The effect of nutritional fetal programming on post-pubertal male reproduction in sheep

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ABSTRACT

There is evidence that the *in utero* environment affects or programmes postnatal development and performance of offspring. Previous investigations have been conducted to establish the effects of dam nutrition on the development and reproductive performance of rams following various nutritional regimes of the ewe during pregnancy. This study further examines the effect of nutritional treatments of ewes during pregnancy on post-pubertal male reproductive performance. Sixty-two ram offspring, obtained from twin-bearing ewes that were fed at one of three different nutritional treatments in early pregnancy (Day 21-50, Low (L_{D21-50}) vs. Maintenance (M_{D21-50}) vs. High (H_{D21-50})), and one of two different nutritional treatments in mid to late pregnancy (Day 51-140, Maintenance ($M_{D51-140}$) vs. High ($H_{D51-140}$), were utilised in this study. Reproductive performance was measured using the accepted indicators of scrotal circumference, and semen quality and quantity (visual density, motility, quantitative sperm density and morphology) to establish if there was any effect of maternal nutrition on these parameters. The influence of seasonality was also investigated. Ewe nutrition during D 21–50 or D 51–140 had no effect on scrotal circumference, semen quality nor quantity. The rams in this study generally conformed to previously described seasonal patterns of reproductive activity. In conclusion, the present study demonstrates that under these conditions, post-pubertal male reproductive function and capacity and therefore fertility appear to be unaffected by prenatal maternal nutrition, and that rams maintain their cyclical reproductive response to seasonal cues.

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LH	Luteinising hormone
FSH	Follicle stimulating hormone
GnRH	Gonadotrophin-releasing hormone
DHT	Dihydrotestosterone
CNS	Central Nervous System
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
GDNF	Glial cell line derived neurotrophic factor
AR	Androgen receptor
MAP	Mitogen-activated protein
cAMP	Cyclic adenosine monophosphate
CREB	cAMP response element binding protein
H_{D21-50}	*ad libitum* grazing conditions that results in ewe average weight gain of 100g/day
M_{D21-50}	no change in total ewe liveweight
L_{D21-50}	loss in total ewe liveweight of 100g/day
$H_{D51-140}$	*ad libitum* grazing conditions
$M_{D51-140}$	ensures total ewe liveweight increased at similar level to that of expected conceptus mass

CHAPTER 1: INTRODUCTION

The growth and development of the fetus *in utero* is under the influence of many factors. A major determinant and limiting factor to fetal growth is the intrauterine environment, and it is suggested that nutrition in particular plays the most critical role (Barker & Clark, 1997; Wu et al., 2004). Human epidemiological studies link the effects of fetal undernutrition to the incidence of disease later in life (Barker, 1995, 1998b). This evidence supports the theory of "fetal programming" which states that "a stimulus or insult at a critical period of development has long-term effects through a process called programming" (Godfrey & Barker, 2000).

There has recently been increased interest in fetal programming in production animals. Dam nutrition during critical periods of prenatal development plays a fundamental role in the regulation of fetal development of sheep with consequences to postnatal and lifetime productivity and health (Bell, 2006; Symonds et al., 2006; Wu et al., 2006). Nutritional effects during gestation may be of greater consequence to animals carrying multiple fetuses because of the higher nutritional demand of twin pregnancies compared with singletons (Cleal et al., 2007; Kenyon et al., 2011). This is of particular importance with the trend in the sheep industry of selection for increasing litter size with the intent of improving lambing percentage and therefore profitability.

Another challenge to production is periods of nutrient inadequacy that are commonly experienced in extensive ruminant production. In fact, it is noted that often the greatest period of feed shortage, in extensive grazing systems of temperate regions, such as in New Zealand, occurs during winter and so coincides with the gestational period of the sheep (Mathews et al., 1999). This combination of factors has significant potential to influence offspring development in both short and long term production with great economic effects on the industry. As such, livestock production success is obviously dependent on the ability to optimise productivity and profitability. Postnatal growth and

development are key factors for efficient production characteristics of livestock, and optimal fetal growth, its precursor, is mandatory to ensure maximal productivity is achieved (Redmer et al., 2004).

It has been established that the nutritional status of the ewe during pregnancy is linked to fetal reproductive development *in utero* and performance post-birth (Rhind, 2004). Male reproductive performance and fertility is highly dependent on the success of spermatogenesis, and this in turn requires that normal and complete sexual differentiation and development has taken place (McLachlan, 2000). Fetal gonadal development begins as early as the first stage "trimester" of gestation in sheep (Bielli et al., 2002), suggesting the possibility that nutritional manipulations during this time might have an influence on the subsequent reproductive performance of offspring. It should also be noted, that although development of reproductive organs begins during gestation, spermatogenesis is primarily a function of adulthood (McLachlan, 2000). Once puberty has been attained and the spermatogenic cycle initiated, it is considered continuous, even though in some seasonally breeding sheep temporary arrests or restrictions of spermatogenesis occur due to seasonal triggers (Martin et al., 1999).

Reproductive inefficiency has always been considered a limiting factor to productivity. Ram performance has significant influence on pregnancy rates. If it were to be established that inadequate nutrition during gestational development affected the fertility of rams that are subsequently used for breeding, this may be another factor that will influence the decision of farmers to investigate the cost-benefits of improved nutrition for pregnant ewes. Thus the outcome of this research has the potential to influence changes in current farming practices and may possibly have applications to human gestational nutrition and health concepts.

The aim of this research was to investigate the effects of early (Day 21–50) and mid- to late (Day 50–140) dam pregnancy nutrition on the post-pubertal reproductive capacity

of twin-born male offspring, and to observe if the effects of maternal nutrition on the reproductive function varies, depending on the season. Therefore, the following literature review will describe firstly the basic process and control of spermatogenesis. Seasonality effects as they pertain to male reproductive performance will then be discussed. Finally, the review will explore the effects of dam nutrition during pregnancy, most importantly the fetal programming effects on male reproductive performance with a focus on these effects in rams.

2.1 CONTROL OF SPERMATOGENESIS

Spermatogenesis is the process by which the testis produces sperm with the potential to become capable of fertilisation. To date, there is considerable information that indicates that this process involves a complex interaction between the structural elements of the testis and the endocrine system (McLachlan, 2000; Holdcraft & Braun, 2004). Whilst spermatogenesis can be considered primarily a process of adulthood, the success of this interplay between hormones and testicular cells is predicated on there being normal and complete male sexual differentiation and development during the fetal and peri-pubertal period (McLachlan, 2000). Thus, it may be considered that the first influence of hormonal control on this process occurs during the fetal developmental stage of the male offspring, with resultant effects on the development of the primary structures of the gonad necessary for successful sperm output.

Spermatogenesis has been widely studied and documented in various reviews and textbooks (Amann & Schanbacher, 1983; Sharpe, 1994; de Kretser et al., 1998; Parkinson, 2009), with the majority of information on the organisation and control of the process derived from studies done in the laboratory rat. The emphasis of Sharpe's (1994) review is on findings from rats and humans; however, correlations of the hormonal control of the spermatogenic process in other species including sheep are also discussed. It is important to understand the process and duration of sperm production, from gestational development through to the actions that occur within the seminiferous epithelium and other tissues of the testis, in order to provide context for the actions of environmental, nutritional and other external stimuli on the outcome of this process. For the purpose of this thesis, this review section on spermatogenesis will focus on a simple summary of the previously mentioned reviews.

2.1.1. Endocrine control and development of testicular structures

The endocrine control of male reproductive physiology is primarily modulated by the pituitary gonadotrophins luteinising hormone (LH) and follicle stimulating hormone (FSH). These two hormones are secreted in response to the regulating hormone gonadotrophin-releasing hormone (GnRH) (Lincoln, 1979). GnRH is produced by neurons in the hypothalamus in a pulsatile manner. This signal results in the production of LH and FSH from the gonadotrophe cells of the anterior pituitary, which then act on targets within the testis to regulate spermatogenic potential. The development of the hypothalamo-pituitary axis and production of these hormones occurs early in the gestational life of the fetus; in fetal sheep GnRH neuronal systems develop between Day 35 to 85 of gestation (Caldani et al., 1995). As such, there is evidence of effects of these hormones on the testicular cells at this stage (Thomas et al., 1994) that may have subsequent implications for spermatic yield.

The development of testicular structures is of importance in laying the foundation for successful spermatogenesis in adult life. The proper development and function of the hypothalamo-pituitary axis, and timely occurrence of the onset of steroidogenesis and the associated secretion of fetal gonadotropins in response to hypothalamo-pituitary signals are necessary to ensure this occurs (Rhind et al., 2001). Thomas et al. (1994) demonstrated that GnRH positively regulates LH and FSH synthesis and secretion in fetal sheep. Further to this, they reported evidence of the role of fetal gonadotrophins in the development and growth of fetal testes. This is corroborated by the work of Brooks and Thomas (1995) who stated that the pulsatile LH secretion by the fetal pituitary plays an essential role in the normal development of the testis.

It appears that the contribution of FSH to the control of spermatogenesis begins in the fetal stage of development. Studies using animal models have demonstrated the role of FSH to stimulate Sertoli cell proliferation in both mice (Heckert & Griswold, 2002) and rats (Orth, 1984). This is of particular note, as the number of Sertoli cells determines the

number of germ cells that can be supported through spermatogenesis (Sharpe, 1994), thus having significant implications on spermatogenic capacity, including whether sufficient numbers of sperm are produced for the subsequent fertility of the male in adulthood (Orth, 1984; Holdcraft & Braun, 2004).

2.1.2 Endocrine control of normal adult spermatogenesis

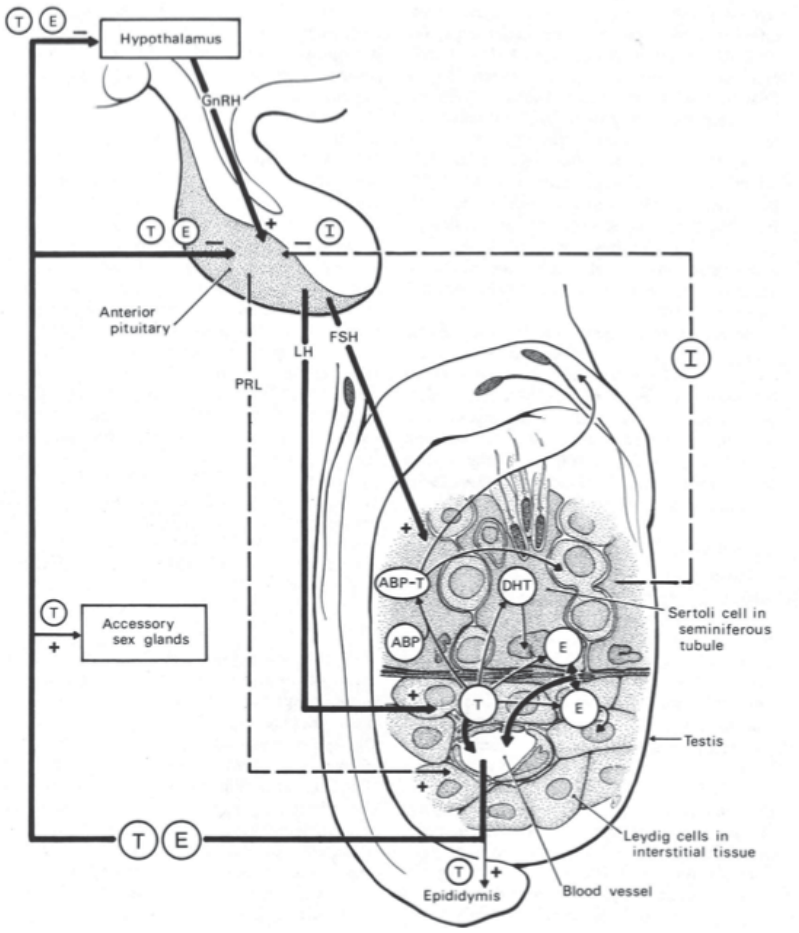
FSH has other key roles in spermatogenesis apart from its developmental role to ensure adequate Sertoli cell number. These roles include ensuring the maturation of Sertoli cells at puberty, maintenance of their cell junctions, and maintenance of spermatogonial development in the post-pubertal male (McLachlan, 2000). It should be noted that while FSH plays a major role influencing Sertoli cell support of germ cells, regulation of spermatogonial development is under the additional influence of testosterone, which is regulated through the action of LH.

LH is secreted in a pulsatile manner from the pituitary, in response to episodic stimulation by GnRH. Its primary action is to control testosterone synthesis by the Leydig cells. It is accepted that while LH may play other roles in the regulation of spermatogenesis, its role in the regulation of testosterone production is of particular importance, such that it is indispensible to the function of the adult testis (Holdcraft & Braun, 2004). The production of testosterone by these cells in response to LH is mediated through adenylate cyclase, which regulates steroidogenesis through a number of intermediate steps. Testosterone is further converted by 5α-reductase into 5α-dihydrotestosterone (DHT), which is considered to be a more biologically active and potent androgen that testosterone itself. It has long been recognised that there is very little reductase activity in the Leydig cells and this, coupled with the fact that FSH stimulates this conversion, suggests that this is an activity that occurs in the Sertoli cells (Bardin et al., 1994). Testosterone, and its reduced product DHT, play important roles in the production of sperm from the primordial germ cells, their subsequent maturation in the epididymis, accessory sex gland function and development of male secondary sexual

characteristics (Griffin, 2000). It is well established however, that while DHT is essential for the activity of the epididymis and accessory glands, testosterone is the androgen of importance in the testis for spermatogenesis (Walker & Cheng, 2005). The mechanism through which these hormones control spermatogenesis occurs through a complex relationship of negative and positive feedback mechanisms as in the schematic Figure 2.1.

Empirical evidence has shown the need for both testosterone and FSH for the successful completion of spermatogenesis. While FSH has particular influence at the developmental level in ensuring structural adequacy within the testis in preparation for spermatogenesis in adulthood, Sertoli cell multiplication is independent of testosterone. The support of spermatogenesis after puberty through the Sertoli cells is maintained by either of these hormones (Walker & Cheng, 2005), however, in sheep some steps (spermatogonial divisions) are only supported by FSH (Kilgour et al., 1998). In contrast, there is evidence that FSH may not be required and that testosterone may be even more crucial for meiosis (Singh et al., 1995). This argument, that only testosterone is needed for complete spermatogenesis, is further corroborated by work by Kumar et al. (1997) who also demonstrated that in cases of FSH deficiency (via knockout of the FSHβ-subunit gene) in mice spermatogenesis proceeded to completion. Although it is established that both FSH and testosterone have shared signals and responding functions, there are also differences in their actions. These differences may account for the fact that testosterone is capable of maintaining spermatogenesis independently of FSH but not vice versa (Walker & Cheng, 2005).

Figure 2.1: Diagram showing inter-relationship among hormone production in the Leydig cells and the seminiferous tubules, and the feedback control of gonadal hormones on the hypothalamus and anterior lobe of the pituitary gland. (T- testosterone; I- inhibin; E- oestrogen; PRL- prolactin; ABT- androgen-binding protein) (Amann & Schanbacher, 1983)



2.1.3 Other hormones involved in the control of spermatogenesis

The main control of spermatogenesis occurs through the negative and positive feedback mechanisms that exist between GnRH, LH, FSH, testosterone (and steroids derived from it), and gonadal peptides (see Figure 2.1). These peptides and hormones that play a role in this process include inhibin (known to play a role in feedback mechanism of FSH), and oestradiol (formed by aromatisation of testosterone within the Leydig and Sertoli cells

(Carreau et al., 1999) and CNS (Naftolin et al., 1975) and involved in feedback of LH secretion via hypothalamic GnRH). This does not preclude other intra-testicular paracrine and autocrine factors that act at the local level (e.g. activin which appears to act mostly at a paracrine level in the testis, but is also associated with stimulation of FSH production at the pituitary level (de Kretser et al., 2004)). These factors are beyond the scope of this review, but may include growth factors, cytokines and insulin.

Cellular processes underpinning spermatogenesis commence before birth, with male sexual development and continuing through puberty into adulthood guided by the hypothalamo- pituitary hormonal axis (Thomas et al., 1994; Brooks et al., 1995b; Rhind et al., 2001). The role of hormones in adulthood on spermatogenesis is of as much significance to sperm production as it is during pre-adult development. Evidence suggests that the withdrawal of gonadotrophins in adulthood results in abolition of sperm production in all species (McLachlan, 2000). McLachlan (2000) stated that although questions continue to arise with regards to the specific roles and site of action of the gonadotrophins, four main steps of spermatogenesis which are under the control of these hormones have been described: (i) spermatogonial proliferation and differentiation (described above with respect to FSH), (ii) spermatocyte development encompassing meiosis, (iii) spermiogenesis, and (iv) spermiation. These steps will be described in more detail in the following section of this review.

2.2 PHYSIOLOGY OF THE TESTIS

Male fertility is dependent on the successful production of large numbers of spermatozoa that are capable of fertilizing ova. The main physiologic function of the testis is to produce normal yet immotile and infertile, haploid, elongated spermatozoa (Brooks, 1983). Spermatozoa are produced in the seminiferous tubules through the process of spermatogenesis and spermiogenesis, but these immature, non-motile spermatozoa are incapable of fertilizing ova (Cosentino & Cockett, 1986). It is then

through passage through the epididymis that the properties that ensure fertility are conferred onto the spermatozoa before storage (Amann, 1987; Cooper, 2011). Each of these steps is needed for a successful spermatogenic process. However, failure of the process is possible due to defects or perturbations of any of these steps (de Kretser et al., 1998). This may lead to production of defective spermatozoa and reduction or absence of sperm production. For the purpose of this thesis, the following summary will cover a general overview of the process. Comprehensive descriptions can be found in the following reviews: de Kretser and Kerr, 1994; Eddy and O'Brian, 1994; Sharpe, 1994; Lie et al., 2009; Cheng et al., 2010; Cooper, 2011.

2.2.1 Stage 1 of spermatogenesis

Spermatogenesis takes place through the continuous replication of stem cells that results in the production of cohorts of cells. These cohorts then proceed through various changes finally resulting in haploid cells (de Kretser & Kerr, 1994). The first step of spermatogenesis is stem cell renewal. This occurs through the process of mitosis. In the mature animal spermatogenesis begins with differentiated spermatogonia. These are primordial germ cells that migrated into the genital ridge, replicated and differentiated and are divided into A, intermediate and B classes. These classes are further divided dependent on the level of differentiation and morphological characteristics. In the ram, A0, A1, A2, A3, Intermediate, B1 and B2 spermatogonia are present (Hochereau-de-Reviers, 1976). Initial division of the stem cell A0 spermatogonia give rise to two daughter cells, one enters the process of further mitotic divisions, the other remains as a stem cell (Sharpe, 1994). These mitotic divisions finally end with the onset of meiosis I, and the formation of two primary spermatocytes.

2.2.2 Stage 2 of spermatogenesis

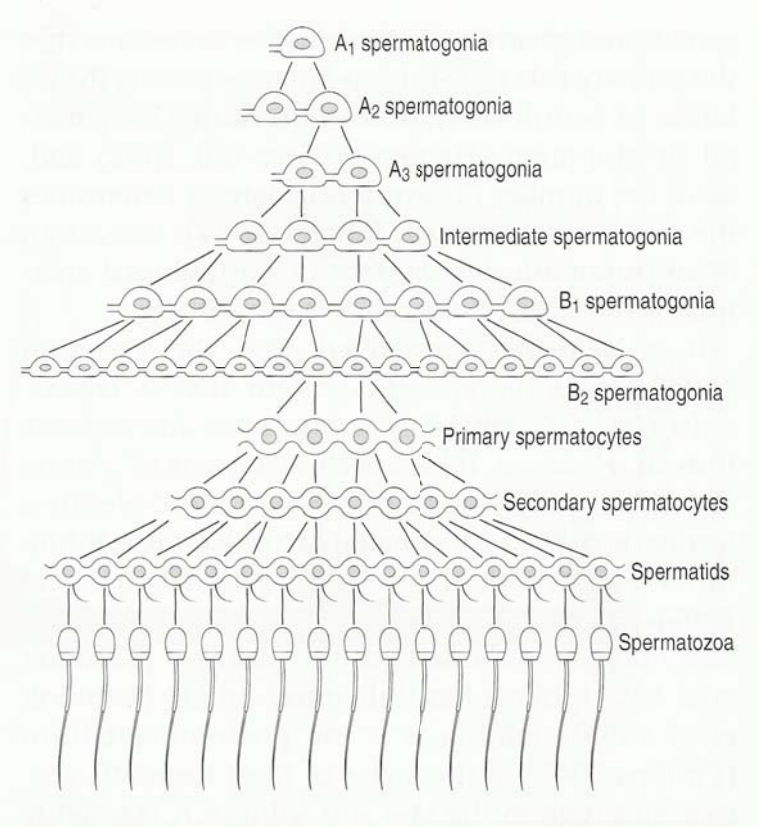
The first meiotic division take the primary spermatocytes through preleptotene, leptotene, zygotene, pachytene and diplotene stages to produce secondary spermatocytes; this is followed by a second meiotic division producing round haploid

spermatids (de Kretser & Kerr, 1994). This multiplication of cells from A1 spermatogonia to spermatids is seen in Figure 2.2. It is the process of DNA and RNA synthesis that occurs during these meiotic divisions that results in the formation of four haploid spermatids out of every diploid, pachytene spermatocyte (Sharpe, 1994). The number of spermatids produced from the completion of this process is closely related to the number of spermatozoa that will be eventually released from the Sertoli cell. The zygotene and pachytene stages have been identified as critical periods at which the final number of sperm that are released can be altered (Martin-du Pan & Campana, 1993). It is thought that these periods are particularly sensitive to noxious stimuli resulting in reduced numbers of secondary spermatocytes available to enter into the second meiotic division. This is consonant with work by other authors that show that this is a period of possible degeneration (Sinha-Hikim & Swerdloff, 1993). As such, it is thought to be a major rate-limiting step in the process of spermatogenesis.

Indeed, degeneration and loss occurs throughout any of the stages of spermatogenesis (mitosis, first and second meiotic divisions, and differentiation of spermatids to spermatozoa during spermiogenesis). The impact of degeneration or loss of developing spermatozoa at the various stages directly affects “efficiency of spermatogenesis” which is comparable between species and measured as “daily sperm production per gram of testicular parenchyma” (Johnson et al., 2000, p. 474). Of the species examined, humans are noted to have the lowest efficiency of spermatogenesis (4-6 $x10^6$/g), followed by the bull (12 x 10^6/g), while rats (20-24 $x10^6$/g), rams (21 x 10^6/g) and horses (16-19 $x10^6$/g) are considered to have greater efficiency. Furthermore, this efficiency is dependent on the effect of losses at the different developmental steps of spermatogenesis, such that for each species there may be stages at which losses are more likely to occur (e.g. rats have no significant losses during any of the stages, whereas degeneration is most likely to be observed at the end of meiosis in boars and humans (Johnson et al., 2000)). In addition, it has been reported that these losses are influenced by pubertal development, season of the year and aging (Johnson et al., 2000).

Furthermore, Sharpe (1994) states that spermiation is one of the most important aspects of the process of spermatogenesis as it is noted that it is a highly vulnerable process and is easily perturbed. Not surprisingly, the most common defect that can occur in spermiation is the failure of sperm to be released from the Sertoli cells into the lumen of the seminiferous tubule (O'Donnell et al., 2011). Sperm that fail to be released are phagocytosed by the Sertoli cell. Although the mechanisms by which impaired spermatogenesis results in failures in sperm release are unknown, it is thought that the unsuccessful spermiation could possibly be as a result of a failure at an earlier stage with phagocytosis at that point. Spermiation failure has been associated with negative stimuli associated with defects at other stages of spermatogenesis including inadequate hormone support or exposure to chemicals or other toxicants, and often spermiation failure may be observed concurrently with perturbations at other spermatogenic stages (Russell, 1991).

Figure 2.2: Multiplication of cells during spermatogenesis (bull). A1 spermatogonia undergo mitotic divisions to produce A2, A3, intermediate, B1, and B2 spermatogonia. Primary spermatocytes, produced by the final mitotic division, then enter into meiosis, producing secondary spermatocytes (first meiotic division), followed by spermatids (second meiotic division). Spermatids differentiate into spermatozoa without further division (Parkinson, 2009).



2.2.3 Stage 3 of spermatogenesis

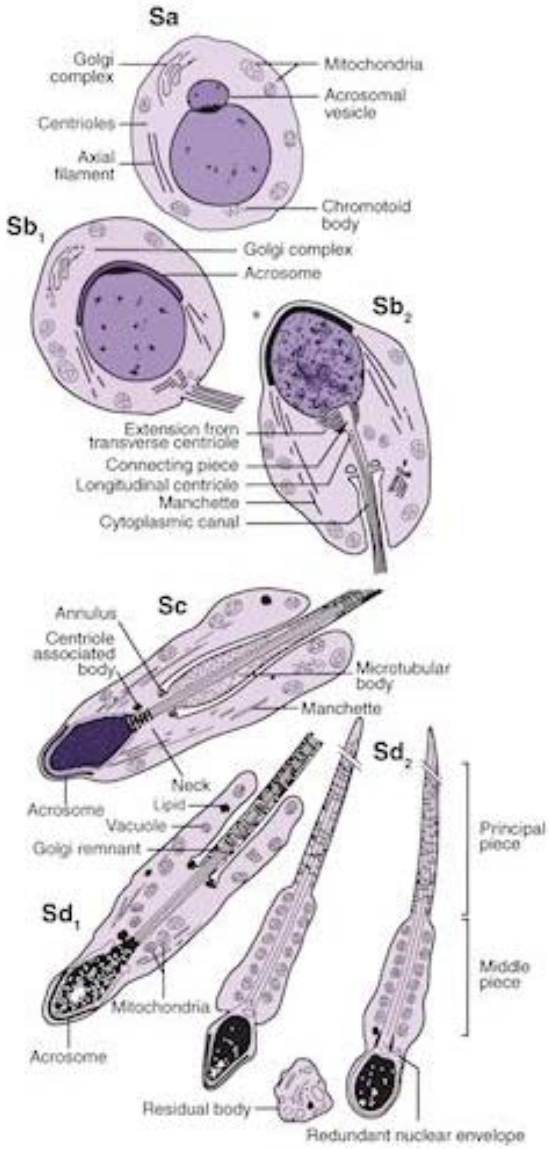
The third stage, 'spermiogenesis', describes the process through which the round spermatid differentiates into an elongated, highly condensed spermatozoon before being released into the lumen of the seminiferous epithelium. This change takes place through a series of morphological changes or events (Figure 2.3) and is summarised as follows (de Kretser et al., 1998):

- Nuclear condensation and movement of the nucleus to the periphery of the cell

- Formation of a modified lysosome, the acrosome, which attaches to the surface of the nucleus in apposition to the cell membrane
- Flagella formation: development of a core of microtubules, the axoneme, formed from one of the centrioles of the round spermatid which becomes lodged at the abacrosomal pole of the nucleus at what would form the 'neck' of the spermatozoon
- The axoneme is modified in the formation of the tail by development of a series of electron dense fibres (outer dense fibres) in the region of the mid piece of the spermatozoon and distally by the formation of a fibrous sheath in the region of the principal piece.
- The spermatid then sheds a large part of cytoplasm as a residual body that is phagocytosed by the Sertoli cell.

The resultant spermatozoon is then released from the apex of the Sertoli cell into the lumen of the cytoplasm. This is known as spermiation (stage 4).

Figure 2.3: The changes during spermiogenesis involving the transformation of a round spermatid to a mature spermatozoon (de Kretser & Kerr (1994) as redrawn in O'Donnell & de Kretser, 2013).

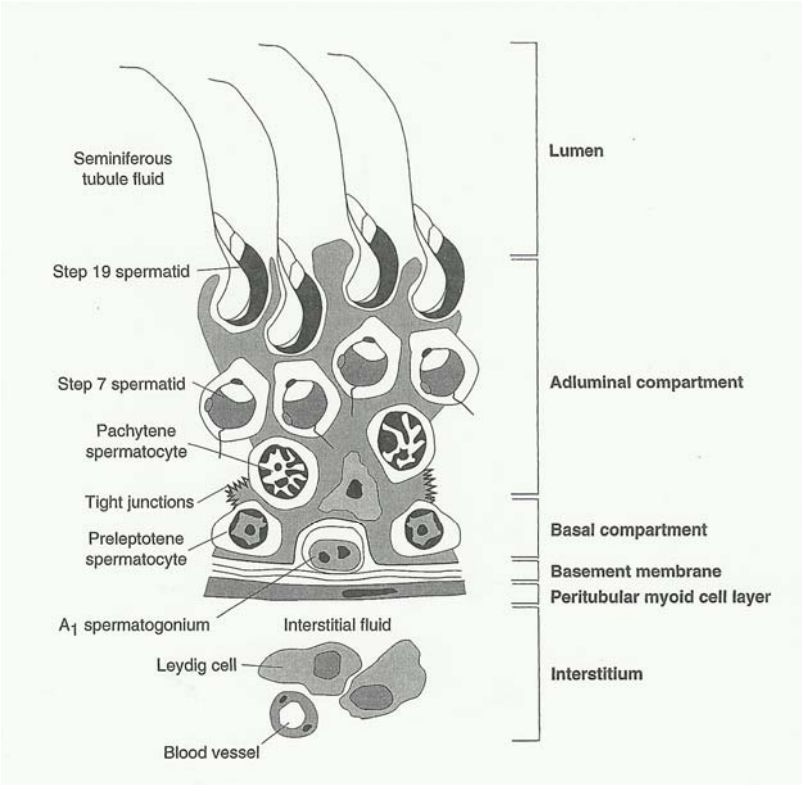


2.2.4 The role of the Sertoli cell in spermatogenesis

The success of spermatogenesis is highly dependent on the relationship that germ cells and their resultant progeny have with the cells that make up the seminiferous tubule.

The somatic Sertoli cells make up a major portion of the seminiferous tubule, resting on the basement membrane and extending through the entire thickness of the seminiferous epithelium. This allows germinal cells that are at all the different stages of spermatogenesis to remain in close contact with them. Figure 2.4 illustrates this relationship between a Sertoli cell and different stages of germ cells. It is long accepted that Sertoli cell numbers are highly correlated to sperm output (Hochereau-de-Reviers et al., 1984; Orth et al., 1988). It is reported that this particular effect may be as a result of the numbers of germ cells they are able to support as reflected by the correlation of A1 to Sertoli cell ratio (Hochereau-de-Reviers et al., 1987). In addition, it is established that it is "not only number but also function of the Sertoli cell that is crucial for the successful development and completion of spermatogenesis" (de Kretser et al., 1998, p. 6).

Figure 2.4: Illustration of a Sertoli cell and its association with different stages of germ cells (rat); and the division of the cell into basal and adluminal compartments (Sharpe, 1994).



The function of the Sertoli cells in spermatogenesis is not fully understood, however it is accepted that one of its primary roles is to provide the required microenvironment necessary for germ cell development, both as physical and nutritional support (Griswold, 1998). Of additional importance to the efficiency of spermatogenesis is the arrangement of Sertoli cells within the seminiferous tubules to form 'junctional' complexes. These tight cell junctions divide the seminiferous tubule into basal and luminal compartments (Setchell et al., 1994), forming the blood-testis barrier (Hochereau-de-Reviers et al., 1990) (Figure 2.4). This barrier serves to maintain the environment of the lumen necessary for spermatogenesis and separate dividing cells from the immune system.

It is primarily through the action of gonadotrophic hormones FSH and testosterone that spermatogenesis occurs. Within the seminiferous tubules only Sertoli cells contain receptors for both FSH and testosterone and as such these cells are the primary targets for these hormones. Thus, it appears they are essential for the normal function of Sertoli cells and play a major role in regulating spermatogenesis (de Kretser et al., 1998; Walker & Cheng, 2005). The mechanisms through which these effects occur are still unclear, although recent data suggest that these hormones act by switching on a number of different genes within the Sertoli cell that regulate or support spermatogenesis (Walker & Cheng, 2005).

FSH binds to receptors on the plasma membrane of Sertoli cells stimulating adenylate cyclase, resulting in increased intracellular cAMP. It is through the action of cAMP, and other signalling pathways that FSH exerts its genomic action to support spermatogenesis (Walker & Cheng, 2005). Recently, microarray assay has allowed the successful demonstration of as many of as 300 genes in Sertoli cells that have been implicated in up or down regulation by FSH (McLean et al., 2002; Sadate-Ngatchou et al., 2004a). This does not preclude genes that have been identified by other studies including: FSH receptor (Maguire et al., 1997), glial cell line derived neurotrophic factor

(GDNF) that regulates germinal stem cell proliferation (Tadokoro et al., 2002) and stem cell factor associated with survival of spermatogonia (Taylor et al., 1996). Likewise, genomic testosterone action has been shown to occur through androgen receptor (AR) expression (Holdcraft & Braun, 2004). The Pem gene is recognised as one such gene that is regulated through AR expression (Lindsey & Wilkinson, 1996). Furthermore, continued investigations using microarray analysis has identified some genes within the Sertoli cells that are thought to be directly associated with the action of testosterone on spermatogenesis (Sadate-Ngatchou et al., 2004b). However, it has recently been proposed that non-genomic pathways independent of AR binding play an equally or possibly more important role in the support and success of spermatogenesis by testosterone. This includes testosterone-mediated elevation of intracellular Ca^{2+} concentration and activation of MAP kinase pathway and induction of cAMP response element binding protein (CREB)-regulated gene expression (Walker & Cheng, 2005).

2.2.5 The physiology of the epididymis

The sperm that are released into the lumen of the seminiferous tubule are immature, non-motile and lacking the capacity to successfully fertilise an ovum. These properties are acquired through transit through the epididymis (Amann, 1987; Eddy & O'Brian, 1994). These authors report that the acquisition of motility and fertilising ability occurs within the body of the epididymis, since sperm harvested from the head do not demonstrate any of these properties. In addition, sperm exhibit morphological changes during their passage through the epididymis. The addition and/or modification of surface glycoproteins to the plasma membrane is noteworthy as these changes are thought to confer stability to the acrosome, reduce surface immunogenicity and enhance the ability of the sperm to bind to the zona pellucida (Amann, 1987; Hammerstedt & Parkes, 1987). Other morphological changes include cholesterol and phospholipid loss, which are associated with remodelling of the plasma membrane, and acquisition of motility and fertilising capacity (Jones, 1998a).

The epididymis is divided into 2 regions, a proximal (made up of the caput and corpus regions) and distal (or cauda) region with differing functionalities in each region (Hammerstedt et al., 1979). The proximal segment is the area in which sperm maturation takes place, whereas the distal segment is the primary site of storage and transport (Jones, 1998b). Transit time differs between the two regions, and it is well established that the function of the epididymis is androgen dependent (Amann, 1987; Brooks, 1983). Consequently, if androgen levels are supressed, epididymal function is immediately impaired (Robaire et al., 2006).

2.2.6 The physiology of the accessory glands

Finally, it should be noted that the accessory glands, although of limited importance to this thesis, play an important physiological role associated with ensuring the fertility of spermatozoa particularly in their passage through the female reproductive tract (Brooks, 1990). These glands all require androgens, particularly DHT for their function. Their primary purpose is to produce secretions that make up most of the volume of the ejaculate; however, the functions of the individual constituents are still debated. It is postulated that they provide energy, maintain osmotic pressure, buffer, as well as provide immunosuppression and facilitate sperm motility within the female genital tract (Brooks, 1990). These are all thought to be necessary properties allowing sperm to survive within the female genital tract and accomplish successful fertilisation of the ovum.

It is obvious, therefore, that reproductive success of the male is dependent on the efficient production of normal, adult, motile sperm capable of fertilisation. This is highly dependent on the success of the described major cellular events of spermatogenesis and functional support of the epididymis and accessory glands, all which depend on gonadotrophic support. In the post pubertal male in most species the spermatogenic cycle is continuous once initiated and conditions are optimal. However in some seasonal

breeds such as sheep it has been shown that spermatogenesis is temporarily arrested or reduced due to seasonal triggers. The following section will explore this effect further.

2.3 SEASONALITY EFFECTS ON MALE REPRODUCTION

The environment in which animals live directly influences their behaviour and adaptability in order to ensure survival of the species. In particular, seasonal fluctuations in environmental conditions can result in periods when food availability is limited and conditions are detrimental to survival, alternating with fertile periods when environmental conditions are conducive to optimal growth and survival. Daily photoperiod and annual temperature cycles are important examples of such environmental factors in temperate regions, whereas annual rainfall cycles and consequent food availability are considered important variables in the tropical regions (Vivien- Roels & Pévet, 1983). The result of these alternating favourable/non-favourable periods has put pressure on animals to develop coping strategies to give birth when conditions are favourable for offspring survival and performance, conferring a seasonal pattern to their reproductive activity. Thus, in many species, reproductive activity or breeding is restricted to certain times of the year (Wayne et al., 1989). In temperate climates, the period of optimum growth usually corresponds with spring or early summer.

Photoperiod is the most widely used signal or environmental cue to synchronise reproductive pattern with climatic season (Karsh et al., 1984). Sheep breeds originating in temperate regions are seasonal breeders and use photoperiod to regulate breeding activity. They fall into the category of “short-day” breeders, meaning that they become sexually active in response to decreasing day-length in late summer to early autumn. There is a plethora of literature describing seasonality effects on the ewes, characterising behavioural, endocrine and ovulatory changes in response to photoperiodic changes with season (Poulton & Robinson, 1987; Barrell et al., 1992;

Karsh et al., 1993). In fact, two distinct periods have been identified: a breeding season in which regular oestrous behaviour and ovulation is displayed, alternating with a anoestrous period, characterised by cessation of sexual activity (Yeates, 1949). It has also been reported that fluctuations in sexual behaviour and characteristics are observed in rams in response to seasonal changes (D'Occhio & Brooks, 1983; Martin et al., 1999). These response/ behavioural changes are of equal importance to sheep production as are those demonstrated by the ewe. These effects may have a direct impact on the ability of farmers to manipulate and maximise rams for the benefit of increasing profitability of the industry. Therefore, for the purpose of this thesis, the following section will focus briefly on the reported seasonal effects in rams, and mention will be made of the neuroendocrine mechanisms that control seasonality in this species.

2.3.1 Photoperiodic control of reproductive cyclicity

Seasonal reproduction in sheep is primarily regulated by photoperiod. The response to photoperiod has been shown to vary with breed. In males timing and magnitude of seasonal cycles of testicular size, mating activity and gonadotrophin and testosterone secretion differ between breeds (Islam & Land, 1977; Pelletier et al., 1982; D'Occhio & Brooks, 1983; Poulton & Robinson, 1987; Lincoln et al., 1990; Mandiki et al., 1998), which leads to the conclusion that some breeds are more “photoperiod-responsive” than others (Martin et al., 2002). It is noted, however, that all breeds, irrespective of their sensitivity to photoperiod or other environmental cues, demonstrate an “endogenous annual rhythm or cycle” of reproductive activity that is strikingly similar in timing and duration (Martin et al., 1999). Some investigations have shown that there may be other environmental cues that entrain this endogenous rhythm, including nutrition (feed availability), temperature and social cues (Martin et al., 1994). These may be of greater influence to sheep found in lower latitudes (35°N–35°S), which appear to be less sensitive to photoperiods, and regions where annual cycles of food supply and photoperiod are poorly synchronised, or are incompatible in terms of the “classical”

seasons of reproduction (Martin et al., 1994). Therefore, the combined influence of multiple environmental factors (nutrition, temperature and social stimuli), along with day-length, to entrain the seasonal cycle in sheep living outside in a natural environment, should not be dismissed. However, in the present review, photoperiodic entrainment of seasonal reproductive effects will be the focus considering that the breed selected for this study (Romney) is thought to be highly photoperiod responsive (Hafez, 1952; Poulton & Robinson, 1987), and the location in which the study was performed lies within higher latitude regions in which clearly defined seasons (photoperiodic, temperature and climatic changes) exists.

The pineal gland plays a vital role in the entrainment of reproductive cyclicity by transducing the signal of changes in day-length into neuroendocrine signals via the release of the indoleamine, melatonin (Lincoln & Short, 1980; Karsh et al., 1984). Melatonin is secreted during periods of darkness, and the changes in duration of the period of melatonin secretion due to the seasonal changes in day-length then result in changes to the pulsatile secretion of GnRH from the hypothalamus and subsequent influence on the pituitary-gonadal axis. The action of melatonin to influence secretion of GnRH is thought to involve a complex circuit of interneurons (Malpaux et al., 1996). It is demonstrated via the placement of melatonin micro-implants that melatonin action specifically targets the medio-basal hypothalamus and thus relays photoperiod signals to regulate the timing of ram reproductive cycles (Lincoln & Maeda, 1992) via dopaminergic, serotoninergic and excitatory amino-acidergic neurons (Malpaux et al., 1996).

Variations in response to seasonality are not sex linked, although it has been suggested by Ortavant et al. (1988) that these variations are less pronounced in the ram than in the ewe. Whereas it appears that the ewe responds to seasonal fluctuations with complete arrest of ovulation and oestrus (strong negative feedback of oestrogen resulting in the LH pulse frequency falling below the threshold to induce ovulation

during the non breeding (long-day) period (Legan & Winans, 1981)), spermatogenesis and sexual activity in rams appears not to cease, retaining some degree of fertility throughout the year, even in those breeds in which seasonality is most pronounced. In fact it is recognised that there is wide variation in the degree of regression of spermatogenesis in the non-breeding season between species and strains of seasonal mammals (Lincoln, 1989). It is thought that this variation in response is partly related to the latitude at which they had evolved. This variation in spermatogenic response is a reflection of the degree to which episodic release of gonadotrophins and testosterone fluctuate in response to day-length (D'Occhio et al., 1984; Lincoln et al., 1990). Thus, it is not surprising that even among rams of differing breeds, a range of responses to seasonal cues may occur. Generally, however, there is evidence of observable seasonal fluctuations in hormonal activity, gametogenesis, and physical and behavioural changes in sexual characteristics in response to the effects of changing day-length in most breeds of temperate latitudes.

2.3.2 Manifestation of seasonality in the ram

In response to the shortening day-lengths of late summer/early autumn the pituitary testicular axis is more active, as evidenced by the corresponding increases in LH and FSH and testosterone in response to increased GnRH pulsatility during the breeding season (D'Occhio et al., 1984). Other researchers corroborate these findings of increased gonadal activity and demonstrate the subsequent effect of increased testicular size and associated mating behaviour as a consequence of the stimulus of day-length (D'Occhio & Brooks, 1983; Pelletier et al., 1982; Poulton & Robinson, 1987; Lincoln et al., 1990; Martin et al., 2002). In contrast, the opposite occurs in response to long day-lengths; that is, gonadotrophin secretion is inhibited, followed by lower testosterone production and subsequent testicular regression (Lincoln & Short, 1980; D'Occhio & Brooks, 1983; Lincoln et al., 1990) as is seen in the non-breeding season of spring into summer.

These seasonal effects also affect quantity and quality of spermatozoa. Ortavant et al. (1988) reported changes in spermatogenic efficiency with season, such that an average of 8.5 x 10^6 spermatozoa/g of testicular parenchyma were produced in spring versus 12.2 x 10^6/g in autumn. Likewise, earlier studies by Dacheux et al. (1981) demonstrated similar findings in daily sperm output (1 x10^9 at the minimum in spring and up to 4.8 x 10^9 at late summer). Morphologically, a higher percentage of abnormal cells are seen when day-length increases (spring) than when it decreases (autumn) and subsequent fertilising ability is higher in autumn than spring (Colas, 1979).

Furthermore, seasonal effects on spermatogenic efficiency of rams may be explained by greater degeneration of spermatogonia following long day illumination (Ortavant (1958) as cited in Johnson, 1986). This is consonant with reports by Hochereau-de-Reviers (1976) that a reduction occurs in the number of A1 spermatogonia as a result of degeneration after each seasonal breeding season in the adult ram. Compensatory replenishment of A1 from A0 then occurs just before the beginning of the following breeding season allowing for sufficient precursors to enter the spermatogenetic production line.

Although it appears that the rams' sensitivity to photoperiod is different from ewes', and the response axiomatically less extreme, the synchronicity of seasonal effects between the male and female would seem to be of great importance. Stimulation of sexual activity in response to decreasing day-length is usually observed 1–1.5 months earlier in the ram (Ortavant et al., 1988), ensuring that the rams have already achieved a high level of sexual activity in preparation for when the cycle of the ewes starts (Rosa & Bryant, 2003). This lag time is coincident with the 45 days needed for a ram to complete spermatogenesis, in comparison to the ewes' almost immediate ability to ovulate within a few days after hormonal stimulation (Rosa & Bryant, 2003).

It is therefore evident that season, primarily photoperiod, acts through the hormonal control of spermatogenesis in the ram to achieve maximal sperm output ensuring well-timed and efficient reproduction. As described in previous sections, the ability of the ram to produce normal, adult, motile sperm capable of fertilisation is imperative. It is also obvious that as many opportunities for perturbations exist, as there are steps and factors that influence this process, which possibly could result in a non-favourable outcome. It is suggested that the actions of environment, and nutritional and external stimuli can be exerted on this process as early as gestation with both positive and negative results. These effects will be covered in the following sections of the review.

2.4 EFFECTS OF FETAL NUTRITION

There are many factors that influence the growth of the fetus *in utero*. It is accepted that fetal genes may be the primary determinant of fetal growth and development (McCance & Widdowson (1974) as cited in Godfrey & Barker, 2001; Wu et al., 2004). However, increasing evidence suggests that the intrauterine environment is an additional major determinant and limiting factor to fetal growth (Barker & Clark, 1997; Wu et al., 2004). The effect of physical constraints imposed by maternal uterine size has been demonstrated in embryo transfer studies, which show that it is the recipient mother that limits fetal growth. Thus, a fetus transferred to a larger uterus (as defined as the recipient dam being larger both in weight and size than that of the embryo donor) achieves a larger birth size (Brooks et al., 1995a) and vice versa (Sharma et al., 2009). It is suggested that although genomic and physical limitations are important determinants of growth potential *in utero*, it now appears that these may be secondary to the nutritional supply and hormonal milieu to which the fetus is exposed, in particular nutrient and oxygen supply (Godfrey & Barker, 2000). Thus, among all the potential intrauterine factors that may affect growth, more and more evidence seems to suggest that nutrition is one of the more important factors influencing fetal growth. As such, some of the most compelling data provided by studies of the Dutch famine (Roseboom

et al., 2000) (an extreme case of maternal undernutrition during pregnancy) has provided crucial information on the long-term effects on health of offspring born to mothers who were pregnant during the famine, and incited interest in further investigations to determine how the intrauterine environment and particularly nutrition affects fetal development.

2.4.1 Maternal nutrition and fetal growth and development

Some studies indicate that maternal nutrition plays a critical role in fetal growth and development (Wu et al., 2004). Yet this hypothesis is often contested as other work suggests that changes in maternal nutrition have little effect on fetal development, except in cases of extreme famine (Barker & Clark, 1997). Maternal nutrient supply may differ in terms of total nutrient intake or specific nutrient availability and, in practical farming conditions, this is often dependent on feed availability (quantity and/or quality), perturbations of intake, and environmental conditions (Caton & Hess, 2010). It is important to understand that observed impairment of fetal growth and development occur when fetal demand for nutrients is not met by maternoplacental capacity to meet that demand (Godfrey & Barker, 2000). This 'supply line', which links maternal diet at one end to fetal uptake at the other, is instrumental in understanding the mechanisms by which fetal growth may be affected. It includes maternal nutrient uptake, maternal metabolism and endocrine milieu, umbilical blood flow and placental transfer and metabolism (Harding, 2001). Therefore, fetal nutrition is not only determined by the combination of maternal dietary intakes and nutrient stores, but also delivery of nutrients via the placenta, and the transfer capabilities of the placenta may have an important role in meeting fetal nutritional demand (Harding, 2001). Furthermore, Harding (2001) suggests that the interactions and properties of the fetal supply line allow a large margin of safety for fetal growth even in cases of relatively large changes in maternal nutrition. Thus, it is thought that the maternal and placental systems have regulatory mechanisms that work to ensure that fetal growth and development should

be unaffected by variations in maternal nutrient intake. However, to date, epidemiological and experimental evidence challenges this view.

Any occurrence of a perturbation in the process of fetal growth (accretion or differentiation) of tissues or organs may have adverse consequences on neonatal viability and possible long-term effects on health in adult life (Fowden, 1995). It has been demonstrated that changes in nutrient supply during critical periods of embryonic and fetal life, resulting in alterations to the plane of nutrition or specific dietary needs, impart different changes in growth and development affecting neonatal survival and adult performance (Robinson et al., 1999; Bloomfield et al., 2006). This literature review, therefore, will focus on the effects of a reduced substrate supply to fetal nutrition, as a result of maternal undernutrition, on long-term effects in postnatal life rather than on the direct effects of physiological constraints imposed by maternal, placental or vascular limitations.

2.4.2 Evidence of effects of maternal nutrition: Human epidemiological studies

Studies following the Dutch famine at the end of World War II, provide insight into the effects of severe gestational undernutrition on disease risk later in life, and help to define variations in the effects dependent on the trimester at which the insult was experienced. It was observed that offspring born to women who were exposed to famine in early gestation were born with normal birthweights but had higher risk of coronary heart disease accompanied by abnormal lipid profiles (Roseboom et al., 2000) later in life. However, those born to women exposed to famine in late gestation had lighter birthweights with increased risk of glucose intolerance (Ravelli et al., 1998).

Other epidemiological studies link the effects of fetal undernutrition to the incidence of diseases in adult life in humans. Such responses appear to be the result of fetal adaptations to undernutrition that were directed toward short-term survival, even though they seem to have permanent negative effects on body structure and function,

and consequential long-term detrimental effects upon health (Barker, 1995, 1998b). Substantial evidence linking undernutrition during gestation to the incidence of chronic disease in adult humans has been demonstrated, as reviewed by Barker and co-workers (Barker et al., 1993a; Osmond et al., 1993; Barker, 1995, 1998a, 1998b). These authors have demonstrated an association between low growth rates and birthweight, as a result of undernutrition during gestation, and the occurrence of coronary heart disease, in adult humans (Barker et al., 1993b). The increased prevalence of risk factors for cardiovascular disease (blood pressure (Barker et al., 1990), plasma concentration of glucose and insulin (Hales et al., 1991), and fibrinogen and other clotting factors (Barker et al., 1992)) are convincingly associated with low fetal and infant growth rates, further linking the effects of undernutrition *in utero* to the occurrence of chronic disease. These researchers definitively point out that these *in utero* undernutrition effects were seen in adults who as babies were born small for their gestational age rather than premature births; further qualified as babies of known intrauterine growth retardation, small compared to placental size, thin at birth, and disproportionately short compared to head size with below average infant weight gain. In addition, they suggest that the timing of undernutrition may play a significant role in the occurrence of these metabolic abnormalities as different effects are exerted on the various organs involved depending on their stage of development (Barker et al., 1993a). Similar associations between low birth weight and coronary heart disease have been reported in other studies in Sweden (Leon et al., 1998), USA (Rich-Edwards et al., 1997), and India (Stein et al., 1996).

2.4.3 Evidence of the effect of maternal nutrition: Animal models

The long-term effects of nutritional manipulation during gestation are further supported by numerous experiments in animals (Bell, 2006; Greenwood et al., 2010). Maternal gestational diets low in protein and/or energy have been associated with responses of glucose intolerance/ insulin resistance in guinea pigs (Kind et al., 2003), and increased blood pressure in guinea pigs (Kind et al., 2002), and rats (Langley & Jackson, 1994). Similarly, in sheep, glucose resistance in offspring was observed following restricted

maternal diets (Smith et al., 2010). Moreover, nutrition during pregnancy not only has effects on fetal growth, but can also have effects post birth on growth and other characteristics such as carcass and meat quality, and steroidogenesis. The following list of studies is only a small portion of the body of work in which these effects are demonstrated: Wallace, 1948; Schinckel and Short, 1961; Everitt, 1967; Gunn et al., 1995; Kelly et al., 1996; Krausgrill et al., 1999; Bielli et al., 2001; Vonnahme et al., 2003; Gardner et al., 2005; Kelly et al., 2006; Ford et al., 2007.

2.4.4 Fetal origins of disease

The evidence cited above supports the theory of "fetal origins of disease", whereby negative exposure or restrictive stimuli during the period of growth and development causes physiological, neuroendocrine or metabolic adaptations, which enable the fetus to survive a period of uterine deprivation, but cause permanent effects on the development and differentiation of key tissues and organs leading to pathological consequences in adult life (Barker, 1999, 2004a, 2004b). The process whereby "a stimulus or insult at a sensitive or critical period of development has long-term effects is termed *programming*" (Godfrey & Barker, 2000, p. 1344s). Nutrition in fetal life is now widely accepted as the primary candidate that causes a programming effect (Harding, 2001). Indeed, the influences of various stimuli during gestation are widely accepted as having effects on fetal growth and development, but it appears that nutritional effects *in utero* are of great interest, certainly because of the ease by which this can be manipulated and the universal influence this factor has on all species including man. In addition, it is apparent that these nutritional fetal programming effects are not limited to increasing the risk of adult disease.

It is proposed that there are implications for effects on the development and growth of other organs that may affect the productive life of offspring, including reproductive performance, and that such effects are dependent on the stage of gestation at which the insult is applied. Numerous studies have focused on the effects of reduced maternal

nutrition on changes in birthweight/fetal growth and specific organ development. It is concluded that the period at which the insult is applied affects the organ that is in a rapid phase of growth at that period of gestation (Harding & Johnston, 1995). The obvious effect of this is that short term or long term outcomes will vary depending on which organ or system is in the process of development when the nutritional insult is applied. This has incited interest in investigating these relationships further, particularly in production species.

Based on the long-standing evidence of effects of nutrition *in utero* on postnatal development and susceptibility to disease in adult life, it is not surprising that there is increasing interest in the possible effects of fetal programming in production animals. However, little is known about the *in utero* nutritional effect on reproductive development and performance in (first-generation) lamb offspring, nor how this might influence production efficiency in adulthood. In this respect, this review will examine the effects of maternal nutrition during gestation on reproductive development in male offspring.

2.5 FETAL PROGRAMMING EFFECTS ON MALE REPRODUCTION

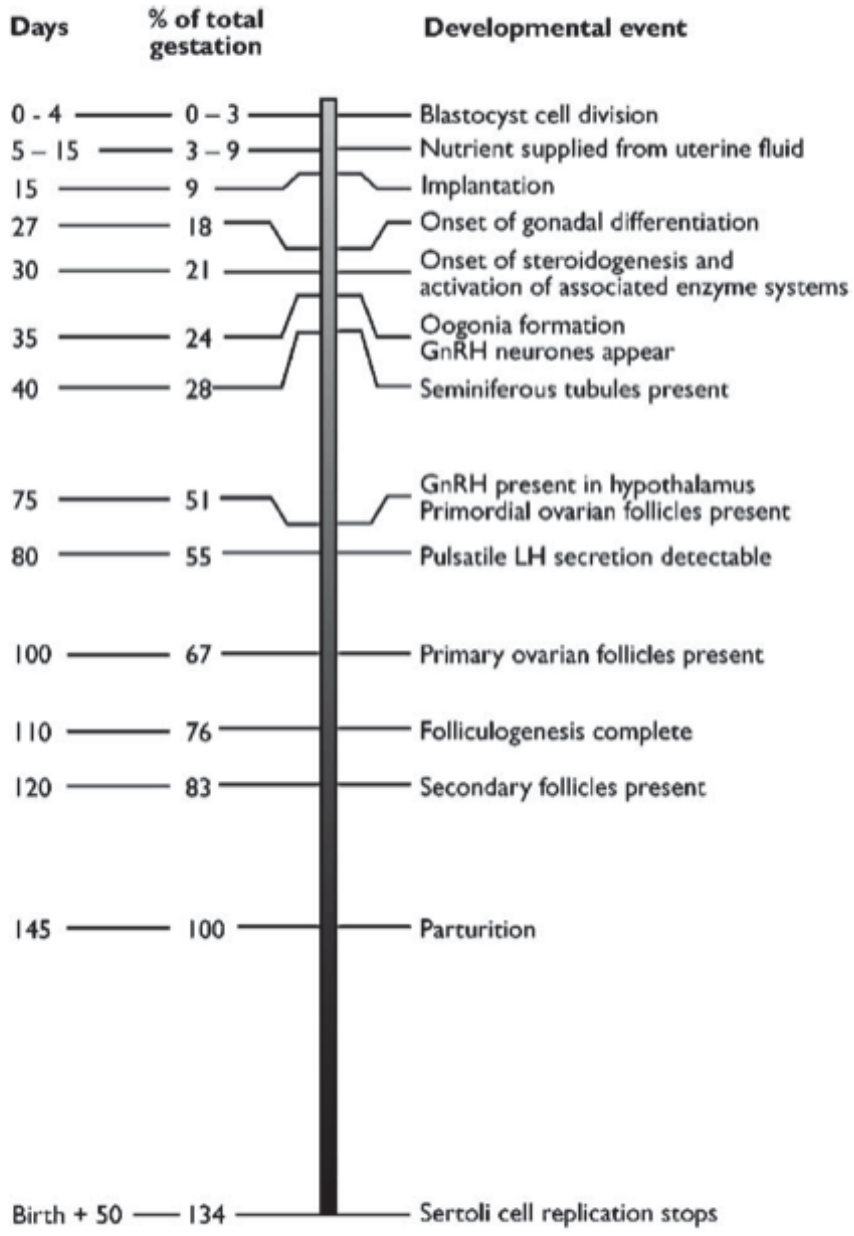
It is established that prenatal programming can occur at many different stages of development. Different organs develop at different stages of gestation and so it follows that this process affects many organs, including gonads. Because gametogenesis takes place during early gestation it is not surprising that perturbations to the uterine environment would cause an effect on reproductive organ development with possible long-term effects on fertility. Impaired gonadal development has been reported in intrauterine growth restricted (IUGR) female (Da Silva et al., 2002) and male (Asmad et al., in press) sheep fetuses. The following sections will outline the effects of fetal programming on development of gonads and subsequently reproductive performance in male offspring. A general account of critical periods of development and effects in other

species will be described, however the material covered (as it refers to this thesis) will focus on maternal nutritional as a limiting factor in male reproductive development, specifically in rams.

2.5.1 Critical periods of development

It is suggested that in both male and female fetuses there are critical windows of development for each organ system (Rhind et al., 2001; Rhind, 2004; Nathanielsz, 2006; Symonds et al., 2006). These periods of vulnerability differ for specific organs, systems and tissues and are dependent on the developmental stage and timing. Proper development of the testis during these developmental stages is imperative to ensure maximum reproductive capacity in adult life (Dufour et al., 2002). In the male, the processes of germ cell migration, steroidogenesis, gonadal development, folliculogenesis and Sertoli cell replication take place in distinct periods (Rhind, 2004; Magre & Vigier (2001) as cited in Dupont et al., 2012). These critical periods are seen in the timeline of developmental events in Figure 2.5 and are exemplified by the work of Töhönen et al. (2003), who state that a range of male sub-normal fertility symptoms can partly be caused by disturbances in the prenatal environment during differentiation of the testis.

Figure 2.5: Approximate timings of developmental events (sheep) expressed as days gestation and percentage gestation length (Rhind, 2004).



2.5.2 Potential factors causing fetal programming effects

Uterine environment (restricted uterine capacity) not only has a significant effect on liveweight, but also on testicular diameter and blood testosterone levels. It does not

appear to affect semen characteristics of adults that are exposed to a restricted uterine capacity during gestation (Jafariahangari et al., 2012). While the outcome of this experiment is of value in determining *in utero* developmental effects, it appears to focus on physical limitations to growth as denoted by comparative size of donor fetus to recipient uterus. It should be noted, however, that many other properties of the uterine environment are of consequence and may be exerting effects on the fetus. Thus, factors that influence fetal growth are not limited to just a spatial effect of the uterus; but placental attachment sites, uterine blood flow, maternal nutrient restriction or excess, and overall capacity to allow growth must not be ignored.

Some human epidemiological studies also suggest maternal gestational factors have an effect on male reproductive capacity. A study of 328 men (follow up from a Danish pregnancy cohort study) showed a trend towards a detrimental influence of high maternal Body Mass Index (obesity) on semen quality and plasma Inhibin B concentration (Ramlau-Hansen et al., 2007) of their male offspring. This suggests that there may be a programming effect of maternal overweight on development of the male fetus that is observed in adult life. Ramlau-Hansen et al. (2007) proposed that a higher level of oestrogen exposure on the fetus (due to greater hormonally active adipose tissue of obese mothers) might interfere with the development of the male urogenital organs, with possible effects on fertility in later life. This pilot study reiterates the fact that there are many facets to fetal programming effects, and therefore it should be noted that maternal factors may act on various levels to cause effects on fetal development.

On the other hand, specific challenges like maternal nutritional imbalances during these critical windows can result in changes to these male reproductive developmental processes, with short- and long-term effects, which may later manifest as perturbations in fertility in postnatal life (Rhind et al., 2001; Rhind, 2004; Dupont et al., 2012).

2.5.3 The effect of nutrition in utero *on male reproduction*

Nutrition of the dam can have an effect on the fetal reproductive system at all stages of development. Early studies on the effect of nutrition focused on the third trimester as this is the time when the fetus gains most of its weight and its nutrient demands are highest. However, the development of the reproductive and other associated organs (e.g. neuroendocrine glands) usually occur at a much earlier stage of gestation. Therefore, it is possible that maternal nutrition could have effects on fetal development regardless of the stage of gestation. Possibly the greatest influence on reproductive performance could be exerted within the first trimester when gonadal differentiation (Day 27) and onset of steroidogenesis (Day 30) take place (Figure 5). Subsequently, the effects may be experienced during both the prenatal and postnatal periods. It has been suggested that the effects of under-nutrition may be expressed at many stages of development, even before a certain organ, cell type or receptor has developed, possibly through action on precursor cells (Rhind et al., 2001). For the purpose of this review, the focus will be on effects that are demonstrated postnatally, particularly those seen or having potential to be expressed in adult life. However, some mention will be made of structural and physiological changes seen in the gonads and related organs during gestational development.

2.5.4 Indirect effects of maternal nutrition on gonadal development/the effects of hormones

Maternal nutrition can act on fetal gonadal development directly and indirectly. An alteration in the structure and/or function of the hypothalamo-pituitary-gonadal system, or other endocrine organs *in utero* may reflect an indirect effect on fetal gonadal development due to maternal nutrition. The development and function of testes in sheep fetuses depend on circulating fetal gonadotrophin concentrations (Brooks et al., 1995b). In fact, it has been shown that the growth of testes, and Sertoli cell number, in male sheep fetuses, are dependent on adequate gonadotrophin secretion during fetal life (Thomas et al., 1994). However, (Deligeorgis et al., 1996)

suggested that this may be dependent on a critical stage during which they exert their influence, or special duration or level of exposure that is necessary for gonadal development, since no effect was observed on fetal gonadal weight at 55 days of age in spite of an observed reduction in fetal pituitary activity following maternal undernutrition. Rae et al. (2002c) reported altered pituitary function in males following undernutrition of the dam, that could influence reproductive development in spite of the lack of a demonstrable effect on fetal gonadal weight at Day 119 of gestation. Similar results were observed by Rae et al. (2002b). However, in contrast, other work done by Rae et al. (2002a) suggest that the effects observed due to maternal undernutrition in both male and female offspring is not associated with changes in gonadotrophin profiles or pituitary responsiveness. Little is known on the effects of undernutrition on fetal testes development in the gestational period prior to the development of a functioning fetal pituitary; however, this does not preclude the fact that programming effects on reproductive capacity may occur through changes in the hypothalamus-pituitary gland or the gonads (Rhind et al., 2001). As such, it may be suggested that the potentially direct effects of nutrient supply to the gonad, expressed as structural differences in gonadal development, may be of more consequence to reproductive function and performance in adult life.

2.5.5 Gestational nutrition effect on male offspring in species other than sheep

In many species the effect of maternal nutrition on postnatal male reproductive parameters of offspring has been demonstrated. Fifty per cent maternal food restriction in the Rat-like hamster (*Cricetulus triton*) was demonstrated to reduce both the size of the epididymis and the concentration of testosterone in F1 generation offspring (60 days of age); 70% restricted diet significantly reduced the size of male reproductive organs and hormone concentrations (Liang & Zhang, 2006). Delayed onset of puberty was demonstrated in male rats that were subjected to gestational malnutrition (Engelbregt et al., 2000) and, in other studies, undernutrition during the fetal to pubertal periods in rats resulted in changes in testicular structure associated with lower

Sertoli cell numbers in adult life that may be associated with lowered sperm production (Genovese et al., 2010). Gestational protein-restricted diets in rats delayed sexual maturation, impaired sperm counts (Zambrano et al., 2005), reduced Sertoli cell numbers and sperm motility counts, and increased number of sperm presenting with morphological abnormalities (Toledo et al., 2011) in male offspring. These effects all suggest impairment of sperm quality and possibly reduced fertility or reproductive capacity in adulthood. Low protein diets fed to dams in early and mid gestation has also been shown to lead to increased pre-pubertal plasma FSH and testicular weight in young bulls (Sullivan et al., 2010). The evidence cited here is of immense value; however, for the purpose of this study the focus on maternal nutritional effects seen in sheep reproductive development must be considered and will be discussed in the following section.

2.5.6 The effect of nutrition in utero *on the ram*

Gestation in sheep is approximately 145–150 days and can be equally divided into three distinct stages ("trimesters"). The nutrient requirements vary with the growth and development of the fetus in each trimester. Specific gonadal structures in the sheep fetus begin to develop in the first trimester, and the neuroendocrine (hypothalamic-pituitary) systems at about Day 35–85 (Kotsampasi et al., 2009). Gonadal differentiation begins at around Day 34 of pregnancy (Bielli et al., 2002). This would suggest that nutritional manipulations at this time might have an influence on the subsequent reproductive performance of both male and female offspring. To date, there exists substantial evidence of responses in female offspring to nutritional manipulations during various periods of gestation which include: a delay in ovarian follicular development (early to mid (Rae et al., 2001), late (Da Silva et al., 2002)); retarded fetal ovarian development (early (Borwick et al., 1997)); and reduced ovulation rate (early to mid (Rae et al., 2002a)), that corresponds with earlier evidence that nutrition in (late) pregnancy influences the subsequent reproductive performance in female sheep (Gunn et al., 1995). However, little is known about reproductive effects in ram offspring.

It has been shown that postnatal testicular diameter and blood testosterone differed at Days 120, 150, 180 and 240 in response to different maternal uterine environments (Jafariahangari et al., 2012). In contrast, this study also demonstrated that uterine environment had no effect on semen characteristics of the same study population at 150–240 days of age. Effects on gonadal development due to prenatal under-nutrition for the first 95 days of gestation have also been investigated in male sheep, and no effect on scrotal size nor semen quality was observed at 55 days of age (Rae et al., 2002a). Other studies have focused on onset of puberty. Da Silva et al. (2001) demonstrated lowered testosterone concentrations and testicular volume as an indication of delayed onset of puberty in ram-lambs that had been growth restricted *in utero*. This study compared the effects of High (ad libitum) vs. Moderate (fed to achieve 75g/d liveweight gain) nutritional treatments from Day 1 to 100 of gestation, using the "adolescent pregnancy model". In this model, over-nutrition of adolescent ewes throughout gestation results in rapid maternal growth rates at the expense of nutrient requirements of the uterus and developing fetus. As a result, major placental growth and fetal growth restriction occurs (Wallace et al., 1996).

The effects on Sertoli cells and gonadal structure may be of more importance in defining the influence of maternal nutrition on male reproductive capacity in rams. Sperm production and output is highly correlated to Sertoli cell numbers (Orth et al., 1988), and, as a result, changes to Sertoli cell numbers in response to nutritional treatments during gestation might directly affect reproductive performance in terms of sperm quantity and/or quality. No effect of maternal nutrition was seen on seminiferous cord nor Sertoli cell numbers in testes harvested from Day 103 fetuses (Da Silva et al., 2003). The "adolescent pregnancy model" was used in this study. In contrast, reduced Sertoli cell numbers have been observed in ram-lambs as early as birth in response to maternal under-nutrition (Bielli et al., 2002; Kotsampasi et al., 2009). This concurs with the previously cited response in pubertal rats as described by Genovese et al. (2010). However, the importance of the effect on Sertoli cell number at this early postnatal

stage should be considered carefully, as it may be disputed that this structural effect may not be found to carry forward into adult-life nor affect reproductive capacity at the later post-pubertal stage. A study by Bielli et al. (2001) showed that Sertoli cell numbers in ram offspring respond positively to supplementation or improved nutrition during gestation. This evidence would suggest that due to the link between Sertoli cells numbers and sperm output, this cell type would successfully demonstrate the effect of prenatal nutrition on ram reproductive development and production in adulthood. Thus, it may be suggested that since the effect exerted *in utero* may be expressed at a later stage (i.e. sexual maturity/adulthood), the period that would be most indicative of an effect would be in the post-pubertal period of the ram's life. It is during this period that effects may be expressed as changes to sperm output and quality as well as other sexual characteristics. These effects are summarised in Table 2.1.

Rae et al. (2002a) measured scrotal circumference, and observed no difference in size between control (dams fed to maintenance) and treatment (dams fed 50% of maintenance) rams, thus concluding that under-nutrition *in utero* has no effect on male reproductive development. Total size may not give a true picture of the effects of *in utero* nutritional treatments as the testicle consists of various cell types including Sertoli cells. There is evidence that the critical period during which the complement of Sertoli cells that populates the adult testis is established occurs during fetal development (Hochereau-de-Reviers et al., 1995). However, it is noted that Sertoli cell multiplication does continue between birth and puberty, with an increase by a factor of 5–10 before spermatogenesis commences (Hochereau-de-Reviers et al., 1987). Further to this, Monet-Kuntz et al. (1984) reported that in sheep Sertoli cell size (cellular and nuclear) increases with development of the seminiferous epithelium during the period between birth and puberty. This is associated with a high correlation observed between Sertoli cell size and germinal cell numbers, possibly explaining why no effects are observed on testicular size in post-pubertal rams. This may have implications for adult reproductive performance, as possible recovery from deleterious manipulations *in utero* may occur

postnatally due to increased Sertoli cell numbers during this postnatal “recovery” period, consequently resulting in adequate sperm output during the post-pubertal period. In addition, it is apparent that there is variation in results between studies for other measured variables and the effects seen in the fetus and young animal are not observed in the adult; indeed suggesting that recovery and/or other factors may play an important role in the development of adult male reproductive capacity.

Table 2.1: The effect of maternal nutritional regimen and timing of nutritional regimen on post-parturition and fetal reproductive traits of male sheep offspring. Adapted from: Kenyon (2008)

Reference	Timings of nutritional regimens	Nutritional regimens	Reproductive traits
Rae et al., 2002a	D1 to D95	0.5M[1] vs. 1.0M	No effect on semen quality nor scrotal size at 20 months of age
Deligeorgis et al., 1996	D30 to parturition	0.9M vs. 1.1M	0.9M reduced response to GnRH No effect on male gonadal weight at 55 days of age
Kotsampasi et al., 2009	D1 to D30 (R1[2]) D31 to D100 (R2[3])	1.0M vs. 0.5M	No effects on onset of puberty No effect on testis weight at 10 months of age Sertoli cell numbers were lower in R2 group Smaller seminiferous tubule diameter in R2 group
Bielli et al., 2002	Week 10 to parturition	0.7M vs. 1.1M	0.7M lower testis weight and lower Sertoli cell number at 2 days of age
Bielli et al., 2001	mid to late pregnancy	1.0M vs. >M	1.0M lower testis weight and tendency to lower Sertoli cell numbers at 99 days of age
Da Silva et al., 2001*	D1 to D100	High (*ad lib*) vs. Moderate (75g/d liveweight gain)	Lower testosterone concentration and testicular volume at birth and weeks 28, 35 of age in High (growth restricted lambs)
Rae et al., 2002b	D1 to D30, D31 to D50, D31 to D35, D31 to D110	0.5M vs. 1.0M	No effect on fetal testis mass Some effect on fetal steroidogenic capacity in early pregnancy
Rae et al., 2002c	D1 to d119	0.5M vs. 1.0M	No effect on fetal gonadal weight at Day 119 of gestation Effects on pituitary function (GnRH response)
Da Silva et al., 2003*	D1 to D100	High vs. Moderate	No effect on fetal plasma gonadotrophin levels and no effect on seminiferous cord or Sertoli cell numbers at Day 103 of gestation

*Adolescent Pregnancy Model – High nutritional regimen of dam results in fetal nutritional restriction
[1]Maintenance; [2]Restricted group 1; [3]Restricted group 2

2.6 SUMMARY AND CONCLUSIONS FROM THE LITERATURE REVIEW

Ewe nutrition plays a fundamental role in the regulation of fetal development with consequences on health and production in later life through the process of programming (Bell, 2006; Symonds et al., 2006; Wu et al., 2006). These effects may be of greater consequence to ewes carrying multiple fetuses because of the higher nutritional demand of twin pregnancies compared to singletons (Cleal et al., 2007; Kenyon et al., 2011). In addition, in extensive grazing systems of temperate regions, gestation usually coincides with winter, during the period of greatest feed shortage (Mathews et al., 1999) when low grass growth rates do not meet livestock demands. Thus, there is significant potential to have an influence on offspring development and both short- and long-term production, and this is of great economic importance.

Male reproductive performance is highly dependent on the success of spermatogenesis. Spermatogenesis involves a complex interplay between hormones and testicular cells to produce large numbers of normal sperm capable of fertilisation. Once the spermatogenic cycle of the ram is initiated it is thought to be continuous, but undergoes periods of temporary arrest due to seasonal triggers, with the consequence that, over a yearly period, fertility differs with season. Most importantly, however, the success of the process of spermatogenesis (and thus male fertility) is highly dependent on there being normal and complete male sexual differentiation and development (McLachlan, 2000). During early pregnancy (Day 0–50) the onset of gonadal development and differentiation and steroidogenesis takes place (Rhind, 2004). Thus the greatest influence of maternal nutrition on the development of reproductive tissues is likely to occur during this period with potential consequences to reproductive performance in adulthood.

There were some inconsistencies in the studies reviewed that make it difficult to identify whether the effects demonstrated would persist into adulthood. Bielli et al. (2001) measured Sertoli cell numbers at 2 days post-partum, whereas Bielli et al. (2002) and Kotsampasi et al. (2009) measured at 10 months and 99 days respectively. Rae et al.

(2002a) assessed ram offspring at 20 months of age. It should be noted that the nutritional treatments differed between studies in both the rations provided and the gestational period in which they were administered, making it difficult to compare the results. Additionally, comparisons may be difficult as maternal nutrition may affect other factors that influence male reproductive development, such as the hypothalamic-pituitary response (as suggested by Rae et al. (2002b; 2002c) and Bielli et al. (2002)). At the steroidogenic level, there is evidence that the physiology of development of sheep fetal testes was significantly altered by nutrient restriction in early pregnancy (Rae et al., 2002b), although no changes in fetal testis mass was observed. However, there is still considerable debate about the actual link between steroidogenic development/ hormonal effects on gonadal development and subsequent reproductive capacity in males. Furthermore, other factors that may confer intra-uterine restrictions may be of consequence in comparing the results e.g. use of twin bearing dams in some studies (Kotsampasi et al., 2009) vs. single bearing in others (Bielli et al., 2001; Bielli et al., 2002) or both (Rae et al., 2002a).

The existing evidence therefore suggests that the effect of undernutrition of the ewe would be more likely to be deleterious to the fetal reproductive system compared to a ewe maintained on a high nutritional plane during (early) gestation. It is clear, however, from the epidemiological and experimental data cited in this review that maternal nutrition has the potential to adversely impact on offspring reproductive development and maturation. To date, studies examining the effects of maternal nutrition on male reproductive performance are limited to effects observed on late gestation fetuses and neonates. Little information exists on the effects in post-pubertal or adult life. Thus, although it may be thought that the effects demonstrated may be extrapolated to this later period, there is a need to demonstrate if indeed these changes are expressed as changes to fertility in the post- pubertal period.

Therefore, the purpose of this study was to examine the effects of maternal nutritional treatments at early (Day 21 to 50) and mid- to late (Day 50 to 140) gestation on the reproductive characteristics of twin ram offspring; and to determine if there is variation in the reproductive parameters with season. Twin-bearing ewes were used due to their economic importance to the sheep industry in New Zealand and their high vulnerability to maternal undernutrition. It was postulated that rams born to dams underfed during early pregnancy would have underdeveloped reproductive organs resulting in reduced fertility factors/reproductive performance in the post-pubertal period.

3.1 BACKGROUND INFORMATION

This study utilised 65 twin-born ram offspring from a larger study examining the long-term effects of maternal nutrition (Kenyon et al., 2011). In brief, a group of 1169 Romney ewes (average liveweight 66.3 ± 0.18kg (s.e.[1]) and condition score of 2.96 ± 0.02) from a commercial flock were synchronised by vaginal insertion of a controlled internal progesterone releasing device (CIDR, 0.3g progesterone, Pharmacia & UpJohn, Auckland, New Zealand) for 13 days (Days -14 to 0). On Day -2 CIDRs were removed from half of the flock and on Day 0 these ewes were artificially inseminated, via intra-uterine laparoscopy, using semen randomly allocated from one of five Romney rams. The remaining ewes underwent the same procedure staggered by one day (Day -1 removal of CIDR, Day 1 artificial insemination). The cohorts were then merged. On Day 5, 12 crayon-harnessed, entire, Romney rams were introduced to the ewes. The joined group was managed under commercial conditions. They were offered herbage at a minimum post-grazing mass of 1200kg DM/ha until Day 20, to allow unrestricted feed intake (Kenyon & Webby, 2007).

The entire rams and "return-to-service" ewes (i.e. ewes that were displaying harness marks on their rumps) were removed at Day 21. The remaining ewes were then randomly allocated to one of three nutritional treatment groups until Day 50 (Early gestation treatment period Day 21–50: High (H_{D21-50}) vs. Maintenance (M_{D21-50}) vs. Low (L_{D21-50})). The aims of these treatment groups were as follows: H_{D21-50}: to provide *ad libitum* grazing conditions and an average weight gain of 100g/day; M_{D21-50}: to ensure no change in total ewe liveweight; L_{D21-50}: to achieve a loss in total ewe live weight of 100g/day.

Pregnancy scanning via transabdominal ultrasonography was performed on all the ewes at Day 48. Non-pregnant, single- and triple-bearing ewes and ewes with incomplete data

[1] standard error

were removed at this point, and the remaining twin-bearing ewes were randomly allocated to one of two further nutritional treatments until Day 140 (mid-late gestation treatment period Day 50–140: High ($H_{D50-140}$) vs. Maintenance ($M_{D50-140}$)). Animals from each of the three Day 21–50 treatment groups were included in each of these two treatment groups. The aim of these treatment groups were either to provide *ad libitum* grazing conditions ($H_{D50-140}$), or to ensure that total ewe liveweight increased at a level similar to that of expected conceptus mass (Rattray et al., 1974)($M_{D50-140}$).

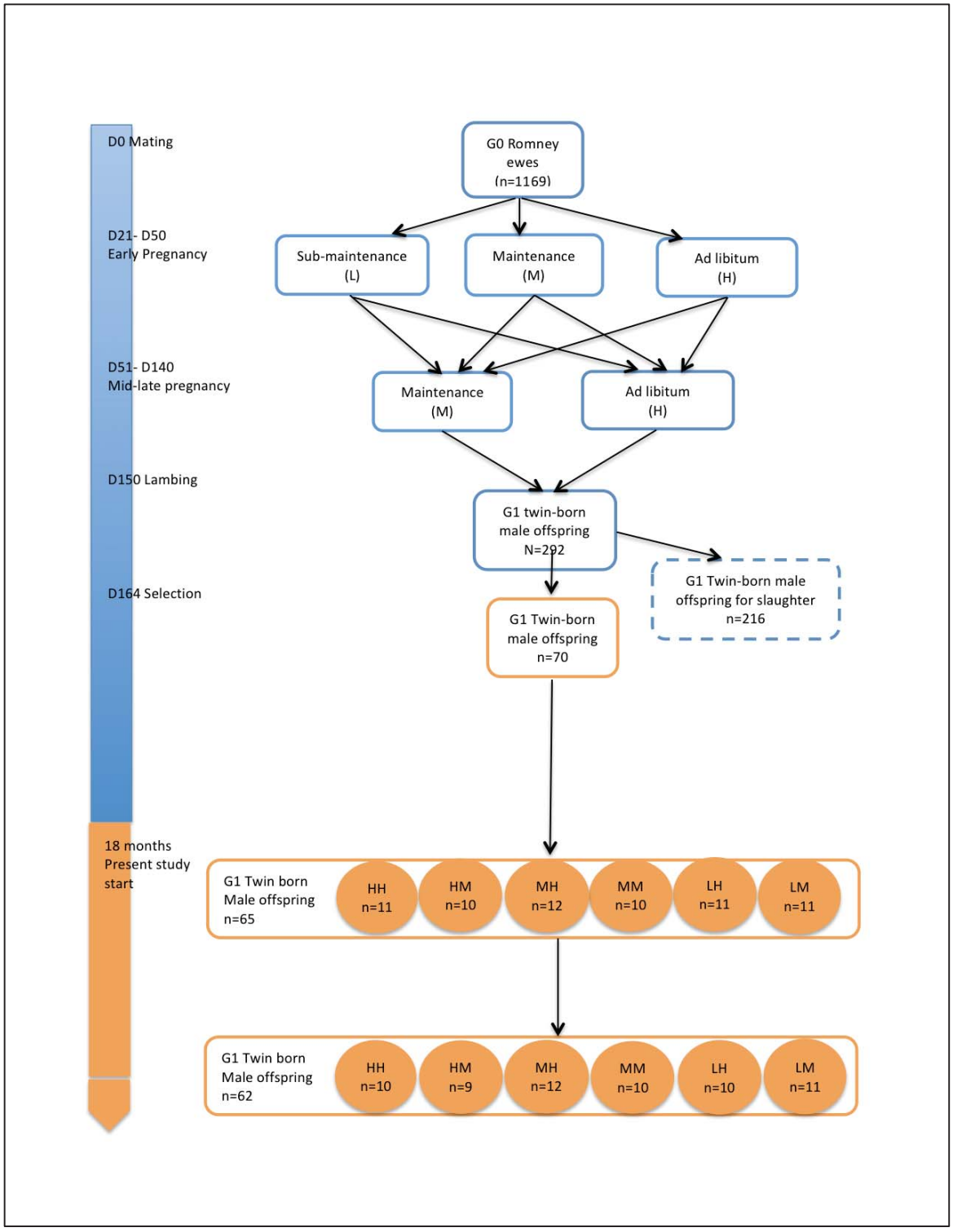
On Day 140, the nutritional treatments were merged and all the ewes were placed in paddocks for lambing, at a rate of 12.1 ewes/ha, with an average herbage mass of 1558 ± 73 (s.e.) kg DM/ha (Kenyon et al., 2011). Offspring were ewe reared until weaning. From weaning, all twin-born male lambs (n=292) from both mixed and same sex sets were managed together under commercial grazing conditions. On Day 164 post birth, 70 male twins were retained for this study while 216 males were sent to slaughter (Asmad et al., 2011). The 70 rams were randomly selected from within all the treatment groups. These rams continued to be managed as one group under commercial grazing conditions from this time-point and for the duration of the study. Five rams were lost from the group before the start of the present study.

3.2 THE PRESENT STUDY

3.2.1 Experimental model and animals

This study only reports on twin-born ram offspring. Trial design and timeline is shown in Figure 3.1. This study took place at Massey University Sheep and Beef farm/Haurango Shed (5km south of Palmerston North, New Zealand), from March 2011 to March 2012 (winter solstice 21 June). The study and all animal-handling procedures were approved by the Massey University Animal Ethics Committee, Palmerston North, New Zealand.

Figure 3.1: Trial design and timeline



3.2.2 Measurements

A total of 62 rams were evaluated in the present study. Incomplete data were collected from three rams due sickness or losses due to death; therefore they were omitted from the final report. The number of rams in each treatment group is shown in figure 3.1. The data collection ran continuously for 13 months. The age of the rams at the start of the trial was 18 months, running to 31 months at the end of the trial. Liveweight was measured monthly and scrotal circumference was assessed at two-week intervals. Semen collection was performed at two-monthly intervals for the duration of the study period.

Live weight measurements

Individual rams were restrained in a weigh-box located at the end of a race. The tag number of the ram was identified and entered into the electronic digital scale and the scale automatically recorded the ram's weight.

Scrotal circumference measurements

Measurements of the maximum circumference of paired testes of standing rams were made using a standard scrotal circumference tape. The testes were pulled firmly into the base of the scrotum and the loop of the tape slipped over the scrotum and its contents, and pulled snugly around the point of greatest diameter. A single reading at this point was taken for each ram.

Semen analysis: Semen collection

Semen samples from the rams were collected via electro-ejaculation using a New Zealand Ruakura Probe. Rams (non-sedated) were held in lateral recumbency with hind-legs extended slightly caudally. The penis was extruded and held with gauze bandage and electro-ejaculation performed using the lubricated probe inserted into the rectum (Evans & Maxwell, 1987). Semen was collected into 50mL collection pottles. Preparations of fresh semen morphological sides were made using eosin-negrosin (Merck, Germany) stain for further examination, and a portion of the fresh semen samples were also diluted

1:1 using 1% formal phosphate buffered saline solution for further evaluation of quantitative sperm density (sperm concentration).

Semen analysis: Visual characterisation

Semen characteristics of visual density, wave (gross) and forward motility were assessed immediately on site post-collection (Evans & Maxwell, 1987). Visual density was classified based on the appearance of the collected sample. Wave motility was assessed under phase contrast light microscopy at X10 and a portion of the sample diluted with phosphate buffered solution was examined for forward motility at X40 magnification. The visual density was classified as creamy, milky or watery. Wave motility was classified on a scale of 0–10; 0: no wave motility observed, 10: very good, dense, very rapidly moving waves. Forward motility was also classified on a scale of 0–10; 0: no sperm observed moving in a progressive (forward) manner, 10: 10 out of every 10 sperm observed moving in a progressive manner (Evans &Maxwell, 1987).

Semen analysis: Sperm morphology

Sperm morphology was determined via microscopic examination of the prepared slides at X1000 magnification under oil immersion. Major and minor spermatozoal defects were identified and categorized (Blom, 1983). Major sperm defects were listed as: acrosome defects, head defects (small, abnormal, detached-abnormal, or double heads), mid-piece defects (corkscrew, thickened or other mid-piece defects), tail defects (coiled, dag defects), or distal mid-piece reflex (looped tails with enclosed cytoplasmic droplets) proximal cytoplasmic droplets and teratospermia. Minor sperm defects were listed as: head defects (small or narrow, giant or broad, detached-normal heads), detached acrosomal membranes, fractured necks, tail defects (terminally looped, looped and two tailed), and distal cytoplasmic droplets.

Semen analysis: Quantitative semen analysis

The semen samples that were diluted to 50% using formal phosphate buffered saline were further diluted to 2% and the quantitative sperm density (sperm/ml) was determined using a haemocytometer (Evans & Maxwell, 1987).

3.3 STATISTICAL ANALYSIS

Complete ram data were collected from 62 rams and the statistical analysis was only undertaken on these animals. The study utilised a 3x2 factorial design resulting in six nutritional groups. Repeated measure analysis of variance with respect to treatment and time in which individual animals were nested within treatment groups was used to analyse the data for ram liveweight, scrotal circumference and quantitative sperm density (concentration). The data were analysed using SAS (version 9.3, SAS Institute Inc., Cary, NC, USA) mixed model procedure. The data for quantitative sperm density was not normally distributed so a square root transformation was used to achieve a normal distribution for statistical analysis. There was incomplete data collected for ram liveweight for September 2011; therefore the liveweight data for this month is not included in the analysis. The models used to analyse ram liveweight, scrotal circumference and quantitative sperm density included the fixed effects of nutritional treatments during D21 to 50 (early gestation), D50 to 140 (mid- to late gestation) and their interaction. Date of collection was fitted as a fixed effect. Non-significant two-way interactions between the two nutritional regimens were removed.

The data for forward motility and morphology (percentage abnormal sperm) were not normally distributed and could not be normalised. Non-parametric Kruskal-Wallis one-way analysis of variance was conducted to detect treatment effects and followed by the Wilcoxon rank sum post hoc test (Bonferroni correction) to identify significant differences where a treatment effect was identified. Data for these variables are

presented as medians rather than means as non-parametric tests were used for the analysis.

Percentage abnormal sperm was converted to a binomial distribution to reflect fertility/ non-fertility and this was analysed using a generalised model allowing for repeated measures. This data for both nutritional treatments (D21–50 and D51–140) could not be analysed concurrently due to all rams in the $M_{21\text{-}50}$ followed by $M_{51\text{-}140}$ maternal nutrition treatment group reaching the threshold for not fertile at the November time-point, therefore, the generalised model was conducted separately for each nutritional treatment.

The data for visual density and wave (gross) motility of the semen was not normally distributed and could not be analysed. There were certain categories/scores within some time-points for the maternal nutritional treatment groups at which none of the semen of study rams fit the criteria for that score or observation. The raw data is presented for these characteristics for each maternal nutritional regimen in Table A.1 and A.2 in the appendix.

4.1 LIVEWEIGHT

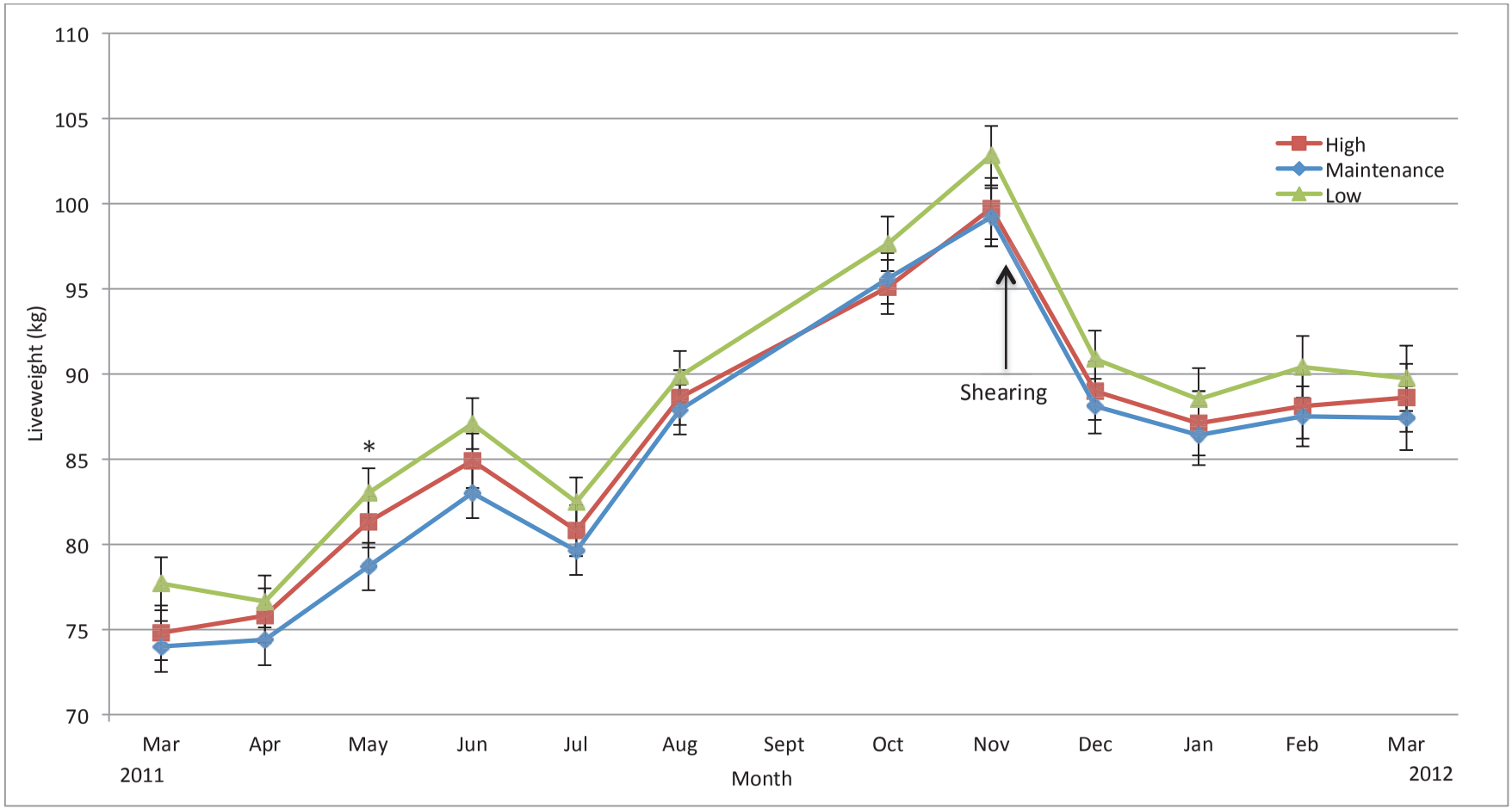
Changes of liveweight of rams between 18 (March 2011) and 31 (March 2012) months of are shown in Figure 4.1a, 4.1b and Table A.3 (in appendix). Ram liveweight increased from March 2011 to November 2011; this was followed by a decrease in liveweight in early December. There were no interactions (P>0.05) between the D21-50 and D51-140 maternal nutritional treatments for post pubertal ram liveweight. During the period March 2011 to March 2012 the only effect of maternal nutrition on ram offspring occurred in May, when rams born to ewes fed $M_{21\text{-}50}$ were lighter (P<0.05) than those born to ewes on the $L_{21\text{-}50}$ diet. Ewe nutrition between D21 to D50 and between D51 to D140 of gestation did not have an effect on liveweight of the rams at any other time-point.

4.2 SCROTAL CIRCUMFERENCE

Changes in scrotal circumference of rams between 18 and 31 months of age are shown in Figure 4.2a, 4.2b and Table A.4. There were no interactions (P>0.05) between the maternal nutritional treatments during D21-50 and D51-140 of gestation for scrotal circumference. At 22 months (early July), scrotal circumference was greater (P<0.05) in rams born to ewes fed $M_{21\text{-}50}$ than those born to ewes fed $L_{21\text{-}50}$. At 25 months (October 2011), the scrotal circumference of rams born to $H_{51\text{-}140}$ fed ewes were greater (P<0.05) than those born to ewes fed $M_{51\text{-}140}$. At no other time points did the ewe nutrition, in either D21–50 or D51–140 of gestation affect ram scrotal circumference.

In December 2011, mean scrotal circumference of the rams was smaller (P<0.05) than throughout the rest of the study (Figure4.2a, 4.2b). Following this time-point scrotal circumference increased to a maximum in March 2012 which was greater than all the other months (P<0.05) except late February 2012.

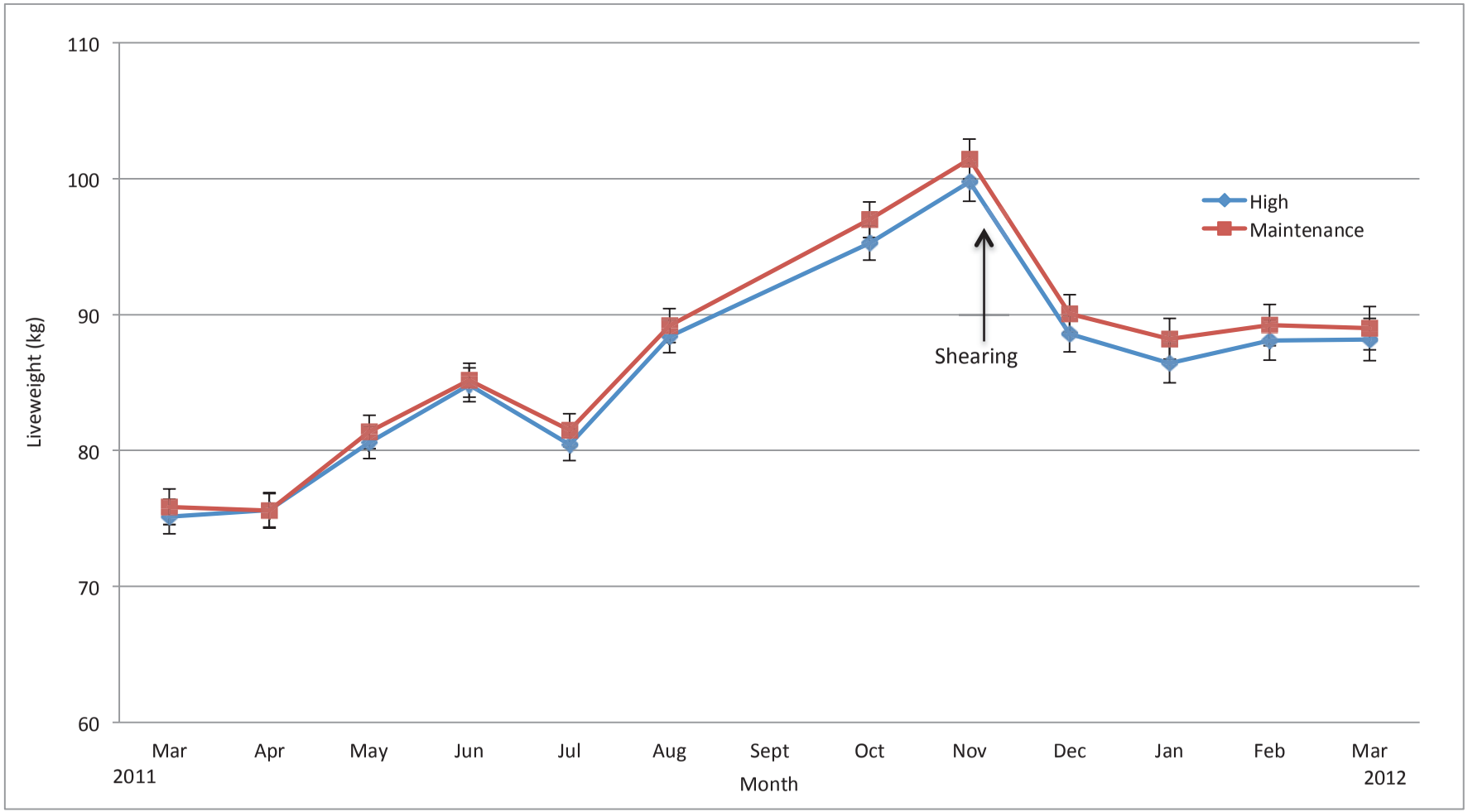
Figure 4.1a: Liveweight (kg) of post-pubertal rams from 18 to 31 months of age (March 2011 to March 2012) that were born to ewes fed either High (H) or Maintenance (M) or Low (L) nutritional levels during early pregnancy (D21–50).



Values are least squares means ± standard error of the mean.

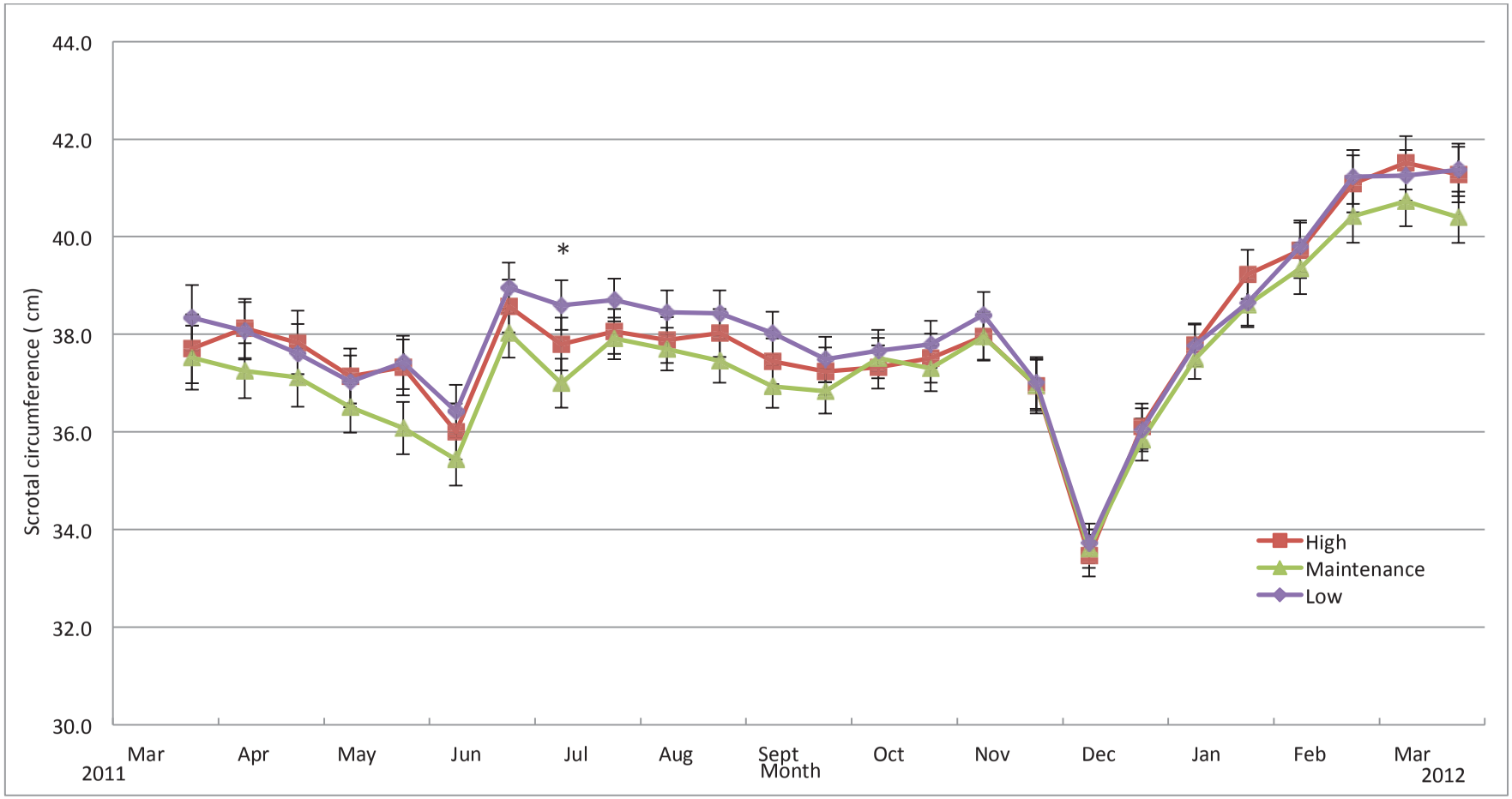
* Indicates time-point at which significant differences were observed (P<0.05).

Figure 4.1b: Liveweight (kg) of post-pubertal rams from 18 to 31 months of age (March 2011 to March 2012) that were born to ewes fed either High (H) or Maintenance (M) nutritional levels during mid-late pregnancy (D51–140).



Values are least squares means ± standard error of the mean.

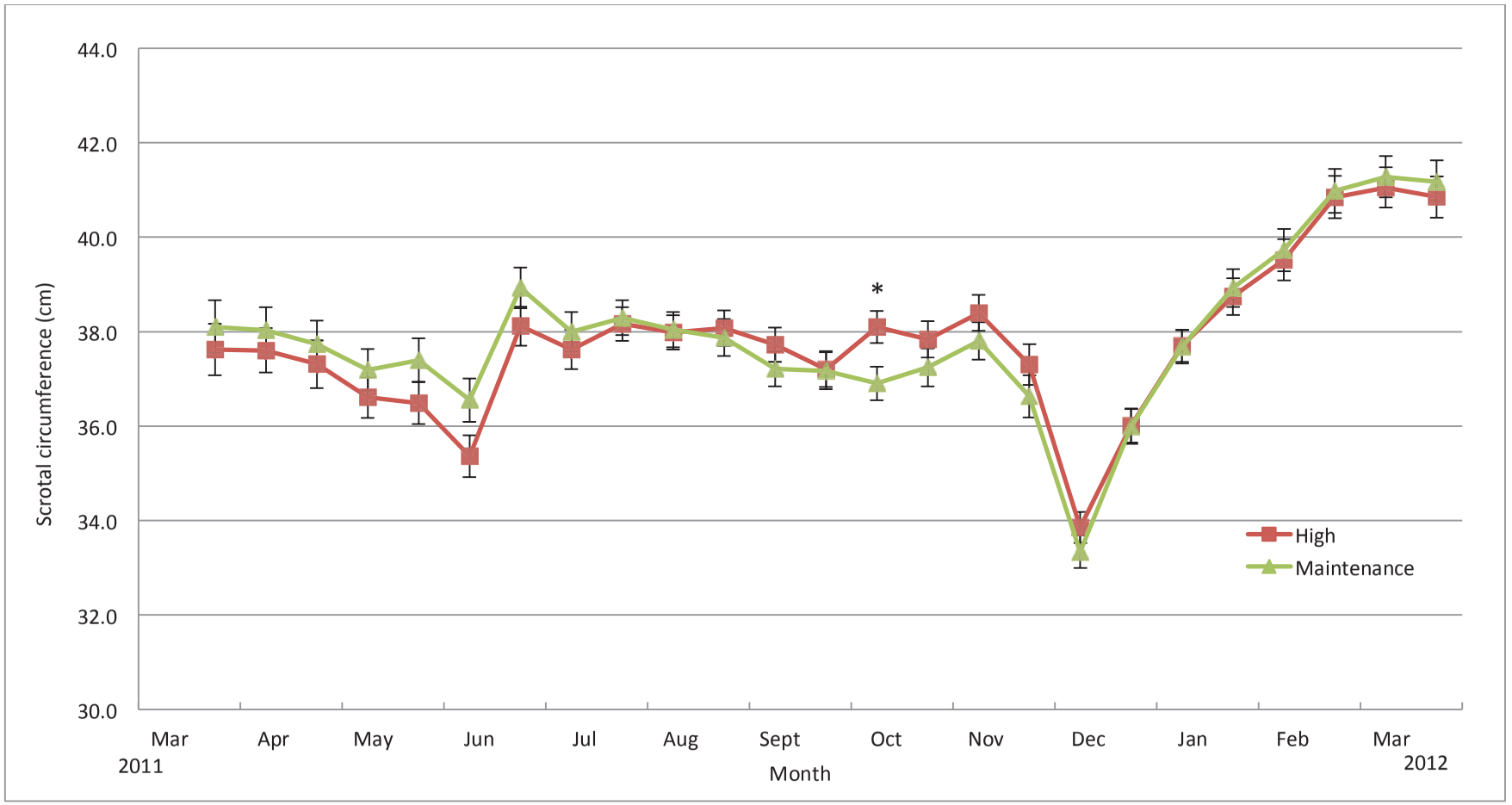
Figure 4.2a: Scrotal circumference (cm) of post-pubertal rams from 18 to 31 months of age (March 2011 to March 2012) that were born to ewes fed either High (H) or Maintenance (M) or Low (L) nutritional levels during early pregnancy (D21–50).



Values are least squares means ± standard error of the mean.

* Indicates time-point at which significant differences were observed (P<0.05).

Figure 4.2b: Scrotal circumference (cm) of post-pubertal rams from 18 to 31 months of age (March 2011 to March 2012) that were born to ewes fed either High (H) or Maintenance (M) nutritional levels during mid-late pregnancy (D51–140).



Values are least squares means ± standard error of the mean.

* Indicates time-point at which significant differences were observed (P<0.05).

4.3 VISUAL CHARACTERISTICS OF SEMEN

4.3.1 Forward Motility

The data for semen characteristics of rams (visual and quantitative) between 18 and 31 months of age are shown in Tables 4.1, 4.2, 4.3 and 4.4 and Figures 4.3, 4.4 and 4.5. There were no ($P>0.05$) interactions between the two maternal nutritional treatment regimens for forward motility scores. Median forward motility scores did not differ ($P>0.05$) between maternal nutritional treatment groups at any time-point for either D21–50 or D50–140 of gestation (Table 4.1).

4.4 QUANTITATIVE SPERM DENSITY (sperm x10^9/ml)

There were no significant interactions ($P>0.05$) between the maternal nutritional treatments in D21- 50 and D51-140 of gestation for quantitative sperm density. At 22 months of age (July) rams born to ewes fed $H_{21\text{-}50}$ had lower ($P<0.05$) sperm density that those born to ewes fed $M_{21\text{-}50}$ and $L_{21\text{-}50}$ (Table 4.2). At 31 months (March 2012), rams born to ewes fed $L_{21\text{-}50}$ had lower ($P<0.05$) sperm density than rams born to ewes fed $H_{21\text{-}50}$. Maternal nutrition during D21 to D50 had no effect on sperm density at any other time point. At 26 months (November) rams born to ewes fed $H_{51\text{-}140}$ had higher sperm density ($P<0.05$) than those born to ewes fed $M_{51\text{-}140}$. Maternal nutrition during D51 to D140 had no impact ($P>0.05$) on quantitative sperm density at any other time-point.

Sperm density differed ($P<0.05$) between monthly time points for all the rams (Figure 4.3). Sperm density was greatest ($P<0.05$) in September 2011 than any of the other months; this was followed by July 2011 and January 2012, which were in turn greater than May 2011, November 2011 and March 2012. The month in which the sperm density was the least was March 2011 ($P<0.05$).

Table 4.1: Median[1] values of forward motility scores of sperm in semen of post-pubertal rams from 18 to 31 months of age (March 2011 to March 2012) that were born to ewes fed either High (H) or Maintenance (M) or Low (L) nutritional levels during early pregnancy (D21–50) and High (H) or Maintenance (M) nutritional levels during mid-late pregnancy (D51–140).

	D21-50			D51-140	
	H	M	L	H	M
n	19	22	21	32	30
March 2011	8.0	8.0	9.0	8.5	8.0
May 2011	8.0	8.0	9.0	8.0	8.0
July 2011	8.0	8.0	8.0	8.0	8.0
September 2011	9.0	9.0	9.0	9.0	9.0
November 2011	8.0	8.0	9.0	9.0	9.0
January 2012	9.0	9.0	9.0	9.0	9.0
March 2012	10.0	9.0	10.0	10.0	9.0

No significant differences (P>0.05).

[1]Data could not be normalised so forward motility scores are presented as median values.

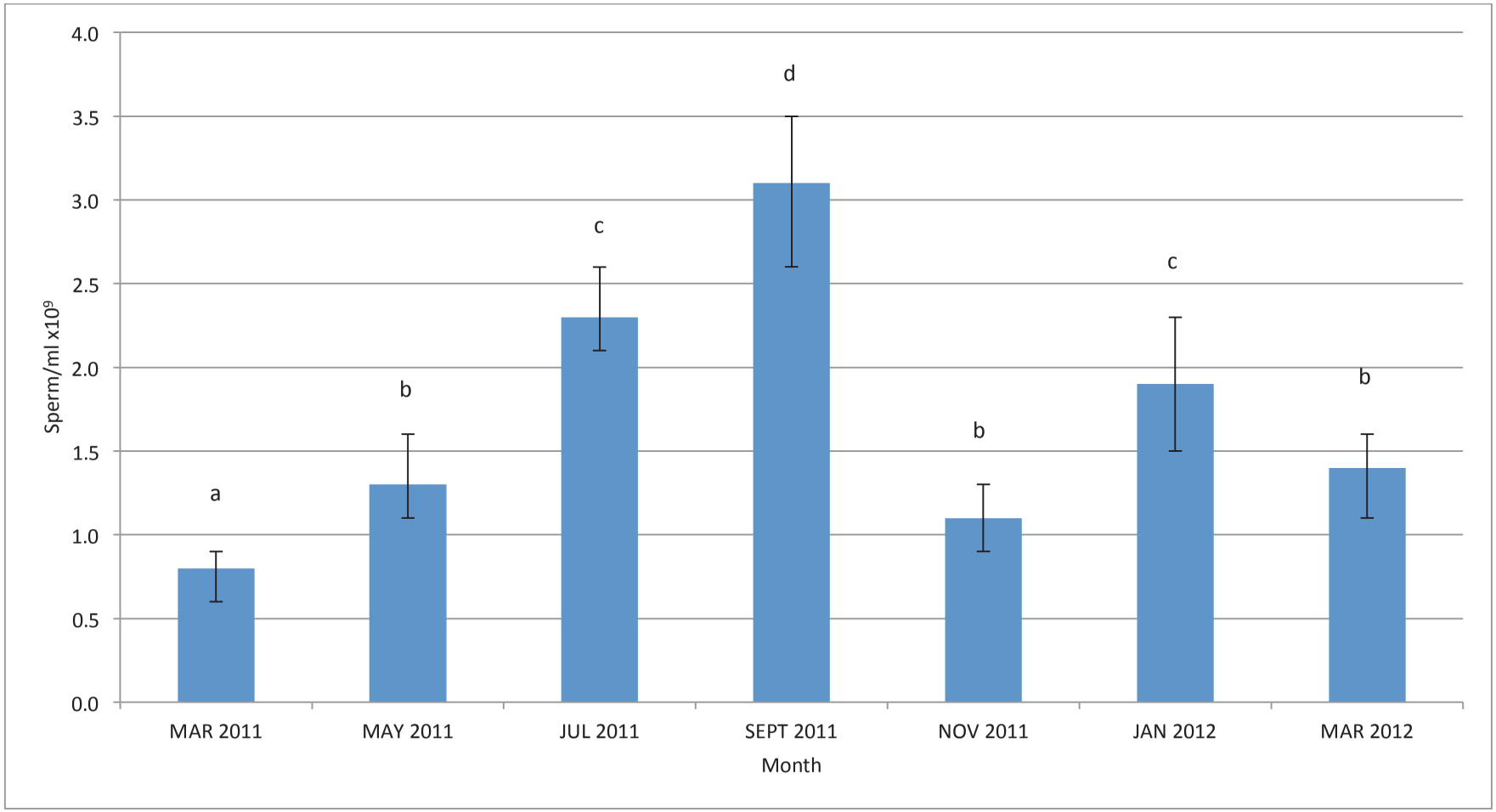
Table 4.2: Quantitative sperm density (sperm x10^9/ml) of semen of post-pubertal rams from 18 to 31 months of age (March 2011 to March 2012) that were born to ewes fed either High (H) or Maintenance (M) or Low (L) nutritional levels during early pregnancy (D21–50) and High (H) or Maintenance (M) nutritional levels during mid-late pregnancy (D51–140).

	D21-50			D51-140	
	H	M	L	H	M
n	19	22	21	32	30
March 2011	0.7 (0.4- 1.0)	0.7 (0.4- 1.0)	0.9 (0.7- 1.3)	0.6 (0.4- 0.9)	0.9 (0.7- 1.2)
May 2011	1.4 (1.0- 1.9)	1.3 (1.0- 1.8)	1.3 (0.9- 1.7)	1.4 (1.1- 1.8)	1.2 (0.9- 1.6)
July 2011	1.8 (1.3- 2.2)[a]	2.8 (2.3- 3.3)[b]	2.6 (2.1- 3.1)[b]	2.5 (2.1- 2.9)	2.2 (1.8- 2.6)
September 2011	3.1 (2.3- 4.0)	2.8 (2.1- 3.6)	3.3 (2.5- 4.2)	3.3 (2.6- 4.0)	2.9 (2.3- 3.5)
November 2011	1.0 (0.8- 1.4)	1.1 (0.8- 1.4)	1.2 (0.9- 1.5)	1.3 (1.1- 1.6)[b]	0.9 (0.7- 1.1)[a]
January 2012	1.5 (0.9- 2.3)	2.1 (1.4- 2.9)	2.0 (1.3- 2.8)	1.7 (1.2- 2.3)	2.0 (1.5- 2.7)
March 2012	1.8 (1.3- 2.3)[b]	1.3 (0.9- 1.7)[ab]	1.1 (0.7-1.5)[a]	1.3 (1.0- 1.7)	1.4 (1.0- 1.8)

Values are back transformed least squares means + 95% Confidence Intervals. Different superscripts within main effects and rows indicate significant differences ($P<0.05$).

Data was square root transformed for analysis.

Figure 4.3: Quantitative sperm density (sperm x10^9/ml) of semen of post-pubertal rams from 18 to 31 months of age (March 2011 to March 2012) as it varies by season/month irrespective of nutritional treatment.



Values are least squares means + 95% Confidence Intervals. Different letters indicate significant differences (P<0.05) between time-points.

4.5 SPERM MORPHOLOGY (% abnormal sperm)

There were no significant interactions (P>0.05) between the two nutritional treatment regimens for percent abnormal sperm. Maternal nutrition during D21 to D50 and D51 to D140 had no effect (P>0.05) on percent abnormal sperm at any of the monthly time points (Table 4.3). At 26 months (November 2011) rams born to ewes fed $M_{21\text{-}50}$ were less likely (P<0.05) to be categorised as having excellent fertility (i.e. semen contains ≤10% abnormal sperm (Kimberling & Parsons, 2007)), than those born to ewes fed $H_{21\text{-}50}$ and $L_{21\text{-}50}$ (Table 4.4).

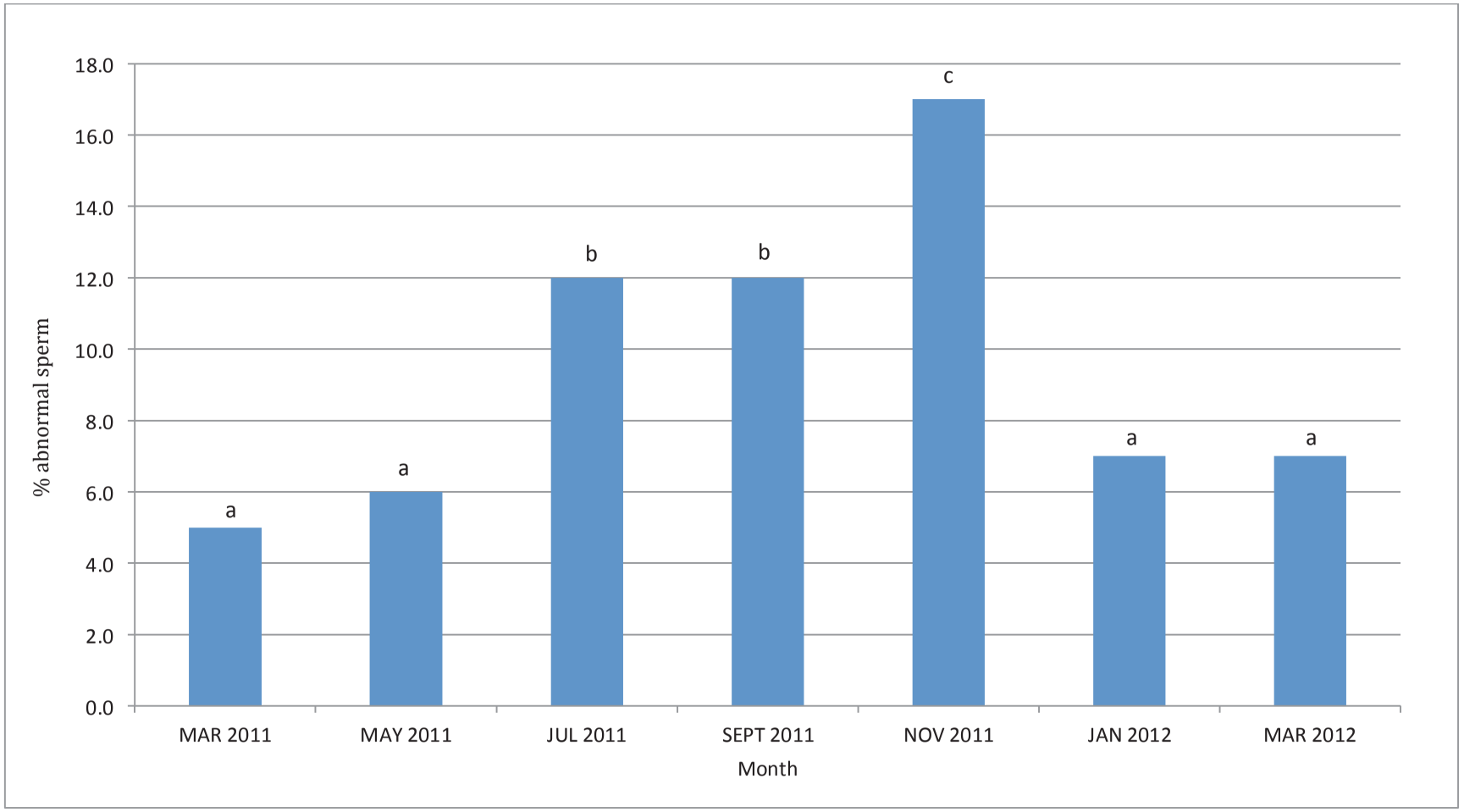
Overall median values for per cent abnormal sperm was greatest (p<0.05) in November 2011 (17%) (Figure 4.4). The second highest median values were observed in July 2011 and September 2011 (both 12%). Correspondingly, the probability of post-pubertal rams having excellent fertility was lowest (P<0.05) in November 2011 (0.18) (Figure 4.5) followed by July and September.

Table 4.3: Median values for percent abnormal sperm in semen of post-pubertal rams from 18 to 31 months of age (March 2011 to March 2012) that were born to ewes fed either High (H) or Maintenance (M) or Low (L) nutritional levels during early pregnancy (D21–50) and High (H) or Maintenance (M) nutritional levels during mid-late pregnancy (D51–140).

	D21-50			D51-140	
	H	M	L	H	M
n	19	22	21	32	30
March 2011	4.0	6.5	4.0	5.5	5.0
May 2011	8.0	6.0	6.0	7.0	6.0
July 2011	14.0	10.5	12.0	11.5	14.0
September 2011	13.0	11.0	11.0	14.0	11.5
November 2011	16.0	17.0	20.0	14.5	18.0
January 2012	5.0	8.0	7.0	6.5	7.0
March 2012	6.0	9.5	6.0	7.0	6.0
ALL	9.0	9.0	10.0	9.0	9.0

No significant differences (P>0.05).

Figure 4.4: Median values for percent abnormal sperm in semen of post-pubertal rams from 18 to 31 months of age (March 2011 to March 2012) as it varies by season/month and irrespective of nutritional treatment.



Different letters indicate significant differences (P<0.05) between time-points.

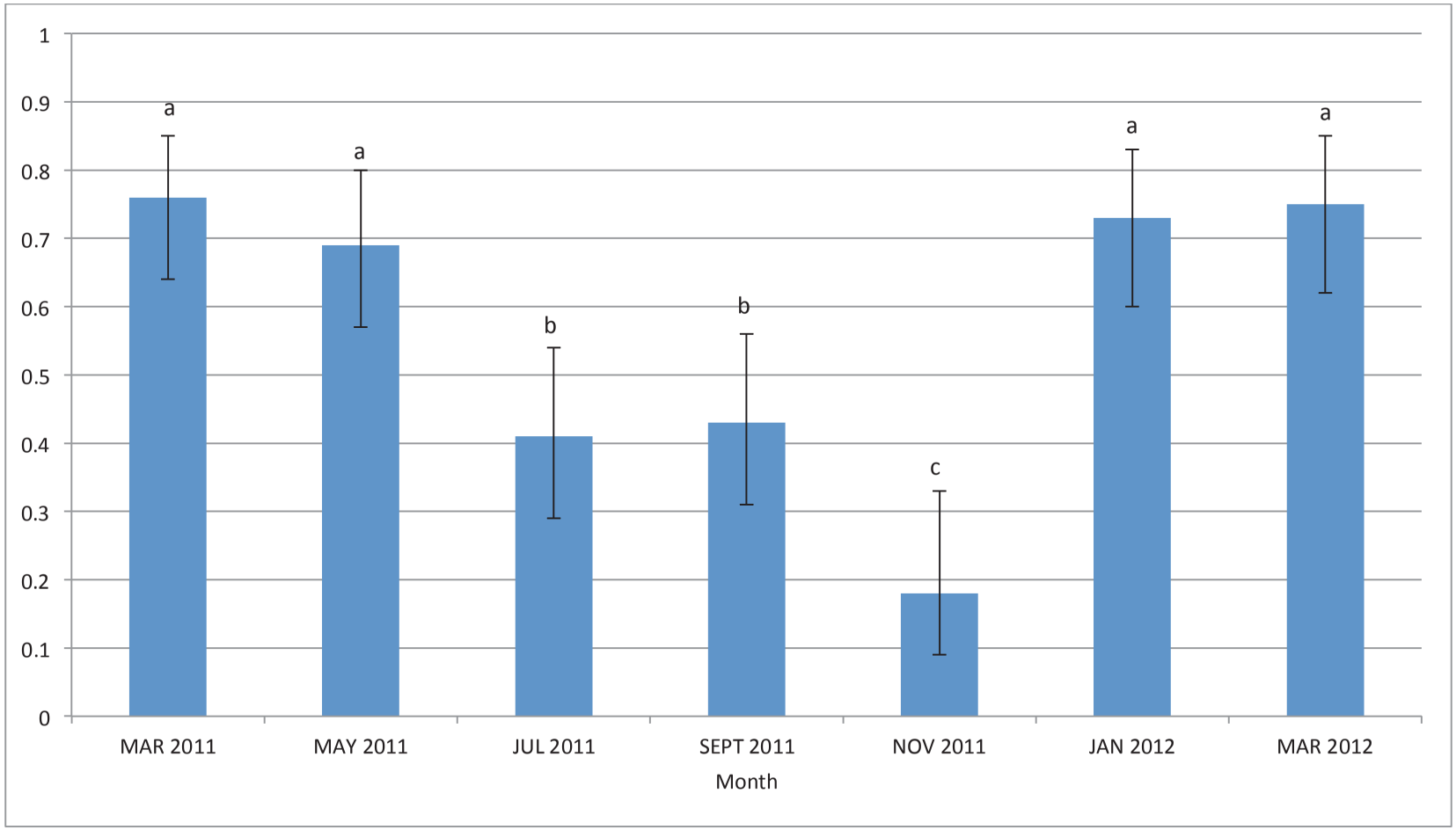
Table 4.4: Probability that post pubertal rams 18 to 31 months of age (March 2011 to March 2012) that were born to ewes fed either High (H) or Maintenance (M) or Low (L) nutritional levels during early pregnancy (D21–50) and High (H) or Maintenance (M) nutritional levels during mid-late pregnancy (D51–140) may be categorised as having "excellent"[1] fertility (i.e. semen contains ≤10% abnormal sperm).

	D21-50			D51-140	
	H	M	L	H	M
n	19	22	21	32	30
March 2011	0.8 (0.6- 0.9)	0.7 (0.5- 0.9)	0.8 (0.5- 0.9)	0.7 (0.5- 0.8)	0.8 (0.7- 0.9)
May 2011	0.7 (0.5- 0.9)	0.7 (0.5- 0.9)	0.7 (0.5- 0.8)	0.6 (0.5- 0.8)	0.8 (0.6- 0.9)
July 2011	0.3 (0.1- 0.5)	0.5 (0.3- 0.7)	0.5 (0.3- 0.7)	0.5 (0.3- 0.6)	0.4 (0.2- 0.6)
September 2011	0.4 (0.2- 0.6)	0.5 (0.3- 0.7)	0.4 (0.2- 0.6)	0.4 (0.3- 0.6)	0.4 (0.3- 0.6)
November 2011	0.3 (0.2- 0.6)[a]	0.1 (0.0- 0.3)[b]	0.3 (0.2- 0.6)[a]	0.3 (0.2- 0.5)	0.2 (0.1- 0.3)
January 2012	0.7 (0.5- 0.9)	0.7 (0.5- 0.8)	0.8 (0.6- 0.9)	0.7 (0.5- 0.9)	0.7 (0.6- 0.9)
March 2012	0.8 (0.6- 0.9)	0.6 (0.3- 0.7)	0.9 (0.6- 1.0)	0.7 (0.5- 0.8)	0.8 (0.6- 0.9)

Values are least squares means + 95% Confidence Interval. Different superscripts within main effects and rows indicate significant differences (P<0.05).

[1](Kimberling & Parsons, 2007)

Figure 4.5: Probability that post-pubertal rams from 18 to 31 months of age (March 2011 to March 2012) may be categorised as having "excellent"[1] fertility (i.e. semen contains ≤10% abnormal sperm).



Values are least squares means + 95% Confidence Intervals. Different letters indicate significant differences (P<0.05) between time-points.

[1](Kimberling & Parsons, 2007)

CHAPTER 5: DISCUSSION

The primary aim of this study was to investigate the effects of maternal nutrition during early (Day 21 to 50) and mid- to late (Day 50 to 140) pregnancy on the post-pubertal reproductive characteristics and liveweight of twin-born ram offspring. In addition the effect of season on reproductive characteristics of the ram progeny was investigated. This preliminary investigation followed on from the studies conducted by Kenyon et al. (2011) and Asmad et al. (2011), and data were collected and analysed for 62 rams over the period of 18 to 31 months of age (March 2011 to March 2012). The study was designed to demonstrate the fertility of post pubertal rams at a phenotypic level using non-invasive and accepted indicators of fertility. As such, the number of rams utilised in each treatment group cohort was adequate to test the effects using the methods chosen for the present study. The rams' age fit within the scope of the study, as they were over the established thresholds for weight and scrotal circumference for puberty, although they had not reached their maximum mature weight. Using this study design, post-pubertal rams born to ewes that were underfed during early pregnancy did not show evidence of reduced reproductive capacity. Thus, the fertility of these rams was not affected by maternal undernutrition under these experimental conditions.

In the present study there were generally no maternal nutritional treatment effects on mean liveweight of the post-pubertal ram offspring during the period of 18 to 31 months of age. Early studies suggested that chronic restriction of maternal nutrition during pregnancy can have detrimental effects on the liveweight of offspring in adulthood (Schinckel & Short, 1961; Everitt, 1967). However, more recent evidence concurs with the present findings that there are no liveweight effects on offspring as adults due to maternal nutrition during gestation (Gunn et al., 1995; Rae et al., 2002a; Borwick et al., 2003; Gopalakrishnan et al., 2004; Gardner et al., 2005; Corner et al., 2006; Kelly et al., 2006). Some of these studies demonstrated lower birthweights of

offspring born to dams that were nutritionally restricted (Gunn et al., 1995; Borwick et al., 2003); however, in all cases when the offspring reached adulthood no differences in liveweight were observed. In a previous study using the larger cohort, from which the rams used in the present study were selected, it was reported that there was no effect of the maternal nutritional treatments on their liveweight from birth to 343 days of age (Asmad et al., 2011). It is not surprising therefore, that no liveweight differences were observed in the rams at adulthood.

It has been proposed that any adverse effects of ewe nutrition applied during early pregnancy can by overcome by adequate maternal nutrition during mid- to late gestation (Taplin & Everitt, 1964). This is of particular consequence to the present study as the dam nutritional treatments in mid- to late pregnancy were either at, or above, the level required for pregnancy maintenance. This may have compensated for any potential adverse effects of low nutrition in early pregnancy. The lack of an effect of mid- to late pregnancy maternal nutrition on liveweight of the rams may simply therefore be explained by the fact that neither maternal treatment (i.e. maintenance or *ad libitum*) resulted in a negative energy balance of the ewe, such as has been associated with lower fetal growth rates during this period and subsequently low birthweight (Tygesen et al., 2007).

Scrotal circumference was not affected by maternal nutritional treatment. This concurs with findings of Rae et al. (2002a) who reported no effect of maternal undernutrition on scrotal circumference of ram offspring at both 6 weeks and 20 months of age. It is widely accepted that testis size, an acceptable indicator of sperm production, is largely determined by the number of Sertoli cells (Sharpe et al., 2003). Interestingly, it appears that no other studies have investigated the effects of maternal nutrition during pregnancy on the adult performance of ram offspring in terms of structural changes to the gonads, nor in terms of scrotal circumference measurements.

Maternal nutrition has previously been shown to affect testicular structure in fetal and neonatal rams at both a gross and histological level: including decreased testicular size in nutritionally restricted fetuses (Rae et al., 2002b); and decreased number of Sertoli cells, but not testicular volume in offspring that were born to ewes which were nutritionally restricted in mid to late gestation at 2 days old (Bielli et al., 2002), and 10 month old offspring (Kotsampasi et al., 2009); and lowered testicular volume at 35 weeks of age in growth-restricted offspring (Da Silva et al., 2001). The histological findings of the previous studies suggest that there is potential for the structural effects that are observed in early life to be carried forward, resulting in defects in function later in adult life. The testis of the population of rams in the present study was not examined at a histological level. However, given the lack of effect of the maternal nutritional treatments tested on reproductive function, histological examination may not be warranted. On the other hand, there may be merit to histological examination in future investigations, to demonstrate if indeed structural changes persists into adult life and result in functional changes as others have suggested, or whether the existence of possible buffering effects compensate for any structural changes, thus having implications for further investigations and previous extrapolations/conclusions.

The present study provided no evidence to indicate that maternal nutrition during early or mid- to late pregnancy affected ram reproductive capacity in terms of effects upon semen quality. Forward motion, quantitative sperm density and percentage of abnormal sperm all did not differ between nutritional treatments. This is consistent with the findings of Rae et al. (2002a), who also reported no effect of maternal nutrition on quantitative sperm density or sperm motility. However, Rae et al. (2002a) pointed out that simple semen analysis does not provide a totally adequate means of evaluating the contribution of rams to fertility and fecundity of flocks. Further, they indicate that ram mating behaviour is an important factor contributing to ram performance. This could have warranted investigation in the present group of rams. The findings of the present study, however, indicate that no effects on structure or function are likely, in terms of

sperm-producing ability. Thus, functional reproductive capacity and the potential for successful fertilisation by the study rams was not affected by maternal nutritional restriction imposed on them as fetuses.

To date, the present study appears to be the first to examine whether there is any effect of maternal nutrition on male reproductive performance of male offspring in sheep, and therefore, simple evaluations of semen characteristics and sperm quality may be considered adequate. The parameters that were investigated are recognised as a commonplace means of identifying the fertility of rams during selection for breeding; from a practical and clinically-relevant point of view the findings provide reliable and usable evidence allowing farmers to confidently select breeding rams without concerns about the effect of maternal nutrition on fertility. However, the present investigation also aimed to establish whether fetal programming effects have occurred. While it appears that functionally there are no effects, it may be debated that more in-depth investigations of sperm output would have had the potential to demonstrate another facet of functional reproductive capacity. Future investigators may consider testing spermatogenic capacity or recovery following depletion of sperm reserves by serial ejaculations to exhaust the epididymal reserves. In addition, quantitative testicular histology, measuring spermatocyte and spermatid numbers, and measurement of epididymal or rete testis fluid sperm numbers and quality, may be more definitive measures of spermatogenic function than that measured in ejaculated samples. Therefore, it may be worthwhile to include use of these more invasive methods.

Furthermore, most studies, including the present, focused on the possible effects of nutrition during early pregnancy on reproductive development. While this approach has merit considering that the critical window of gonadal development occurs at around Day 34 of pregnancy (Bielli et al., 2002), it is possible that in this case, the nutritional treatments of maintenance and above in mid- to late pregnancy may have compensated for any deleterious effects imposed on reproductive function. Bielli et al.

(2002) and Kotsampasi et al. (2009) both demonstrated lower numbers of Sertoli cells without accompanying changes in testicular weight at 2 days and 10 months post-partum respectively, following restricted maternal nutrition in mid- to late pregnancy. As such, the possibility exists that negative effects on the reproductive performance of the rams may have been observed if below maintenance feeding levels were continued through mid- to late pregnancy. This warrants further investigation.

Indeed, while the focus of the present study on adult reproductive function was demonstrated by testicular, semen and sperm characteristics, functional effects at the gonadotrophin level were not examined. Changes to the hypothalamic-pituitary-testis axis have been demonstrated in response to maternal undernutrition although unaccompanied by structural effects in prenatal and neonatal life (Rae et al., 2002a; 2002c). It is accepted however, that any changes to the hypothalamo-pituitary-gonadal axis during development is generally only observed at puberty or in adult reproductive life (Pereira, 2003). Kotsampasi et al. (2009) demonstrated gonadotrophin and histological effects at 10 months of age following maternal undernutrition in late pregnancy. These authors propose that the effect observed was primarily influenced by the timing and duration of the nutritional insult. The hypothalamic-pituitary development systems develop between Day 35–85 of pregnancy (Kotsampasi et al., 2009), and a functional hypothalamic-pituitary link is not established until Day 60 (Miller et al., 1998). As such, there is considerable uncertainty in the link between the effects of maternal undernutrition on the development of the reproductive endocrine axis and reproductive function in adult life. Therefore, although it was beyond the scope of this study, the role of the hypothalamo-pituitary-testicular axis in reproductive function, the effect of maternal nutrition on its development, and the potential for indirect effects on gonadal function should not be ignored.

Independently of the effects of maternal nutrition during pregnancy on ram reproductive capacity, the present study aimed to investigate if there were any effects

of season on reproductive parameters. Over the 13 month study period, liveweight increased from March 2011 (18 months of age) to November 2011 (31 months of age). This was not unexpected as the rams were still likely to be growing during this period. Rams were shorn in early December 2011, which likely accounts for the apparent drop in liveweight observed at that time.

Seasonal factors did not appear to differentially affect scrotal circumference of the rams from March 2011 to November 2011 according to maternal gestational diets. Although there was a significant difference in the scrotal circumference in early June 2011 (winter solstice 21 June) compared to the other measurements during this time period, mean scrotal circumference remained within the range of 34 to 39 cm. This is considered to fall in the range of "satisfactory" to "excellent" for fertility of the ram in standard breeding soundness evaluation examinations (Kimberling & Parsons, 2007). Other authors have reported a decrease in the size of the testes during the non-breeding season in response to the increasing day-length of spring and summer (Islam & Land, 1977; Mickelsen et al., 1981; Lincoln et al., 1990; Mandiki et al., 1998). Similarly, in the present study, scrotal circumference decreased from late October through to December. The lowest recorded mean scrotal circumference was observed in early December.

The correlation between testicular size/weight and spermatogenic capacity/ fertility has long been reported (Amann, 1970). The seasonal effect on scrotal circumference observed in the present study supports other findings on semen/sperm characteristics that were observed in response to seasonal cues. Together, these observations reflect the changes to the fertility of the rams over the study period. In fact, lowered fertility as a result of increased abnormal spermatozoa occurs earlier in November, during the period of decline of testicular tissue, and is discussed below. However, it is also possible this significant decrease from late November to early December in scrotal circumference may have been exacerbated by the effects of shearing in early December. Tilbrook et al.,

(2002) have described the possibility of a detrimental effect of stress (in cases of management practices) on reproductive function via activation of the hypothalamo-pituitary axis associated with reduced secretion of LH. Additionally, studies done on rams have shown the effects of restraint stress to cause depression of the LH response to GnRH (Matteri et al., 1984). It is possible therefore, that these endocrine responses may be manifested as the changes seen in scrotal circumference.

It is noted that the testis does not consists solely of one cell or tissue type; whereas Sertoli cells have been implicated as important to both composition and function of the testis, the decrease in scrotal circumference may not be directly as a result of decreased Sertoli cell numbers. In fact, Sharpe (1994) states that the majority of evidence suggests that Sertoli cell numbers in seasonally breeding rams remain constant in the breeding and non- breeding season. That is not to say that size and activity has not decreased. Rams therefore, would likely retain a measure of fertility, albeit decreased, year round, having adequate spermatogenic tissue regardless of season. It is of note that mean circumference measurements in March 2012 were larger than those of March 2011, which likely is explained by the maturity of the rams at 31 months of age in March 2012 (autumn).

It was difficult to assess the semen characteristics of sperm quality with respect to seasonality. It is accepted that visual density, forward and gross motility are affected by many factors that contribute to the subjective nature of these variables. For example, it is suggested that motility is affected by temperature and therefore, it should not considered a reliable indicator of fertility on its own (Kimberling & Parsons, 2007). However, motility is easily assessed and with careful management of the ejaculate, it can be successfully used as a predictor of fertility. It is noted that use of the electro-ejaculator is known to influence sperm density as it can result in a sample of higher volume and lower sperm concentration (Mattner & Voglmayr, 1962; Sinha et al., 1983). Other studies that used the artificial vagina method of collection (Dufour et al., 1984;

Kafi et al., 2004) generally displayed higher sperm concentrations regardless of season in comparison to this study. Therefore, in spite of these differences electro-ejaculation is commonly and easily used, and requires little training with successful results; whereas, use of the artificial vagina method requires that the rams be trained and sperm losses do occur, within the artificial vagina, that need to be compensated for. None the less all of these (motility, electro-ejaculation, artificial vagina) have been consistently and successfully used to identify fertility, and in the case of the present study all precautions were taken to reduce any possible errors due to the methods chosen. In the present study, sperm density increased between March 2011 and September 2011 as the rams moved from the breeding season into the non- breeding season. This may have been the result of operator differences while performing the electro-ejaculation procedure or changes in diet associated with feed availability during winter months.

The least dense sperm was obtained in November 2011 (non-breeding season). Low sperm concentration has been previously reported during spring in comparison to late summer (Dacheux st al., 1981). These authors examined sperm concentrations in rete testis fluid as opposed to ejaculated semen and although a comparable seasonal effect was observed, the concentrations generally were higher in the study conducted by Dacheux et al. (1981). Interestingly, this seasonal effect occurred with no corresponding decrease in scrotal circumference (and possibly sperm-producing testicular tissue) across the population. In addition, it is noted that at this point the study rams had significant wool cover, and possible effects of heat stress on testicular function may be considered (even though November is not considered a particularly hot month (13.5°C national average)). The effects of heat stress on semen quality has been long recognised (Dutt & Hamm, 1957). Sperm density in March 2012 was greater than in March 2011. This corresponds with the increased testicular tissue as seen as an increase in scrotal circumference between these two time-points.

Sperm morphology is considered to be an objective measure of ram fertility, and can be considered an important indicator of normal sperm motility and production (Barth & Oko, 1989). In the present study, seasonal effects were observed on the percentage of abnormal sperm. There was an increase in percentage of abnormal sperm from the breeding season March 2011 through to the non-breeding season September/November 2011. As the next breeding season January/March 2012 approached there was a subsequent decrease in the percentage of abnormal sperm and therefore, fertility of the study population of rams increased. Strongly seasonal breeds have been reported to produce semen with an elevated percentage of abnormalities in spring compared to autumn (Baril et al. (1993) as cited in Mandiki et al., 1998). This is corroborated by earlier reports by (Mickelsen et al., 1981) of high percentages of abnormal sperm during spring. In the present study, the greatest percentage of abnormal sperm occurred in November 2011, indicating that it was the period at which the rams were at their lowest potential for fertility. This was expressed as the probability of the study rams fitting the criteria for "excellent" fertility or having less than 10% abnormal sperm. In addition the most commonly observed abnormalities were: major: proximal cytoplasmic droplets, acrosome defects, dag defect and distal mid-piece reflex; minor: detached normal heads, distal cytoplasmic droplets and looped tails.

In summary, under the conditions of this experiment: rams born to ewes exposed to sub-maintenance, maintenance, or *ad libitum* nutritional levels in early pregnancy and/or maintenance or *ad libitum* nutritional levels in mid- to late pregnancy did not show any effect on post-pubertal fertility. While the present study aimed to examine the effects of nutritional fetal programming as described by Barker (1997; 2004a) the findings herein show that at a phenotypic level, using the parameters of scrotal circumference, semen and sperm characteristics, there are no observable effects on reproductive function.

CHAPTER 6: CONCLUSION

Fertility and reproductive performance of a ram is dependent on numerous factors. Of particular importance is the appropriate development and function of a ram's reproductive organs. Previous studies have shown that maternal nutrition can affect the structure of the fetal reproductive organs. There has, however, been a lack of information on whether changes in functionality of adult reproduction could occur. In this study maternal nutrition in early and mid- to late pregnancy had no effect on testicular size, semen and sperm characteristics for rams between the ages of 18 and 31 months. Therefore, under the conditions of this present study apparent fertility of the rams appears not to be affected by the maternal nutritional treatments. This suggests that farmers do not need to take into account maternal nutrition when selecting rams for use as sires when conditions are at maintenance or above in mid- to late pregnancy.

It is also noted, that the present feeding conditions in mid- to late pregnancy were at a level for pregnancy maintenance or above. It is possible that under poorer nutritional treatments in this period, effects on reproductive performance may be observed. Further studies may wish to examine this. In addition, it should be noted that reproductive efficiency of a ram is also dependent on libido and mating behaviour, which will ultimately impact on number of ewes mated and offspring sired. It may be prudent therefore, to investigate these variables in further studies.

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APPENDIX

MATERIALS AND METHODS

Visual Characteristics of Semen

The following tables present the raw data for visual characteristics of semen. This data was non-normally distributed and could not be normalised, therefore it has not been analysed.

Table A.1: Visual density of semen of post-pubertal rams from 18 to 31 months of age (March 2011 to March 2012) that were born to ewes fed either High (H) or Maintenance (M) or Low (L) nutritional levels during early pregnancy (D21–50) and High (H) or Maintenance (M) nutritional levels during mid-late pregnancy (D51–140).

	D21-50									D51-140					
	H			M			L			H			M		
	C[1]	Mk[2]	W[3]	C	Mk	W	C	Mk	W	C	Mk	W	C	Mk	W
n		19			22			21			32			30	
March 2011	13	4	3	8	11	3	13	3	5	20	6	7	14	12	4
May 2011	13	4	3	12	8	2	11	8	2	20	11	2	16	9	5
July 2011	16	1	3	20	2	0	21	0	0	31	1	1	26	2	2
September 2011	14	3	3	16	3	3	18	2	1	25	5	3	23	3	4
November 2011	19	0	1	20	1	1	15	4	2	29	3	1	25	2	3
January 2012	12	3	5	19	0	3	13	4	4	21	3	9	23	4	3
March 2012	16	0	3	15	4	3	12	1	8	22	4	6	21	1	8

Values are number of rams within each treatment group that fall into each category.
[1]Creamy
[2]Milky
[3]Watery

Table A.2: Median values of gross (wave) motility scores of semen of post pubertal rams from 18 to 31 months (March 2011 to March 2012) that were born to ewes fed either High (H) or Maintenance (M) or Low (L) nutritional levels during early pregnancy (D21–50) and High (H) or Maintenance (M) nutritional levels during mid-late pregnancy (D51–140).

	D21-50			D51-140	
	H	M	L	H	M
n	19	22	21	32	30
March 2011	9.0	8.0	9.0	8.0	9.0
May 2011	8.0	8.0	8.0	8.5	8.0
July 2011	8.0	9.0	9.0	9.0	9.0
September 2011	9.0	9.5	10.0	9.5	9.0
November 2011	7.0	9.0	8.0	8.0	8.0
January 2012	8.0	9.0	8.0	8.0	9.0
March 2012	9.0	7.5	7.0	8.5	8.0

Gross motility scores range from 0-10

RESULTS

The following tables present results that have been described in the Results chapter.

Table A.3: Liveweight (kg) of post-pubertal rams from 18 to 31months of age (March 2011 to March 2012) that were born to ewes fed either High (H) or Maintenance (M) or Low (L) nutritional levels during early pregnancy (D21–50) and High (H) or Maintenance (M) nutritional levels during mid-late pregnancy (D51–140).

	D21-50			D50-140	
	H	M	L	H	M
n	19	22	21	32	30
March 2011	74.8 ±1.6	74.0 ±1.5	77.7 ±1.6	75.1 ±1.3	75.9 ±1.3
April 2011	75.8 ±1.6	74.4 ±1.5	76.6 ±1.5	75.6 ±1.3	75.6 ±1.3
May 2011	81.3 ±1.5^{ab}	78.7 ±1.4^{a}	83.0 ±1.5^{b}	80.6 ±1.2	81.4 ±1.2
June 2011	84.9 ±1.6	83.0 ±1.5	87.1 ±1.5	84.8 ±1.2	85.2 ±1.3
July 2011	80.8 ±1.5	79.6 ±1.4	82.5 ±1.4	80.4 ±1.2	81.5 ±1.2
August 2011	88.6 ±1.6	87.9 ±1.5	89.9 ±1.5	88.4 ±1.2	89.2 ±1.2
October 2011	95.1 ±1.6	95.6 ±1.5	97.6 ±1.6	95.3 ±1.3	97.0 ±1.3
November 2011	99.7 ±1.8	99.2 ±1.7	102.8 ±1.8	99.8 ±1.4	101.4 ±1.5
December 2011	89.0 ±1.7	88.1 ±1.6	90.9 ±1.6	88.6 ±1.3	90.1 ±1.4
January 2012	87.1 ±1.9	86.4 ±1.8	88.5 ±1.8	86.4 ±1.5	88.2 ±1.5
February 2012	88.1 ±1.9	87.5 ±1.8	90.4 ±1.8	88.1 ±1.5	89.2 ±1.5
March 2012	88.6 ±2.0	87.4 ±1.9	89.7 ±1.9	88.2 ±1.6	89.0 ±1.6

Values are least squares means ± standard error of the mean. Different superscripts within main effects and rows indicate significant differences (P<0.05).

Table A.4: Scrotal circumference (cm) of post-pubertal rams from 18 to 31 months of age (March 2011 to March 2012) that were born to ewes fed either High (H) or Maintenance (M) or Low (L) nutritional levels during early pregnancy (D21-50) and High (H) or Maintenance (M) nutritional levels during mid-late pregnancy (D51-140).

	D21-50			D51-140	
	H	M	L	H	M
n	19	22	21	32	30
25Mar11	37.7 ±0.7	37.5 ±0.7	38.3 ±0.7	37.6 ±0.5	38.1 ±0.6
11Apr11	38.1 ±0.6	37.3 ±0.6	38.1 ±0.6	37.6 ±0.5	38.0 ±0.5
21Apr11	37.8 ±0.6	37.1 ±0.6	37.6 ±0.6	37.3 ±0.5	37.7 ±0.5
6May11	37.1 ±0.6	36.5 ±0.5	37.0 ±0.5	36.6 ±0.4	37.2 ±0.4
23May11	37.3 ±0.6	36.1 ±0.5	37.4 ±0.5	36.5 ±0.4	37.4 ±0.5
7Jun11	36.0 ±0.6	35.4 ±0.5	36.4 ±0.5	35.4 ±0.4	36.5 ±0.5
24Jun11	38.6 ±0.5	38.0 ±0.5	39.0 ±0.5	38.1 ±0.4	38.9 ±0.4
8Jul11	37.8 ±0.5^{ab}	37.0 ±0.5^{a}	38.6 ±0.5^{b}	37.6 ±0.4	38.0 ±0.4
22Jul11	38.1 ±0.5	37.9 ±0.4	38.7 ±0.4	38.2 ±0.4	38.3 ±0.4
12Aug11	37.9 ±0.5	37.7 ±0.4	38.5 ±0.4	38.0 ±0.4	38.0 ±0.4
25Aug11	38.0 ±0.5	37.5 ±0.5	38.4 ±0.5	38.1 ±0.4	37.9 ±0.4
9Sept11	37.4 ±0.5	36.9 ±0.4	38.0 ±0.4	37.7 ±0.4	37.2 ±0.4
23Sept11	37.2 ±0.5	36.8 ±0.5	37.5 ±0.5	37.2 ±0.4	37.2 ±0.4
7Oct11	37.3 ±0.4	37.5 ±0.4	37.7 ±0.4	38.1 ±0.3^{b}	36.9 ±0.4^{a}
21Oct11	37.5 ±0.5	37.3 ±0.5	37.8 ±0.5	37.8 ±0.4	37.2 ±0.4
7Nov11	38.0 ±0.5	37.9 ±0.5	38.4 ±0.5	38.4 ±0.4	37.8 ±0.4
28Nov11	36.9 ±0.6	37.0 ±0.5	37.0 ±0.5	37.3 ±0.4	36.6 ±0.4
9Dec11	33.5 ±0.4	33.6 ±0.4	33.7 ±0.4	33.9 ±0.3	33.3 ±0.3
22Dec11	36.1 ±0.5	35.8 ±0.4	36.0 ±0.4	36.0 ±0.4	36.0 ±0.4
9Jan12	37.8 ±0.4	37.5 ±0.4	37.8 ±0.4	37.7 ±0.3	37.7 ±0.4
31Jan12	39.2 ±0.5	38.6 ±0.5	38.7 ±0.5	38.7 ±0.4	38.9 ±0.4
17Feb12	39.7 ±0.6	39.4 ±0.5	39.8 ±0.5	39.5 ±0.4	39.7 ±0.5
2Mar12	41.1 ±0.6	40.4 ±0.5	41.2 ±0.6	40.8 ±0.5	41.0 ±0.5
16Mar12	41.5 ±0.5	40.7 ±0.5	41.3 ±0.5	41.1 ±0.4	41.3 ±0.4
27Mar12	41.3 ±0.6	40.4 ±0.5	41.4 ±0.5	40.9 ±0.4	41.2 ±0.5

Values are least squares means ± standard error of the mean.
Different superscripts within main effects and rows indicate significant differences ($P<0.05$).