SHORT-TERM NUTRITION AND ITS EFFECT ON OVULATION IN THE EWE

by

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SUMMARY

The studies reported in this thesis were undertaken to examine the physiological basis of the known ability of lupin grain to increase ovulation rate in the ewe. The major findings from these studies can be summarised as follows:

To determine whether lupins act by providing a source of post-ruminal protein or additional energy to the ewe, animals were fed supplements of either lupin grain, formaldehyde treated casein, formaldehyde treated casein plus wheat starch or wheat starch . The last three supplements were fed at levels calculated to supply similar amounts of protein post-ruminally and/or digestible energy to that provided by supplementing with lupin grain. In this experiment, casein but not wheat starch produced a similar increase in ovulation rate to lupin grain, indicating that the lupin response is more likely to be related to an increase in the amount of protein digested post-ruminally than to energy availability. To explore whether the effect of casein was due simply to an improvement in the animal's protein status or to the improved availability of specific amino acids, a second experiment was undertaken to compare formaldehyde treated gelatine with casein to provide a different spectrum of amino acids for digestion post-ruminally. In this experiment, the ovulatory response to feeding gelatine was similar to that obtained with the casein, indicating that the quantity rather than the quality of protein is important in the response to lupin grain.

To allow more detailed studies of the effect of lupins, a second series of experiments was undertaken to determine precisely the period which lupins need to be fed in order to increase ovulation rate. In the first of these studies, ewes were fed lupin grain for seven days commencing on either day 3, 7 or 11 of the oestrous cycle and then induced to ovulate on the sixth day of supplementation by PG injection. Ovulation rate was increased in all groups, demonstrating that the early induction of luteolysis does not interfere in the response to lupins and that the response is initiated near luteolysis. To determine the minimum period of lupin feeding needed to increase ovulation rate, lupin grain was fed for 1, 2, 3 or 7 days commencing on day 11 of the cycle. Ovulation rate tended to be higher in all four supplemented groups indicating that the minimum period required for feeding to increase ovulation rate may be less than four days.

To examine the endocrine basis of the response to lupin grain a series of experiments were carried out to relate the increase in FSH secretion on day 13 of the oestrous cycle reported previously to the ovulatory response. In the first of these studies, it was found that the administration of 4 mg of FSH at this stage of the cycle can produce a similar ovulatory response to lupin feeding. In a second experiment, lupins appeared to increase FSH in ovariectomised ewes supporting the hypothesis that lupin grain acts directly at the hypothalamic/pituitary axis to increase FSH secretion. In this experiment, 3 of the 9 ewes showed marked increases in FSH secretion within 24 hours of commencing feeding suggesting also that there may be a genetic basis for the variability to this response.

The final experiment reported in this thesis examined the dynamics of the final stages of follicular growth in lupin-fed ewes. In this experiment, the

number of follicles recruited together with the number of these that were selected to ovulate was examined using serial laparoscopy. No difference was evident between control and lupin-fed ewes in either the number of follicles > 2 mm in diameter at the start of luteolysis or in the number of these that were recruited to continue growing. However, at the time the selection of the preovulatory follicle is thought to be complete, the number of recruited follicles still growing was 40% higher in the lupin-fed group, indicating that increases in ovulation rate in lupin-fed ewes are a result of an increase in the number of follicles selected to ovulate.

In summary, increases in ovulation rate in lupin-fed ewes appear to be mediated by the availability of increased amounts of protein post-ruminally near the time of luteolysis. This effect of protein is most probably mediated via a mechanism involving central activity at the hypothalamic/pituitary axis which increases FSH secretion in a proportion of ewes. In turn, increases in FSH secretion appear to increase the number of follicles selected to ovulate.

DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person, except where due reference is made in the text.

I consent to this thesis being made available for photocopying and loan if accepted for the award of the degree.

M. B. Nottle

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PUBLICATIONS

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Abstracts

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Nottle, M. B., Armstong, D. T., Setchell, B. P. and Seamark, R. F. (1986a). Lupin supplementation, FSH secretion and ovulation rate in the ewe. Proc. Aust. Soc. Reprod. Biol. 18: 49

Nottle, M. B., Setchell, B. P. and Seamark R. F. (1986b). Supplementation with lupin grain can increase induced ovulation rate in the ewe. Proc. Nutr. Soc. Aust. 11:139.

Nottle, M. B., Setchell, B. P. and Seamark, R. F. (1987). Protein, amino acids and ovulation rate. Proc. Nutr. Soc. Aust. 12: 89.

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Papers

Nottle, M. B. Hynd, P. I., Seamark, R. F. and Setchell, B. P. (1988). Increases in ovulation rate in lupin-fed ewes are initiated by increases in protein digested post-ruminally. J. Reprod. Fert. (in press).

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CHAPTER 1. PREFACE.

Nutrition has long been implicated in the control of ovulation rate in the ewe. However, despite a voluminous literature on this subject, very little is known about the physiological mechanisms whereby the various effects of nutritional status are mediated within the animal. As stated by Smith (1985);

" ... while the hormonal changes or responses that govern the number of follicles that ovulate remain unresolved the mechanism of action of nutrition will also continue to be elusive".

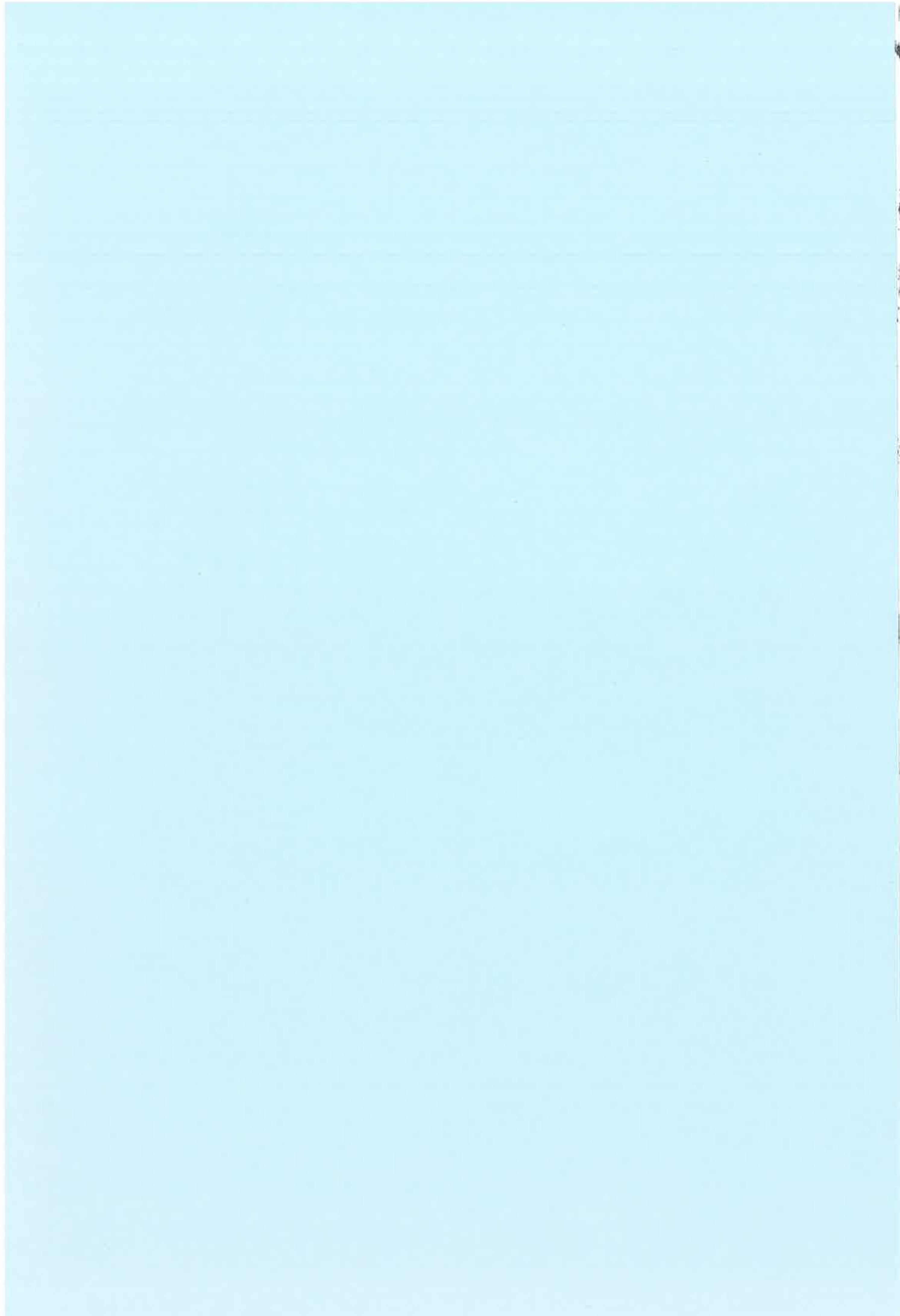
The lack of progress in the understanding of the interaction between nutritional status and ovulation rate is in part due to the problem falling between two well developed disciplines of study, namely, nutrition and reproductive biology. Of all the models used by reproductive biologists to study the control of ovulation rate in the ewe such as unilateral ovariectomy, immunisation against steroids and comparisons between prolific and non-prolific breeds, nutrition is seldom used despite its being perhaps one of the least invasive approaches and the one which is based on the fewest assumptions. The lack of progress in understanding the interaction between nutrition and ovulation rate then, is perhaps because;

" Most such studies to date have been dominated by nutritionists (who have investigated many and variable nutrients in respect to a single endpoint, O.R.) or by physiologists (who have studied crude liveweight effects on follicular dynamics). An investigation incorporating detailed

nutrition partitioning and follicular studies , simultaneously could prove more productive."

Cahill (1984)

The purpose of the present study therefore, was to re-examine the previously demonstrated ability of lupin grain to increase ovulation rate in the ewe within six days of commencing feeding (Oldham and Lindsay, 1984) using a similar approach, in an attempt to understand further the interaction between nutrition and ovulation rate.



CHAPTER 2. LITERATURE REVIEW.

2.1. Introduction

The ovary of the adult ewe contains a large pool of non-growing primordial follicles which provides the source of a much smaller number of growing follicles. Once initiated to grow, a follicle grows through a series of stages until it ultimately develops into a preovulatory follicle or as is the fate of more than 99% of all follicles undergoes atresia at some intermediate stage. Follicular growth is a continuous process throughout the life of the animal and occurs irrespective of its reproductive state. In the ewe, folliculogenesis takes approximately six months and can be divided into four phases (Turnbull *et al.*,1977, Cahill and Mauleon, 1980);

(1) initiation of growth

(2) slow growth or pre-antral phase (130 days)

(3) antrum formation and rapid growth phase (34-45 days)

(4) preovulatory growth and maturation (3-5 days)

There is considerable evidence in the literature to suggest that nutrition can influence all four phases of follicular growth. This evidence, together with the literature concerning folliculogenesis is discussed in the present review. Because of a voluminous literature for both areas this review is by no means exhaustive. Rather, work prior to 1985 when the present study

commenced is used in an attempt to provide a physiological basis for the various nutritional influences that have been described.

2.2. Initiation of follicular growth

2.2.1. Follicular growth

The number of primordial follicles in the sheep ovary reaches a maximum soon after birth (Trounson *et al.* 1974; Worthington and Kennedy, 1976). In form they consist of an oocyte arrested in meiotic prophase surrounded by a single layer of granulosa cells. Primordial follicles are morphologically heterogeneous and have been divided into two categories based on oocyte and follicular parameters (Cahill and Mauleon, 1981). The first category includes the majority of follicles which are thought to be dormant because of the lack of a relationship between oocyte size and follicle diameter.[1] At a follicular diameter of approximately 0.03 mm the number of granulosa cells and the diameter of the ooycte begin to increase and it is from this so-called transitory category that follicles are believed to be recruited into the slow growth or pre-antral phase (Cahill and Mauleon, 1981).

Follicular growth commences prenatally around day 70 in sheep with new follicles commencing growth daily (Turnbull *et al.* 1977; Cahill and Mauleon, 1980). After the first weeks of post-natal life the number of growing follicles in the ovaries of lambs decreases to a level which remains

[1] (Cahill and Mauleon, 1981)

relatively constant in the mature animal (Kennedy *et al.*, 1974). This decrease in the number of growing follicles is coincident with both the first follicles forming an antrum and a decrease in FSH secretion (De Reviers and Mauleon, 1972). Cahill and Mauleon (1981) have interpreted this to mean that factors present in the follicular fluid act locally to influence the number of follicles initiating growth. In rodents, initiation of growth appears to occur independently of gonadotrophins as this process is unaffected by blocking the action of gonadotrophins by injecting antiserum (Eshkol *et al.*, 1970), by hypophysectomoy (Smith, 1930), or if exogenous gonadotrophins are injected (Peters *et al.*, 1973). In the ewe however, hypopyhsectomy results in a marked reduction in the number of follicles in all size classes (Dufour *et al.,* 1979) and is thought to mean that gonadotrophins are important in facilitating the recruitment of follicles into the pre-antral or slow growth phase (Cahill and Mauleon, 1981)

Entry may also be influenced by the size of the pool of primordial follicles (Jones and Krohn, 1961; Pedersen, 1969; Krarup *et al.*1969). Negative correlations between the total oocyte population and ovulation rate have been demonstrated in neonates in studies comparing prolific and non-prolific breeds of sheep (Land, 1970; Trounson *et al.,* 1974; Cahill *et al.* 1979; Cahill *et al.*, 1982) However in the adult ewe this relationship may be reversed as some prolific breeds such as the Romanov (Cahill *et al.*, 1979) and the Booroola (Cahill *et al* .,1982) have fewer primordial follicles but more growing follicles than their non-prolific counterparts.

More follicles also appear to enter the growth phase during the anoestrous season however, the reason(s) for this phenomenon is unknown (Cahill and Mauleon, 1980).

2.2.2. Nutrition and the initiation of follicular growth.

It is uncertain whether nutrition *in utero* or soon after birth can influence life-time ovulation rate. The few reports that have examined this question (see review by Gunn, 1982) suggest that there are no long-term effects of nutrition *in utero* or early in post-natal life on ovulation rate where compensatory growth has followed the removal of a nutritional constraint. However, where the restriction is particularly severe and/ or of long duration compensatory growth can not overcome the initial handicap and ovulation rate is affected (Gunn, 1977; Allden, 1979). Restricting the nutrition of ewes for the first 12 months of post-natal life has been shown to decrease subsequent lambing performance over five years by an average of 30% (Gunn, 1977). This has been confirmed in a subsequent study by Allden (1979) who reported that restricting nutrition in the first 14 months of life also decreased the numbers of lambs born in subsequent years by 14%.

Of particular relevance to the present discussion is the finding by Allden (1979) that ewes given access to unrestricted nutrition for the first eight weeks of life prior to a twelve month period of restricted nutrition have a higher reproductive potential compared with those whose nutrition is restricted from birth. A similar result has been reported by Reardon and Lambourne (1966), suggesting that there may be a critical period for follicular development in the first few weeks of the animal's life which is particularly sensitive to nutrition and which influences its life-time ovulation rate. This is consistent with the finding that the number of primordial

follicles is set soon after birth (Trounson *et al.,*1974; Worthington and Kennedy, 1976).

2.3. Slow growth or preantral phase

2.3.1. Follicular growth.

After leaving the transitory pool, a follicle enters the slow growth or pre-antral phase and grows by an increase in the size of the ooycte and multiplication of the granulosa cells. In the ovary of the adult ewe there are approximately twice as many preantral follicles as there are antral follicles. The time taken for a follicle to pass through this phase has been estimated to be 130 days and as such preantral follicles are thought to act as a "buffer" between the dormant primordial population and growing antral follicles (Cahill and Mauleon, 1980).

A number of studies in laboratory rodents have demonstrated that growth during this phase is under the control of gonadotrophins, in particular, FSH. Blocking the action of gonadotrophins for example, by injection of antiserum has been shown to result in abnormal granulosa and thecal cell development in neonatal rats (Eshkol *et al.* 1970). In these animals FSH alone stimulated follicular growth (Eshkol and Lunenfeld 1972). These findings are supported by autoradiographic studies in the rat ovary which have shown that receptors for FSH appear very early in development

(Zelzenik *et al.,* 1974; Richards and Midgley, 1976). The marked reduction in the number of preantral follicles that occurs following hypophysectomy in the ewe (Dufour *et al.,* 1979) also suggests that gonadotrophins are important for growth at this stage.

The ability to synthesise oestradiol appears to be particularly important for further growth at this stage. Preantral follicles have been shown to have a limited capacity to produce oestradiol, despite the presence of an active aromatase system (Erickson and Hseuh, 1978; Richards,1980). Estrogens however, have been shown to stimulate granulosa cell proliferation in hypophysectomised rats as well as to increase the incorporation of FSH by these cells (Goldenberg *et al.,* 1972). Hence, Richards and Midgley (1976) have suggested that it is only when preantral follicles gain an ability to produce significant amounts of oestrogen, that they proceed to the antral phase.

Very little atresia is evident in preantral follicles (Cahill *et al.* 1979, Turnbull *et al.* 1977).

2.3.2. Nutrition and the growth of preantral follicles

An effect of nutrition on the recruitment of follicles into the preantral phase can be interpreted from the experiment of Fletcher (1974). In this experiment, severe undernutrition imposed for a period of seven months was found to decrease ovulation rate by 30% after ewes had returned to their initial liveweights five months later. Furthermore, there appeared to be

no uniformly graded effect of high, medium or low levels of previous nutrition on subsequent ovulation rate. Rather, Fletcher concluded "that there was a threshold level of previous nutrition below which ovulation rate was adversely affected and above which there was no effect." The mechanism whereby severe undernutrition affects recruitment is unknown. However, Lamming (1966) has suggested that severe undernutrition produces a "pseudohypophsectomy" which is consistent with the suggestion by Cahill and Mauleon (1981) that in the ewe, recruitment is facilitated by gonadotrophins.

2.4. Antrum formation and rapid growth phase

2.4.1. Follicular growth

In the ewe, the first signs of antrum formation are observed in follicles approximately 0.2 mm in diameter (Turnbull *et al.*,1977; Cahill and Mauleon, 1980). Soon after, the follicle enters a phase of rapid growth where the rate of division (mitotic index) of the granulosa cells increases rapidly to reach a maximum in follicles 0.7 mm in diameter and then declines slowly to be almost zero at the time of ovulation.[1] Antral volume also increases rapidly during this phase until the antrum occupies some 90% of the volume of the preovulatory follicle.[2] During this rapid phase of growth a follicle grows from approximately 0.3 mm to a diameter > 2 mm in 34 - 45 days (Turnbull *et al.,* 1977; Cahill and Mauleon, 1980). Three to

[1] (Turnbull *et al.*, 1977) [2] (Cahill and Mauleon, 1980)

four follicles enter this growth phase per day (Turnbull *et al.,* 1977) resulting in 5 - 24 antral follicles being present in the ovary on any one day of the oestrous cycle (McNatty, 1982).

FSH has long been implicated in antrum formation (Evans *et al.* 1932). In rodents, FSH has been shown to bind to specific receptors in the granulosa cells and together with oestradiol stimulate the formation of an antrum and granulosa cell division (Richards, 1979). Whether these hormones directly stimulate granulosa cell proliferation has not been elucidated. Various growth factors present in follicular fluid also stimulate granulosa cell proliferation *in vitro* (see review by Hammond, 1981) and therefore a synergistic action between these hormones and growth factors may be involved (Richards, 1980). In the ewe, changes in the pattern of gonadotrophin receptors are also associated with follicular growth. Granulosa cells of follicles < 2 mm in diameter can bind FSH but not LH (Carson *et al.*, 1979). In sheep, LH receptors are present in the theca cells of antral follicles but do not appear in the granulosa cells until follicles reach a diameter of approximately 2 mm (Carson *et al.*, 1979; Webb and England 1982a). The action of FSH and LH on these cells has been shown in rats to be mediated through the adenylate cyclase system and specific protein kinases (Richards, 1980). In rats, the initial appearance of LH receptors in the granulosa cells appears to be mediated by FSH and oestradiol (Richards and Midgley, 1976). In the rat, as the preovulatory follicles grow the number of LH receptors increases (Richards, 1979). A similar pattern in the number of LH receptors has also been observed in the preovulatory follicle(s) in the sheep during the follicular phase (Webb and England, 1982a). In the sheep, only those follicles that have LH receptors on their

granulosa cells ovulate (Carson et al., 1979; Webb and England, 1982).

As follicles grow they develop an ability to convert androgens to oestrogens (England *et al.*, 1981; Webb and England 1982a,b). Follicular androgens are produced in the thecal layer and are converted to oestrogen in the granulosa cells (Moor, 1977; Baird, 1977; Armstrong *et al.*, 1981). Small antral follicles have a limited ability to secrete oestradiol (Armstrong *et al.*, 1981; Webb and England 1982a). This is not due to a lack of androgens as evident from the high intra-follicular concentrations found *in vitro* (Webb and England, 1982a). Rather an inability to convert androgens to oestrogens is thought to be due to a deficiency in aromatase activity in the granulosa cells of these follicles (Baird, 1983). In turn aromatase activity has been shown to be regulated by FSH (Erickson and Hseuh, 1978; Moon *et al.*, 1975, Leung and Armstrong, 1980). In the rat, oestrogen treatment has been reported to enhance the ability of FSH to increase the number of LH receptors on the granulosa cells in hypophysectomised rats and has been interpreted to mean that oestradiol participates in the mechanism of induction of LH receptors on these cells (Richards, 1979). In turn, it has been proposed (Richards, 1980; Baird, 1983) that the appearance of LH receptors in the granulosa cells gives a follicle the ability to respond to the preovulatory gonadotrophin surge and ovulate. In sheep, over 90% of the oestradiol entering the ovarian vein originates from the largest non-atretic follicle (Moor *et al.*, 1975; Baird and Scaramuzzi, 1976)

At the end of this rapid phase of growth the follicle has enlarged to approximately 2 mm with maximum increases in both the thecal and granulosa layers; formed an antrum filled with fluid and begun to acquire

receptors for LH on the granulosa cells enabling it to respond to the preovulatory LH surge.

2.4.2. Nutrition and the growth of antral follicles

It has long been recognised that increasing the level of nutrition for an extended period prior to joining can improve lambing performance (as distinct from ovulation rate).[1] This practice has been termed "flushing" and appears to have evolved as a husbandry practice early in the 19th century in the British Isles;

"It had been observed that the lambing percentage of ewes on lowland country was higher than that for the same or similar ewes in the highlands and that these lowland ewes were in better condition. Further, the annual drafting of ewes from the poor nutritional environment of the highlands to the better environment of the lowlands takes place shortly before mating. This presumably led sheepfarmers to improve deliberately the nutrition of their flocks before mating - in other words to flush them in the expectation of obtaining a higher lambing percentage."

(Coop, 1966b)

The early literature on flushing has been reviewed by Moule (1962) and is largely subjective in nature having been based on surveys of farmer opinion together with an analysis of production records. These studies confirmed that flocks in good condition and those which were "flushed" did

[1] (see reviews by Moule, 1962; Coop, 1966b)

have higher lambing percentages. Experimental evidence for this effect appears first to have become available in the 1930's. McKenzie and Terrill (1937) for example, reported that flushing resulted in ewes with higher liveweights and ovulation rates compared with controls. Furthermore they concluded that "good nutrition seemed to influence reproductive phenomena only to the extent that it allows full expression of the inherited capacity", a fact which has been largely overlooked by subsequent workers.

This early work appears to have been forgotten until its rediscovery in the early 1960's by Coop (1962), who analysed some 11,000 production records of reproductive performance and mating liveweight and reported that the incidence of twin births increased on average by 6 per cent for each 4.5 kg increase in mean liveweight. In this study the incidence of barren ewes was relatively independent of liveweight above 41 kg but increased rapidly below this weight, suggesting that severe undernutrition in the medium term can also interfere directly with the functioning of the hypothalamic/pituitary axis (Lamming, 1966).

There have been numerous reports published since Coop's confirming these findings (e.g. Coop, 1966a; Killeen, 1967; Edey, 1968; Suiter and Fels, 1971; Fletcher, 1971). In a review of most of the available published data, Morley *et al.,* (1978) concluded that the relationship between ovulation rate and liveweight at mating between breeds on a flock basis is similar with there being an increase in ovulation rate of 2 percent for each kg. of mean increase in liveweight . As described by Gunn (1982) in his review, this relationship exists over a certain range. Below a certain liveweight the number of ewes that do not ovulate increases markedly and above a certain liveweight no further increases in ovulation rate are

possible because ewes are already expressing their maximum genetic potential. A similar relationship to that for ovulation rate and liveweight has also been described for ovulation rate and condition score (Gunn and Doney, 1979).

The fact that medium term changes in nutrition can be readily correlated with liveweight indicates that this effect is primarily a response to changes in energy intake. This agrees with numerous reports suggesting that energy and not protein is the major factor limiting ovulation rate (see reviews by Coop,1966b; Gunn, 1982; Morley *et al.*, 1978). Coop (1966b) for example, considered "that there is no evidence that there is any specific feed factor such as high protein which is conducive to flushing". However, it is perhaps incorrect to consider energy and protein as separate entities in ruminants, since protein in the form of amino acids for example, can meet as much as 50% of the ruminant animals obligatory glucose requirement (see review by Armstong and Hutton, 1975). Hence, it may be more appropriate to consider this effect in terms of an animal's "endogenous catabolic sources" of energy and protein (Lindsay, 1976).

Whether energy intake influences ovulation rate by increasing the number of pre-antral follicles recruited, their subsequent growth rate or by reducing the incidence of atresia in antral follicles remains to be determined. Allen and Lamming (1961) have reported that ewes fed submaintenance diets for 3 months have fewer follicles < 2 mm in their ovaries compared with controls. This finding together with the suggestion by (Coop, 1966b) that changes in the level of nutrition need to operate for least six weeks before joining suggests that energy intake acts to influence the entry of preantral follicles into the rapid phase of growth. Thus, Fletcher (1971)

concluded that ovulation rate is better correlated with an animal's liveweight several weeks prior to mating than at mating. Support for the present suggestion also comes from comparative studies between prolific and non-prolific breeds in which differences in the number of antral follicles has been related to increases in the recruitment of preantral follicles into this phase (Cahill *et al.* 1979; Cahill *et al.* 1982). The precise mechanism that governs the rate of entry of preantral follicles into the antral phase is unknown. However, the involvement of oestradiol and FSH in antrum formation and in the rapid increase in growth that takes place soon after,[1] suggests that both may be involved. In particular it has been suggested that it is only when pre-antral follicles gain the ability to produce significant amounts of oestradiol that they can proceed to the antral phase (Richards and Midgley, 1976). Various growth factors present in follicular fluid may also be important at this stage of development (see review by Hammond, 1981).

2.5. Preovulatory growth and maturation

2.5.1. Follicular growth

The final stages of growth and maturation of the preovulatory follicle in the ewe are initiated at luteolysis. One to three antral follicles > 2 mm can be

1 (see review by Richards, 1980)

found on the surface of the ovaries throughout the luteal phase. The majority of these undergo atresia except those present at luteal regression. (Smeaton and Robertson, 1971; Tsonis *et al.*,1982; Driancourt and Cahill 1984). These follicles are active steroidogenically and secrete oestradiol into ovarian venous blood resulting in peaks of oestradiol secretion on days 2-6, 8-11 and 14 -17 of the cycle (Cox *et al.,* 1971; Holst *et al.* 1972; Hauger *et al.,* 1977; Herriman *et al.*, 1979). FSH appears to fluctuate in two waves during the luteal phase in response to increasing oestradiol and inhibin secretion from these "oestrogenic" follicles (Miller *et al.,* 1981; Bister and Paquay, 1983) and may be responsible for reported waves in follicular growth (Smeaton and Robertson 1971; Brand and de Jong, 1973). The development of antral follicles results in increased oestradiol and inhibin secretion thereby depressing FSH secretion via negative feedback to the hypothalamic/pituitary axis (Knobil, 1974; Goodman et al., 1981). However, an ovulatory surge does not occur, as both tonic LH secretion and LH pulse frequency are inhibited by progesterone secreted by the corpus luteum (Baird and Scaramuzzi, 1976; Hauger *et al.,* 1977; Karsch *et al.*, 1977). In the absence of increased LH levels follicles undergo atresia allowing FSH levels to rise (Miller *et al.*, 1981; Bister and Paquay, 1983). This continuous follicular activity ensures that a pool of antral follicles is always present in the sheep ovary (Turnbull *et al.*, 1977; Cahill and Mauleon, 1980) which can be induced to ovulate if the influence of the corpus luteum is removed (Smeaton and Robertson,1971; Tsonis *et al.*, 1982). Thus, it would appear that follicular growth to 2 mm in the sheep can occur independently of the major hormonal events of the oestrous cycle (Dufour *et al.*, 1979).

2.5.2 Recruitment and selection of the preovulatory follicle

The differentiation of the preovulatory follicle is a two step process (di Zerega and Hodgen, 1981). Antral follicles are brought forward from a pool of follicles when exposed to sufficient gonadotrophin stimulation. The development of this cohort of follicles is called recruitment. From amongst these, a follicle(s) continues to grow and develop and is ultimately ovulated instead of degenerating. This process is called selection and as a consequence the selected follicle is said to become dominant as it exerts a major influence on the growth of the other follicles recruited.

In the ewe, recruitment occurs around 12 hours after the start of luteolysis (Driancourt and Cahill, 1984). Various studies in sheep (e.g. Smeaton and Robertson, 1971, Tsonis *et al.,* 1982; Driancourt and Cahill, 1984) have demonstrated that recruitment begins with follicles > 2 mm in diameter at the start of luteolysis. About half of the follicles at luteolysis are atretic (Brand and de Jong, 1973: Cahill *et al.,* 1979) and it has been argued (Driancourt and Cahill, 1984) that recruitment is simply the regression of these follicles. If so, the number of follicles capable of being recruited would be determined before luteolysis .

In the rat, recruitment is considered to be a gonadotrophin dependent process (Richards, 1980). Support for this suggestion in the sheep comes from studies showing that preovulatory enlargement can be induced during anoestrous by injection of hCG or LH in amounts that mimic plasma concentrations during the follicular phase (Goodman and Karsch, 1980; McNatty *et al.,* 1981a). However these follicles must be primed by FSH before they can respond to LH (Oussaid *et al.,* 1982, McNatty *et al.,* 1984)

which is consistent with the finding that FSH together with oestradiol induces receptors in the granulosa cells for LH allowing the follicle to respond to the preovulatory gonadotrophin surge (Richards, 1979)

One of two sets of criteria is generally used to determine if selection has occurred : (1) the presence of a large antral follicle secreting enough oestradiol which is seen as a difference between ipsi and contralateral ovarian venous blood concentration (McNatty *et al.,* 1982) and/or (2) the binding of LH on both the thecal interna and granulosa cells (Webb and England, 1982a). The concentration of oestradiol and the steroidogenic capacity of the dominant follicle increases soon after luteolysis and reaches a peak before the LH surge (McNatty *et al.,* 1981b). Only those follicles with LH receptors in both the theca and granulosa cells show increased oestradiol concentrations in the antral fluid prior to the LH surge and only these follicles ovulate (Webb and England,1982a).

In the sheep selection of the preovulatory follicle is thought to be complete 54 hours after the start of luteolysis (Driancourt and Cahill,1984). The main feature of the timing of selection in sheep is its variability. In the study of Driancourt and Cahill (1984) for example, the preovulatory follicle was the largest in 0/7, 1/7, 1/7, 4/7, 5/7 and 5/7 ewes at 0, 4, 8, 12, 24 and 48 hours respectively, after prostaglandin injection. In the sheep, FSH has been suggested by Baird (1983) to be responsible for the selection of the dominant follicle and the atresia of others at this stage. The fall in FSH concentration which promotes atresia appears to be quite small. In the sheep the increase in the number of atretic follicles > 2 mm in diameter from 50 % on day 12 to more than 80% 24 hours after luteal regression is accompanied by only a 30 % fall in the concentration of FSH (Baird *et al.*,

1981). This decline in the number of healthy follicles can be offset by PMSG (Dott *et al.*, 1979; McNatty *et al.*, 1982) supporting a role for FSH in the selection process. The mechanism however, that allows one follicle to continue growing while the others undergo atresia is still unclear. The ability of the selected follicle to respond to gonadotrophins, negative feedback between the dominant follicle and the pituitary gland, and the secretion of inhibitory factors from the dominant follicle have all been implicated in this process.(see review by Baird, 1983).

Initiation of follicular growth has been suggested to be asynchronous (Lintern-Moore and Moore, 1979) and hence, not all growing follicles may be at the same stage of development at the time of luteolysis. One reason, therefore that antral follicles do not continue to grow may be that they are at an inappropriate stage of development to respond to the selection stimulus. This could be because of differences in the number of granulosa and thecal cells together with differences in the number of gonadotrophin receptors on these cells (see review by Richards, 1980).

Selection is also likely to involve negative feedback between a dominant follicle secreting oestradiol and inhibin and the anterior pituitary secreting FSH (see review by Baird, 1983). In sheep the dominant follicle has an enhanced capacity to synthesize oestradiol (Baird, 1977; McNatty *et al.*, 1981b). In turn, high intrafollicular levels of oestradiol are thought to be essential for further growth at this stage (Richards, 1980; Baird, 1983). Only those follicles > 2 mm in diameter can convert significant amounts of androgens to oestrogens because of the increase in aromatase activity in their granulosa cells (Baird, 1983). At the time of luteal regression in the sheep there is an increase in the frequency of LH pulse which stimulates

increased androgen synthesis by the theca and oestradiol secretion into the ovarian vein (Baird, 1978). In the sheep, the increase in oestradiol secretion by the dominant follicle at this stage supresses FSH secretion which in turn, is thought to ensure that those follicles at a less advanced stage of development are deprived of FSH (see review by Baird, 1983).

It has also been suggested that the dominant follicle secretes a range of paracrine factors which effectively inhibit the growth of the other follicles recruited (di Zeregra and Hodgen, 1980). Many factors with a wide range of biological activities have been described in follicular fluid including inhibitors of gonadotrophin binding, steroidogenesis and oocyte maturation (see review by Hammond, 1981). Evidence for such an effect in the ewe comes from the recent finding that steroid- free ovine follicular fluid can inhibit follicular growth and increase atresia in hypophysectomised-PMSG treated ewes (Cahill, 1984). This inhibitor ("follicular growth inhibitor") is either very potent or has a long half-life as only relatively small doses of follicular fluid (2-4 ml) injected subcutaneously were required to inhibit folliculogenesis in this experiment.

In summary, gonadotrophins regulate recruitment. However it is uncertain whether recruitment occurs before luteolysis. FSH, oestradiol and inhibin appear to be the major factors involved in selection but this is probably also facilitated by growth factors present in the follicular fluid of the dominant follicle.

2.5.3. Follicular atresia

As a consequence of selection all recruited follicles except one become atretic. The proportion of follicles that exhibit signs of atresia increases from almost none prior to antrum formation to approximately two thirds of all follicles 1 - 2 mm in diameter (Brand and de Jong, 1973; Turnbull *et al.* 1977; Cahill *et al.* 1979). The earliest structural change in atretic follicles in sheep is the appearance of pyknotic nuclei (Turnbull *et al.* 1977). Whilst the mechanism of atresia in the ewe is largely unknown, various studies have implicated gonadotrophins in a central regulatory role. Hypophysectomy in the ewe for example, greatly increases the number of atretic follicles (Dufour *et al.* 1979) Studies with oestrogen- treated hypophysectomised rats have also implicated androgens as being important in atresia (Payne and Runsen, 1958; Louvet *et al.* 1975;). Support for this suggestion also comes from studies in sheep in which immunisation against androstenedione has been shown to increase ovulation rate (Scaramuzzi *et al.* 1977; Scaramuzzi, 1979). Receptors for androgens have been demonstrated in granulosa cells of oestrogen-treated hypophysectomised rats indicating that androgens can act directly on granulosa cells to induce atresia (Schreiber and Ross, 1976; Schreiber *et al.* 1976). This suggests that androgens interfere with the FSH or oestradiol response systems in these cells (Richards, 1979). DHT for example, has been shown to inhibit the induction of LH receptors in developing antral follicles in immature rats (Farookhi, 1980).

Evidence from the rat, suggests that thecal androgen production increases only in developing preovulatory follicles (Richards, 1979). Therefore androgens may be responsible for atresia in only these large antral

follicles which because of the decline in FSH during the follicular phase (Welschen and Dullaart, 1976) may not have sufficient aromatase activity to convert androgens to oestrogens (Erickson and Hseuh, 1978; Moon *et al.*, 1975; Leung and Armstrong, 1980). In the sheep, the majority of follicles however, appear to undergo atresia at an earlier stage of development (Brand and de Jong, 1973: Turnbull *et al.*, 1977; Cahill *et al.,* 1979; Carson *et al.*, 1979). Therefore, conditions other than increased androgen production may explain the failure of these follicles to continue to grow and develop. In the sheep, basal concentrations of gonadotrophins appear sufficient to maintain the growth of follicles only to the small antral stage (Dufour *et al.,* 1979). Richards (1980) has proposed that in the absence of sustained increases in gonadotrophin secretion, these follicles are unable to enter the preovulatory stage of development and degenerate.

2.5.4. Nutrition and preovulatory growth and maturation.

Short-term nutrition i.e. for less than six weeks, also influences ovulation rate. In one of the first experiments to measure ovulation rate, Allen and Lamming (1961) reported that flushing for one or two cycles could increase ovulation rate. However the term " flushing" has not been defined. Some workers have considered it to mean a process whereby ewes are in good condition at the time they are mated while others have considered it to mean a process whereby liveweight is rapidly increased immediately prior

to joining[1]. Prior to the 1960's however, none of the flushing experiments had determined which of these processes operate in response to an improvement in the level of nutrition.

Subsequently Coop(1962a)concluded that the classical concept of flushing should be modified to one in where the static body condition or liveweight of the sheep and a rapid increase in liveweight are of equal importance. He referred to these two effects as "static" and "dynamic" and defined them (Coop, 1966b) as

"(a) static effect -the increase in liveweight brought about by flushing. This is not specifically related in time to mating. An increase in liveweight brought about at any time of the year if maintained until mating will cause a higher lambing percentage.

(b) dynamic effect- the effect of the dynamic processes of change in liveweight or condition."

The static effect described by Coop is almost certainly the effect of energy intake in the medium term discussed previously (Section 2.4.2.). The existence of the second component of the flushing response namely a dynamic effect remains controversial. The flushing experiments of Coop (1966a) were the first designed to determine whether there existed a true flushing effect as a result of increasing liveweight. In this experiment ewes actively gaining weight were shown to have a higher twinning rate than controls who had similar liveweights at conception.

The existence of a separate dynamic effect has been suggested by several workers since Coop's initial experiments (e.g. McInnes and Smith, 1966;

1 (see reviews by Moule, 1962; Coop, 1966b; Lindsay, 1976)

Killeen, 1967). However this has not been confirmed by other workers (see review by Morley *et al.,* 1978) suggesting that changes in liveweight are an inadequate criterion of this effect and cannot consistently explain the observed increases in ovulation rate. As stated by Lindsay (1976) liveweight

" ...is a crude inaccurate criterion which because it describes only long-term changes in feeding is incompatible with studies on many components of the reproductive process such as oestrus, ovulation, transport of gametes, etc., which takes place over a few days or even hours. "

That increasing the level of nutrition can increase ovulation rate without increasing liveweight was first demonstrated by workers in Western Australia (Knight *et al.*,1975). These workers observed that the ovulation rates of ewes at joining were lower than their liveweights would otherwise suggest (Lindsay *et al.,* 1975). They also noted that the time of joining corresponded to a period of reduced wool growth which had been attributed previously to the relatively low protein content of wilted pasture (Stewart *et al.*, 1961). Subsequently these workers showed that supplementary feeding with lupin grain- a grain legume relatively high in protein, at joining could markedly increase ovulation rate without necessarily altering liveweight.

The minimum period required for lupin feeding to increase ovulation rate has been defined by Oldham and Lindsay (1984; cited by Lindsay, 1976). Merino ewes with regular unsynchronised cycles were fed lupin grain over a 14 day period. On the morning of feeding, those ewes marked by vasectomised rams since the previous day were removed and ovulation

rate determined by laparoscopy 1 week later. In this experiment, an increase in ovulation rate was first observed in those ewes fed lupin grain for six days. From this study, these workers concluded that lupin grain "induced a significant increase in ovulation rate when feeding commenced six days before ovulation, or around day 10 of their oestrous cycle". This finding appears to be at variance with those from studies involving unilateral ovariectomy which have been interpreted to mean that ovulation rate is determined at luteolysis or soon after (Land, 1973; Findlay and Cumming, 1977). In these studies unilateral ovariectomy on day 14 of the cycle did not depress ovulation rate because of compensatory hypertrophy by the remaining ovary resulting in it ovulating at the same rate as intact controls. Ovariectomy carried out on day 16 however, caused a decrease in ovulation rate. As it is not possible to determine from the design of Oldham and Lindsay's experiment whether the response to lupin grain is initiated near the time of luteolysis, the possibility remains that the minimum period required for feeding is less than six days.

The rapidity of the lupin response has been confirmed by other workers (Lightfoot *et al.*, 1976; Radford *et al.*, 1980). The latter have also shown this response to be reversible with ewes responding repeatedly to supplementation (see Scaramuzzi and Radford, 1983). The rapidity of lupin response suggests that short-term nutrition influences the number of follicles recruited and/or the number of these that are selected to ovulate. This view, together with the finding that the effect of lupin grain is reversible also indicates that short-term changes in the level of nutrition can act as a final regulator of ovulation rate.

The component(s) in lupin grain responsible for increasing ovulation rate has not been determined. In the experiments of Knight *et al.*, (1975) the feeding of a barley plus urea supplement which provided 25% more digestible energy than the lupin supplement and similar nitrogen levels, but with 50% of the nitrogen in the form of urea resulted in no increase in ovulation rate. From this study these workers concluded that;

" ... the effect of lupin supplementation is not simply explicable in terms of its input of either nitrogen and/or energy but may be related to a significant amount of the lupin protein escaping ruminal proteolysis."

However, attempts to confirm this suggestion by increasing the amount of protein digested post-ruminally have produced equivocal results (Corbett and Edey,1977; Fletcher,1981; Davis *et al.,* 1981). The finding by Smith (1981) that the response to protein is threshold in nature may explain much of the confusion concerning the role of protein in this response.

Whether an increase in the amount of protein digested post-ruminally would also provide an explanation for the dynamic effect of flushing proposed by Coop (1966) is uncertain. However, it is interesting to recall Coop's (1962) original remarks concerning this effect;

"... liveweight gain or loss at mating either directly or as insinuated from the time and abundance of the autumn flush of pasture is also important. In other words there is strong presumptive evidence in this work that there is something specific in the rapidly rising condition"

Increases in post-ruminal protein supply may also provide a basis for other reports which indicate that short-term changes in feed quality (Rattray *et al.*, 1978, 1980, 1981) and feed intake (Gunn *et al.,* 1979) can influence ovulation rate. The reason that protein , in particular post-ruminal protein has been overlooked as the "something specific" in the flushing response is perhaps because it is only recently that its importance has been recognised (ARC 1980). The results of Smith *et al.*, (1981) however, also suggest that short-term changes in the level of digestible energy intake can influence ovulation rate. Hence, increases in ovulation rate in lupin-fed ewes may be the result of combined increases in protein and digestible energy intake. This would also explain the lack of unanimity amongst workers concerning the role of protein in this response since none of the aforementioned studies have provided post-ruminal protein and digestible energy in amounts commensurate with that supplied by feeding lupin grain. More recently however, Teleni *et al.,* (1984) have suggested that the effect of lupin grain can be explained entirely by increases in digestible energy intake.

Whilst it remains uncertain as to how short-term changes in nutrition influences ovulation rate, increases in ovulation rate in lupin-fed ewes have been associated with an increased concentration of FSH in the plasma on day 13 of the oestrous cycle (Brien *et al.,* 1976; Davis *et al.,* 1981; Knight *et al.,* 1981). Supplementation with lupin grain however, appears not to influence LH secretion (Radford *et al.,* 1980). Whether these changes in FSH are involved in the control of ovulation rate remains to be established. However, it is known that administration of PMSG at this stage of the cycle

can increase ovulation rate (Robinson, 1957, Bindon *et al.,* 1971; Allison, 1975).

2.5.5. Hormonal correlates of ovulation rate

While the factors involved in ovulation in the ewe are well described the exact determinates of ovulation rate are unknown. Administration of pituitary extracts, PMSG and FSH can all increase ovulation rate and;

"Consequently it is hardly surprising that a considerable effort has gone into attempts to uncover the hormonal correlates of ovulation rate. It is widely believed (perhaps wrongly) that a knowledge of the hormonal correlates of ovulation rate will rapidly lead to a better understanding of ovulation rate and its control mechanism(s)."

Scaramuzzi and Radford (1983)

However most recent reviews (e.g. Bindon and Piper, 1982; Scaramuzzi and Radford, 1983) have concluded that circulating levels of gonadotrophins do not provide in themselves an adequate explanation for differences in ovulation rate. This is not to say that differences in gonadotrophin secretion are unimportant as determinants of ovulation rate, since differences in the individual animal are likely to be much smaller than differences between individuals (e.g. FSH, Findlay and Cumming, 1977). Brown (1978), for example has argued that within the same individual the difference in the dose of FSH between no effect and the

stimulation of follicular development may be as little as 20%. Furthermore because of feedback mechanisms operating in the intact animal, Baird (1983) has suggested a difference in circulating levels in gonadotrophins may occur only for a short period or can only be detected when the influence of ovarian feedback is removed.

Follicles greater than 2 mm in diameter are present in the ovaries of sheep throughout the oestrous cycle (Turnbull *et al.*, 1977; Cahill and Mauleon, 1980). The number of follicles in this class appear to be higher in the ovaries of twin ovulators compared with single ovulators within the same breed (Turnbull *et al.*, 1977) and in prolific breeds compared with non-prolific breeds (Cahill *et al.*, 1979; Cahill *et al.*, 1982). This suggests that there may be a mechanism that controls ovulation rate which operates independently of the major changes in hormonal secretion during the oestrous cycle.

Support for this suggestion comes from a study involving the measurement of peripheral FSH following ovariectomy in the prolific Finn breed and the non-prolific Scottish Blackface (Webb *et al.*, 1982). In this study there was a significant difference in FSH concentrations between breeds in long-term ovariectomised ewes, with Finns having higher FSH levels. This difference was reversed in short-term ovariectomised animals with the Scottish Blackface having higher concentrations of FSH than the Finn breed. From this study these workers concluded that there is an underlying breed difference in FSH release and possibly pituitary function and that an ovarian factor with apparent long-term effects is involved in the control of pituitary function in the ewe. Similar differences in circulating levels of FSH

have also been reported recently in comparative studies in intact ewes between the prolific D'man and non-prolific Timahdite breeds near the time of luteolysis (Lahlou-Kassi *et al.,* 1984). If, as previously argued there is a requirement for FSH throughout follicular growth including the establishment of the dominant follicle, then small but significant changes in circulating gonadotrophins may be important for ovulation (Brown, 1978; Richards, 1980).

The ovarian factor involved in the long-term control of pituitary function may be inhibin. Inhibin has been shown to reduce FSH release in the ewe (Goodman *et al.,* 1981; Miller *et al.,* 1979 Cummins *et al.,* 1983). The last mentioned authors have also suggested that the feedback relationship between inhibin and FSH is different in the Booroola compared with control ewes. In particular, a deficiency in inhibin production may play a key role in the Booroola's high ovulation rate. The abnormalities of the Booroola follicle including the reduced granulosa cell population[1] are compatible with inhibin deficiency since these cells are the site of inhibin synthesis (Henderson and Franchimont, 1983). Similar differences in the feedback relationship between inhibin and FSH in other breeds have not been determined. Indirect evidence for this suggestion also comes from recent studies that have demonstrated that administration of steroid-free ovine follicular fluid which contains inhibin can depress FSH levels (Cummins *et al.*, 1983) and that immunisation against ovine follcular fluid can increase ovulation rate (O'Shea *et al.*, 1982).

Finally, although the control mechanisms may be acting via hypothalamic/ pituitary and ovarian feedback mechanisms, direct effects of ovarian steroids and proteins on the ovary cannot be excluded. It has recently been

[1] (Baird *et al.*, 1982)

shown (Cahill, 1984) that steroid-free ovine follicular fluid contains a substance ("follicle growth inhibitor ") that inhibits ovulation in PMSG-treated hypophysectomised ewes. This factor cannot be inhibin but rather is thought to act locally within the follicle to prevent mitotic division in granulosa cells. Hence local ovarian factors may also be involved in the control of terminal follicular growth in addition to any feedback effects via the hypothalamus / pituitary axis. In particular, it may be that this factor and others present in follicular fluid are secreted by the dominant follicle to ensure that the growth of the other follicles recruited is prevented (Richards, 1980).

2.6. Conclusion and working hypothesis.

Ovulation rate appears to be determined by the number of follicles that have developed sufficient maturity by the time of luteolysis to respond to the preovulatory gonadotrophin surge. This development incorporates structural growth of the follicle, the acquisition of LH receptors on the granulosa cells and the ability to convert androgens to oestradiol. In turn, the number of "oestrogenic" follicles is dependent on the balance between the number of growing follicles, their growth rate and the incidence of atresia, all of which can be influenced during the six months required for growth up to the point of ovulation. Gonadotrophins, in particular FSH, are required throughout folliculogenesis for follicular growth but their role in the control of ovulation rate is uncertain. However, there is increasing evidence in the sheep to suggest that FSH levels near the time of luteolysis are

important in the control of ovulation rate. This may reflect an underlying difference in FSH secretion between breeds or particular strains of animals or may be the stimulus that converts a small, healthy antral follicle into an oestrogenic one capable of ovulating. Hence, ovulation rate may be the result of small differences in FSH secretion which determine the number of growing follicles and/or the number of these that continue preovulatory growth following luteal regression. However, a direct or causal relationship between circulating levels of FSH and ovulation rate remains to be demonstrated.

There is considerable evidence to suggest that nutrition can influence all stages of follicular growth. How it does this is open to speculation. Severe undernutrition can influence the initiation of follicular growth and also subsequent development and appears to do so by interfering directly with the functioning of the hypothalamic/pituitary/gonadal axis. Such a suggestion is consistent with a considerable literature demonstrating a requirement for gonadotrophins throughout folliculogenesis.

The effect of nutrition in the medium term is readily correlated with liveweight with energy being the most likely determinant of this effect. In turn, changes in energy intake appear to influence the recruitment of preantral follicles into the antral phase. How this effect is mediated within the animal is unknown. However, FSH and oestradiol may be involved since both are required for antrum formation and the rapid increase in the growth of the follicle that takes place soon after.

Changes in the level of nutrition in the short-term also influence ovulation rate. Whether this effect is mediated by changes in protein intake, in

particular post-ruminal protein is uncertain. Post-ruminal protein however, has been implicated in a number of physiological processes in ruminants including feed intake regulation (see review by Egan, 1980). Associated increases in FSH secretion immediately prior to luteolysis as demonstrated in lupin-fed ewes may be responsible for this effect. If so there is a parallel in prolific breeds. The rapidity of the lupin effect suggests that short-term nutrition influences the number of follicles that become dominant either as a result of recruitment or selection or both.

In summary, apart from severe undernutrition at least two separate effects of nutrition can be identified that influence follicular growth during the six months it takes a follicle to grow to the preovulatory stage. A similar conclusion can be drawn from Lindsay's (1976) nett nutritional hypothesis;

" ovulation rate in ewes is related to their nett nutritional status- the sum of endogenous catabolic sources of nutrients and uptake of exogenous nutrition from the gut."

Hence, as argued by Lindsay, heavy ewes given poor quality feed may still produce a reasonable number of twin ovulations because they have a reasonable "endogenous source of energy and protein". On the other hand, poor ewes temporarily well fed will also ovulate well because of the contribution of the "exogenous nutrition" to an animal's "nett nutritional status". In terms of folliculogenesis therefore, "endogenous catabolic sources" would determine the number of antral follicles capable of becoming dominant whereas, the number of these that ovulate is determined largely by "exogenous nutrition". These effects appear to operate independently of each other at different stages of folliculogenesis

to ensure that an animals ovulation rate is matched to its "nett nutritional status".

2.7. The aim of the present study.

From the preceding discussion it would have become apparent that a systematic study of the known ability of lupin grain to increase ovulation rate in the ewe would not only furnish information about this effect but also about the influence of nutrition in general and the control of ovulation rate. The present study therefore, was undertaken to examine the physiological basis of this response in mature Merinos under conditions of maintenance energy and protein. The major objectives of the present study being;

1). To determine the component(s) in lupin grain responsible for increasing ovulation rate.

2). To determine when in the oestrous cycle this response is initiated.

3). To determine how the response to lupin grain is mediated within the animal.

CHAPTER 3. MATERIALS AND METHODS.

3.1. Location

Experiments 1, 2, 3, 4, and 7 were conducted at "Glenthorne", O'Hallaron Hill which is located approximately 20 km. south of Adelaide (Lat. 34° 58' S. Long. 138° 38' E.). Experiment 5 was conducted at the Mortlock Experimental Station located 120 km. north of Adelaide and situated near the township of Clare. Experiment 6 was conducted at the Waite Agricultural Research Institute located approximately 7 km. south east of Adelaide. All three areas experience a similar mediterranean type climate.

All seven experiments were conducted during the spontaneous breeding season which is generally considered to extend from February to May for Merinos in the southern hemisphere (Watson, 1952; Fletcher and Geytenbeek, 1970, Oldham,1980).

3.2. Sheep

The animals used, with the exception of experiment 5, were 6 year-old cast for age medium wool Merino ewes which were obtained from Martindale Holdings. Since its inception in 1850 Martindale has been run virtually as a closed stud having been based on Peppin sirelines from Wonganella in New South Wales. The marking percentage of these ewes has remained

relatively constant at between 80-90% since the property has been managed by The University of Adelaide.

In July of each of three years, 500 ewes were drafted off at lamb marking and run as one mob until they were transported to Glenthorne in January of the following year. Soon after their arrival, animals were drenched for internal parasites and ear tagged. Prior to the commencement of experiments ewes grazed 40 hectares of cereal stubble and were subjected to normal management. Animals were "teased" during the last two weeks of January by running 2% vasectomised rams with the ewe flock. The 1020 ewes used in six of the seven experiments over three years had an initial liveweight at the time of sponge insertion of 47.3 $\pm$ 0.6 kg (mean $\pm$ s.e.m.) and a condition score (Jefferies, 1961) of 2.71 $\pm$ 0.05 (mean $\pm$ s.e.m.)

3.3. Nutrition

Groups housed outdoors were maintained under feedlot conditions (Plate 3.1.). Feedlots were approximately 0.5 hectares in area and were constructed on cereal stubble which had been heavily grazed and mowed prior to the start of the breeding season. The remaining stubble available to ewes in the paddocks was considered to be of little nutritive value and did not exceed 1000kg per hectare in the three years the experiments were undertaken.

Plate 3. 1. In the majority of experiments animals were maintained outdoors under feedlot conditions. Each paddock was approximately O.5 ha. in area.

Plate 3. 2. In the field experiments, supplements were fed out in troughs. O.5 m of troughing was allowed for each ewe.

Groups were placed in the feedlot 14 days prior to the commencement of an experiment. During this time and throughout the experiments animals were fed wheaten hay (1.2% crude protein, 9.6 MJ digestible energy per kg; determined *in vitro* , see Chapter 4. for details) on a daily basis at a rate calculated to meet maintenance requirements (~1100g; MAFF, 1975) and also thought to meet maintenance protein requirements via ruminal microbial protein synthesis (Egan and Walker, 1975). Water was freely available from automatic-fill troughs located in each paddock.

3.4. Oestrous cycle synchronisation

Oestrous cycles were synchronised by means of intravaginal progestagen sponges (Repromap, Upjohn, Rydalmere, New South Wales) inserted for twelve days. Oestrus (day 0) was considered to occur 36 hours after sponge removal. This method results in cycles of normal length i.e 17 days in more than 80 per cent of ewes (unpublished results).

Cloprostenol (100 ug i. m. ; PG; Estrumate, ICI, Melbourne) was used to resynchronise oestrus in Experiment 4 and to induce luteolysis in Experiments 3 and 7.

3.5. Assignment to treatments

Ewes were assigned to treatments on the basis of liveweight. Animals were fasted overnight before being weighed on the morning of sponge insertion. Ewes were ranked in descending order according to liveweight and then assigned to each treatment in rotation. Two vasectomised rams were run with each group throughout the experiments.

3.6. Supplementation

Animals were fed 500g of lupin grain per ewe per day for periods of 1-14 days in the experiments. Details of feeding are described in the individual experiments. The cultivar Uniwhite (29% crude protein, 12.8 MJ digestible energy per kg; determined *in vitro* , see Chapter 4. for details) was used in all experiments. The feeding of the other supplements is detailed in the relevant chapters. Supplements were fed out daily in troughs with 0.5m of troughing allowed for each ewe. All supplements were readily eaten by the ewes (Plate 3.2.).

3.7. Liveweight change

Liveweight change during the experiments was determined by weighing animals on the day of sponge removal and then again on the day of endoscopy after fasting groups overnight. Animals were weighed using a

Plate 3.3. Changes in liveweight were determined by weighing ewes on the day of sponge removal and then again on the day of endoscopy.

Plate 3.4. Ovulation rate was determined using endoscopy 5-6 days after the second oestrous following sponge removal.

transportable scale which was standardised prior to each weighing using a 20 kg. weight (Plate 3.3.). Ewes were weighed to the nearest 0.5 kg.

3.8. Ovulation rate

Ovulation rate was calculated on a per ewe present basis.

Ovulation rate was determined using laparoscopy. The method used was essentially the same as that described by Oldham *et al.*, (1976). Ewes were fasted overnight before being subjected to laparoscopy 5-6 days after the expected date of the second oestrus following sponge removal. Animals were restrained in a laparotomy cradle modified from that described by Lamond and Urquhart (1961) and had their belly wool removed using electric hand shears. At each of two sites, anterior to the udder on either side of the midline, 2.5 ml 2% xylocaine (Astra Pharmaceuticals, Melbourne) was injected subcutaneously and the skin swabbed with benzalkonium chloride solution (2%; Zepharin, Winthrop Laboratories, Sydney). Animals were insufflated with medical air via a pneumoperitoneal needle inserted into the abdominal cavity. A 7 mm trocar and cannula was inserted to the right of the midline. The trocar was removed from the cannula and a 30 ^{o}, 6.5 mm laparoscope (Stortz, West Germany) inserted . Atraumatic manipulating forceps were inserted similarly at the other site and the ovaries of the ewe examined (Plate 3.4.). At the end of this procedure puncture wounds were swabbed with antiseptic cream (Savlon, ICI, Melbourne). When not in use instruments were kept in benzalkonium solution.

3.9. Statistical analyses

All data were tested for normality using a maximum likelihood ratio test. Differences in liveweight change between groups were determined by one way analysis of variance. Ovulation rate was analysed using X^2. In particular, r x c contigency tables were used to examine whether the number of ewes with zero, one or two ovulations was independent of treatments. Other statistical analyses are detailed in the relevant chapters.

3.10. Formaldehyde treatment of casein and gelatine

Casein and gelatine were used in experiments 1 and 2 as protein supplements. To render their degradability in the rumen similar to that previously measured for lupin protein (c. 35%; Hume, 1974) both proteins were treated with formaldehyde (Ferguson *et al.*, 1967).

3.10.1. Preliminary experiment

3.10.1.1. Introduction

While numerous workers have reported that formaldehyde can be used to render casein resistant to degradation in the rumen no such information existed for gelatine. A preliminary experiment therefore was undertaken to determine the concentration of formaldehyde required to render casein and gelatine degradable to the same extent as lupin protein.

3.10.1.2. Method

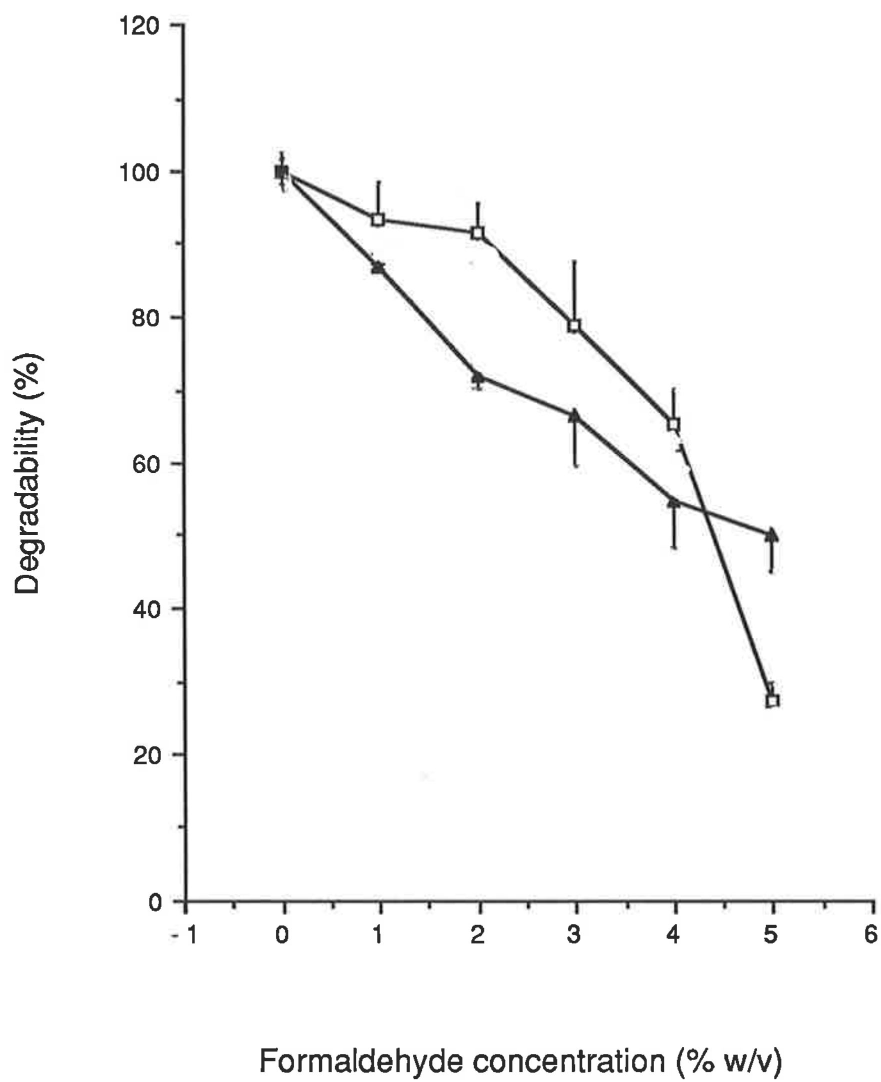
Due to gelatine's marked solubility in water the low volume method of Hemsley *et al.*, 1973 was used. 1 kg quantities of casein (Farmer's Union, Adelaide) or gelatine (Park Davis, Melbourne) were mixed with 0, 1, 2, 3, 4 and 5% w/v formaldehyde solutions in a kitchen mixer. Solutions were sprayed on at a rate of 100 mls per kg with a hand spray pump. The resulting product was then sealed in plastic bags and the reaction allowed to continue for 48 hours.

Degradability was assessed *in vitro* by determining ammonia levels after incubation in a solution of rumen fluid for 24 hours at 39 ^{o}C as described by Hemsley *et al.*, (1973). Samples were incubated in triplicate.

3.10.1.3. Results

The results of the *in vitro* trial are shown in Figure 3.1. Values for degradability are expressed as the percentage of dry matter degraded. In general as the concentration of formaldehyde increased degradability was reduced for both proteins.The concentration at which formaldehyde

Figure 3. 1. The *in vitro* degradability of casein (□) and gelatine (▲) treated with 0, 1, 2, 3, 4 or 5% formaldehyde. Values are the mean ± s.e.m. of three replicates.


120
100
80
60
40
20
0
Degradability (%)
-1
0
1
2
3
4
5
6
Formaldehyde concentration (% w/v)

rendered casein and gelatine degradable to the same extent as described by Hume (1974) for lupin protein was 4 and 3% respectively.

3.10.1.4. Discussion.

The results of the present study demonstrate that formaldehyde can be used to treat casein and gelatine so that their degradability in the rumen is similar to that for lupin protein. The reason why more formaldehyde is required for casein to give a similar degradability is perhaps explained by the nature of the reaction of formaldehyde with protein. The principal reaction of formaldehyde is the formation of intermolecular and intra-molecular methylene bridges (Ferguson *et al.*, 1967). Hence, because of marked differences in the amino acid composition of the two proteins it may be that gelatine exhibits a greater degree of binding to formaldehyde compared with casein.

3.10.2. Large scale treatment

For use in Experiments 1 and 2 10 kg batches of casein and gelatine were mixed with formaldehyde solutions in a dough mixer. Solutions of formaldehyde were sprayed on with a spray gun. The product was then sealed in plastic bags and the reaction allowed to continue for a further 48 hours. After this time the different batches of casein and gelatine were mixed to give one uniform batch for each treated protein. As a check the degradability of casein and gelatine prepared in this manner was

determined against those of the first *in vitro* by the above method and was found to be similar (casein, small scale 34.7 ± 5.3%,large scale 37.6 ± 3.5%; gelatine, small scale 33.6% ± 6.9%, large scale 39.4 ± 4.1%; mean ± s.e.m., n=6)

3.11. Blood sampling

Blood samples for hormone determinations were obtained either via jugular venipuncture using a 19 G needle attached to a 10 ml syringe or via indwelling catheters when repeated sampling was required. Catheters were constructed from polyethylene tubing (Medical Grade; I. D. 1.00 mm, O. D. 1.5 mm; Dural Plastics and Engineering, Dural, New South Wales, Australia). Samples of 5 ml were collected into 12 x 75 mm glass tubes. After drawing a blood sample catheters were flushed with 0.9 % sterile saline containing heparin (50 I. U./ml). Blood samples were allowed to clot at room temperature for 1-2 hours, transferred to 4° C for twelve hours and then centrifuged at 2000 rpm for twenty minutes. The serum harvested after centrifugation was stored at -20° C until required for assay.

3.12. Radioimmunoassays for gonadotrophic hormones

All chemicals used were analytical grade and obtained from the chemical house Ajax (Sydney, New South Wales, Australia) unless otherwise stated.

3.12.1. Phosphate buffers

(A) 0.5 M Phosphate-buffered saline (pH 7.5)

1.42 g Na_2HPO_4 (anhydrous)

0.37 g EDTA

1.00 g NaN_3

8.18 g NaCl

pH adjusted to 7.5 with 1N HCl

made up to 1 litre with Type 1 water

(B). 0.05 M Gel PBS

as above with gelatine added to give a 2% solution

pH adjusted to 7.5 with 1N NaOH

3.12.2. Iodination of gonadotrophic hormones

Gonadotrophic hormones were iodinated by the Chloramine-T method (Greenwood *et al.*, 1963).

(i) Preparation of Bio-Gel p60 columns for gel filtration of iodinated hormones.

(A) Preparation of columns

1. 10 ml disposable glass pipettes (Crown Corning, Corning, New York, U. S. A.) were used.

2. The pipettes were thoroughly washed and dried.

3. The pipettes were siliconised by immersing them in Cotasil (Ajax Chemicals, Sydney, New South Wales, Australia), rinsed and then dried .

(B) Preparation of Bio-Gel

1. 300 ml PBS was added to 10 g of Bio-gel (Bio-Rad laboratories, Richmond, California, U.S.A.) and mixed by continuous stirring for 3 hours.

2. The solution was allowed to settle and the excess buffer removed from the gel. A new. volume of PBS was added to the gel and stirring recommenced for 15 minutes. This was repeated 3 times to wash the gel solution.

(C) Preparation of columns

1. A 10 ml disposable siliconised glass pipette was secured in the upright position and a glass bead placed at the bottom. A short piece of silastic tubing with clamp attached was secured to the lower end of the pipette

2. The column was rinsed with PBS and filled with Bio-Gel slurry. The column was kept wet at all times with PBS

3. The Bio-Gel was allowed to settle to the 10ml mark.

4. The clamp was released and the column was washed with 20ml PBS.

5. 1.5 mls of 5% bovine serum albumin (BSA; Sigma, St. Louis, Missouri, U.S.A.) in PBS was gently pipetted into the 1 ml PBS layer above the column bed.

6. The column was washed with 20 mls PBS and capped with parafilm and stored at 4° C. Prior to use the column was allowed to warm to room temperatue.

(ii) Reagents

1. Hormone

Highly purified hormone preparations of FSH and LH were used. A description of each hormone is given separately.

2. Radioactive iodine

For each iodination 0.5 mCi of sodium iodide (^{125}I; Amersham, Sydney, New South Wales, Australia) was used.

3. Prior to each iodination a solution of Chloramine-T (Sigma, St. Louis, Missouri, U. S .A) was prepared by dissolving 6 mg Chloramine-T in 15 ml PBS.

4. Sodium metabisulphite ($Na_2S_2O_5$)

A solution was prepared by dissolving 10 mg of $Na_2S_2O_5$ in 10 ml PBS.

5. Transfer solution

The transfer solution was prepared by dissolving 50 mg of potassium iodide (KI) in 5ml of PBS containing 16% sucrose.

6. Rinse solution

The rinse solution was prepared by dissolving 50 mg KI in 5 ml PBS containing 8 % sucrose

(iii) Iodination procedure

1. Five ug of LH or 5.0 ug of FSH dissolved in 25 ul of PBS in an Eppendorf tube was used.

2. 0.5 mCi of ^{125}I was added to the hormone and mixed by gently tapping on the tube.

3. Four ug (10ul) of Chloramine-T was added and mixed by gentle tapping on the tube for one minute.

4. Fifty ul (50ug) of sodium metabisulphite was added usng a TB syringe to stop the reaction.

5. One minute later, 100 ul of transfer solution was added, and the mixture was transferred into the PBS layer above the surface of the Bio-Gel column. The Eppendorf tube was rinsed with 100 ul of rinse solution and the solution transferred onto the column.

6. The outlet of the column was opened and ten fractions of 0.5 ml each were collected in 12 x 75 mm glass tubes containing 100 ul PBS containing 5% BSA.

7. 10 ul aliquots from each fraction were counted for radioactivity in a gamma-counter (Ria Gamma 1271, LKB-Wallac, Finland). The one or two fractions with the highest radioactivity were retained and

used after they were diluted in gel PBS to give 20000 counts per minute per 100ul

3.12.3 FSH radioimmunoassay

The FSH assay used in the present study was essentially the same as that described and validated for use in sheep previously (McNeilly *et al.*, 1976).

(i) Purified hormone

Highly purified ovine FSH (oFSH-LER-1976-A2) was used for iodination and was a gift from Dr. L. E. Reichert Jr., Albany Medical College, Albany, New York, U.S.A. Purified ovine FSH (NIADDK-oFSH-16) for standard preparations was a gift from the National Institute of Arthritis, Diabetes, Digestive & Kidney Diseases (NIADDK), Bethesda, Maryland, U.S.A. Standards of 0, 0.39, 0.78, 1.56, 3.12, 6.25, 12.5, 25.0, 50.0 and 100.0 ng oFSH each in 200 ul gel PBS were used. Standards were made up by serial dilution of the 100 ng stardard.

(ii) FSH antiserum

The serum against FSH (M 94) was a gift from Dr. S. S. Lynch, Birmingham and Midland Hospital for Women, Birmingham, U.K., and was raised in rabbits against human FSH. Details of the production of this antisera have been described previously (Lynch and Shirley 1975). The antiserum has a cross reactivity of 100% with oFSH. Cross

reactivity with ovine LH, TSH, GH and prolactin is less than O.2% (McNeilly *et al.*, 1976).

The sensitivity of the assay was 0.39 ng per tube , based on the definition of sensitivity given by Chard (1982), i.e. the least concentration of unlabelled hormone which can be distinguished from a sample containing no unlabelled hormone (zero standard), the distinction being based on the confidence limits (mean ± 1.96 SD) of the estimate of the zero standard on the one hand and the standard on the other.

Intra-assay coefficients of variation were calculated from eight replicates (each in duplicate) of a sample run in the same assay. One sample of low and one sample of high concentration of the hormone, each run in duplicate in all assays , were used to calculate inter- assay coefficients of variation. For FSH, the inter- and intra-assay coefficients of variation were 9.2% and 6.3% respectively.

(iii) Assay method

200 ul of serum or standard hormone preparation and 400 ul of anti-serum (1:30 000) containing 0.002% rabbit gamma-globulin were dispensed into 12 x 75 mm glass tubes using a reagent dispenser (Micromedic Systems, Horsham, Pennsylvania, U.S.A.) and mixed. The samples were assayed in duplicate and the standards in triplicate. Another dispenser was then used to add iodinated hormone (100 ul) immediately afterwards. Tubes were kept at room temperature for tweny four hours. The bound fraction of the hormone was precipitated by the addition of 100 ul diluted serum (1:15 in gel PBS) containing

the second antibody (anti-rabbit gamma globulin raised in sheep; see Niswender *et al.*, (1969) for method). The tubes were then incubated for a further twenty four hours at room temperature and then at 4° C for twelve hours. Following the addition of 1.7 ml PBS, tubes were centrifuged for twenty minutes at 2000 rpm at 4° C, and the supernatant containing the unbound fraction of the hormone decanted. The pellet left containing the bound fraction of the hormone was counted for radioactivity in the gamma counter.

(iv) Calculation of results

The bound fraction contained a non-specific binding (NSB) component which was the proportion of the radioactive hormone bound in the absence of the first antibody. In each assay three tubes each containing 200 ul gel PBS, 100 ul of ^{125}I-FSH and 400 ul gel PBS with 0.002% rabbit gamma-globulin , were included in order to estimate the NSB component. The percentage of total counts bound to the antibody after the correction for NSB, was calculated as follows :

$$\text{percentage bound} = \frac{\text{total counts bound to antibody} \times 100}{\text{total counts added to tube}}$$

Standard curves were constructed using a spline plot curve (Chard,1982). The counting and calculations were carried out in a LKB-Wallac automatic microcomputer- controlled two channel gamma-counter.

3.12.4. LH radioimmunoassay

The LH assay used in the present study was essentially the same as that described and validated for use in sheep previously (Niswender *et al.*, 1969).

(i) Purified hormone

Highly purified ovine LH (oLH-LER-1056-C2) was used for iodinations and was a gift from Dr. L. E. Reichert Jr., Albany Medical College, Albany, New York, U.S.A. Purified ovine LH (NIADDK-oLH-25) used for standard preparations was a gift from the National Institute of Arthritis, Diabetes, Digestive & Kidney Diseases (NIADDK), Bethesda, Maryland, U.S.A. The standards used were 0, 0.039, 0.078, 0.156, 0.312, 0.625, 1.25, 2.5, 5.0 and 10 ng oLH each in 200 ul gel PBS. Standards were made up by serial dilution of the 10 ng standard.

(iii) LH antiserum

The LH antiserum was raised in rabbits and was a gift from Dr. G. D. Niswender, Colorado State University, Fort Collins, Colarado, U.S.A. Details of the production of this antisera and its specificty have been described previously (Niswender *et al.*, 1968). The sensitivity of the assay was 39 pg per tube and inter and intra-assay coefficients of variation were 9.6 % and 5.4 % respectively.

(ii) Assay method

200 ul of samples were assayed in duplicate using a similar method to that described for oFSH (section 3.11.3.) with the following modifications

1. The LH antiserum was used at a dilution of 1 in 150 000.
2. The radioactive hormone was added 24 hours after the antiserum was mixed with the samples or standards and the tubes kept at room temperature for twenty four hours.

(iii) Calculation of results

The results were calculated in the way described for FSH (section 3.10.3)

CHAPTER 4. THE RELATIVE IMPORTANCE OF PROTEIN DIGESTED POST-RUMINALLY AND DIGESTIBLE ENERGY INTAKE IN THE RESPONSE TO LUPIN GRAIN.

4.1. Introduction

Considerable confusion exists in the literature concerning the component or components in lupin grain responsible for increasing ovulation rate in the ewe. From their original experiments Knight *et al.,* (1975) concluded that "...the effect of lupin supplementation is not simply explicable in terms of its input of either nitrogen and/or energy but may be related to a significant amount of the lupin protein escaping ruminal proteolysis". However, attempts to confirm this suggestion have led to different conclusions regarding the importance of protein digested post-ruminally in this response. Corbett and Edey (1977) for example, were unable to increase ovulation rate in ewes by feeding casein which had been treated with formaldehyde to reduce degradation in the rumen and concluded that a change in protein intake was likely to have only a small effect on ovulation rate and that energy was the major limiting factor. In contrast, work by Fletcher (1981) and Davis *et al.*, (1981) have indicated significant effects of protein on ovulation rate, but at different levels of digestible energy intake. As suggested by Smith (1985), much of the confusion concerning the role of protein digested post-ruminally could be explained if the response to protein is threshold in nature. However in the same paper Smith (1985) provided experimental data that showed that ovulation rate could also be

influenced by short-term changes in energy intake, suggesting that for a maximum response, increases in both protein and digestible energy are required. At the same time,Teleni *et al.*, (1984) have argued that the lupin response is due entirely to increases in digestible energy intake as they were able to show increases in ovulation rate following intravenous infusions of glucose. However this experimental approach does not yield unequivocal data as infusions of glucose have been shown elsewhere to markedly increase insulin mediated uptake of amino acids from the blood (see review by Trenkle, 1980), thus infusions of glucose in effect could be simply mimicking protein. To resolve these questions a systematic study is needed to critically examine the relative importance of the increases in protein digested post-ruminally and digestible energy intake associated with feeding lupin grain. The aim of the present study therefore was to determine the relative importance of these components in this response.

4.2. Experimental procedure

Five groups of 40 ewes were maintained outdoors under feedlot conditions and fed daily a maintenance ration of wheaten hay. Four groups were supplemented with either lupin grain, formaldehyde treated casein, wheat starch or formaldehyde treated casein plus wheat starch. The last three supplements were calculated to supply equivalent amounts of protein post-ruminally and/or digestible energy to that provided by feeding lupin grain. The amount of protein supplied post-ruminally and the digestible energy content of the four supplements is shown in Table 4.1. Dry matter

Table 4.1. The digestible energy and post-ruminal protein content of the four supplements.

Supplement	Amount (g/day)	Digestible energy * (MJ/day)	Estimated post-ruminal protein (g/day)**
lupin	500	6.3	62
casein	160	3.5	55
starch	415	6.3	<1
casein + starch	160 + 185	3.5 + 2.8 (6.3)	55 + <1

* Calculated from dry matter organic matter digestability (MAFF 1975).
** Crude protein x *in vitro* degradability values (see text for details).

digestibility was determined *in vitro* (Tilley and Terry, 1963) and crude protein content by micro- Kjeldahl nitrogen (x 6.25). Casein was treated with formaldehyde (section 3.10.) to render its degradability in the rumen similar to lupin protein.The effectiveness of this treatment in increasing the amount of protein digested post-ruminally has been demonstrated previously in nitrogen retention and wool growth studies (Hemsley *et al.*, 1973). In the present study, the degradability of the treated casein and lupin protein were checked *in vitro* (Hemsley *et al.*, 1973; section 3.10.1.2) and found to be similar (37.6 $\pm$ 5.3% and 40.6 $\pm$ 6.7%, respectively; mean $\pm$ s.e.m., n = 6). Part (30%)of the maintenance ration was pelleted to incorporate the supplements. Pellets were fed out once daily in troughs for 14 days commencing on day 4 of the synchronised cycle. Animals were adapted to the supplements by feeding pellets in increasing amounts for the first seven days.

4.3. Results

4.3.1. Liveweight change

All groups had recorded small increases in liveweight by the time of endoscopy. These were no significant differences in liveweight gain between the groups (Table 4.2.)

Table 4.2. The liveweight change of the control and supplemented groups.

Treatment	n	Liveweight change (g/day)
control	40	20 ± 12
lupin	40	30 ± 7
casein	39	28 ± 13
starch	40	16 ± 9
casein + starch	40	26 ± 8

Values are mean ± s.e.m.

4.3.2. Ovulation rate

The ovulation rate of the control and supplemented groups is shown in Table 4.3. Supplementation with lupin grain significantly increased ovulation rate by 0.4 ovulations per ewe by increasing the proportion of twin ovulations. Similar increases in ovulation rate resulted from increasing the amount of protein digested post-ruminally in the casein and casein plus starch supplemented groups. Increasing digestible energy intake alone however did not increase ovulation rate.

4.4. Discussion

The present study confirms previous reports that feeding lupin grain for the second half of the sheep oestrous cycle can increase ovulation rate (Oldham and Lindsay, 1984; Stewart and Oldham, 1986). The data also shows that the lupin response can be mimicked by increasing the amount of protein digested post-ruminally to a level similar to that achieved by lupin feeding. These data, obtained using formaldehyde treated casein, gives strong support to previous reports indicating that changes in the level of protein intake can influence ovulation rate (e.g. Torell *et al.*, 1973; Davis *et al.*, 1981; Fletcher, 1981) and clearly shows that the response to lupin grain could be explained by the amount of lupin protein that is digested post-ruminally (Knight *et al.*, 1975). Whether this response is due to an improvement in the animal's protein status or is initiated by an increase in

Table 4.3. The ovulation rate of the control and supplemented groups

Treatment	n	Ewes with n corpora lutea.			Ovulation rate
		0	1	2	
control	40	2	33	5	1.08 ± 0.07
lupin	40	1	19	20	1.48 ± 0.09 **
casein	39	0	21	18	1.46 ± 0.08 **
starch	40	4	29	7	1.08 ± 0.09
casein + starch	40	2	20	18	1.40 ± 0.08 **

Values are mean ± s.e.m.
**$P < 0.01$ compared with control .

the uptake of one or more essential amino acids from the gut remains to be determined and is the subject of a later experiment described in this thesis. However, the present study does demonstrate that the lupin response is most likely to be nutritionally mediated and not the result of some specific factor present in lupin grain. Smith (1985) has suggested that the ovulatory response to post-ruminal protein is threshold in nature. The amount of protein required to trigger this response therefore would depend to a large extent on its degradability in the rumen. Failure to meet the threshold may partly explain the confusion in the literature concerning the role of protein in the control of ovulation rate. The exact amount of protein required to initiate the ovulatory response in the present study is uncertain. However, since wheaten hay via ruminal bacterial protein synthesis is thought to supply sufficient protein post-ruminally to meet maintenance requirements (Egan and Walker, 1975), it would appear that protein in excess of this requirement is required if ovulation rate is to be increased. Such a suggestion is consistent with the conclusion by Fletcher(1981) that additional protein can increase ovulation rate providing that the initial intake is close to maintenance.

The present study provides no evidence to support the view that the increase in digestible energy intake associated with feeding lupin grain is important in the ovulatory response. This is in accord with reports from other workers who have undertaken ovulation rate studies with ewes fed lupin and cereal grains (e. g. Rizzoli *et al.*, 1976; Knight, 1980; Denney, 1983). On the basis of data derived form several studies Smith (1985) has formulated equations which describe the relationship between energy intake and the percentage of ewes with multiple ovulations as continuous.

In the present study there was an apparent increase of 6 % in the percentage of ewes with two ovulations in the starch supplemented group which agrees closely with a predicted value of 5 % from these equations, confirming that increases in energy intake of this magnitude are unlikely to trigger the response to lupin grain. However, much higher levels of digestible energy intake can increase ovulation rate in the short-term (e.g. Gunn, 1982; Smith 1985). It is perhaps incorrect though, to consider short-term increases in energy and protein intake as having separate effects on ovulation rate as up to half of the animal's glucose requirement is met by amino acids via gluconeogenesis (Armstrong and Hutton, 1975). To explain the continuous nature of the ovulatory response to digestible energy intake and the discontinuous or threshold nature of the response to protein (Smith, 1985) therefore, it is proposed that the level of energy intake determines the extent to which amino acids are catabolised in gluconeogenesis. Evidence for this effect comes form several studies in the sheep (e.g. Nissen and Ostaszewski, 1985; Orskov and Hovell, 1986; Mahyuddin and Teleni, 1988). It follows, therefore, that the amount of protein required to increase ovulation rate depends not only on the amount of protein required to meet the threshold requirement but also on the extent to which amino acids are used for gluconeogenesis. On the basis of this supposition, a ewe whose digestible energy intake is in excess of that required for maintenance would require less protein to increase ovulation rate than one fed at maintenance. Thus, at very high levels of energy intake sufficient protein may be available from microbial and dietary pools to increase ovulation rate. However, at relatively low levels of energy intake it may be necessary to increase the amount of protein digested post-ruminally to increase ovulation rate. Within any given flock, maintenance energy requirements are likely to be normally

distributed. Therefore, while most sheep would have a similar energy requirement and therefore exhibit a similar protein threshold, some ewes would require increased or reduced amounts of protien to increase ovulation rate. This would explain both the threshold response of ovulation rate to protein intake and the continuous nature of the lesser response to energy intake (Smith, 1985). The current proposal would also provide an alternative explanation as to why intravenous infusions of glucose can also increase ovulation rate (Teleni *et al.,* 1984) as glucose could also be expected to spare amino acids from gluconeogenesis which, in itself, may be sufficient to trigger an increase in ovulation rate. However, it should be noted that these workers, on the basis of subsequent studies of glucose kinetics in the ewe (Leury *et al.*, 1986), have concluded that glucose infused intravenously "may not necessarily mimic the lupin response".

Increases in the amount of protein digested post-ruminally also provides an explanation for the dynamic effect of flushing proposed by Coop (1966b) together with other reports that feed quality (Rattray *et al.*, 1978, 1980, 1981) and feed intake (Gunn *et al.*, 1979; Rhind *et al.*, 1985) can increase ovulation rate. The failure to acheive the threshold for protein available to be digested post-ruminally under various diets would explain much of the confusion in the literature concerning the existance of a separate dynamic effect. As proposed by Corbett and Edey (1977), failure to demonstrate a true flushing response could also be due to the basal ration supplying sufficient protein to meet the threshold requirement. Alternatively, ovulation rate may already be maximised because of an animal's endogenous reserves of protein and energy as a result of its previous nutrition ("static effect", Coop 1966b) and this status would unlikely to be influenced

markedly by changes in short-term nutrition unless these are particularly severe (e.g. fasting, Killeen, 1982). However such proposals may not provide a complete explanation since in Oldham and Lindsay's (1984) experiment it was found that it was still possible to increase ovulation rate in ewes in "forward store to fat" condition which grazed a pasture of sub-clover considered not to be limiting in terms of energy. Hence it may be that an animal's protein status has a more general effect on ovulation rate. In particular, the partitioning of post-ruminal protein within the animal may act as a homeostatic mechanism which regulates ovulation rate. Hence, the amount of protein digested post-ruminally may not only determine whether ovulation rate is to be increased, but also if it is to be maintained or decreased. Such a suggestion is not unreasonable, since the ratio between protein and energy intake has been shown to be important in the homeostatic control of feed intake in ruminants (see review by Egan, 1980).

In summary, the present study clearly demonstrates that increases in ovulation rate in ewes fed lupin grain for less than one cycle can be explained by an increase in the amount of protein digested post-ruminally associated with feeding but not by the increase in digestible energy intake. The amount of protein required to trigger this response however, is likely to be influenced by an animal's overall energy intake together with its body reserves of protein and energy. In particular, it is suggested that post-ruminal protein acts as a short-term regulator which matches the animal's ovulation rate to its "nett nutritional status" (Lindsay, 1976).

CHAPTER 5. THE RELATIVE IMPORTANCE OF ESSENTIAL AMINO ACID UPTAKE COMPARED WITH PROTEIN *PER SE* IN THE RESPONSE TO LUPIN GRAIN.

5.1. Introduction

In the previous experiment, it was demonstrated that increases in ovulation rate in lupin-fed ewes can be mimicked by using formaldehyde treated casein to increase the amount of protein digested post-ruminally. Whether this response was due to protein *per se* or to an increase in the uptake of one or more amino acids from the gut remained undetermined. Physiologically, there are reasons to believe the response is more likely to be a consequence of an effect of individual dietary amino acids rather than protein *per se.* Support for this notion comes from studies in rats, which have shown the dietary intake of tyrosine and tryptophan influences the turnover of dopamine and serotonin, respectively, in the brain (see review by Wurtman, 1976). Both these neurotransmitters have been implicated in the control of gonadotrophin secretion (Kamberi *et al.*, 1970, 1971a, b). Further evidence to support the suggestion that specific amino acids may influence the ovulatory response to protein also comes from a previous study (Nottle, 1982). In this experiment weaner sheep were fed a basal ration of wheaten hay which was supplemented with either formaldehyde-treated casein or gelatine to increase the availability of protein post-ruminally (Ferguson *et al.*, 1967). Both proteins increased voluntary intake

of the basal ration with the increase in feed intake being greater with casein than with gelatine. This appparent difference was interpreted as indicating that the mechanism controlling feed intake was responsive to amino acid supply as gelatine is relatively deficient in essential amino acids compared with casein (Block and Weiss, 1956). This suggestion was supported by the finding that the infusion of a small amount of essential amino acids into the duodenum could increase feed intake in the gelatine-supplemented group (Nottle, 1982). On the basis of these data it could be argued that if one physiological process, namely, the control of feed intake, can be influenced by amino acid supply then it may also be possible to influence ovulation rate in a similar manner. If so, supplementation of gelatine-fed ewes with post-ruminal infusions of one or more amino acids thought to be limiting in this regard may provide a model whereby the role of amino acids in the control of ovulation rate could be investigated. The present study therefore was undertaken with this viewpoint in mind and served as a preliminary investigation designed to determine whether the response to protein described in the previous chapter was to increases in protein *per se* or could be influenced by altering the supply of amino acids post-ruminally.

5.2. Experimental procedure

The design of the experiment was similar to that in the previous chapter. Four groups of 40 ewes were maintained outdoors under feedlot conditions and fed daily a maintenance ration of wheaten hay. Three groups were supplemented with either 500g of lupin grain or isonitrogenous amounts of

Table 5.1. The amino acid composition of lupin grain, casein and gelatine.

Amino acid	lupin	casein	gelatine
* alanine	3.14	2.74	10.07
arginine	10.36	3.71	8.52
aspartic acid	9.88	5.74	6.31
cystine	1.36	0.35	0.22
glutamic acid	24.17	20.81	11.18
glycine	3.99	1.85	24.23
* histidine	2.63	2.65	0.88
* isoleucine	3.68	5.83	1.77
* leucine	6.41	8.92	3.54
* lysine	4.62	7.24	4.43
* methionine	0.60	2.91	1.00
* phenylalanine	3.43	5.12	2.43
proline	3.47	10.9	14.16
serine	4.90	5.56	4.42
* threonine	3.35	3.96	2.89
* tryptophan	0.90	1.32	0.00
tyrosine	3.37	5.56	1.00
* valine	3.78	6.54	3.10

Values are expressed as a percentage of the protein in the dry matter.
* Considered to be essential in the ruminant
Source: Leche et al., (1984); Block and Weiss (1956)

5.3.1. Liveweight change

All groups had recorded small increases in liveweight by the end of the experiment. There were no signifcant differences in liveweight gain between the groups (*P* > 0.1; Table 5.2.)

5.3.2. Ovulation rate

The mean ovulation rate of the control and supplemented groups is shown in Table 5.3. Supplementation with lupin grain significantly increased ovulation rate. Increases in ovulation rate in lupin-fed ewes were similar to that in the previous study and were the result of an increase in the proportion of ewes with two ovulations. Increasing the amount of protein digested post-ruminally in the casein supplemented group also increased ovulation rate. Supplementation with gelatine resulted in an ovulation rate which was intermediate and not significantly different from either the control or other supplemented groups. However, when this analysis was performed on a per ewe ovulating basis the difference between the gelatine-supplemented and the control group was significant and the apparent difference in ovulation rates between gelatine and the other supplemented groups was no longer evident (Table 5.3.).

Table 5.2. The liveweight change of the control and supplemented groups.

Treatment	n	Liveweight change (g/day)
control	40	35 ± 14
lupin	39	22 ± 15
casein	40	37 ± 13
gelatine	40	46 ± 15

Values are mean ± s.e.m.

Table 5.3. The ovulation rate of the control and supplemented groups.

Treatment	n	Ewes with n corpora lutea			Ovulation rate	
		0	1	2	per ewe present	per ewe ovulating
control	40	2	31	7	1.13 ± 0.07 [a]	1.18 ± 0.06 [a]
lupin	39	1	17	21	1.51 ± 0.09 [b]	1.55 ± 0.08 [b]
casein	40	0	20	20	1.50 ± 0.08 [b]	1.50 ± 0.08 [b]
gelatine	40	3	22	15	1.30 ± 0.10 [a,b]	1.41 ± 0.08 [b]

Values are mean ± s.e.m.
Within each column means with different superscripts are significantly different ($P < 0.05$).

5.4. Discussion

In the present experiment, supplementation with gelatine resulted in an ovulation rate which was intermediate and not different from that either for the control or the other supplemented groups. However, when the data for ovulation rate was analysed on a per ewe ovulating basis the ovulation rate of the gelatine group did differ significantly from that for the control group. The exclusion of non-ovulators in the analysis of ovulation rate has been suggested by several workers (Oldham, 1980; Scaramuzzi and Radford, 1983) and appears to be valid in the present study since gelatine tended to increase ovulation rate and was therefore unlikely to have been responsible for the higher number of non-ovulators in this group.

The present study confirms the finding in the previous study that the increase in ovulation rate in lupin-fed ewes can be explained by an increase in the amount of protein digested post-ruminally. The experiment also provides further information about the effect of lupin grain and protein in particular, in regard to the control of ovulation rate. The current finding that gelatine and casein can increase ovulation rate to a similar extent demonstrates that the response to protein described in the previous chapter is to protein *per se* in the first instance. In other words, ovulatory responses to increases in post-ruminal protein are unlikely to be influenced by differences in the amino acid composition of the particular protein fed. This finding supports the view that the ruminant animal has evolved a pattern of usage of amino acids which is different compared with the amino acid requirements of non-ruminants (see review by Egan, 1980). Specifically,

the use of individual amino acids by the ruminant animal appears to be linked to the supply of microbial protein which is generally considered not to be as well balanced in relation to the amino acid requirements of non-ruminants (Egan, 1980). This does not exclude the involvement of individual amino acids in the response to lupin grain. Rather, it is suggested that their importance is not readily demonstrated using the present experimental approach for the following reasons. Firstly, given that there exists a threshold for protein digested post-ruminally (Smith, 1985), then it is also possible that individual responses to individual amino acids are also threshold in nature. Hence, in much the same way as energy was suggested to preferentially spare amino acids from being catabolised in gluconeogenesis (see previous chapter), increasing the general supply of gluconeogenic amino acids may spare specific gluconeogenic amino acids such as tyrosine and tryptophan and lower the threshold requirement for any specific activities they may have. Therefore, even though proteins such as gelatine may be deficient in certain amino acids, the additional amount made available for post-ruminal digestion together with contributions from microbial protein and other endogenous sources may be sufficient to trigger an increase in ovulation rate under conditions of high protein intake. Secondly, the uptake of tyrosine and tryptophan by the brain has been shown to be actively promoted by increases in carbohydrate intake via insulin secretion and influenced by the ratio of these amino acids to the neutral amino acids (see review by Wurtman,1976). An excess of gluconeogenic amino acids may also therefore be acting via insulin secretion to promote the uptake of these thereby lowering their threshold requirement even further. Evidence for such an effect in the ewe comes

from a recent study (Hinch and Roelofs,1986) showing that insulin infused intravenously between days 10-14 of the cycle can increase ovulation rate in Merinos fed sorghum. However, whether this effect is similar to that for lupin grain is uncertain since insulin did not increase FSH levels in this experiment, whereas lupin grain did (G. N. Hinch-personal communication). Hence, insulin may be acting via a different mechanism to influence ovulation rate. Possibly, insulin may be exerting direct effects on the ovary since insulin and insulin-like growth factors have been reported to directly effect the developing follicle (see review by Tsafriri, 1988). The fact that glucose infused intravenously also increases ovulation rate (Teleni *et al.*, 1984) can be interpreted to be consistent with this view since this experimental approach could also be expected to increase insulin secretion .

In conclusion, the present study suggests that differences in the amino acid acid profile of the protein digested post-ruminally do not appear to influence the response to protein described in the previous chapter. Specific amino acids may be important mediators in the lupin response but an experimental approach different from that in the present study is required to determine this. Post-ruminal or intravenous infusions of individual amino acids may provide one such approach, however the design of such an experiment would necessarily be complex to allow for the interaction between amino acids . Alternatively, further elucidation of the role of centrally acting neurotransmitters in the control of ovulation and the use of specific agonists and antagonists of these may provide useful insights into this question .

CHAPTER 6. THE EFFECT OF SHORT-TERM SUPPLEMENTATION WITH LUPIN GRAIN ON OVULATION RATE IN EWES INDUCED TO OVULATE DURING THE LUTEAL PHASE.

6.1. Introduction.

There is now compelling evidence based on the results of several studies using a variety of different approaches including unilateral ovariectomy (Land, 1973; Findlay and Cumming, 1977) and the administration of exogenous hormones (e.g. PMSG; Robinson, 1959; Bindon *et al.*, 1971; Allison, 1975) to support the view that the final decision regarding ovulation rate in the sheep is made near the time of luteolysis. By contrast, the study by Oldham and Lindsay (1984) suggests lupin grain needs to be fed for the last six days of the oestrous cycle including the follicular phase to increase ovulation rate. This conclusion may not be entirely correct however, since in their experiment ewes were fed lupin grain until they were detected in oestrus, a period of sufficient length to have included luteolysis. The possibility remains therefore, that feeding lupin grain over the very short, putative period prior to luteolysis could be all that is required to increase ovulation rate. The present study therefore was undertaken to test this hypothesis that is , that the response to lupin grain is determined late in the luteal phase near the onset of luteolysis. To do this animals were fed lupin grain for seven days commencing on day 3, 7 or 11 of the oestrous cycle and were then induced to ovulate on the sixth day using PG . PG injection has been widely used as a means of synchronising luteal

regression between ewes to facilitate various studies including the monitoring of preovulatory growth (e.g. Driancourt and Cahill, 1984), but it has not been determined whether the use of PG at this stage of the cycle interferes in any way with the ovulatory response to lupin grain. The present study therefore, also addresses this question.

6.2. Experimental procedure.

The design of the experiment is shown in Figure 6.1. Six groups of 40 ewes were maintained outdoors under feedlot conditions and fed daily a maintenance ration of wheaten hay. Three groups were also fed 500g of lupin grain for 7 days commencing on either days 3, 7 or 11 of the oestrous cycle. Oestrus was synchronised by means of intravaginal progestagen sponges inserted for twelve days (section 3.4). Supplemented groups were adapted to lupins by feeding these in increasing amounts for the first three days. Luteolysis was induced by injecting PG on the sixth day of supplementation. The remaining three groups served as corresponding controls.

6.3. Results.

6.3.1. Liveweight change

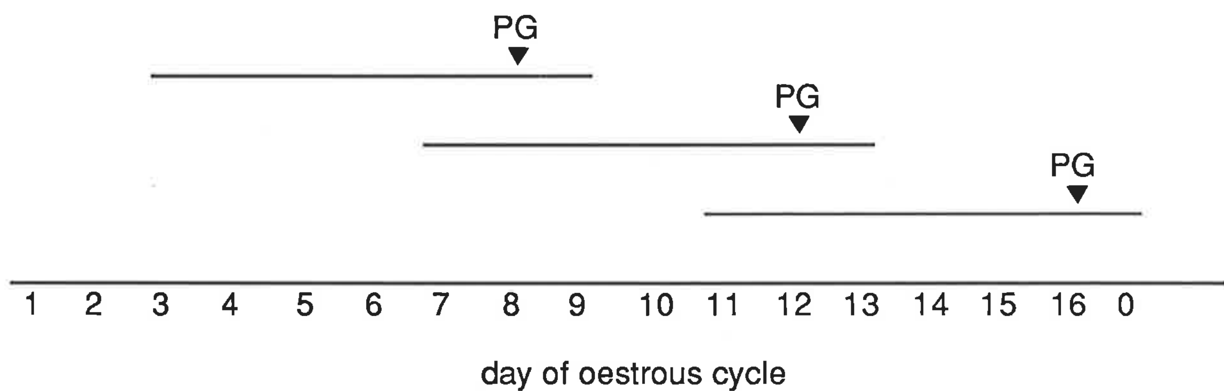


Figure 6. 1. The design of the experiment. Three groups of 40 ewes were fed 500g of lupin grain per ewe/per day for seven days commencing on either days 3, 7 or 11 of the oestrous cycle. Horizontal lines represent the periods of supplementation. Oestrus was synchronised by means of intravaginal progestagen sponges inserted for twelve days. Luteolysis was induced on the sixth day of supplementation by PG injection. Three other groups served as corresponding controls.

Table 6.1. The liveweight change (g/day) of the control and lupin-fed groups

Treatment	Day of oestrous cycle		
	3 - 9	7 - 13	11 - 0
control	10 ± 13	16 ± 14	14 ± 13
lupin	8 ± 14	15 ± 17	23 ± 14

Values are mean ± s.e.m. of 40 ewes.

Both the supplemented groups and their corresponding controls recorded similar increases in liveweight for the experiment ($P > 0.1$; Table 6.1.).

6.3.2. Ovulation rate

The mean ovulation rate of the control and lupin-fed groups is shown in Table 6.2. Supplementation with lupin grain significantly increased mean ovulation rates regardless of when in the oestrous cycle feeding commenced. This was due to an increase in the proportion of ewes with twin ovulations ($P < 0.01$). There were no significant differences in ovulation rate between the three control groups or between the three supplemented groups.

6.4. Discussion

The present study has demonstrated that supplementation with lupin grain for seven days followed by the induction of luteolysis with PG six days after the commencement of feeding can increase ovulation rate. This response did not apear to depend on when in the oestrous cycle supplementation commenced or at what stage of the luteal phase luteolysis was induced. These findings lend good support to the hypothesis developed at the beginning of this study that feeding lupin grain immediately prior to luteolysis can increase ovulation rate.

Table 6.2. The ovulation rate of the control and lupin-fed groups.

Treatment	Day of oestrous cycle		
	3 - 9	7 - 11	11 - 0
control	1.03 ± 0.06	1.08 ± 0.08	1.10 ± 0.07
lupin	1.43 ± 0.08 **	1.53 ± 0.08 ***	1.40 ± 0.08 *

Values are mean ± s.e.m. of 40 ewes.

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared with control.

The finding that ovulation rate was the same in all control groups and increased to a similar extent in the three supplemented groups regardless of when luteal regression occurred, suggests that there was always a sufficient number of follicles present in the ovaries throughout the oestrous cycle which were capable of ovulating. This view is consistent with that suggesting that folliculogenesis in the sheep is a continuous process that occurs throughout the oestrous cycle (Smeaton and Robertson, 1971; Turnbull *et al.*, 1977; Cahill *et al.*, 1979).

The results from a number of studies using a variety of different experimental approaches including unilateral ovariectomy (Land, 1973; Findlay and Cumming, 1977) and the electrocautery of follicles (Tsonis *et al.,* 1982) have been interpreted to mean that ovulation rate in the sheep is finalised near luteolysis. The present finding that supplementation with lupins immediately prior to the induction of luteal regression can increase ovulation rate suggests that short-term changes in the level of nutrition also influence ovulation rate at this stage of the oestrous cycle. Evidence to support this view is available from other nutritional studies. Smith *et al.*, (1979) for example, have reported coumestrol-lucerne can only reduce the incidence of multiple ovulations if it is fed prior to day 12 of the oestrous cycle. Similarly, Killeen (1982) has reported a decrease in ovulation rate for ewes fasted between days 7-13 of the oestrous cycle.

The present proposal that short-term changes in the level of nutrition need to have occurred by luteolysis if they are to influence ovulation rate, may provide an explanation for the failure of infusions of glucose and amino acids immediately prior to oestrus to increase ovulation rate (Goerke and

Dutt, 1972). In contrast, ovulation rate in sows and gilts can be increased by increasing energy intake immediately prior to (Zimmerman *et al.*,1960), or during oestrus (Heap *et al.*, 1967; Lodge andHardy 1968; Brooks *et al.*, 1972). Hence, there may be a fundamental difference between these two species in relation to when in the oestrous cycle ovulation rate is determined.

Since the effect of lupin grain appears to be manifest at the time of luteolysis, it is unlikely that lupins need to be fed other than at this stage. Rather, the finding by Oldham and Lindsay (1984) that feeding lupins for the last six days of the oestrous cycle can increase ovulation rate together with the results from the present study that feeding lupin grain immediately prior to the induction of luteolysis can also increase ovulation rate, suggests that lupins need to be fed only for a relatively short period immediately prior to luteolysis to increase ovulation rate. Evidence to support this suggestion comes from a recent study by Stewart and Oldham (1986). In their experiments, feeding lupin grain for 8 to 5 days prior to ovulation increased ovulation rate whereas supplementation from 4 to 1 days before ovulation did not. From their experiments, these workers concluded that there is a critical stage in the luteal phase during which time supplementation needs to take place in order for ovulation rate to be increased. The present finding that feeding lupin grain immediately prior to the induction of luteolysis at different stages of the luteal phase can increase ovulation rate suggests that this critical phase is not temporally related to when in the cycle lupin grain is fed but to luteolysis.

The present finding that the early induction of luteolysis does not interfere in the ovulatory response to lupin grain, would allow the method used by

Driancourt and Cahill (1984) to monitor preovulatory follicular growth to be used in lupin-fed ewes. This may provide insights into the follicular mechanism that operates in response to lupin feeding and warrants further investigation.

In conclusion, the present study provides good evidence to support the hypothesis that feeding lupin grain immediately prior to the onset of luteolysis can increase ovulation rate. This view is consistent with the results from other studies using a variety of different experimental approaches which have been interpreted to mean that ovulation rate in the sheep is finalised near luteolysis.

CHAPTER 7. THE MINIMUM PERIOD REQUIRED FOR FEEDING LUPINS TO INCREASE OVULATION RATE.

7.1. Introduction

In the previous chapter it was concluded that the response to lupin grain is initiated near the time of luteolysis. This suggestion is consistent with the recent finding by Stewart and Oldham (1986) that feeding lupin grain from approximately days 10 to 13 of the oestrous cycle can increase ovulation rate whereas feeding from day 14 to oestrus does not. The overlap between feeding lupin grain from day 10 to 13 in the experiments of these workers and feeding from day 11 in the previous study, suggests that feeding for less than four days may be sufficient to increase ovulation rate. Furthermore, since the half-life of lupin grain and particulate matter in general in the rumen is around 36 hours (Church, 1979), the relatively slow release of lupin protein post-ruminally may further shorten the number of days necessary for feeding. The present study therefore was undertaken to define further the minimum period required for feeding lupin grain in order to increase ovulation rate.

7.2. Experimental procedure

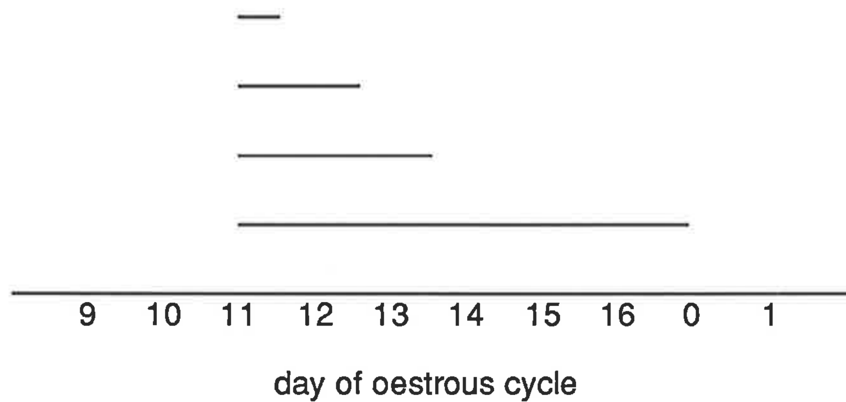


Figure 7. 1. The design of the experiment. Four groups of 40 ewes were fed 500g of lupin grain for 1, 2, 3 or 7 days commencing on day 11 of the oestrous cycle. Horizontal lines represent the periods of supplementation. Oestrus was synchronised by means of intravaginal progestagen sponges inserted for twelve days. A fifth group served as an unsupplemented control.

The design of the experiment is shown in Figure 7.1. and used animals from the previous experiment except those from the day 11-0 supplemented group because of the possibility of a carry-over effect of lupin grain (Gherardi and Lindsay, 1982; Stewart and Oldham, 1986). Ewes were re-assigned to five groups of 40 and had their oestrous cycles re-synchronised by injecting PG during the middle of the luteal phase. Groups were maintained under feedlot conditions and fed daily a maintenance ration of wheaten hay. Four groups were also fed 500g of lupin grain per ewe per day from day 11 of the synchronised cycle for 1, 2, 3 or 7 days. The fifth group served as an unsupplemented control.

7.3. Results

7.3.2. Liveweight change

The mean liveweight change of the control and supplemented groups is shown in Table 7.1. All groups had recorded similar increases ($P > 0.1$) in liveweight gain by the end of the experiment.

7.3.2. Ovulation rate

Table 7.1. The liveweight change of the control and lupin-fed groups.

Treatment	n	Liveweight change (g/day)
control	40	10 ± 9
day 11	40	14 ± 8
day 11 - 12	39	15 ± 11
day 11 - 13	40	3 ± 12
day 11 - 0	40	15 ± 9

Values are mean ± s.e.m.

Table 7.2. The ovulation rate of the control and lupin-fed groups.

Treatment	n	Ewes with n corpora lutea			Ovulation rate
		0	1	2	
control	40	1	33 (6)	6 (1)	1.13 ± 0.06
day 11	40	0	30 (5)	10 (2)	1.25 ± 0.07
day 11 - 12	39	1	27 (7)	11 (1)	1.26 ± 0.08
day 11 - 13	40	4	19 (5)	17 (1)	1.33 ± 0.10
day 11 - 0	40	2	25 (4)	13 (2)	1.28 ± 0.09

Values are mean ± s.e.m.
Values in brackets are the number of ewes within the classification given that had corpora lutea at an inappropriate stage of development.

The ovulation rate of the four supplemented groups tended to be 10-20% higher than the control group ($P > 0.1$; Table 7.2.). Within all six groups approximately 20% of animals had corpora lutea which were at an inappropriate stage of development. In other words, based on personal experience together with photographic records of the development of copora lutea in sheep (Oldham and Lindsay, 1980), a number of corpora lutea were judged as being either too early or too late in their development to be considered appropriate for that normally seen when laparoscopy is performed 5-6 days after oestrus The number of these animals in each group is shown in Table 7.2.

7.4. Discussion

The results of the present study are somewhat disappointing given the consistency of the increases in ovulation rate in the previous three experiments. The reason for the apparent failure of lupin grain to increase ovulation rate in the present study is almost certainly due to resynchronisation with PG causing a cycle of abnormal length. This was indicated by the observation at laparoscopy that some ewes had corpora lutea at inappropriate stages of development compared with those normally seen when laparoscopy is performed on days 5 and 6 of the oestrous cycle. In these ewes supplementation is unlikely to have coincided with that stage of the oestrous cycle deemed critical by Stewart and Oldham (1986) for lupin feeding to increase ovulation rate. This would explain the poor response to lupins in the present study.

A degree of luteal failure following oestrous synchronisation with PG has also been reported for superovulated goats (Armstrong *et al.*, 1983) but appears not to have been observed in non- superovulated sheep before.

Oestrus synchronisation with PG may have also been a contributing factor in the apparent failure of lupin grain to increase ovulation rate in other studies. Knight (1980) for example, reported that feeding lupin grain for 14 to 16 days did not increase ovulation rate in Romney ewes and suggested that injecting PG 4 and 14 days after the start of feeding to synchronise oestrus may have been responsible for this. PG may have also been responsible, in part at least, for the variability in the response to lupin feeding in the experiments of Stewart and Oldham (1986), since it was injected 14 days apart to induce 15 day cycles. In their experiments, responses to lupins were similar to that in the present study but because of a larger number of animals (c. 70) were shown to be statistically significant.

In conclusion, the present study warrants repeating using progestagen sponges to synchronise oestrus since there was the suggestion that feeding lupin grain for less than four days immediately prior to luteolysis can increase ovulation rate. However, at the time this experiment was undertaken similar studies were also being undertaken by Stewart and Oldham (1986) in Western Australia and it was decided to end this line of investigation.

CHAPTER 8. THE EFFECT OF SMALL DOSES OF FSH INJECTED BETWEEN DAYS 12-14 OF THE OESTROUS CYCLE ON OVULATION RATE.

8.1. Introduction.

The mechanism whereby the response to lupin grain is mediated within the ewe is unknown. Several workers have reported that feeding lupin grain for one or two cycles can increase the concentration of FSH in the plasma on day 13 of the cycle (Brien *et al.*, 1976; Davis *et al.*, 1981; Knight *et al.*, 1981). Small transient increases in the secretion of FSH are also thought to be responsible for the compensatory hypertrophy of the remaining ovary and the maintenance of ovulation rate following unilateral ovariectomy (Findlay and Cumming, 1977). That FSH is essential for the growth and maturation of follicles is well known, but direct evidence for a positive or causal relationship between endogenous FSH concentrations and ovulation rate has been equivocal (see reviews by Driancourt, 1987; Bindon and Piper 1986; Webb and Gauld, 1987). Indirect evidence however, is available from studies in which relatively large doses of FSH have been used to markedly increase ovulation rate in superovulation studies (e.g. Armstrong and Evans, 1983; Evans *et al.*, 1984; Nottle *et al.*, 1984). Furthermore, treatments which decrease ovulation rate, such as the injection of steroid-free follicular fluid (Cummins *et al.*, 1983; Cahill *et al.*, 1985) have also been shown to lower FSH. The present study therefore

was undertaken to determine whether injecting small doses of FSH around day 13 of the oestrous cycle could duplicate the ovulatory response to lupin feeding.

8.2. Experimental procedure.

8.2.1. Design

The experiment was conducted at the Mortlock Experimental Station and used six year old strong wool Merino ewes (Bungaree strain) which had an initial liveweight of 51 $\pm$ 0.4 kg (mean $\pm$ s.e.m.) and a condition score of 2.91 $\pm$ 0.04 (mean $\pm$ s.e.m.). Animals were assigned to four groups of 40 ewes. Twenty animals from each group were housed indoors in individual pens under conditions of natural lighting and fed daily a maintenance ration of chopped wheaten hay. The remaining twenty animals from each group were housed outdoors under feedlot conditions because of a lack of pen space. One group was supplemented with lupin grain at a rate of 500 g per ewe per day for 10 days beginning on day 8 of the oestrous cycle with increasing amounts for the first three days. Oestrus was synchronised by means of intravaginal progestagen sponges inserted for twelve days (section 3.4.). Two groups were injected with a total dose of 2 or 4 mg of FSH from day 12 to day 14 of the cycle.

8.2.2. FSH

The FSH (Folltropin) was a gift from Vetrepharm (London, Ontario, Canada). Its preparation and the measurement of its potency by radioreceptor assay have been described elsewhere (Armstrong and Opaskvy,1988). Folltropin is purified from porcine pituitaries and has a FSH biopotency of 4.2 U/ mg (1 U = 1 mg NIH-FSH-S1) and a LH biopotency of 0.022 U/mg (1 U = 1 mg NIH-LH-S1). A total dose of either 2 or 4 mg of FSH per ewe in sterile saline (1 mg/ml) was injected subcutaneously per ewe in a series of four injections on day12 (1700h), day 13 (0800 and 1700h) and on day 14 (0800h).

Twenty animals from each group were bled via jugular using venipuncture 3 x daily at 0700,1130 and 1600 hours from days 11 to 15 of the cycle to determine FSH levels. FSH injections were given immediately after a blood sample was taken. Because of a lack of freezing facilities, samples were bulked on a daily basis for individual animals. FSH was measured by radioimmunoassay as described in section 3.12.3. Follitrophin has been shown to cross-react in this assay (P.H. Sharpe, M. B. Nottle and D. T. Armstrong- unpublished results). As this assay has not been fully validated for porcine FSH , the level of immunoreactive FSH in the Folltropin-treated groups was measured purely to provide some indication of the effectiveness of these two treatments in raising circulating FSH.

8.2.3. Statistical analyses

Mean FSH concentrations for the four groups were compared using a two-way analysis of variance. Data were transformed to log 10 to equalise

variances before analysis . Individual means were tested using Duncan's new multiple range test .

8.3. Results.

There were no significant differences in liveweight gain or ovulation rate within treatments between those animals housed indoors and those maintained outdoors and the data for these two sub-groups were combined.

8.3.1. Liveweight change

All four groups recorded similar increases in liveweight for the experiment ($P > 0.1$; Table 8.1.).

8.3.2. Ovulation rate

The ovulation rate of the four groups is shown in Table 8.2. Supplementation with lupin grain significantly increased ovulation rate by increasing the proportion of ewes with two (and three) ovulations . A similar increase in the number of twin ovulating ewes was recorded by the 4 mg

Table 8.1. The liveweight change (g/day) of the control, lupin-fed and FSH treated groups.

Treatment	n	Liveweight change
control	40	23 ± 15
lupin	39	18 ± 26
2mg FSH	40	21 ± 13
4mg FSH	40	26 ± 12

Values are mean ± s.e.m.

Table 8.2. The ovulation rate of the control, lupin-fed and FSH treated groups.

Treatment	n	Ewes with n corpora lutea				Ovulation rate
		0	1	2	3	
control	40	1	27	12	0	1.28 ± 0.08
lupin	39	2	8	27	2	1.74 ± 0.10 *
2mg FSH	40	2	22	16	0	1.35 ± 0.09
4mg FSH	40	0	14	26	0	1.65 ± 0.08 *

Values are mean ± s.e.m.
* Significantly different from control ($P < 0.001$).

FSH treated group. The ovulation rate of the 2 mg FSH treated was not significantly different from the control group.

8.3.3. FSH

The mean FSH concentration on days 11 to 15 of the oestrous cycle for the four groups is shown in Table 8.3. Supplementation with lupin grain significantly increased FSH on day 13 of the cycle but not on any of the other days samples were taken.

Injecting Folltropin from days 12-14 of the cycle increased the concentration of immunoreactive FSH in the serum on days 13 and 14 of the oestrous cycle in both the 2 and 4 mg treated groups. The level of immunoreactive FSH was also increased in the 2 mg group on day 15 of the cycle.

Within the lupin-fed group those ewes with two ovulations had signifcantly higher levels of FSH on days 14 and 15 of the oestrous cycle (Table 8.4.) compared with those with one ovulation. Within the control group FSH did not differ on these days when a similar comparison was made (Table 8.5.).

8.4. Discussion

Table 8.3. The FSH concentration (ng/ml) of the control, lupin-fed and FSH - treated groups.

Treatment	Day of oestrous cycle				
	11	12	13	14	15
control	14.9 ± 0.7 [a]	13.4 ± 0.8 [a]	13.6 ± 0.8 [a]	14.0 ± 0.8 [a]	13.7 ± 0.6 [a]
lupin	13.1 ± 0.8 [a]	14.2 ± 0.9 [a]	16.6 ± 0.7 [b]	14.6 ± 0.6 [a]	13.7 ± 0.7 [a]
2mg FSH	15.6 ± 0.8 [a]	15.5 ± 0.8 [a]	17.8 ± 0.7 [b]	19.1 ± 0.8 [b]	16.4 ± 1.1 [b]
4mg FSH	13.2 ± 0.7 [a]	13.1 ± 0.7 [a]	22.9 ± 1.0 [c]	26.3 ± 1.1 [c]	15.7 ± 0.6 [a]

Values are mean ± s.e.m. of 20 ewes.
Within each column means with different superscripts are significantly different ($P < 0.05$).

Table 8.4. The FSH concentration (ng/ml) of the lupin-fed group classified according to number of corpora lutea.

n corpora lutea	Day of oestrous cycle				
	11	12	13	14	15
1	11.7 ± 1.0	13.8 ± 1.2	14.3 ± 1.3	11.88 ± 1.0	11.1 ± 0.4
2	13.8 ± 1.0	14.5 ± 1.2	17.0 ± 0.8	14.50 ± 0.6 *	13.5 ± 0.9 *

Values are mean ± s.e.m. of 7 and 13 ewes respectively.
* Significantly different compared to ewes with one corpora lutea ($P < 0.05$).

Table 8.5. The FSH concentration (ng/ml) of the control group classified according to number of corpora lutea.

n corpora lutea	Day of oestrous cycle				
	11	12	13	14	15
1	13.2 ± 1.0	13.4 ± 1.1	15.1 ± 1.1	14.4 ± 1.1	13.2 ± 0.8
2	10.6 ± 0.9	12.5 ± 1.0	14.6 ± 0.9	13.2 ± 1.9	14.5 ± 1.1

Values are mean ± s.e.m. of 12 and 7 ewes respectively.

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The present study confirms previous reports (Brien *et al.*, 1976; Davis *et al.*, 1981, Knight *et al.*, 1981) that feeding lupin grain can increase the concentration of FSH in the serum on day 13 of the oestrous cycle. In the present experiment mean FSH levels in the lupin-fed group were 22% higher at this stage of the cycle in the lupin-fed group compared with control values and is within the range of those values reported by the above workers. These increases are likely to be the result of an increase in the secretion of FSH from the pituitary since the metabolic clearance of FSH has been shown to be unaffected by nutrition (Findlay and Cumming, 1976). In the present study similar increases in circulating levels of FSH to those in the lupin-fed group were mimicked by injecting 2 mg of FSH but these injections failed to increase the ovulation rate and it was necessary to double the amount of FSH to increase ovulation rate. This increase was the result of more ewes with two ovulations and was not the result of a mild superovulatory response as has been reported previously with relatively low doses of PMSG (e.g. Bindon *et al.*,1971; Smith 1976). While it remains to be determined whether the nature of the FSH response achieved in the present study reflected the purity of the FSH porcine preparation used, or the injection protocol employed, the administration of FSH as in the present experiment, may prove useful in the development of more reliable methods for the induction of twinning and superovulation in the ewe.

Why it was necessary to raise the circulating level of FSH higher by exogenous means than that seen in the lupin-fed group to give a similar ovulatory response is uncertain. However, discussions based on

immunoreactivity are somewhat meaningless unless the question of biological activity- defined here as the ability to increase ovulation rate, is also addressed. Ideally, ovine FSH should have been used in the present study. However, at the time the experiment was undertaken, it was virtually impossible to obtain purified ovine FSH in the amounts needed from Australian sources and the use of overseas material was precluded by quarantine regulations. A similar result to that in the present study has been reported by Mc Natty and co-workers (1985) for ovine FSH. In their experiments the incidence of twin ovulations in Romney ewes was increased by hourly intravenous injections of NIAMDD-FSH-S15 (1.6ug/h) for 24 hours prior to a cloprostenol-induced luteolysis. Taken together, the results from their experiments and the present study strongly suggest that the increase in the concentration of FSH in the plasma that occurs around day 13 of the oestrous cycle in ewes fed lupin grain can increase ovulation rate.

Various FSH preparations have been used in recent times to induce superovulation in sheep (e.g. Evans *et al.*, 1984; Nottle *et al.*, 1984). It is generally agreed amongst workers that this effect is dose-dependent. That is, the higher the dose the greater the number of ovulations per ewe up until the point where it is no longer possible to increase ovulation further. Evidence for a dose dependent effect for ovulation rate in sheep treated with Folltropin was obtained in preliminary investigation (unpublished results, appended as appendix A). In this experiment, increasing the dose of Folltropin from 4 to 8 mg resulted in a degree of superovulation- defined here as more than three ovulations per ewe. A dose response curve for NIADDK-oFSH-16 infused intravenously around the time of luteolysis has

also been reported (Henderson *et al.*, 1988). A direct comparison between these two preparations in regard to the amount of each preparation required to duplicate the ovulatory response to lupin grain would provide further information concerning the biological activity of Folltropin. However, the present finding that treatment with 4 mg of Folltropin does not result in superovulation suggests that the ovulatory response obtained was the result of a physiological manipulation rather than a pharmacolgical one.

It has been suggested that similar increases in FSH secretion to those found in lupin-fed ewes underlie the increased ovulation rate in prolific breeds (see review by Bindon and Piper, 1986) and in twin ovulators within the same breed (McNatty *et al.*, 1985). These recent data and the present finding that the injection of relatively low doses of FSH at day 13 can increase the number of twin ovulations clearly support the assumption that an increased secretion of FSH at this particular stage of the cycle is an important determinant of ovulation rate. The failure of numerous studies in the past to reveal this relationship probably reflects the wide differences between individual animals in plasma concentrations of FSH. Hence statistical differences in mean FSH levels which correlate with increased ovulation rate, are only likely to be detected when large numbers of animals are sampled. Brown (1978) for example, has argued that a 20% increase in FSH above the threshold requirement for follcular growth can increase ovulation rate whereas the between animal variation in the concentration of FSH in sheep has been shown to contribute as much as 80% of the total variation in FSH measurements (Findlay and Cumming, 1976). Furthermore, Baird (1983) has suggested that because of the feedback

mechanisms operating in the intact animal, a difference in FSH levels may occur for a short period only when the system is perturbed. This "window" appears to occur around day13 of the cycle when the influence of the corpus luteum is waning and before sufficient oestradiol is secreted from the dominant follicle to inhibit FSH secretion.

How an increased secretion of FSH in lupin-fed ewes provides the stimulus for another follicle to become dominant is uncertain. At the same time as the present study was undertaken, McNatty *et al.*, (1985) reported that even smaller doses of FSH than those used in the present study, when injected intravenously at hourly intervals for 24 hours prior to the induction of luteolysis, can increase ovulation rate. As discussed previously, luteolysis is also the stage of the cycle by which recruitment of the preovulatory follicle is thought to be evident (Driancourt and Cahill, 1984). Thus, FSH may be acting to increase the number of follicles recruited. This is consistent with studies demonstrating that preovulatory enlargement and the induction of ovulation in anoestrous ewes requires FSH to prime a follicle before it can ovulate in response to exogenous LH (Oussaid *et al.*, 1982; McNatty *et al.*, 1984). However, Driancourt and Cahill (1984) have argued on the basis of earlier morphological studies (Brand and de Jong, 1973; Cahill *et al.*, 1979) that the reduction in the number of follicles within the preovulatory size range at recruitment is due to the regression of follicles that are already atretic prior to luteolysis. This conclusion is also supported by previous studies showing that in the late luteal phase the number of follicles containing LH receptors in the theca and granulosa cells (England *et al.*, 1981) or with intrafollicular oestradiol concentrations > 367 nM (McNatty, 1982) is equal to the ovulation rate at the subsequent ovulation. Hence

*Further clarification on this point awaits the accurate determination of when recruitment of the preovulatory follicle(s) occurs in the sheep. A similar case can also be made for selection. Until these two times are determined, discussions regarding the recruitment and selection of the preovulatory follicle(s) will continue to be speculative.The convention adopted in this thesis is the same as that used by Driancourt and Cahill (1984), namely, recruitment is the growth of follicles greater than 2 mm in diameter after the start of luteolysis with selection being the further growth of one or more of these resulting in ovulation.

recruitment may have already occurred before FSH is increased in the lupin-fed ewe.*

FSH may therefore be acting to increase the number of follicles selected to ovulate. A mean time for selection in the ewe has not been determined (see review by Driancourt *et al.,* 1985b). However, since it is possible to identify the preovulatory follicle soon after the start of luteolysis in at least a proportion of ewes (Driancourt and Cahill, 1984) selection may occur as early as luteal regression. Selection therefore may be influenced by an increased secretion of FSH at this stage. In particular, an increase in FSH secretion may convert a small healthy antral follicle into an "oestrogenic" one (Baird, 1983), thereby allowing it to escape the atresia-promoting effect of androgens produced at this stage. Support for this suggestion comes from studies in rodents which have shown that the induction/activation of the aromatase system is regulated by FSH (Erickson and Hseuh, 1978; Moon *et al.*, 1975; Leung and Armstrong, 1980). It may be therefore, that different follicles have different absolute requirements for FSH possibly because they are at slightly different stages of development (Lintern-Moore and Moore, 1979). Stated simply, the higher the secretion of FSH the more follicles that are likely to be selected.

Higher levels of FSH during the first half of the follicular phase may also be important in the response to lupin grain. Welschen and Dullart (1976) have suggested the atresia of antral follicles > 2 mm during the follicular phase is the result of the decline in FSH secretion at this time. In the present study FSH was higher in the lupin-fed group in those ewes with two ovulations on days 14 and 15 of the cycle . Hence, an increase in the level of FSH during the first half of the follicular phase may effectively rescue a follicle

from atresia. How it does this is uncertain. McNatty *et al.*, (1985) have reported that FSH can increase aromatase activity in granulosa cells of 3-4.5 mm diameter follicles to levels similar to those in cells from preovulatory follicles > 5 mm in diameter. One possible explanation therefore may be that FSH influences follicular viability by maximising oestradiol secretion.

These last two suggestions as to how FSH might be acting in the lupin-fed ewe may not be mutually exclusive. High levels of FSH may be required to induce aromatase activity at luteal regression which is maximised or maintained by FSH during the follicular phase. FSH may also be acting directly within the follicle to influence its viability, since it is present in higher concentrations in this follicle compared with any other (McNatty, 1982).

In conclusion the present study demonstrates that increases to circulating levels of FSH on days 13-14 of the cycle result in a similar ovulatory response to that in lupin-fed ewes. Whether this response is due to an increase in the number of follicles recruited or the number of these that are selected to ovulate cannot be determined from the present study. Alternatively, both mechanisms may operate in the lupin-fed ewe. In a recent study, Fry *et al.*, (1986) reported that ovulation rate in high liveweight ewes (medium-term nutrition) is the result of an increase in the number of follicles recruited together with an increase in the number of these selected to ovulate. The monitoring of preovulatory growth during the follicular phase following short-term supplementation with lupin grain would distinguish between these possibilities.

CHAPTER 9. THE EFFECT OF LUPIN GRAIN ON FSH SECRETION IN OVARIECTOMISED EWES.

9.1. Introduction

In the previous experiment, evidence was obtained to suggest that increases in ovulation rate in lupin-fed ewes are mediated by increases in FSH secretion. Whether these increases are initiated centrally at the hypothalamic/pituitary axis or are the result of changes in the sensitivity of this axis to the negative feedback effects of oestradiol and inhibin remains to be determined. Indirect evidence to suggest that the response is centrally mediated comes from studies which have demonstrated that improving the nutrition of undernourished, ovariectomised lambs can lead to a rapid increase in LH pulse frequency (Foster and Olster, 1985). However, lupin grain may be acting indirectly and involve changes in the sensitivity of the hypothalamic/pituitary axis to oestradiol. Knight *et al.*, (1981) for example showed that elevated FSH levels in lupin-fed ewes coincide with elevated oestrogen levels and have interpreted this to mean that lupin feeding elevates the threshold to the negative feedback between oestradiol and FSH. Increases in FSH secretion could also be due to a reduction in inhibin secretion (Goodman *et al.*, 1981) or positive feedback from the ovary involving the secretion of the recently discovered substance, activin (Ling, 1986). The present study therefore was undertaken in an attempt to determine the origin of the change to FSH secretion in lupin-fed ewes. For this purpose ewes were ovariectomised and the effect of ovarian feedback

removed, to test the hypothesis that lupin grain acts directly at the hypothalmic/pituitary axis to increase FSH secretion.

9.2. Experimental procedure

9.2.1. Animals

Twenty ewes were paired according to liveweight and ovariectomised via mid-ventral laparotomy. Laparotomy was performed under general anaesthesia which was induced using pentothal and maintained using halothane/oxygen. Only ewes with one corpus luteum present at ovariectomy were used in the present study. Ewes were housed indoors under conditions of natural lighting and fed daily a ration of chopped wheaten hay calculated to meet maintenance energy requirements. Animals were allowed two weeks to recover from laparotomy and to adapt to conditions indoors.

9.2.2. Design

The experiment consisted of three one week periods. In the first week both groups were fed the maintenace ration. In the second week one group also received 500 g of lupin grain per head per day. In the third week these

animals were fed the maintenance ration only. Animals were bled 3 x daily for FSH via indwelling jugular catheters and samples bulked on a daily basis for individual animals. Serial samples for LH were obtained on the second day of each 7 day period by bleeding animals for 4 hours at 12 minute intervals. FSH and LH were determined by radioimmunoassay using procedures described previously (section 3.11.).

9.2.2. Statistical analyses

FSH concentrations were analysed using an analysis of variance of a split plot design.[1] LH parameters were analysed using a two-way analysis of variance. Hormone data not normally distributed were transformed to log 10 before analysis. Basal LH concentrations were computed from values not associated with either the ascending or descending portions of LH peaks. Mean LH levels represent the average of all 12 min. samples collected during the 4 h period. LH pulses were defined according to the method described by Baird *et al.*,(1981), i.e. a pulse was considered to have occurred when two consecutive values were higher than the two preceeding sample values and when the value of the highest exceeded the mean basal value by at least four times the coefficient of variation of the assay. LH pulse amplitude was defined as the highest point minus the basal LH concentration.

9.3. Results

[1] (GENSTAT, 1984)

One ewe was removed from the control group for health reasons. One ewe in the lupin-fed group refused to eat the lupin supplement and was not included in the results.

9.3.1. Liveweight change

Both groups had recorded small increases in liveweight by the end of the experiment (Table 8.1). There were no significant differences in liveweight gain between the two groups.

9.3.2. FSH

Mean FSH concentration did not differ significantly between the control and supplemented groups during the three week period (Table 9.2.). However, within the lupin-fed group, the concentration of FSH appeared to be higher in the lupin-fed group during the second week ($P < 0.1$; Figure 9.1.). This apparent increase in FSH secretion appeared to be due largely, to an increase FSH secretion in three ewes (nos. 22, 23 and 27; Figure 9.2.). However, as no significant differences in FSH were detected, either, between treatments or between weeks within treatments using the analysis of variance outlined above, it was not possible to test for differences in individual FSH secretion.

Table 9.1. The liveweight change of the control and lupin-fed groups.

Treatment	n	Liveweight change g/day
control	9	26 ± 15
lupin	9	31 ± 10

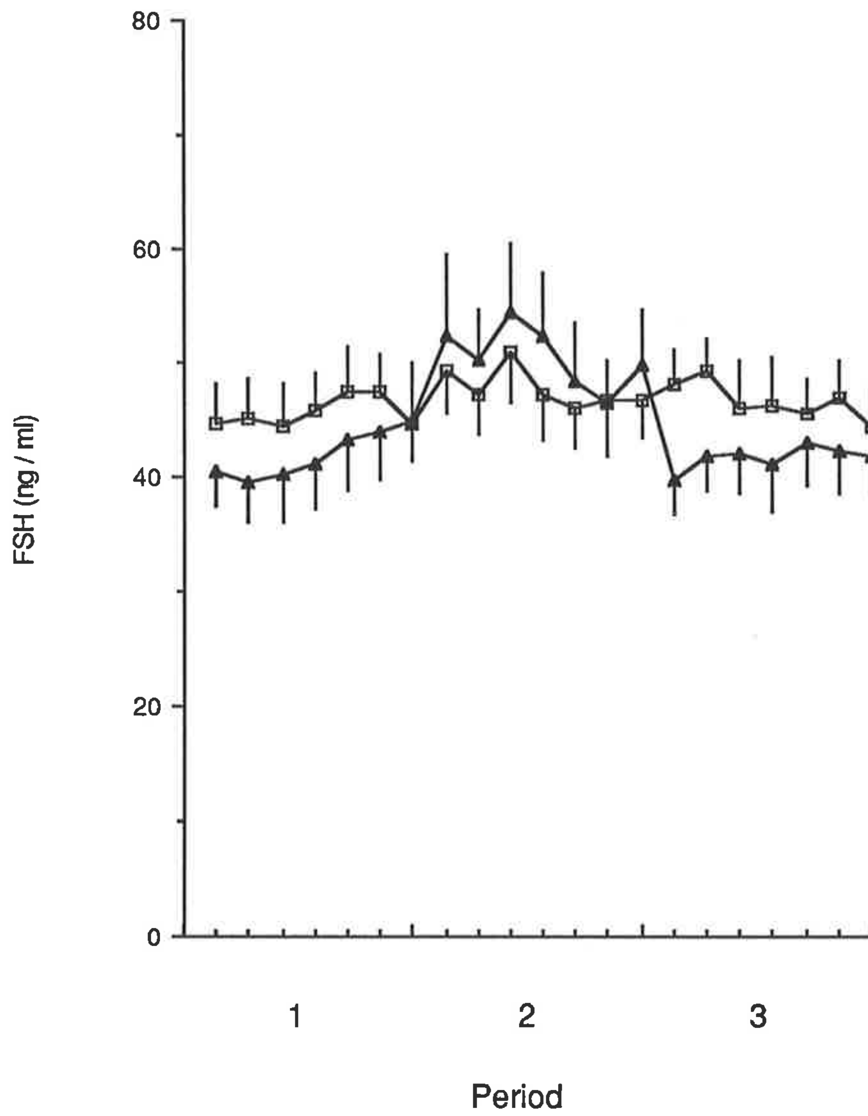
Values are mean ± s.e.m.

Table 9.2. The FSH concentration of the control and lupin-fed groups.

Treatment	FSH (ng/ml)		
	period 1	period 2	period 3
control	45.6 ± 3.3	47.8 ± 3.6	46.6 ± 3.4
lupin	41.8 ± 3.9	50.5 ± 5.2	41.7 ± 3.3

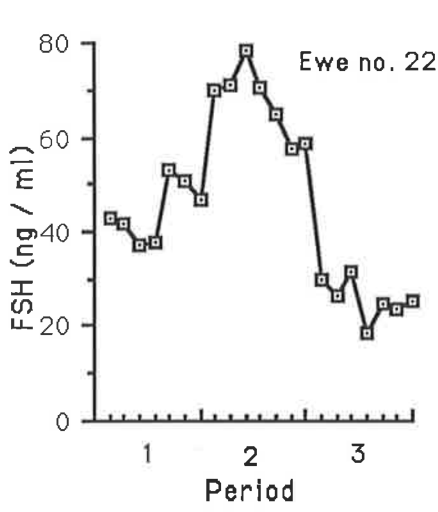
Values are mean ± s.e.m. of 9 ewes

Figure 9. 1. The concentration of FSH in the peripheral serum of the control (□) and lupin-fed groups (▲). Blood samples were collected three times daily and the serum bulked on a per ewe basis. Lupin-fed ewes were fed 500g lupins/ewe/day for seven days from the start of Period 2. Values are the mean ± s. e. m. of 9 ewes.



80
60
40
20
0
FSH (ng / ml)
1
2
3
Period

Figure 9.2. The concentration of FSH in the peripheral serum of individual ewes from the lupin-fed group. 500g lupins/ewe/day were fed for 7 days from the start of Period 2.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FSH (ng / ml)
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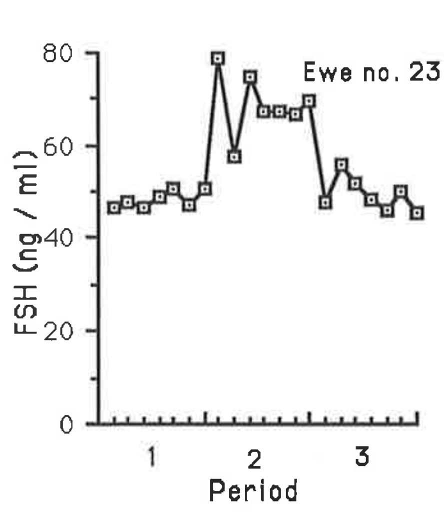
1
2
3
Period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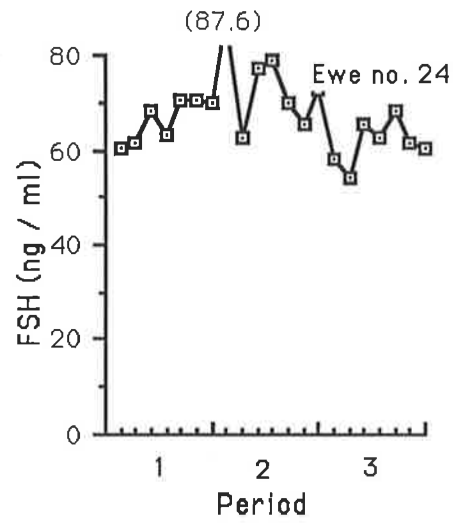
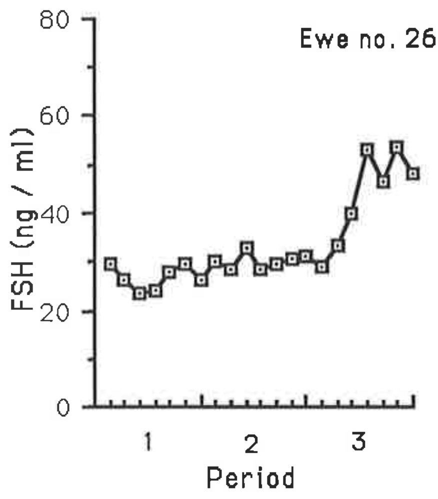
20
0
FSH (ng / ml)
Ewe no. 23
1
2
3
Period


(87.6)
80
60
40
20
0
FSH (ng / ml)
Ewe no. 24
1
2
3
Period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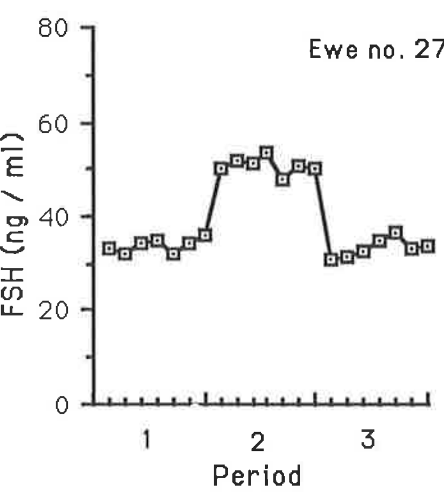
60
40
20
0
FSH (ng / ml)
Ewe no. 26
1
2
3
Period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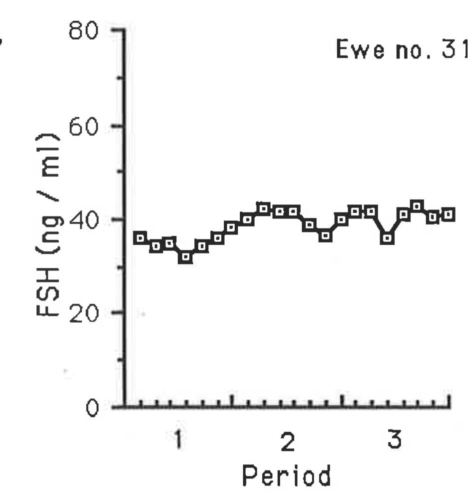
40
20
0
FSH (ng / ml)
Ewe no. 27
1
2
3
Period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60
40
20
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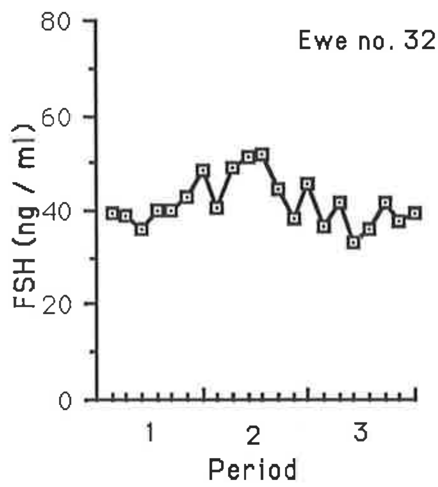
FSH (ng / ml)
Ewe no. 31
1
2
3
Period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FSH (ng / ml)
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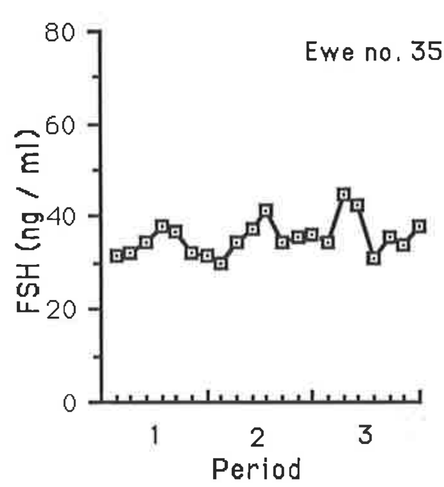
2
3
Period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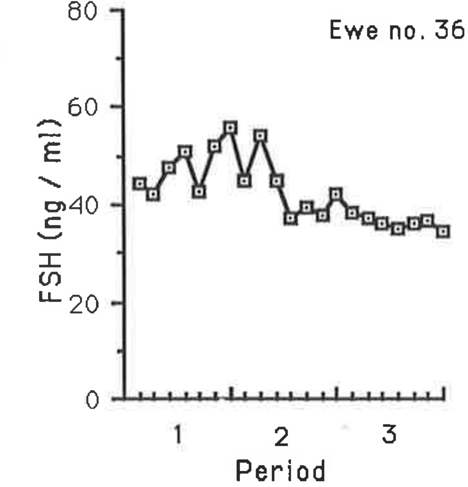
FSH (ng / ml)
Ewe no. 35
1
2
3
Period


80
60
40
20
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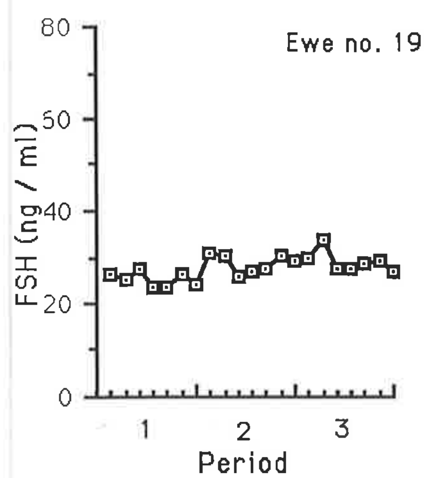
FSH (ng / ml)
Ewe no. 36
1
2
3
Period

Figure 9.3. The concentration of FSH in the peripheral serum of individual ewes from the control group.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FSH (ng / ml)
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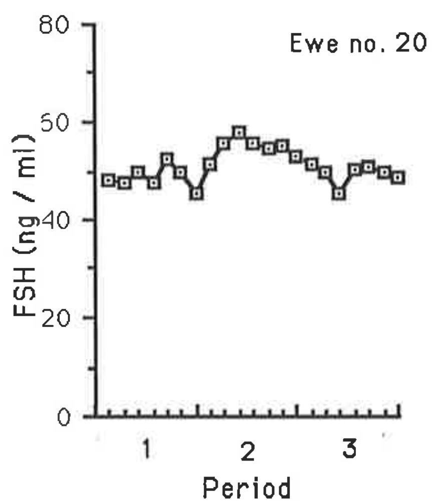
50
40
20
0
1
2
3
Period


Ewe no. 20
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20
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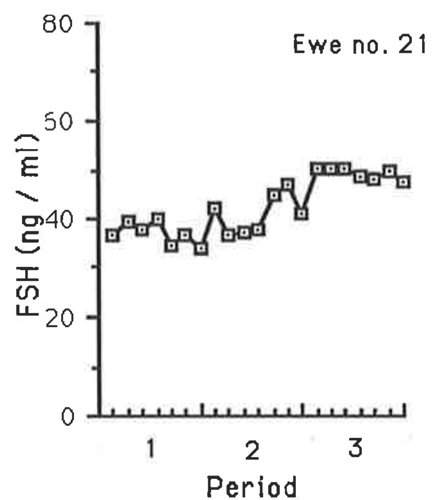
1
2
3
Period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FSH (ng / ml)
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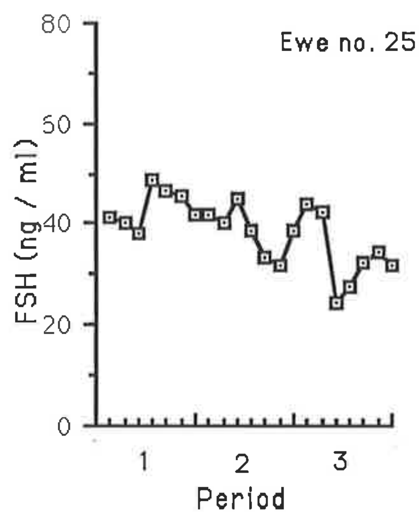
40
20
0
1
2
3
Period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FSH (ng / ml)
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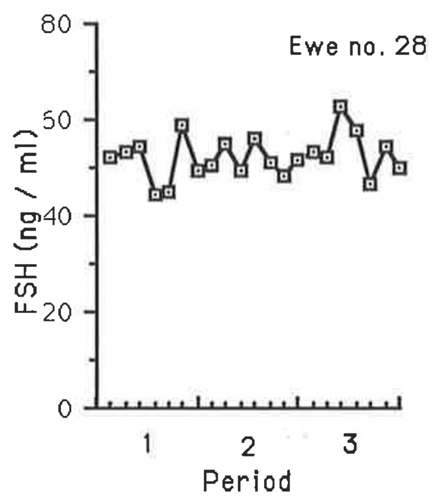
50
40
20
0
1
2
3
Period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FSH (ng / ml)
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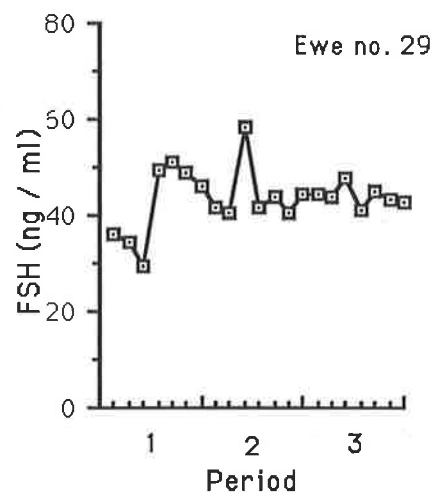
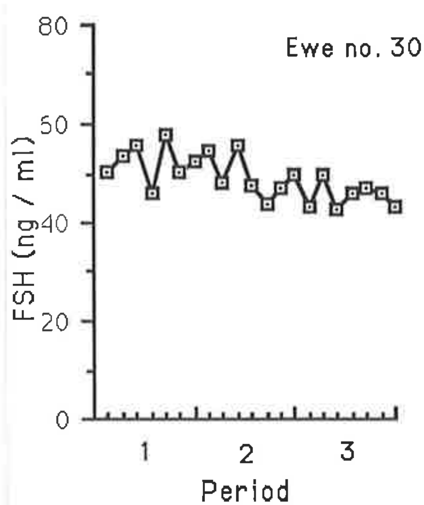
50
40
20
0
1
2
3
Period


Ewe no. 29
FSH (ng / ml)
80
50
40
20
0
1
2
3
Period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FSH (ng / ml)
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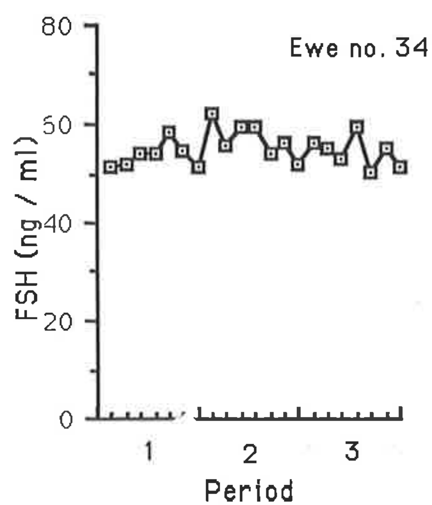
50
40
20
0
1
2
3
Period


Ewe no. 34
FSH (ng / ml)
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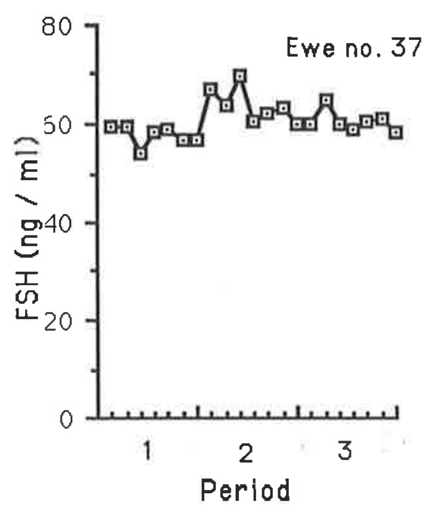
50
40
20
0
1
2
3
Period


Ewe no. 37
FSH (ng / ml)
80
50
40
20
0
1
2
3
Period

9.3.3. LH

Mean LH levels declined between periods 1 and 2 in both groups (*P* < 0.01; Table 9.3.). This was the result of a significant decrease in basal LH and pulse amplitude as pulse frequency did not differ significantly between the two sampling periods (Tables , 9.4., 9.5. and 9.6, respectively). There were no significant differences in basal LH, mean LH, pulse amplitude or pulse frequency between the control and lupin-fed groups at any of the three sampling periods.

9.4. Discussion.

The present study does not provide unequivocal evidence as to the reason for the increased secretion of FSH in lupin-fed intact ewes observed in the previous chapter. However, within the lupin-fed group FSH levels tended to be higher during the week of lupin feeding compared with pre-and post-feeding levels, suggesting that lupins can also increase FSH secretion in ovariectomised ewes. It remains possible however, that ovarian factors such as oestradiol and inhibin are also involved in the response. A similar result to that in the present study has also been reported for ovariectomised

Table 9.3. The mean LH concentration of the control and lupin-fed groups.

Treatment	LH (ng/ml)		
	period 1	period 2	period 3
control	2.31 ± 0.40	1.35 ± 0.14	1.23 ± 0.15
lupin	2.69 ± 0.33	1.25 ± 0.12	1.44 ± 0.18

Values are mean ± s.e.m. of 9 ewes.

Table 9.4. The basal LH concentration of the control and lupin-fed groups.

Treatment	LH (ng/ml)		
	period 1	period 2	period 3
control	2.08 ± 0.29	1.21 ± 0.12	1.10 ± 0.13
lupin	2.37 ± 0.22	106 ± 0.10	1.25 ± 0.21

Values are mean ± s.e.m. of 9 ewes.

Table 9.5. The LH pulse amplitude of the control and lupin-fed groups.

Treatment	LH pulse amplitude (ng/ml)		
	week 1	week 2	week 3
control	1.95 ± 0.38	0.88 ± 014	1.71 ± 0.12
lupin	2.38 ± 0.31	0.99 ± 0.11	0.86 ± 0.10

Values are mean ± s.e.m. of 9 ewes.

Table 9.6. The LH pulse frequency of the control and lupin-fed groups.

Treatment	LH pulse frequency (pulses/hr.)		
	period 1	period 2	period 3
control	0.78 ± 0.09	0.92 ± 0.10	0.75 ± 0.11
lupin	1.00 ± 0.08	0.92 ± 0.07	0.81 ± 0.07

Values are mean ± s.e.m. of 9 ewes.

ewes implanted with oestradiol and fed lupin grain (Nottle *et al.*, 1987; appended as appendix B). Interestingly however, the increase in FSH secretion in the former study appeared to be sustained several days after the ending of supplementation, suggesting that lupin grain may be acting to elevate the threshold to oestradiol negative feedback (Knight *et al.*, 1981). While both studies need to be repeated with larger numbers of animals to confirm these various suggestions, together, they provide evidence to suggest that the increase in FSH secretion seen in lupin-fed intact ewes is initiated at the level of the hypothalamic/pituitary axis.

Combined, the data from both studies also suggest that the secretion of FSH can be increased soon after the commencement of lupin feeding. Confirmation on this point may provide a physiological basis for the rapidity of the ovulatory response to lupin feeding. Also, further evidence to support the present suggestion that FSH is increased in some but not in all ewes may explain, partly, the failure consistently to show differences in FSH levels in lupin-fed ewes. Ritar and Adams (1988) for example, were unable to detect a significant difference in FSH levels between control and lupin-fed ewes immediately following ovariectomy. While this result is probably explained by the erratic nature of the post-castration rise in gonadotrophin secretion, these workers did find a significantly higher between animal variation within their lupin-fed group suggesting that FSH may have been increased in some but not in all ewes.

LH parameters were unaffected by supplementation in the present study. This finding is consistent with that by Radford *et al.*, (1980) who have reported that LH levels are not affected in intact ewes by feeding lupin grain. In contrast, Rhind *et al.*, (1985) have reported significant increases in

LH pulse frequency during the follicular phase in response to short-term increases in the level of nutrition and have suggested that this may be a possible mechanism. However, these workers did not measure FSH on day 13 but did find higher levels of FSH on days 14 and 15 of the cycle. Moreover, there is increasing evidence to suggest that alterations in LH levels do not affect ovulation rate *per se*. For example, increasing the level of LH in the follicular phase by injection or infusion of LH or GnRH does not affect ovulation rate (McNatty *et al.,* 1981a; McLeod and Haresign, 1984). The finding that exogenous LH does not increase ovulation rate above that obtained with FSH alone (Oussaid *et al.*, 1982) also supports this view. The reason for the general decline in LH pulse amplitude in the present study is uncertain. However, since the experiment ended in mid May it is possible that the general decline in mean LH may indicate the transition between the period of oestrous and anoestrous (Karsch, 1984).

The individual ewe variability in FSH suggests that there may be a genetic basis to the lupin response. However, unlike the Booroola where increases in FSH are thought to be due to a single gene (Piper and Bindon, 1982), the lupin effect is probably due to a number of closely linked genes whose effects are additive (see review by Spearow, 1985). Such an explanation would explain the overall consistency in the ovulatory responses to lupins in the present series of experiments. Evidence to suggest that this effect has a genetic basis also comes from a study by Oldham (1980), demonstrating that the ability of ewes to respond to lupin grain is dependent on their inherent potential for multiple ovulations. The identification of those animals within a flock who respond to lupin grain and confirmation that they do so repeatedly would, as suggested by Oldham (1980), allow more intensive

intensive physiological studies of the lupin effect to be undertaken than are currently possible because of the need for large numbers of ewes per treatment to establish significant differences in mean ovulation rate. The measurement of individual FSH secretion in ovariectomised ewes would refine this a model further by reducing the relatively long time needed to undertake ovulation rate experiments (c. 5 weeks) during the relatively short time that is the breeding season. Furthermore, the possibility of using such a model outside the breeding season would make it even more attractive.

In conclusion, the present study provides evidence to suggest that increases in FSH secretion in lupin-fed ewes are initiated centrally at the hypothalamic axis but only in a proportion of ewes. This possibility now needs to be confirmed in experiments with larger numbers of animals since such an effect not only has important connotations in understanding the ovulatory response to lupin grain, but also for the role of FSH in the control of ovulation rate in general.

CHAPTER 10. THE EFFECT OF LUPIN GRAIN ON THE RECRUITMENT AND SELECTION OF THE PREOVULATORY FOLLICLE .

10.1. Introduction

In the previous two chapters, evidence was obtained to suggest that increases in FSH secretion are important in mediating the response to lupin grain. How these increases influence terminal follicular growth and hence, ovulation rate remains to be determined. Several workers (Allen and Lamming, 1961; Haresign, 1981; Rhind *et al.*, 1985) have reported increases in the number of follicles greater than 2 mm in the ovaries of "flushed" ewes examined at laparotomy or slaughter and have interpreted this to mean that flushing influences the number of large antral follicles that become atretic. However, the differentiation of the preovulatory follicle in the ewe has been shown to involve the recruitment of follicles > 2 mm in diameter at the start of luteolysis and the selection of one of these which continues to grow and is ultimately ovulated (Driancourt and Cahill, 1984). Hence, an increase in the number of antral follicles in "flushed" ewes could be the result of an increase in the number of follicles recruited at the start of luteolysis and/ or in the number of these selected to ovulate. All three mechanisms have been recently reported to operate in prolific breeds (Driancourt *et al.*, 1986). The present study therefore was undertaken to determine whether increases in ovulation rate in lupin-fed ewes are the result of one or both of these processes.

*Driancourt (1985) has shown that the mean deviation in the diameter of follicles greater than 2 mm measured at the surface of the ovary compared with their actual size after dissection does not exceed 10%. This finding suggests that the measurement of the diameter of follicles at the ovarian surface provides a good estimate of a follicle's actual size. The other assumption that this technique makes is that enlarging follicles are not atretic. While these workers have provided no direct evidence to support this assumption, indirect evidence comes from their studies which have demonstrated that in more than 80% of ewes, the follicle(s) that ovulated was one identified on the surface of the ovary that was greater than 2 mm in diameter at the start of luteolysis and which continued to grow throughout the follicular phase. The assumption that growing follicles are not atretic therefore would appear to be a valid one.

10.2. Experimental procedure

10.2.1. Design

Two groups of 40 ewes were maintained outdoors in feedlots and fed daily a maintenance ration of wheaten hay. One group was also fed lupin grain for 10 days beginning on day 4 of the oestrous cycle. Ewes were adapted to lupin grain by feeding increasing amounts for the first three days.

10.2.2. Measurement of follicular growth

Ink labelling at successive laparotomies has been used to study preovulatory follicular enlargement (Driancourt and Cahill, 1984; Driancourt *et al.*, 1985a, 1986). Such a technique allows the growth and the regression of follicles > 2 mm in diameter which form the population of recruitable follicles to be monitored (Driancourt and Cahill, 1984).* The method used to measure follicular growth in the present study was similar to that of these workers but used laparoscopy instead of laparotomy to monitor preovulatory follicular growth. Both groups were induced to ovulate during the luteal phase by injecting PG on day 12 of the synchronised cycle. Twenty animals selected on a liveweight basis, then underwent

Figure 10.1. The position of follicles > 2 mm on the surface of the ovary was recorded by dividing the ovary into nine regions of approximately equal size. The position of each of these regions was standardised by regarding the pole of the ovary proximal to the uterus as the top.

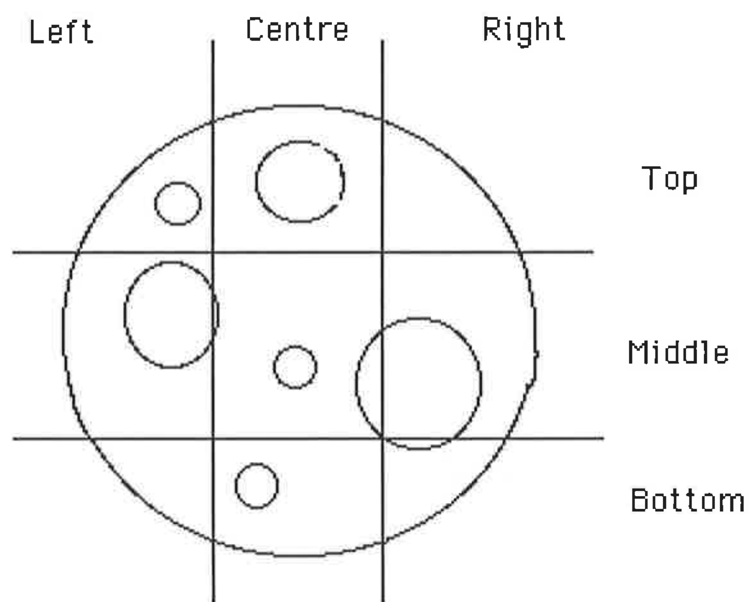

Left
Centre
Right
Top
Middle
Bottom

Plate 10. 1. The diameter of follicles at the surface of the ovary was estimated by reference to 2 mm lengths marked along the grasping mechanism of a pair of atraumatic forceps.

successive laprascopies at 0, 24 and 54 hours after PG injection to assess the growth of individual follicles > 2 mm. The position and size of follicles > 2 mm on the surface of each ovary was recorded at 0 h. The size of these follicles was again measured 24 hours later together with any other follicles that were greater than 2 mm in diameter. The number of preovulatory follicles (i.e. "labelled and still growing"; Driancourt and Cahill, 1984) was determined at 54 hours post PG. The position of follicles > 2mm on the surface of the ovary was recorded by dividing each ovary into nine regions of approximately equal size as shown in Figure 10.1. The position of each of these regions was standardised by regarding the pole of the ovary proximal to the uterus as the top of the ovary. The diameter of follicles was estimated by reference to 2 mm lengths marked along the grasping mechanism of a pair of atraumatic forceps (Plate 10.1.). Enlarging follicles between successive laparoscopies were considered to be healthy, while regressing ones were assumed to be atretic. Animals underwent a final laparoscopy at 120 hours post PG to determine ovulation rate.The experiment was conducted as a double blind experiment. The remaining 20 animals in each of the two groups served as ovulatory controls to assess the degree of stress associated with the above procedure.

10.2.3. Statistical analyses

Differences in liveweight gain and ovulation rate were determined by methods described previously (section 3.9.) . Because of a lack of normal

distributions the follicular data was analysed using the non-parametric Mann Whitney U-test.

10.3. Results.

There were no significant differences in either liveweight gain or ovulation rate between the animals that underwent serial laparoscopy and those that did not within the two groups and the data for these animals has been pooled.

10.3.1. Liveweight change.

Both groups had recorded small increases in liveweight by the end of the experiment (Table 10.1.). Liveweight gain was similar for both groups ($P > 0.1$).

10.3.2. Follicle number

The number of follicles > 2 mm diameter at the time PG was injected,together with the number of these still growing 24 and 54 hours post PG is shown in Table 10.2. The number of follicles > 2 mm in diameter

Table 10.1. The liveweight change of the control and lupin-fed groups.

Treatment	n	Liveweight change (g/day)
control	40	29 ± 13
lupin	40	36 ± 17

Values are mean ± s.e.m.

Table 10.2. The number of follicles recruited and selected to ovulate in the control and lupin-fed groups.

Time post PG	Treatment		P
	Control	Lupin-fed	
0 hours			
number of follicles > 2 mm	3.75 ± 0.30	3.55 ± 0.23	$P > 0.1$
24 hours			
number of follicles > 2 mm still growing	2.20 ± 0.25	2.15 ± 0.25	$P > 0.1$
additional follicles not identifed at 0h.	0.15 ± 0.08	0.25 ± 0.10	$P > 0.1$
total number of follicles recruited	2.35 ± 0.25	2.40 ± 0.24	$P > 0.1$
54 hours			
number of recruited follicles still growing	1.25 ± 0.12	1.75 ± 0.19	$P < 0.1$
incidence of atresia following recruitment	46.8%	27.1%	

Values are mean ± s.e.m. of 20 ewes.

*In the 20 ewes from both groups in which follicular growth was monitored, 16 control ewes and 17 lupin-fed ewes ovulated. Of these, the ovulatory follicle(s) came from those follicles that formed the recruitment pool and continued to grow in 15 control ewes and 14 lupin-fed ewes ($P < 0.01$). In one ewe from the control group the ovulatory follicle regressed slightly prior to ovulation. In three animals from the lupin-fed group a similar reduction in the size of the ovulatory follicle was observed in three animals.

were similar for both groups at the time PG was injected. Twenty four hours after PG injection there was a 40% decrease ($P < 0.01$) in the number of these follicles in both the control and lupin-fed groups. This was because a number of follicles which were > 2 mm at O hours had ceased growing and had started to regress . The total number of growing follicles at 24 h post-PG was similar in both the control and lupin-fed groups. Fifty- four hours after PG injection there was a further reduction in the number of growing follicles in both groups ($P < 0.01$). In the lupin-fed group, the number of growing follicles at this time tended to be higher compared with the control group ($P < 0.1$).*

10.3.3. Ovulation rate

Supplementation with lupin grain significantly increased ovulation rate. (Table 10.3.). Within both groups ovulation rate tended to be higher in the ewes that did not undergo serial laparoscopy ($P = 0.1$)

10.4. Discussion

The results of the present study are consistent with those reported previously (Driancourt and Cahill, 1984; Driancourt *et al.*,1985a,1986) confirming that the differentiation of the preovulatory follicle is a two step process involving the recruitment of follicles > 2 mm soon after the start of luteolysis and the selection of one of these which ovulates. In the present

Table 10.3. The ovulation rate of the control and lupin-fed groups.

Treatment	Ovulation rate		
	non-laparoscoped	laparoscoped	total
control	1.15 ± 0.11 [a] (20)	0.90 ± 0.12 [a] (20)	1.03 ± 0.08 [a] (40)
lupin	1.50 ± 0.13 [a] (20)	1.20 ± 0.15 [a] (20)	1.35 ± 0.10 [b] (40)

Values are mean ± s.e.m.
Numbers in brackets indicate number of ewes within each column.
Within each group means with different superscripts are significantly different ($P < 0.05$).

*In the majority of ewes in which follicular growth was monitored, the follicle(s) that ovulated came from those follicles which were greater than 2 mm in diameter at the start of luteolysis and which had increased in size at 54 hours post-luteolysis. This finding supports the assumption made by Driancourt and Cahill (1984) in their studies that follicles which are greater than 2mm in diameter at the start of luteolysis and which continue to grow are not atretic and form the recruitment pool from which the preovulatory follicle(s) is selected.

study, there was a significant reduction in the number of follicles > 2 mm at two times following PG injection. The first of these occurred 24 hours after the administration of PG. This may be due to the regression of follicles which are already atretic since approximately half of the follicles present during the luteal phase are thought to be atretic (Brand and de Jong, 1973; Cahill *et al.*, 1979). In the present study a further reduction in the number of growing follicles had occurred in both groups 54 hours after PG. Between 24 and 54 hours post PG no new follicles grew up to replace those that had become atretic presumably because of the decrease in FSH secretion that occurs at this time (Welschen and Dullart, 1976; Baird, 1983). The number of follicles still growing at 54 hours was similar to the ovulation rate of both groups confirming that the selection of the pre-ovulatory follicle is complete by this time (Driancourt and Cahill, 1984).[*]

In the present study there was no difference between the control and supplemented groups in either the number of follicles > 2 mm at the time luteolysis was induced , or in the number of these still growing at 24 hours afterwards. Short-term feeding of lupin grain therefore, appears not to influence recruitment. Fifty- four hours after PG injection however, there were 40% more follicles still growing in the lupin-fed group compared with the control group. This apparent difference was not significant but was reflected in a significantly higher ovulation rate in the lupin-fed group. Thus just as groups of 40 or more animals are required per treatment to establish significant differences in ovulation rate similar numbers would also appear to be necessary for the monitoring of preovulatory growth. In this context the present method of monitoring follicular growth by laparoscopy would appear to be more practical than serial laparotomy. Within both groups

however, ewes subjected to serial laparoscopy tended to a have lower ovulation rate than those who did not undergo laparoscopy. This suggests that there is some stress associated with this procedure. In contrast, there appears to be no significant stress associated with repeated measurements performed under general anaesthesia induced with a mixture of alphaxalone and alphadolone chosen because it does not interfere with LH pulsatility (Driancourt and Cahill, 1984). The present method therefore perhaps needs to be performed using this anaesthetic. Interestingly however, the stress associated with serial laparoscopy appears not to affect either the recruitment or the final selection of follicles, since the number of follicles selected to ovulate in both groups was similar to the ovulation rate of the non-laparascoped controls. Rather, stress appears to influence the rupture of these follicles. The reasons for this effect are uncertain. Matteri *et al.*, (1984) have demonstrated that restraint stress in sheep can reduce LH responsiveness to exogenous LHRH. Hence one reason why these follicles failed to ovulate may be that stress in some way interfered with the LH surge in these animals.

The results of the present study suggest that increases in ovulation rate in lupin-fed ewes are the result of an increase in the number of follicles selected to ovulate. A similar result has been reported by Rhind *et al.*, (1985) who have suggested that increases in feed intake (short-term nutrition) induce differences in the proportion of large follicles that ovulate and not the number of follicles that develop. The present view also agrees with that by Haresign (1981) that flushing reduces the incidence of late atresia amongst large antral follicles in the last 30 hours before ovulation.

How short-term changes in the level of nutrition effectively rescues a follicle from atresia is uncertain. In the previous two chapters evidence was obtained to suggest that FSH may be involved in this response. In particular, it was suggested that FSH converts a recruited follicle into one capable of secreting significant amounts of oestradiol (Baird, 1983; McNatty *et al.*, 1985) thereby allowing it to escape both the atresia promoting effects of androgens and a decrease in FSH secretion. This process may also involve various intraovarian factors present in follicular fluid (see reviews by Driancourt, 1987, Webb and Gauld, 1987). The role of these factors is thought to be to prevent other recruited follicles from becoming dominant. If so, it will be interesting to determine how a second follicle becomes co-dominant.

Recently, Fry *et al.*, (1986) using ink labelling and repeated laparotomy have suggested that increases in ovulation rate in high liveweight ewes (medium-term nutrition) are the result of an increase in the number of follicles recruited together with an increase in the number of these selected to ovulate. In contrast, the present study provides no evidence to suggest that nutrition in the short-term can influence recruitment. While both experiments need to be repeated with larger number of animals they do lend support to hypothesis developed earlier that medium and short -term nutrition operate via different mechanisms to influence ovulation rate. Support for this suggestion also comes from the finding by Knight *et al.*, (1981) that FSH and oestradiol are increased in lupin-fed ewes but not high liveweight ewes. That these two effects operate via different mechanisms has also been suggested by other workers, the most recent being Rhind and an co-workers (1985, 1986) who have suggested different hormonal

mechanisms for the effects of condition score (medium-term nutrition) and feed intake (short-term nutrition). However, direct effects of these hormonal changes on ovulation rate remain to be demonstrated. Support for two separate effects of nutrition also comes from the recent demonstration by Driancourt *et al.*, (1986) that the follicular mechanisms that operate are different for the different prolific breeds suggesting that more than one mechanism can control ovulation rate. Hence, future nutritional studies are likely to be more informative if they include the monitoring of preovulatory growth. Under this context the refinement of the laparoscopic method described in the present study when used in conjunction with groups of 40 or more animals would be particularly useful.

Chapter 11. General Discussion.

In reviewing the literature at the beginning of this study an attempt was made to rationalise the interaction between nutrition and ovulation rate in the ewe. In summary, three different nutritional influences were proposed. The first of these, severe undernutrition was identified as inhibiting all stages of follicular growth by acting centrally at the hypothalamic/pituitary axis to suppress gonadotrophin secretion to a level below that necessary for the maintenance of folliculogenesis. The second influence proposed, operates in the medium term and can be readily correlated with liveweight, suggesting that its effect is mediated by changes in energy intake. This influence is almost certainly analagous to the static effect of liveweight proposed by Coop (1966b) and probably acts by modulating the number of preantral follicles that enter the antral phase, since it is generally considered that this effect needs to operate for at least six weeks to be effective (Smith, 1985). The third effect relates to the dramatic impact the level of nutrition has in the period immediately preceeding ovulation in determining the number of antral follicles that ovulate. Despite the obvious importance of nutrition at this time very little is known about how this effect is mediated within the animal. The present study therefore, was undertaken to determine this latter response in detail using the known ability of lupin grain fed at this time to increase ovulation rate.

Short-term changes in the level of nutrition and their effect on ovulation rate, appear to be communicated via changes in the amount of protein digested post-ruminally. This was confirmed in the present study which

demonstrated that increasing the amount of protein digested post-ruminally to a similar extent to that provided by feeding lupins, has a comparable effect on ovulation rate. Smith (1985) has proposed that this response is threshold in nature and depends on the level of dietary protein intake together with the extent to which this is degraded in the rumen. This requirement for protein almost certainly varies depending on the partitioning of protein in the animal, with it is proposed, energy preferentially sparing amino acids from gluconeogensis. Hence, an animal whose digestible energy intake exceeds maintenance is likely to have a lower requirement for post-ruminal protein than one whose energy intake is below this level. This interpretation is arguably more realistic than that by Smith (1985) that short-term changes in protein and energy intake can act separately to influence ovulation rate. The more recent finding that intravenous infusions of glucose and acetate can increase ovulation rate (Teleni *et al.*, 1984) may therefore be more of a pharmacological effect than a physiological one. However, because of the degree of interconversion between protein and energy in the ruminant animal, resolution of this controversy probably awaits the demonstration of responses to specific amino acids. Although ovulation rate can be increased by increasing the amount of protein digested post-ruminally, it is probably more meaningful to think of post-ruminal protein as a final nutritional check which matches ovulation rate to the animal's "nett nutritional status" (Lindsay, 1976). Hence, the partitioning of post-ruminal protein in the animal may not only determine if ovulation rate is to be increased but also if it is to be maintained or decreased.

The rapidity of the response to lupin grain provides strong support for the present view that short-term changes in the level of nutrition determine ovulation rate by influencing the final stages of follicular growth. This effect on terminal follicular growth was demonstrated in the present study to be temporally related to luteolysis. Supplementation with lupin grain needs to occur for a maximum of four days prior to this time for ovulation rate to be increased (Stewart and Oldham, 1986). It is perhaps surprising then, that lupin feeding has not become more widely accepted as a means of increasing ovulation rate at the commercial, or farm level. Theoretically, given the consistent increases in ovulation rate of around 30% acheived in this and other studies with lupins and even allowing for a loss of 20% of embryos (Edey, 1969), it should still be possible to increase lamb numbers by 20% . However, the results of field trials with lupins have been variable and it has been suggested that this is due to the failure to define nutritional conditions in which lupin grain can increase ovulation rate (e.g. Croker *et al.*, 1985). However, under most circumstances in southern Australia it is unlikely that pastures would supply sufficient protein to maximise ovulation rate at the time Merinos are normally mated,from October through to January. Oldham and Lindsay (1984) for example, were able to show that lupin grain could increase ovulation rate even in ewes in "forward store to fat condition" grazing sufficient dry sub clover pasture to maintain their liveweights. Rather, it is perhaps because in the absence of oestrus synchronisation, lupin grain would need to be fed for 3 or more weeks to be effective, that has deterred most sheep farmers from feeding lupin grain. One practical solution to this problem may be to use the "ram-effect" (see review by Oldham and Pearce, 1986) to partially synchronise the mating flock thereby allowing lupin grain to be fed for a more economical period. A

economical period. One strategy may be to feed lupins for 14 days commencing seven days before the first peak of oestrus activity that occurs approximately 18 days after ram introduction. This would ensure that those ewes that are mated at this time are fed lupins for 8 to 5 days prior to ovulation (Stewart and Oldham, 1986). Supplementation for 14 days would also ensure that those ewes that are mated during the second peak of oestrus activity that occurs approximately 24 days after ram introduction are also fed lupins during the critical phase. Since it is generally agreed that the majority of ewes normally conceive during these two peaks of oestrus activity, this strategy may provide a economical way of increasing the number of lambs born. Alternatively, the possibility of using lupin grain lupin grain earlier in life to increase lifetime ovulation rate may be more attractive to the producer. In particular, the suggestion by Allden (1979) that nutrition soon after birth influences a ewe's life-time ovulation rate warrants further investigation.

The mechanism whereby short-term changes in the level of nutrition alter ovulation rate remains to be fully elucidated. However, the present study provides strong evidence to suggest that the effect is mediated through changes in the secretion of FSH immediately prior to luteolysis on day 13 of the cycle. In particular, the present study showed that as little as 4 mg of FSH injected at this stage of the oestrous cycle can effectively mimic the ovulatory response to lupin feeding. Evidence to suggest that lupins can increase FSH secretion in ovariectomised ewes was also obtained in the present study. This suggests that the effect of lupins on FSH secretion is centrally mediated at the hypothalamic/pituitary axis. Evidence to suggest that these changes occur soon after the commencement of feeding was

also obtained. Confirmation of these findings may provide a physiological basis for the rapidity of the lupin response. Furthermore, lupin feeding did not appear to influence FSH secretion in all ovariectomised ewes in this study, suggesting that there may be a genetic basis to this response. This variability in FSH secretion now needs to be explored with larger numbers of animals. Alternatively the identification of animals that respond to lupin feeding prior to ovariectomy, may allow this experiment to be repeated with a similar number of animals. The use of known responders would also allow more intensive types of experiments of the lupin response to be undertaken than are currently possible.

Regardless of how the various nutritional influences proposed are mediated within the animal their final effect on ovualtion rate is seen at the follicular level. Reports by other workers have suggested that short-term increases in the level of nutrition or flushing, increase ovulation rate by reducing the number of large antral follicles that become atretic during the follicular phase. More recent studies of follicular dynamics however, have shown the differentiation of the preovulatory follicle to involve the recruitment and selection of large antral follicles (Driancourt and Cahill, 1984) and that one or both of these processes can increase ovulation rate (Driancourt et al., 1986). The results of the present study suggest that short-term nutrition influences ovulation rate by determining the number of follicles that are selected to ovulate. In the ewe, there appears to be no single time during the follicular phase at which selection occurs (Driancourt et al., 1985b) rather, it is proposed that the level of FSH immediately prior to luteolysis determines the number of follicles that are selected to ovulate. This view together with that by Fry *et al.*, (1986) that medium term nutrition

also influences recruitment , supports the original hypothesis that apart from severe undernutrition there are at least two other effects of nutrition that influence ovulation rate. Since feeding lupin grain prior to the induction of luteolysis had no effect on the number of follicles recruited in the present study it would appear that recruitment is determined prior to luteolysis. Interestingly, in both the present study and that by Fry *et al.*, (1986) the number of recruited follicles was in excess of ovulation rate suggesting that short-term nutrition is the more important of these two influences.

In summary, the present study has confirmed previous reports that increases in the level of nutrition in the short-term can increase ovulation rate. This effect appears to be related primarily to changes in protein intake which together with ruminal degradation determines the amount of protein available to the animal. Increases in the amount of protein digested post-ruminally appear to act near the time of luteolysis to directly influence FSH secretion from the anterior pituitary. In turn, changes in FSH secretion appear to influence terminal follicular growth. In particular, it is suggested that the absolute level of FSH is important in increasing aromatase activity in individual follicles and that this enhanced ability to produce oestradiol effectively allows a follicle to escape atresia and ovulate.

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APPENDICES

Introduction

Two appendices are included in this thesis to lend support to the major conclusions drawn in chapters 8 and 9.

The first appendix details a pilot study undertaken to determine whether the administration of FSH near the time of luteolysis could increase ovulation rate in the ewe in a similar manner to that when lupins are fed. The details for this experiment including the FSH used and its method of administration etc., were the same as that given in chapter 8. The results from this study are included as evidence to support the arguement in chapter 8 that the ovulatory response following treatment with 4mg of Folltropin was the result of a physiological manipulation rather than a pharmacological one.

The second appendix is a copy of an abstract presented elsewhere (Nottle *et al.,* 1987). It is included as an appendix to lend support to the major conclusion drawn in chapter 9 that the increase in FSH secretion in lupin-fed intact ewes is initiated centrally at the hypothalamic/pituitary axis. The details for this experiment were similar to that given in chapter 9. In particular, the same FSH assay was used. However, it was realised that the experiment did not answer fully the question as to the origin of the increase in FSH secretion. In particular, it could be argued that the increase in FSH secretion seen in ovariectomised ewes implanted with oestradiol was the result of an increase in the metabolic clearance of oestrdiol. Thus, it was decided to repeat the experiment using non -implanted ewes.

Appendix A

Table A.1. The ovulation rate of ewes treated with different amounts of Folltropin.

Amount (mg)	Ewes with n corpora lutea 0	1	2	3	>3	ovulation rate
0	1	7	2	0	0	1.10 ± 0.18^a
1	2	6	2	0	0	1.00 ± 0.21^a
2	1	5	4	0	0	1.30 ± 0.21^a
4	1	3	6	0	0	1.50 ± 0.22^a
8	0	2	4	1	3 (4,6,7)+	3.00 ± 0.65^a

Values are mean ± s.e.m. of 10 ewes
Means with the same superscript are not different ($P>0.05$)
+No. of corpora lutea per ewe.

Appendix B

SHORT-TERM SUPPLEMENTATION WITH LUPIN GRAIN INCREASES SERUM FSH IN THE OVARIECTOMISED, OESTRADIOL-IMPLANTED EWE.

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Increases in ovulation rate (OR) in lupin-supplemented ewes are thought to be mediated by increases in FSH secretion between days 12-14 of the oestrous cycle (1,2). The present study was undertaken to determine whether similar increases in FSH could be demonstrated in ovariectomised ewes in which post-castrational rises had been inhibited by oestradiol (E_2).

20 mature Merino ewes (43.2±1.0 kg liveweight) which had been paired according to liveweight were used in the experiment which began in April. 3cm E_2 implants reported to produce similar levels to those in intact ewes (3) were inserted subcutaneously at ovariectomy. Animals were housed indoors in individual pens and fed a maintenance ration of wheaten hay. Supplemented animals received 500g of lupin grain per ewe per day for seven days (days 0-6). FSH was measured by radioimmunoassay and data analysed by ANOVA procedures. Mean serum FSH prior to, during and after supplementation are summarised in the following table.

Table 1. Mean (± SE) serum FSH (ng/ml) in control and lupin-supplemented ewes.

	day-1	day 0	day 1	day 6	day 13
Control	29.8 ± 3.7^{a}	29.3 ± 3.0^{a}	28.9 ± 3.1^{a}	28.9 ± 2.7^{a}	27.2 ± 2.1^{a}
Supplemented	28.0 ± 2.2^{a}	29.1 ± 2.4^{a}	40.4 ± 2.8^{b}	40.1 ± 3.3^{b}	37.3 ± 3.6^{b}

Within each column means with different superscripts are significantly different (P<0.05).

Supplementation with lupin grain increased serum FSH within 24 hours (P<0.02). FSH levels remained elevated thereafter for the period of supplementation and for at least seven days afterwards.

In conclusion, the results provide further evidence to suggest that increases in OR in lupin-supplemented ewes are mediated by increases in FSH secretion. Furthermore, the present study suggests that such increases are initiated at the level of the hypothalamus or pituitary.

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