted until later stages of pregnancy. The measurement of ovarian follicles during adult age was done to investigate if the effects observed during fetal life persisted into adult life.

7.3.3 Morphological study of fetal and adult ovaries

Fetal ovaries from Day 65 of gestation

From each fetal ovary, five sections were cut (5 µm thick) midway through the ovary. These sections were stained with H&E. The numbers of dividing and non dividing oogonia were counted under 1000x magnification (four 100 x 100 µm areas/fields per section resulting in 20 fields per ovary) (Figure 7.1.).

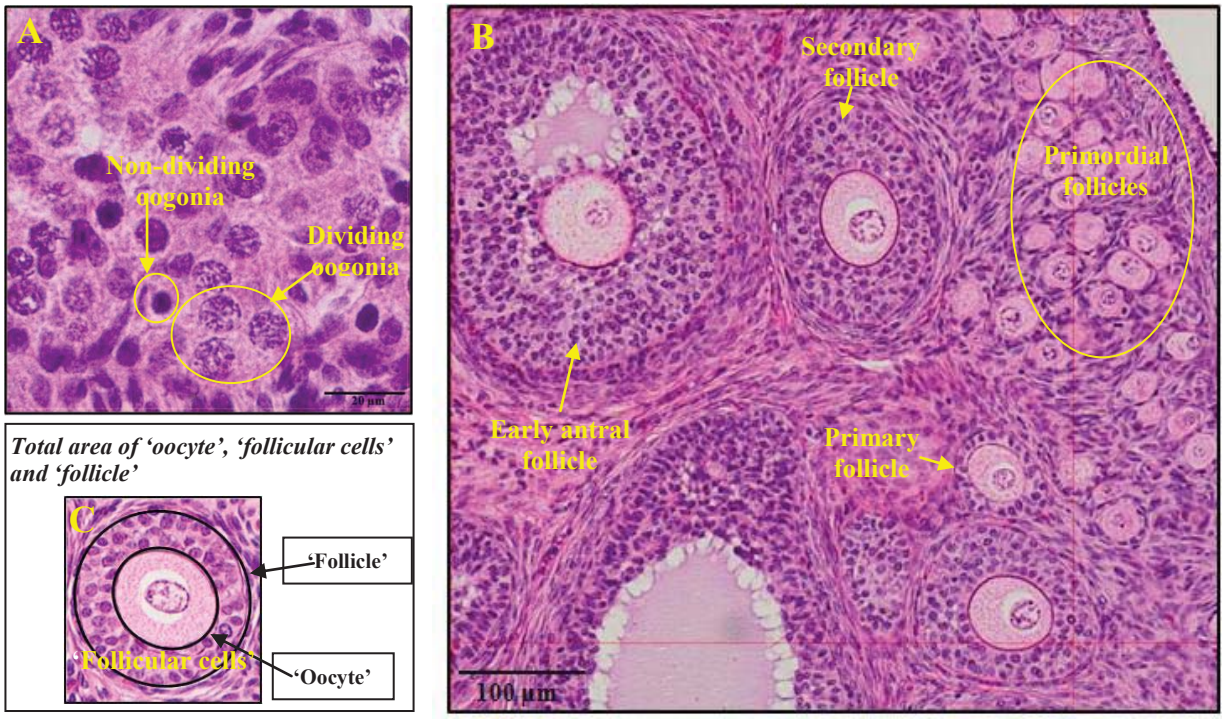
Fetal ovaries from Day 100 and 140 of gestation,

From each fetal ovary, five sections were cut (5 µm thick) midway through the ovary and were stained with H&E (Figure 7.1.). The number of pre-antral and antral follicles were counted in each section (four 500 x 500 µm areas/fields resulting in 20 fields per ovary (under 200x magnification)), each area encompassing the cortex), including only those follicles cut through the plane of the oocyte nucleolus (Rae *et al*., 2001). Thus, if

the follicle was not sectioned through the nucleolus, it was not counted and so the risk of counting a given follicle more than once was eliminated.

Ovaries from 6 year-old ewes

From the left ovary, five sections were cut (5 µm thick, 50 µm apart from each section) midway through the ovary and stained with H&E (Figure 7.1.) (under 200x magnification). The number of pre-antral and antral follicles was counted from whole ovaries.



A = Ovary at Day 65 of gestation (under 1000x magnification)
B = Ovary at Day 140 of gestation (under 200x magnification)
C = Total area of 'oocyte', 'follicular cells' and 'follicle' of each follicle measured

Figure 7.1. The measured histological variables of (A) total number of dividing and non-dividing oogonia at Day 65 of gestation, and (B) total number of pre-antral (primordial, primary and secondary) and antral follicles (tertiary follicles), and (C) total area of 'oocytes', 'follicular cells' and 'follicle' of the counted follicles at Day 100 and 140 of gestation.

Classification of follicles

Follicles were classified by the criteria of Smith *et al*. (1994) as preantral (primordial follicles germ cells surrounded by flattened follicular cells), primary (enlarged oocyte completely surrounded by one or two layers of cubodial follicular cells), secondary (enlarged oocyte surrounded by two or more concentric layers of cubodial cells) or antral (an oocyte surrounded by multiple layers of cuboidal granulose cells and containing antral spaces, cumulus oophorus and theca layer). The cross-sectional area of each follicle (follicle, follicular cells and oocyte area) was measured using ImageJ software (Rasband, 1997).

7.3.4 Immunohistochemical localisation of AMH and GDF9 in fetal ovaries at Day 140 of gestation

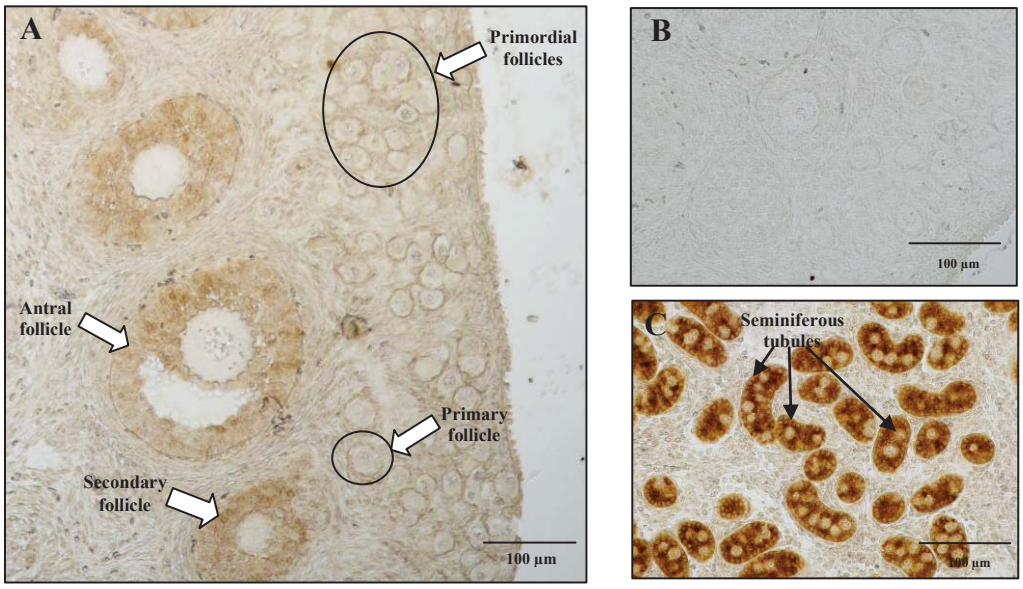
Sections were cut (5 μm thick) of fetal ovarian tissues (singles and twins) (n = 24; HA = 6, HM = 6, LA = 6 and LM = 6) at P140. All ovarian tissue sections were deparaffinised, dehydrated and incubated in a waterbath with citrate buffer (pH 6.2 = well-suited pH for paraffin-embedded tissue sections mounted on glass slide) for 30 minutes at 95°C for antigen retrieval. The slides were then cooled for 30 minutes at room temperature. For AMH, testicular tissues from Day 140 of gestation were included with each run as a positive control. These sections were treated in the same way as ovarian tissue, except that antigen retrieval was done for 10 minutes at 95°C. The endogenous peroxidase activity was blocked by incubating the slides with 3% H_2O_2 in methanol for 12 minutes at room temperature (RT), and nonspecific binding was blocked by incubating them with 10% bovine serum albumin (BSA) for 15 minutes at RT.

Sections were thereafter incubated overnight at 4°C with a dilution of 1:100 of following primary antibodies: AMH (polyclonal; sc-6886, Santa Cruz Biotechnologies) and GDF9 (polyclonal; sc-12244, Santa Cruz Biotechnologies). Instead of primary antibodies, 1.5% BSA was used in negative controls. All sections (i.e. those destined for either AMH or GDF9 detection) were incubated with a 1:200 dilution of peroxidase-labelled donkey anti-goat IgG (sc-2020; Santa Cruz Biotechnologies) for 1 hour. Detection of AMH and GDF9 staining was performed using chromogen 3.3-diaminobenzidine (DAB; Dako North America, USA) according to the manufacturer's recommendation. Blocking of the endogenous peroxidase activity was confirmed by incubating some sections with DAB alone (negative control).

For AMH, photographs of the ovaries were taken at 200 x magnification. These sections were counterstained with hematoxylin, dehydrated, mounted and further photographs were taken. For GDF9 analysis, photographs of the ovaries were taken after counterstaining with haematoxylin at 200x magnification (Figure 7.2. and 7.3.). For evaluation of AMH and GDF9 staining, images of individual follicles were extracted from the 200x photographs. Because the GDF9 staining was confined to the oocyte, only the oocyte area was cropped from the image for analysis (Figure 7.3.). Likewise, because AMH staining was confined to granulosa cells, the images were cropped to the edge of the follicle (or, if theca cells were present, to the edge of the granulosa) and the oocyte was excluded from the image. The intensity of staining was subjectively scored on a scale of 1 (lowest) to 10 (highest).

Thereafter, these subjective scores were quantified (integrated intensity) using computer-based image analysis (MetaMorph^{TM} (Molecular Devices) Version 6.2.6). Because AMH staining was distributed evenly throughout the granulosa cells, a single score was generated for a representative area of follicular area (i.e. the follicle was

scored as a whole). However, because GDF9 staining was confined to the ooplasm, the score was generated from a representative area of the ooplasm of each individual oocyte (oocyte images were taken at 400x magnification for analyses). Because the distribution of staining within the ooplasm was not homogenous, the software counted each point of staining and combined those counts to provide an overall score for the selected area.



A = Ovary at Day 140 of gestation (under 100x magnification)
B = Ovary at Day 140 of gestation – negative control (under 200x magnification)
C = Testis at Day 140 of gestation – positive control (under 200x magnification)

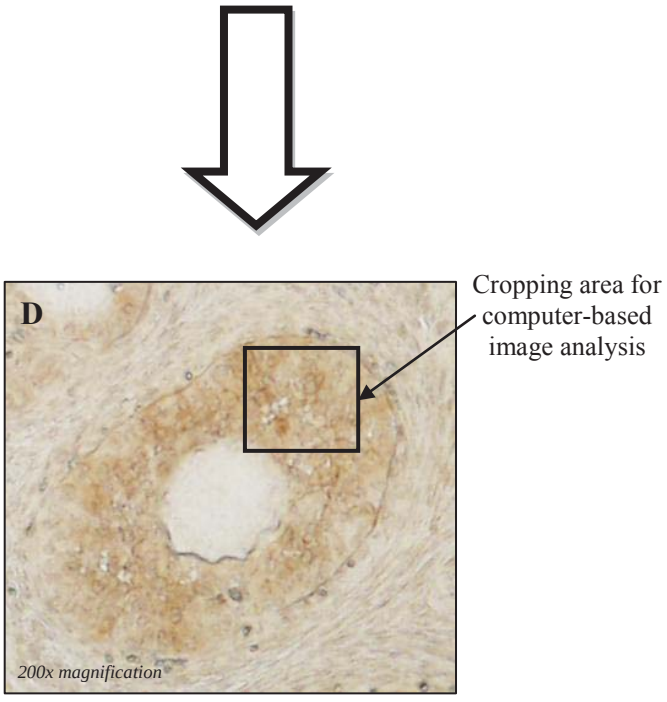
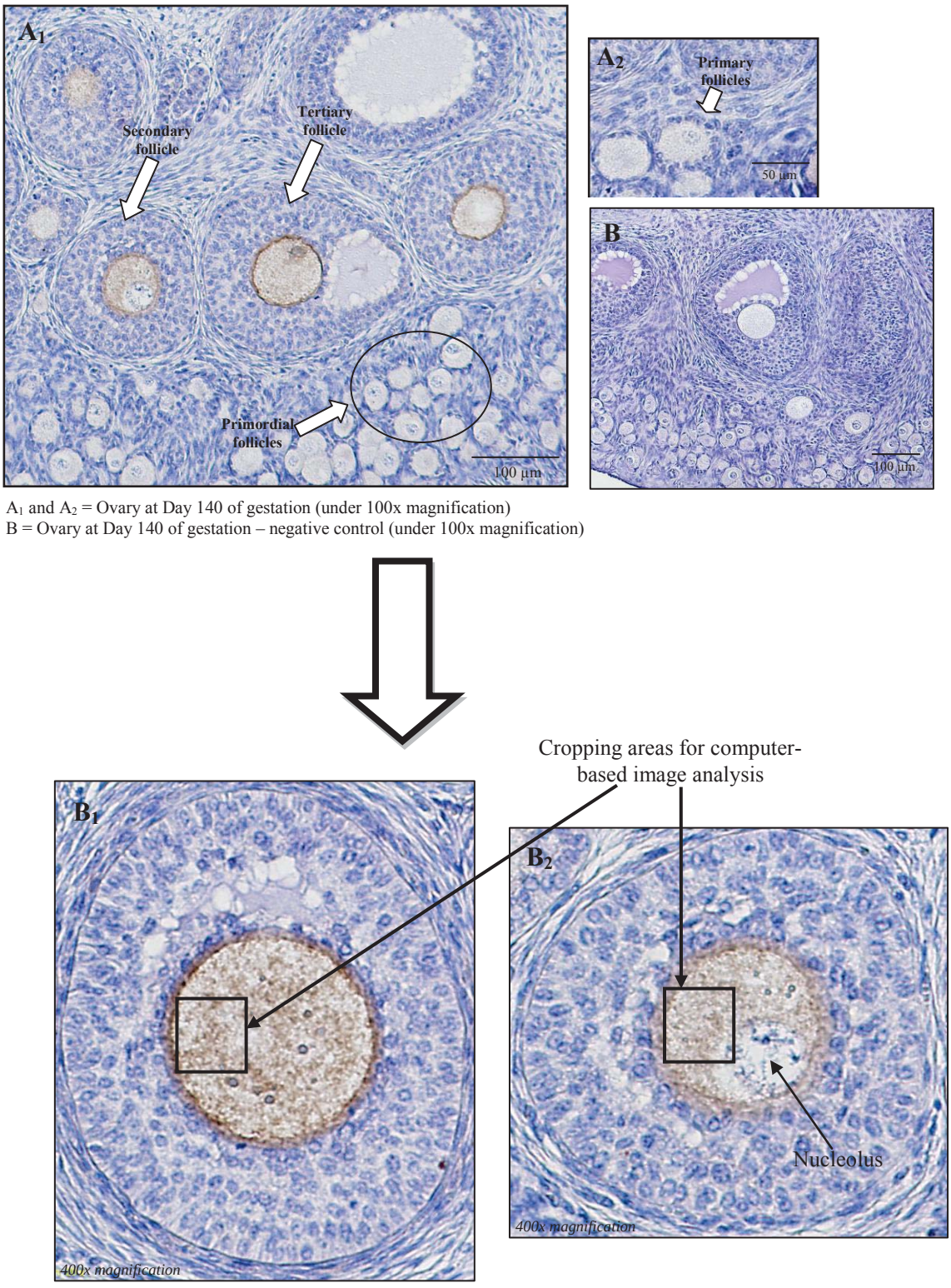


Figure 7.2. Immunohistochemical localisation of anti-Müllerian hormone (AMH) in fetal ovaries (testis used as positive control) at Day 140 of gestation showing localisation of AMH in different type of follicles (A), negative control ovary (B), positive control (C) and cropping area for computer-based image analysis (D).



A_1 and A_2 = Ovary at Day 140 of gestation (under 100x magnification)
B = Ovary at Day 140 of gestation – negative control (under 100x magnification)

Figure 7.3. Immunohistochemical localisation of growth differentiation factor 9 (GDF9) in fetal ovaries at Day 140 of gestation showing localisation of GDF9 in different type of follicles (A_1 and A_2), negative control ovary (B), and cropping areas for computer-based image analysis (B_1 and B_2).

7.3.5. Statistical analysis

Statistical analyses were carried out using the Statistical Analysis System (SAS, 2008; SAS 9.2, SAS Institute, North Carolina, USA). Fetal weight was included as a covariate for all analyses. Dam size and nutrition and the interaction between dam size and nutrition remained in all models to allow for testing of the study design.

Morphological study

Mean values for number of dividing and non-dividing oogonia (P65), number of antral and pre-antral follicles (P100, P140 and ~6 years of age), and total area of follicles, oocytes and follicular cells (P100 and P140) in fetal and adult ovaries were analysed using a linear mixed model including fixed effects of co-twin (Female-Female *vs* Female-Male; P100, P140 and ~6 years of age), fetal rank (singleton *vs* twin; P140), dam size (heavy *vs* light), dam nutrition (*ad libitum vs* maintenance). All two-way and three-way interactions were included in the initial model. This analysis was undertaken using the MIXED procedure.

Immunohistochemistry study

Mean values for the degree of expression of AMH and GDF9 in follicular cells and oocytes, respectively from each animal were analysed using linear mixed models including fixed effects of fetal rank (singleton *vs* twin), dam size treatment (heavy *vs* light), dam nutrition (*ad libitum vs* maintenance). All two-way and three-way interactions were included in the initial model. This analysis was undertaken using the MIXED procedure.

Further, combining intensity data of AMH and GDF9 in all animals (without separating them into different dam size and nutrition treatment), the degree of staining intensity of AMH and GDF9 in different types of follicle were measured. The degree of staining intensity of AMH and GDF9 in different type of follicles (primary, secondary and antral follicles) were analysed using linear mixed models including fixed effect of fetal rank (singleton *vs* twin) and follicle type (primary *vs* secondary *vs* antral). All two-way and three-way interactions were included in the initial model. Follicle type remained in all models to allow for testing of the study design. This analysis was undertaken using the MIXED procedure.

Type III mean square values were used to determine the significance of fixed effects. Data are presented as least square means ± standard error (SE).

7.4. Results

The sampling choice was to represent the three main periods of pregnancy (i.e. early (Day 65), mid (100) and late pregnancy (140)). The measurement of ovarian follicles during adult age was undertaken to investigate if any effect observed in the fetus persists into adult life.

7.4.1. Total number and size of follicles

At P65 of gestation, fetuses from H-dams displayed more (P=0.05) dividing oogonia and fewer non-dividing oogonia (P=0.05) than fetuses from L-dams (Table 7.1.). There was an interaction between dam size and nutrition at P100 for the number of pre-antral follicles whereby fetuses from LA-dams had more (P<0.05) pre-antral follicles than fetuses from HA- and LM-dams. At P140, fetuses from M-dams had a greater

(P=0.016) number of pre-antral follicles and an increased total number of follicles compared to fetuses from A-dams. There was no interaction (P>0.05) between dam size and nutrition on the number of dividing and non dividing oogonia at P65, or the number of follicles at P140.

There was no effect (P>0.05) of either dam size and nutrition, nor an interaction between dam size and nutrition for total area of follicle, number of oocytes or follicular cells at P100 (Table 7.2.). However, at P140, fetuses from A-dams had larger (P=0.027) antral follicles than fetuses from M-dams (Table 7.3.). There was no effect (P>0.05) of dam size, nor was there an interaction between dam size and nutrition, on total area of follicles, number of oocytes, or number of follicular cells of antral and pre-antral follicles at P140.

In adult ovaries, daughters from H-ewes had greater total number of antral follicles (P=0.018) and greater total number of all follicles (P=0.05) than daughters from L-ewes (Table 7.4.). There was no effect (P>0.05) of neither dam nutrition nor an interaction between dam size and nutrition on the number of follicles. There was no effect (P>0.05) of fetal rank on ovarian follicle number and size.

Table 7.1. Least squares means ± standard error of the mean per field (P65 = 10000 μm^2; P100 and P140 = 250,000 μm^2) for number of dividing and non-dividing oogonia (P65), pre-antral follicles (P100), and pre-antral, antral follicles and total number of follicles (P140) for each dam size, dam nutrition and dam size by nutrition group.

Treatment	P65 (oogonia)			P100 (follicles)		P140 (follicles)			
	n	Dividing	Non-dividing	*n*	Pre-antral	*n*	Pre-antral	Antral	Total follicles
Size									
Heavy (H)	*7*	6.0 ± 0.8[b]	16.5 ± 1.0[a]	*12*	16.0 ± 1.7	*17*	10.5 ± 1.1	0.17 ± 0.0	10.7 ± 1.1
Light (L)	*7*	3.1 ± 1.10[a]	20.2 ± 1.3[b]	*10*	14.7 ± 2.1	*20*	10.9 ± 1.0	0.08 ± 0.0	11.0 ± 1.0
Nutrition									
Ad libitum (A)	*9*	4.8 ± 0.7	16.9 ± 0.9	*15*	17.0 ± 1.5	*19*	8.5 ± 1.1[a]	0.17 ± 0.0	8.7 ± 1.1[a]
Maintenance (M)	*5*	4.3 ± 1.2	19.8 ± 1.4	*7*	13.7 ± 2.3	*18*	12.9 ± 1.1[b]	0.08 ± 0.0	13.0 ± 1.1[b]
Size x Nutrition									
HA	*4*	5.3 ± 1.1	16.5 ± 1.4	*7*	13.9 ± 2.2[a]	*8*	8.1 ± 1.7	0.21 ± 0.1	8.3 ± 1.7
HM	*3*	6.6 ± 1.2	16.5 ± 1.5	*5*	18.2 ± 2.5[ab]	*9*	13.0 ± 1.5	0.12 ± 0.1	13.1 ± 1.5
LA	*5*	4.3 ± 0.9	17.4 ± 1.1	*8*	20.2 ± 2.0[b]	*11*	9.0 ± 1.4	0.13 ± 0.1	9.1 ± 1.3
LM	*2*	2.0 ± 2.0	23.1 ± 2.3	*2*	9.3 ± 4.0[a]	*9*	12.8 ± 1.6	0.03 ± 0.1	12.8 ± 1.6

[ab]Different letters within columns represent the groups that are significantly different (P<0.05)

Table 7.2. Least squares means ± standard error of the mean for total area ($x10^3 \mu m$) of follicle, oocytes and follicular cells at Day 100 of gestation for each dam size, dam nutrition and dam size by nutrition group.

Treatment		P100		
	n	Follicle	Oocyte	Follicular cells
Size				
Heavy (H)	*7*	3.6 ± 0.4	1.9 ± 0.2	1.7 ± 0.2
Light (L)	*7*	3.4 ± 0.5	1.8 ± 0.2	1.6 ± 0.3
Nutrition				
Ad libitum (A)	*9*	3.2 ± 0.4	1.7 ± 0.2	1.5 ± 0.2
Maintenance (M)	*5*	3.9 ± 0.6	2.0 ± 0.3	1.9 ± 0.3
Size x Nutrition				
HA	*4*	3.8 ± 0.5	2.0 ± 0.2	1.8 ± 0.3
HM	*3*	3.5 ± 0.6	1.8 ± 0.3	1.7 ± 0.4
LA	*5*	2.6 ± 0.5	1.5 ± 0.2	1.1 ± 0.3
LM	*2*	4.3 ± 1.0	2.2 ± 0.4	2.1 ± 0.6

Table 7.3. Least squares means ± standard error of the mean for total area ($x10^3 \mu m$) of follicle, oocytes and follicular cells for pre-antral and antral follicles at Day 140 of gestation for each dam size, dam nutrition and dam size by nutrition group.

Treatment		Pre-antral			Antral		
	n	Follicle	Oocyte	Follicular cells	Follicle	Oocyte	Follicular cells
Size							
Heavy (H)	*17*	12.4 ± 1.2	3.6 ± 0.3	8.7 ± 1.0	85 ± 10.6	9.4 ± 1.2	52 ± 5.8
Light (L)	*20*	12.3 ± 1.2	3.7 ± 0.2	8.6 ± 1.0	57 ± 11.9	7.3 ± 1.3	38 ± 6.5
Nutrition							
Ad libitum (A)	*19*	13.6 ± 1.2	3.9 ± 0.2	9.7 ± 1.0	92 ± 10.5[b]	9.8 ± 1.2	54 ± 5.7
Maintenance (M)	*18*	11.1 ± 1.2	3.4 ± 0.2	7.7 ± 1.3	50 ± 12.9[a]	7.0 ± 1.4	36 ± 7.1
Size x Nutrition							
HA	*8*	14.7 ± 1.9	4.1 ± 0.4	10.6 ± 1.6	110 ± 14.3	10.6 ± 1.6	62 ± 7.8
HM	*9*	10.0 ± 1.5	3.1 ± 0.3	6.9 ± 1.3	61 ± 16.4	8.3 ± 1.8	41 ± 9.0
LA	*11*	12.6 ± 1.5	3.7 ± 0.3	8.8 ± 1.2	74 ± 15.0	8.9 ± 1.7	45 ± 8.2
LM	*9*	12.1 ± 1.9	3.7 ± 0.4	8.4 ± 1.6	39 ± 19.1	5.7 ± 2.1	32 ± 10.4

[ab]Different letters within columns represent the groups that are significantly different ($P<0.05$)

Table 7.4. Least squares means ± standard error of the mean ($x10^3$) per field (250,000 μm^2) for number of pre-antral, antral and total number of follicles at 6 years of age for each dam size, dam nutrition and dam size by nutrition group.

Treatment	*n*	Pre-antral	Antral	Total number of follicle
Size				
Heavy (H)	*12*	31.3 ± 4.2	7.1 ± 0.9[b]	38.2 ± 4.3[b]
Light (L)	*14*	22.2 ± 4.1	3.9 ± 0.8[a]	26.2 ± 4.1[a]
Nutrition				
Ad libitum (A)	*12*	24.7 ± 4.2	6.5 ± 0.9	31.1 ± 4.3
Maintenance (M)	*14*	28.9 ± 4.0	4.5 ± 0.8	33.3 ± 4.1
Size x Nutrition				
HA	*5*	30.5 ± 6.6	9.2 ± 1.4	39.7 ± 4.3
HM	*7*	31.9 ± 5.5	4.8 ± 1.1	36.7 ± 5.7
LA	*7*	18.6 ± 5.7	3.8 ± 1.2	22.4 ± 5.9
LM	*7*	25.7 ± 5.5	4.2 ± 1.1	29.9 ± 5.6

[ab]Different letters within columns represent the groups that are significantly different (P<0.05)

7.4.2. Immunohistochemistry study of AMH and GDF9

Visual observation

At P140 there was an interaction (P=0.049) between dam size and nutrition on the staining intensity of GDF9 in secondary follicles, whereby fetuses from HA-dams showed increased GDF9 staining compared to fetuses from LA-dams (Table 7.5.). However, there was no effect (P>0.05) of either dam size or nutrition, nor any interaction between dam size and nutrition, on the intensity of GDF9 staining in primary and antral follicles. There was no effect (P>0.05) of either dam size and nutrition or interaction between dam size and nutrition on the intensity of AMH staining at P140 in either secondary or antral follicles of fetuses' ovaries.

The antral follicles (5.0 ± 0.2) showed higher intensity of GDF9 staining (P<0.05) than secondary (3.2 ± 0.2) and primary follicles (1.1 ± 0.2), however, there was no difference in staining intensity of AMH between secondary (4.3 ± 0.3) and antral follicles (4.7 ± 0.4). There was no effect (P>0.05) of fetal rank on ovarian AMH and GDF9 staining intensity.

Table 7.5. Least squares means ± standard error of the mean for visual observation (score 1 to 10) of anti Mülerian hormone (AMH) and growth differentiation factor 9 (GDF9) staining intensity in primary, secondary and antral follicles at Day 140 of gestation for each dam size, dam nutrition and dam size by nutrition group.

Treatment		AMH		GDF9		
		Secondary follicle	Antral follicle	Primary follicle	Secondary follicle	Antral follicle
Size	*n*					
Heavy (H)	*12*	4.2 ± 0.4	4.8 ± 0.4	1.1 ± 0.1	3.4 ± 0.3	5.0 ± 0.4
Light (L)	*12*	4.3 ± 0.4	4.8 ± 0.5	1.2 ± 0.1	2.9 ± 0.3	4.5 ± 0.5
Nutrition						
Ad libitum (A)	*12*	4.4 ± 0.4	4.3 ± 0.4	1.2 ± 0.1	3.3 ± 0.3	4.9 ± 0.5
Maintenance (M)	*12*	4.1 ± 0.4	5.3 ± 0.5	1.2 ± 0.1	3.1 ± 0.3	4.6 ± 0.4
Size x Nutrition						
HA	*6*	4.8 ± 0.6	4.7 ± 0.5	1.2 ± 0.1	4.0 ± 0.4^{b}	5.4 ± 0.6
HM	*6*	3.5 ± 0.6	4.9 ± 0.7	1.0 ± 0.1	2.9 ± 0.4^{ab}	4.6 ± 0.6
LA	*6*	4.1 ± 0.6	4.0 ± 0.5	1.2 ± 0.1	2.6 ± 0.4^{a}	4.4 ± 0.7
LM	*6*	4.6 ± 0.6	5.6 ± 0.8	1.1 ± 0.1	3.2 ± 0.4^{ab}	4.6 ± 0.7

^{ab}Different letters within columns represent the groups that are significantly different (P<0.05)

Image analysis

At Day 140 of gestation there was an interaction (P=0.024) between dam size and nutrition on the staining intensity of AMH in secondary follicle, whereby fetuses from HM-dams had greater staining intensity of AMH compared to fetuses from LM-dams

(Table 7.6.) However, there was no effect (P>0.05) of either dam size or nutrition, nor any the interaction between dam size and nutrition, on AMH staining intensity in antral follicles. There was no effect (P>0.05) of either dam size and nutrition, nor any interaction between dam size and nutrition, on GDF9 staining intensity in primary, secondary and tertiary follicles.

Secondary follicles (4.6 ± 0.1) showed greater (P<0.05) AMH staining intensity than antral follicles (4.2 ± 0.1), and antral (127.8 ± 7.3) and secondary follicles (116 ± 6.0) showed greater (P<0.05) GDF9 staining intensity than primary follicle (68 ± 5.7). There was no effect (P>0.05) of fetal rank on ovarian AMH and GDF9 staining intensity.

Table 7.6. Integrated staining intensity (least squares means ± standard error of the mean) per μm^2 of anti Mülerian hormone (AMH) and growth differentiation factor 9 (GDF9) in primary, secondary and antral follicles at Day 140 of gestation for each dam size, dam nutrition and dam size by nutrition group.

Treatment		AMH (whole image analysis, $x10^6$)		GDF9 (count nuclei analysis $x10^3$)		
		Secondary follicle	Antral follicle	Primary follicle	Secondary follicle	Antral follicle
Size	*n*					
Heavy (H)	*12*	4.7 ± 0.1	4.4 ± 0.1	65 ± 7.6	117 ± 9.3	136 ± 14.5
Light (L)	*12*	4.5 ± 0.1	4.1 ± 0.1	72 ± 6.4	117 ± 9.3	121 ± 16.2
Nutrition						
Ad libitum (A)	*12*	4.5 ± 0.1	4.2 ± 0.1	64 ± 6.5	107 ± 8.8	125 ± 14.7
Maintenance (M)	*12*	4.6 ± 0.1	4.2 ± 0.1	74 ± 6.5	126 ± 9.9	133 ± 15.2
Size x Nutrition						
HA	*6*	4.5 ± 0.1[ab]	4.3 ± 0.1	62 ± 9.1	107 ± 12.5	122 ± 17.9
HM	*6*	4.8 ± 0.1[b]	4.5 ± 0.2	68 ± 9.0	127 ± 13.9	150 ± 21.6
LA	*6*	4.6 ± 0.1[ab]	4.2 ± 0.2	65 ± 9.1	108 ± 12.5	127 ± 23.0
LM	*6*	4.4 ± 0.1[a]	4.0 ± 0.2	79 ± 9.1	126 ± 13.8	115 ± 23.1

[abc]Different letters within columns represent the groups that are significantly different (P<0.05)

7.5. Discussion

A number of studies have documented the effects of maternal nutrition on reproductive function (Rhind *et al*., 2001; Léonhardt *et al*., 2003; da Silva Faria *et al*., 2010; Dupont *et al*., 2012; Mossa *et al*., 2013), however, most of these studies have only focused on a limited period of time and have not continuously monitered the animal and assessed lifetime reproductive performance. The meta-analysis study (Chapter 4) showed that maternal undernutrition can reduce the concentration of luteinising hormone but does not alter follicular cell count in female sheep offspring. In Chapter 5, it was observed that at P140, male fetuses (i.e. the male fetal cohorts to the female fetuses used in the present chapter) from H-dams had a higher number of gonocytes than did fetuses from L-dams. Therefore, gaining a better understanding of the interaction between follicular cell development and the hormonal and growth factor regulation in the ovaries would be beneficial to determining mechanisms for the observed effects and could help in the development of interventions to improve reproductive efficiency.

Histological morphology results showed that fetuses from H-dams had more dividing oogonia at P65, and that adult daughters from H-dams had more follicles, than offspring from L-dams. However, the differences of maternal size that appeared at P65 and adult age on ovarian follicles were not apparent at P100 and P140. Furthermore, H-fetuses tended to have bigger oocytes, follicular cells and follicles than L-fetuses (although these differences were not significantly different). These results suggest that the size of the dam can potentially play an important role in the ovarian growth and development of the offspring. In addition, the number of oogonia in fetal life shows a potential positive correlation with ovarian follicle number in adult life. Further study is required to examine the extent to which dam size affects lifetime reproductive performance

measures since follicular cell development may be correlated with ovulation rate and consequently the number of offspring produced.

Nutrition of pregnant ewes at a maintenance level resulted in fetuses with more follicles but smaller antral follicles than fetuses from *ad libitum* fed ewes, but there was no difference in ovarian follicular cells as an adult. Similarly, Da Silva *et al*. (2002; 2003) found that fetuses from dams on a moderate plane of nutrition had more follicles in their ovaries at P131 than did fetuses from dams on a high plane of nutrition. Moreover, Borwick *et al*. (1997) showed that maternal nutrition restriction to 50% maintenance during pregnancy resulted in a higher number of germ cells in the ovaries of their fetuses at P47 and P62 compared to fetuses from ewes fed 150% maintenance nutrition. In contrast, Rae *et al*. (2001) reported that a 50% maintenance maternal nutrition restriction during pregnancy resulted in fewer germ cells in the ovaries of their fetuses at P65, while Murdoch *et al*. (2003) reported no difference in germ cells concentration in undernourished offspring. Given the lack of difference found in the present study in adult tissue it might be expected that no difference would be observed in lifetime performance. These results do, however, indicate that differences that appear during fetal life do not necessarily alter ovarian cell development during adult life.

Immunohistochemical staining was used as a semi-quantitative measure of expression. Immunohistochemical analysis showed that in secondary follicles, fetuses from HM-dams had greater intensity of AMH than fetuses from LM-dams, whilst fetuses from HA-dams had greater staining intensity of GDF9 than fetuses from LA-dams. This concurs under condition in which the histological morphology examination showed that fetuses from H-dams had higher numbers of dividing oogonia and greater total numbers of follicles. Thus, the present results show a potential positive correlation between follicle numbers and AMH and GDF9 staining intensity on dam size. Other studies

have shown that the concentration of AMH is a reflection of the number of growing follicles in the ovary (Anderson, 2012). Additionally, AMH serum concentration is a strong indicator of ovarian follicle growth (Gruijters *et al*., 2003). Together, these observations support the greater staining intensity of AMH in H-daughters in the present experiment. Furthermore, GDF9 is known to have an important role in the regulation of folliculogenesis in the ovary especially for the transition of primordial to primary follicles (Vitt *et al*., 2000). Therefore, the higher number of dividing oogonia and follicles observed in the offspring from H-dams is potentially related to GDF9 concentration in the ovaries. On the other hand, a study by Bernal *et al*. (2010) found that 50% maintenance restriction of maternal nutrition during pregnancy resulted in lower ovarian GDF9 mRNA levels in female offspring. The absence of an effect in the present study maybe due to the fact that the pregnancy maintenance feeding regimen used was insufficient to cause an effect, whilst the negative effect found in Bernal *et al*. (2010) maybe due to the greater level of maternal undernutrition imposed.

Higher staining intensity of GDF9 in antral compared to secondary or primary follicles is expected, as GDF9 regulates the transition of pre-antral to antral follicles during folliculogenesis (Orisaka *et al*., 2006). As expected, the expression of AMH was higher in secondary follicles due to the increasing number of granulosa cells which are the site of AMH production (Gruijters *et al*., 2003) and was lower in larger follicles as estrogen production starts to escalate and causes a decrease in the sensitivity of the follicles to AMH (Visser *et al*., 2006; Anderson, 2012).

7.6 Conclusions

In conclusion, this study suggests that dam size can alter fetal ovary development and the differences created during fetal life persist into adult life. Conversely, pregnancy maintenance versus *ad libitum* planes of nutrition had little effect upon the development of the fetal ovary and no effect on the adult tissue. This suggests little impact on the reproductive potential of ewe progeny. However, given the influence of dam size on ovarian development, further study is warranted to investigate the effect of dam size on lifetime reproductive performance.

7.7 Acknowledgements

The authors thank Gravida, National Centre for Growth and Development and Massey University for providing funding for this project. The first author is funded by Ministry of Higher Education (Malaysia) Doctoral Scholarship. The authors would like to thank to M. Dragunow and P. Narayan for their help with computer-based image analysis.

7.8 References

Anderson, R. A. (2012). What does anti- Müllerian hormone tell you about ovarian function? *Clinical Endocrinology, 77*(5), 652-655.

Bernal, A. B., Vickers, M. H., Hampton, M. B., Poynton, R. A., & Sloboda, D. M. (2010). Maternal undernutrition significantly impacts ovarian follicle number and increases ovarian oxidative stress in adult rat offspring. *PLoS ONE, 5*(12), e15558.

Blair, H. T., van der Linden, D. S., Jenkinson, C. M. C., Morris, S. T., Mackenzie, D. D. S., Peterson, S. W., Firth, E. C., & Kenyon, P. R. (2011). Do ewe size and nutrition during pregnancy affect foetus and foetal organ weight in twins? *Livestock Science, 142*(1-3), 99-107.

Borwick, S. C., Rae, M. T., Brooks, J., McNeilly, A. S., Racey, P. A., & Rhind, S. M. (2003). Undernutrition of ewe lambs *in utero* and in early post-natal life does not affect hypothalamic–pituitary function in adulthood. *Animal Reproduction Science, 77*(1-2), 61-70.

Borwick, S. C., Rhind, S. M., McMillen, S. R., & Racey, P. A. (1997). Effect of undernutrition of ewes from the time of mating on fetal ovarian development in mid gestation. *Reproduction Fertility and Development, 9*(7), 711-715.

da Silva Faria, T., de Bittencourt Brasil, F., Sampaio, F. J. B., & da Fonte Ramos, C. (2008). Maternal malnutrition during lactation alters the folliculogenesis and gonadotropins and estrogen isoforms ovarian receptors in the offspring at puberty. *Journal of Endocrinology, 198*(3), 625-634.

Da Silva, P., Aitken, R. P., Rhind, S. M., Racey, P. A., & Wallace, J. M. (2002). Impact of maternal nutrition during pregnancy on pituitary gonadotrophin gene expression and ovarian development in growth-restricted and normally grown late gestation sheep fetuses. *Reproduction, 123*(6), 769-777.

Da Silva, P., Aitken, R. P., Rhind, S. M., Racey, P. A., & Wallace, J. M. (2003). Effect of maternal overnutrition during pregnancy on pituitary gonadotrophin gene expression and gonadal morphology in female and male foetal sheep at day 103 of gestation. *Placenta, 24*(2-3), 248-257.

Dong, J., Albertini, D. F., Nishimori, K., Kumar, T. R., Lu, N., & Matzuk, M. M. (1996). Growth differentiation factor-9 is required during early ovarian folliculogenesis. *Nature, 383*, 531-535.

Dupont, C., Cordier, A. G., Junien, C., Mandon-Pépin, B., Levy, R., & Chavatte-Palmer, P. (2012). Maternal environment and the reproductive function of the offspring. *Theriogenology, 78*(7), 1405-1414.

Eckery, D. C., Tisdall, D. J., Heath, D. A., & McNatty, K. P. (1996). Morphology and function of the ovary during fetal and early neonatal life: A comparison between the sheep and brushtail possum (*Trichosurus vulpecula*). *Animal Reproduction Science, 42*(1-4), 551-561.

Grazul-Bilska, A. T., Caton, J. S., Arndt, W., Burchill, K., Thorson, C., Borowczyk, E., Bilski, J. J., Redmer, D. A., Reynolds, L. P., & Vonnahme, K. A. (2009). Cellular proliferation and vascularization in ovine fetal ovaries: effects of undernutrition and selenium in maternal diet. *Reproduction, 137*(4), 699-707.

Gruijters, M. J. G., Visser, J. A., Durlinger, A. L. L., & Themmen, A. P. N. (2003). Anti-Müllerian hormone and its role in ovarian function. *Molecular and Cellular Endocrinology, 211*(1–2), 85-90.

Jefferies, B. C. (1961). Body condition scoring and its use in management. *Tasmanian Journal of Agriculture, 32*, 19-21.

Juengel, J. L., Hudson, N. L., Heath, D. A., Smith, P., Reader, K. L., Lawrence, S. B., O'Connell, A. R., Laitinen, M. P. E., Cranfield, M., Groome, N. P., ritvos, O., & McNatty, K. P. (2002). Growth differentiation factor 9 and bone morphogenetic

protein 15 are essential for ovarian follicular development in sheep. *Biology of Reproduction, 67*(6), 1777-1789.

Kenyon, P. R., & Webby, R. W. (2007). Pastures and supplements in sheep production systems. In P. V. Rattray, I. M. Brookes, & A. M. Nicol (Eds.), *Pasture and Supplements for Grazing Animals* (pp. 255-273): New Zealand Society of Animal Production Occasional Publication.

Kenyon, P. R., Blair, H. T., Jenkinson, C. M. C., Morris, S. T., Mackenzie, D. D. S., Peterson, S. W., Firth, E. C., & Johnston, P. L. (2009). The effect of ewe size and nutritional regimen beginning in early pregnancy on ewe and lamb performance to weaning. *New Zealand Journal of Agricultural Research, 52*(2), 203-212.

Léonhardt, M., Lesage, J., Croix, D., Dutriez-Casteloot, I., Beauvillain, J. C., & Dupouy, J. P. (2003). Effects of perinatal maternal food restriction on pituitary-gonadal axis and plasma leptin level in rat pup at birth and weaning and on timing of puberty. *Biology of Reproduction, 68*(2), 390-400.

Montgomery, G. W., Galloway, S. M., Davis, G. H., & McNatty, K. P. (2001). Genes controlling ovulation rate in sheep. *Reproduction, 121*(6), 843-852.

Moot, D.J., Matthew, C., & Kemp, P. D. (2007). Pastures and supplements in sheep production systems. In P. V. Rattray, I. M. Brookes, & A. M. Nicol (Eds.), *Pasture and Supplements for Grazing Animals* (pp. 13-22): New Zealand Society of Animal Production Occasional Publication.

Mossa, F., Carter, F., Walsh, S. W., Kenny, D. A., Smith, G. W., Ireland, J. L. H., Hildebrant, T. B., Lonergan, P., Ireland, J. J., & Evans, A. C. O. (2013). Maternal undernutrition in cows impairs ovarian and cardiovascular systems in their offspring. *Biology of Reproduction, 88*(4), 92, 91-99.

Murdoch, J. W., Edward, A. V. K., Vonnahme, K. A., & Ford, S. P. (2003). Ovarian responses to undernutrition in pregnant ewes, USA. *Reproductive Biology and Endocrinology, 1*, 6-8.

Orisaka, M., Orisaka, S., Jiang, J. Y., Craig, J., Wang, Y. F., Kotsuji, F., & Tsang, B. K. (2006). Growth differentiation factor 9 is antiapoptotic during follicular development from preantral to early antral stage. *Molecular Endocrinology, 20*(10), 2456-2468.

Rae, M. T., Palassio, S., Kyle, C. E., Brooks, A. N., Lea, R. G., Miller, D. W., & Rhind, S. M. (2001). Effect of maternal undernutrition during pregnancy on early ovarian development and subsequent follicular development in sheep fetuses. *Reproduction, 122*(6), 915-922.

Rasband, W.S., (1997). ImageJ, Image processing and analysis in Java. U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/ (accessed 22 April 2013).

Rhind, S. M. (2004). Effects of maternal nutrition on fetal and neonatal reproductive development and function. *Animal Reproduction Science, 82-3*, 169-181.

Rhind, S. M., Rae, M. T., & Brooks, A. N. (2001). Effects of nutrition and environmental factors on the fetal programming of the reproductive axis. *Reproduction, 122*(2), 205-214.

SAS. (2008). Statistical Analysis System. SAS Institute Incorporation, Cary, North Carolina, USA.

Sawyer, H. R., Smith, P., Heath, D. A., Juengel, J. L., Wakefield, S. J., & McNatty, K. P. (2002). Formation of Ovarian Follicles During Fetal Development in Sheep. *Biology of Reproduction, 66*(4), 1134-1150.

Smith, P., Braw-Tal, R., Corrigan, K., Hudson, N. L., Heath, D. A., & McNatty, K. P. (1994). Ontogeny of ovarian follicle development in Booroola sheep fetuses that are homozygous carriers or non-carriers of the FecB gene. *Journal of Reproduction and Fertility, 100*(2), 485-490.

Visser, J. A., de Jong, F. H., Laven, J. S. E., & Themmen, A. P. N. (2006). Anti-Müllerian hormone: a new marker for ovarian function. *Reproduction, 131*(1), 1-9.

Vitt, U. A., McGee, E. A., Hayashi, M., & Hsueh, A. J. W. (2000). *In vivo* treatment with GDF-9 stimulates primordial and primary follicle progression and theca cell marker CYP17 in ovaries of immature rats. *Endocrinology, 141*(10), 3814-3820.

Weenen, C., Laven, J. S. E., von Bergh, A. R. M., Cranfield, M., Groome, N. P., Visser, J. A., Kramer, P., Fauser, B. C. J.M., & Themmen, A. P. N. (2004). Anti-Müllerian hormone expression pattern in the human ovary: potential implications for initial and cyclic follicle recruitment. *Molecular Human Reproduction, 10*(2), 77-83.

Chapter 8

Effects of dam size and nutrition during pregnancy on the lifetime performance of female offspring

8.1 Abstract

The objective of this study was to examine the effects of maternal size and nutrition during pregnancy upon the subsequent growth and reproductive performance of the daughter up to 6 years of age. In 2005, Romney ewes (G_0) were selected as heavy (H; n=450, mean live weight = 60.8 kg ± 0.18) or light (L; n=450; mean live weight = 42.5 kg ± 0.17) based upon live weight at the time of mating, and then randomly allocated to receive pregnancy maintenance (M) or *ad libitum* (A) feeding treatment from day 21 to 140 of pregnancy; resulting in 4 treatment groups (HA, HM, LA and LM). Performance data were recorded in the daughter progeny over the period 2007 to 2011 (580 to 2298 days of age) and the performance of their lambs (grand progeny) to weaning. Daughter live weights and body condition scores were inconsistently affected by dam size and nutrition over time and at most measurement points no differences were found. Whilst splines function analysis showed no difference of dam size and nutrition on either live weights or body condition scores. A-daughters at 2050 days of age had higher ($P<0.05$) proportion that ovulate than M-daughters and A-daughters gave birth to more lambs than M-daughters, but there was no differences ($P>0.05$) found in relation to number or weight of lambs weaned. Daughter longevity (the daughters that still present in the flock) at the end of the study was not affected by dam size or nutrition. There were no interactions ($P>0.05$) between dam size and nutrition for any parameters of lifetime reproductive performance data collected. This study suggests that dam size and maintenance and *ad libitum* nutrition during pregnancy has minimal impact on subsequent daughter lifetime performance.

8.2 Introduction

Epidemiological studies of humans have shown that the *in utero* environment influences development and can have long-term health outcomes of offspring (Barker *et al*., 1993; Godfrey and Barker, 2000). Similarly, in production animals such as sheep, the *in utero* environment to which a fetus is subjected can have both short- and long-term consequences on growth and reproductive performance (Rhind *et al*., 2001; Rhind, 2004; Wu *et al*., 2006; Kenyon, 2008; Gardner *et al*., 2009; Dupont *et al*., 2012). Nutrition of the dam has been shown to influence fetal growth (Edward and McMillan, 2002; Vonnahme *et al.,* 2003), birth size (Kelly *et al.,* 2006; Neville *et al*., 2010), growth post birth (Deliogeorgis *et al.,* 1996; Borwick *et al.,* 2003), and the ovulation rate (Rae *et al.,* 2002) of offspring. Likewise, dam size can influence the supply of nutrients and substrates to the developing fetus (Redmer *et al.,* 2004; Gardner *et al.,* 2007; Gootwine *et al.,* 2007) which may have both short- and long-term consequences.

In 2005, 900 ewes were selected, from within a single flock, to represent two live weight extremes. These ewes were subsequently offered either pregnancy maintenance or *ad libitum* nutrition between Days 21 and 140 of pregnancy (Kenyon *et al.,* 2009), to represent feeding practice that commonly employed by New Zealand sheep farmers during pregnancy. At weaning the ewe progeny were retained and their early performance recorded (van der Linden *et al.,* 2007). Maternal nutrition treatment influenced fetal development (Blair *et al.,* 2011; Kenyon *et al.,* 2011b), fetal mammary gland weight (van der Linden *et al.,* 2009), lamb birth weight and growth rates from birth to weaning (van der Linden *et al.,* 2007; Kenyon *et al.,* 2011a), metabolism and first lactational performance (van der Linden *et al*., 2009; van der Linden *et al*., 2010b; van der Linden *et al*., 2010c; Blair *et al*., 2010) and live weight of the first two sets of grand-offspring (van der Linden *et al*., 2010a; Blair *et al*., 2010). In addition, dam size

affected fetal mammary duct area (van der Linden *et al.,* 2009), number of ovarian follicles in fetal and adult life (Chapter 7), lamb live weights (Kenyon *et al.,* 2009; 2011a), milk production at their first but not second lactation (van der Linden *et al.,* 2009; Blair *et al*., 2010), and grand offspring live weight and onset of puberty (van der Linden *et al.,* 2010a; Blair *et al.,* 2010).

There are a number of studies that have examined the effect of maternal nutrition on daughter offspring, however, the vast majority of these studies have been relatively short term (Gunn *et al*., 1995; Rhind *et al*., 2001; Rae *et al*., 2002; Rhind, 2004; Munoz *et al*., 2009). While it is of interest to note short-term effects on the offspring, it is the impact on the offsprings lifetime performance that will influence farmers' decisions of whether to manipulate the nutrition or the size of their ewes to influence the performance of future generations.

Therefore, this paper reports on the lifetime performance of daughters born to ewes of two sizes and which offered one of two feeding levels in pregnancy. Lifetime performance data were collected from daughters between 1.5 to 6 years of age and included their five lambing events. It was postulated that being born to small ewes or ewes fed at pregnancy maintenance, would have little effect on the live weight of the daughters. In contrast, based on previous studies, and differences observed in the daughter's early life, it was postulated that differences in lifetime reproductive performance of the daughters would be observed.

8.3 Materials and methods

The study was conducted at the Massey University Keeble Sheep and Beef farm (latitude 41° 10'S longitude 175° 35'E), 5 km south of Palmerston North, New Zealand.

All experimental animal procedures were approved by the Massey University Animal Ethics Committee (MUAEC 09/18), Palmerston North, New Zealand.

8.3.1 Background

Two groups, each of 450 ewes, were selected from a single commercial flock of 2900 Romney ewes to provide subsets of the flock that were of heavy ((H; 60.8 kg ± 0.18) or light (L; 42.5 kg ± 0.17) live weights at mating, as described by Kenyon *et al.* (2009). Briefly, the dams were bred using artificial insemination to one of four Suffolk rams and, between Days 21 and 140 (D21 – D140) of pregnancy, were randomly allocated to receive either pregnancy maintenance (M) or *ad libitum* (A) feeding regimens under New Zealand pastoral grazing conditions), to represent feeding practice that commonly employed by New Zealand sheep farmers during pregnancy. From Day 140 of pregnancy through to weaning, all dams and their lambs were provided with *ad libitum* pasture (Kenyon *et al.*, 2009). The average pre- and post-grazing pasture covers during the period between Days 21 and 140 of pregnancy were 1330 ±140.0 and 804 ±133.4 kg DM/ha for the maintenance regimen, and 2304 ± 156.8 and 1723 ± 149.7 kg DM/ha for the *ad libitum* regimen (Kenyon *et al*., 2009). After weaning, all female daughter offspring were managed and fed as one group under commercial conditions for the remainder of their lifetime. Therefore, this study utilised a two-by-two factorial design, including two dam-size treatments (H *vs* L) and two dam-nutrition treatments (M *vs* A); resulting in four treatment groups (HA, HM, LA and LM).

8.3.2 Present study

The present study evaluated the lifetime effects of dam size and nutrition during pregnancy on the performance data of the daughters born in the study of Kenyon *et al*. (2009). The daughters were approximately 19 months (average age 580 days) in June 2007 when the present study started at the time of their first breeding. The study continued until the daughters were (average age 2298 days) approximately 6 years and 5 months at the time of the weaning of their fifth set of lambs in December 2011. The study was conducted at the Massey University Keeble Sheep and Beef Farm, 5 km south of Palmerston North, New Zealand. All experimental procedures on animal were approved by the Massey University Animal Ethics Committee, Palmerston North, New Zealand.

Daughter live weight and body condition score

In each of the five years 2007-2011, daughters were weighed and body condition scored (Jefferies 1961; scale 1 – 5, with 1 = emaciated and 5 = obese) at breeding (approximately one month prior to mating; average ages of 580 (2007), 946 (2008), 1311 (2009), 1671 (2010) and 2041 (2011) days), mid-pregnancy (average age of 671 (2007), 1382 (2009), 1740 (2010) and 2123 (2011) days), pre-lambing (approximately two weeks pre-lambing (average age of 733 (2007), 1099 (2008), 1464 (2009), 1810 (2010) and 2181 (2011) days); and at weaning of their lambs ('grand progeny') (approximately 100 days after the mid-point of lambing; average age of 839 (2007), 1205 (2008), 1570 (2009), 1927 (2010) and 2298 (2012) days).

Daughter breeding performance and pregnancy diagnosis

In 2007 (609 days), 2008 (975 days), 2009 (1320 days), 2010 (1678 days) and 2011 (2061 days), the daughters were bred after synchronisation with a progesterone controlled internal drug released devise (CIDR, 0.3g progesterone, Pharmacia & UpJohn, Auckland, New Zealand), to Romney rams (approximate ram:ewe ratio 1:20) for five days. Following this, rams at an approximate ratio 1:100 remained with the daughters for a further 17 days. Pregnancy status was then diagnosed by ultrasound scanning approximately 50 days after the end of the breeding period, where daughters were diagnosed as non-pregnant or bearing single-, twin- or triplet-fetuses for each year.

Daughter ovulation rate

In the April 2010 (2050 days, 5 years 8 months) breeding season, an estimation of ovulation rate was conducted by counting the number of corpora lutea present using trans-rectal ultrasounography (B-mode scanner, Mindray DP 6600, 50mm linear 7.5 mHz probe) (Viñoles *et al*., 2010).

Progeny live weight

All lambs (grand-progeny) were weighed within 24 h of birth, identified to their mothers and their birth rank and their sex determined. Lambs were weighed again at weaning (average age of 76 (2007), 113 (2008), 116 (2009), 111 (2010) and 104 (2011) days).

8.3.3 Statistical analysis

Where appropriate data (live weight, body condition score, pregnancy diagnosis, lambs born, weaned and weaned weights, and longevity) were subjected to repeated measures analysis of variance with respect to dam size (heavy *vs* light) and feeding regimen (*ad libitum vs* maintenance), sire and birth rank (singleton *vs* twin), in which individual daughters were nested within treatment. All two-way and three-way interactions were included in the initial model, but interactions were removed if found non-significant ($P<0.05$) and the model was then re-fitted. The models also included the daughter's birth date as a covariate. The dam size and nutrition treatments and interaction between size and nutrition treatments remained in all models irrespective of whether they were significant or not to allow for testing of the study design. These analyses were carried out using either mixed linear model (proc MIXED) for continuous data or general linear model (proc GENMOD) for categorical data in the Statistical Analysis System (SAS, 2008; SAS 9.2, SAS Institute, North Carolina, USA) for analysis of variance. Type III mean square values were used to determine the significance of fixed effects.

Daughter live weight and body condition score

Two different analyses were used for daughter's live weight and body condition score data. The first models were analysed using the mixed linear model (proc MIXED) to determine the performance of daughters' live weight and body condition score at difference measurement points (from 580 to 2298 days of age). Whilst the second analysis was modelled using a spline function over the age range of 568 to 2304 days (this range was selected based on the actual age of original data) with general linear

mixed model (proc GLIMMIX) for prediction of overall live weight and body condition score.

Daughter pregnancy diagnosis

To determine a proxy for 'lifetime' (2007–2011) reproductive performance for each daughter present at the beginning of the study (2007), each daughter's pregnancy scanning data was collated (non-pregnant, single-, twin- and triplet bearing) for each year. These data were analysed as follows:

'Lifetime daughters data 2007 to 2011': daughters that were not present for any year (i.e. those that had been removed from the flock or had died during 2007 to 2011) were also given a nominal value of 0 for the remaining years' pregnancy scanning data. The data were analysed using the general linear model (proc GENMOD). The model also included the date of birth of the daughter as a covariate.

'Lifetime daughters present 2011': the lifetime reproductive performance of only those daughters that were present for the entire study (i.e. those that had complete pregnancy ultrasound scanning data for all years) was analysed using the general linear model (proc GENMOD). The model also included the date of birth of the daughter as a covariate.

'Lifetime daughters present with output 2011': the lifetime reproductive performance of only those daughters that were present for the entire study and had either a single-, twin- or triplet pregnancy each year (i.e. were pregnant every year) was also analysed using the general linear model (proc GENMOD). The model also included the date of birth of the daughter as a covariate.

Daughter ovulation rate

To examine the apparent ovulation rate of daughters, the total number of corpora lutea present in the ovaries of each ewe was recorded at day 2050 days (5 years 7 months) of age. The data were analysed using the general linear model (proc GENMOD) with date of birth as a covariate.

To determine the proportion of daughters that actually ovulated at day 2050, daughters with at least one corpus luteum were given a nominal value of 1 (ovulated) and daughters without a corpus luteum were given a nominal value of 0 (non-ovulated). The data were analysed using the general linear model (proc GENMOD) with date of birth as a covariate.

Lambs born and weaned per daughter and the weaning live weights of those lambs

The lifetime efficiency performance of daughters (2007–2011) was determined as lifetime and yearly totals of (i) *the number of lambs born,* (ii) *the number of lambs weaned,* and (iii) *the weight of lambs weaned for each daughter present at the beginning of the study* (2007). Daughters that were not present for any year (i.e. those that had been removed from the flock or had died during 2007 to 2011) were also given a nominal value of 0 for the lambs born, weaned or weaning weight data for the corresponding years that data was not present ('Lifetime daughters data 2007 – 2011').

For only those daughters that were present for the entire study that either gave birth (1, 2 or 3 lambs) or not (0 lamb) each year were included in *'Lifetime daughters present 2011'*. In addition daughters that were present for the entire study and gave birth to at

least one lamb each year each were included in *'Lifetime daughters present with output 2011'*.

Daughter efficiency

Daughter efficiency was calculated as the total lamb weaning weight per estimated total maintenance MJME consumed by each daughter for each year from 2007 to 2011. These data were analysed using the mixed linear model (proc MIXED). Estimated metabolisable energy requirement per year for maintenance (MJME) of the daughters were calculated using the following equation:

0.48 MJME/kg $W^{0.75}$ (Nicol and Brookes, 2007)

where W is the weight of the animal.

Each year, four live weights (at breeding, mid-pregnancy, pre-lambing and weaning) were recorded per daughter. The estimated average live weight for each daughter was calculated as a weighted average of the live weights collected for that year. Live weight at breeding was calculated as the live weight for breeding to mid-pregnancy (February to April: average 90 days), live weight at mid-pregnancy as the weight for mid-pregnancy to pre-lambing (May to July; average 90 days), live weight at pre-lambing as the weight for pre-lambing to weaning (August to September; average 60 days) and live weight at weaning as the live weight for weaning to the next breeding (October to January; average 125 days) for each year.

Daughter longevity

The percentage of daughters present at breeding each year was used as a proxy for longevity within the flock, with the number present at breeding in 2007 being the base value (i.e. 100%). Daughter survival was analysed as a binomial trait using the general linear model proc GENMOD. The LA group had no ewe deaths prior to 2009 so could only be analysed from 2010. If a daughter was not present at a given breeding (i.e. due to culling or death) she was considered as no longer being part of the flock.

8.4 Results

8.4.1 Daughter live weight

Figure 8.1. indicates that ewe size and feeding treatment had very little impact on the live weight of daughter offspring with differences only observed at four time points (between 733 and 1927 days of age). Daughters born to ewes from the H-dams were heavier ($P<0.05$) at 1205 days of age (weaning 2008) than those born to L-dams (H: 72.7 ± 1.3 kg, L: 66.2 ± 1.4 kg) (Figure 8.1a.). At an average age of 1464 days (pre-lambing 2009), daughters born to A-dams were heavier ($P<0.05$) than those born to M-dams (A: 78.4 ± 2.2 kg, M: 70.6 ± 2.1 kg). There were interactions ($P<0.05$) between dam size and nutrition at two ages: at 733 days of age (pre-lambing 2007), daughters born to HA-, HM- and LA-dams (HA: 74.8 ± 1.3 kg, HM: 77.5 ± 1.3 kg LA:74.5 ± 1.4 kg) were heavier ($P<0.05$) than those born to LM-dams (LM: 70.4 ± 1.5 kg); at 1927 days of age (weaning 2010) daughters born to HM- and LA-dams (HM: 72.3 ± 2.9 kg LA: 70.1 ± 3.6 kg) were heavier than those born to LM-dams (60.3 ± 3.3 kg), whilst daughters born to HA-dams (68.5 ± 2.9 kg) did not differ ($P>0.05$) from all other groups. In support of the minor differences observed the overall predicted daughter live

weight using the spline function did not differ (P>0.05) between either dam feeding regimen (A *vs* M) or size (L *vs* S) treatments (Figure 8.1b.).

8.4.2 Daughter body condition score

Differences in body condition score were only observed at two time points (Figure 8.2a.). Daughters born to L-dams had higher (P<0.05) mean body condition score at 1570 days (weaning, 2009) compared to those born to H-dams (L: 2.6 ± 0.1, H: 2.9 ± 0.1) (Figure 8.2a.). There was an (P<0.05) interaction between dam size and nutrition treatments at 1927 days of age (weaning 2010), such that daughters born to HM- and LA-dams had greater condition scores (HM: 2.5 ± 0.1, LA: 2.5 ± 0.2) than those born to LM-dams (LM: 2.1 ± 0.1); while daughters born to HA-dams (HA: 2.3 ± 0.1) did not differ (P>0.05) from any other groups. The overall predicted daughter body condition score using the spline function showed no difference (P>0.05) between groups (Figure 8.2b.).

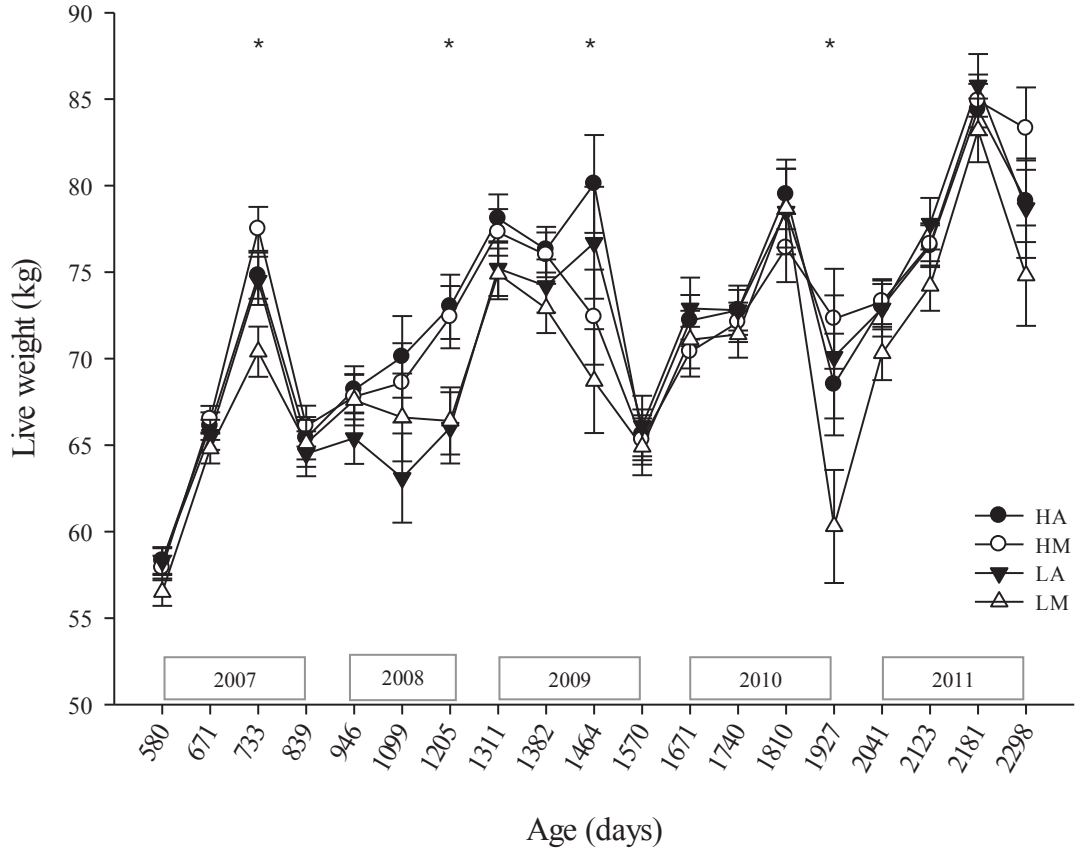


Figure 8.1a. Effect of dam size (heavy (H) *vs* light (L)) and nutrition (*ad libitum* (A) *vs* pregnancy maintenance (M)) during pregnancy on daughter live weight (means ± SE) during the period 580 to 2298 days of age (from 2007 to 2011; at breeding, mid-pregnancy (except in 2008), pre-lambing and weaning). The * indicates a significant difference at $P<0.05$.

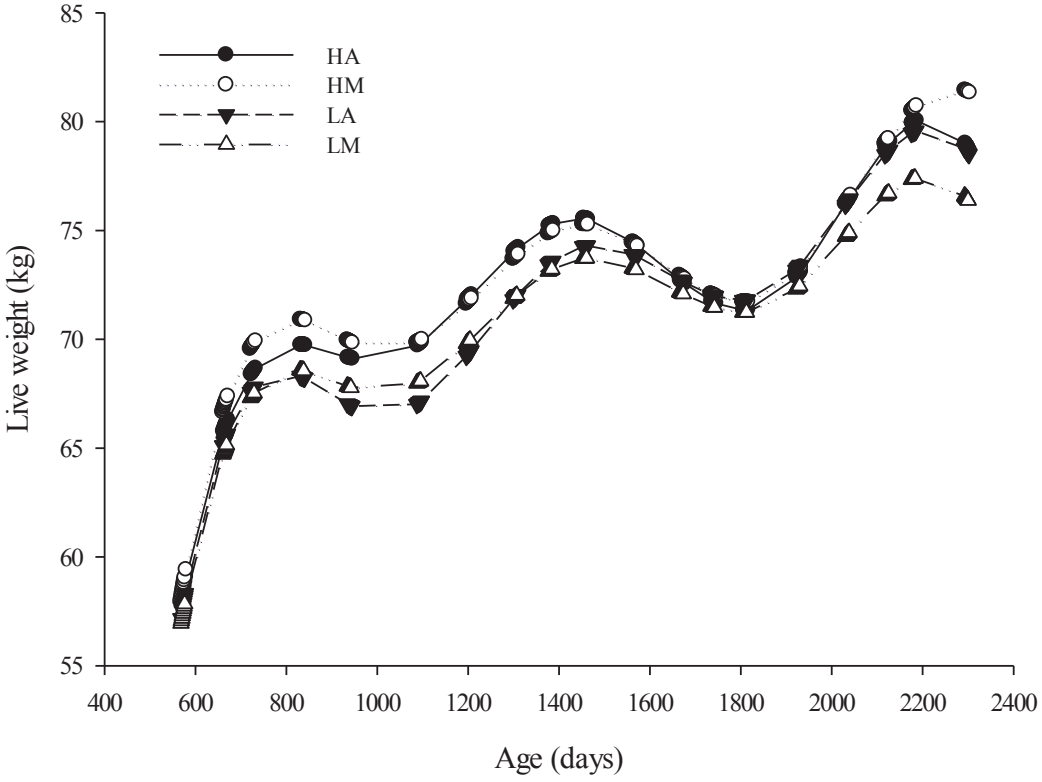


Figure 8.1b. Effect of dam size (heavy (H) *vs* light (L)) and nutrition (*ad libitum* (A) *vs* pregnancy maintenance (M)) during pregnancy on predicted daughter live weight using spline function during the period 568 to 2304 days of age.

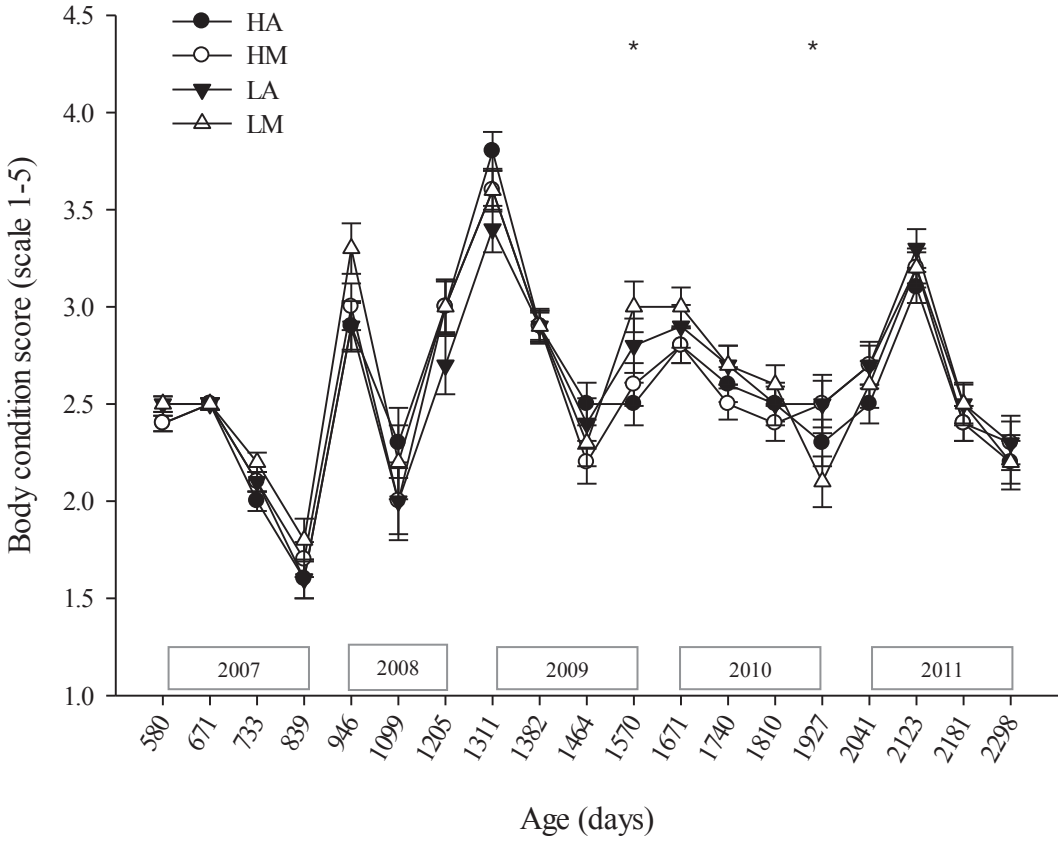


Figure 8.2a. Effect of dam size (heavy (H) *vs* light (L)) and nutrition (*ad libitum* (A) *vs* pregnancy maintenance (M)) during pregnancy on daughter body condition score (means ± SE) during the period 580 to 2298 days of age (from 2007 to 2011; at breeding, mid-pregnancy (except in 2008), pre-lambing and weaning). The * indicates a significant difference at $P<0.05$. Figures show least square means (±SE).

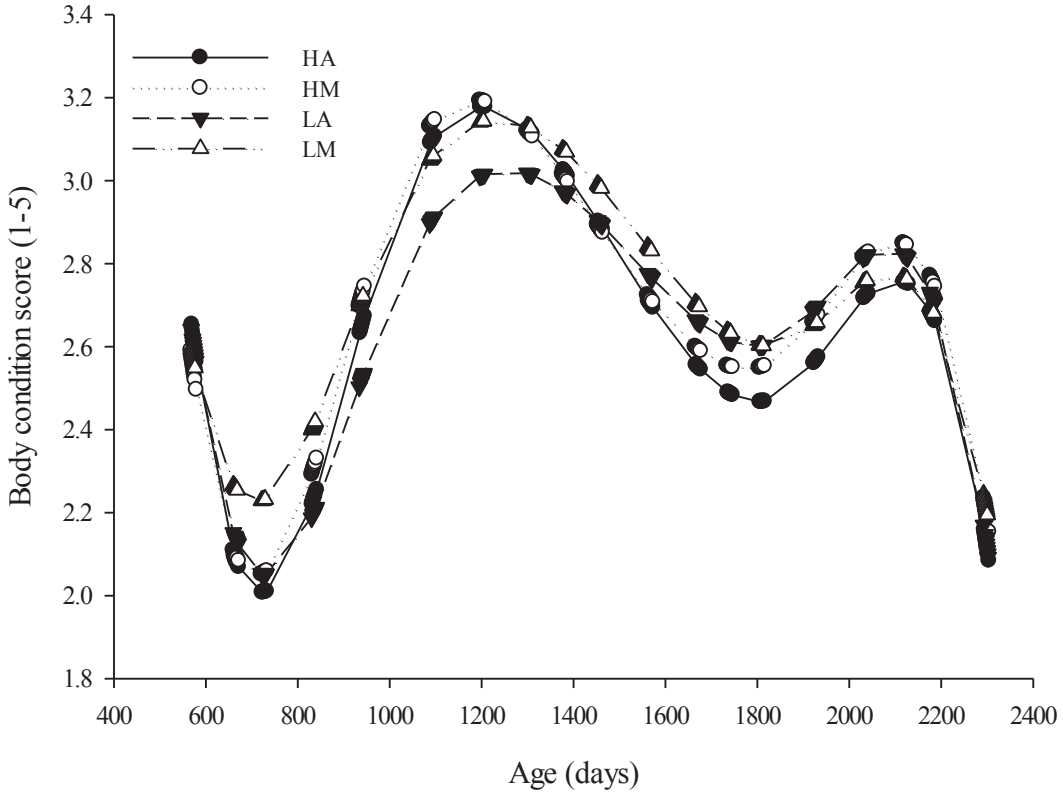


Figure 8.2b. Effect of dam size (heavy (H) *vs* light (L)) and nutrition (*ad libitum* (A) *vs* pregnancy maintenance (M)) during pregnancy on daughter predicted body condition score using spline function during the period 568 to 2304 days of age.

8.4.3 Daughter ovulation rate in 2010

There was no affect (P>0.05) of dam size or nutrition on daughter ovulation rate. A higher (P<0.05) proportion of daughters born to A-dams ovulated compared to those born to M-dams (Table 8.1.). There was no effect (P>0.05) of dam-size on the proportion of daughters ovulating. Further, there was no (P>0.05) interaction between dam-size and nutrition for the proportion of daughters ovulating or ovulation rate.

Table 8.1. Ovulation rate and the proportion that ovulated after oestrus synchronisation in daughters of dams that were fed *ad libitum* (A) or pregnancy maintenance (M) during pregnancy, or were heavy (H) or light (L) at the start of pregnancy. The daughters were of an average age of 2050 days. Table shows least square means (±SE).

Treatment	*n*	Ovulation rate	*n*	Proportion that ovulated
Size				
H	*93*	2.1 ± 0.1	*94*	1.0 ± 0.0
L	*53*	2.2 ± 0.1	*56*	0.9 ± 0.0
Nutrition				
A	*78*	2.2 ± 0.1	*78*	1.0 ± 0.0[b]
M	*68*	2.1 ± 0.1	*72*	0.9 ± 0.0[a]
Size x Nutr				
HA	*48*	2.3 ± 0.1	*48*	1.0 ± 0.0
HM	*45*	2.0 ± 0.1	*46*	1.0 ± 0.0
LA	*30*	2.2 ± 0.1	*30*	1.0 ± 0.0
LM	*23*	2.1 ± 0.1	*26*	0.9 ± 0.0

[ab]Different letters within columns represent the groups that are significantly different (P<0.05) from each other.
Size x Nutr: interaction between dam size and dam nutrition treatments

8.4.4 Number of fetuses per daughter

The total number of fetuses per daughters did not differ (P>0.05) between dam size or nutrition treatment groups, regardless of whether this was calculated on the basis of 'Lifetime daughter data 2007 to 2011', 'Lifetime daughters present 2011', or 'Lifetime daughters present with output 2011. There were no interactions (P>0.05) between dam size and nutrition treatments (Table 8.2.).

8.4.5 Total number of lambs born and weaned per daughter

Daughters of H-dams gave birth to a greater (P<0.05) number of grand-progeny compared to daughters those born to L-dams according to the criteria for 'Lifetime

daughter data 2007 to 2011' (Table 8.3a.). For the criteria of 'Lifetime daughters present 2011', daughters of A-dams gave birth to a greater number of grand progeny than did daughters of M-dams (P=0.05). There was no effect (P>0.05) of either dam size or nutrition for the numbers of grand progeny born according to the criteria of 'Lifetime daughters present with output 2011'. There were no (P>0.05) interactions between dam size and nutrition treatments for the numbers of grand progeny born to daughters according to the criteria of either 'Lifetime daughter data 2007 to 2011', 'Lifetime data daughters present 2011', or 'Lifetime daughters present with output 2011'.

There was no effect (P>0.05) of either dam size or nutrition treatments nor any interactions (P>0.05) for the total grand progeny weaned to daughters according to the criteria of either 'Lifetime daughter data 2007 to 2011', 'Lifetime data daughters present 2011' or 'Lifetime daughters present with output 2011' (Table 8.3b.).

8.4.6 Total weight of grand-progeny weaned per daughter

There was no effect (P>0.05) of dam size or nutrition or an interaction between size and nutrition treatments for the total weight of grand progeny (Table 8.4.).

8.4.7 Daughter efficiency 2007 to 2011

There was no effect (P>0.05) of dam size or nutrition treatments nor any interactions for daughters' efficiency (Table 8.5.).

8.4.8 Daughter longevity

Dam nutrition had no effect (P>0.05) on the proportion of daughters that were present in the flock during 2007 to 2011. In 2011 there was a greater (P<0.05) proportion of daughters born to H-dams present than daughters born to L-dams (H: 88.1, L: 73.5%), but this difference was not (P>0.05) evident in the other years (Figure 8.3.). There was no interaction (P>0.05) between dam size and nutrition treatments for daughter longevity. In December 2011 (daughters' average age of 2298 days) the percentage of daughters that were still present in the flock were 75.7% *vs* 63.5% for H- and L-dams, and 74.9% *vs* 64.4% for A- and M-dams, respectively.

Table 8.2. Effect of dam size (heavy (H) *vs* light (L)) and nutrition (*ad libitum* (A) *vs* pregnancy maintenance (M)) during pregnancy on the lifetime reproductive performance (number of fetuses identified per ewe at pregnancy scanning) for all daughters (Lifetime 2007 to 2011), for those daughters that had pregnancy data for each year (Lifetime daughters present 2011) or only those daughters that had at least one fetus each year (Lifetime daughters present with output 2011). Table shows least square means (±SE).

Treatment	*n*	Lifetime 2007 to 2011	*n*	Lifetime daughters present 2011	*n*	Lifetime daughters present with output 2011
Size						
H	*113*	7.8 ± 0.3	*99*	8.9 ± 0.2	*76*	9.1 ± 0.2
L	*85*	7.2 ± 0.3	*62*	8.2 ± 0.3	*48*	9.1 ± 0.2
Nutrition						
A	*97*	7.6 ± 0.3	*79*	8.5 ± 0.3	*63*	9.2 ± 0.2
M	*101*	7.4 ± 0.3	*82*	8.1 ± 0.3	*61*	9.0 ± 0.2
Size x Nutr						
HA	*55*	8.0 ± 0.4	*49*	8.6 ± 0.3	*38*	9.2 ± 0.3
HM	*58*	7.7 ± 0.4	*50*	8.2 ± 0.3	*38*	9.1 ± 0.3
LA	*42*	7.3 ± 0.4	*30*	8.5 ± 0.4	*25*	9.3 ± 0.3
LM	*43*	7.1 ± 0.4	*32*	7.9 ± 0.4	*23*	8.9 ± 0.3

Size x Nutr: interaction between dam size and dam nutrition treatments

Table 8.3. Effect of dam size (heavy (H) *vs* light (L)) and nutrition (*ad libitum* (A) *vs* pregnancy maintenance (M)) during pregnancy on (**A)** the total lambs born, and (**B)** weaned all daughters (Lifetime 2007 to 2011), only those daughters that were present each year (Lifetime daughters present 2011) or only those daughters that gave birth and weaned at least one lamb each year (Lifetime daughters present with output 2011). Tables show least square means (±SE).

A (Total lambs born)

Treatment	*n*	Lifetime 2007 to 2011	*n*	Lifetime daughters present 2011	*n*	Lifetime daughters present with output 2011
Size						
H	*113*	7.4 ± 0.3[b]	*99*	7.8 ± 0.2	*57*	9.1 ± 0.2
L	*85*	6.4 ± 0.3[a]	*62*	7.5 ± 0.3	*35*	8.6 ± 0.2
Nutrition						
A	*97*	7.1 ± 0.3	*79*	8.1 ± 0.3[b]	*50*	8.8 ± 0.2
M	*101*	6.7 ± 0.3	*82*	7.3 ± 0.3[a]	*42*	8.9 ± 0.2
Size x Nutr						
HA	*55*	7.7 ± 0.4	*49*	8.3 ± 0.3	*31*	9.0 ± 0.3
HM	*58*	7.0 ± 0.4	*50*	7.3 ± 0.3	*26*	9.3 ± 0.3
LA	*42*	6.4 ± 0.4	*30*	7.8 ± 0.4	*19*	8.7 ± 0.3
LM	*43*	6.4 ± 0.4	*32*	7.2 ± 0.4	*16*	8.4 ± 0.4

[ab]Different letters within columns represent the groups that are significantly different (P<0.05)
Size x Nutr: interaction between dam size and dam nutrition treatments

B (Total lambs weaned)

Treatment	*n*	Lifetime 2007 to 2011	*n*	Lifetime daughters present 2011	*n*	Lifetime daughters present with output 2011
Size						
H	*113*	5.4 ± 0.3	*99*	5.8 ± 0.2	*32*	8.2 ± 0.3
L	*85*	5.0 ± 0.3	*62*	6.0 ± 0.3	*22*	7.8 ± 0.3
Nutrition						
A	*97*	5.3 ± 0.3	*79*	6.0 ± 0.3	*29*	8.0 ± 0.3
M	*101*	5.2 ± 0.3	*82*	5.8 ± 0.3	*25*	8.0 ± 0.3
Size x Nutr						
HA	*55*	5.5 ± 0.4	*49*	5.8 ± 0.3	*16*	8.3 ± 0.3
HM	*58*	5.3 ± 0.4	*50*	5.8 ± 0.3	*16*	8.1 ± 0.4
LA	*42*	5.0 ± 0.4	*30*	6.1 ± 0.4	*13*	7.8 ± 0.3
LM	*43*	5.0 ± 0.4	*32*	5.9 ± 0.4	*9*	7.8 ± 0.4

Size x Nutr: interaction between dam size and dam nutrition treatments

Table 8.4. Effect of dam size (heavy (H) *vs* light (L)) and nutrition (*ad libitum* (A) *vs* pregnancy maintenance (M)) during pregnancy on the total weight of lambs weaned for all daughters (Lifetime 2007 to 2011), only those daughters that were present each year (Lifetime daughters present 2011) or only those daughters that had the weight of lamb weaned at least one lamb each year (Lifetime daughters present output 2011). Tables show least square means (±SE).

Treatment	*n*	Lifetime 2007 to 2011	*n*	Lifetime daughters present 2011	*n*	Lifetime daughters present with output 2011
Size						
H	*113*	168 ± 8.2	*99*	181 ± 7.6	*32*	256 ± 6.8
L	*85*	156 ± 8.9	*62*	187 ± 9.1	*22*	244 ± 7.2
Nutrition						
A	*97*	163 ± 9.1	*79*	186 ± 8.9	*29*	252 ± 6.9
M	*101*	161 ± 8.7	*82*	183 ± 8.4	*25*	248 ± 8.0
Size x Nutr						
HA	*55*	172 ± 11.8	*49*	183 ± 10.8	*16*	258 ± 8.9
HM	*58*	164 ± 11.5	*50*	179 ± 10.7	*16*	254 ± 9.4
LA	*42*	153 ± 13.0	*30*	189 ± 13.4	*13*	246 ± 9.1
LM	*43*	159 ± 12.7	*32*	186 ± 12.7	*9*	241 ± 11.1

Size x Nutr: interaction between dam size and dam nutrition treatments

Table 8.5. Effect of dam size (heavy (H) *vs* light (L)) and nutrition (*ad libitum* (A) *vs* pregnancy maintenance (M)) during pregnancy on daughter efficiency (kg/MJME/year) from 2007 to 2011 for all daughters (Lifetime 2007 to 2011), only daughters that had efficiency data for each year (Lifetime daughters present 2011) or only daughters whose efficiency data greater than zero (>0) each year (Lifetime daughters present with output 2011). Table shows least square means (±SE).

Treatment	*n*	Lifetime 2007 to 2011 *($x10^3$)*	n	Lifetime daughters present 2011 *($x10^3$)*	n	Lifetime daughters present with output 2011 *($x10^3$)*
Size						
H	*95*	10 ± 0.4	*70*	10 ± 0.4	*31*	12 ± 0.4
L	*71*	9 ± 0.4	*43*	9 ± 0.5	*19*	12 ± 0.4
Nutrition						
A	*82*	9 ± 0.4	*59*	9 ± 0.5	*26*	12 ± 0.4
M	*84*	10 ± 0.4	*54*	10 ± 0.5	*24*	12 ± 0.5
Size x Nutr						
HA	*46*	10 ± 0.4	*36*	9 ± 0.6	*15*	12 ± 0.5
HM	*49*	9 ± 0.5	*34*	10 ± 0.6	*16*	12 ± 0.5
LA	*36*	9 ± 0.6	*23*	9 ± 0.7	*11*	11 ± 0.5
LM	*35*	10 ± 0.6	*20*	10 ± 0.7	*8*	12 ± 0.6

Size x Nutr: interaction between dam size and dam nutrition treatments

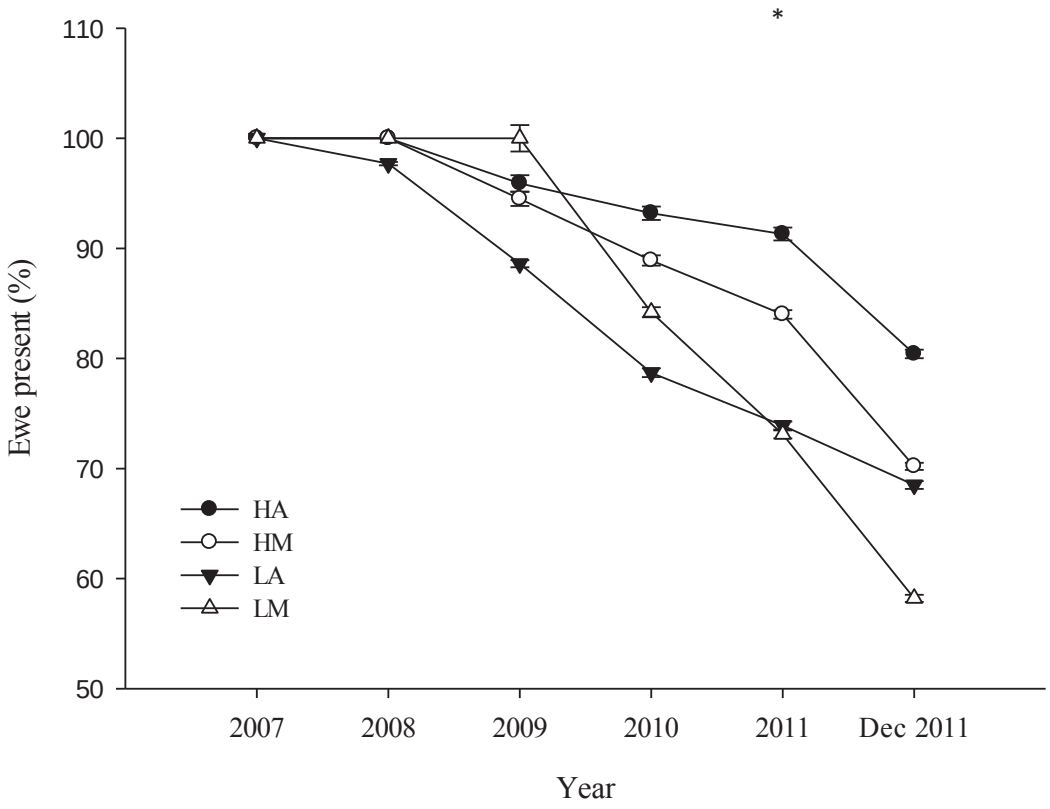


Figure 8.3. Effect of dam size (heavy (H) *vs* light (L)) and nutrition (*ad libitum* (A) *vs* pregnancy maintenance (M)) during pregnancy on the proportion (%) of daughters present in 2007, 2008, 2009, 2010 and 2011. The * indicates a significant difference at $P<0.05$.

8.5 Discussion

The aim of this study was to investigate the effect of dam size and nutrition during pregnancy on the lifetime performance of their daughters to the weaning of their fifth set of lambs (grand-progeny) as a 6-year-old ewe. The hypothesis of this study that differences in lifetime reproductive performance of the daughters would be observed in daughters born to small ewes or ewes fed at pregnancy maintenance was rejected.

8.5.1 Live weight and body condition

The present study showed only minor and transient effects of dam size and nutrition on the live weight and body condition score of daughters from 580 and 2298 days of age. Previous studies using these animals have shown dam size and nutrition affected their fetal size (Blair *et al*., 2011) and their live weight from birth and 100 days of age (van der Linden *et al*., 2007; Kenyon *et al*., 2009; van der Linden *et al*., 2009). However, after weaning these live weight differences were no longer apparent in these daughters (van der Linden *et al.,* 2010b) supporting the general lack of effects as they aged. Evidence from Gunn *et al*. (1995), Da Silva *et al*. (2001), Borwick *et al*. (2003) and Neville *et al*. (2010) are in agreement with the present collective studies, that demonstrated any live weight effect which appeared during early life (birth or weaning weight) did not persist as the animals matured. It is probable that the effects observed of maternal nutrition on early life live weights are due to either the carryover effects of changed lamb birth weight or differential ewe lactational performance (O'Doherty and Crosby, 1996; Bizelis *et al*., 2000; Kenyon *et al*., 2004). In addition, a better buffering ability of a heavier dam against undernutrition (Rattray and Trigg, 1979) and a possible genetic difference for birth weight, due to the dam size might also contribute to this effect (Blair *et al*., 2010).

Perhaps the minor year-to-year differences in daughters' performance can be explained with reference to meteorological events, which might identify periods when the ewes could have been exposed to a period of natural stress such as a drought or persistently cold weather, and during which the groups might react differentially. Figure 8.1a. shows that the LM group was significantly lighter than the other 2 groups (LA and HM) at weaning in 2010. Consideration of local meteorological records suggests that the August to December 2010 was wet and cold resulting in poor pasture growth and likely

feed restriction for lactating ewes. However, if the LM-daughters were to be better adapted to the stressful uterine environment they were exposed to in 2005, it would be expected that their mean live-weight would be heavier rather than lighter than the other groups. It would seem that these results are not consistent with a predictive adaptive response (Gluckman and Hanson, 2004). In other words, such considerations reinforce the view showed that dam nutrition and size restriction may change early life development of female offspring but does not alter their lifetime growth performance.

8.5.2 Reproductive performance

Pregnancy diagnosis and ovulation rate

Investigation of ovarian development using the fetal and adult counterparts to the daughters used in this study suggest ewe nutrition during pregnancy appears to affect fetal (fetuses from M-dams had higher number of follicles than fetuses from A-dams) but not adult follicular cell number (Chapter 7), and fetal ovarian weight (ovaries were lighter in fetuses from M-dams than A-dams) at day 140 of gestation (Blair *et al*., 2011). The present study demonstrated that differing dam nutrition did not alter ovulation rate of their female offspring at 2050 days of age or total lifetime number of fetuses. Van der Linden *et al*. (2010a) has previously reported in these daughters that there was no effect on number of fetuses present at 18 months of age. These results are in agreement with Parr *et al*. (1986) and Gunn *et al.* (1995), who all reported no effect of maternal nutrition (during mid-to-late pregnancy and weaning) on ovulation rate of the offspring. The present study reinforces the suggestion of Parr *et al*. (1986) and Gunn *et al*. (1995) that differences occurring at an ovary cellular level do not necessarily result in changes in ovulation rate and number of fetuses.

Ewe size in the present study had no effect on daughter's ovulation rate and number of fetus. However, there was an effect of dam size on adult cohort data, where daughters from H-dams have more follicles than daughters from L-dams (Chapter 7). The absence of an effect in the present study on ovulation might also be a reflection of there being minimal effects on live weight and body condition score between progeny born to dam of disparate size. Ewe reproductive performance is known to be positively related to their live weight (Smith, 1991; Scaramuzzi *et al*., 2006). In addition, Ferguson *et al*. (2011) showed that the reproductive rate (lambs scanned in utero per wew joined) of individual ewes increased with live weight at mating. Thus, given the similar weights of offspring it is not surprising there is no detectable difference in reproduction measures of number of fetuses and ovulation rate.

Number of lambs born and weaned per daughter and grand offspring live weights

Lifetime reproductive success of females is commonly determined by the number of offspring weaned (Gaillard *et al.*, 2000). Steinheim *et al.* (2002) demonstrated that lighter dams can lead to a reduced lifetime reproductive success in their daughters measured as the number of offspring (grand progeny) produced. In the present study, there was a modest effect of dam size on the total number of grand progeny born based on all daughters present in 2007 only. However overall, there was no effect of dam size or nutrition on total lifetime number of grand offspring weaned, the total lifetime kg of lamb weaned or on the lifetime performance efficiency of the daughter. Van der Linden *et al.* (2010a) reported that dam size and nutrition did not affect the total number of grand offspring born or weaned in the daughter's first lactation, however, daughters born to M-dams had a greater weight of lamb weaned, likely due to their higher milk

production in that lactation (van der Linden *et al*., 2009) compared to daughters born to A-dams. In contrast in their second lactation, grand-progeny of HA-dams were heavier at weaning than were the grand progeny of HM-, LA- and LM-dams, but in that study lactational performance was not influenced (Blair *et al*., 2010). These conflicting milk and progeny live weight results from the first and second parities and the lack of difference in number of lambs weaned likely explain the absence of lifetime difference productivity in the daughters.

Longevity

The percentage of the daughters still present in the flock at the end of the study was not affected by either dam size or nutrition. Studies examining the effects of maternal manipulation on daughter longevity in sheep are not numerous. Coop and Clark (1955) reported a slight increase in death rate and a reduction in longevity of sheep due to nutritional restriction in early life. Bérubé *et al*. (1999) found that longevity in Bighorn ewes was positively correlated with body weight and Morgan-Davies *et al*. (2008) reported that the survival of ewes within the flock was influenced by body condition score. Therefore, given the general absence of differences in live weight and body condition score in the daughters the lack of a difference in longevity might be expected.

8.6 Conclusions

The lifetime performance results of the daughters studied in this experiment indicated only minor inconsistent effects on live weight and proportion that ovulated due to dam size and nutrition during pregnancy. Overall, in terms of the number and weight of

grand offspring, daughter efficiency and longevity, dam size and nutrition during pregnancy had no observable effect. This might be due to the nutrition applied in the present study was not severe enough to affect daughters' performance. However, if the offspring are continuously exposed to nutrition treatment (nutritional mismatch) after birth, their later life performance could potentially be affected. These results suggest that under the conditions of the present study, farmers do not need to take into account dam size and nutrition when selecting replacement ewes.

8.7 Acknowledgements

The authors are grateful to the Gravida, National Centre for Growth and Development and Massey University for providing funding for this project. The first author is funded by Ministry of Higher Education (Malaysia) Doctoral Scholarship. The authors would like to thank to M.F.P. Loureiro who assisted with ovulation rate data collection.

8.8 References

Barker, D. J., Gluckman, P. D., Godfrey, K. M., Harding, J. E., Owens, J. A., & Robinson, J. S. (1993). Fetal nutrition and cardiovascular disease in adult life. *Lancet, 341*(8850), 938-941.

Bérubé, C. H., Festa-Bianchet, M., & Jorgenson, J. T. (1999). Individual differences, longevity, and reproductive senescence in bighorn ewes. *Ecology, 80*(8), 2555-2565.

Bizelis, J. A., Charismiadou, M. A., & Rodkais, E. (2000). Metabolic changes during the perinatal period in dairy sheep in relation to level of nutrition and breed. II. Early lactation. *Journal of Animal Physiology & Animal Nutrition, 84*(3/4), 73-84.

Blair, H. T., Jenkinson, C. M. C., Peterson, S. W., Kenyon, P. R., van der Linden, D. S., Davenport, L. C., Mackenzie, D. D. S., Morris, S. T., & Firth, E. C. (2010). Dam and granddam feeding during pregnancy in sheep affects milk supply in offspring and reproductive performance in grand-offspring. *Journal of Animal Science, 88*(13), E40-E50.

Blair, H. T., van der Linden, D. S., Jenkinson, C. M. C., Morris, S. T., Mackenzie, D. D. S., Peterson, S. W., Firth, E. C., & Kenyon, P.R. (2011). Do ewe size and nutrition during pregnancy affect foetus and foetal organ weight in twins? *Livestock Science, 142*(1-3), 99-107.

Borwick, S. C., Rae, M. T., Brooks, J., McNeilly, A. S., Racey, P. A., & Rhind, S. M. (2003). Undernutrition of ewe lambs *in utero* and in early post-natal life does not affect hypothalamic–pituitary function in adulthood. *Animal Reproduction Science, 77*(1-2), 61-70.

Coop, I. E., & Clark, V. R. (1955). The influence of method of rearing as hoggets on the lifetime productivity of sheep. *New Zealand Journal of Science and Technology, 37A*, 214-228.

Da Silva, P., Aitken, R. P., Rhind, S. M., Racey, P. A., & Wallace, J. M. (2001). Influence of placentally mediated fetal growth restriction on the onset of puberty in male and female lambs. *Reproduction, 122*(3), 375-383.

Deligeorgis, S. G., Chadio, S., & Menegatos, J. (1996). Pituitary responsiveness to GnRH in lambs undernourished during fetal life. *Animal Reproduction Science, 43*(2-3), 113-121.

Dupont, C., Cordier, A. G., Junien, C., Mandon-Pépin, B., Levy, R., & Chavatte-Palmer, P. (2012). Maternal environment and the reproductive function of the offspring. *Theriogenology, 78*(7), 1405-1414.

Edwards, L. J., & McMillen, I. C. (2002). Impact of maternal undernutrition during the periconceptional period, fetal number, and fetal sex on the development of the hypothalamo-pituitary adrenal axis in sheep during late gestation. *Biology of Reproduction, 66*(5), 1562-1569.

Ferguson, M. B., Thompson, A. N., Gordon, D. J., M. W. Hyder, Kearney, G. A., Oldham, C. M., & Paganoni, B. L. (2011). The wool production and reproduction of Merino ewes can be predicted from changes in liveweight during pregnancy and lactation. *Animal Production Science* (51), 763-775.

Gaillard, J. M., Festa-Bianchet, M., Delorme, D., & Jorgenson, J. (2000). Body mass and individual fitness in female ungulates: bigger is not always better. *Proceedings of the Royal Society B-Biological Sciences, 267*(1442), 471-477.

Gardner, D. S., Buttery, P. J., Daniel, Z., & Symonds, M. E. (2007). Factors affecting birth weight in sheep: maternal environment. *Reproduction, 133*(1), 297-307.

Gardner, D. S., Ozanne, S. E., & Sinclair, K. D. (2009). Effect of the early-life nutritional environment on fecundity and fertility of mammals. *Philosophical Transactions of the Royal Society B-Biological Sciences, 364*(1534), 3419-3427.

Gluckman, P. D., & Hanson, M. A. (2004). Living with the past: Evolution, development, and patterns of disease. *Science, 305*(5691), 1733-1736.

Godfrey, K. M., & Barker, D. J. (2000). Fetal nutrition and adult disease. *The American Journal of Clinical Nutrition, 71*(5), 1344S-1352S.

Gootwine, E., Spencer, T. E., & Bazer, F. W. (2007). Litter-size-dependent intrauterine growth restriction in sheep. *Animal, 1*(04), 547-564.

Gunn, R. G., Sim, D. A., & Hunter, E. A. (1995). Effects of nutrition in-utero and in early-life on the subsequent lifetime reproductive-performance of Scottish blackface ewes in 2 management-systems. *Animal Science, 60*, 223-230.

Jefferies, B. C. (1961). Body condition scoring and its use in management. *Tasmanian Journal of Agriculture, 32*, 19-21.

Kelly, R. W., Greeff, J. C., & Macleod, I. (2006). Lifetime changes in wool production of Merino sheep following differential feeding in fetal and early life. *Australian Journal of Agricultural Research, 57*(8), 867-876.

Kenyon, P. R. (2008). A review of in-utero environmental effects on sheep production. *Proceedings New Zealand Society of Animal Production, 68*, 142-155.

Kenyon, P. R., Blair, H. T., Jenkinson, C. M. C., Morris, S. T., Mackenzie, D. D. S., Peterson, S. W., Firth, E. C., & Johnston, P. L. (2009). The effect of ewe size and nutritional regimen beginning in early pregnancy on ewe and lamb performance to weaning. *New Zealand Journal of Agricultural Research, 52*(2), 203-212.

Kenyon, P. R., Morel, P. C. H., & Morris, S. T. (2004). Effect of liveweight and condition score of ewes at mating, and shearing mid-pregnancy, on birthweights and growth rates of twin lambs to weaning. *New Zealand Veterinary Journal, 52*(3), 145-149.

Kenyon, P. R., van der Linden, D. S., Blair, H. T., Morris, S. T., Jenkinson, C. M. C., Peterson, S. W., Mackenzie, D. D. S., & Firth, E. C. (2011a). Effects of dam size and nutritional plane during pregnancy on lamb performance to weaning. *Small Ruminant Research, 97*(1-3), 21-27.

Kenyon, P. R., van der Linden, D. S., Jenkinson, C. M. C., Morris, S. T., Mackenzie, D. D. S., Peterson, S. W., Firth, E. C., & Blair, H. T. (2011b). The effect of ewe size and nutritional regimen beginning in early pregnancy on development of singleton foetuses in late pregnancy. *Livestock Science, 142*(1-3), 92-98.

Morgan-Davies, C., Waterhouse, A., Pollock, M. L., & Milner, J. M. (2008). Body condition score as an indicator of ewe survival under extensive conditions. *Animal Welfare, 17*(1), 71-77.

Munoz, C., Carson, A. F., McCoy, M. A., Dawson, L. E. R., Wylie, A. R. G., & Gordon, A. W. (2009). Effects of plane of nutrition of ewes in early and mid-pregnancy on performance of the offspring: Female reproduction and male carcass characteristics. *Journal of Animal Science, 87*(11), 3647-3655.

Neville, T. L., Caton, J. S., Hammer, C. J., Reed, J. J., Luther, J. S., Taylor, J. B., Redmer, D. A., Reynolds, L. P., & Vonnahme, K. A. (2010). Ovine offspring growth and diet digestibility are influenced by maternal selenium supplementation and nutritional intake during pregnancy despite a common post-natal diet. *Journal of Animal Science, 88*(11), 3645-3656.

Nicol, A. M., & Brookes, I. M. (2007). The metabolisable energy requirements of grazing livestock. In P. V. Rattray, I. M. Brookes & A. M. Nicol (Eds.), *Pasture*

and Supplements for Grazing Animals (pp. 151-172): New Zealand Society of Animal Production Occasional Publication.

O'Doherty, J. V., & Crosby, T. F. (1996). The effect of diet in late pregnancy on progesterone concentration and colostrum yield in ewes. *Theriogenology, 46*(2), 233-241.

Parr, R. A., Williams, A. H., Campbell, I. P., Witcombe, G. F., & Roberts, A. M. (1986). Low nutrition of ewes in early pregnancy and the residual effect on the offspring. *The Journal of Agricultural Science, 106*(01), 81-87.

Rae, M. T., Kyle, C. E., Miller, D. W., Hammond, A. J., Brooks, A. N., & Rhind, S. M. (2002). The effects of undernutrition, *in utero*, on reproductive function in adult male and female sheep. *Animal Reproduction Science, 72*(1-2), 63-71.

Rattray, P. V., & Trigg, T. E. (1979). Minimal feeding of pregnant ewes. *Proceeding of the New Zealand Society of Animal Production, 39*, 242-250.

Redmer, D. A., Wallace, J. M., & Reynolds, L. P. (2004). Effect of nutrient intake during pregnancy on fetal and placental growth and vascular development. *Domestic Animal Endocrinology, 27*(3), 199-217.

Rhind, S. M. (2004). Effects of maternal nutrition on fetal and neonatal reproductive development and function. *Animal Reproduction Science, 82-83*, 169-181.

Rhind, S. M., Rae, M. T., & Brooks, A. N. (2001). Effects of nutrition and environmental factors on the fetal programming of the reproductive axis. *Reproduction, 122*(2), 205-214.

SAS. (2008). Statistical Analysis System. SAS Institute Incorporation, Cary, North Carolina, USA.

Scaramuzzi, R. J., Campbell, B. K., Downing, J. A., Kendall, N. R., Khalid, M., Munoz-Gutierrez, M., & Somchit, A. (2006). A review of the effects of supplementary nutrition in the ewe on the concentrations of reproductive and metabolic hormones and the mechanisms that regulate folliculogenesis and ovulation rate. *Reproduction Nutrition Development, 46*(4), 339-354.

Smith, J. F. (1991). A review of recent development on the effect of nutrition on ovulation rate (the flushing effect) with particular reference to research at Ruakura. *Proceedings of the New Zealand Society of Animal Production, 51*, 15-23.

Steinheim, G., Mysterud, A., Holand, O., Bakken, M., & Adnoy, T. (2002). The effect of initial weight of the ewe on later reproductive effort in domestic sheep (*Ovis aries*). *Journal of Zoology, 258*, 515-520.

van der Linden, D. S., Kenyon, P. R., Blair, H. T., Lopez-Villalobos, N., Jenkinson, C. M. C., Peterson, S. W., & Mackenzie, D. D. S. (2009). Effects of ewe size and nutrition on fetal mammary gland development and lactational performance of offspring at their first lactation. *Journal of Animal Science, 87*(12), 3944-3954.

van der Linden, D. S., Kenyon, P. R., Blair, H. T., Lopez-Villalobos, N., Jenkinson, C. M. C., Peterson, S. W., & Mackenzie, D. D. S. (2010b). Effects of ewe size and

nutrition during pregnancy on glucose metabolism, fat metabolism and adrenal function of postpubertal female twin offspring. *Animal Production Science, 50*(9), 869-879.

van der Linden, D. S., Kenyon, P. R., Lopez-Villalobos, N., Jenkinson, C. M. C., Peterson, S. W., & Blair, H. T. (2010a). Effects of ewe size and nutrition during pregnancy on performance of 2-year-old female offspring. *Journal of Agricultural Science, 148*, 465-475.

van der Linden, D. S., Kenyon, P. R., Blair, H. T., Lopez-Villalobos, N., Jenkinson, C. M. C., Peterson, S. W., & Mackenzie, D. D. S. (2010c). Relationships between early post-natal growth and metabolic function of 16-month-old female offspring born to ewes exposed to different environments during pregnancy. *Journal of Developmental Origins of Health and Disease, 1*(1), 50-59.

van der Linden, D. S., Kenyon, P. R., Jenkinson, C. M. C., Peterson, S. W., Lopez-Villalobos, N., & Blair, H. T. (2007). The effects of ewe size and nutrition during pregnancy on growth and onset of puberty in female progeny. *Proceedings of the New Zealand Society of Animal Production, 67*, 126-129.

Viñoles, C., Gonzalez de Bulnes A., Martin G. B., Sales, F., & Sale, S. (2010). Sheep and goats. In L. Des Coteaux, J. Colloton, & G. Gnemi (Eds.), *Atlas of ruminant and camelid reproductive ultrasonography*. Ames, Iowa, US Wiley-Blackwell.

Vonnahme, K. A., Hess, B. W., Hansen, T. R., McCormick, R. J., Rule, D. C., Moss, G. E., Murdoch, W. J., Nijland, M. J., Skinner, D. C., Nathanielsz, P. W., & Ford, S. P. (2003). Maternal undernutrition from early- to mid-gestation leads to growth retardation, cardiac ventricular hypertrophy, and increased liver weight in the fetal sheep. *Biology of Reproduction, 69*(1), 133-140.

Wu, G., Bazer, F. W., Wallace, J. M., & Spencer, T. E. (2006). Board-Invited Review: Intrauterine growth retardation: Implications for the animal sciences. *Journal of Animal Science, 84*(9), 2316-2337.

.

Chapter 9

Effects of dam nutrition during pregnancy on fetal ovarian oxidative stress and reproductive performance of ewe progeny

9.1 Abstract

Fetal programming studies have shown that maternal nutrition during pregnancy can affect the reproductive performance of ewes' daughters during both their pre-natal and post-natal life. The aim of this study was to determine the effect of maternal nutritional treatments (*ad libitum* (A_1; to provide unrestricted access to pasture forage) *vs* maintenance (M_1; to ensure no change in total ewe live weight) *vs* sub maintenance (Sm_1; to achieve a loss in total ewe live weight og 0.1 kg/day)) between Days 21 and 50 (P21-50) of pregnancy and nutritional treatments between P50 and P139 (A_2 *vs* M_2) on the oxidative stress level in fetal ovaries on P140, and on the reproductive performance of the daughters. On P140, twin-bearing dams were euthanised (n=44) and fetal ovaries collected. The fetal ovaries were examined for levels of protein carbonyl and hyperoxidised peroxiredoxin 3 (Prx 3) as biomarkers of oxidative stress. In addition, ewe reproductive performance at nine, 20 and 22 months of age were determined. There was no effect of either P21-50 or P50-139 nutritional treatments on protein carbonyl level (P=0.614) or hyperoxidised Prx 3 in fetal ovaries, or the percentage of ewe lambs reaching puberty, ovulation rate, pregnancy diagnosis and number of fetuses in two-year-old daughters. These results suggest that the levels of maternal nutrition applied to ewes in the present study do not appear to affect the reproductive development and performance of their daughters. This suggest under the conditions of the present study farmers may not need to take into account dam nutrition when selecting replacement ewes.

9.2 Introduction

Under New Zealand's spring lambing pastoral conditions, late pregnancy occurs during winter, when pasture growth and availability can be low (Moot *et al*., 2007; Kenyon and Webby, 2007). This may create a situation where optimal maternal feeding levels are not met, thereby affecting fetal nutrient supply especially for multiple-bearing ewes. Evidence in the literature suggests that restricted maternal nutrition can either increase (Da Silva *et al*., 2002) or reduce (Bernal *et al*., 2010) fetal follicular cell development, reduce age at puberty (Sloboda *et al*., 2009), and reduce ovulation rate (Rae *et al*., 2002) in female offspring.

A growing pool of evidence in sheep (Murdoch *et al*., 2003) and rodents (Bernal *et al*., 2010; Igosheva *et al*., 2010; Tarry-Adkins *et al*., 2010) shows that undernutrition during pre-natal life is associated with alteration of oxidative stress levels in the offspring. Bernal *et al*. (2010) showed that nutritional status of the mother during pregnancy altered both ovarian follicular cell development and the oxidative stress level of the offspring. Increased levels of oxidative stress were associated with decreased ovarian follicle numbers (Bernal *et al*., 2010). This notion is supported by Agarwal *et al*. (2005) who showed that oxidative stress can influence the fertility of the female offspring, due to effects on the modulation of reproductive functions including oocyte maturation, ovarian steroidogenesis, luteal function and luteolysis.

It is therefore postulated that alteration of oxidative stress level during pre-natal life due to altered maternal nutrition of twin-bearing ewes would alter later-life reproductive performance of female offspring. Thus, the objectives of this study were to examine the effects of maternal nutrition during pregnancy on fetal ovarian oxidative stress level and post-natal reproductive performance of twin female sheep offspring.

9.3 Materials and methods

The study was conducted at the Massey University Keeble Sheep and Beef farm (latitude 41° 10'S longitude 175° 35'E), 5 km south of Palmerston North, New Zealand. All experimental animal procedures were approved by the Massey University Animal Ethics Committee (MUAEC 09/18), Palmerston North, New Zealand.

9.3.1 Experimental animal

A full description of experimental design has been published by Kenyon *et al.* (2011) and previously described in Chapter 6. Romney ewes (n = 1169, average live weight 66.3 kg ± 0.18, condition score 2.96 ± 0.02) from commercial flock were artificially inseminated using fresh semen from one of five Romney rams (Day 0) in April (i.e. autumn) 2009 (Kenyon *et al*., 2011). Twenty one days after insemination ewes were randomly allocated to one of three nutritional treatments from Days 21 to 50 (P21-50) of pregnancy.

1) sub maintenance (Sm_1 (P21 = 66.0 ± 0.58, P30 = 63.8 ± 0.65, P50 = 62.2 ± 0.56 kg) with the aim of achieving a loss in mean ewe live weight of 0.1 kg/d through restricting access to forage with a pre- and post-grazing mass of 996 ± 89.3 and 814 ± 54.2 kg DM/ha)
2) maintenance (M_1 (P21 = 65.6 ± 0.55, P30 = 66.8 ± 0.61, P50 = 65.1 ± 0.53 kg) with the aim of achieving no change in mean live weight; through managing access to forage with a pre- and post-grazing mass of 1479 ± 107.7 and 1112 ± 59.4 kg DM/ha)
3) *ad libitum* (A_1 (P21 = 66.2 ± 0.55, P30 = 66.6 ± 0.61, P50 = 69.5 ± 0.53 kg) to provide unrestricted pasture intake conditions; through managing access to

forage with a pre- and post-grazing mass of 2331 ± 82.0 and 1649 ± 54.2 kg DM/ha.

On P50, non pregnant and single and triplet bearing ewes were removed for the remainder of the study. The remaining twin bearing ewes were randomly re-allocated to one of two further nutritional treatments until P139 (Kenyon *et al.*, 2011):

1) M_2 (P69 = 64.9 ± 0.47, P113 = 71.8 ± 0.48, P137 = 82.6 ± 0.53 kg) to achieve a total ewe live weight increased similar to that of the expected conceptus mass (Rattray *et al.*, 1974); through managing access to forage with a pre- and post-grazing mass of 1450 ± 83.9 and 1011 ± 32.8 kg DM/ha
2) A_2 (P69 = 75.4 ± 0.48, P113 = 80.5 ± 0.48, P137 = 88.2 ± 0.53 kg) to provide unrestricted pasture intake conditions; through managing access to forage with a pre- and post-grazing mass of 1828 ± 76.0 and 1301 ± 37.8 kg DM/ha)

Therefore, this study had a 3 x 2 factorial design (A_1A_2, A_1M_2, M_1A_2, M_1M_2, Sm_1A_2 and Sm_1M_2). From P139, all ewe groups were merged and managed under commercial grazing conditions until weaning (Kenyon, *et al.*, 2011).

The nutritional regimens divided the timing of the maternal nutritional treatments into two different windows; early (P21 – P50) and mid-to-late pregnancy (P50 – P139) and were chosen due the duration of the nutritional restrictions studied in Chapter 5, 7 and 8 (the dam size and pregnancy nutrition study) being relatively long (P21 – P140) making it difficult to define the exact periods of pregnancy that resulted in the observed effects.

Forty-four twin-bearing ewes were euthanised on P140, resulting in 60 female fetuses (A_1A_2 n = 9; A_1M_2 n = 8; M_1A_2 n = 10; M_1M_2 n = 8; Sm_1A_2 n = 14 and Sm_1M_2 n = 11 (Martín *et al.*, 2012). Post weaning, all ewe progeny were managed under commercial conditions and their reproductive performance measured to 678 days of age. Therefore,

the present study examines two cohorts of female offspring from the study of Kenyon *et al*. (2011). Firstly fetuses at 140 days age and young ewes to twenty-two months of age.

9.3.2 Measurement of oxidative stress in fetal ovarian tissue

Fetal ovaries (one per fetus) from each group were randomly selected, dissected, weighed and snap-frozen in liquid nitrogen and stored at -80°C. Frozen ovarian tissues were thawed and homogenized in 100 µl of phosphate buffer saline pH 7.4 (PBS) containing 10 µM diethylene triamine pentaacetic acid (DTPA) and 20 µM of butylated hydroxytoluene (BHT). Protein concentration was measured using the Bradford dye-binding procedure (Bradford, 1976) and expressed as µg/µL. Aliquots of the homogenate (70-100 µL) were set aside for protein carbonyl detection. The remainder was treated with Complete^{TM} protease inhibitor (Roche Applied Science, Mannheim, Germany) and used for the detection of Prx 3 hyperoxidation using Western blotting.

Protein carbonyl ELISA assay

Protein carbonyl concentrations were determined by an enzyme-linked immunosorbent assay (ELISA) after derivatisation of samples with 2,4-dinitrophenylhydrazine (DNP) using the standard procedure suggested in the BioCell Protein Carbonyl Assay kit (BioCell, Papatoetoe, New Zealand). The concentrations of protein carbonyl were read at 450 nm.

Peroxiredoxin 3 (Prx 3) western blotting analysis

Thirty fetal ovarian samples (5 animals per group) were randomly selected for the detection of Prx 3 hyperoxidation. Protein samples (10 µg) were separated on 12% SDS-polyacrylamide gel under non-reducing conditions and blotted onto Hybond PVDF membranes (GE Healthcare Life Sciences, Piscataway, NJ, USA). The membranes were blocked in 5% skim milk TBS-T_{20} (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, and 0.05% Tween 20) for 1 hour at room temperature. These membranes were then incubated overnight at 4°C with rabbit anti-Prx 3 polyclonal antibody (1:10,000; Ab Frontier, Seoul, Korea). After washing 3 times for 5 minutes each (TBS-T_{20}), the membranes were incubated with peroxidise-conjugated goat anti-rabit secondary antibody and visualised using the ECL Plus Western Blotting Detection Reagents (GE Healthcare Life Sciences, Piscataway, NJ, USA) with the Chemidoc XRS gel documentation system and Quantify One software (BioRad laboratories, Hercules, CA, USA) to quantify the density of the relevant bands (Figure 9.1.).

9.3.3 Puberty attainment at approximately 9 months of age

Eight crayon-harnessed vasectomised rams were placed with the ewe-lambs (n = 370) at 261 days of age (27th May 2010, winter solstice 21 July) for a period of 17 days. Crayon marks displayed on the rump of ewe lambs were used as an indication of puberty attainment.

9.3.4 Ovulation rate of ewe at approximately 20 months of age

At 607 days of age (April 2011) the ewe progeny (n = 252) underwent oestrus synchronization using intravaginal progesterone-releasing inserts (CIDR, 0.3 g progesterone, Pharmacia & Upjohn, Auckland, New Zealand) for 12 days. The ewes were then bred with Romney rams eight days after CIDR removal, each ovary was viewed using a transrectal ultrasound probe (B-mode scanner, Mindray DP 6600, 50mm linear 7.5 mHz probe) and the total number of corpora lutea (CL) were counted as proxy for ovulation rate (Viñoles, *et al*., 2010).

9.3.5 Pregnancy diagnosis

In July 2011 (at 672 days of age) pregnancy status was diagnosed by transabdominal ultrasound scanning, and the ewes were classified as being non-pregnant, single-, twin- or triplet-bearing.

9.3.6 Statistical analysis

Statistical analysis was carried out using the Statistical Analysis System (SAS, 2008; SAS 9.2, SAS Institute, North Carolina, USA) for analysis of variance. The main effects i.e. feeding treatment during early (P21-50) and mid-to-late pregnancy (P50-139), and the interactions between early and mid-to-late pregnancy remained in all models irrespective of whether they were significant or not to allow for testing of the study design.

Protein carbonyl concentration

Data for protein carbonyl concentration in the present study could not be normalised. Therefore, the data was analysed using a non-parametric test (Kruskal-Wallis Test). Data were then analysed for mean values with nutritional treatments as fixed effects to obtain median and confidence limits of the mean for each nutritional treatment in each time period and the interaction between early and mid-to-late pregnancy nutrition treatments. These analyses were undertaken using the NPAR1WAY and MEANS procedures, respectively.

The level of Prx 3 could not be statistically analysed due to no band being detected on the 20 kDa of western blot membrane.

Puberty attainment and ovulation rate

The proportion of ewe lambs that displayed estrus (indicating puberty attainment) between 261 to 278 days of age was analysed using a general linear model. Fixed effects included: sire, nutrition treatments in early and mid-to-late pregnancy. Two-way and three-way interactions were included in the initial model but removed if found to be non-significant ($P<0.05$) and the model re-fitted; with and without live weight at puberty as a covariate. Date of birth was also included as a covariate. Post-hoc differences between the groups were detected using least significant differences. The puberty attainment data was logit transformed prior to analysis and back-transformed using inverse-logit to obtain the mean proportions. This analysis was undertaken using the GENMOD procedure.

Ovulation rate and proportion of daughters that ovulated were analysed using a general linear model. To examine the apparent ovulation rate of daughters, the total number of corpora lutea present in the ovaries of each ewe was recorded at 607 days (approximately 2 months) of age. To determine the proportion of daughters that actually ovulated at 607, daughters with at least one corpus luteum were given a nominal value of 1 (ovulated) and daughters without corpus luteum were given a nominal value as 0 (non-ovulated). Fixed effects included: sire, nutrition treatments in early and mid-to-late pregnancy. Two-way and three-way interactions were included in the initial model but removed if found to be non-significant ($P<0.05$) and the model re-fitted. Date of birth was included as a covariate. Post-hoc differences between the groups were detected using least significant differences. This analysis was undertaken using the GENMOD procedure.

Pregnancy diagnosis and number of fetuses

Pregnancy diagnosis and number of fetuses conceived were analysed using general linear model. Fixed effects included: sire, nutrition treatments in early and mid-to-late pregnancy. Two-way and three-way interactions were included in the initial model but removed if found to be non-significant ($P<0.05$) and the model re-fitted. Date of birth was included as a covariate. Post-hoc differences between the groups were detected using least significant differences. This analysis was undertaken using the GENMOD procedure.

9.4 Results

9.4.1 Protein carbonyl concentration and hyperoxidised Peroxiredoxin 3

There was no effect (P>0.05) of dam nutrition during either early or mid-to-late pregnancy nor was there an interaction (P>0.05) between nutrition treatment periods, on the level of protein carbonyl (Table 9.1.) or hyperoxidised Prx 3 (Figure 9.1.) in fetal ovaries on P140.

9.4.2 Puberty attainment and ovulation rate

The proportion of daughters that reach puberty was not affected ($P > 0.05$) by either dam nutrition during early pregnancy, mid-to-late pregnancy or nor was there an interaction between early and mid-to-late pregnancy (Table 9.2.).

There was no effect (P>0.05) of dam nutrition during either early (P21-50) or mid-to-late pregnancy (P50-139), nor was there an interaction (P>0.05) between nutrition treatment period on the proportion of daughters that ovulated (Table 9.2.). Likewise, there was no effect (P>0.05) of dam nutrition during either early or mid-to-late pregnancy, nor was there an interaction (P>0.05) between nutrition treatment periods, on the ovulation rate of their daughters.

9.4.3 Pregnancy diagnosis and number of fetuses

There was no effect (P>0.05) of dam nutrition during early and mid-to-late pregnancy, nor was there an interaction (P>0.05) between nutrition treatment periods, on either pregnancy diagnosis or the number of fetuses carried by their daughters (Table 9.3.).

Table 9.1. Effect of dam nutrition during early pregnancy (P21-50; either sub maintenance (Sm_1), maintenance (M_1) or *ad libitum* (A_1) nutrition) and mid-to-late pregnancy (P50-140; either maintenance (M_2) or *ad libitum* (A_2) nutrition) on the protein carbonyl content (nmol/mg) in fetal ovaries at Day 140 of gestation (P140). Median and 95% confidence limits (CL) data are provided

Treatment	n	Median (min – max)	95% CL for mean (lower-upper limit)
Early pregnancy			
A_1	17	0.12 (0.06 – 0.21)	0.10 – 0.14
M_1	18	0.13 (0.04 – 0.30)	0.11 – 0.17
Sm_1	24	0.12 (0.04 – 0.33)	0.11 – 0.19
Mid-to-late pregnancy			
A_2	32	0.12 (0.04 – 0.33)	0.11 – 0.17
M_2	27	0.12 (0.05 – 0.33)	0.11 – 0.16
Early x Mid-to-late pregnancy [(1)]			
A_1A_2	9	0.12 (0.09 – 0.21)	0.10 – 0.17
A_1M_2	8	0.09 (0.06 – 0.16)	0.07 – 0.13
M_1A_2	10	0.15 (0.04 – 0.30)	0.09 – 0.20
M_1M_2	8	0.12 (0.09 – 0.19)	0.10 – 0.16
Sm_1A_2	13	0.10 (0.04 – 0.33)	0.09 – 0.20
Sm_1M_2	11	0.13 (0.05 – 0.33)	0.10 – 0.22

[(1)] Early x Mid-to-late pregnancy: interaction between treatments.

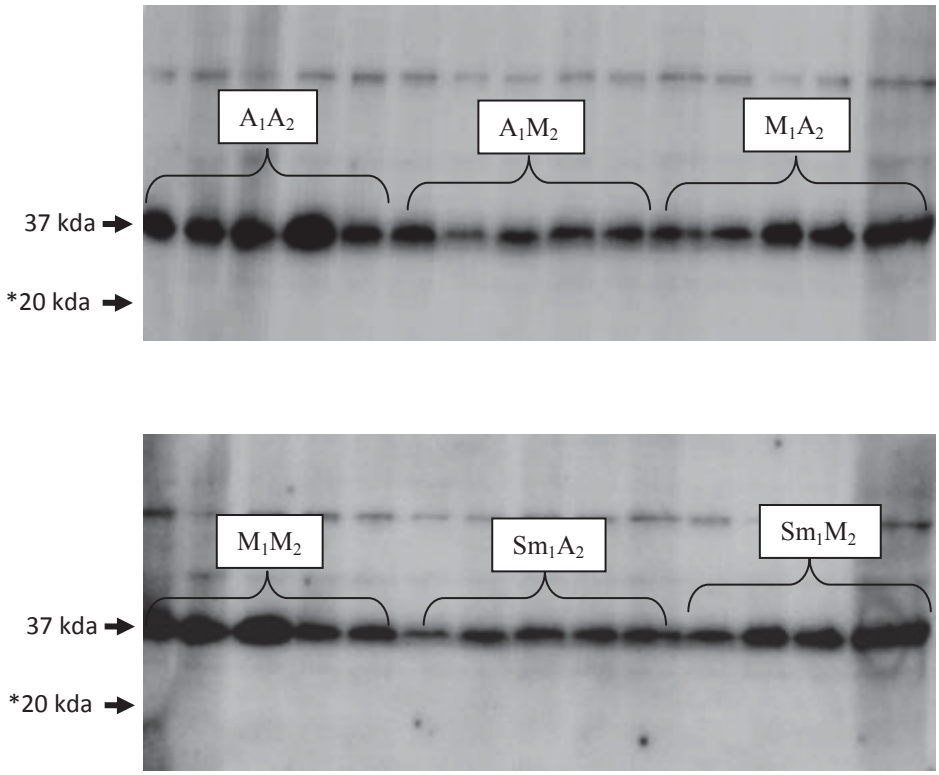


Figure 9.1. Effect of dam nutrition during early (P21-50; either sub maintenance (Sm_1), maintenance (M_1) or *ad libitum* (A_1) nutrition) and mid-to-late pregnancy (P50-140; either maintenance (M_2) or *ad libitum* (A_2) nutrition) on the hyperoxidised peroxiredoxin 3 (Prx 3) of fetal ovaries at Day 140 gestation. Representative non-reducing Western blot of Prx 3; Prx 3 is visualised as a 37kDa band, hyperoxidised Prx 3 is visualised as a low molecular weight band (20 kDa) The columns represent the 30 animals that were used for the detection of Prx 3 hyperoxidation. (*no band was observed on the 20 kDa, which show no hyperoxidised form of Prx 3).

Table 9.2. Effect of dam nutrition during early (P21-50; either sub maintenance (Sm_1), maintenance (M_1) or *ad libitum* (A_1) nutrition) and mid-to-late pregnancy (P50-140; either maintenance (M_2) or *ad libitum* (A_2) nutrition) on back-transformed percentages (± 95% confidence interval) of ewe lambs that attained puberty (during Days 261 to 278 post partum), the apparent ovulation rate (at Day 607 post partum) and the percentage which ovulated post-synchronisation (at Day 607 post partum). Table shows least square means (±SE) for ovulation rate and proportion that ovulated.

Treatment	*n*	Puberty attainment by Day 278 post partum (%) (Confidence intervals in parenthesis)	*n*	Ovulation rate	*n*	Proportion that ovulated
Early pregnancy						
A_1	*144*	51.9 (50.8, 53.2)	*90*	1.8 ± 0.1	*94*	1.0 ± 0.0
M_1	*124*	37.6 (36.3, 38.8)	*93*	2.0 ± 0.1	*94*	1.0 ± 0.0
Sm_1	*102*	37.1 (35.7, 38.4)	*62*	1.9 ± 0.1	*64*	1.0 ± 0.0
Mid-to-late pregnancy						
A_2	*174*	36.2 (35.0, 37.3)	*111*	1.9 ± 0.1	*114*	1.0 ± 0.0
M_2	*196*	48.2 (47.2, 49.3)	*134*	1.8 ± 0.1	*138*	1.0 ± 0.0
Early x Mid-to-late pregnancy [(1)]						
A_1A_2	*70*	46.5 (44.8, 48.3)	*43*	1.8 ± 0.1	*44*	1.0 ± 0.0
A_1M_2	*74*	57.3 (55.5, 58.9)	*47*	1.8 ± 0.1	*50*	0.9 ± 0.0
M_1A_2	*57*	30.5 (28.8, 32.2)	*41*	1.9 ± 0.1	*42*	1.0 ± 0.0
M_1M_2	*67*	45.2 (43.5, 46.9)	*52*	2.0 ± 0.1	*52*	1.0 ± 0.0
Sm_1A_2	*47*	32.4 (30.2, 34.5)	*27*	2.0 ± 0.1	*28*	1.0 ± 0.0
Sm_1M_2	*55*	42.2 (40.2, 44.0)	*35*	1.8 ± 0.1	*36*	1.0 ± 0.0

[(1)] Early x Mid-to-late pregnancy: interaction between treatments

Table 9.3. Effect of dam nutrition during early pregnancy (P21-50; either sub maintenance (Sm_1), maintenance (M_1) or *ad libitum* (A_1) nutrition) and mid-to-late pregnancy (P50-140; either maintenance (M_2) or *ad libitum* (A_2) nutrition) on pregnancy rate and number of fetuses of their daughters at pregnancy scanning (Day 672 post partum). Table shows least square means (±SE).

Treatment	*n*	Pregnancy rate	*n*	Number of fetuses
Early pregnancy				
A_1	*100*	0.99 ± 0.11	*100*	1.75 ± 0.14
M_1	*92*	0.99 ± 0.10	*92*	1.58 ± 0.13
Sm_1	*70*	0.97 ± 0.13	*70*	1.69 ± 0.17
Mid-to-late pregnancy				
A_2	*122*	0.99 ± 0.10	*122*	1.71 ± 0.13
M_2	*140*	0.98 ± 0.09	*140*	1.63 ± 0.12
Early x Mid-to-late pregnancy [(1)]				
A_1A_2	*52*	0.97 ± 0.15	*52*	1.79 ± 0.20
A_1M_2	*48*	1.00 ± 0.15	*48*	1.71 ± 0.20
M_1A_2	*41*	1.00 ± 0.16	*41*	1.59 ± 0.20
M_1M_2	*51*	0.99 ± 0.14	*51*	1.57 ± 0.18
Sm_1A_2	*29*	1.00 ± 0.20	*29*	1.76 ± 0.27
Sm_1M_2	*41*	0.94 ± 0.17	*41*	1.61 ± 0.22

[(1)] Early x Mid-to-late pregnancy: interaction between treatments

9.5 Discussion

The aim of this study was to investigate the effect of twin-bearing dam nutrition during early and mid-to-late pregnancy on reproductive traits in female fetuses (oxidative stress) during late gestation, and ewes' daughters to 2-years of age (puberty attainment, ovulation rate, pregnancy diagnosis and number of fetuses). This study examined the effect of oxidative stress in fetal ovaries and its potential interaction with maternal

nutrition on ovarian development as previous findings showed that oxidative stress affects the quality of gametes through reaction of free radicals such as reactive oxygen species (ROS) that can influence the physiological process of oocytes maturation, fertilization, embryo development and pregnancy (Agarwal *et al*., 2005). The present study demonstrated that maternal nutrition during pregnancy did not alter the levels of protein carbonyl or hyperoxidised Prx 3 in fetal ovaries in late gestation. This is in contrast to three studies in rodents that showed maternal undernutrition increased the level of protein carbonyl and hyperoxidised Prx 3 in the offspring at 3 months (Tarry-Adkins *et al*., 2009), 5 months (Bernal *et al*., 2010) and 3 and 15 months of age (Tarry-Adkins *et al*., 2010). However, these studies applied 50% nutrition (Bernal *et al*., 2010) and 60% protein restriction from control animals (Tarry-Adkins *et al*., 2009; 2010). Thus, this may indicate that severe under-nutrition can adversely affect the oxidative stress of the fetal ovary, whilst pregnancy sub maintenance diets (small restriction at early pregnancy) or maintenance (no restriction of nutrition) or above maintenance diets do not alter the oxidative stress of the fetal ovary. In addition, species effect might potentially affect on how offspring response with undernourished environment due to physiological differences.

Under the nutritional conditions of the present study the results showed that neither early nor mid-to-late pregnancy affected puberty onset at 19 months of age. This is in agreement with Da Silva *et al*. (2001) and Kotsampasi *et al*. (2009), both of whom showed that maternal nutrition restriction did not alter onset of puberty in ewe-offspring. However, previous findings did show that ovarian development, steroid hormones and follicle development were affected by maternal maintenance nutrition (Da Silva *et al*., 2002). Da Silva *et al*. (2001) and Kotsampasi *et al*. (2009) suggested that ovarian cellular development and/or hormone levels at an early age may not

necessarily result in changes in the timing of attaining puberty. Moreover, the time of attaining puberty is positively related to live weight (Mukasa-Mugerwa *et al*., 1991), and there was no effect of maternal nutritional treatments on live weight at puberty in the present study (Paten *et al*., 2011). In addition, the fetus and the newborn can potentially express compensatory growth which might eliminate the effects of undernutrition (Ryan, 1990).

This study demonstrated that maternal nutrition during early and mid-to-late pregnancy did not alter ovulation rate or numbers of CL present in female offspring at 20 months of age. This result is in agreement with Parr *et al*. (1986) and Gunn *et al.* (1995), who found no effect of maternal nutrition during mid-to-late pregnancy on ovulation rate of the offspring. However, Rae *et al*. (2002) found a reduced ovulation rate in 20-month-old female progeny from dams that were undernourished (50% ME requirement) during early-to-mid pregnancy (P1 – P95). The present study and that of Parr *et al*. (1986) and Gunn *et al*. (1995) suggest that differences at an ovarian cellular level may not influence ovulation rate in ewe-offspring. The effect on ovulation rate reported by Rae *et al*. (2002) might be due to the level of undernutrition, suggesting that significant levels of restriction during pregnancy could alter ovulation rate in female offspring. It is known that, oxidative stress modulates oocyte maturation and ovarian steroidogenesis (Agrawal *et al*., 2005) and that this can influence the ovulation process. Thus, the absence of an effect on ovulation rate is consistent with there being no difference observed in oxidative stress levels in the fetal ovaries.

The economic effect of the reproductive performance of females can be measured by the number of offspring produced (Gaillard *et al.*, 2000). Under the conditions of the present study maternal nutrition did not alter pregnancy rate and number of fetuses carried by their daughters. This view is supported by Chapter 8, which demonstrated

that there was no effect of maternal size and pregnancy nutrition on ovulation rate, number of fetuses and total number of lambs weaned at approximately 6 years of age. Furthermore, Munoz *et al*. (2009) also showed that restriction of nutrition did not alter conception rate in female offspring. The absence of an effect on pregnancy rate and number of fetuses in the present study was not unexpected given the previously demonstrated lack of an effect on ovulation rate (Waldron and Thomas, 1992).

9.6 Conclusions

In summary, maternal nutrition during pregnancy did not alter fetal ovarian oxidative stress or the reproductive performance of ewes' progeny, nor, consequentially was there any adverse effect upon ovary functionality. It is possible that the sub maintenance nutritional regimen applied during early pregnancy and the maintenance nutrition applied during mid-to-late pregnancy, which are commonly practiced by New Zealand sheep farmers were not sufficiently severe enough to adversely affect reproductive development in the female offspring; perhaps indicating that the mother had the ability to buffer the fetus from the detrimental effects of nutritional restriction applied in these studies or potential compensatory growth catch up of the fetal and newborn offspring occurred.

9.7 Acknowledgements

The authors thank the National Research Centre for Growth and Development and Massey University for providing funding for this project. The first author is funded by Ministry of Higher Education (Malaysia) Doctoral Scholarship. The authors would like

to thank to M.F.P. Loureiro who assisted with ovulation rate data collection, and M.B. Hampton, T.J. Moccatta, R.A. Poynton and I. Khalilova, University of Otago for their assistance with oxidative stress measures.

9.8 References

Agarwal, A., Gupta, S., & Sharma, R. (2005). Oxidative stress and its implications in female infertility – a clinician's perspective. *Reproductive BioMedicine Online, 11*(5), 641-650.

Bernal, A. B., Vickers, M. H., Hampton, M. B., Poynton, R. A., & Sloboda, D. M. (2010). Maternal undernutrition significantly impacts ovarian follicle number and increases ovarian oxidative stress in adult rat offspring. *PLoS ONE, 5*(12), e15558.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry, 72*(1–2), 248-254.

Da Silva, P., Aitken, R. P., Rhind, S. M., Racey, P. A., & Wallace, J. M. (2001). Influence of placentally mediated fetal growth restriction on the onset of puberty in male and female lambs. *Reproduction, 122*(3), 375-383.

Da Silva, P., Aitken, R. P., Rhind, S. M., Racey, P. A., & Wallace, J. M. (2002). Impact of maternal nutrition during pregnancy on pituitary gonadotrophin gene expression and ovarian development in growth-restricted and normally grown late gestation sheep fetuses. *Reproduction, 123*(6), 769-777.

Gaillard, J. M., Festa-Bianchet, M., Delorme, D., & Jorgenson, J. (2000). Body mass and individual fitness in female ungulates: bigger is not always better. *Proceedings of the Royal Society B-Biological Sciences, 267*(1442), 471-477.

Gunn, R. G., Sim, D. A., & Hunter, E. A. (1995). Effects of nutrition in-utero and in early-life on the subsequent lifetime reproductive-performance of Scottish blackface ewes in 2 management-systems. *Animal Science, 60*, 223-230.

Igosheva, N., Abramov, A. Y., Poston, L., Eckert, J. J., Fleming, T. P., Duchen, M. R., & McConnell, J. (2010). Maternal diet-induced obesity alters mitochondrial activity and redox status in mouse oocytes and zygotes. *PLoS ONE, 5*(4), e10074.

Kenyon, P. R., Pain, S. J., Hutton, P. G., Jenkinson, C. M. C., Morris, S. T., Peterson, S. W., & Blair, H. T. (2011). Effects of twin-bearing ewe nutritional treatments on ewe and lamb performance to weaning. *Animal Production Science, 51*(5), 406-415.

Kenyon, P. R., & Webby, R. W. (2007). Pastures and supplements in sheep production systems. In P. V. Rattray, I. M. Brookes, & A. M. Nicol (Eds.), *Pasture and*

Supplements for Grazing Animals (pp. 255-273): New Zealand Society of Animal Production Occasional Publication.

Kotsampasi, B., Chadio, S., Papadomichelakis, G., Deligeorgis, S., Kalogiannis, D., Menegatos, I., & Zervas, G. (2009). Effects of maternal undernutrition on the hypothalamic–pituitary–gonadal axis function in female sheep offspring. *Reproduction in Domestic Animals, 44*(4), 677-684.

Martín, N. P., Kenyon, P. R., Morel, P. C. H., Pain, S. J., Jenkinson, C. M. C., Hutton, P. G., Morris, S. T., Peterson, S. W., Firth, E. C., & Blair, H. T. (2012). Ewe nutrition in early and mid- to late pregnancy has few effects on fetal development. *Animal Production Science, 52*(7), 533-539.

Moot, D.J., Matthew, C., & Kemp, P. D. (2007). Pastures and supplements in sheep production systems. In P. V. Rattray, I. M. Brookes, & A. M. Nicol (Eds.), *Pasture and Supplements for Grazing Animals* (pp. 13-22): New Zealand Society of Animal Production Occassional Publication.

Mukasa-Mugerwa, E., Kasali, O. B., & Said, A. N. (1991). Effect of nutrition and endoparasitic treatment on growth, onset of puberty and reproductive activity in Menz ewe lambs. *Theriogenology, 36*(2), 319-328.

Munoz, C., Carson, A. F., McCoy, M. A., Dawson, L. E. R., Wylie, A. R. G., & Gordon, A. W. (2009). Effects of plane of nutrition of ewes in early and mid-pregnancy on performance of the offspring: Female reproduction and male carcass characteristics. *Journal of Animal Science, 87*(11), 3647-3655.

Murdoch, J. W., Edward, A. V. K., Vonnahme, K. A., & Stephen, P. F. (2003). Ovarian responses to undernutrition in pregnant ewes, USA. *Reproductive Biology and Endocrinology, 1*(6), 1-8.

Parr, R. A., Williams, A. H., Campbell, I. P., Witcombe, G. F., & Roberts, A. M. (1986). Low nutrition of ewes in early pregnancy and the residual effect on the offspring. *The Journal of Agricultural Science, 106*(01), 81-87.

Paten, A. M., Asmad K., Loureiro M. F. P., Kenyon P. R., Pain S. J., Peterson S. W., Pomroy W. E., Scott, I., & Blair, H. T. (2011). The effects of dam nutrition during pregnancy on the post-natal growth and puberty attainment of ewe progeny. *Proceedings New Zealand Society of Animal Production, 71*, 56-58.

Rae, M. T., Kyle, C. E., Miller, D. W., Hammond, A. J., Brooks, A. N., & Rhind, S. M. (2002). The effects of undernutrition, *in utero*, on reproductive function in adult male and female sheep. *Animal Reproduction Science, 72*(1-2), 63-71.

Rattray, P. V., Garrett, W. N., East, N. E., & Hinman, N. (1974). Growth, development and composition of the ovine conceptus and mammary gland during pregnancy. *Journal of Animal Science, 38*(3), 613-626.

Ryan, W. J. (1990). Compensatory growth in cattle and sheep. *Nutrition Abstracts and Reviews. Series B, Livestock Feeds and Feeding, 60*(9), 653-664.

SAS. (2008). Statistical Analysis System. SAS Institute Incorporation, Cary, North Carolina, USA.

Sloboda, D. M., Howie, G. J., Pleasants, A., Gluckman, P. D., & Vickers, M. H. (2009). Pre- and post-natal nutritional histories influence reproductive maturation and ovarian function in the rat. *PLoS ONE, 4*(8), 1-8.

Tarry-Adkins, J. L., Chen, J. H., Smith, N. S., Jones, R. H., Cherif, H., & Ozanne, S. E. (2009). Poor maternal nutrition followed by accelerated post-natal growth leads to telomere shortening and increased markers of cell senescence in rat islets. *Faseb Journal, 23*(5), 1521-1528.

Tarry-Adkins, J. L., Chen, J.-H., Jones, R. H., Smith, N. H., & Ozanne, S. E. (2010). Poor maternal nutrition leads to alterations in oxidative stress, antioxidant defense capacity, and markers of fibrosis in rat islets: potential underlying mechanisms for development of the diabetic phenotype in later life. *Faseb Journal, 24*(8), 2762-2771.

Viñoles, C., Gonzalez de Bulnes A., Martin G. B., Sales, F., & Sale, S. (2010). Sheep and goats. In L. Des Coteaux, J. Colloton, & G. Gnemi (Eds.), *Atlas of Ruminant and Camelid Reproductive Ultrasonography*. Ames, Iowa, US Wiley-Blackwell.

Waldron, D. F., & Thomas, D. L. (1992). Increased litter size in Rambouillet sheep: I. Estimation of genetic parameters. *Journal of Animal Science, 70*(11), 3333-3344.

Chapter 10

General discussion

10.1. Overall aim

The studies presented in this thesis were based upon the hypothesis that the *in utero* environment affects the development of the reproductive system in male and female offspring, and their subsequent performance as an adult. This hypothesis was investigated by examining two models;

3) Dam size (heavy *vs* light) and dam nutrition for a prolonged period in pregnancy (*ad libitum vs* maintenance; P21-140)
4) Dam nutrition during early (*ad libitum vs* maintenance *vs* sub maintenance; P21-50) and mid-to-late pregnancy (*ad libitum vs* maintenance; P50-139).

The hypothesis of these studies that differential maternal size and/or nutrition (maintenance or moderately restrict feed) during pregnancy can lead to the alteration of the growth and reproductive development of offspring was rejected. These studies suggest that the feeding practices typically employed by New Zealand sheep farmers during ewe pregnancy appear to be adequate and do not negatively affect offspring growth and reproductive performance. Additionally *ad libitum* levels of feeding during pregnancy were not found to confer any advantage to either the dam or her offspring

10.2. Summary of results

10.2.1 Chapter 3 and 4

These chapters describe two meta-analyses examining the effect of maternal nutritional on the growth and reproductive development of offspring in order to better elucidate the overall conclusions from the current body of literature. The meta-analyses suggested that dam nutrition during pregnancy can influence male and female sheep offspring

growth, testosterone concentrations (male) and LH concentrations (female). However, dam nutrition during pregnancy did not appear to alter testis weight or testicular cell development in male offspring nor ovarian weight or ovarian cell development in female offspring. The lack of any significant effect on organ weight and cell development may be due to the limited number of studies that specifically examine the effects of maternal nutrition during pregnancy on sheep offspring reproductive development available for meta-analysis. The meta-analyses reported here suggested that additional studies were warranted.

10.2.2 Chapter 5 and 6

In these chapters two models were used to examine maternal effects on the development of their male offspring: (i) the effect of dam size and pregnancy nutrition on fetal testicular cell development in late gestation (Chapter 5), and (ii) the effect of dam nutrition during early and mid-to-late pregnancy on the growth and reproductive performance of male offspring (Chapter 6). Results showed that dam size affected the number of gonocytes in the male fetuses, but neither dam size nor nutrition affected other testicular cell characteristics. There was a minor effect of dam nutrition during early and mid-to-late pregnancy on carcass weight (8 months) or scrotal circumference (27 months). No effects of maternal nutrition or dam size in relation to the growth and the other reproductive parameters measured were observed in male offspring up to 2 years of age. These results suggest that the sub maintenance nutritional treatment used in the present study was relatively minor and only applied during early pregnancy and probably not severe enough to affect fetal testicular cell development and post-natal growth and reproductive performance of male offspring. However, further studies are

needed to investigate the effect of dam size on gonocyte number, as this is an early indicator of fertility potential in males.

10.2.3 Chapter 7 and 8

These chapters examined the effects of dam size and pregnancy nutrition on fetal ovarian cell development (Chapter 7) and lifetime performance of female offspring until 6 years of age (Chapter 8). Results demonstrated that dam size affected the number of oogonia in fetal ovaries and the number of follicles in adult ovaries, whilst dam nutrition had minor effects on fetal ovarian development. Lifetime performance results showed that daughters born to *ad-libitum* fed dams gave birth to more lambs and had a higher proportion of ovulated at 2050 days of age than daughters born to maintenance fed dams, but no difference was found for lifetime performance on total number or weight of lambs weaned. There were no interaction between dam size and nutrition for any parameters of lifetime reproductive performance. These results indicate that lighter dam live weight at breeding affects fetal and adult ovary structure, however, nutrition at or above maintenance during pregnancy is unlikely to negatively affect fetal ovarian development. The absence of an effect in the present study on lifetime reproductive performance might be a reflection of the fact that there were also minimal effects on live weight and body condition score between daughters. This study suggests that dam size and nutrition levels during pregnancy used in the present study do not have a significant impact on daughter lifetime performance.

10.2.4 Chapter 9

This chapter examined the effect of dam nutrition during early and mid-to-late pregnancy on fetal ovarian oxidative stress and daughter reproductive performance. Results showed that dam pregnancy nutrition had no effect on oxidative stress levels in fetal ovaries, and also had no effect on puberty attainment, ovulation rate, pregnancy diagnosis and number of fetuses in two-year-old daughters. The absence of an effect on daughter reproductive performance is consistent with there being no differences observed in oxidative stress levels in the fetal ovaries, as oxidative stress modulates oocyte maturation and ovarian steroidogenesis (Agrawal *et al*., 2005). The results suggest that the sub maintenance nutritional regimen applied during early pregnancy and the maintenance nutrition applied during mid-to-late pregnancy was not sufficiently severe to adversely affect reproductive development in the female offspring.

10.3 Overall concluding remarks from the present study results

New Zealand agricultural livestock industries rely on reproduction and to not only generate a product for sale, but also to breed good quality replacement animals. Viable, healthy offspring need a optimal environment to grow and develop in during pregnancy (Robinson *et al*., 1997). There is a substantial body of research in both humans and animals that has shown maternal nutrition during pregnancy can influence the development of various organs and systems in the growing fetus, and subsequently affect productive performance in later life (Barker *et al*., 1993; Gunn *et al*., 1995; Bielli *et al*., 2001; Da Silva *et al*., 2001; Rhind *et al*., 2001; Rae *et al*., 2002a; Redmer *et al*., 2004; Kenyon, 2008; Kotsampasi *et al*., 2009; Bernal *et al*., 2010; Dupont *et al*., 2012; Chadio *et al*., 2013). However, in this thesis the effects of maternal nutrition during

pregnancy were only observed in the fetus and were not apparent in the productive performance of the adult offspring.

The difference in results observed in this thesis compared to previous studies is probably related to differences in maternal pregnancy nutrition treatments utilised. Many studies reported in the literature exposed pregnant females to levels of undernutrition that were substantially below maintenance and were more severe than the sub maintenance nutrition treatment applied in the second model (Chapter 6 and 9) of current research. This more severe maternal undernutrition may be have been responsible for the changes of productivity level changes observed in offspring in previous studies. The levels of nutrition applied in the present study (typically pregnancy maintenance and above maintenance) are those more likely to be commonly encountered on New Zealand sheep farms. Thus, a more extreme nutrient restriction during pregnancy may cause permanent productive level changes in the offspring. However, it is unlikely that such extreme levels of feeding will be purposely applied under New Zealand feeding conditions. In conclusion, this thesis has shown that the feeding practices that are most likely employed by New Zealand sheep farmers during ewe pregnancy are unlikely to negatively affect the productive performance of the offspring.

10.4. Limitations with the studies in this thesis

There are some limitations in the present studies. These include:

1) In Chapter 5, collection of testis samples was conducted only during pre-natal life with no tissue collection during post-natal life. Therefore, morphological studies could not be done to examine whether the difference in testicular

gonocytes observed during fetal life still existed as in the adult animal. Bielli *et al*. (2001) found the difference in testis weight that appeared at birth persisted until 99 days of age. With information at both fetal and adult ages, a better understanding and conclusion about the dam size effects on offspring testicular cell development could be made.

2) In Chapter 9, gonadal samples were not embedded in medium suitable for morphological evaluation, due to the effects of freezing (-196°C) (Appendix 2). However, these samples may be suitable for other analysis.
3) Independence of replicates in animals managed as flocks. However, in large studies such as these the individual animal can be considered as the replicate.

10.5. Recommendation for further research

The potential future works that could be done in the present models and future further studies have been listed below based on their priorities.

10.5.1. Potential future work of present models

In this thesis, no study was conducted to investigate the potential molecular changes induced by either dam size and nutrition regimens during pregnancy or dam nutrition during early and mid-to-late pregnancy. Studies such as gene expression and, perhaps, DNA methylation would be useful tools to investigate how the maternal environment affects offspring development at a molecular level, as there is evidence in the literature showing that maternal nutrition can induce changes in fetal DNA methylation thereby modifying gene expression in the rat (Guerrero-Bosagna *et al*., 2013) and sheep

offspring (Lan *et al*., 2013). Therefore, investigation of the effect of maternal pregnancy environment on a range of genes examples given below in fetal and/or adult offspring testis and ovary might uncover between treatment differences in testicular and ovarian development at the molecular level. Many genes have been identified in previous studies which have an important role in the regulation of gonadal development in sheep (Sweeney *et al*., 1997; Fowler *et al*., 2008), human (Robinson *et al*., 2001; Carlsson *et al*., 2006) and rodents (Itman and Loveland, 2008; Otsuka *et al*., 2011). Potential genes include:

Ovary:

- c-kit and kit ligand
- GDF9
- BMP15
- 3β-hydroxysteroid dehydrogenase (3βHSD)

Testis:

- c-kit and kit ligand
- SMAD protein
- 3β-hydroxysteroid dehydrogenase (3βHSD)
- CYP11A1

Evidence in the literature demonstrates transgenerational effects of maternal nutrition on offspring development in rats (Liang and Zhang, 2006) and humans (Roseboom and Watson, 2012; Susser *et al*., 2012). This was supported with study in sheep by Blair *et al*. (2010), using the same cohort of animals from the dam size and pregnancy nutrition study described in Chapter 5, 7 and 8 in which they showed that granddam pregnancy nutrition affected grand offspring birth weight and the proportion of granddaughters reaching puberty before one year of age. This shows potential transgenerational effects of maternal nutrition on offspring development in sheep. Thus, it might be worthwhile to further investigate maternal effects on the reproductive performance of subsequent generations.

However, due to the lack of effects on phenotype observed in the present study, it may be that there are little or no differences to be observed at the molecular level or in subsequent generations.

10.5.2. Potential further studies

Critical window of maternal nutrition treatment

The duration of nutritional restrictions studied in Chapter 5, 7 and 8 (the dam size and pregnancy nutrition study) was relatively long (P21 – P140) making it difficult to define the exact periods of pregnancy that resulted in the observed effects. This was improved by the studies on Chapter 6 and 9 (early and mid-to-late pregnancy dam nutrition study) which had divided the timing of the maternal nutritional treatments into two different windows; early (P21 – P50) and mid-to-late pregnancy (P50 – P139). However, further dividing the maternal nutrition treatments is likely to be essential, as it has been demonstrated by many studies in various species that there are three distinct stages of pregnancy that are related to fetal developmental processes; early-, mid- and late-pregnancy (Rhind *et al*., 2001; Rhind, 2004). Placental growth is completed by mid-prenancy and fetal growth is greatest during late pregnancy (Redmer *et al*., 2004), whilst reproductive development of male and female offspring is almost complete by mid pregnancy (Rhind *et al*., 2001; Rhind, 2004). In addition, Holst *et al*. (1986) showed the importance of early post-natal nutrition on offspring survival and growth. Thus, to narrow down the 'critical window' of nutritional intervention, it would be of interest to examine the exact point of time during pregnancy or during early post-natal life at which the reproductive development of offspring is affected. In an ideal situation, the nutritional regimen of the dams would be split into three periods during gestation

where most of the reproductive development in the fetus occurs (Rhind *et al*., 2001; Rhind, 2004) (from breeding (P0) to P30, then from P31 to P60, and from P60 to P100) and these nutritional windows would be followed with a nutrition mismatch after birth.

In the present study nutritional mismatch post birth was not tested to determine whether continuous application of a particular nutrition treatment or the opposite treatment to the offspring after birth could affect their later life productive performance. To proceed with such post birth this mismatch studies would require a much larger number of animals, which was not possible in the present studies.

Degree of maternal nutrition treatment

In the present study the three different nutrition levels that were used for comparison resulted in few and minor effects upon the growth and reproductive development of offspring. It could be argued that it is unknown if the sub maintenance or maintenance maternal nutrition treatments decreased performance or in the *ad libitum* maternal nutrition treatment increased performance when observed. Common practise by New Zealand sheep farmers to aim to feed at pregnancy maintenance where possible (on occasion this may fall to slightly below pregnancy sub maintenance) during early pregnancy and to pregnancy requirements during late pregnancy due to seasonal feed availability and increasing energy requirements for fetal growth (Robinson *et al*., 1999; Kenyon and Webby, 2007). Therefore, the levels of nutrition applied in this study should be applicable for farmers in New Zealand. However, it might be worthwhile for further studies to consider a more severe maternal nutrient restriction (e.g. 70% or 50% restriction) in order to determine whether greater restriction of the mother during

pregnancy could alter the growth and reproductive development of her offspring to a greater duration of time.

Type of nutrients

In extensive production systems, there is typically little or no supplement provided for grazing ruminants (Fontaneli *et al.*, 2005) which could lead to below optimal nutrient uptake of grazing ewes. There are different types of nutrients (e.g. energy (fat and carbohydrates), protein, minerals and vitamins) that are essential for growth, production and reproduction of animals (Rinehart, 2008). In this thesis, the studies were focussed on the energy requirement of the dam during pregnancy; the dams were given either *ad libitum*, pregnancy maintenance or pregnancy sub maintenance under pastoral grazing conditions. However, specific types of nutrients were not investigated. There is growing evidence in the literature showing the importance of protein in the diets of ruminants (Chew *et al*., 1984; Hoaglund *et al*., 1992; McNeill *et al*., 1997; Bell *et al*., 2000). Protein regulates growth and development through repairing old tissues and building new tissues (Schaefer, 1946; Zeman and Stanbrough, 1969; Toledo *et al*., 2011). Previous studies have shown that maternal protein diet during pregnancy in sheep can affect birth weight, kidney growth and had minor effects on lamb performance through weaning (Lloyd, 2013; Van Emond *et al*., 2014). Thus, it could be beneficial to investigate whether different levels of protein in the feed during pregnancy (e.g. by providing different levels of protein during pregnancy; 60% *vs* 80% *vs* 100% metabolisable protein requirements) could affect offspring growth and reproductive development. Then to fit with New Zealand sheep farming industry, an estimation

measurement of protein content in the feed (herbage) used by farmers within the breeding to lambing period would be beneficial to be determined.

Dam size

This thesis reported that the size of the dam at the time of mating appears to influence offspring development. However, the difference in dam weight/size was based on condition score and live weight of the animals at mating and there was no information as to whether this difference in size/weight was due to genetic or environmental factors, or perhaps both. Thus, it might be worthwhile for future studies to further investigate the genetic effect per se of dam size/weight on offspring productive performance.

Dam parity and birth rank

Work by Loureiro *et al*. (2012) showed that dam parity can affect offspring's live weight at birth and up to 12 months of age. This suggests that dam parity or dam age could potentially be another parameter that needs to be investigated in future studies to determine the effects of having either a nulliparous/young or multiparous/old mother on offspring growth and reproductive development.

Multifetal pregnancy can influence birth weight of the offspring (Gootwine, 2005) and may impair fetal growth (Greenwood *et al*., 2000).Thus, the number of fetuses carried by the dam (ie, single *vs* twin *vs* triplet pregnancy) is another potential parameter that needs to be examined, as this can significantly influence the *in utero* environment to which the fetus is exposed, particularly via effects upon the placenta (Redmer *et al*., 2004). In the present study the effect of dam nutrition only being investigated on single

and twin offspring, but no study was conducted to examine the effect of dam nutrition on triplet offspring.

Potential techniques for future studies

Growing evidence in the literature has shown that the maternal environment can affect offspring development at a molecular level (Wu *et al*., 2006; Bernal *et al*., 2010) and that these effects can be expressed in subsequent generations (Susser, *et al*., 2012). This thesis has shown that modest feed restriction and pregnancy maintenance nutrition do not have sufficient effects to warrant detailed molecular studies, but that such studies would be a useful component of an investigation under more severe feed restriction. Further, in the present studies, the time limit of the thesis period was a significant constraint, as further investigation of maternal effects on offspring productive performance at a cellular or molecular level would require a substantial amount of time.

Immunohistochemical studies (e.g. using Western Blot analysis) would be beneficial to be investigated for further determination the localisation of genes of interest which would enable a better understanding on the regulation of the related genes due to maternal nutritional effect.

Blood collection of the dams and fetuses during different stages of pregnancy (early, mid and late), and from offspring at an early age (from birth to weaning), puberty and as an adult would be useful to determine the regulation of hormones (e.g. GnRH, FSH, LH, estrogens, androgens, growth hormones etc) and reproductive development of animals due to maternal *in utero* environmental effects. The regulation of hormones is highly correlated with gonadal development during pre-natal and post-natal age (Collaer and Hines, 1995; Juengel *et al*., 2002; Rae *et al*., 2002b). For an example, in females,

follicular cell development is an early indicator for fertility potential. Measurement of circulating AMH concentrations at each stage of offspring development would be a suitable and convenient method for indication of ovarian pool, as AMH concentration represents the number of growing follicles in an ovary (Anderson, 2012; Lahoz *et al*., 2012). In the present studies blood collection for measurement of testosterone concentrations was done in adult males. There were no other blood collections from offspring conducted in either pre-natal or post-natal life. Therefore, understanding regulation of the concentration of hormones (e.g. androgens, estrogens, LH, FSH, and etc.) and their correlation with gonadal development would be useful for determination of reproductive potential in animals. This would enable a better discussion and understanding of maternal effects during pregnancy on the regulation of hormones and their relationship with gonadal development, as assessed by morphological studies of gonadal cell development during both pre-natal and post-natal ages in the present studies.

10.6. Concluding statement

The results in this thesis indicate that dam size at the time of conception affects the reproductive development of the offspring, whilst dam nutrition during pregnancy at the levels imposed in this study are unlikely to affect offspring growth and reproductive performance. This thesis added valuable information to the current body of knowledge and also helped to indicate what could be investigated in the future to increase our knowledge and improve animal production through better understanding the effects of maternal environmental on offspring growth and reproductive development.

10.7 References

Agarwal, A., Gupta, S., & Sharma, R. (2005). Oxidative stress and its implications in female infertility – a clinician's perspective. *Reproductive BioMedicine Online, 11*(5), 641-650.

Anderson, R. A. (2012). What does anti-Müllerian hormone tell you about ovarian function? *Clinical Endocrinology, 77*(5), 652-655.

Barker, D. J., Gluckman, P. D., Godfrey, K. M., Harding, J. E., Owens, J. A., & Robinson, J. S. (1993). Fetal nutrition and cardiovascular disease in adult life. *Lancet, 341*(8850), 938-941.

Bell, A. W., Burhans, W. S., & Overton, T. R. (2000). Protein nutrition in late pregnancy, maternal protein reserves and lactation performance in dairy cows. *Proceedings of the Nutrition Society, 59*, 119-126.

Bernal, A. B., Vickers, M. H., Hampton, M. B., Poynton, R. A., & Sloboda, D. M. (2010). Maternal undernutrition significantly impacts ovarian follicle number and increases ovarian oxidative stress in adult rat offspring. *PLoS ONE, 5*(12), e15558.

Bielli, A., Katz, H., Pedrana, G., Gastel, M. T., Morana, A., Castrillejo, A., Lundeheim, N., Forsberg, M., & Rodriguez-Martinez, H. (2001). Nutritional management during fetal and post-natal life, and the influence on testicular stereology and Sertoli cell numbers in Corriedale ram lambs. *Small Ruminant Research, 40*(1), 63-71.

Blair, H. T., Jenkinson, C. M. C., Peterson, S. W., Kenyon, P. R., van der Linden, D. S., Davenport, L. C., Mackenzie, D. D. S., Morris, S. T., & Firth, E. C. (2010). Dam and granddam feeding during pregnancy in sheep affects milk supply in offspring and reproductive performance in grand-offspring. *Journal of Animal Science, 88*(13), E40-E50.

Carlsson, I. B., Laitinen, M. P. E., Scott, J. E., Louhio, H., Velentzis, L., Tuuri, T., Aaltonen, J., Ritvos, O., Winston, R. M. L., & Hovatta, O. (2006). Kit ligand and c-kit are expressed during early human ovarian follicular development and their interaction is required for the survival of follicles in long-term culture. *Reproduction, 131*(4), 641-649.

Chadio, S., & Kotsampasi, B. (2013). The role of early life nutrition in programming of reproductive function. *Journal of Developmental Origins of Health and Disease, FirstView*, 1-14.

Chew, B. P., Murdock, F. R., Riley, R. E., & Hillers, J. K. (1984). Influence of prepartum dietary crude protein on growth hormone, insulin, reproduction, and lactation of dairy cows. [Article]. *Journal of Dairy Science, 67*, 270-275.

Collaer, M. L., & Hines, M. (1995). Human behavioral sex differences: A role for gonadal hormones during early development? *Psychological Bulletin, 118*(1), 55-107.

Da Silva, P., Aitken, R. P., Rhind, S. M., Racey, P. A., & Wallace, J. M. (2001). Influence of placentally mediated fetal growth restriction on the onset of puberty in male and female lambs. *Reproduction, 122*(3), 375-383.

Dupont, C., Cordier, A. G., Junien, C., Mandon-Pépin, B., Levy, R., & Chavatte-Palmer, P. (2012). Maternal environment and the reproductive function of the offspring. *Theriogenology, 78*(7), 1405-1414.

Fontaneli, R. S., Sollenberger, L. E., Littell, R. C., & Staples, C. R. (2005). Performance of Lactating Dairy Cows Managed on Pasture-Based or in Freestall Barn-Feeding Systems. *Journal of Dairy Science, 88*(3), 1264-1276.

Fowler, P. A., Cassie, S., Rhind, S. M., Brewer, M. J., Collinson, J. M., Lea, R. G., Baker, P. J., Bhattacharya, S., & O'Shaughnessy, P. J. (2008). Maternal smoking during pregnancy specifically reduces human fetal desert hedgehog gene expression during testis development. *Journal of Clinical Endocrinology & Metabolism, 93*(2), 619-626.

Gootwine, E. (2005). Variability in the rate of decline in birth weight as litter size increases in sheep. *Animal Science, 81*, 393-398.

Greenwood, P. L., Hunt, A. S., Hermanson, J. W., & Bell, A. W. (2000). Effects of birth weight and post-natal nutrition on neonatal sheep: II. Skeletal muscle growth and development. *Journal of Animal Science, 78*, 50-61.

Guerrero-Bosagna, C., Savenkova, M., Haque, M. M., Nilsson, E., & Skinner, M. K. (2013). Environmentally induced epigenetic transgenerational inheritance of altered Sertoli cell transcriptome and epigenome: molecular etiology of male infertility. *PLoS ONE, 8*(3), e59922.

Gunn, R. G., Sim, D. A., & Hunter, E. A. (1995). Effects of nutrition in-utero and in early-life on the subsequent lifetime reproductive-performance of Scottish blackface ewes in 2 management-systems. *Animal Science, 60*, 223-230.

Hoaglund, C. M., Thomas, V. M., Petersen, M. K., & Kott, R. W. (1992). Effects of supplemental protein source and metabolizable energy intake on nutritional status in pregnant ewes. *Journal of Animal Science, 70*(1), 273-280.

Holst, P., Killeen, I., & Cullis, B. (1986). Nutrition of the pregnant ewe and its effect on gestation length, lamb birth weight and lamb survival. *Australian Journal of Agricultural Research, 37*(6), 647-655.

Itman, C., & Loveland, K. L. (2008). SMAD expression in the testis: an insight into BMP regulation of spermatogenesis. *Developmental Dynamics: An Official Publication of the American Association of Anatomists, 237*(1), 97-111.

Juengel, J. L., Hudson, N. L., Heath, D. A., Smith, P., Reader, K. L., Lawrence, S. B., O'Connell, A. R., Laitinen, M. P. E., Cranfield, M., Groome, N. P., Ritvos, O., & McNatty, K. P. (2002). Growth differentiation factor 9 and bone morphogenetic protein 15 are essential for ovarian follicular development in sheep. *Biology of Reproduction, 67*(6), 1777-1789.

Kenyon, P. R. (2008). A review of in-utero environmental effects on sheep production. *Proceedings of the New Zealand Society of Animal Production, 68*, 142-155.

Kenyon, P. R., & Webby, R. W. (2007). Pastures and supplements in sheep production systems. In P. V. Rattray, I. M. Brookes, A. M. Nicol (Eds.), *Pasture and Supplements for Grazing Animals* (pp. 255-273): New Zealand Society of Animal Production Occasional Publication.

Kotsampasi, B., Balaskas, C., Papadomichelakis, G., & Chadio, S. E. (2009). Reduced Sertoli cell number and altered pituitary responsiveness in male lambs undernourished *in utero*. *Animal Reproduction Science, 114*(1-3), 135-147.

Lahoz, B., Alabart, J. L., Monniaux, D., Mermillod, P., & Folch, J. (2012). Anti-Müllerian hormone plasma concentration in prepubertal ewe lambs as a predictor of their fertility at a young age. *BMC Veterinary Research, 8*(1), 118-126.

Lan, X., Cretney, E. C., Kropp, J., Khateeb, K., Berg, M. A., Peñagaricano, F., Magness, R., Radunz, A. E., & Khatib, H. (2013). Maternal diet during pregnancy induces gene expression and DNA methylation changes in fetal tissues in sheep. *Frontiers in Genetics, 4*, 49-49.

Liang, H., & Zhang, Z. (2006). Food restriction affects reproduction and survival of F1 and F2 offspring of Rat-like hamster (*Cricetulus triton*). *Physiology & Behavior, 87*(3), 607-613.

Lloyd, L. J. *The effect of a maternal low protein diet on renal development and function in the offspring*. Doctor of Philosophy, University of Nottingham, UK.

Loureiro, M. F. P., Pain, S. J., Kenyon, P. R., Peterson, S. W., & Blair, H. T. (2012). Single female offspring born to primiparous ewe-lambs are lighter than those born to adult multiparous ewes but their reproduction and milk production are unaffected. *Animal Production Science, 52*(7), 552-556.

McNeill, D. M., Slepetis, R., Ehrhardt, R. A., Smith, D. M., & Bell, A. W. (1997). Protein requirements of sheep in late pregnancy: Partitioning of nitrogen between gravid uterus and maternal tissues. *Journal of Animal Science, 75*, 809-816.

Otsuka, F., McTavish, K. J., & Shimasaki, S. (2011). Integral role of GDF-9 and BMP-15 in ovarian function. *Molecular Reproduction and Development, 78*(1), 9-21.

Rae, M. T., Kyle, C. E., Miller, D. W., Hammond, A. J., Brooks, A. N., & Rhind, S. M. (2002a). The effects of undernutrition, *in utero*, on reproductive function in adult male and female sheep. *Animal Reproduction Science, 72*(1-2), 63-71.

Rae, M. T., Rhind, S. M., Kyle, C. E., Miller, D. W., & Brooks, A. N. (2002b). Maternal undernutrition alters triiodothyronine concentrations and pituitary response to GnRH in fetal sheep. *Journal of Endocrinology, 173*, 449-455.

Redmer, D. A., Wallace, J. M., & Reynolds, L. P. (2004). Effect of nutrient intake during pregnancy on fetal and placental growth and vascular development. *Domestic Animal Endocrinology, 27*(3), 199-217.

Rhind, S. M. (2004). Effects of maternal nutrition on fetal and neonatal reproductive development and function. *Animal Reproduction Science, 82-3*, 169-181.

Rhind, S. M., Rae, M. T., & Brooks, A. N. (2001). Effects of nutrition and environmental factors on the fetal programming of the reproductive axis. *Reproduction, 122*(2), 205-214.

Rinehart, L. (2008). Ruminant nutrition for graziers. *NCAT Agriculture Specialist*, IP318.

Robinson, J. J., Sinclair, K. D., & McEvoy, T. G. (1999). Nutritional effects on foetal growth. *Animal Science, 68*, 315-331.

Robinson, J. S., Hartwich, K. M., Walker, S. K., Erwich, J., & Owens, J. A. (1997). Early influences on embryonic and placental growth. *Acta Paediatrica, 86*, 159-163.

Robinson, L. L. L., Gaskell, T. L., Saunders, P. T. K., & Anderson, R. A. (2001). Germ cell specific expression of c-kit in the human fetal gonad. *Molecular Human Reproduction, 7*(9), 845-852.

Roseboom, T. J., & Watson, E. D. (2012). The next generation of disease risk: Are the effects of pre-natal nutrition transmitted across generations? Evidence from animal and human studies. *Placenta, 33*(Supplement 2), e40-e44.

Schaefer, H. C. (1946). The role of proteins in animal nutrition. *Oil and Soap, 23*(12), 375-379.

Susser, E., Kirkbride, J. B., Heijmans, B. T., Kresovich, J. K., Lumey, L. H., & Stein, A. D. (2012). Maternal pre-natal nutrition and health in grandchildren and subsequent generations. *Annual Review of Anthropology, 41*, 577-610.

Sweeney, T., Saunders, P. T. K., Millar, M. R., & Brooks, A. N. (1997). Ontogeny of anti-Müllerian hormone, 3β-hydroxysteroid dehydrogenase and androgen receptor expression during ovine fetal gonadal development. *Journal of Endocrinology, 153*(1), 27-32.

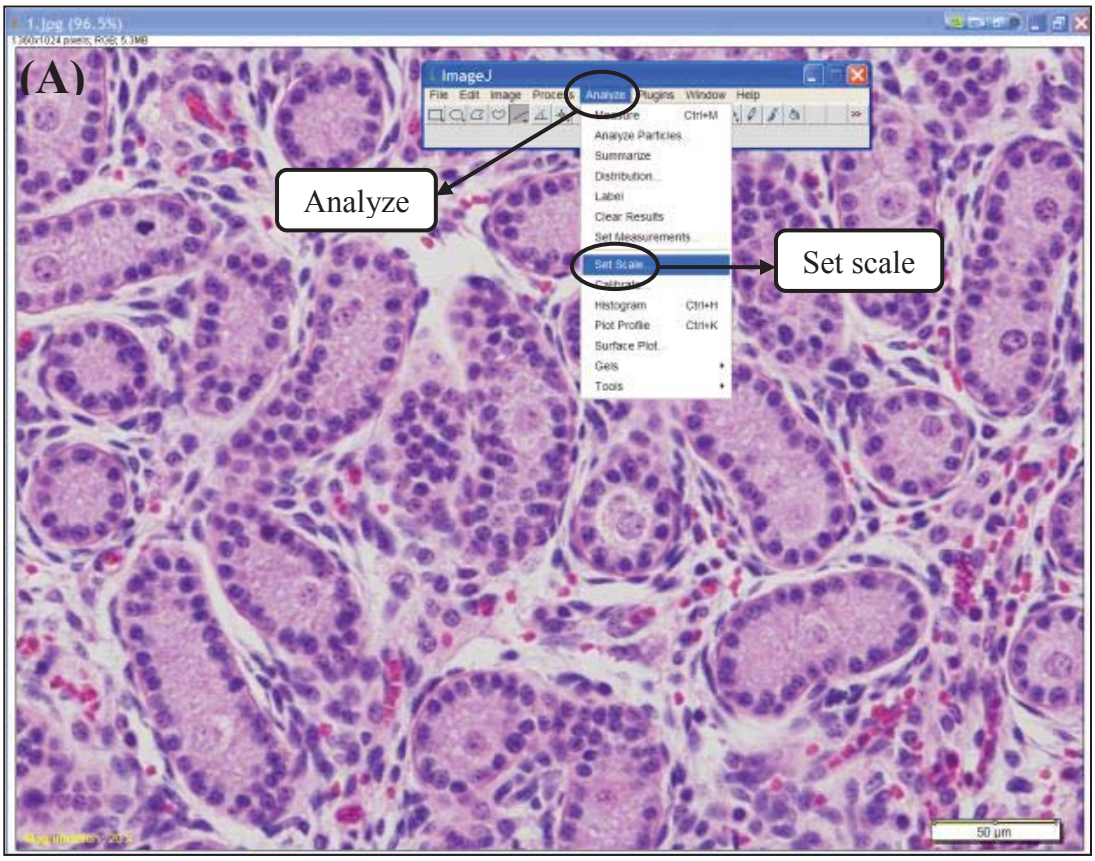
Toledo, F., Perobelli, J., Pedrosa, F., Anselmo-Franci, J., & Kempinas, W. (2011). *In utero* protein restriction causes growth delay and alters sperm parameters in adult male rats. *Reproductive Biology and Endocrinology, 9*(1), 94.

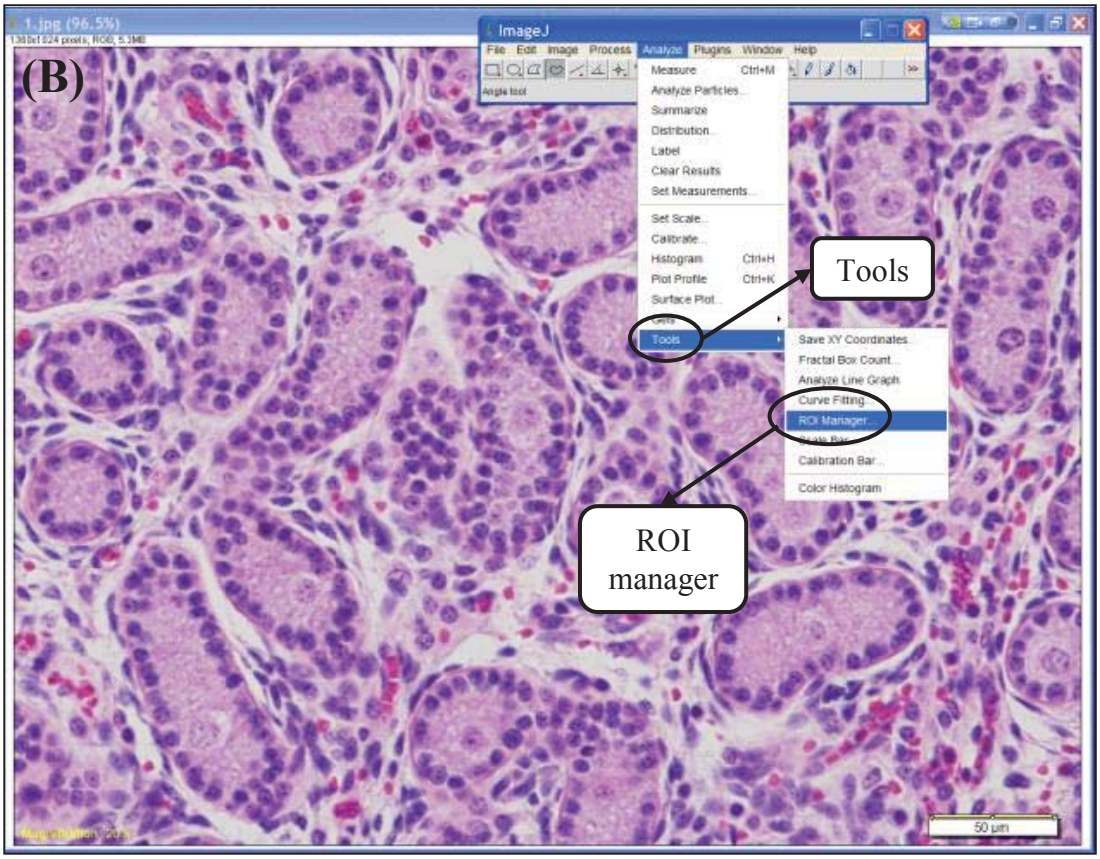
Van Emon, M. L., Schauer, C. S., Lekatz, L. A., Eckerman, S. R., Maddock-Carlin, K., & Vonnahme, K. A. (2014). Supplementing metabolizable protein to ewes during late gestation: I. Effects on ewe performance and offspring performance from birth to weaning. *Journal of Animal Science, 92*(1), 339-348.

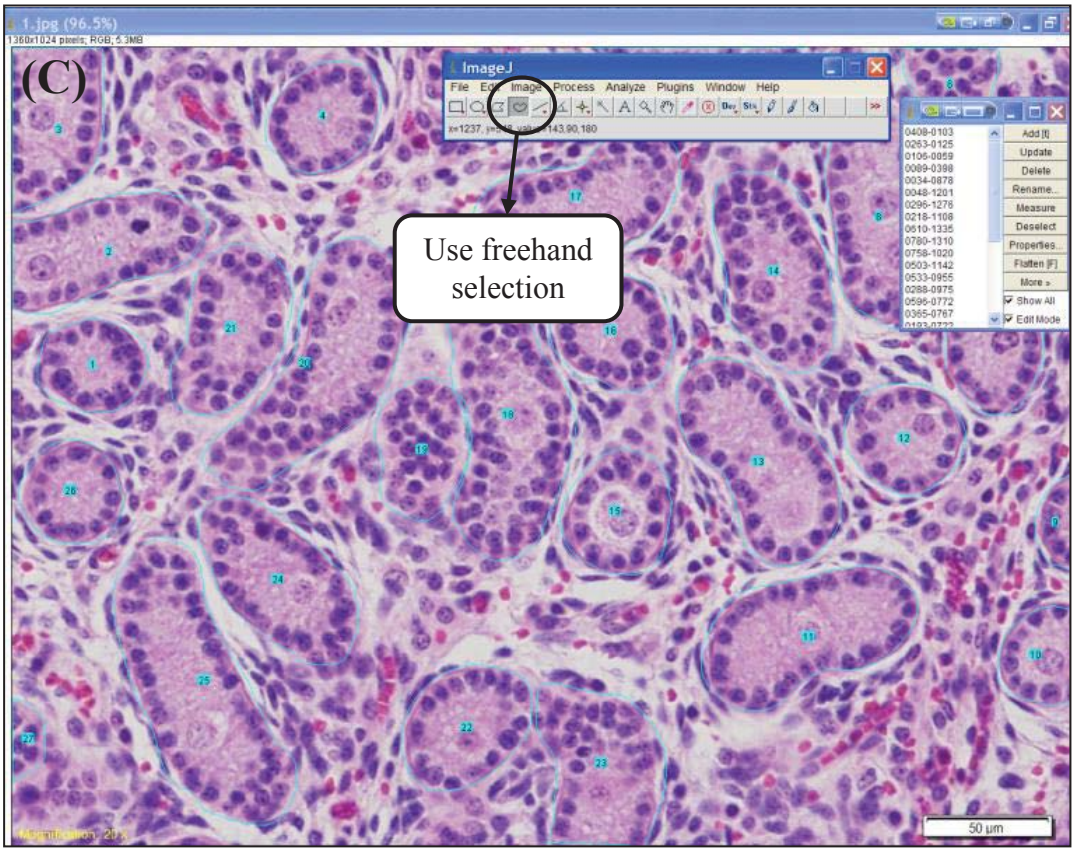
Wu, G., Bazer, F. W., Wallace, J. M., & Spencer, T. E. (2006). Intrauterine growth retardation: Implications for the animal sciences. *Journal of Animal Science, 84*(9), 2316-2337.

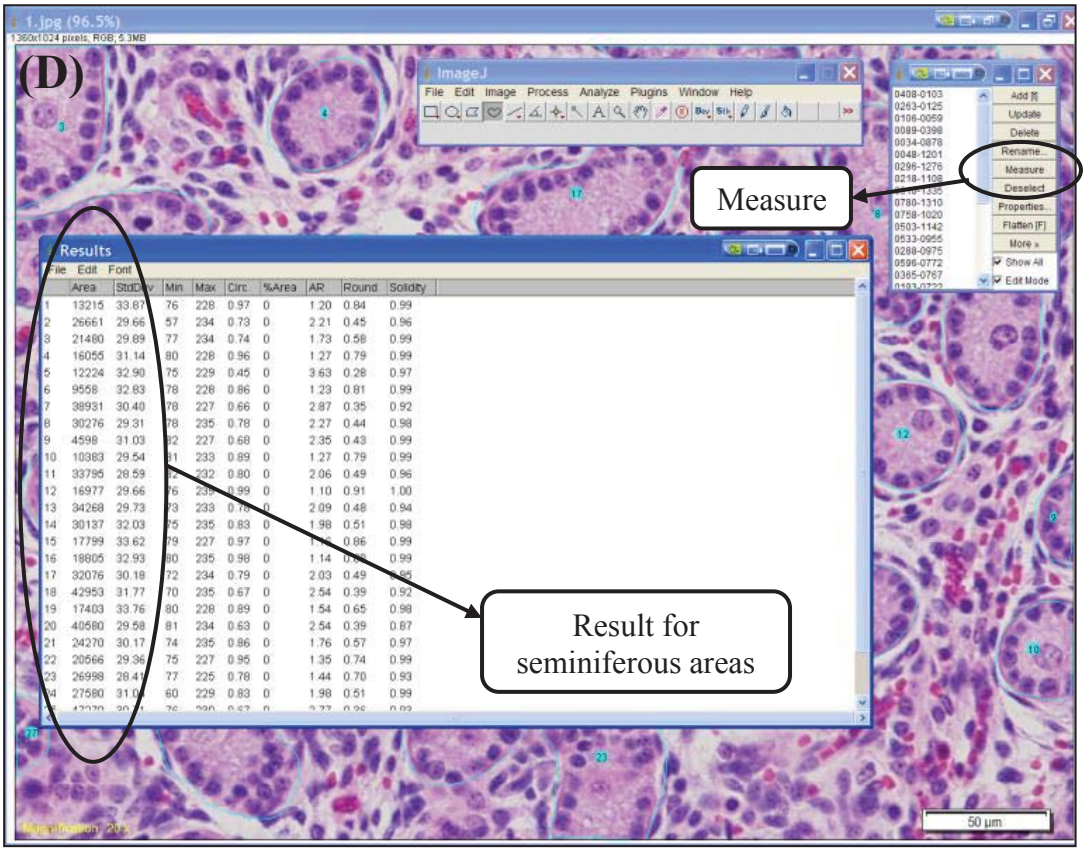
Zeman, F. J., & Stanbrough, E. C. (1969). Effect of maternal protein deficiency on cellular development in the fetal rat. *The Journal of Nutrition, 99*(3), 274-282.

Appendices

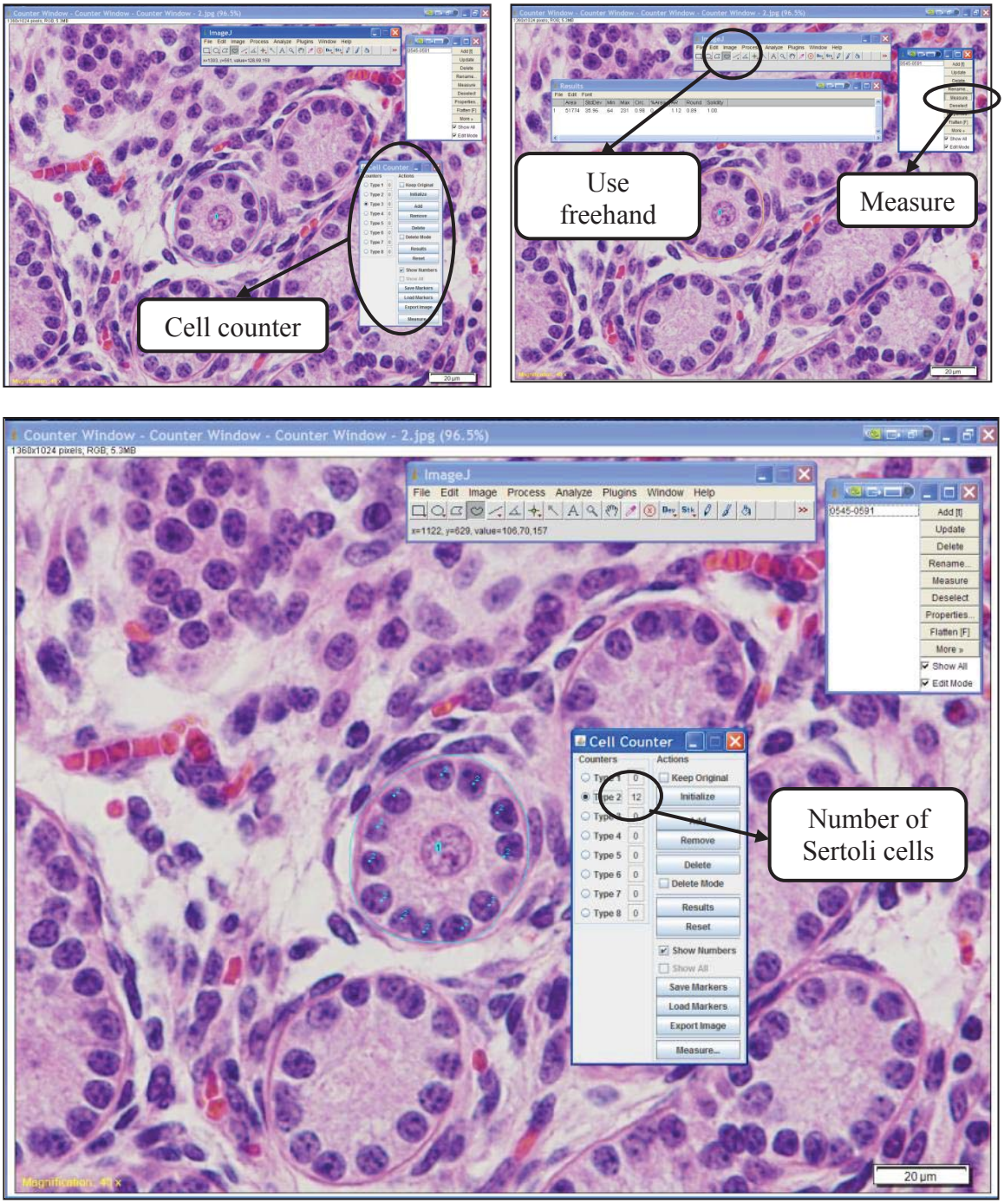

(A)
ImageJ
File Edit Image Process Analyze Plugins Window Help
Measure Ctrl+M
Analyze Particles...
Summarize
Distribution...
Label
Clear Results
Set Measurements...
Set Scale...
Calibrate...
Histogram Ctrl+H
Plot Profile Ctrl+K
Surface Plot...
Gels
Tools
Analyze
Set scale
50 µm


(B)
ImageJ
File Edit Image Process Analyze Plugins Window Help
Measure Ctrl+M
Analyze Particles...
Summarize
Distribution...
Label
Clear Results
Set Measurements...
Set Scale...
Calibrate...
Histogram Ctrl+H
Plot Profile Ctrl+K
Surface Plot...
Tools
Save XY Coordinates...
Fractal Box Count...
Analyze Line Graph...
Curve Fitting...
ROI Manager...
Calibration Bar...
Color Histogram
Tools
ROI manager
50 µm

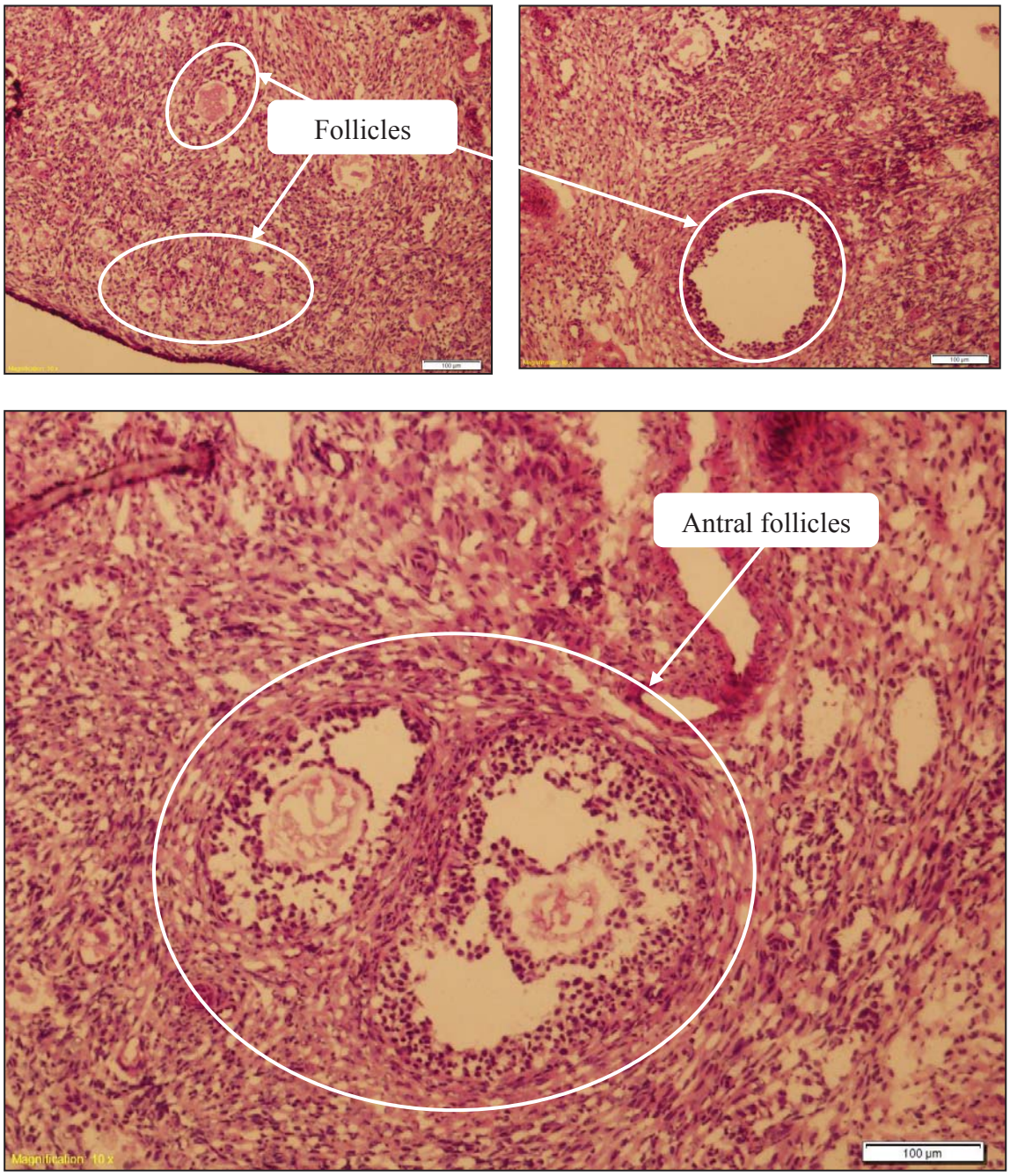




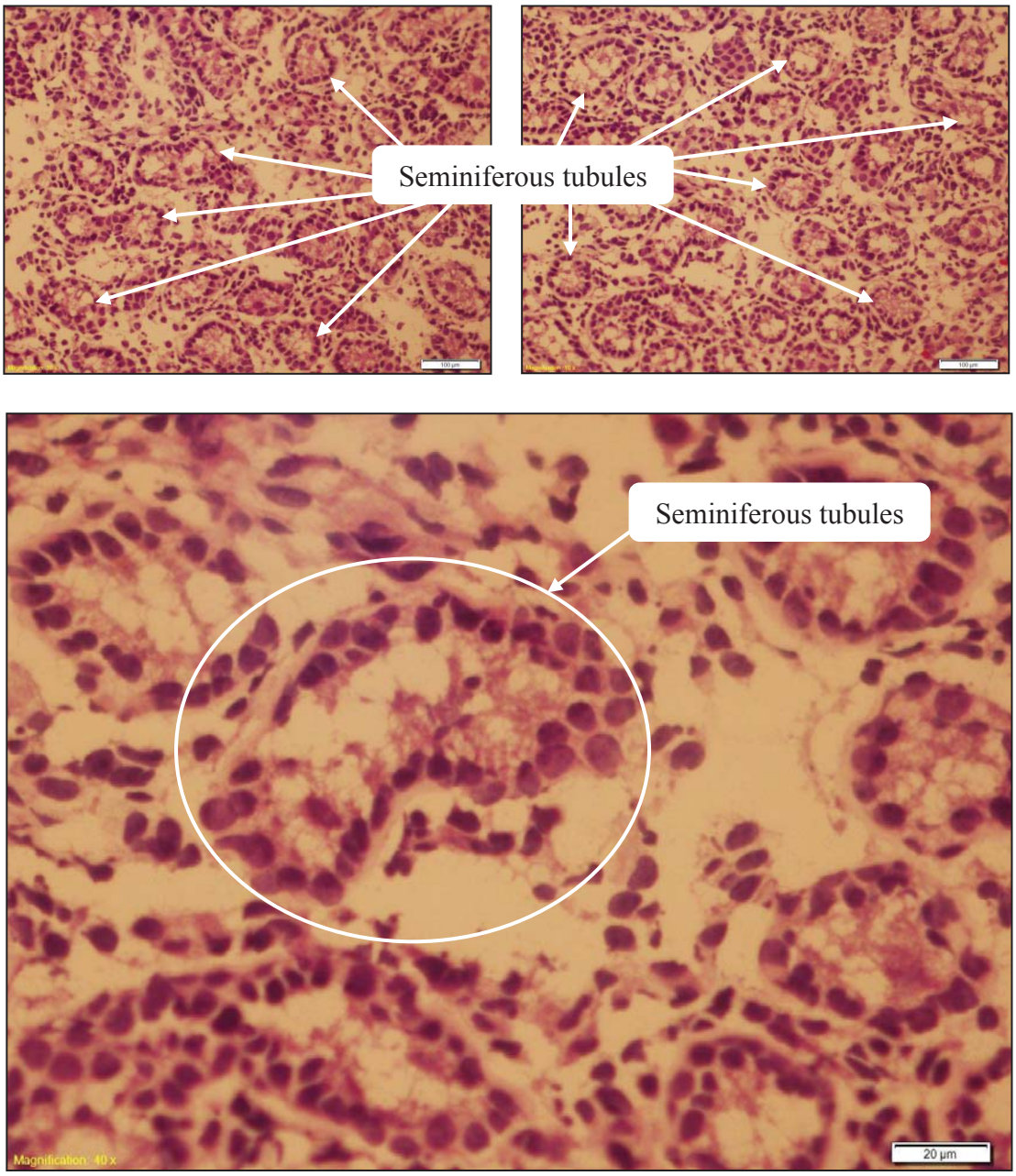
Appendix 1a. Measurement of seminiferous tubules total area using ImageJ software (Chapter 5).



Appendix 1b. Measurement of seminiferous tubule circumference and Sertoli cell counts using ImageJ software (Chapter 5).



Appendix 2a. Defects of fetal ovarian cell structure in OCT compound due to freezing effect (Chapter 9).



Appendix 2b. Defects of fetal testicular cell structure in OCT compound due to freezing effect (Chapter 9).

Day 1

1. Deparaffinise in graded alcohol.

2. Antigen retrieval (citrate buffer).
   - Water bath (30 minutes at 95°C).
   - Cool for 30 minutes at room temperature.

3. Incubate with 3% H_2O_2 in methanol (block endogenous peroxidise activity) for 12 minutes at room temperature.

4. Wash 3 x (5 minutes each) with PBS + 0.05% Tween 20.

5. Incubate with 10% BSA (block non-specific binding) for 15 minutes at room temperature.

6. Primary antibody (incubate overnight at 4°C).
   - Dilution 1:100 in 1.5% BSA

Day 2

7. Wash 3 x (5 minutes each) with PBS + 0.05% Tween 20

8. Secondary antibody (incubate 1 hour at room temperature)
   - Dilution 1:200 in 1.5% BSA

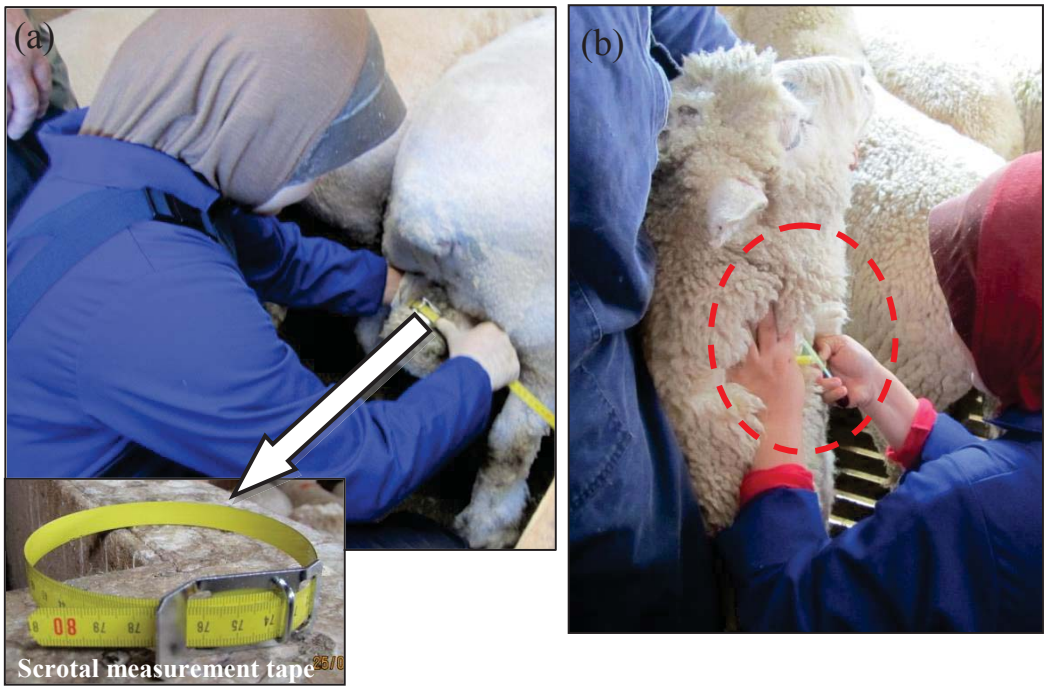
9. Wash 3 x (5 minutes each) with PBS + 0.05% Tween 20

10. Stain with DAB (±10 minutes)
    - 2.5 ml distilled water
    - 1 drop of buffer (mix well)
    - 2 drops of DAB (mix well)
    - 1 drop of HPS (mix well)

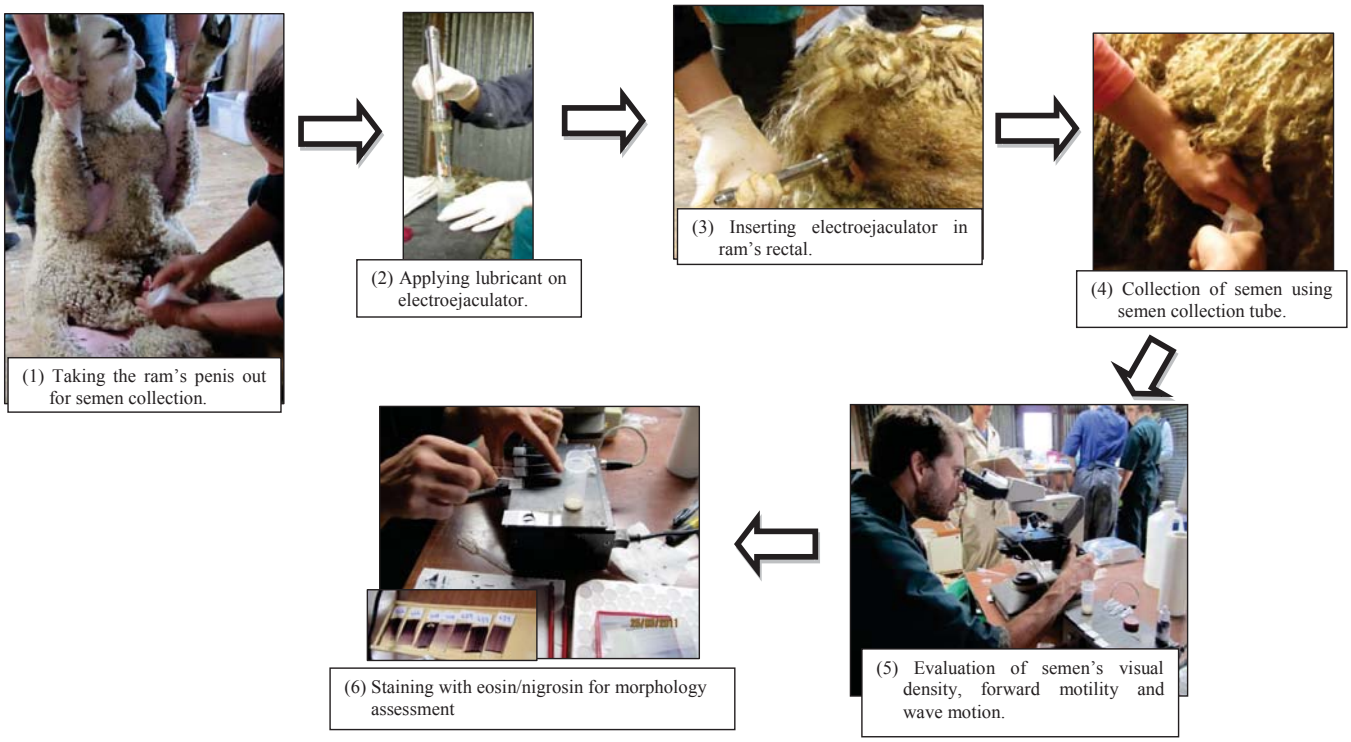
11. Counterstain with Haematoxylin

Appendix 3. Immunohistochemistry basic protocol for the evaluation of anti Müllerian hormone (AMH) and Growth Differentiation Factor 9 (GDF9) expression in fetal ovaries (Chapter 7).

Appendix 4a. Rams from the study of the effect of maternal nutrition during pregnancy (early and mid-to-late pregnancy) on the offspring growth and reproductive performance (Chapter 6).



Appendix 4b. Measurement of scrotal circumference using scrotal measurement tape (a) and blood collection from jugular vein for testosterone concentration analysis (b) of male offspring (Chapter 6).



Appendix 4c. Semen collection and evaluation (Chapter 6).