Use of nutritional supplementation to improve responses to the ‘ram effect’ in the Merino ewe

Phillip Clemens Khaiseb

Supervisors:

Professor Graeme B. Martin (University of Western Australia, Perth)

Dr Penny A. R. Hawken (University of Western Australia, Perth)

A thesis submitted to fulfil the requirements for the degree of Master of Agricultural Science

School of Agriculture and Environment
University of Western Australia, Perth, Australia

December 2016
Table of Contents

Declaration ........................................................................................................................................... i

Acknowledgements ........................................................................................................................... ii

Summary ............................................................................................................................................ iii

Chapter 1 ........................................................................................................................................... 1

1.1 General Introduction ..................................................................................................................... 1

Chapter 2 ........................................................................................................................................... 4

2.1 Review of the literature .................................................................................................................. 4

2.2 The Reproductive System .............................................................................................................. 4

2.2.1 The hypothalamo-hypophyseal axis ......................................................................................... 4

Figure 1: Schema of the hypothalamic-pituitary axis in sheep. ......................................................... 5

2.3 Folliculogenesis ............................................................................................................................ 5

Figure 2: The structure of an ovary. ..................................................................................................... 6

Figure 3: A model for folliculogenesis in the ewe. ............................................................................. 7

2.4 Primordial follicles ........................................................................................................................ 8

2.5 Committed follicles ........................................................................................................................ 8

2.6 Gonadotrophin-responsive follicles .............................................................................................. 8

2.7 Gonadotrophin-dependent follicles .............................................................................................. 8

2.8 Ovulatory follicle .......................................................................................................................... 9

2.9 Steroidogenesis ............................................................................................................................ 10

Figure 4: A pictorial illustration of ovarian steroidogenesis. ............................................................. 10

2.10 Feedback Mechanisms ................................................................................................................ 11

2.11 The negative feedback ............................................................................................................... 11

2.12 Positive feedback ....................................................................................................................... 12

Figure 5: Negative and positive feedback mechanisms. .................................................................. 12

2.13 The Oestrous Cycle .................................................................................................................... 13

Figure 6: Oestrous cycle of the ewe. .................................................................................................. 13

2.14 "Male effect" for controlled breeding ......................................................................................... 14
5.2 Methods and Materials........................................................................................................................................... 39

5.3 Experimental design............................................................................................................................................... 40

**Figure 12: Schematic experimental design.** ............................................................................................................. 40

5.4 Results................................................................................................................................................................. 41

**Figure 13: Average live weight of ewes.** .................................................................................................................. 41

Table 3: Reproductive variables:................................................................................................................................. 42

**Figure 14: Oestrus distribution of anovular ewes induced at the 'male effect'.** ..................................................... 43

5.5 Discussion.............................................................................................................................................................. 44

Chapter 6 ..................................................................................................................................................................... 47

6.1 General Discussion............................................................................................................................................... 47

References..................................................................................................................................................................... 50
DECLARATION

This thesis has been composed by myself and has not been accepted in any previous application for a degree. The work, of which this is a record, has been done by myself and all sources of information have been cited.

Phillip Clemens Khaiseb

Date: December 06, 2016
Acknowledgements

I wish to take this opportunity to sincerely thank Graeme Martin for his enormous patience with me through unwavering guidance and wisdom he passed on to me. I have learned a great deal from him for his art in writing, especially communication in science. It requires absolutely different skills altogether to communicate scientifically, but in a simple manner to ensure that the reader understands everything – and it is something which I shall continue to learn. Penny Hawken has been a silent contributor to my learning process during my time at UWA, but she has been very effective in sharing her knowledge with me. I valued her teaching which was in an unstressed environment and I fully came to grasp with the reproductive physiology in mammals during my first year. Thank you to both Graeme and Penny and memories will remain with me forever. Importantly, I would like to thank the AUSAID Scholarship for having granted me this once in a lifetime opportunity to upgrade myself academically and having spent 24 months at UWA, which I learned immensely both academically and socially. Finally, to my beloved wife, Fealofani Khaiseb, my two daughters, Leilani A. Khaiseb and Anisa T. Khaiseb. You allowed me to endeavour this project and you were also very understanding not to spend long periods with you for me to achieve my objectives. I am indebted to you for all the support you afforded me in whatever shape and forms they came. Fani, you also pushed me to get this thesis behind me, thanks a lot for that.
Summary

The pressure from world’s societies to develop technologies in animal production systems has prompted researchers around the world to focus on three things. We use an approach that is hormone-free, environmentally friendly as well as taking care of animal welfare. This approach has led to the development of ‘clean, green and ethical’ concept in Australia. The important tool to induce ovulation in anovular ewes which were previously isolated from ram induces a physiological response, a phenomenon coined the ‘ram effect’. This effect is sufficient to override the seasonal suppression of the hypothalamic-pituitary axis to induce in the ewes.

The objective in the first experiment was to develop a regime for short-term nutritional supplementation before and after ram induce oestrus cycle at the onset of the natural breeding season to improve reproductive performance of ewes. Nutritional supplementation was not possible to test as ovulation was observed in all ewes with only 6% of ewes which did not ovulate in the controls. However, there was an increase in the ovulation rate in the group which received nutritional supplementation during the ram induced oestrous cycle (luteal phase). The synergistic effects of the ‘ram effect’ and nutritional supplementation did not reduce the proportion of ewes experiencing a short cycle. The experiment showed that nutritional supplementation seems to have a greater effect on follicle stimulation during the oestrous cycle.

The second experiment was conducted to investigate the effect of short-term nutritional supplementation on the response of anovular ewes during the non-breeding season following the ‘ram effect’. Similar to the first experiment, ovulation was poor in the treatment group while the controls showed low ovulation which confirms that nutritional supplementation does not affect ovulation. The ovulation rate was increased in the treatment group at the first ovulation, which was evident on Day 10, but it subsequently dropped to the similar level of the controls once the nutritional supplementation was discontinued. Therefore, the results of the first and second experiments confirm that nutritional supplementation is critical just prior to the preovulatory period which suggest that the length rather than the timing of the nutritional supplementation is important. The luteal failure is independent of the nutritional stimulus because there is consistency in the increased ovulation rate at the first ovulation and the expected stimulation of folliculogenesis by the lupin supplements.
In conclusion, we have demonstrated through the repeatability of two separate experiments at the onset of the breeding season and during the non-breeding season that nutritional supplementation does not have an effect on the number of ewes ovulating, but increases ovulation rate. Such supplementation should be sustained until the preovulatory stage at the ram induced oestrous cycle.
CHAPTER 1

1.1 General Introduction

Namibia is situated in the south-west of the African continent bordering the Atlantic Ocean on the west. Rainfall has a wide regional variation, from less than 20 mm in the western Namib and coastal zones to more than 700 mm at the eastern end of the Caprivi strip (Sweet, 1998). The communal farmers occupy 41% of the total land area, in regions that receive less rain relative to that occupied by commercial farmers, who occupy ‘prime land’ about 44% of total land area. The production systems in the communal areas are based on pastoralism and agro-pastoralism, and the majority of households are subsistence-based and labour intensive with limited use of technology and external inputs. It is important to improve small ruminant production in these subsistence systems so these farmers can access high price markets.

The main problems that subsistence farmers encounter are: (i) poor or no management strategies for feeding leading to livestock in poor condition at mating; (ii) no subdivision of land into paddocks to ensure that males are kept separate from females during the non-breeding season; (iii) also, as a result of the lack of subdivision, there is overgrazing, with effects exacerbated by erratic rainfall; and, (iv) low income that prevents the use of expensive technologies. Therefore, it is logical for sheep and goat farmers in semi-arid and arid conditions to adopt low cost management strategies that give them greater reproductive output, yet also allow flexibility so mating and lambing can be timed to coincide with abundant food. In current Namibian conditions, farmers simply rely on the natural seasonality of their animals to dictate the onset and succession of the breeding season.

The hierarchy of factors controlling the onset of reproductive activity varies greatly around the world. Photoperiod is the primary driver of reproductive activity and in high latitudes; it dominates completely, leading to strict patterns of seasonal breeding (Adams, N. R.; Atkinson, S.; Martin G. B.; Briegel, J. R.; Bouklig, R.; Sanders, 1993). In regions closer to the Equator, the power of photoperiod fades so rainfall and associated abundance of food dictate the onset of the breeding season (Martin et al., 2004b). Breeds of sheep and goats native to lower latitudes, such as Merinos, are very opportunistic and the onset of reproductive activity is equally affected by changes in food availability and reduced day length (Chemineau et al., 1988). Effective coordination of reproductive opportunistic breeding patterns will still be hampered by year-to-year variations in the timing of the rainy periods, but they offer the best option for farmers in regions such as Namibia.
It is important for late pregnancy and lactation to coincide with most of the rainy season. In Namibia, this generally means that the breeding season should commence in spring instead of autumn. For genotypes that are strongly controlled by photoperiod, the peak in annual food supply will be “out of phase” (Martin et al., 1994). Although mating out of the breeding season can lead to lower lambing percentage, better lamb survival rates will be achieved (Parsons and Hunter, 1965). Mating in spring is feasible because social-sexual stimuli are also able to induce the reproductive activity by over-riding both circannual rhythm and the inhibitory effects of photoperiod (Martin et al., 2002; Martin et al., 1999). Controlled breeding using the ‘male effect’ could therefore play an important role in strategies to improve reproductive performance where “out of phase” breeding is advantageous.

The ‘male effect’ is predominantly of pheromonal stimulus that increases LH pulse frequency and stimulates the growth of ovarian follicles, leading to ovulation (Atkinson and Williamson, 1985). However, it is not an effective tool on its own because the proportion of ewes ovulating, ovulation rate and proportion of ewes experiencing a short cycle are variable from season to season. On average, 50% of the flock experiences a normal cycle and the other 50% a short cycle. The short cycle is the result of poorly developed corpus luteum which only lasts for 6 to 9 days at second ovulation (Martin, 1979; Oldham and Martin, 1979). It is important to improve or prevent the occurrence of the short cycle because it will allow farmers to have better control over timing of the breeding season, planning of pregnancy and subsequently the lambing season to coincide with the food availability.

The general theory is that the problem is at the ovary level where the preovulatory follicles do not develop fully to produce a viable corpus luteum. The sudden introduction of rams causing acute LH pulse frequency and secretion within 10 minutes, provides insufficient time for the transformation of the gonadotrophin-dependent follicle into an ovulatory follicle. The concept “window of opportunity” was developed which is understood to be the period of time when the sensitivity of the gonadotrophin-dependent follicle to FSH stimulus is adequate so that it will resist atresia and develop into an ovulatory follicle (Scaramuzzi et al., 1993).

The response to the “ram effect” can be improved with nutritional supplementation (Stewart and Oldham, 1986; Oldham and Lindsay, 1984; Knight et al., 1983; Knight et al., 1979; Lightfoot and Marshall, 1976; Lightfoot et al., 1976). Nutritional supplementation stimulates follicle growth and the follicles will be afforded that “window of opportunity” to grow further even with little amounts of FSH concentrations. The overall effect of short-term lupin
supplementation is the immediate increase of insulin concentrations after feeding that leads to increased supply of glucose to the follicles, mediating nutritionally-induced stimuli in ovulation rate (Downing et al., 1995). Therefore, nutritional supplementation before the “ram effect” may play an important role in preventing the short cycle, hence, synchrony of the majority of the ewes within a three-day period.

In 1995, the Farming Systems, Research and Extension (FSRE) programme was implemented by the Ministry of Agriculture, Water and Forestry (MAWF) to improve the efficiency and productivity of sheep and goat farming in Namibia. The FSRE aims to make research outcomes more accessible to farmers, since most research into reproductive strategies is done under controlled conditions and not necessarily directly applicable to farming systems. Namibian farmers are in an ideal position to benefit from the increasing demand for “clean, green and ethical” (CGE) agriculture (Martin et al., 2004a) since their management strategies are extensive and rarely use hormones or chemicals.

The proposed research project will investigate how two CGE management tools, the ‘male effect’ and focussed feeding can be used to influence the reproductive performance of small ruminants. I am optimistic that this information will help me to develop strategies for controlling reproduction in sheep that are both directly applicable and easily accessible to communal farmers in Namibia. The general hypothesis is that nutritional supplement will improve the response to the ‘male effect’ and the subsequent reproductive performance of Merino ewes.
CHAPTER 2

2.1 Review of the literature

In the hypothalamic-pituitary axis, there is a convergence of various external and internal inputs that influence outputs that control many essential bodily functions, including reproduction. For reproduction in the ewe, for example, the external and internal inputs are processed and the outcome determines the pattern of secretion of the neurohormone, gonadotrophin-releasing hormone (GnRH) which, in turn, regulates the secretion of the gonadotrophins from the pituitary gland. The gonadotrophins stimulate the ovaries which then send signals (primarily sex steroid hormones) back to the brain to exert feedback on GnRH/gonadotrophin secretion and elicit sexual behaviour. This system, linking hypothalamus, pituitary gland and ovaries, is responsible for the generally close temporal relationship between ovarian function (ovulation) and sexual behaviour.

In addition to the primary sex steroids (oestrogens and progestagens), the internal factors include metabolites and metabolic hormones that convey information to the reproductive control centres about body reserves (including the demands of lactation) and stress responses. Among the external factors are those that reflect season (night-length) and nutrition, to which the reproductive centres generally respond by ensuring that lambing occurs at that time of the year when the food supply is adequate for offspring survival.

This review of the literature discusses the physiological processes that are linked to the control of reproduction in the female, with a focus on sheep, and explores the possibility of using nutritional management to improve the reproductive performance.

2.2 The Reproductive System

2.2.1 The hypothalamo-hypophyseal axis

Reproductive activity is primarily controlled by the activity of the neurons in the brain that produce and secrete gonadotrophin-releasing hormone (GnRH). In sheep, the GnRH cell bodies are located in the hypothalamic-preoptic continuum (Caldani et al., 1988). The synthesis of GnRH by these neurones is transported along the axons to the median eminence where it is released, in pulses rather than as a continuous stream, into the hypophyseal portal system that connects with the anterior pituitary gland (Fig. 1). The arrival of GnRH pituitary tissue stimulates the synthesis and secretion of the gonadotrophins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), two of the major regulators of gonadal function.
(Lincoln, 1979) that are then transported to the target tissues, the ovaries, to stimulate gonadal activity.

**Figure 1: Schema of the hypothalamic-pituitary axis in sheep.**

The pulsatile release of GnRH stimulates the secretion of LH pulses (on a 1:1 basis), whereas it elicits a more continuous stream of FSH secretion. In the brain, the important regions are the preoptic area (POA), anterior hypothalamic area (AHA), the mediobasal hypothalamus (MBH) and the median eminence. Adopted from Thiéry & Martin (1991) and Caldani et al. (1988).

### 2.3 Folliculogenesis

Folliculogenesis, the process of follicle growth and development, is a complex series of events leading to an increase in number of follicles through the differentiation of somatic and germ cells within the ovary. This proliferation of follicles commences in late stages of foetal development, and the subsequent maturation of small cohorts takes place continuously, through puberty and throughout the adult life. The maturation phase takes 4-6 months in cattle and sheep (Hunter et al., 2004b; Souza, Campbell & Baird, 1997), but just over three months in pigs (Hunter et al., 2004a). The final stages of maturation are dynamic and rapid and, in the sheep, culminate in the production of only 1-3 (usually) mature, ovulatory (‘Graafian’) follicles during the follicular phase of the oestrous cycle (Fig. 2; Pineda, 2003a).
The whole process, from differentiation of large numbers of primordial follicles through to the ovulation of a few at the end of each oestrous cycle, is driven by numerous signalling and gene expression events (McNatty et al., 2006). The follicle tissues (theca and granulosa cells) and the oocyte that each follicle carries, communicate continuously with each other through molecules such as growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15), both secreted in the oocyte, sustain development and maturation of the follicle (McNatty et al., 2006; McNatty et al., 2004).

The initiation of growth in a primordial follicle involves many multiplications of granulosa cells which eventually form more than one layer around the oocyte (Fig. 2) and ultimately create an environment in which an antrum forms, a space full of fluid (Webb & Campbell, 2006; McNatty et al., 2006). The antral follicles in sheep range between 200 and 400 μm in diameter (Turnbull, Braden & Mattner, 1977). The antral fluid contains large quantities of ovarian steroids (androgens, oestrogens, progestagens), electrolytes, growth factors and cytokines (Pineda, 2003a). As a result of the increasing volume of the follicular fluid in the antrum, the follicle begins to bulge from the surface of the ovary.

**Figure 2: The structure of an ovary.**

Clockwise changes during the oestrous cycle of the sheep, starting at the hilum as it recurs on average every 16-17 days. Primordial follicles go through a complex maturation process that ends with atresia for most of them, and ovulation of a mature oocyte for a few of them. As the preovulatory follicle enlarges, it secretes large amounts of oestrogen and inhibin and these two hormones act synergistically to suppress the development of other follicles through inhibitory feedback on FSH secretion (this is 'follicle selection' and leads to 'follicle dominance'). Following ovulation, the remnants of the follicle are transformed into a corpus luteum (luteinization). Adapted from Porterfield (2001a).
After ovulation, when the ovum is released for potential fertilisation by the sperm, the corpus luteum forms and, if there is no pregnancy, regresses to allow a new cycle to begin.

The process of follicular development is controlled by a highly co-ordinated endocrine and paracrine signals that ultimately determine whether a follicle ovulates and also whether more than one follicle achieves this outcome (ovulation rate). The endocrine signals are between the pituitary gland and the ovary, whereas the paracrine signals are among the follicular cells, viz. the oocyte, cumulus, granulosa and thecal cells (Juengel & McNatty, 2005; Shimasaki et al., 2004; Matzuk et al., 2002). The process of follicular development is continuous but is nowadays divided into functional (rather than morphological) stages: primordial, committed, gonadotrophin-responsive, gonadotrophin-dependent and ovulatory (Fig. 3). These stages also involve changes in capacity for steroidogenesis, an integral part of the ovarian cycle.

**Figure 3: A model for folliculogenesis in the ewe.**

Cascade of development during which follicles emerge from a pool of primordial follicles to enter a process of growth and development that is continuous and ends in either atresia or ovulation. In most mammals, this process is approximately linear to the gonadotrophin-responsive stage and, especially in ruminants, becomes wave-like in the gonadotrophin-dependent stage. Adapted from Scaramuzzi et al. (2011).
2.4 Primordial follicles

Folliculogenesis begins with the development in the foetus of a pool of quiescent primordial follicles that are about 0.03 mm in diameter (Scaramuzzi et al., 2011). With little selection taking place, these follicles are primarily gonadotrophin-independent (Lopez-Sebastian et al., 1993; Cahill & Mauléon, 1980; Dufour, Cahill & Mauléon, 1979; Turnbull et al., 1977; Hutchinson & Robertson, 1966). Young ewes have between 40,000 to 300,000 primordial follicles.

2.5 Committed follicles

In an apparently ordered sequence, gradually and regularly, primordial follicles leave the resting pool and start to grow under the control of stimulatory and inhibitory feedback mechanisms between the ovaries and the hypothalamo-hypophyseal axis (Cahill, Mariana & Mauléon, 1979). On entering this phase, they are ‘committed’ to full-scale development that will, ultimately, end in atresia for all except the few that ovulate, although very few die at this early stage. The process seems to have evolved to ensure that high numbers of follicles which enter the gonadotrophin-responsive stage, increasing the likelihood of recruitment for ovulation.

2.6 Gonadotrophin-responsive follicles

After becoming committed, the follicles become gonadotrophin-responsive and develop receptors for LH on the theca cells and for FSH on the granulosa cells (Scaramuzzi et al., 1993, 2011). There is some doubt about whether the gonadotrophins are essential for development beyond the committed stage, but the follicles are classed as gonadotrophin-responsive because they can respond to gonadotrophins by synthesising cyclic adenosine monophosphate (cAMP) and steroids (McNatty et al., 1992). Furthermore, the granulosa cells synthesise and secrete proteoglycans in response to FSH, suggesting that they are perhaps involved in the formation of the antrum by preventing early luteinization in the later stage of the antral follicle development (Findlay et al., 1993; Baird, 1984; Ax & Ryan, 1979). About 25 follicles with diameters of 1.0-2.5 mm emerge from this phase, but most are apparently too immature to be gonadotrophin-dependent and thus ovulate in the absence of exogenous gonadotrophins (Henderson et al., 1988; Tsonis et al., 1984).

2.7 Gonadotrophin-dependent follicles

In this phase, the follicles develop an antrum and become tertiary and Graafian follicles in a process that leads to wave-like patterns of follicle growth during the cycle primarily under
gonadotrophic control (Bartlewski et al., 1998; Ginther, Kot & Wiltbank, 1995; Ginther & Kot, 1994; Ravindra et al. 1994; Noel, Bister & Paquay, 1993). The waves are due to interactions between FSH, inhibin and oestradiol, and are associated with the phenomenon of follicle ‘dominance’ (Viñoles et al., 2010; Scaramuzzi et al., 2011). For the follicles to grow larger than 2.5 mm in diameter, they need FSH and LH and, because the largest follicle(s) produce the most oestradiol and inhibin A, they inhibit FSH secretion and drive other gonadotrophin-dependent follicles into atresia (Scaramuzzi et al., 1993; Gonzalez-Añover et al., 2007). The dominant follicles have very high concentrations of oestradiol in their follicular fluid so they are often classified as ‘oestrogenic’ (Fortune, Rivera & Yang, 2004; Scaramuzzi et al., 1993; McNeilly, Jonassen & Fraser, 1986).

This, however, is a critical phase for the dominant follicles too – they are also totally dependent on gonadotrophic control to avoid atresia so they overcome the decreasing concentration of FSH by shifting their dependency to LH; they develop LH receptors on the granulosa cells and thus no longer need FSH to produce aromatase and convert androgen to oestrogen (Campbell, Scaramuzzi & Webb, 1995). Because of their vulnerability to atresia, and thus the process of follicle selection, the number of gonadotrophin-responsive and ovulatory follicles declines markedly and only 1-8 follicles remain (Scaramuzzi et al., 1993).

2.8 Ovulatory follicle

In the final stages of the follicular phase of the oestrous cycle, FSH concentrations are very low but the actual values might still be critical for final maturation of the granulosa cells (Henderson et al., 1988). Variations in the FSH concentration at this time are probably due to variability within and between animals (Scaramuzzi et al., 1993). The granulosa cells in ovulatory follicles present with large number of LH receptors and they become highly responsive to both LH and FSH (Webb & England, 1982). The aromatase activity per granulosa cell reaches a maximum, leading to massive increases in the production and secretion of oestradiol and inhibin, finally suppressing FSH concentrations below the critical threshold required by the gonadotrophin-dependent follicles, all of which undergo atresia (Scaramuzzi et al., 1993). In rats, the oestradiol also increases the responsiveness of the ovulatory follicle to gonadotrophins by enhancing the ability of FSH to induce LH and FSH receptors (Richards, 1980). Furthermore, in humans and rats, local peptides like inhibin enhance the ability of LH to stimulate androgen production by thecal cells (Hillier et al., 1991; Hsueh et al., 1987).
At this stage, only a few follicles remain and enter the ovulatory process. This number controls the ovulation rate of the animal – for a Merino ewe, usually, only one or two ova are shed at ovulation.

2.9 Steroidogenesis

Implicit in folliculogenesis is the synthesis of sex steroids by the theca and granulosa cells (Fig. 4), a process that is best explained by the two-cell theory in which the theca and granulosa cells complement each other (Falck, 1959). Importantly, the granulosa cells cannot produce the androgens as they lack the enzyme 17α-hydroxylase. Therefore, the theca interna cells produce androgen as the precursor for oestrogen production by aromatase in the granulosa cells (Porterfield, 2001a). The thecal cells have LH receptors (LH-R) linked to 17α-hydroxylase activity throughout all stages of antral follicular development (Porterfield, 2001a; Hillier, 1985) and thus androgenic steroids are synthesised during active periods of antral growth (McNatty, 1982; Moor, 1977). The granulosa cells, on the other hand, are the predominant site of oestradiol-17β production and are the only cells that have FSH receptor (FSH-R) through which FSH stimulates the production of inhibin and the production of oestrogen by aromatization of androgens that have diffused in from the theca cells (Pineda, 2003a; Hillier, 1985; Porterfield, 2001a).

![Diagram of ovarian steroidogenesis](image)

**Figure 4: A pictorial illustration of ovarian steroidogenesis.**

*From the precursors (cholesterol pregnenolone, progesterone) theca cells produce androgens that then diffuse into the granulosa cells where they are converted to oestrogens by aromatase. In antral follicles, the granulosa cells develop LH receptors so do not need to rely on FSH for the production of aromatase.*
2.10 Feedback Mechanisms

Ovarian function is dependent on the actions of pituitary LH and FSH to stimulate follicular growth, and the secretion of LH and FSH is, in turn, controlled by inhibitory and stimulatory feedback through a range of interactions that orchestrate the stages of the reproductive cycle (Pineda, 2003b).

2.11 The negative feedback

The negative feedback occurs when the concentration levels of progesterone, oestradiol and inhibin are sufficiently high to suppress the response to the hypothalamic-pituitary axis. Progesterone synthesised and secreted from the corpus luteum synergises with oestradiol-17β to inhibit hypothalamus for the stimulation of GnRH.

It has been demonstrated that either oestradiol or progesterone alone cannot inhibit the LH release and that they require a collective effort (Martin & Thomas, 1990). The ovarian oestrogens and progestagens as well as other locally produced ovarian peptide factors (inhibin A and follistatin) feed back to the hypothalamus and pituitary to regulate the secretion of the gonadotrophins (Fig. 5) (Findlay et al., 1993). Particularly, inhibin which is a dimeric polypeptide factor, acting directly on the anterior pituitary gland and oestradiol-17β which acts on both the hypothalamus and the anterior pituitary gland inhibit the secretion and release of LH and FSH by adenohypophysial cells (Pineda, 2003a).

The feedback in the female is complicated by the combinations that can arise because there are two endocrine organs in the ovary that produce different hormones with different targets in the hypothalamo-pituitary axis.

*Negative Feedback Loop 1*  
Gonadotrophin-releasing hormone pulse frequency is inhibited by oestrogen (from the follicles) and progesterone (from the corpora lutea) acting synergistically on the hypothalamus;

*Negative Feedback Loop 2*  
Follicle-stimulating hormone secretion is inhibited by oestrogen and inhibin acting synergistically on the anterior pituitary gland.

Between them, these two negative feedback loops can explain nearly all the events of the female ovulatory cycle. For instance, the transition from the luteal phase (when progesterone is present) to the follicular phase (when progesterone has disappeared) is explained by loss of
Feedback Loop 1. This allows GnRH pulse frequency to ‘escape’, stimulate the secretion of LH and FSH, and drive follicular growth and maturation. As the follicles grow and mature, they produce lots of oestrogen, but oestrogen is a very weak agent of negative feedback, so GnRH pulse frequency is not affected.

Feedback Loop 2 is most important during follicle selection because the main source of oestradiol and inhibin, the dominant follicle(s), drive FSH secretion down, depriving other follicles of this essential hormone and thus causing atresia.

As oestrogen builds up during the follicular phase it triggers a ‘switch’ in the hypothalamus and pituitary gland and a large surge of GnRH, LH and FSH is produced which drives the last stage of follicle development and leads any fully mature follicles to burst and release their ova. The high concentration of oestrogen at this time also induces oestrous behaviour.

2.12 Positive feedback

The secretion of FSH stimulates growth and differentiation of ovarian follicles during the preantral and early antral stages. Unlike LH, however, FSH release is not in pulses but it occurs in a constant manner (Martin, Rodger & Blache, 2004).

For the release of LH, each GnRH pulse results in an equivalent pulse of LH and each pulse of LH, in turn, stimulates the follicles to secrete a pulse of oestrogen (Martin, 1984). The effect of LH is two-fold: i) it acts on theca interna cells and luteal cells; and, ii) late in the follicular phase, it acts on granulosa cells to sustain further growth of the large antral follicles (oestrogenic) (Porterfield, 2001a; Baird et al., 1991; Baird, 1984).

![Figure 5: Negative and positive feedback mechanisms.](image)

*Figure 5: Negative and positive feedback mechanisms.*

A schematic illustration of the regulation of ovarian function via negative and positive feedback mechanisms in female sheep. Behavioural effects such as oestrus exhibition due to increased oestradiol concentrations are important during mating.
2.13 The Oestrous Cycle
The length of the average oestrous cycle is 17 days with a modal range of 14 to 19 days in most breeds of sheep (Fig. 6). As a matter of convenience, the beginning of the oestrous cycle is counted from the time when the ewe has been marked by the male and is designated as Day 0. In a cyclic ewe, ovulation is preceded by oestrus behaviour (sexual receptivity) and preovulatory LH surge which all these three phases occur within 72 hours (Martin et al., 1986; Martin, Scaramuzzi & Lindsay, 1983; Oldham & Knight, 1979). The follicle that ovulated is luteinized which becomes the corpus luteum, hence, called the luteal phase.

Figure 6: Oestrous cycle of the ewe.
Relationships among hormone levels, sexual receptivity and the time of ovulation, demonstrating the key role played by PGA-F2α in inducing luteolysis. Adapted from Pineda (2003b).

The progesterone avoids further ovulation in anticipation of an embryo implantation which its concentration levels begin to rise rapidly and are detectable 3 days after ovulation. It reaches maximum concentrations on Day 8 and remains elevated until Day 11 to 12 (Pineda, 2003b). The cycle is therefore dominated by a luteal phase which lasts for 12 to 14 days and these elevated progesterone concentrations keep the gonadotrophin at basal levels, particularly LH (Pineda, 2003b). Inter-wave folliculogenesis occurs every 2 to 4 days in sheep and goats during the luteal phase (de Castro et al., 1999; Ginther et al., 1995; Ginther & Kot, 1994; Smeaton & Robertson, 1971). However, in most instances, the waves do not produce ovulatory follicles and all follicles undergo atresia. Only the last wave of folliculogenesis is
vital as it emerges at the end of the luteal phase and the dominant follicles from this wave are candidates for ovulation. The inter-wave folliculogenesis is self-regulated by oestradiol, inhibin and progesterone in a negative feedback mechanism between the hypothalamus, pituitary and the ovary (Scaramuzzi et al., 1993). Since oestradiol levels are secreted by the large antral follicles, the blood levels of oestradiol remain low during most of the luteal phase, but at times of inter-wave follicular growth, oestradiol levels do rise until atresia of these non-ovulatory follicles occur (Pineda, 2003b).

In the absence of embryo implantation (no pregnancy), luteolysis is initiated as a consequence of ovarian and posterior pituitary oxytocin release that stimulates endometrial secretion of prostaglandin F$_2$a (PGA-F$_2$a) from the uterus (Pineda, 2003b). Prostaglandin F$_2$a is a natural luteolysin (Porterfield, 2001b; Baird, 1984) and its luteolytic activity has been described to: (i) constrict the utero-ovarian vessels causing ischemia (inadequate blood supply) and starvation of the luteal cells; (ii) interfering with progesterone synthesis; (ii) competing with LH for the receptor sites; and (iv) destructing the LH receptor sites (Porterfield, 2001b). Progesterone levels in the peripheral blood decrease to less than 1.0 ng/mL (Pineda, 2003b). The alleviation of gonadotrophin suppression due to the low blood levels of progesterone, oestradiol and inhibin, FSH synergises with LH to facilitate follicular growth and oestradiol production from Day 14 to 16, called the follicular phase. The rapidly rising levels of oestradiol towards the end of Day 16 induces oestrus behaviour in the ewe which is shortly followed by preovulatory LH surge (Pineda, 2003b; Martin, 1984). Ovulation within hours of LH surge signifies the start of the new cycle as a new corpus luteum is formed and progesterone blood levels start to rise again.

2.14 “Male effect” for controlled breeding

There is sufficient evidence that demonstrated the effectiveness of teasing anoestrous ewes elicit an acute physiological response when exposed to novel males (Martin, Oldham & Lindsay, 1980; Oldham & Knight, 1979). Such male contact causes ewes to ovulate, but oestrous cycle synchrony is not what we exactly want (Oldham, 1979). This knowledge has shifted us into an era of looking at how nutritional supplementation could improve the ewe response to the male effect. The following sections have therefore been revisited and will be briefly discussed: i); the importance of isolating ewes from the males ii) ewe response to the “male effect”; and, iii) our understanding so far with regards to the short cycle.
Our understanding of the aforementioned factors has made the use of male effect very important as farmers can choose when to commence with the breeding season.

### 2.15 Isolation of ewes from males

There is abundance of evidence that previously isolated ewes respond spontaneously to the introduction of males while continuous association causes females to familiarise with the males and the “male effect” concept proves ineffective. The principle of pre-conditioning disapproves of the farming system currently managed by subsistence farmers in Namibia. In the Merino sheep that are also under a system of continuous association of sexes, the seasonal breeding pattern of the breed is restricted and seems to be under light control (Schinckel, 1954b). Also, yearling ewes that continuously cohabited with males took on average 45 days longer to start mating relative to when ewes were previously isolated (Notter, 1989).

In addition, continuous association can also mean that females show photoperiodic-driven reproductive behaviour under such a system and their opportunistic strategies to coincide the lambing and lactation with feed availability become out of phase (Martin et al., 2004). Namibian breeds have a similar origin (Mediterranean) as the Australian breeds and the “male effect” by isolating males and bucks from female sheep and goats should in theory have the same effect, except that feeding strategies that are poorly applied in Namibian conditions need to be improved on. In fact, isolation means that all the males are kept out of sight of the females because the male odour, visual or sound can apparently mediate the “male effect” (Gelez & Fabre-Nys, 2006; Pearce & Oldham, 1988).

### 2.16 Ewe response to the “male effect”

When previously isolated anoestrous ewes come in contact with males, ovulation within 3 days occurs that is preceded by an increased LH pulse frequencies and rapid LH surge which is within 27 h (Oldham & Knight, 1979), 35 h (Knight, Peterson & Payne, 1979) and 36.7 h (Ungerfeld et al., 2002). Ovulation varies from as few as 40 h (Oldham & Knight, 1979), 3 days (Martin et al., 1980; Knight et al., 1979), 5 days (Oldham, Boyes & Lindsay, 1984), to 6 days (Oldham, 1979; Schinckel, 1954a). These variations may be subject to (i) the nutritional status of the animals at the time of male introduction; (ii) the breed genotype; (iii) experimental methodology; (iv) time of season; (v) age of maturity: and (vi) environmental factors. There is a two-peak cycle in one half of the group and one peak cycle in the other half of the group (Oldham, 1979; Schinckel, 1954a). The two-peak cycle is a characteristic of
two LH surges within the space of 9 days, hence the short cycle. Ewes with a normal cycle only have one LH surge within 72 h (Fig. 7).

Interestingly, short-term studies of the “male effect” on LH concentrations showed that after 24 h of exposure, the LH concentrations returned to basal levels similar to LH concentration levels of the control group. On the other hand, FSH concentration levels fell within 2 hours of male introduction and remain below basal levels of the control group for the remainder of the experiment (Atkinson & Williamson, 1985).

All females are unable to exhibit manifest sexual receptivity at their first ovulation of male introduction due to the prematurely regressing corpus luteum (Oldham & Pearce, 1984; Martin, 1979; Oldham, 1979; Robinson, 1959; Schinckel, 1954b). Oestrus detection by males and subsequent mating will only occur between 17 to 19 days and 23 to 25 days post male introduction in the ewes experiencing the normal cycle and in ewes with the short cycle, respectively (Fig. 7).

![Graph showing oestrous cycles](image)

Figure 7: **Two types of oestrous cycles induced in anovulatory ewes.**

Response of anovulatory ewes after first ovulation within 72 h of male exposure. The first normal cycle in one proportion of the ewe flock (top graph) and a short cycle of 6 days (bottom graph) in the other proportion of the flock are both accompanied by silent heat at both the first ovulation and, in ewes showing the short cycle, at the second ovulation. Ewes showing a normal cycle will display their first oestrus on Day 19 and ewes having a short cycle will display their first oestrus 6 days later. Adapted from (Martin et al. 1986).
2.17 The short cycle
At this stage, not much is known about what causes the short cycle in ewes. Though, it was discovered that the inadequacy is not at the hypothalamic-pituitary axis level because immunisation against androstenedione, to stimulate tonic secretion of LH (and not FSH) and follicular activity does not prevent the short cycle nor does supplementation with oestradiol (Martin, Scaramuzzi & Lindsay, 1981; Martin, 1979). This finding has shifted the attention to the ovary and we now know that the follicle maturation does not complete and that results in a poor quality corpus luteum which only lasts for 9 to 10 days after male-induced second ovulation.

The period of delay from male introduction to LH surge is important to allow follicular growth and it was suggested the possibility of a relationship between the LH surge and the type of corpus luteum formed (Pearce, Martin & Oldham, 1985). Importantly, priming ewes with progesterone does not block the LH synthesis and secretion, but it only delays its surge (Martin et al., 1983). As this will present a long follicular phase, sufficient time is afforded to synchronise follicle growth with other endocrine processes (Martin et al. 1986). Therefore, the “window of opportunity” is a prerequisite, allowing FSH concentrations to remain elevated so that gonadotrophin-dependent follicles continue to grow into ovulatory follicles. Unfortunately, to achieve this, using natural methods to improve reproductive performance in animals is a tall order because ovarian activity or the state of follicles varies from ewe to ewe leading to a high degree of randomness.

2.18 Nutrition
Over the past 90 years, many workers had made numerous advances in animal nutrition and it is undoubtedly the most important environmental factor to improve the reproductive performance in small ruminants (Smith & Stewart, 1990; Hafez, 1952). Nutrients boost programming and expression of metabolic pathways that enable animals to achieve their genetic potential for reproduction. In other words, the feeding regimes will have a long-term or permanent change in the subsequent reproductive performance (Lucas, 1992). The suggestion was that poor nutrition may have consequences on the number of gonadotrophin-dependent follicles (Scaramuzzi et al., 1993). Thus, changing levels of nutrition at a critical or sensitive period of reproduction, are essential and its effects in the efficiency of most stages of reproductive processes are by far the most important on ovulation rate (Smith, 1985). Research on nutrition and ruminant fertility concentrates from the whole animal responses to particular and complex functions of cellular and molecular processes that control
gamete production, ovulation, fertilization, embryo development and survival, conceptus implantation and growth (Robinson et al., 2006; Holst, Killeen & Cullis, 1986).

2.18.1 Types of nutrition

Two types of nutritional effects, *dynamic* and *static effects*, were invented where increased ovulation rate is associated with change in bodyweight (Coop, 1966; Coop, 1962). In contrast, Lindsay et al., 1975 discovered the *acute effect* using lupins in short-term nutritional strategies to significantly increase ovulation rate. The three effects of nutrition have been adequately explained by Smith & Stewart, 1990 and will be briefly discussed for the purpose of the choice for this thesis.

2.18.1.1 Acute effect

This approach uses short-term feeding strategies to increase ovulation rate. It is so acute that increased ovulation rate becomes apparent long before the changes in body condition have their effects (Fig. 8). For example, when ewes are fed lupin supplements: (i) plasma glucose, acetate and propionate increase; (ii) entry rates of glucose, acetate and carbon dioxide into the cells are accelerated; and (iii) glucose oxidation increases (Teleni et al., 1989a). On the other hand, intravenous glucose infusion only caused increased glucose entry rates at the highest infusion rate of 46.8 mmoles h\(^{-1}\) (cf. 31 mmoles h\(^{-1}\) due to lupins) (Teleni et al., 1989a) and 60 – 65 mmoles h\(^{-1}\) for 5 days in the late luteal phase of the oestrous cycle (Downing, Joss & Scaramuzzi, 1995). The energy levels provided by lupins prove adequate to increase ovulation rate compared to the intravenous infusion of glucose.

Supplementing ewes with lupins for as few as 4 and 6 days prior to ovulation can significantly increase the ovulation rate (Stewart & Oldham, 1986; Oldham & Lindsay, 1984). This was in agreement when ovulatory response to lupin grain supplements was initiated near the time of luteolysis (Nottle, Seaman & Setchell, 1990). Short-term supplementation from Days 8 to 14 of the oestrous cycle increases ovulation rate in ewes with a moderate to high body condition due to acute changes in the blood glucose, insulin and leptin concentrations (Viñoles et al., 2003; Viñoles, 2003). These critical time ranges of short-term nutritional supplement close to luteolysis is when the ovulatory wave emerges (Viñoles, 2003). A lupin diet with protein (34\%) and metabolizable energy contents 13.7 MJ/kg (Milton, 2001) can induce high ovulation rate which promotes the consumption of increased digestible energy presumably mediated via biological pathways associated with the
synthesis and utilisation of glucose for reproduction (Leury, Murray & Rowe, 1990; Teleni et al., 1989b; Murray & Rowe, 1984).

![Figure 8: Acute effect of feeding.](image)

The result leads to positive energy balance which, in turn, increases leptin and insulin concentrations in the blood subsequently increasing the glucose uptake by the cells. These effects seem to have a direct effect on the ovary which are linked to increased folliculogenesis and ovulation rate (arrow indicating acute). The arrow designated undetectable illustrates that change in body weight is imperceptible at the equivalent time when increased folliculogenesis and ovulation rate are detected. Adapted from Scaramuzzi et al. (2006).

2.18.1.2 Static effect

Static effect is the body condition of ewes within the same genotype and female size that enter the breeding season which is indicative of a stable metabolic status. Depending on their different levels of body condition, ewes will have different ovulation rates. Body condition affected the size of the large follicles (≥4 mm diameter) during the late luteal and follicular phases (Rhind & McNeilly, 1986) and ewes in high body condition had higher FSH and low oestradiol (E2) concentrations during the follicular phase than ewes in a low body condition (Viñoles et al. 2002). Rhind & McNeilly, 1986, however, found that the difference in mean FSH concentrations during the follicular phase was unclear but concluded that there is a greater suppression of FSH in ewes in good condition. They indicated that such suppression is the result of higher oestrogen and inhibin concentrations synthesised by the greater number of large oestrogenic follicles present. This is a possible explanation because the serum
concentrations of FSH decline at maximum follicular growth between 2 to 3 days prior to oestrus (L'Hermite et al., 1972). It is the time when large oestrogenic follicles exert a negative feedback on the hypothalamic-hypophyseal axis to restrict the further growth of other developing follicles (Baird et al., 1975).

The “static effect” of nutrition on ovulation rate in ewes in a higher average body condition of 2.86 had a higher ovulation rate (1.8 v 0.9) and more large follicles with ≥4 mm in diameter than ewes in a lower average body condition of 1.84, but the numbers of small follicles between 1 mm – 4 mm in diameter were similar in both groups (Rhind et al., 1989; Rhind & McNeilly, 1986). This was further supported when ewes with high body condition (4.1) had high levels of FSH concentration during the follicular phase, thus, allowing a longer period of follicle recruitment (Viñoles et al., 2002).

Ewes with a low live weight of 41.6 kg compared to heavier ewes of 56.5 kg: (i) had low ovulation rate; (ii) came into oestrous later upon the withdrawal of the sponges; (iii) had more follicles ≥2 mm in diameter in late luteal phase; (iv) had less number of follicles that were recruited early in the follicular phase; and (v) a high intensity of selection through atresia (Xu, McDonald & McCutcheon, 1989).

2.18.2 Acute versus Static Effect

The diagrams in Scaramuzzi et al., 2006; Viñoles et al., 2005 illustrate the comparisons of immediate and static effects on hormones, glucose, insulin and leptin concentrations that rise rapidly with peak levels experienced on day three after supplementation commenced. Static effect is responsible for: (1) increased FSH concentrations mediated via the hypothalamus; (2) development of more follicles; and, (3) decreased oestradiol (E2) concentrations. At the ovarian level, FSH stimulates the development of more follicles which eventually promotes an increase in ovulation rate. During this period of high FSH levels, E2 production by the follicles is low which is reported to be the result of high leptin concentrations that inhibit steroidogenesis in ewes with high body condition. The lower oestradiol concentrations reduce negative feedback at the hypothalamus and pituitary gland that lead to increased circulating FSH concentrations.

Contrary to the static effect, the immediate effect of nutritional supplement promotes acute responses of: (1) increased glucose concentrations; (2) increased insulin concentrations; (3) increased leptin concentrations; and, (4) therefore stimulation of follicular growth. This is indicative of positive energy balance and these changes increase folliculogenesis and
ovulation rate in sheep as consequence of the direct effect on the ovary (Scaramuzzi et al., 2006).

2.18.2.1 Dynamic effect

Ewes at any given live weight have different ovulation rates caused by the varying levels of body condition preceded by a period of supplementation (e.g. weeks) and this is known as the ‘dynamic effect’. Providing extra feeding for several weeks has proved to increase reproductive performance in breeding stock (Kiyma et al., 2004; Adams et al., 1997; Nottle et al., 1997; Blache et al., 1996; Boukhliq, Adams & Martin, 1996; Nottle et al., 1990; Stewart & Oldham, 1986; Lightfoot & Marshall, 1976; Lightfoot, Marshall & Croker, 1976; Knight, Oldham & Lindsay, 1975). However, timing of feeding and type of feeding appear to play a role to elicit increased ovulation rate. It took approximately three weeks to observe ovulation rate responses when high pasture allowances were fed to ewes (Smith, Jagusch & Farquhar, 1983). Coop, 1966 demonstrated though that ewes need to be fed high energy supplement for one entire oestrous cycle if increased ovulation rate is to be expected. This was supported by Dufour & Matton, 1977 who found that feeding such diet for less than one oestrous cycle does not result in increased ovulation rate. The shortened supplementation can only be achieved when nutrients, e.g. glucose and acetate are infused for nine days to produce an energy intake equivalent to that of a maintenance diet plus 750 g of lupins (Teleni et al., 1989b; Teleni & Rowe, 1986). It is almost inconceivable to suggest that farmers have to supplement for the entire 17 days of the oestrous cycle to achieve high ovulation rates. Moreover, although high ovulation rate is achieved with supplementation, it does not necessarily mean high conception rate, thus, feeding animals for such a long period may not produce good economical returns.

2.19 Nutrition to increase ovulation rate

The physiological processes that control folliculogenesis will produce in majority of breeds between 1 and 2 dominant follicles (Scaramuzzi & Radford, 1983) and, in other prolific breeds such as Booroola Merino, Dahman, Finn and Romanov, as many as 3 or more dominant follicles that are destined to ovulate (Scaramuzzi & Radford, 1983; Lahlou-Kassi & Marie, 1981; Bindon et al., 1979; Bradford, Quirke & Hart, 1971). This is termed ovulation rate which determines fecundity (e.g. litter size) in breeds. Ovulation rate is defined as the number of ovulations in a flock divided by the number of ewes ovulating which is a characteristic that displays a considerable degree of genetic variation. Ovulation rate is determined by counting the number of corpora lutea appearing on both ovaries of each ewe.
The ovulation rate, whether it is single or multiple ovulations, is apparently driven by several factors that work in harmony and they are: 1) the number of immediately available gonadotrophin-dependent follicles to be transformed into gonadotrophin-responsive follicles; 2) the individual requirement for FSH by gonadotrophin-dependent follicles; and, 3) the degree of response of hypothalamic-pituitary axis to inhibin and oestradiol inhibitory effects (Scaramuzzi et al., 1993). The next section will concentrate on the influence of nutrition on ovulation with reference to the types of nutritional effects.

2.20 Nutrition to improve response to the male effect
Comparing the results of various experiments with lupin supplements to ewes for 35 days before mating and another 18 days during mating showed significant increases in ovulation rate, lambing and twinning rate. Different rates of lupins (0, 125, 250 and *ad libitum* g/ewe/day) and different durations of feeding (0, 7, and 14 days prior to joining) showed no significant difference when feeding ewes for 7 or 14 days, but it was higher than ewes that received supplementation on Day 0 of mating (Lightfoot *et al.*, 1976). Interestingly, the rate of feeding at 250g/ewe/per day for all three durations had higher ovulation rate than feeding at *ad libitum*. In addition, supplements with 250g/ewe/per day of sweet lupin for 14 days before mating until Day 17 of mating had a variation of -14 to +21% in terms of lambs born (Croker, Johns & Johnson, 1985). Importantly, supplementing at 500g/ewe/per day did not alleviate the problem of variable response. This supported Lightfoot *et al.*, 1976 who suggested that there is perhaps a threshold at which nutrition stops influencing ovulation rate. In goats, supplementary feeding for seven days prior to buck exposure increased ovulation rate at first male-induced ovulation in the treated group compared to the control group, but nutrition had no effect in the subsequent ovulation (De Santiago-Miramontes *et al.*, 2007). They further found that the proportion of does with the short cycle was higher in the treated group than the control group.

2.21 Summary
The literature review has highlighted the importance of nutritional supplementation to the overall reproductive performance in small ruminants. Many reporters have demonstrated the synergistic effects of nutrition and the “male effect” to induce ovulation in a higher proportion of anovular ewes to increase ovulation rate in higher proportion of ewes with twin ovulations. The use of the “male effect” is an environmentally friendly and non-hormonal tool to control the breeding season. This allows farmers to adopt strategies to maximise on
the reproductive performance of the breeding stock. For example, a farmer can change the breeding season from autumn to spring mating so pregnancy and lactation overlap with the rainy season. Although this may lead to low lambing percentages the benefit of lamb survival and possibly heavier lambs as a result of sufficient food availability outweighs most factors of autumn mating.

Improved responses to the “male effect” is possible with short-term nutritional supplementation as opposed to prolonged feeding strategies. Despite this, the short-term nutritional supplements are easily feasible by studying the ovarian activity with laparoscopy and ultrasound techniques during the oestrous cycle only and not during an otherwise quiescent ovary. For example, nutritional supplements are applied at the emergence of the last follicular wave (Days 8 to 13 of the cycle), just before luteolysis, to take advantage of the time of the synchronous growth of the antral follicles under the influence of FSH. More work needs to be conducted in anoestrous ewes to fully understand when it is the best time to supplement ewes to stimulate follicle development. Much of the research is now focussed on the effects of external and internal inputs that converge into a common pathway to induce ovulation in sheep and goats. Further understanding of these pathways could help to manipulate nutrition and the “male effect” strategies, especially with regard to the short cycle.
CHAPTER 3

3.1 General Methods and Materials

This experiment was carried out in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (8th Edition, 2013) and was approved by the Animal Ethics Committee of The University of Western Australia under RA 03/100/596.

3.1.1 Area of study

Both experiments were conducted at Allandale Farm, a field station of The University of Western Australia (UWA) that lies between 110º and 120º W and 30º and 40º S north, 60 km northeast of Perth, Western Australia. The farm has mixed-age Merino ewes ranging from 3 to 10 years old, but in this experiment only ewes in the age range 3-6 years old were selected. Experiment 1 was begun in mid-January, the start of the normal breeding period at the farm, just before the onset of the normal breeding season for Merinos. Experiment 2 was carried out during the non-breeding season at the end of spring (September-November) and the beginning of the summer (December-February). The climate is Mediterranean and the studies were conducted under natural light. In both experiments, animals were kept in paddocks.

Grazing is mainly extensive with occasional feeding of hay to alleviate nutritional stress during the dry spells of the year (late October to early May). Rainfall normally starts late autumn and carries through winter until early spring.

Experimental animals

In all experiments, Merino sheep were used to define the effects of lupin grain and the ‘male effect’ on reproductive performance. All the animals selected for the experiments were sexually experienced and had lambed at least once.

3.2 Experiment 1

Before the experiment commenced, all the ewes were running on dry summer pastures and were fed hay *ad libitum*. Transrectal ultrasonography using a real-time B-mode machine (Aloka SSD500, Aloka Co. Ltd., Tokyo, Japan) equipped with a 7.5 MHz linear array Transducer was performed to determine the proportion of the flock that was cyclic (corpora lutea on either or both ovaries) and to allow selection of acyclic ewes (no corpora lutea). The acyclic ewes were allocated among three treatment groups; some were subsequently removed when retrospective analysis of oestrus observations suggested that they were cyclic but not detected by ultrasound examination. Prior to randomised allocation into three groups, the ewes were weighed and condition-scored (scale 1 to 5, with 1 being emaciated, 2.5 medium
fat and 5 very fat; Russel, Doney & Gunn, 1969; Jefferies, 1961). The three experimental groups were: Control (\(n = 32\); not fed any supplement); Lupin-supplemented before male introduction (L-Pre; \(n = 23\)); Lupin-supplemented after male introduction (L-Post; \(n = 30\)). Experiment 1 was split into 2 parts.

**Part 1**

Prior to male introduction, the L-Pre group was run separately and fed at a rate of 500 g lupin grain per ewe per day for 6 days, while the Control and L-Post groups were run together. All groups were combined on the final day of L-Pre supplementation and testosterone-treated wethers fitted with harnesses and crayons were introduced. Any ewes that showed oestrus within the 14 days from introduction of the wethers were removed from the experiment. The end of 14 days was also the start of the breeding programme on the farm and the wethers were replaced with testis-intact rams fitted with different crayon colours at a 6% ratio. The colours of the crayons on the rams were changed on Days 21 and 28.

**Part 2**

The L-Post group was separated from the Control and L-Pre groups and they were fed lupin grain at 500 g per ewe per day for 12 days from Days 11 to 22 of the oestrous cycle, which was after male introduction. On Day 55, trans-abdominal (3.5 MHz linear-array transducer) ultrasonography was used to determine pregnancy and to count the number of foetuses per pregnant ewe.

**3.3 Experiment 2**

The Merino ewes (\(n = 112\)) had all lambed once before, but not in the preceding lambing season to ensure that ewes were anovulatory. As in Experiment 1, ultrasound was used to separate cyclic from acyclic ewes, and the acyclic ewes were allocated among two equal-sized groups: Control (\(n = 29\)) and L-Pre (\(n = 29\)). Throughout the experiment, ewes were weighed and condition scored every 7 seven days, as described in Experiment 1.

The two treatment groups were run in separate paddocks. The Control group was fed hay *ad libitum* on dry pastures; the L-Pre group was fed 500 g of lupin grain per ewe per day prior to male introduction, from Day –5 to Day 0. At the end of nutritional supplementation, on Day 6, the groups were combined and vasectomized rams fitted with harnesses and crayons, at a ratio of 1:10, were introduced.
Oestrus observations

In Experiment 1, oestrus distribution was determined by recording marked ewes in the morning and late afternoon every day, from Day 16 to Day 26 after initiation of the ‘male effect’. In Experiment 2, all ewes marked within the first 14 days after male introduction were recorded, but only data from ewes with distinct crayon marks on the rumps were retained for analysis. Crayons were replaced on Day 14 after ram introduction and then every 7 days until Day 28. Oestrus observations were performed every day between Days 16 and 26.

Examination of the ovaries

All experimental animals were kept overnight in the yards with no feed or water. Ovulation rate was determined by counting the number of corpora lutea on both ovaries per ewe ovulating by directly viewing the ovaries with a laparoscope (Oldham and Lindsay 1980).

Five minutes before laparoscopy was started, 2 mg xylazine was injected intramuscularly for partial sedation. Ewes were placed in a crate in a vertical, dorsal recumbent position. At the end of the laparoscopy, 5 mL of benzacillin was injected intramuscularly and the wound was sprayed with vetericyn, all supplied by the veterinarian who performed the laparoscopy for this experiment.

Statistical analyses

We have based the analysis of treatment effects only on data collected from ewes that were deemed to have responded to the ‘male effect’, as evidenced by the occurrence of oestrus between Days 16 and 26. The number of corpora lutea per ewe ovulating (ovulation rate) was analysed using Chi-squared tests (χ²) in a 2 x 2 contingency table. Oestrus distributions for short versus normal cycles were determined using histograms with fits and groups in the Minitab 14 programme.
CHAPTER 4

4.1 Introduction

Sheep farmers place a lot of emphasis on maximising lamb output per ewe reproductive lifespan and a major contributor to lamb output is the ovulation rate of the ewe, the ultimate factor that determines fecundity (litter size). The upper limit for ovulation rate is set by genotype, but the degree to which a ewe can express her genetic potential is determined largely by nutrition. For decades, researchers have been intrigued by the mechanisms through which nutrition influences ovulation rate and, consequently, how nutrition can be optimally utilised to increase fecundity. Most of the research on sheep and goats has used a combination of ‘medium- and long-term’ feeding regimes (2 to 8 weeks), before and during mating, to boost energy levels (Coop, 1966; Findlay & Cumming, 1976; Fletcher, 1981; Lightfoot & Marshall, 1976; Lightfoot, Marshall & Croker, 1976; McInnes & Smith, 1966; Molle et al., 1995; Molle et al., 1997; Radford, Donegan & Scaramuzzi, 1983; Smith, Jagusch & Farquhar, 1983). However, in extensive and subsistence production systems, the costs of long periods of high-level feeding are prohibitive except when pasture is abundant, so it is not economically viable to feed animals so they can reach their genetic potential at all times of the year. This means that the manipulation of nutrition is restricted to short-term supplementation.

Research has shown that, if they are carefully timed, short-term supplementation can enable animals to achieve their genetic potential by directly stimulating the metabolic processes that control ovulation (Smith & Stewart, 1990). The ultimate refinement of this concept is ‘focus feeding’ in which supplements fed for only a few days are used to increase ovulation rates (Nottle et al., 1992; Nottle, Seamark & Setchell, 1990; Oldham & Lindsay, 1984; Viñoles et al., 2003).

Short-term nutritional supplements, such as lupin grain (*Lupinus angustifolius*), and related nutritional stimuli, such as glucose infusion, stimulate follicle development by promoting FSH-stimulated intracellular signalling pathways in the granulosa cells, yet somehow reduce the secretion of oestradiol. This leads to changes in the balance of the negative feedback loops in the hypothalamo-pituitary-ovarian axis that control gonadotrophin secretion, so that FSH concentrations can be sustained during the follicular phase of the oestrous cycle even when more follicles are growing and developing (Scaramuzzi et al., 2006; Somchit et al., 2007; Viñoles et al., 2005).
The general consensus is that the critical time during which nutrition can influence ovulation is around the end of the 12-day luteal phase (Nottle et al., 1990; Stewart & Oldham, 1986) when the last wave of follicular development culminates in the production of ovulatory follicles (Viñoles et al., 2003a). For such accurate timing of supplementation, the time of the next expected ovulation needs to be known. Until now, this has been feasible through the use of exogenous hormones (progestagen, prostaglandin, gonadotrophin) to control the cycle, although these treatments are also too expensive or impractical for subsistence and extensive production systems. In anoestrous ewes, an alternative that is theoretically feasible and affordable is the induction of ovulation with the ‘ram effect’. However, there are three problems with the ram effect:

i) There is considerable variation among individuals, flocks and genotypes in the responsiveness of the ewes to the ram effect (Martin et al., 1986);

ii) On average, after ram-induced ovulation, 50% of the ewes in a flock experience a short cycle and the other 50% a normal cycle, so cycle synchrony is far from perfect (Oldham, 1979; Schinckel, 1954);

iii) The ‘ram effect’ itself contributes to variation in ovulation rate (Cognie et al., 1980) with unknown outcomes under variations in short-term nutrition.

All three of these problems can be linked to the processes controlling the growth and development of the follicles in the ovary. Follicular waves continue throughout anoestrus, with follicles emerging, developing and becoming atretic, but the rate of development may be dampened in deeply anoestrous ewes compared to ewes with a shallow anoestrus. The short luteal phase is reported to be caused by either poor transformation of the ovulating follicles into corpora lutea, or by premature regression of the corpora lutea (Martin et al., 1986). In both cases, the underlying cause may be the status of the follicles at the time of ram introduction. Finally, the number of follicles that ovulate (ovulation rate) at the ram-induced ovulation is also determined by variations in the processes of folliculogenesis (Scaramuzzi et al., 1993).

Because nutrition exerts a wide range of largely beneficial effects on folliculogenesis, perhaps it could be used to improve the outcomes of the ram effect. Therefore, in the present study, we tested whether a nutritional supplement, fed for 6 days before the introduction of the males will improve the outcomes for the male-induced ovulation. In the subsequent male-induced oestrous cycle, we also tested whether supplementation for 12 days in the late luteal and follicular phases would affect ovulation rate
at the first oestrus (second/third ovulation), based on the knowledge that some farmers in Western Australia apply this nutritional strategy during the breeding season.

The specific hypotheses tested were:

a) Nutritional supplementation for six days before male introduction will increase number of ewes that ovulate in response to the ‘male effect’;

b) Nutritional supplementation for six days before male introduction will prevent short cycles;

c) Nutritional supplementation during the male-induced cycle will increase ovulation rate at the first oestrus.

4.2 Materials and Methods

The ewes selected for the experiment were part of the main breeding flock at Allandale, the farm of the University of Western Australia. The experiment was begun in mid-January, the start of the normal breeding period at the farm, just before the onset of the normal breeding season for Merinos in the southern hemisphere (Pearce and Oldham 1988) and ended on April 30. Before the experiment commenced, all the ewes were on dry summer pastures and were fed hay *ad libitum*. Transrectal ultrasound was performed on 60 ewes, eight days before the experiment started, to determine the proportion of the flock that was cyclic and to allow selection of ewes deemed as acyclic. Cyclic ewes were described as such if they had corpora lutea on either or both ovaries whereas acyclic ewes had no corpora lutea. The acyclic ewes were allocated among three treatment groups and then some ewes were subsequently deleted when retrospective analysis of oestrus observations suggested that they were cyclic but not detected by ultrasound examination. The final three groups were: Control (*n* = 32) not fed any supplement; Lupin-supplemented before male introduction (L-Pre; *n* = 23); Lupin-supplemented after male introduction (L-Post; *n* = 30).

The experiment consisted of two parts: in Part 1, we compared the Control and L-Pre groups, and Part 2 we compared the Control and L-Post groups. Initially, the Control and L-Post groups were run together, with L-Pre group in a separate field. In all fields, the animals were fed hay *ad libitum* on dry pastures. All ewes were weighed and condition scored every seven days. Ovulation was induced by the introduction of wethers (6 per 100 ewes) that had received three subcutaneous injections of 2 mL of testosterone at 7-day intervals.
4.3 Part 1

For 6 days, the L-Pre ewes were fed as a group with 500 g lupin grain per ewe daily (Fig. 9). On the final day of L-Pre supplementation (= Day 0 of the male-induced cycles), all three groups were placed in the same field and testosterone-treated wethers were introduced. The wethers had harnesses with crayons to mark ewes that showed oestrus. The ewes marked within the first 14 days were removed from the experiment as retrospective calculation of the timing of oestrus indicated that they were cyclic prior to the wether treatment. Day 14 was also start of the normal breeding programme on the farm and the wethers were replaced with entire rams, carrying a different colour crayon, at the same ratio (6%). On Days 21 and 28, the colours of the crayons carried by the rams were changed so we could detect the week within which the ewes came into oestrus.

Figure 9: A schematic illustration of the experimental design.

Laparoscopy procedure

Ovulation was determined by counting the number of corpora lutea on both ovaries of each ewe on Day 15 in the L-Pre and C groups only, by directly viewing the ovaries with a laparoscope (Oldham and Lindsay 1980).
4.4 Part 2
The L-Post group underwent the same protocol as above except the feeding regime (lupin grain at 500 g per ewe daily) ran for 12 days, from Day 11 to Day 22 (inclusive) after male introduction. The L-Post group was run separately from the L-Pre and Control ewes during the treatment period, after which all three groups were again recombined. In relation to the two potential outcomes of male-induced ovulation, the timing of L-Post supplementation would have been either, a) from the second ovulation of the male-induced ovulatory cycle for the ewes having a normal cycle only, or b) from the third male-induced ovulatory cycle for the ewes experiencing the short cycle. The timing of this feeding regime was in anticipation when the supplement would have an effect on the ovulation.

Oestrus distribution was determined by recording marked ewes in the morning and late afternoon every day, from Day 16 to Day 26 after the ‘male effect’. On Day 32 of the experiment, laparoscopy was again used to determine ovulation rate. On Day 55, ultrasound was used to detect pregnant ewes and to count the number of foetuses per pregnant ewe.

Statistical Analysis
We have based the analysis of treatment effects only on data collected from ewes that had most probably responded to the ‘male effect’, as evidenced by the occurrence of oestrus between Days 19 and 29. The number of corpora lutea per ewe ovulating (ovulation rate) was analysed using Chi-squared tests ($\chi^2$) in a 2 x 2 contingency table. Oestrus distributions for short versus normal cycles were determined using histograms with fits and groups in the Minitab 15 programme.

4.5 Results

Live weight
The live weight data are presented in Figure 10. The weights did not differ among the three groups at the beginning or at the end of the experimental period ($P > 0.05$). There were similar trends of slight weight gains of 0.91 g, 0.61 g and 0.76 g for Control, L-Pre and L-Post, respectively. The L-Post group tended to be heavier throughout the experiment than Control and L-Pre groups.
Figure 10: The mean live weights of the ewes.

Weighing was done prior to and at the end of the experimental period, in the L-Pre (short broken lines), L-Post (solid line) and Control (long broken lines) groups. There were no significant differences (P > 0.05).

Ovulation and oestrus

The data for oestrus, ovulation and ovulation rate, and the proportions of ewes with short and normal cycles after the ram-induced ovulation, are shown in Table 1. In all three groups, almost all ewes ovulated so there was no effect of treatment on the proportion of ewes responding to the male effect. The percentage of ewes ovulating was closely followed by the percentage of ewes showing oestrus and more ewes ovulated than displayed oestrus in all three groups.

Ovulation Rate

The ovulation rate tended to be higher in the L-Pre group than in the Control (P > 0.05) at the first ovulation and was significantly higher in the L-Post group than in the Control (P < 0.05) at laparoscopy on Day 32, at the second ovulation for ewes with a normal cycle and at third ovulation for ewes with short and normal cycle.
Oestrus Distribution

Figure 11 shows the distribution of oestrus for each group with the bars representing the frequency of ewes marked on each day of observation.

Table 1: Reproductive variables:

Reproductive variables in ewes induced to ovulate using the “male effect” at the onset of the breeding season. Data were collected at laparoscopy on Day 15 for L-Pre and Day 30 for L-Post. Percentages are based on n; different superscripts indicate significance (P < 0.05).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>L-Pre</th>
<th>L-Post</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>32</td>
<td>23</td>
<td>30</td>
</tr>
<tr>
<td>Ewes ovulating (%)</td>
<td>94</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Ovulation Rate</td>
<td>1.37&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.48&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.66&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Short cycle (%)</td>
<td>34&lt;sup&gt;a&lt;/sup&gt;</td>
<td>39&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Normal cycle (%)</td>
<td>41&lt;sup&gt;a&lt;/sup&gt;</td>
<td>48&lt;sup&gt;a&lt;/sup&gt;</td>
<td>67&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Oestrus (%)</td>
<td>75</td>
<td>87</td>
<td>90</td>
</tr>
</tbody>
</table>

Figure 11: Oestrus distribution curves in ewes.

Distribution curves illustrating the number of ewes with a normal cycle (clear bars) and a short plus a normal cycle (shaded bars) and days of oestrus occurrence in a) Control, b) L-Pre and c) L-Post groups.
Following male-induced ovulation, the pattern of oestrus was similar for L-Pre and Control (P < 0.05). In all groups, more ewes were marked between Days 19 and 20 than between Days 21 and 29, indicating a concentration of normal cycles.

**Oestrous Cycle**

Figure 4.3 shows the percentage of ewes in each group that expressed normal and short cycles. The proportion of ewes with the normal cycle was higher than the number of ewes with the short cycles in the L-Post group (P < 0.05). This difference was not seen in the C and L-Pre (P < 0.05). Consequently, 67% of the ewes experiencing a normal cycle in L-Post group were significantly more than the ewes with a normal cycle in C and L-Pre groups (P < 0.05).

**Pregnancy Rate**

The pregnancy data are shown in Table 2. Pregnancy rates did not differ among the groups. There were significantly more twin (and thus less single) foetuses in L-Post than in the other two groups.

**Table 2: Singles, twins and overall pregnancy rates:**

Data from ultrasound pregnancy diagnosis on Day 50 after male-induced ovulation. Different superscripts indicate significant differences (P < 0.05).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>L-Pre</th>
<th>L-Post</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>32</td>
<td>23</td>
<td>30</td>
</tr>
<tr>
<td>Singles (%)</td>
<td>74</td>
<td>63</td>
<td>45</td>
</tr>
<tr>
<td>Twins (%)</td>
<td>26(^a)</td>
<td>37(^a)</td>
<td>55(^b)</td>
</tr>
<tr>
<td>Pregnant (%)</td>
<td>72</td>
<td>83</td>
<td>73</td>
</tr>
</tbody>
</table>
4.6 Discussion

The use of nutrition to increase the number of ewes ovulating was effectively not tested because ovulation was observed in all ewes in the L-Pre and L-Post groups and 94% of Control ewes, suggesting a strong response to the male effect and perhaps a major contribution from spontaneous ovulations. The second of these possibilities is supported by the fact that 10% of ewes in the flock were observed to be cycling when the experimental animals were selected just before the experiment. The time of the season probably played a major role – the study was begun around the expected onset of the normal, photoperiod-induced breeding season when we would expect there to be an increasing number of ewes becoming spontaneously cyclic. These observations indicate that the Merinos on Allandale Farm have a shallower anoestrus than expected, and are thus highly sensitive to the male effect. Other observations of this flock support this contention (P Hawken, T Jorre de St Jorre 2008, pers. Comm., 2008), suggesting that a reduction in seasonality has developed over recent years in the University flock. In previous studies with Merino ewes, generally, less than 100% ovulated in response to the ram effect in the absence of lupin supplementation (Fletcher & Geytenbeek, 1970; Pearce & Oldham, 1988; Wheeler & Land, 1977). In breeds that are more seasonal than Merinos, the response tends to be lower (review: Martin et al 1986). In these genotypes, it would be worthwhile testing again the hypothesis that nutritional supplements can improve the ability of the ram effect to induce ovulation.

On the other hand, the nutritional supplementations appear to have stimulated follicle growth, as evidenced by increases in ovulation rate. The effect of supplementations on ovulation rate was thought to be more apparent when ewes had previously been kept on poor quality pasture (Gherardi & Lindsay, 1982). This certainly applies to the current experiment because the ewes were run on dry summer pasture and, although they were given hay ad libitum, it was poor quality. Therefore, even with small numbers of experimental animals, we observed a response to the second period of supplementation as natural seasonal grazing conditions were deteriorating.

The hypothesis that a nutritional supplement could be used to prevent short cycles in ewes responding to the ‘male effect’ was rejected because 39% of L-Pre and 41% of Control ewes experienced short cycles. However, we need to be cautious in this interpretation because the power of our experimental design was limited by feeding the ewes in groups rather than in individual pens. This could pose a problem because there can be large differences among
ewes in the intake of lupins. In addition, group feeding behaviour, leading interactions among
the animals, could increase this variation. At the extremes of this variation, we would expect
major differences in the way the follicles respond and grow to nutritional stimulus (Murray &
Rowe, 1984). On the other hand, we did detect significant responses to the second
supplementation for ovulation rate, so folliculogenesis was affected by the treatment. At this
stage, we therefore conclude that a nutritional supplementation is not likely to reduce the
likelihood of induction of short-life-span corpora lutea.

A smaller proportion (23%) of ewes in the L-Post group had a short cycle, compared to
around 50% in the other groups. This is probably a chance observation because the
supplement for L-Post ewes was not begun until 11 days after the ‘male effect’, by which
time the distribution among short and normal cycles should have been determined. It is
feasible that the 12-day nutritional supplementation may have facilitated an extension of the
follicular phase of the oestrous cycle. As an a posteriori observation, this needs to be tested
again as an a priori hypothesis.

The data for oestrus distribution showed that, in all three groups, the ewes that experienced a
normal cycle following male-induced ovulation came into oestrus in a brief period, mostly
over Days 19 and 20. In contrast, there was a considerable variability among the ewes
experiencing a short cycle, with oestrus exhibited over a 9-day period (Days 21-29). These
ewes could not have been cyclic before the ‘ram effect’ as they were not marked in the first
14 days after male introduction. In all groups, small proportions of ewes ovulated but failed
to show oestrus, or showed oestrus but apparently did not ovulate, or conceived without being
detected in oestrus. Such variations in the response to the ‘male effect’ have been previously
reported for Corriedale ewes (Ungerfeld et al., 2002) although it is difficult to rule out
technical problems such as errors in detection of oestrus. Interestingly, 24-hourly repeated
short-term exposure of ewes to rams in temperate regions led to improved synchrony of the
oestrous cycle, thus a compact and earlier lambing. This suggests that continuous presence of
rams is not essential, especially during the transition of the anoestrous into the breeding
season (Hawken, Evans & Beard, 2007).

Nutritional supplementation for 12 days during the oestrous cycle improved reproductive
performance, as evidenced by the increased percentage of twin foetuses observed at scanning.
This supports the conclusions drawn for ovulation rate. Therefore, an additional period of
feeding to the 6-day nutritional supplementation before the ‘male effect’ is recommended. However, supplementation for only 6 days rather than 12 days, during the oestrous cycle may prove sufficient to sustain the nutritional influence on ovulation rate and this needs to be tested because it would reduce costs.

In conclusion, we have provided evidence that a short-term nutritional supplement, perhaps through its effects on ovarian follicular activity can improve the ovulation rate of ewes responding to the male effect, but not the incidence of short cycles. The effect on ovulation rate was not significant with supplementation before male introduction, but this needs to be re-tested under better experimental conditions. The outcome was favourable when the supplement was supplied during the male-induced oestrous cycle.
CHAPTER 5

5.1 Introduction

Seasonal breeding patterns are caused primarily by the effects of photoperiod on the pulsatility of GnRH and thus gonadotrophin secretion (Gallegos-Sánchez et al., 1998; Martin, 1984). When the day length is decreasing during the autumn, the inhibition of GnRH secretion by oestradiol is weak allowing the ewe to experience oestrous cycles and when day length is increasing in spring and summer, oestradiol gains a strong ability to inhibit the hypothalamo-hypophyseal system and thus inhibit GnRH secretion, preventing ovulation and oestrus (Gallegos-Sánchez et al., 1998; Bittman et al., 1985; Martin et al., 1983).

However, the ‘commercial breeding season’ in Western Australia, when farmers place rams with ewes can commence in late spring and continue until late autumn (Knight et al., 1975), so it is not well coordinated with the photoperiod-driven natural breeding season. This is feasible because most of the ewes are Merinos, one of the least seasonal of all sheep breeds – a proportion of the ewes in a flock ovulates continually throughout the anoestrous season (Pearce and Oldham, 1988a; Wheeler and Land, 1977; Fletcher and Geytenbeek, 1970). This phenomenon of ‘shallow anoestrus’ allows us to use the ‘ram effect’ (‘teasing’) to induce fertile mating and thus advance the mating season into late spring and early summer. The seasonality of the Merino genotype still causes problems because of the depth of anoestrus can vary within a ewe as well as a flock of ewes, from season to season (Goodman, 1996). Also, anovulatory ewes that have been successfully ‘teased’ will return to anoestrous within a cycle or two if they fail to conceive – a phenomenon attributed to the lengthening daylight hours during late spring and early summer (Oldham and Cognie, 1980).

Using the ‘ram effect’ to manage reproduction in ewes continues to present two problems: first, the stimulus by the ram often seems insufficient to induce ovulation, so the response of ewes varies among and within breeds; second, at the level of the ovary, the follicles destined to ovulate might not be fully developed and, after ovulation, produce corpora lutea that regress within 6 days, the ‘short cycle’ phenomenon (Martin et al., 1986).

In the Merino genotype, nutrition is as important as photoperiod for reproduction (Martin et al., 2002). For most of the summer-autumn commercial breeding season in Western Australia, feed supply is poor in quality because pasture growth is driven by winter rains.
For this reason, nutritional supplementation (or ‘flushing’) is used at the beginning of the mating period to boost the energy balance of the ewe and improve reproductive performance (Scaramuzzi and Martin, 2008). Nutritional supplementation initiates metabolic processes that increase circulating concentrations of leptin and insulin, allowing supply of more glucose to the follicles (Scaramuzzi et al., 2006; Leury et al., 1990; Teleni et al., 1989a; Teleni et al., 1989b). Glucose is the major source of energy for the ovary and the presence of glucose transporters increases the availability of glucose and other metabolic hormones for follicle growth (Muñoz-Gutiérrez et al., 2005; Muñoz-Gutiérrez et al., 2004; Williams et al., 2001). Insulin stimulates the proliferation of granulosa and theca cells while leptin acts on both the hypothalamo-pituitary axis and the ovary (Muñoz-Gutiérrez et al., 2005). In females that are already ovulating, short-term nutritional supplementation does not seem to affect the concentrations of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Downing et al., 1995b); however, the nutritional signals act directly via the ovarian mechanism (Scaramuzzi and Martin, 2008; Viñoles et al., 2005) to make follicles more responsive to FSH, allowing extra follicle development.

It is therefore possible that, through actions at ovarian level, nutritional supplementation might improve the responses to the ‘ram effect’. We tested two hypotheses:

i) That nutritional supplementation will increase the proportion of a flock of ewes that ovulate in response to teasing in the non-breeding season;
ii) That nutritional supplementation will reduce the proportion of ewes experiencing the short cycle following ovulation induced by the ‘ram effect’.

5.2 Methods and Materials
The experiment was conducted at Allandale Farm from early December until mid-January using 112 Merino ewes, all which had lambed at least lambed once, but not in the preceding lambing season. Prior to the commencement of the experiment (Day -3), laparoscopy was used to detect cyclic ewes (those with corpora lutea) and acyclic ewes (no corpus luteum present at the time of laparoscopy). Five minutes before laparoscopy was started, 2 mg xylazine was injected intramuscularly for partial sedation. At the end of the laparoscopy, 5 mL of benacillin was injected intramuscularly and the wound was sprayed with vetericyn, all supplied by the Veterinarian who performed the laparoscopy for this experiment.
5.3 Experimental design

Cyclic ewes were immediately removed from the experiment. For the duration of the study, experimental ewes were weighed and condition scored once weekly. The experimental protocol is shown in Figure 12.

**Figure 12: Schematic experimental design.**

**The Control group** (n = 29) was fed hay *ad libitum* on dry pastures. The experimental treatments involved nutritional supplements on top of the hay ration.

**The treatment group (L-Pre)** (n = 29) was fed a supplement before the male effect – lupin grain (*Lupinus angustifolius*) at 500g/ewe/day from Day -5 to Day 0.

The Control and L-Pre groups were run separately from Day -5 to Day 0 (the ‘ram effect period’). On Day 6 of nutritional treatment, Day 0 of the ‘ram effect’, both groups were placed together in the same paddock and 6 vasectomised rams were introduced (i.e., 1 ram per 10 ewes). Harnesses with crayons were fitted to the rams for oestrus detection. Ewes marked within the first 14 days were recorded, but only ewes with distinct crayon marks on their rumps were part of the data collection and analysis. Crayon colours were changed on Day 14 and every 7 days thereafter until Day 28. Oestrus observations were also performed from Days 16 to 26, the expected period of oestrus display by the ewes.
Ovulation rate (ovulations per ewe ovulating) was determined at laparoscopy by counting the number of corpora lutea on both ovaries of each ewe on Day 10 for first ovulation and on Day 30 for second ovulation in both groups. Ewes with normal cycle and short cycle were determined with countback of days from first visible oestrus display.

**Statistical analysis**

We have based the analysis of treatment effects only on data collected from ewes that had most probably responded to the ‘male effect’, as evidenced by the occurrence of oestrus between Days 16 and 26. The number of corpora lutea per ewe ovulating (ovulation rate) was analysed using Chi-squared tests ($\chi^2$) in a 2 x 2 contingency table. Oestrus distributions for short versus normal cycles were determined using histograms with fits and groups in the Minitab 14 programme.

### 5.4 Results

*Live weight*

Figure 13 shows the live weight data of both groups. The weights were similar for the two groups throughout the experimental period ($P > 0.05$).

![Figure 13: Average live weight of ewes.](image)

*Average (± 0.34) live weight of the Control (unbroken line) and L-Pre (broken line) groups during the experimental period of 42 days ($P > 0.05$).*
**Ovulation**

Table 5.1 shows the percentage of ewes ovulating. In the Control group, the same proportion 16/29 (55%) of ewes exhibited oestrus as ovulated. In contrast, only 10/29 (34%) ewes ovulated in the L-Pre group. This discrepancy was largely due to 21% of ewes that failed to show oestrus and they were subsequently excluded from the data.

**Ovulation rate**

The data for ovulation rate and oestrus for Days 10 and 30 after the ‘male effect’ are also shown in Table 3. Ovulation rate was significantly higher in L-Pre ewes than in Controls on Day 10 (P < 0.05), but declined by the second ovulation on Day 30 to be similar to the Control value.

**Table 3: Reproductive variables:**

Reproductive variables in Merino ewes induced to ovulate using the ‘ram effect’ during the anoestrous season. Ovulation data were collected at laparoscopy on Day 10 and Day 30. Percentages are based on n; different superscripts indicate significant differences (P < 0.05).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>L-Pre</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>29</td>
<td>29</td>
</tr>
<tr>
<td>Ewes ovulating</td>
<td>16 (55%)</td>
<td>10 (34%)</td>
</tr>
<tr>
<td>Ovulation Rate¹ (Day 10)</td>
<td>1.31ᵃ</td>
<td>1.68ᵇ</td>
</tr>
<tr>
<td>Ewes showing oestrus</td>
<td>16 (55%)</td>
<td>15 (51%)</td>
</tr>
<tr>
<td>Ewes showing a short cycle</td>
<td>3 (10%)ᵃ</td>
<td>5 (17%)ᵃ</td>
</tr>
<tr>
<td>Ewes showing a normal cycle</td>
<td>13 (45%)ᵃ</td>
<td>10 (34%)ᵇ</td>
</tr>
<tr>
<td>Ovulation Rate² (Day 30)</td>
<td>1.40ᵃ</td>
<td>1.35ᵃ</td>
</tr>
</tbody>
</table>

**Ovulatory cycles**

Table 5.1 shows the percentage of ewes with normal and short cycles in the two groups. Less than 50% of the ewes experienced a normal cycle in both groups, but was higher in Control than in L-Pre (P < 0.05).

**Oestrus distribution**

Figure 14 shows the number of ewes marked on each day of observation. The ram-induced ovulation resulted in similar patterns of oestrus for L-Pre and Control (P > 0.05). In both groups, more ewes were marked between Days 16 and 18 (i.e., a normal cycle length
following the ram effect) than between Days 23 and 24 (ewes experiencing a short cycle after the ram effect followed by a normal cycle). More ewes 16/29 (55%) showed oestrus in the Control group than in the L-Pre group 15/29 (51%), but the difference was not significant (P > 0.05).

![Graph showing oestrus distribution](image)

**Figure 14: Oestrus distribution of anovular ewes induced at the ‘male effect’**.

Distribution of ram-induced oestrus in animals experiencing a normal cycle (clear bars) or short cycle (shaded bars). a) Control: an even distribution of oestrus over three days (ewes experiencing a normal cycle); ewes which experienced the short cycle showed oestrus on Days 23 and 24. b) L-Pre: an apparently skewed curve in favour of oestrus being displayed on Day 16 after male exposure; all ewes that experienced the short cycle showed oestrus on Day 23.
5.5 Discussion

The nutritional supplementation did not affect the percentage of ewes ovulating so the first hypothesis was rejected because the treatment group showed poor ovulation while 38% of the ewes did not ovulate at all. There was a low response to the ‘ram effect’ in the Control group, effectively providing the perfect scenario for testing the hypothesis because, if all the Control ewes had ovulated, there would be no room for improvement with the lupin supplement in the L-Pre group. This observation confirms other studies with ewes in temperate regions showing that nutritional supplementation did not respond to the ‘ram effect’, although it seems likely that increasing the body condition score (BSC) could improve the outcome (Scaramuzzi, 2013, Johson, 2011, Vinoles, 2002). Consequently, a longer period of nutritional supplementation leading to a high BSC could be a valuable management option (Johnson, 2011).

We used laparoscopy to detect ewes that ovulated by direct visualisation of corpora lutea. The alternative method of ultrasound scanning might present a better understanding of the ovarian follicle development, even if it provides less predictive value for follicles < 4 mm in diameter (Viñoles et al., 2004). On the other hand, our use of laparoscopy strengthens our observation of ewes that did not ovulate (Johnson, 2011; Pearce and Oldham, 1988a; Oldham and Lindsay, 1980; Wheeler and Land, 1977; Fletcher and Geytenbeek, 1970).

We expected nutritional supplementation to improve the responsiveness of ovarian follicles at the ram stimulus. Our understanding of how small follicles in ewes respond to nutritional supplementation is still poor and the current information is not consistent. For example, follicle sizes between 2 and 6 mm in diameter have been reported to escape atresia following nutritional supplementation, mainly due to changing levels of glucose and metabolic hormones in cyclic ewes (Ying et al., 2011; Viñoles et al., 2005; Muñoz-Gutiérrez et al., 2002). In contrast, Rhind and McNeilly (1998) found that nutritional supplementation had no effect on follicles > 2.5 mm in diameter, but increased the number of follicles 1.0–2.5 mm in diameter in both follicular and luteal phases.

A small proportion of ewes responding to the ram effect might be due to poor exposure to the ram stimulus – full physical contact appears to improve the efficacy in ram-induced ovulation compared to exposure to odour or visual cues (Abecia et al., 2002; Pearce and Oldham, 1988b). It is feasible that tactile cues play a major role and the social interaction among ewes, as well as between ewes and rams could be important for enhancing the response of ewes to...
the ‘ram effect’ (Zarco et al., 1995) and in goats (Alvarez et al., 2007). On the other hand, other reports showed that direct contact with rams was not necessary and have suggested that the smell and sound are sufficient to induce ovulation (Watson and Radford, 1960). Previous studies are often very difficult to interpret with confidence because, in the descriptions of methodology, little is often said about the novelty of the rams used to stimulate the ewes, a factor we now know to be critical for the ram effect (Jorre De St Jorre, Hawken, & Martin, 2014).

In the present experiment, short-term nutritional supplementation increased ovulation rate at the first ovulation, as observed on Day 10. Ovulation rate subsequently returned to control values after the withdrawal of the nutritional supplementation. Together with the results of the previous experiment, this observation shows the importance of nutritional supplementation during the immediate preovulatory period. Glucose and metabolic hormones at their peak concentrations seem to be major factors in the determination of ovulation rate (Viñoles et al., 2005; Downing and Scaramuzzi, 1997; Downing et al., 1995a). Nutritional supplementation lengthens the lifespan of the last non-ovulatory follicle and apparently prevents the initiation of another follicular wave (Viñoles et al., 2005). In addition, more follicles of larger size develop when ewes are fed a lupin supplement during the luteal phase of the oestrus cycle (Somchit et al., 2007; Viñoles et al., 2005). The feeding regime we used in Chapter 4 supports these findings and the 33% decrease in multiple ovulations in the L-Pre group at the second ovulation suggests that the withdrawal of the supplement led to fewer follicles growing to the preovulatory stage. Therefore, nutritional supplementation needs to continue until after the occurrence of the ovulation that is important for the reproductive efficiency of the flock.

The increase in ovulation rate at the first ovulation is coherent with an expected stimulation of follicle development by the lupin supplement, but this outcome was not associated with a reduction in the proportion of ewes experiencing short cycles. We conclude that luteal failure is independent of the level of nutrition. The occurrence of the short cycle is likely due to issues at molecular level during luteinization between Day 3 and Day 7 after the ‘ram effect’ (Brown et al., 2014). The uterus also seems to play a role in luteal failure because hysterectomized ewes showed no evidence of short luteal phase after ram exposure (Lassoued et al., 1997; Chemineau et al., 1993). This problem is critical because it is preventing farmers from maximising the value of ram-induced ovulation in flock management (Martin, 2014).
In conclusion, a short-term nutritional supplement such as lupin grain can be used to improve ovulation rate following the ‘ram effect, but has no benefit for number of ewes ovulating or the occurrence of short cycles. It would be worthwhile to test longer periods of supplementation because both the proportion of the flock that ovulates and the proportion of ovulating ewes that go on to show normal cycles are major limitations in the use of the ram effect for ‘clean, green and ethical’ flock management.
CHAPTER 6

6.1 General Discussion

We use the ‘male effect’ as a management tool to induce ovulation in ewes without resorting to hormone treatments. The general hypothesis of this thesis was that the response of female Merino sheep to the ‘male effect’ and their subsequent reproductive performance would be improved by a short-term nutritional supplement. The experimental findings, in combination with the literature, offer broad support for the hypothesis. However, it is clear that, to maximise ewe fertility, short-term nutritional supplementation should be fed twice, once before the introduction of rams and again before the second ovulation, during the ram-induced oestrous cycle.

We expected short-term nutritional supplementation to increase the number of ewes that ovulate in response to the ‘male effect’, but the results did not support this hypothesis. The experiment might have been compromised because it was done at the start of the mating season and many ewes were beginning to ovulate spontaneously. However, even in the ewes that were still anovulatory, nutritional supplementation did not improve the outcome. Our knowledge on how nutritional supplementation affects ovarian dynamics is not complete, especially for anovulatory sheep, but it is still likely that nutritional stimulus increased follicular activity (Viñoles et al., 2005; Somchit et al., 2007). The most logical interpretation is that the outcome of the male effect, in terms of whether the female ovulates or not, is not determined at the level of the ovary, but most probably at the level of the brain-pituitary systems that control gonadotrophin secretion.

After ovulation is induced by the male effect, the next question is whether the female has a single or multiple ovulations. In this case, the importance of nutrition is very well documented for ovulatory Merino sheep. We can now extend this to ovulation induced in anovulatory ewes because, in both experiments, ovulation rate at the ram-induced ovulation was increased by nutritional supplementation. We can therefore conclude that an increase in energy supply enhances ovarian follicular development so more follicles can ovulate in response to the prevovulatory surge, whether the surge is induced by the male effect or during the normal processes of spontaneous ovulation (Scaramuzzi et al., 1993, 2006, 2011).
The overall outcome was an improvement of reproductive performance in ewes by nutritional supplementation at the ram-induced ovulation. Moreover, a nutritional stimulus after the ram-induced ovulation, in the period leading the the second ovulation, also significantly improves ovulation rate, as demonstrated in Chapter 4. These findings suggest that, rather than aiming for precise timing, farmers would be better off ensuring that the duration of the feeding is sufficient to cover the second cycle.

Farmers can be confident in this plan because when nutritional supplementation is maintained in the period between October and March, ovulation rate is also maintained and not affected by season, whereas the response to single, short-term supplements diminishes in December before recovering in March (Gherardi & Lindsay, 1982). As shown in Chapter 4, a second supplement near the time of luteolysis after the first ram-induced ovulation seems to avoid the decrease of ovulation rate experienced at the second ovulation in Chapter 5 where only a single nutritional supplementation was given before the first ovulation induced by the male effect.

One other limitation of the present experiments was the need to run the ewes in treatment groups, with the ewes trail-fed in a paddock. This design is difficult to avoid with field studies and makes it difficult to ensure that each ewe has received an appropriate amount of the lupin grain, with perhaps the most dominant ewes consuming more than the lower members in the hierarchy. On the farm, farmers dealing with large numbers of production animals cannot feed them individually so, although compromised, the experiment reflected reality.

The hypothesis that short-term nutritional supplementation would prevent or reduce the proportion of ewes experiencing a short cycle after the ram-induced ovulation was rejected. As stated above, we have little knowledge on how nutritional supplementation affects ovarian dynamics in anovulatory sheep, but we do know that, in the present experiments, these supplements allow an increase in ovulation rate so there are clear benefits for follicular function. We therefore feel confident that the failure of the ram-induced corpus luteum is not caused by the ovulation of follicles that are poorly developed because of nutrition-dependent factors. In other words, the same nutritional signal that improves ovulation rate at the ram-induced ovulation does not affect the destiny of the the ram-induced corpora lutea. Other factors must therefore be the dominant cause of short cycles, such as premature luteolytic
signals from uterus (Southee et al., 1988; Chemineau et al., 2006) or poor activation of genes that are critical for the development and function of the corpus luteum (Brown et al., 2014). We seem to be gaining an understanding of the cause of short cycles following ram-induced ovulation, but need much more progress towards unpacking the phenomenon if we are to provide a solution relevant to farmers.

Future areas for research

We need more research to help us overcome the shortcomings of the ‘ram effect’, such as the failure of some ewes to respond by ovulating and the short cycle phenomenon. The short cycle is particularly important for practical applications on farm because it ruins an otherwise excellent level of synchrony of ovulation and lambing in the ewe flock. The work of Brown et al., 2014 clearly shows the value of exploring the molecular processes that underpin the phenomenon. A solution would allow us to gain far more value from the ram effect in the implementation of ‘clean, green and ethical’ management of sheep production.

Namibian perspective

The repeatability of the present experiments under Namibian conditions remains to be tested. However, it seems likely that farmers in Namibia could use the male effect to adjust the mating season to ensure that the lambing coincides with the best grazing to support lactation. Indeed, Namibian farmers need to incorporate a ‘smart feeding regime’ that takes into consideration the long dry spells the country experiences each year. There have been some attempts to do this, with breeding programmes commencing in July so that lambing and lactation coincide with the rainy period from early December to late March, but the entire reproductive process has not been followed to provide quantitative information.

To date, most livestock research in Namibia has focussed on the breeding of hardy, well-adapted animals. We can build on this as well as our general understanding of the value of nutrititional supplementation for improving reproductive performance, but we need to design locally-relevant projects that will allow the adoption of the ‘clean, green and ethical’ concept. We also need to investigate the extent of reproductive wastage under arid conditions and identify sources of wastage that can be mitigated. Finally, we need a full understanding of reproductive performance across the broad spectrum of livestock production systems that exist in Namibia.
References


the plasma concentration of LH and FSH and the ovulation rate in Merino ewes. 

gonadotrophins, insulin, and insulin-like growth factor 1 by merion rams supplemented 
with different legume seeds. *Australian Journal of Agricultural Research* 47:843-852.


sheep during anoestrus involve defects in progesterone biosynthesis and luteal 

Cahill, L. P., Mariana, J. C., Mauléon, P. (1979). Total follicular populations in ewes of high 

Cahill, L. P., Mauléon, P. (1980). Influences of season, cycle and breed on follicular growth 

Caldani, M., Batailler, M., Thiéry, J.-C., Dubois, M. P. (1988). LHRH-immunoreactive 


Carson, R. S., Findlay, J. K., Clarke, I. J., Burger, H. G. (1981). Estradiol, testosterone, and 
androstenedione in ovine follicular fluid during growth and atresia of ovarian follicles. 

the estrogen secretion in rhesus monkey *in vivo*. *Endocrinology*, 98:590-597.(Abstract)

Chemineau, P., Pelletier, J., Guerin, Y., Colas, G., Ravault, J. P., Touré, G., Almeida, G., 
Thimonier, J., and Ortavant, R. (1988). Photoperiodic and melatonin treatments for the 

phases: effect of hysterectomy and cellular composition of the corpus luteum. 

induced short oestrous and ovarian cycles in sheep and goats: a working hypothesis. 


Martin, G. B., Scaramuzzi, R. J., Lindsay, D. R. (1981). Induction of ovulation in seasonally anovular ewes by the introduction of rams: effects of progesterone and active


Somchit, A., Campbell, B. K., Khalid, M., Kendall, N. R., Scaramuzzi, R. J. (2007). The effect of short-term nutritional supplementation of ewes with lupin grain (Lupinus luteus), during the luteal phase of the estrous cycle on the number of ovarian follicles


