# Excess dietary rumen degradable protein intake in ewes, its effect on ovulation rate, fertilization and embryo survival both *in vivo* and *in vitro*.

by

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**Declaration**

This thesis is based on work carried out on an original line of research and has not been accepted in any previous application for a degree. All sources of information are referenced and help given by other people is indicated in the acknowledgements.

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**SUMMARY**

The literature on the effects of dietary protein on fertility in ruminants is reviewed and it is concluded that failure to distinguish between rumen degradable and undegradable dietary protein may explain the equivocal nature of the published findings. Thus an experiment was designed in which ewes were used to determine whether or not high levels of rumen degradable protein affect ovulation rate, fertilization and embryo survival *in vivo* and subsequently during *in vitro* culture. In addition the effects of alterations in rumen degradable protein on plasma urea, ammonia, progesterone and luteinizing hormone were investigated.

Thirty Greyface (Border Leicester x Scottish Blackface) ewes were randomly assigned to three treatments and given a basal control diet which met their energy requirements for body weight maintenance. One treatment involved supplementing the basal diet with 24 g of urea/day (low urea) and another with twice this amount (high urea). Both the low and high urea treatment diets exceeded the rumen degradable protein requirements (+ 56.5 g and + 125.5 g/day, respectively). The ewes received the diets throughout the experimental period.

The ewes were put on the experimental diet around 60 days after lambing. Following a single injection of prostaglandin ($Pgf_2$ alpha) each ewe received an intravaginal controlled internal drug release (CIDR) device which supplied exogenous progesterone for a period of 12 days. Ovulation was induced using pregnant mare serum gonadotrophin (PMSG) at the time of CIDR device removal. Plasma samples were taken just before CIDR device insertion and thereafter once daily. These were analysed for progesterone. In the period starting 24 hours after CIDR removal plasma samples were taken at 2 hour intervals for 32 hours and were analysed for luteinizing hormone (LH) to determine LH surge onset and amplitude. These samples were also analysed for plasma urea and ammonia over a time span of 24 hours.

Intrauterine insemination (IUAI) using a laparoscopic technique was used to deposit approximately 100 x $10^6$ spermatozoa in each uterine horn approximately 52 hours after CIDR device withdrawal. Embryo recovery was carried out surgically at 4 days and 11 days after IUAI, with half of

the ewes from each treatment group having their embryos recovered on each day.

Embryos were assessed using a stereomicroscope and where one or more was recovered one embryo was returned to the donor. Remaining embryos recovered at day 4 were cultured *in vitro* for 72 hours prior to a two hour incubation with $^{3}H$ phenylalanine. The embryos recovered on day 11 were only incubated in $^{3}H$ phenylalanine for two hours but were not cultured *in vitro*.

There were no significant differences in ovulation rates which were 4.1 ± 0.92 in the control, 2.9 ± 0.50 in the low-urea and 2.4 ± 0.42 in the high-urea group. The mean percent embryo recovery rates were not affected by `day of recovery' and the overall values were 50.1 ± 10.90 in the control, 40.0 ± 12.4 in the low-urea and 24.7 ± 10.4 in the high-urea groups. At embryo recovery on day 4, two of ten embryos in the control, one of three in the low-urea and three of four in the high-urea groups were considered non-viable. There were more non-viable embryos from ewes in the high urea group (4/4) than the control (3/10) after 72 hours of *in vitro* culture. There was a positive correlation ($r= 0.75$, $p< 0.001$) between $^{3}H$-phenylalanine incorporation and stage of development of embryos following 72 hours of *in vitro* culture.

More pregnancies were sustained in the ewes on the control diets (6/8) than on the high urea diet (1/3).

Plasma urea levels were significantly affected by diet (Control 2 ±0.2, low urea 5 ± 0.3, high urea 7 ± 0.3 mmol/l ; $P < 0.01$) and were elevated throughout the experiment in animals receiving urea supplements. Plasma ammonia levels in the high urea group showed transitory elevations of concentration after feeding and were significantly higher than the control and the low urea groups ($P < 0.05$) for 4 hours after each feed.

There was no treatment effect on plasma progesterone concentration before, during or after CIDR device withdrawal. LH surge onset time and amplitude was not correlated to ovulation rate and was not affected by treatment.

Although the numbers of observations are small, both the subjective and objective measures of embryo viability, coupled with the values for embryo survival following autotransfer, all point to an adverse effect of high circulating concentrations of plasma urea and ammonia on early embryo survival. Furthermore this effect was independent of any alterations in the endocrine balance of the ewes as measured by plasma progesterone and luteinizing hormone concentrations.

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## LITERATURE REVIEW

## INTRODUCTION

In sheep production systems, low producing animals that are solely dependant on low quality forages are often given dietary supplements of urea or other sources of non protein nitrogen (NPN) to alleviate a protein shortage arising from a low yield of microbial protein (Ørskov and Robinson,1981). On the other hand the diets of high producing animals are supplemented with high quality protein that is largely undegraded (UDP) in the rumen or else their diet comprises high quality forage with a level of rumen degradable protein (RDP) that is grossly in excess of that needed by the rumen microbes for maximum microbial protein synthesis. Whether or not the level and quality of protein (undegraded versus readily degradable) has any detrimental effect on reproduction seems to be uncertain. Although there are reports of normal fertility on protein diets there are others in which excess dietary protein has been associated with a reduction in fertility in cows. These include the studies of Jordan and Swanson (1979a), Kaim *et al* (1983), Canfield *et al* (1990), and the very recent work of Elrod and Butler (1993) with heifers.

Where adverse effects have been noted they have on occasion been ascribed to rumen degradable protein (RDP); Folman *et al* (1981);Canfield *et al* (1990). Alternatively,Carroll *et al* (1988) have attributed the reported variation in fertility in various studies:-Jordan and Swanson (1979a), Howard *et al* (1987), Carroll *et al* (1988), Canfield *et al* (1990):-to suboptimal reproductive management (such as treatment of uterine disorders and oestrus detection programmes) during the postpartum period and not to dietary protein.

In their most recent publication, Elrod *et al* (1993) have concluded that the adverse effect on fertility manifests itself regardless of the source and degradability of the protein when crude protein is fed in excess of the animals requirement. They claim that the effect is brought about by changes in uterine pH. However,the mechanism by which this pH change is modulated by excess dietary protein has not been identified.
Despite the claim that excess dietary protein can reduce fertility there is little or no evidence that fertility was reduced in dairy cows feeding on a

diet of protein level as low as 13.2% or as high as 20% (Carroll *et al*, 1988; Howard *et al*, 1987). The fertility criteria measured in these studies were days to first ovulation, number of services per conception, conception rate to first service, pregnancy rate and calving interval. There is, however, a lack of reports of similar studies in sheep.

## 1.1.0 Rumen degradable protein

### 1.1.1 Sources

Dietary protein is divided into protein and non protein components, the latter being amino acids, peptides, nucleic acids, free ammonia, ammonium salts, urea, biuret, nitrate and other nitrogen containing compounds (Huber and Limin Kung, 1981). These non protein nitrogen components and rapidly degradable protein fractions supply most of the rumen degradable nitrogen. Apart from very low protein forages and feed sources that are rich in fermentable carbohydrates but low in protein (e.g. citrus pulp,cassava, maize, etc), most practical diets have sufficient degradable protein for the maximum synthesis of microbial protein and this together with the high turnover of microbial protein in the rumen is sufficient to meet any specific needs for preformed peptides and amino acids in low producing animals (ARC,1980). Under these conditions non protein nitrogen (NPN) is therefore as good a source of rumen degradable nitrogen (RDP) as is the conventional protein fraction of the diet.

### 1.1.2 Degradability and utilization

In low producing ruminants the rumen degradable protein fraction, when synthesized into microbial protein, is the major contributor to the amino acid supply reaching the abomasum and small intestine. In many practical situations it provides three quarters of the total amino acid supply of the animal: the remainder comes from the undegradable protein fraction of the diet (Ørskov and Robinson, 1981: MacRae and Lobley, 1984).

Thus the degradation of protein in the rumen is an important aspect of ruminant nutrition. Not only does it indicate how much feed protein is available for rumen microbes and subsequently for the host animal (Hussein and Jordan, 1991) but it also recognises that feedstuffs do not have a constant degradability value. Rather, degradability varies with the

nature of the basal diet and the level of feeding (ARC, 1980). This latter factor operates via the retention time of the feed in the rumen and therefore the length of time that it is exposed to the degradation process.

### 1.1.3 Requirements for rumen degradable protein

A high protein requirement during rapid growth in early life, in late pregnancy and at peak lactation is well recognised. Under these conditions the microbial protein supply is usually inadequate to meet the needs of the animal (Ørskov, 1970; Robinson, 1983; Hovell and Ørskov, 1989). In order to achieve these higher dietary protein needs it is neccessary to supplement the diet with protein sources that either by-pass the rumen or pass through the rumen undegraded. The latter is referred to as rumen undegraded dietary protein (UDP) (Ørskov and Robinson, 1981). By escaping from the rumen in undegraded state this protein fraction is available for digestion and absorption in the abomasum and small intestine.

Although minimum protein requirements (ARC, 1980 & 1984) are now expressed as RDP and UDP, most dietary supplements of protein provide both RDP and UDP. Thus when they are used to supply additional UDP most of them will inevitably supply excess RDP and thus a higher protein allowance in the diet than the theoretical minimum estimated by the ARC (Robinson, 1987).

### 1.1.4 Efficiency of conversion of RDP to microbial protein

The extent of microbial protein synthesis from rumen degradable protein and dietary NPN sources such as urea depends on the energy available to the rumen microorganisms (ARC, 1980).

In the ARC (1980) publication the net efficiency of conversion of dietary degradable protein to microbial protein is taken as 1.0 while that for urea or other degradable NPN sources is assumed to be 0.8.

### 1.1.5 Protein-energy interactions

The dietary proteins are degraded to nitrogenous compounds which include peptides, amino acids and finally ammonia which is then used by rumen microbes for microbial protein synthesis (Nolan and Leng, 1972). In order to grow and multiply rumen microbes need nitrogen (N) in a form that can readily be used and simultaneously require energy from fermentation of organic matter. Thus protein needs are expressed in relation to energy availability and more specifically in relation to metabolizable energy (ME). For example in a basal diet in which about 80% of the protein is degradable around 10 g crude protein (CP) per MJ of ME yields sufficient RDP for maximum microbial protein synthesis. This microbial protein together with the 20% of the dietary protein that is undegraded can provide the animal with a net protein supply of about 5.5 g/MJ of ME from the 8.0 g available for digestion (Robinson, 1987).

When urea is used in feeding, other microbial requirements should be taken into account such as sulphur (S). Sulphur is normally incorporated into the diet in the approximate ratio to that occurring in the rumen bacteria, i.e. about 14 parts of N to one of S. Phosphorus and other micronutrients are also needed. When these needs are met the amount of N required will largely depend on the amount of available energy.

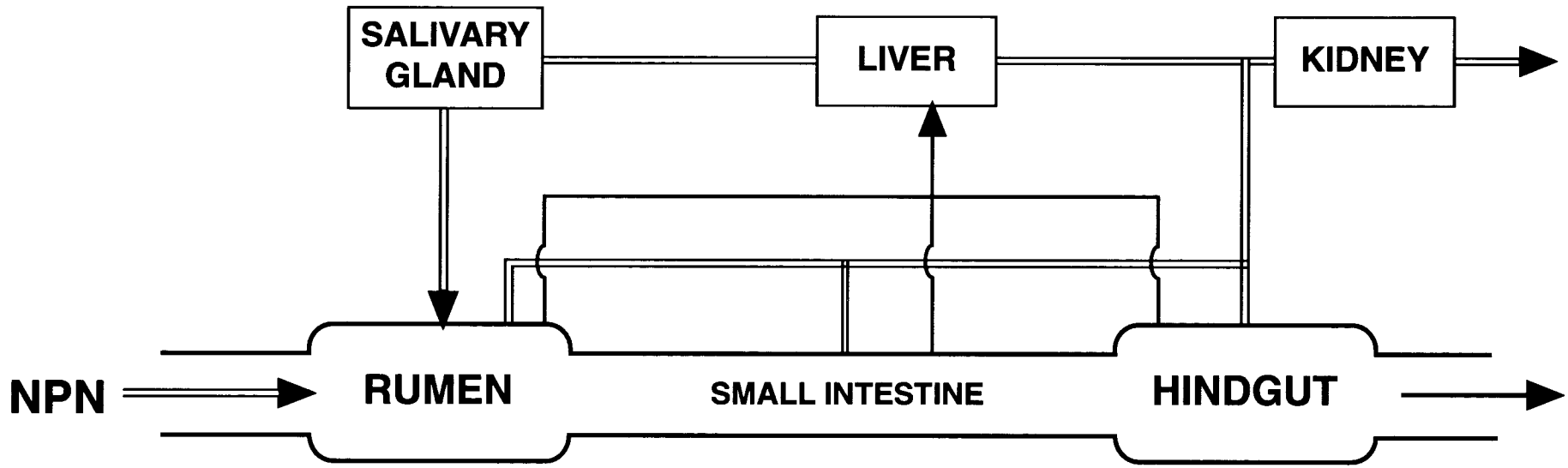
Energy requirements for body weight (BWT) mainteinance vary from 0.35 to 0.45 MJ ME/kgBWT$^{0.75}$/day and the daily nitrogen requirement for mainteinance of body tissue varies from 300 to 400 mg/kgBWT$^{0.75}$ (ARC, 1980).

### 1.2.0 Aspects of urea metabolism in ruminants

### 1.2.1 Urea metabolism

It has been estimated that 70% of the nitrogen ingested daily passes through the urea pool of the body and is excreted in most animal species through the kidneys (Hameyer and Marten,1980). The urea is formed in the liver. In ruminants up to 60 % of plasma urea comes from rumen ammonia; see Figure 1 for an illustration of urea metabolism in the ruminant. Ammonia is of no nutritional value if it is not converted to microbial protein (Chalupa,1984). Hence when high levels of ammonia are absorbed from the rumen they cause biochemical, hormonal and tissue dysfunctions (Chalupa,1984).

**Figure 1: DIAGRAM ILLUSTRATING UREA METABOLISM IN RUMINANTS.**




⟹ – Urea

→ – Ammonia

GI.T – Gastrointestinal tract

Adopted from Harmeyer and Martens (1980) takes into account that only one half of the Urea N- originates from free Ammonia, the other half is provided as nitrogen by aspartate.

### 1.2.2 Urea recycling

Turning to the role of urea recycling in ruminant protein metabolism, the observation that a high protein intake and a high concentration of ammonia in the rumen reduce urea entry from the blood into the rumen led Hameyer and Martens (1980) to suggest that factors other than the concentration of urea in the plasma, may be involved in the control of flow of urea from blood to the gastrointestinal tract (GIT).

For example the saliva of sheep contains 10 to 30 mg N/ 100 ml and, of this 60 to 70 % is urea N. Saliva urea concentrations are estimated to be 60 % of the urea concentration in the plasma (Hameyer and Martens, 1980).

A higher efficiency of protein utilization at low protein intakes has been observed and attributed to nitrogen recycling to the rumen as urea thereby resulting in a reduction in renal losses (Huber and Limin Kung, 1981).

### 1.3.0 High dietary protein levels and reproductive performance

High levels of protein in the diet have been associated with reduced reproductive performance in cattle. This association is examined with regard to ;

(1) the level of protein in the diet,
(2) the source and degradability of the protein and
(3) the parameters that have been used as indicators of reproductive performance.

### 1.3.1 Level of protein and indicators of reproductive performance

In the literature reviewed, the dietary protein levels varied from 12.7 % (Jordan and Swanson, 1979a) on the low protein diets to as high as 25 % of dry matter (Blauwiekel *et al*, 1986). Where the effect of rumen degradable protein has been examined, diets in which rumen degradable protein requirements were exceeded by as much as 50% have been used (Elrod and Butler, 1993).

In the most recent studies, Elrod *et al*, (1993) have shown reduced reproductive performance due to degradable protein intake. This adverse effect however, was demonstrated regardless of the degradability and the

source of the protein and under conditions in which reproductive management was optimal in virgin heifers.

In cattle the link between dietary protein and reproductive performance has been based on the following measurements: days to first oestrus, open days or days between calving and conception, conception to first insemination or conception to mating, and the number of services per conception, and pregnancy rate (Kaim *et al*, 1983: Carroll *et al*, 1988: Howard *et al*, 1987). These measurements of reproductive performance are often then correlated with the concentrations of plasma metabolites created by the different levels of dietary protein ; parameters which have been studied include, for example urea nitrogen, ammonia (Folman *et al*, 1981: Kaim *et al*, 1983), glucose, insulin (Rusche *et al*, 1993), luteinizing hormone (LH) and progesterone (Blauwiekel *et al*, 1986: Rusche *et al*, 1993: Elrod and Butler, 1993), and in some cases changes in the ionic composition of uterine secretions (Jordan *et al*, 1983), pH (Elrod *et al*, 1993) and the concentration of urea and ammonia (Jordan *et al*, 1983: Carroll *et al*, 1988: Elrod and Butler, 1993a).

### 1.3.2 Level of feeding and reproductive performance

High levels of feeding in sheep are generally associated with increases in plasma insulin (Robinson, 1990) and a decline in progesterone (William and Cumming, 1982: McEvoy *et al*, 1993), but usually have little or no effect on gonadotrophins (Robinson ,1990). Thus circulating LH concentrations for example are not usually correlated with ovulation rate (Hunter *et al*, 1992). However, ovulation rate is often enhanced by high-plane feeding. Although the mechanism for the effect is not known it has been suggested that it may arise from a reduction in the inhibitory feedback action of progesterone on gonadotrophin secretion (Parr *et al*, 1992). It is known that high levels of feeding reduce circulatory progesterone concentrations, probably via their stimulatory effect on hepatic blood flow (Mineo *et al*, 1991: Kisauzi *et al*, 1981), which in turn results in an increase in the rate of break-down of plasma progesterone and removes up to 96% of circulating progesterone entering the gut and liver region after one passage (Parr *et al*, 1992).

In contrast progesterone concentrations in plasma have not been consistently found to vary with dietary protein levels in cattle. Blauwiekel *et al* (1986), Elrod and Butler (1993) and Rusche *et al* (1993) did not find any difference in circulating progesterone in relation to dietary protein in contrast to Jordan and Swanson (1979a).

The suggested mechanisms therefore, by which high dietary protein may influence reproductive rate are: local toxic effect on sperm, ovum or embryo; reduced LH binding to ovarian receptors leading to low progesterone at the ovarian level though not detectable in the peripheral circulation; and ammonium toxicity (Carroll *et al*, 1988). The possibility of low concentrations of progesterone acting at the ovarian level yet not being detectable as alterations in the peripheral circulation, led Rhind (1992) to conclude that measurements of peripheral circulatory progesterone concentration may be relatively meaningless.

### 1.3.3 Effects of dietary nitrogen concentration on metabolites and hormone levels.

Differences in dietary protein levels may have effects on hypothalamic and pituitary activities. These effects could be mediated via associated changes in circulating blood metabolites or hormone profiles, although no causal relationship has been identified (Rhind, 1992).

The crude protein content of the diet may raise the concentration of plasma ammonia and plasma urea, phosphorus and potassium and reduce plasma zinc (Jordan *et al*, 1983).

The quantity of daily nitrogen intake is the major determinant of urea synthesis (Harmeyer and Martens, 1980). Levels of 2.6% urea in the diet which resulted in blood urea levels of up to 8.75 mmol/l have been used with no reported signs of toxicity in sheep (Delgado, 1992). However, blood urea itself is not toxic as it is the substance formed on detoxification of ammonia by the liver. A more accurate indicator therefore is plasma ammonia. Toxicity symptoms occur at blood ammonia levels higher than 0.5Êmg/100 ml and become more severe at higher levels, with 4Êmg/100Êml being lethal according to Van Soest (1987).

In general it seems that the body regulates its ammonia levels in blood within certain limits even when excess protein intake leads to high levels of rumen ammonia which cannot be incorporated into microbial protein. This conclusion is based on the observation that blood ammonia concentrations are remarkably stable over a wide range of dietary protein intakes (Elrod and Butler,1993). In contrast blood urea nitrogen (BUN) increases with increased dietary protein (Rusche *et al*, 1993: Elrod *et al*, 1993: Jordan *et al*, 1983).

Rusche *et al*, (1993) found that when a high level of undegradable dietary protein was fed to beef cows, both plasma glucose and urea concentrations were depressed. In contrast a high level of rumen degradable protein resulted in an increase of both plasma glucose and plasma urea but had no effect on LH or progesterone concentrations. The source (i.e. soya bean meal, blood meal, urea, etc) and amount of dietary protein did not have any significant effect on hormonal profiles in beef cows (Rusche *et al*, 1993) or in non lactating cows (Blauwiekel *et al*, 1986). There was no response in serum concentration of insulin, the latter being inconsistent with the findings of Barry et al (1982) and Krysl et al (1987) who noted increased insulin concentration with increased metabolisable protein intake.This is in agreement with the findings of Fernandez *et al* (1988) that hyperglycaemia occurred during hyperammonemia, possibly due to under-utilization of glucose by insulin-sensitive tissues. The increase in insulin due to the "flushing" effect in ewes is not clearly resolved as to whether it is a protein or energy effect (Robinson, 1990).

**Table 1. Alterations in the blood metabolites hormone levels and uterine secretions associated with high levels of dietary protein**

| DIETARY CRUDE PROTEIN | BLOOD | | | | | | UTERINE SECRETIONS | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | BUN / PUN | $NH_4$ | GLUCOSE | LH | PROGESTERONE | pH | UREA | $NH_4$ | | |
| 100 OR 150 % CP High or low escape at each level. | ↓↑ | ↓↑ | ↓↑ | * | * | | | | | | Rusche et al 1993 |
| 50 % RDP above requirement. | ↑ | ↑ | | | ↑ >25d cycle | | ↓ | variable | | | Elrod & Butter 1993 |
| Both DIP & UIP 25 % above requirement. | ↑ | | | | | | ↓ | | | | Elrod et al 1993 |
| 13 % verses 20 % CP | ↑ | | | | ↓ | | | ↑ | | | Carroll et al 1988 |
| 15 % verses 20 % CP | ↑ | | | | | | | | | | Howard et al 1987 |
| 12 % verses 23 % CP | ↑ | ↑ | | | * | P, k ↑ Zu ↓ | | ↑ | ↑ | P, k Mg ↓ | Jordan et al 1983 |
| 15 % verses 25 % CP | | | | * | * | | | | | | Blawiekel et al 1986 |
| 12.7 % verses 19.3 % CP | | | | ↑ | | | | | | | Jordan & Swanson 1979 |
| Requirement +2.6 % urea | ↑ | | ↑ | | | | | | | | Delgado 1992. |

KEY ↑ – Significant increase in high level compared to low level of dietary protein.

↓ – Significant decrease in high level compared to low level of dietary protein.

* – No significant difference demonstrated between the protein levels.

### 1.3.4 Dietary protein levels and ovarian activity

Although effects of energy intake on steroid metabolism are now well documented in ewes (Williams and Cumming, 1982: McEvoy *et al*, 1993), there does not appear to be a comparable effect attributable to dietary protein. For cows the same appears to be true. For example LH and progesterone have not been consistently shown to vary significantly with different dietary protein levels in either heifers or older cows (Rusche *et al*, 1993: Jordan *et al*, 1983: Blauwiekel *et al*, 1979).

## 1.4.0 Endocrine mechanisms controlling ovarian function and embryo survival

Before studying the effects of dietary protein on reproductive performance in the ewe it is important to outline current knowledge on the endocrine mechanisms controlling ovarian function and embryo survival.

### 1.4.1 Ovarian function

Although follicle growth is a continuous process (McNeilly *et al*, 1992), only the one or two large follicles that are destined to ovulate secrete more oestrogen and bind more gonadotrophin in ewes (Hafez, 1987). The production of oestradiol by the granulosa cells of the follicle depends on the presence of an active aromatase system and a supply of androgen precursor (McNeilly *et al*, 1992).

Follicle stimulating hormone (FSH) stimulates granulosa cell mitosis and follicular fluid formation. This is an effect which is enhanced by oestradiol.

Follicle stimulating hormone (FSH) also increases the LH receptor numbers thus increasing sensitivity to LH. The high frequency and high amplitude LH pulses which follow the enhanced sensitivity to LH lead to the preovulatory LH surge (Rawlings and Cook, 1993).

LH pulses in the absence of FSH cannot induce follicle development, but at least at low frequency may be important in controlling follicular growth (Picton and McNeilly ,1991).

The relatively small number of FSH receptors present on granulosa cells led McNeilly *et al*, (1992) to suggest that minor changes in plasma concentrations of FSH or receptor expression too subtle to detect with our current assay methods are all that is necessary to significantly influence follicular growth.

It is generally accepted that during the growth of a dominant follicle, plasma FSH concentrations drop due to the combined negative feed back effect of ovarian oestradiol and inhibin on the pituitary. However, during the normal oestrous cycle, no consistent relationship exists between FSH and LH on the one hand and the number of large oestrogenic follicles or ovulation rate on the other (McNeilly *et al*, 1992).

FSH in the presence of only basal levels of LH can induce follicular growth, oocyte maturation and release of viable oocytes that can be fertilized (Baird *et al*, 1991).

Exogenous gonadotrophin releasing hormone (GnRH) at oestrus has been reported to increase serum progesterone earlier after ovulation and maintain higher concentrations of hormone for up to 40 days. This in turn has been associated with higher embryonic survival at 42 to 56 days after insemination. Michael *et al* (1993) have reported this to be due to an increased proportion of large luteal cells in the CL and possibly to an increased concentration and pulse frequency of FSH secretion.

Alila *et al* (1988) have also reported that large and small luteal cells secrete progesterone but are regulated differently, and only the small cells have receptors for LH. The large cells produce a higher basal output of progesterone but do not respond to LH stimulation.

In dairy cows, Carroll *et al* (1988) and Howard *et al* (1987) did not find a significant effect of crude protein intake or feeding strategy on days to first estimated ovulation. Similarly LH and progesterone did not vary significantly with different dietary protein levels (Rusche *et al*, 1993; Jordan *et al*, 1983; Blauwiekel *et al*, 1986). However, an increase in FSH with high protein intake in ewes associated with an increase in ovulation rate has been reported. It has been suggested that this is an indirect effect on the pituitary gland (Cruickshank *et al*, 1990).

### 1.4.2 Follicular development and ovulation

Three stages of follicular development in cattle have been described, preceding ovulation;

(1) recruitment of the follicle before onset of luteolysis,

(2) selection and growth thereafter until the preovulatory surge of LH, and

(3) final follicular and oocyte maturation after the LH surge and up to ovulation (Dieleman *et al*, 1993).

Full oocyte maturation is regulated by both gonadotrophins and steroids. Appropriate and adequate ratios of FSH and LH in serum are necessary for growth, maturation, ovulation and luteinization of the graafian follicle (Hafez, 1987).

The patterns of plasma FSH concetration in the ewe during the periovulatory period are less well defined due to less sensitive and less specific radioimmunoassays for this hormone (Haresign *et al*, 1983: 1992: McNeilly *et al*, 1992).

In sheep, selection of the follicles that would ovulate occurs throughout reproductive life but the mechanisms underlying selection of large oestrogenic follicles destined to ovulate are unclear. McNeilly *et al* (1992) has reported that in cows there is little evidence of active suppression of growth of other follicles by a factor(s) produced by the dominant or selected follicle.

At the onset of Stage two of follicular development the main steroid is estradiol and its decline coincides with the LH peak. The granulosa cell is depleted of its precursor for oestradiol, leading to a decrease of this steroid around the maturing oocyte six hours after the LH peak. This means that progesterone becomes dominant before ovulation with consequent luteinisation of the follicular tissue. However, the growth of follicles that are destined to ovulate, if given an appropriate LH signal, is determined by plasma FSH concentration (McNeilly *et al*, 1992).

The gonadotrophin surge in ewes has been associated with the turning-off of follicular metabolic pathways resulting in cytoplasmic and nuclear maturation of the oocyte, disruption of cumulus cell cohesiveness in the

granulosa layer and the thinning and rupture of the external follicular wall (Hafez, 1987). The role of prolactin in ovulation is not clear, although there is a preovulatory peak of this hormone, which in many cases is characterized by its biphasic nature.

McNeilly *et al* (1992) have postulated that in an anoestrous ewe when ovulation is induced with luteinizing hormone (LH), it is possible that LH pulses act mainly on the follicles already present and do not cause formation of new preovulatory ones. For this reason, it is necessary to combine LH and either GnRH or FSH, even though the precise interaction between FSH and LH in controlling the number of preovulatory follicles which develop is uncertain. It is generally accepted now that LH pulses may interfere with ability of FSH to induce preovulatory follicle growth, and that the degree of this effect depends on the amplitude of the LH pulses and plasma concentration of FSH.

The effects of dietary protein on ovulation rate may be mediated at the late stage of the oestrous cycle and follicle development. Rhind (1992) did not find that the differences in ovulation rate with level of feed intake were associated with either timing or size of the LH surge.

### 1.4.3 Intraovarian follicular dynamics

In the ovary, growth and maturation of follicles is regulated by several intraovarian factors, intrafollicular factors and hormonal signals. This is probably why the effects of nutrition on ovulation are not always explained by gonadotrophin profiles. Nutrition and intraovarian regulatory substances, some only discovered recently, have a role to play (Tsafriri, 1988).

Proliferation and differentiation of ovarian theca and granulosa cells leading to increased ability to secrete oestradiol are hormonally-induced. Oestradiol production determines the LH receptor gain that is necessary for ovulation and luteinization (Hafez, 1987).

It has been suggested that by understanding the nutritionally induced changes in metabolic hormones and their effects, if any, on intrafollicular peptides and steroids it may be possible to characterise the effects of such

changes on the ovarian response to gonadotrophins (Rhind, 1992). The demonstration of IGF-1 receptors in granulosa cells has led to the suggestion that insulin may act indirectly on the ovary through GH and IGF-1 (Monget *et al*, 1989). More recently with the discovery of the IGF binding proteins (IGFBPs) it has been suggested that they exercise an antigonadotrophic effect [(Ui *et al*, 1992); cited by Adashi, 1992] which may be dependent on an intact IGF, but not FSH binding site (Adashi, 1992). The possible role of plasma IGF-1 in modulating growth and development of follicles in cows experiencing a negative energy balance has been discussed by (Thatcher *et al*, 1992). In cattle the preovulatory LH peak has been associated with induction of further follicular differentiation or oocyte maturation in follicles _8 mm (Kastrop *et al*, 1992).

While FSH controls the number of large oestrogenic follicles that grow and develop in the ewe, the basal level of LH is essential for the ovulation of normal large oestrogenic follicles (McNeilly *et al*, 1992).

Currently there are several groups of suggested intra-ovarian regulators.

### 1.4.4 Induced ovulation and superovulation

In ruminants ovulation induced with gonadotrophin releasing hormone (GnRH) is not accompanied with oestrus and one large single dose will not normally induce additional follicular growth (Hafez, 1987). Hence ovulation induction is based on the use of progesterone to imitate the normal luteal phase and is then followed by gonadotrophin stimulation.

Since the incidence of oestrus cannot be predicted precisely for an individual in a group of randomly cycling ewes, synchronization of oestrus is often adopted (Hansel and Convey, 1983). Two methods used to control oestrus and subsequent ovulation are;

1. Long term progesterone administration to allow natural regression corpora lutea (CL) regression. In this situation the exogenous progesterone exerts a negative feed back effect on LH secretion following the demise of the animals own CL.
2. Use of a luteolytic agent which causes premature luteolysis of the CL some 24 to 72 hours after administration followed by oestrus and ovulation within 2 to 3 days (Hafez, 1987).

In ewes progesterone priming followed by an injection of pregnant mare serum gonadotrophin (PMSG) has been found effective for synchronizing oestrus. A progesterone pessary or implant is given for 12 to 14 days and PMSG is injected at the time of progesterone withdrawal (Hafez, 1987). The controlled internal drug release (CIDR) device containing progesterone maintains plasma levels of this hormone within the normal range found during the normal oestrous cycle (Ainsworth and Downey, 1986). Normal plasma progesterone levels at the time of PMSG administration are critical to ovulation rate and embryo viability in superovulated ewes (Scudamore *et al*, 1992).

Progesterone release from the controlled internal drug release (CIDR) device is influenced by the content and concentration of progesterone in the silicon elastomer and not solely by variation between animals in their capacity to absorb the released progesterone (Macmillan *et al*, 1990).

With regard to superovulation in ewes, FSH administered at high doses ie ≥20 mg can give a good degree of superovulation combined with an acceptable level of ova fertilization and recovery (Rexroad and Powell, 1991). However, FSH preparations unlike PMSG and human chorionic gonadotrophin (hCG) have short biological half lives.

Therefore in contrast to PMSG they have to be given in a series of fairly frequent injections. For a given gonadotrophin preparation the superovulatory response is affected by factors such as individual animal differences, physiological state of the animal at the time of treatment, its age and breed, the time of the year, and the number of consecutive stimulations.

Monniaux *et al* (1983) attributed the variation of response to superovulation to individual differences in the follicle populations of the ovaries both in terms of numbers and stage of development.

The hormonal preparation used to control oestrus and superovulation, causes a reduction in the proportion of ova that are fertilized and the numbers of accessory sperm attached to fertilized ova (Hawk *et al*,1987). It is suggested that this is due to a hostile uterine enviroment for egg development following superovulation (Boland *et al*,1978). Superovulation

in cows can cause follicular steroidogenesis, premature maturation and ovulation of oocytes as well as abnormal systemic hormone profiles (Foote and Ellington,1988; Moore *et al*,1985). Despite these effects an increase in the incidence of chromosomal abnormalities is not a consistent finding in sheep (Murray *et al*,1986).

### 1.4.5 Effect of high dietary protein on oestrus and ovulation

Howard *et al* (1987), Kaim *et al* (1983) and Canfield *et al* (1990) did not find that concentration of dietary protein affected days open (i.e. interval from calving to conception) or time to first observed behavioural oestrus or estimated time of ovulation.

### 1.4.6 In vivo embryo development

In vivo embryo development in normal and superovulated ewes has been determined mostly by subjective assessement of embryos at the time of recovery, often followed by an objective measurement based on short term culture *in vitro*.

The different stages of embryo development within the first 14 days post fertilization are divided into early cleavage stages, morula, blastocyst and elongation stages respectively (Rowson and Moore,1966). These stages have been defined either by examining embryos from animals slaughtered at different stages of early pregnancy or by noting changes in development during *in vitro* culture.

During these stages, progesterone from the corpus luteum dominates the gravid uterine endometrium and stimulates the glandular epithelial development that is necessary for maternal regulation of conceptus growth and development in early pregnancy (Garrett *et al*, 1988). The correct progesterone/oestrogen ratios in maternal plasma and the correct protein content of the uterine fluid are important to blastocyst development (Beier, 1982) . Besides these steroids, a number of polypeptides are released which may be concerned with maintenance of nutrition for the developing conceptus and its attachment and the maintenance of CL function (Geisert *et al*, 1988). Early trophoblastic development in sheep involve cell death and this is part of normal development (Carnergie *et al*, 1985). This cell death may be a process of

over-all remodelling of the blastocyst, or the dying cells may be genetically deficient cells unable to respond to the appropriate cues that stimulate differentiation.

### 1.4.7 Maternal recognition of pregnancy in ewes

Uterine $PGF_2$alpha in ewes is the luteolysin that controls the inter-oestrus interval. It has long been recognised that proteins secreted by the conceptus between days 12 and 21 of pregnancy inhibit $PGF_2$alpha production by uterine endometrium. (Rowson, 1966). Ovine trophoblast protein-1 (oTP-1) for example is one such protein which has been found to inhibit luteolysis at two major levels, *viz* the basal level of secretion and the oxytocin-induced release of $PGF_2$alpha (Knickerbocker *et al*, 1986).

Although GnRH has a luteoprotective function in several species, this is only one of the many embryo-uterine-ovarian interactions. During the first week of pregnancy follicular oestradiol 17-beta and luteal oxytocin are suppressed while pituitary LH secretion is enhanced, causing increasing luteal progesterone release (Thatcher *et al*, 1989).

Oxytocin output begins in the granulosa cells of the preovulatory follicle (Wathes,
1989). Protection against oxytocin effects is prevented by a number of mechanisms which include the cessation of luteal oxytocin secretion which is seen by day 20 after mating in sheep and the maintenance of the oxytocin receptor in the uterus at low levels. It has been sugested for example that progesterone reduces the density of oxytocin receptors in the myometrium (Zhang *et al*, 1992).

In cows another prostaglandin $PGE_2$a is secreted in high quantities by stroma cells. These cells are insensitive to both bovine trophoblast protein-1 (bTP-1) and oxytocin. This has therefore been suggested to play a complementary role to the antiluteolytic effect of bTP-1 on $PGF_2$alpha secretion by epithelial cells. Whether this is the case in sheep is not clear. This does not however imply that there are no other complementary mechanisms.

### 1.4.8 High dietary crude protein and embryo survival

A large proportion of the observed reduction in reproductive efficiency is attributed to embryo loss early in pregnancy and to foetal loss later. High dietary protein levels may be one of the many factors causing early embryo death. The mechanisms of protein effects on fertility may be based on protein metabolism and may include toxic by-products, imbalances in protein and energy supply and nitrogen by-products. The effects may occur together in an additive or synergistic way. The levels in the body at which the effects may be mediated are; the pituitary/ovarian axis, corpus luteal function and the uterine enviroment (Elrod *et al*, 1992). It is possible that poor embryo viability, seen as poor reproductive performance, is a delayed effect triggered at the level of the developing oocyte.

### 1.5.0 Artificial insemination (AI)

### 1.5.1 Methods in sheep

The well known methods are:

(1) conventional method of depositing semen in the os cervix,

(2) transcervical intrauterine insemination of ewes. (the latter gives higher pregnancy rates (Halbert *et al*, 1990) ) and

(3) artificial insemination using a laparoscopic procedure (McKelvey *et al*, 1985) where by semen is deposited directly into the uterine lumen.

In ewes, using 50-100 x $10^6$ viable sperm by intrauterine artificial insemination (IUAI) can overcome the adverse effects of high dose rates of PMSG used in the induction of ovulation as shown by Aitken *et al* (1990). The intrauterine insemination procedure increases pregnancy rates but stress associated with the technique may disrupt the normal collection of the oocytes by the fimbriated infundibulum (Robinson *et al*, 1989). However, the use of sedative just prior to insemination can eliminate this problem (Scudamore *et al*, 1991).

### 1.5.2 Artificial insemination timing and embryo quality

In cows, preovulatory insemination has been found necessary for the establishment of a competent spermatozoa population in the fallopian

tube. Ova remain viable for only 8 to 10 hour after ovulation. For this reason, Hunter (1989) has suggested that the best insemination time is 12 to 18 hours before the estimated time of ovulation. Ovulation in cattle occurs 10 to 15 hours after the end of standing oestrus. This means insemination should be carried out towards the end of the period of behavioural oestrus. Too early insemination puts spermatozoa at risk from aging in the female tract, too late compromises the ova (Hunter, 1985). In the ewe ovulation takes place during standing oestrus, 24 to 30 hours from the begining of standing oestrus. Oestrus itself lasts from 24 to 36 hours and fertilization must take place within 24 hours.

In the non-superovulated ewe following synchronization, insemination twice at 48 and 60 hours after progesterone withdrawal and using the conventional cervical method or natural mating has been found to give the same pregnancy rates as when single intrauterine insemination is carried out either at 48 hours or at 53 ± 3 hours. However, the latter is much more economic in terms of semen use (McKelvey and Robinson ,1986; Robinson *et al*, 1989). This is probably because in ewes, ovulation occurs 21 to 26 hrs after LH surge. Combining IUAI at 40 hours after progesterone withdrawal with natural mating in ewes superovulated using FSH has been reported to give good results in terms of ovulation rate, embryo recovery and fertilization (Rexroad and Powell ,1991).

Intrauterine artificial insemination reduces the chance of failure of fertilization that is commonly observed in natural mating or conventional artificial insemination of ewes (McKelvey and Robinson, 1986: Robinson *et al*, 1989).

### 1.5.3 Parameters for measuring embryo quality

Overstrom (1992) and Butler and Biggers (1989) have reviewed the methods currently in use for assessing embryos and for evaluating the effect of different insults on their development *in vivo*. These are:

(1) morphology;
(2) development in culture;
(3) live/dead cell staining;
(4) fluorescent metabolic probes;
(5) microassays of embryo respiration;

Each of these protocols has its advantages and disdavantages.

Currently the evaluation of bovine and ovine embryos on the basis of morphology is widely used. In this approach, size, shape, symmetry, colour and texture are the parameters measured (Lindner and Wright,1983). The correlation between cell number and morphology is very poor because blastocoele formation is a temporal event independent of cleavage (Batt *et al*,1991). The above methods include counting of nuclei and assessing whether nuclei are normal or pyknotic. Development of embryos in culture is used to assess viability either by noting morphological changes with time or cell division. This possibly coupled with live or dead cell staining, microassays of embryo respiration or flourescent metabolic probes are the methods available.

The metabolism based techniques need to be sensitive enough to measure the incorporation and secretion rates of substrates and metabolites respectively during incubation and the methods must be non-destructive to the embryo and simple to perform. A number of methods that could be used in theory have been suggested. Johnson *et al* (1991) have reported the use of the lactate dehydrogenase (LDH) assay to be a reliable test of embryo viability. This is an example of a metabolic test. The test is based on the bioluminescent assay for lactate dehydrogenase which is an indicator of tissue degeneration and high levels of LDH indicate a degenerating or dying embryo.

Measurement of embryo respiration is another non-invasive method which meets the criteria of an ideal viability assay (Butler and Biggers 1989).

### 1.6.0 Embryo recovery techniques and culture *in vitro*

### 1.6.1 Embryo recovery : surgical versus non-surgical

The methods are either based on surgical or non-surgical techniques and/or a combination of both.

The non-surgical procedure for embryo recovery in ewes has been compared with the surgical procedure (Hackett et al,1992) and found to be less efficient for embryo collection on day five.

A `Through-Flush' technique involving laparoscopic visualization of the uterus was developed and modified by McKelvey *et al* (986). This method, unlike the use of major surgery, can be used to repeatedly recover ova with a reduced incidence of post- operative adhesions.

### 1.6.2 Sheep embryo culture *in vitro*

The ability to culture ovine embryos *in vitro* is limited due to poor understanding of the factors controlling early embryo development. The current knowledge on culture conditions for successful culture of pre-implantation embryos in sheep is reviewed.

Although *in vitro* matured or fertilized bovine oocytes have been shown to develop to the morula stage in chemically defined media, serum factors are necessary for blastocyst development (Pinyopummint and Bavister, 1991). Embryos may be cultured in complete media (i.e. minimum essential media, MEM) or simple media. There is little evidence that zygotes of livestock species develop better in simple media (Wright *et al*, 1976). Simple media have been used in ovine and several livestock species (Walker *et al*, 1992). These media are often supplemented with protein, salts, buffer components and energy sources. Protein sources are sheep serum, bovine serum or albumin and human serum. Serum is considered superior to albumin for culture of zygotes. Whether or not human serum is superior to sheep or cattle serum is debatable (Walker *et al*, 1992).

Some unknown factors in serum are involved in trophoblastic tissue development (Flechon *et al*, 1986). This is rather unfortunate since bovine serum albumin (BSA) or any serum is relatively chemically undefined and varies from batch to batch (Russlerlong *et al*, 1991). In simple media the osmolarity often varies between 276-316 mOsm (Walker *et al*, 1992) . The optimum pH range for media is 7.1 to 7.4. The addition of bovine serum albumin (BSA) or other serum lowers the pH. For pH stabilization bicarbonates are used. Hepes (N-2-hydroxyethyl piperazine-N-2-ethenesulfuric acid) achieves a much better pH stabilization. Phosphate buffered saline (PBS) supplemented with sera for embryo culture has not been found to be successful in sheep and zygotes develop only up to the blastocyst stage.

A gas atmosphere of 5 % oxygen is conducive to sheep embryo development (Walker *et al*,1992). Energy requirements are not well established for sheep embryos. Zygotes can develop in medium with lactate and or pyruvate without glucose. Glucose may be beneficial and up to zygote development the lactate pyruvate ratio may be important (Boone *et al*,1978).

Walker *et al* (1992) suggested that media evaluation should include the assessment of embryo viability after culture since many blastocysts that form *in vitro* are developmentally deficient.

Use of oviduct epithelial or granulosa cells in co-cultures with embryos has been associated with increased developmental rates in cattle (Aoya *et al*,1990) and sheep (Fukui and Ono,1989).

There is evidence now that embryotrophic factors are produced from oviduct cells (Gandolfi *et al*, 1989; Ellington *et al*, 1990; Bavister, 1988) and that these factors are required for very early normal embryo development in sheep (Sutton *et al*, 1984; Gandolf *et al*, 1989). Media supplementation with hormones such as LH, FSH and oestradiol have not been shown to improve fertilization,cleavage and development of embryos *in vitro* (Fukui and Ono, 1989). Zygotes cultured for 5 to 7 days in SOF supplemented with human serum at 20% tend to have a higher incidence cytoplasmic fragmentation (Gandolfi and Moor, 1987) and this higher degree of fragmentation was associated with reduced ability to develop to the blastocyst stage (Walker *et al*, 1992). *In vitro* culture of embryos has been associated with low viability following transfer to recepient ewes. This effect is apparent only by day 50 and not day 14 of pregnancy (Tervit and Rowson, 1974: Walker *et al*, 1992). Sheep embryo development in culture in contrast to development *in vivo*, manifests cytoplasmic fragmentation, early blastocoele formation and about 35% reduction in numbers of nuclei per blastocyst (Walker *et al*, 1992).

### 1.7.0 Objective

The objective of the present study was to investigate the effect of varying levels of dietary urea (rumen degradable protein) during and after the pre-

ovulatory priming period on ovulation, fertilization and the early development of sheep ova both *in vivo* and *in vitro*.

## 2.0 MATERIALS AND METHODS

The experiment was undertaken to study the relationship if any between ruminally degradable protein level in the ruminant diet and ovulation rate, fertilization and embryo survival. Embryo quality was assessed by morphology, on day 4 and day 11 following insemination. Day 4 embryos were also assessed on the basis of growth after 72 hours of *in vitro* culture, and $^{3}H$ phenyalanine incorporation during a two hour incubation period was used to determine rates of protein synthesis in these embryos recovered on Days 4 and 11.

Excess ruminally degradable protein was simulated by feeding 24 g and 48 g urea per day.

### 2.1. Animals

Thirty 2-6 years old Greyface (Border Leicester x Scottish Blackface) ewes were brought indoors during late April with their recently born lambs and maintained on an artificial photoperiod of 12 hours of light: 12 hours of darkness each day. The lambs were weaned at four weeks, a week before the ewes were placed on the experimental diets. The initial body weights of the ewes ranged from 56 to 75 kg and averaged 65.5 kg. The ewes were ranked in ascending order of their weights and each consecutive trio were randomly allocated to the three treatments.

### 2.2 Experimental design

A randomised design with three dietary treatments was used. The treatments were as follows :-

| | |
|---|---|
| (C) basal diet (1600 g) | Control |
| (L) basal diet (1600 g) plus 24 g urea supplement | Low Urea |
| (H) basal diet (1600 g) plus 48 g urea supplement | High Urea |

All diets were weighed separately for each ewe and supplemented with minerals and vitamins. Urea supplements were mixed into the basal diet at feeding time. Sodium sulphate was added as a source of sulphur in the ratio of 14:1 by weight, urea:sulphur. The diet was fed at the rate of 800

g/feed twice daily at 08:00 and 16:00 hours.Water was made available at all times.

Table 2.1 Composition of the basal diet (g/kg).*

| INGREDIENTS | CONTROL g/kg |
|---|---|
| HAY | 832 |
| MOLASSES | 147 |
| DICALCIUM PHOSPHATE | 10 |
| SODIUM CHLORIDE | 10 |
| MIN/VIT # | 1 |

* Fresh basis

# supplied per kg of diet: 0.9 mg retinoyl palmitate, 0.015 mg cholecalciferol, 12 mg DL-_-tocopheryl acetate, 0.12 g MgO, 85 mg $ZnSO_4.7H_2O$, 40 mg $MnSO_4.4H_2O$, 0.12 mg $KIO_3$ and 0.25 mg $CoSO_4.7H_2O$.

## 2.3 Housing and management

Ewes were housed and fed in individual concrete floored pens which were cleaned once daily and bedded with saw dust. The ewes were allowed an adaptation period of one week to adjust to the experimental diets.

## 2.4 Oestrous synchronization and ovulation induction

Each ewe received a single intramuscular injection of 150 µg of prostaglandin $F_2a$ analogue (Cloprostenol; Estrumate, Cooper Animal Health, Crewe, UK). Oestrus was synchronized by using a controlled internal drug release (CIDR) device containing 0.3 g progesterone (Inter-Ag Hamilton, New Zealand). This was inserted into the vagina at 40 hours after the prostaglandin injection and remained in position for a period of 12 days. To induce ovulation an intramuscular injection of 800 IU pregnant mare serum gonadotrophin (PMSG) (manufactured in Holland for Intervet UK Ltd., Cambridge) was given at the time of CIDR withdrawal. The ewes were then exposed to a vasectomised ram at 2-hour intervals beginning 24 hours after CIDR removal for the determination of the time of oestrus onset. Oestrus was defined as the time at which ewes first stood to be mounted by the vasectomised ram.

## 2.5 Semen preparation

Throughout this experiment fresh semen was used for insemination. It was collected from the same Suffolk ram by means of an artificial vagina and immediately diluted in the ratio of 1:1 with phosphate buffered saline (PBS, pH 7.4) held at 37°C. The diluted semen was then kept in an incubator at 37°C for not more than 40 minutes during the period that it was being used for inseminations.

## 2.6 Intrauterine artificial insemination (IUAI) by a laparoscopic technique

IUAI was carriedout approximately 52 hours after CIDR device removal. The 16:00 hour feed on the day preceding intrauterine artificial insemination (IUAI) was given to each ewe as usual but water was withheld from 17:00 hours until after IUAI.

After IUAI at 12:00 hours, the withheld 08:00 hours morning feed was given and water was reintroduced in the pens. Approximately 45 minutes before IUAI the ewes were injected i.m. with 5 mg of the tranquillizer, acepromazine maleate (ACP). IUAI was carried out as described by Scudamore (1991). This method is a modified version of that described by McKelvey *et al* (1985) and involved the use of a laparoscopic cradle to restrain ewes in dorsal recumbency. The abdominal surface cranial to the udder was clipped and sprayed with hibitane solution (15% water, 75% alcohol, 10% v/v hibitane concentrate; ICI Pharmaceuticals, Macclesfield, Cheshire, UK). Local anaesthetic was infiltrated into the abdominal wall in two areas approximately 6 cm cranial to the udder and 4 cm to each side of the udder; 5 mls of Lignavet plus (lignocaine and adrenaline; C-Vet Ltd., Bury St Edmunds, UK) was injected at each site. An intramuscular injection of 3 mls Duphapen-strep (250000 iu procaine penicillin and 250 mg dihydrostreptomycin sulphate; Duphar Veterinary Ltd., Southampton, UK) was given for antibiotic cover.

Following disinfection of the ewe, the cradle was raised so that the ewe was lying head down at an angle of 45° to the horizontal surface. A laparoscope trocar and cannula (Richard Wolf Ltd., Mitcham, Surrey, UK) 7 mm in diameter was inserted on the left side of the mid-line through the body wall at the site of anaesthesia. The procedure was carried out taking care to avoid surface veins. Upon removal of the trocar $CO_2$ was introduced

via the cannula to inflate the abdomen. Another (5 mm diameter) cannula was then introduced through the body wall on the right side of the mid-line. This was used to manipulate the uterus into position. A 7 mm laparoscope, 0° angle of view (Richard Wolf Ltd., Mitcham, Surrey, UK) attached to a 250W light source was introduced down the first cannula. A fine tipped glass pipette (diameter 0.4 cm, length 25 cm), attached by rubber tubing to a 1 ml syringe, containing a cushion of air and approximately 0.2 mls of diluted semen was introduced via the second cannula once the uterus had been viewed. The tip of the pipette was stabbed into the uterine horn and the semen expressed by pushing in the plunger of the syringe. This was repeated for the other uterine horn. Following insemination the laparoscope and inseminating pipettes were withdrawn and the $CO_2$ was released from the abdomen through the cannulae. These were then removed and together with the laparoscope, placed in a sterilizing solution (Marinol blue 50% BK Veterinary Product, Bury St Edmunds, UK). The trocar incisions in the skin were only closed with michael clips (5 mm) if there was a sign of haemorrhage.

## 2.7 Preparation of animals for embryo recovery

Day 4 recoveries

Prior to the surgery water was withheld from the previous evening and the 08:00 hour feed was withheld on the day of surgery. Both feed and water were given again after surgery at approximately 12:00 hours.

Day 11 recoveries

From 17:00 hours on the day preceding the surgery water was withheld and the ewes were not given their 16:00 hour feed. Water was reintroduced after surgery at 12:00 together with the withheld 08:00 hour feed.

## 2.8 Embryo recovery by laparotomy

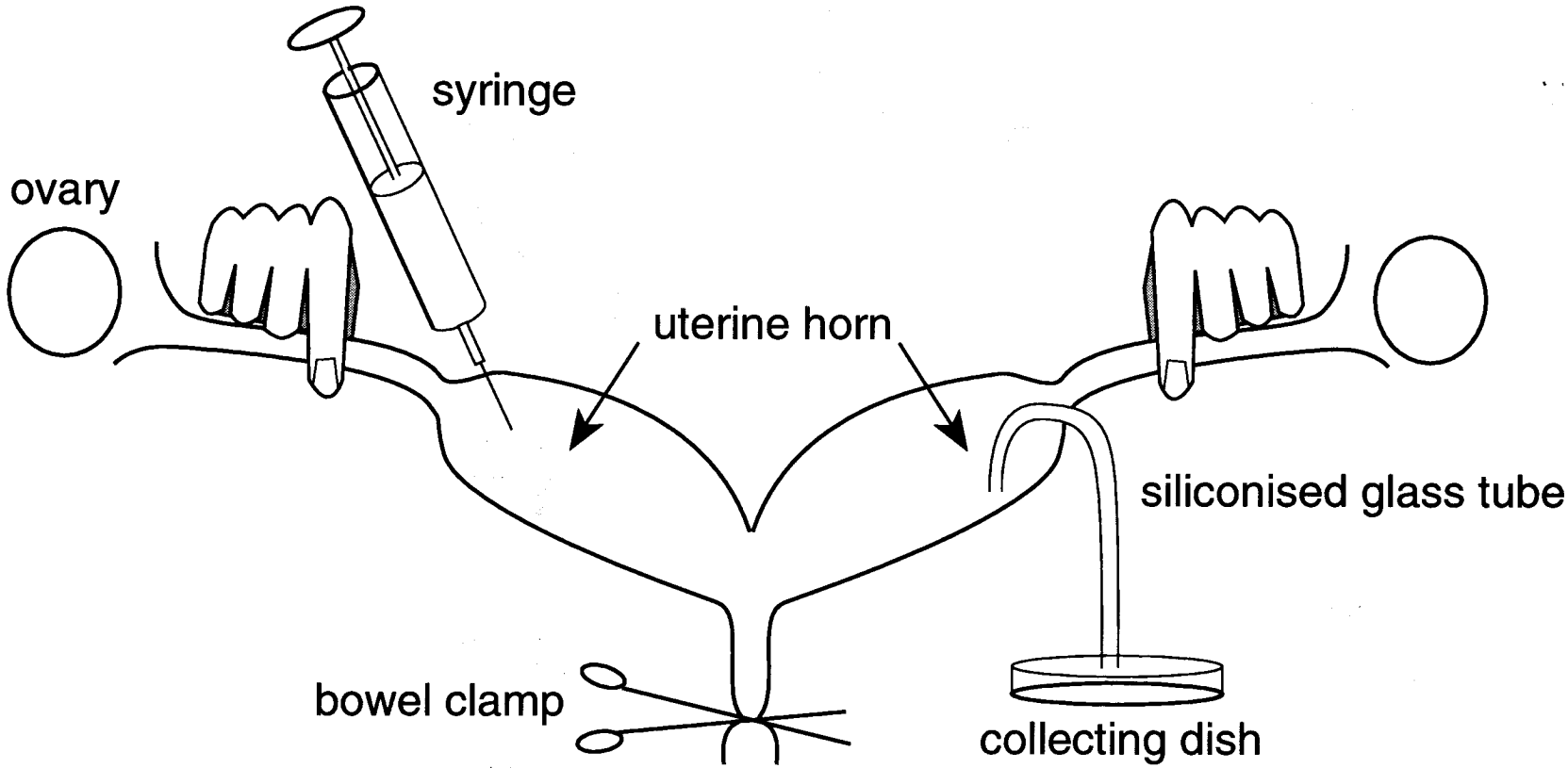
(a) Day 4 recoveries.

Anaesthesia was induced and maintained after intubation using halothane, nitrous oxide and oxygen. At laparotomy the numbers of corpora lutea were counted and the embryos were recovered by retrograde flushing of each oviduct (Hunter *et al*, 1955).

(b) Day 11 recoveries.

Anaesthesia was induced and maintained in the same way as above. Figure 2 is an illustration of the method used to recover embryos at day 11. This method was described by Wallace *et al* (1993). The uterus was exteriorised and clamped at the cervix using bowel clamps. A small tissue forcep was used to puncture the uterine horn close to the utero-tubal junction to allow the introduction of a sterile siliconised glass tube (4 mm i.d) to deliver flushing media from the uterus into a collecting dish. 20 mls of Ovum Culture Medium (OCM) was introduced from the opposite uterine horn using a blunted (18 gauge) needle and manually expressed through the glass tube into the collection dish. Following the autotransfer the puncture wound used to insert the glass tube into the uterine horn was stitched using catgut. In all recoveries at Day 4 and Day 11, one embryo was returned (autotransfer) when one or more embryos were recovered.

**Figure 2. Technique of flushing used to recover embryos at day 11 after Intra-uterine artificial insemination**



(c) Autotransfer

The embryos were located using a stereomicroscope (40 x magnification). When more than one embryo was present in the dish each one was given a code number and one was chosen at random by a person not involved in the location or assessment of the embryos. For the Day 4 transfers a fine glass pipette attached by rubber tubing to a 1 ml syringe containing a cushion of 0.2 mls of air was used to suck up the embryo from the ovum collection dish. The pipette was inserted through a puncture wound created by an 18 gauge blunted needle about 2 cms caudal to the utero-tubal junction. The embryo was gently expelled into the uterine lumen by pressing the plunger of the syringe. For the day 11, embryos a thick glass pipette (0.5 cm diameter drawn to a blunted point, 0.3 cm diameter) was used and this was inserted into the opening in the uterine horn that had been used previously to recover the embryos .

## 2.9 Embryo handling after recovery

(a) 4 Days after IUAI.

In surgery embryos were collected in ovum culture media (OCM) in sterile glass dishes and an intial embryo assessment was made, at (approx. 40 x magnification) using a stereo microscope. When located the ova were placed in OCM supplemented with 10 % v/v heat treated foetal calf serum (HTFCS) at 56°C: 35 min, [wash 1]. This 10% solution was prepared by making up 2 ml HTFCS to 20 mls with OCM in a syringe and then filtering with a sterile acrodisc 0.2 micrometres (low protein binding non-pyrogenic) filter.

Serum was added to prevent embryos adhering to glass. The embryos were then transferred to 4 well dishes with 1.0 ml filtered OCM + 10% FCS, [wash 2]. Preliminary classification was made and, the dish sealed with masking tape to minimize evaporation before being placed on a warm stage at 37°C. Embryos from different donors were kept in separate wells. Further assessment was made in the laboratory using an inverted microscope at 200 x magnification. The stage of development of each individual embryo was determined, and each was categorized and graded according to gross morphological characteristics and the number of

spermatozoa adhering to the zona pellucida was estimated. Prior to culture, all ova in the four-well dish were passed through another series of *washing steps, which involved sequential transfer through two more wells of filtered OCM + 10% FCS* [washes 3 and 4] and through a further two wells containing medium-199 supplemented with 10% FCS, 1% L-Glutamine and 1% penicillin-streptomycin solution.

(b) 11 Days after IUAI.

*The flushing media and washing procedures were the same as at Day 4.* The dimensions (length x width) of the embryos were measured using the microscope eye piece rule.

### 2.10 Culture *in vitro* of day 4 recovered embryos

Day 4 recoveries.

Embryos recovered at Day 4 were cultured for 72 hours in TCM-199 bicarbonate buffered medium at 37°C in an atmosphere of 5% $CO_2$ in air. Except for the auto- transfers, recovered embryos were passed through another series of washing steps involving sequential transfer through filtered OCM + 10% foetal calf serum (FCS), before culture in tissue culture Medium 199 (TCM-199) supplemented with 10% FCS, 1% L-glutamine and 1% penicillin-streptomycin solution. They were then placed in a humidified modular incubator, gassed for 10 minutes with 5% $CO_2$ in air, sealed and placed in a conventional incubator at 37°C. Every 24 hours individual ova were assessed after which the chamber was regassed for 10 minutes. The ova were assessed for development in culture on the basis of morphological changes and graded on a 1 to 5 scale as very good (1), good (2),fair (3), poor (4) or dead (5), based on the criteria described by Lindner and Wright (1983). After 72 hours in culture, ova were washed in OCM. This was followed by a further four washes in Dulbeccos modified essential media (DMEM). A 20 µl carry over during the washing steps was allowed. The ova were finally picked up in 50 µl DMEM $^3H$ Phenylalanine. They were incubated for two hours at 37°C in an atmosphere of 5% $CO_2$. At the end of the incubation period the ova were washed again four times in PBS to remove excess $^3H$ phenyalanine and cold phenylalanine. They were then placed in 50 µl PBS supplemented with serum (FCS) in small vials and

frozen at -20°C for a few minutes before being transferred to -70°C. They were held at -70°C until time for radioisotope count. The serum was added to give a critical mass for protein precipitation.

Day 11 recoveries.

Embryos recovered on Day 11 after IUAI and not returned to the donor ewe were not cultured in the same way as Day 4 ova. They were washed four times in DMEM with a carry over between washing steps of 20 µl before finally being picked up in 50 µl. They were then incubated for two hours in DMEM in the prescence of $^{3}H$- Phenylalanine at 37°C in an atmosphere of 5% $CO_2$ in air. At the end of incubation, they were washed in PBS only, picked up in 50 µl and frozen at -20°C before being transferred to -70°C and held there until time for radioisotope counting.

## 2.11 Preparation of embryonic material for $^{3}H$ phenylalanine incorporation count after 2 hour incubation.

Day 4 and day 11 recovered embryos.

The frozen embryos in 50 µl OCM at -70°C were thawed and 50 µl 0.6M NaOH added to dissolve the tissue; 30 µl 2.0M perchloric acid was then added to alter the pH and thus precipitate protein. After mixing on a vortex mixer the vials were placed on ice for 5 minutes to aid precipitation. The vials were centrifuged for 7 minutes at 9000 rpm. Approximately 130 µl of supernatant was aspirated into 5 mls of scintillating fluid (for internal counting of aqueous and non aqueous samples ; N.E. Technology Ltd Edinburgh, UK) here after considered as fraction A. The pellet in the vial was resuspended in 100 µl of 0.2M perchloric acid, shaken and put on ice for 5 minutes before being centrifuged for 7 minutes at 9000 rpm. The supernatant was then aspirated as before to give fraction B. The pellet was resuspended and the above procedure repeated to give fraction C. The remaining pellet in this last step was resuspended in 100 µl of 0.3M NaOH and the solution aspirated into 5 mls of scintillation fluid. To this solution was added 100 µl of 0.5M hydrochloric acid to ensure acidic pH and to prevent chemiluminescence which occurs in alkaline conditions. This was fraction D.

The ß counts of radioactive $^{3}H$-phenylalanine in fractions A,B,C and D were done on a Minaxi tri-carb 4000 series (United Technologies, Parkard, USA). Whilst the reading in the fraction D is the one which is essential in this procedure, the readings in the other fractions was supposed to indicate whether or not the washing steps were efficient in removing pools of free radioactive isotope.

### 2.12 Blood sampling

Blood samples were collected by jugular vein puncture using vacutainer needles into plastic tubes with added heparin starting just before insertion of the CIDR device and then once daily at 14:00 hours until Day 30 after insemination. These were analyzed for progesterone. Begining twenty-four hours after CIDR device removal and PMSG injection (800 iu) blood samples were also collected every two hours for thirty-two hours before reverting to a single daily sample. The two-hourly samples were taken to determine the time of the LH peak and the duration of sampling was based on previous experience which showed that the LH peak occurred somewhere between 24 and 56 hours after CIDR device removal. Plasma from these blood samples was analyzed for luteinizing hormone. The two-hourly plasma samples spanning a twenty four hour period starting 24 hours after CIDR removal were also analyzed for urea and ammonia.

### 2.13 *Plasma urea determination*

Plasma urea concentrations were determined on an autoanalyzer by a method based on the hydrolysis of urea to ammonia with urease and then reacting ammonia with 2-oxoglutarate to L-Glutamate and subsequent oxidation of NADH to nicotinamide adenine dinucleotide (NAD), a reaction catalysed by the enzyme Glutamate Dehydrogenase. The resulting decrease in absorbance at 340nm is proportional to the level of urea in the sample (Talke and Schubert, 1965).

### 2.14 Plasma ammonia determination

Plasma ammonia concentration was determined on an autoanalyzer by a method based on reductive amination of 2-oxoglutarate, using glutamate dehydrogenase (GLDH) and reduced nicotinamide adenine dinucleotide

(NADH). In this method the decrease in absorbance at 340 nm [$A_{340}$] due to oxidation of NADH to NAD is proportional to the plasma ammonia concentration Talke and Schubert (1965). Methylated amines do not react in this method.

## 2.15 Hormone assays

(a) Progesterone

Plasma was analyzed for progesterone content by radioimmunoassay following extraction as described by Djahanbahkch *et al* (1981).

Extraction

All samples were extracted in singleton with at least 3 quality controls in duplicate. To estimate extraction efficiency of progesterone, 20 µl of $^{3}H$-Progesterone recovery trace (_1000-1500 cpm) (Amersham International Plc., Amersham, Bucks, UK) made up in phosphate gelatin buffered saline (PGBS) was aliquoted together with 100 µl of sample into glass tubes. These tubes were then vortexed and left to stand on the bench for 30 minutes. 2.5 ml diethyl ether (Rhône-Poulene Ltd., Manchester, UK) was added to each tube and the tubes were shaken on a multi-vortexer (Luckham Ltd., Sussex, UK) for 20 minutes. The tubes were then immersed in a methanol bath to freeze the acqueous layer so that the organic layer could be decanted into disposable glass tubes. After placing the tubes on the dry block set at 40-50°C the organic layer was dried down under air. The residue was cooled and reconstituted with 200 µl of PGBS, the tubes were then vortexed and left overnight at 4°C.

Progesterone assay

100 µl of the reconstituted sample or standard (Sigma Chemical Co. Ltd., Poole, Dorset, UK),100 µl of first antibody (sheep antibody, initial dilution 1:20,000) and 100 µl of $^{125}I$-Progesterone tracer (Approx.15000 cpm/100µl, Amersham International Plc., Amersham , Bucks, UK) were added to glass assay tubes. The tubes were vortexed and incubated on the bench for 3 hours after which 100 µl of donkey anti-sheep serum (DASS, SAPU, Carluke, Lanarkshire 1:20 dilution) and 100 µl normal sheep serum (NSS, 1:700 dilution) were added to each tube. The tubes were then vortexed and incubated at 4°C overnight. 1 ml of 0.9% saline was then added to facilitate the decanting process and the tubes were centrifuged at 2000 rpm at 4°C for 30 minutes. The supernatant was drained off and the residue allowed

to dry. A gamma counter (Cobra Auto-gamma, Canberra Parkard) was used to count radio active emissions at 60s/tube.

Progesterone in plasma samples taken immediately before CIDR device withdrawal (sample No. 14) and on Day 18 (Day 0 = oestrus) for pregnancy diagnosis was measured in duplicate, while the samples before, during and after CIDR device removal were measured in singleton.

The lower limit of detection of the assay was 0.15 ng/ml. Since the progesterone measurements were all done in a single assay only the intra-assay coefficient was calculated for 3 quality control pools measured in quadruplicate and was found to be 9%. The mean recovery of progesterone in the extraction process was (± SEM) 61.5 ± 1.02 % or N = 150.

(b) Luteinizing hormone

Plasma LH concentrations were measured in duplicate by a direct double antibody, radioimmunoassay technique as decribed by McNeilly *et al* (1986). To 100 µl sample or standard (NIH-oLH-S25) (NIH, Bethesda, Maryland, USA) with 200 µl buffer was added 100 µl of the first antibody (R29 Rabbit anti-ovine LH, initial dilution 1:120,000). The tubes were incubated at 4°C for 24 hours, after which 100 µl of tracer (LER-1056-C2 iodinated using chloramine-T dilution approx. 15000 cpm tube/60s) was added and then left to incubate at 4°C for 24 hours. 100 µl of second antibody of donkey anti-rabbit serum (DARS dilution 1:32) was added to the tubes and then 100 µl of normal immunised rabbit serum (NIRS dilution 1:200) before being further incubated at 4°C overnight.

1 ml 0.9% saline was added and the tubes were centrifuged at 2500 rpm for 30 minutes at 4°C. The supernatant was decanted quickly and the residue counted on the gamma counter at 60s/tube. Only the samples taken at 2-hourly intervals between 24 and 56 hours after CIDR device removal were assayed for LH to determine the onset and magnitude of the LH surge. Three tubes in duplicate were set up for quality control. The intra-assay coefficient of variation was 10.5%.

## RESULTS

### 3.1 Statistical analysis

The body weights of the ewes, their plasma urea and plasma ammonia concetrations, progesterone levels, LH surge peak and time intervals were compared using student "t" test. Chi-square analysis was applied to investigate treatment differences in the proportion of ewes with and without embryos recovered from them at day 4 and day 11 after insemination. Where the numbers were too small the data are presented without statistical analysis. Correlations were investigated between plasma urea and plasma ammonia, LH surge onset and peak LH and ovulation rate and progesterone concentration before CIDR device removal, also between rate of protein synthesis and embryo stage of development, embryo grade and treatment.

### 3.2 Body weight

The mean initial and final body weights after a period of 40 days of the experiment are given in Table 3. There were no treatment differences in the final body weights of the ewes and during the period of study all ewes maintained their body weights thereby reflecting their maintenance level of feeding.

Table 3 The effect of treatment on mean body weight

| Treatment | No. of ewes | Initial weight (kg ±SEM) | Final weight (kg±SEM) |
|---|---|---|---|
| Control | 10 | 65.5 ±1.61 | 65.5 ±1.76 |
| Low urea | 10 | 65.4 ±1.52 | 65.7 ±1.91 |
| High urea | 10 | 65.5 ±1.55 | 65.5 ±2.10 |
| Overall | 30 | 65.5 ±0.87 | 65.6 ±1.07 |

### 3.3 Progesterone

The mean progesterone concentrations (n = 10) for each treatment just before CIDR device withdrawal were (± SEM) 1.86 ± 0.19, 1.48 ± 0.14 and 1.59 ± 0.12 ng/ml for the control, low urea and high urea groups respectively (see Table 4). There were no signficant differences ($p > 0.10$). Figure 3 provides an example of the progesterone profiles for 2 ewes from the extreme treatments (i.e., one control and one high urea) in the period prior to, during and after CIDR device removal. There was no evidence that urea supplementation altered circulating progesterone concentrations.

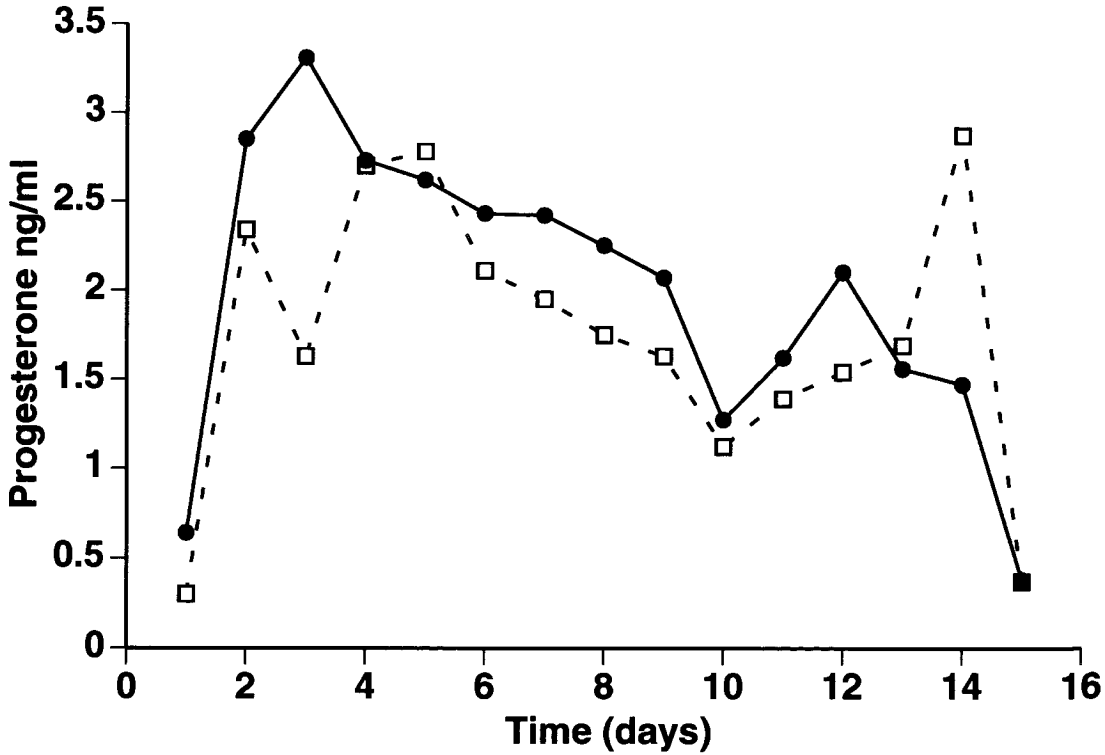



Figure 3.Progesterone profiles for a control (□) and high Urea (●) treatment ewe from immediatly before CIDR insertion to after CIDR removal

### 3.4 Luteinizing hormone (LH)

All the 30 ewes bled to determine the LH surge, its duration, peak height and area under the peak actually exhibited a surge during the sampling period with the individual surges occurring between 28 and 44 hours after CIDR device removal. The onset of the LH surge was not significantly different between the treatment groups ($p<0.10$).

The time of LH peak onset for the high urea group was slightly later 35 ( ± 1.5 s.e.m) hours compared with the low urea (33 ± 0.7) and control (33 ± 1.3) groups. n = 10 per treatment (see Table 4). There was a significant positive correlation ($P < 0.001$) between the times of oestrous onset and LH surge onset ($r = 0.74$).

The time intervals from the LH surge onset to IUAI were also compared (Table 4). Although the high urea group did have a shorter time interval this was not significantly different ($p > 0.10$) from the low urea and the control groups.

There was a significant difference ($p <0.01$) between the low urea and the high urea groups in the LH surge peak heights (see Table 4). In contrast there was no significant difference between the low urea and the control groups.

Although the control group had a 20 % higher LH amplitude than the high urea group, this difference was not statistically significant due to the high variability for the control group (18.5 to 128.2 ng/ml). The area under the curve is shown in Table 4.

Table 4 The effect of treatment on periovulatory events and their timing.

| | Control (±SEM) | Low urea (±SEM) | High urea (±SEM | Overall (±SEM) |
|---|---|---|---|---|
| No. of ewes | 10 | 10 | 10 | 30 |
| Progesterone conc. prior to CIDR device withdrawal | 1.9 ±0.19 | 1.5 ±0.14 | 1.6 ±0.12 | 1.7 ±0.09 |
| CIDR device removal to time of LH surge onset (hrs) | 28 ±1.0 | 27 ±1.1 | 30 ±1.4 | 28 ±0.7 |
| CIDR device removal to time of LH surge onset (hrs) | 33 ±0.7 | 33 ±1.3 | 35 ±1.5 | 34 ±0.7 |
| LH surge amplitude(ng/ml) | 44.6 ±10.73 | 47.2a ±2.85 | 29.0a ±4.16 | 40.3 ±5.68 |
| Area under LH surge | 74 ±19.6 | 81 ±30.6 | 58 ±21.1 | 71 ±13.6 |
| LH surge onset to IUAI interval (hrs) | 19.4 ±0.85 | 19.1 ±1.31 | 17 ±1.47 | 18.5 ±0.72 |

Within rows values with the same letter (a) are significantly different ($p<0.01$)

## 3.5 Plasma urea

Plasma urea concentrations were significantly different between treatments ($p < 0.01$), (Table 6). Table 5 gives mean values for plasma urea at 2-hour intervals over a 24-hour period and Figure 2 provides a clear overview of the treatment profiles in relation to feeding time. The initial (approx. 08:00 hrs) prefeeding urea levels were significantly different ($p < 0.01$) for all three treatments. In the control group the prefeeding level was the highest recorded during the 24 hour period. In contrast the low urea and high urea groups exhibited peak concentrations 4 hours after feeding. In all three groups the level of plasma urea tended to decline

throughout the 24 hour period with the 16:00 hour feed causing little more than a transitory arrest in this decline. In the few hours leading up to the *06:00 hours sample, prefeeding plasma urea concentrations for the control and low urea treatments tended to increase whereas those for the high urea treatment showed a slight but nonsignificant decline* (See Figure 4).

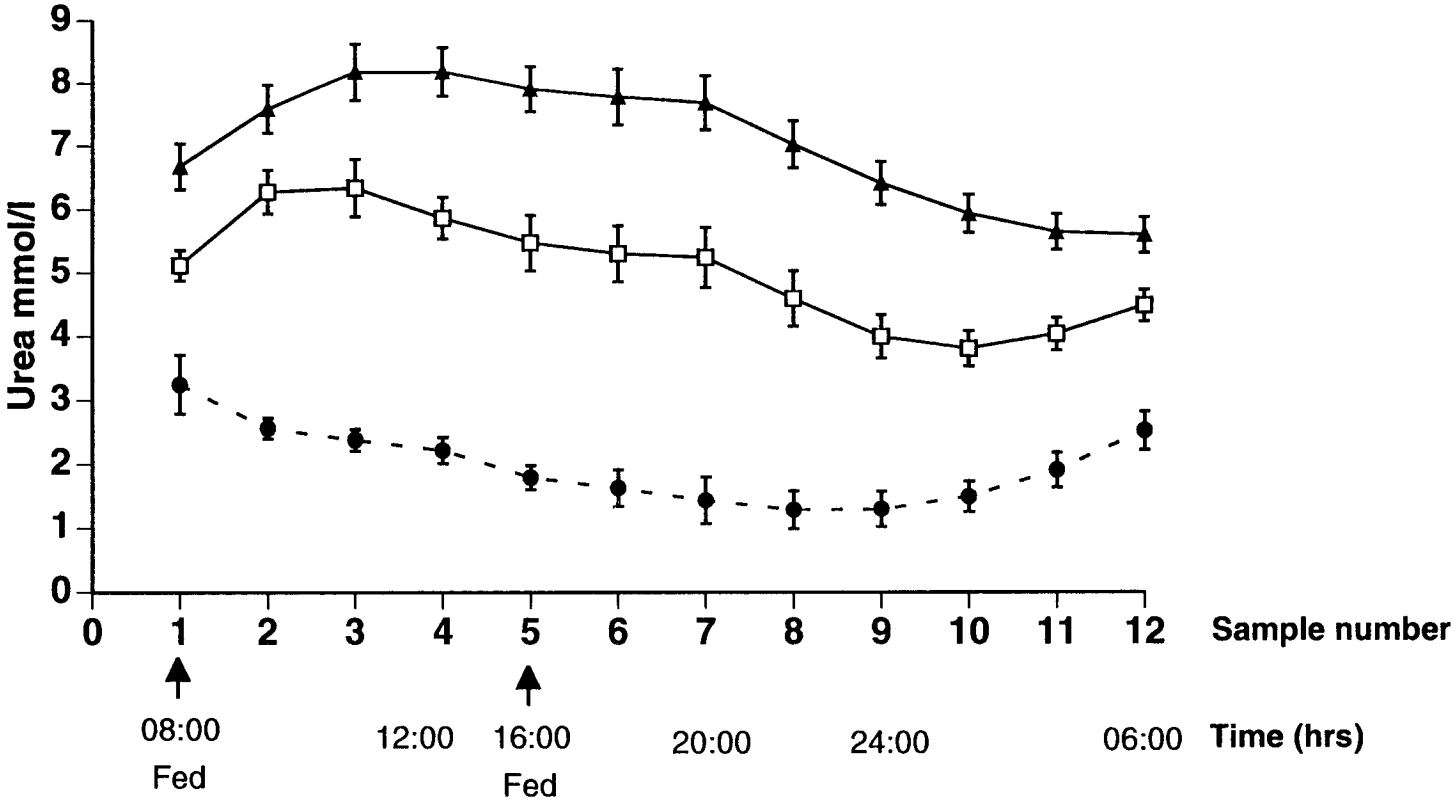



Figure 4. Twenty four hour profiles for plasma Urea in Control (●), low Urea (□) and high Urea (▲) fed ewes. Each value is the mean ± sem for 10 ewes.

### 3.6 Plasma ammonia

The average concentrations for plasma ammonia over the 24-hour sampling period showed that there was a significant difference ($p < 0.01$) between the control and the high urea groups but no significant difference between the control and the low urea groups. The difference between the high urea and the low urea groups was also significant ($p < 0.05$) (Table 5). Table 6 gives the mean plasma ammonia concentrations for the three treatment groups. The prefeeding (08:00 hours) ammonia levels were not significantly different ($p>0.05$) among the three groups. The peak plasma ammonia concentrations in the low urea and high urea groups were reached 2 hours after feeding (Table 6).

Figure 5 provides a picture of the effects of treatment,and the times that ewes were given their feed (08:00 and 16:00 hours each day),on the ammonia profiles in plasma over a 24-hour period. It shows that there was a peak after the morning and the afternoon feeding. These peaks were more prominent in the high-urea group. The levels did not remain highly elevated throughout the 24-hour sampling period. Up to 4 hours after the afternoon feed only the high urea was significantly different from the control ($p < 0.01$) and low urea ($p < 0.02$) groups while the low-urea was not significantly different ($p > 0.10$) from the control group except at 2 hours after morning feeding. Thereafter, there were no significant difference among the groups throughout the rest of the 24-hour sampling period. There was a significant positive correlation between plasma urea and plasma ammonia ($r = 0.75$ ;$p < 0.05$).

Table 7 gives within treatment plasma urea and ammonia levels for ewes from which an embryo/embryos were recovered and those from which embryo recovery was zero. Only in the high-urea group were plasma ammonia levels much higher in ewes without recoveries than in those with recoveries. In contrast differences between the two categories in plasma urea were not significant.

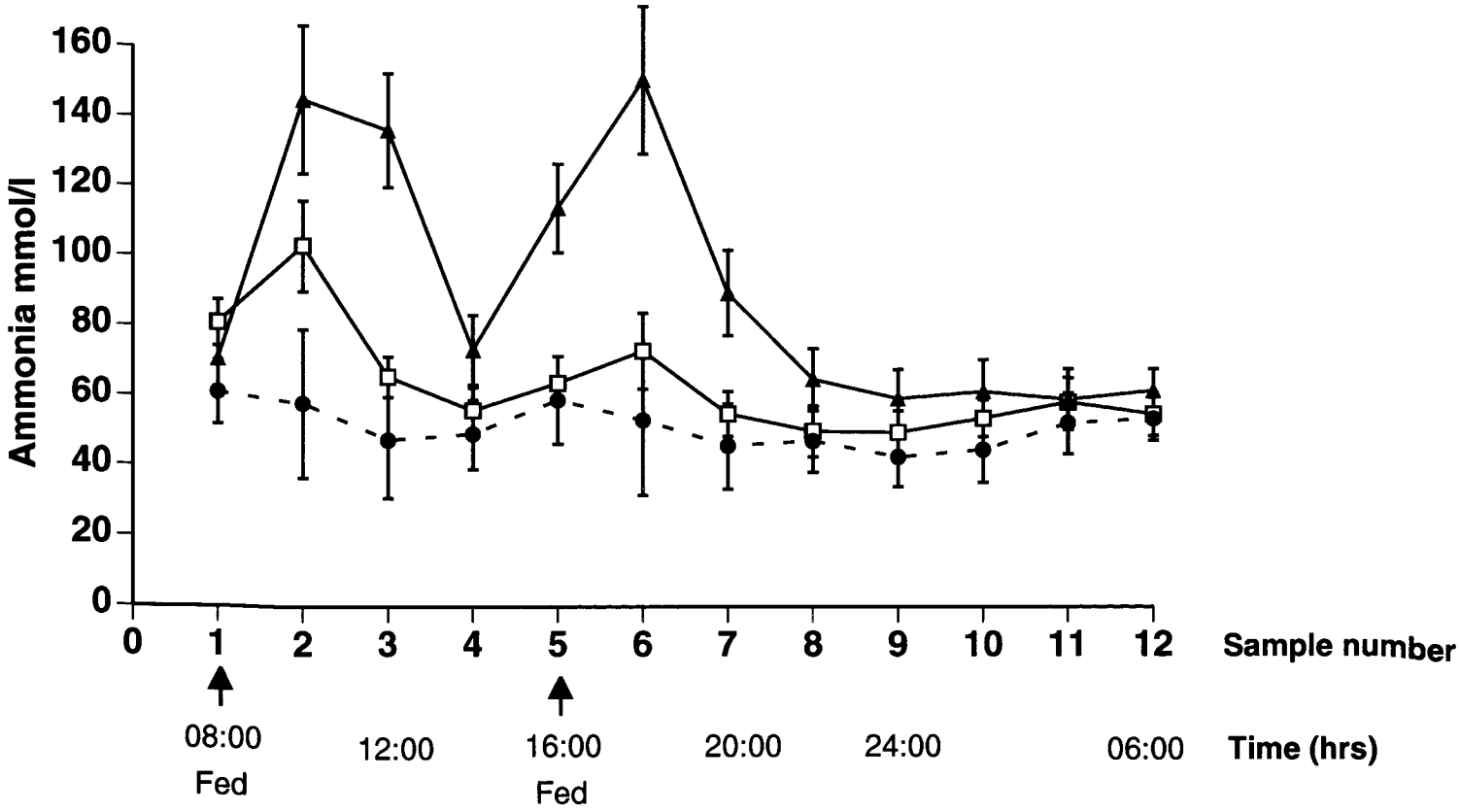



Figure 5. Twenty four hour profiles for plasma Ammonia in Control (●), low Urea (□) and high Urea (▲) fed ewes. Each value is the mean ± sem for 10 ewes.

Table 5. Effect of treatment on plasma urea and plasma ammonia profiles over a 24 hour period.

| Treatment | No. of ewes | 08:00 | 10:00 | 12:00 | 14:00 | 16:00 | 18:00 | 20:00 | 22:00 | 24:00 | 02:00 | 04:00 | 06:00 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Plasma urea (mmol/l) Time of sampling | | | | | | | | | | | |
| Control | 10 | 3.20 | 2.60 | 2.40 | 2.20 | 1.80 | 1.60 | 1.40 | 1.30 | 1.30 | 1.50 | 1.90 | 2.50 |
| (±SEM) | | 0.46 | 0.16 | 0.17 | 0.20 | 0.19 | 0.29 | 0.37 | 0.30 | 0.28 | 0.27 | 0.27 | 0.30 |
| Low urea | 10 | 5.10 | 6.30 | 6.40 | 5.90 | 5.50 | 5.30 | 5.20 | 4.60 | 4.00 | 3.80 | 4.00 | 4.50 |
| (±SEM) | | 0.24 | 0.34 | 0.45 | 0.33 | 0.44 | 0.44 | 0.47 | 0.44 | 0.34 | 0.28 | 0.26 | 0.25 |
| High urea | 10 | 6.70 | 7.60 | 8.20 | 8.20 | 7.90 | 7.80 | 7.70 | 7.00 | 6.40 | 5.90 | 5.70 | 5.60 |
| (±SEM) | | 0.36 | 0.38 | 0.45 | 0.39 | 0.36 | 0.45 | 0.43 | 0.37 | 0.34 | 0.30 | 0.28 | 0.28 |
| | | Plasma ammonia (mmol/l) | | | | | | | | | | | |
| Control | 10 | 61.0 | 58.0 | 47.0 | 49.0 | 59.0 | 53.0 | 46.0 | 47.0 | 42.0 | 44.0 | 52.0 | 53 |
| (±SEM) | | 6.6 | 13.0 | 5.8 | 6.6 | 15.4 | 10.9 | 6.5 | 6.5 | 6.2 | 5.4 | 7.1 | 6.2 |
| Low urea | 10 | 81.0 | 103.0 | 66.0 | 56.0 | 64.0 | 73.0 | 55.0 | 50.0 | 49.0 | 58.0 | 58.0 | 55 |
| (±SEM) | | 7.0 | 13.0 | 6.9 | 7.2 | 9.2 | 6.8 | 5.0 | 6.5 | 6.2 | 7.2 | 7.2 | 4.2 |
| High urea | 10 | 71.0 | 145.0 | 136.0 | 73.0 | 114.0 | 151.0 | 90.0 | 65.0 | 59.0 | 62.0 | 59.0 | 62 |
| (±SEM) | | 9.2 | 21.3 | 16.4 | 10.0 | 12.7 | 21.3 | 12.2 | 8.8 | 8.4 | 9.2 | 8.8 | 6.3 |

| Table 6. | Treatment mean values for plasma urea and plasma ammonia | | | |
|---|---|---|---|---|
| | | Treatment | | |
| | Control (±SEM) | low urea (±SEM) | High urea (±SEM | Overall (±SEM) |
| No. of ewes | 10 | 10 | 10 | 10 |
| Plasma urea (mmol/l) | 2.0a ±0.22 | 5.1a ±0.32 | 7.1a ±0.32 | 4.7 ±0.41 |
| Plasma ammonia (mmol/l) | 53.9c ±7.44 | 63.6b ±5.44 | 90.4bc ±9.22 | 66.7 ±5.42 |
| Within rows values with the same letter are significantly different (a) ($p<0.01$) (b) ($p<0.05$) (c)($p<0.01$) | | | | |

| Table 7 | Treatment mean values (±SEM) for plasma urea and ammonia concentrarions in ewes with and without embryo recovery. | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Treatment n = no.of ewes | Plasma urea (mmol/l) | | | | Plasma ammonia (mmol/l) | | | |
| | with recovery | | zero recovery | | with recovery | | zero recovery | |
| Control | 8 | 2.1 | 2 | 1.6 | 8 | 52.9 | 2 | 57.9 |
| (±sem) | | 0.25 | | 0.54 | | 9.11 | | 12.03 |
| low urea | 7 | 5.5 | 3 | 4.8 | 7 | 65.9 | 3 | 58.5 |
| (±sem) | | 0.44 | | 0.32 | | 6.65 | | 10.74 |
| high urea | 4 | 6.4 | 6 | 7.5 | 4 | 70.4b | 6 | 103.8b |
| (±sem) | | 0.2 | | 0.44 | | 15.33 | | 8.46 |
| Within rows values with the same letter (b) are significantly different ($p<0.05$). | | | | | | | | |

## 3.7 Oestrous onset

The time interval between CIDR device withdrawal and oestrous onset was not significantly different ($p < 0.10$) among the groups. The high urea group was slightly later (30.2 ± 1.4 hrs) compared to the control group (27.8 ± 1.0 hrs) and the low urea group (27 ± 1.1 hrs) (Table 4). All the ewes exhibited oestrus before they were inseminated.

## 3.8 Ovulation rate and embryo recovery rate

Table 8 provides data for the ovulation rates, numbers of embryos recovered per ewe and percent of embryos recovered per ewe in the three treatment groups. The ovulation rates among the groups were not significantly different ($p > 0.05$), similarly the number of embryos

recovered per ewe was also not significantly different between treatments ($p > 0.05$). Although not statistically significant the numbers were such that the control > low urea > high urea groups. There was no significant difference between the low-urea and the control group ($p < 0.10$).

| Table 8 | The effect of treatment on the time interval between CIDR device removal and oestrous onset, ovulation rate and embryo recovery rate on Days 4 and 11 after intrauterine insemination at 52 hours after CIDR device removal. | | | | |
|---|---|---|---|---|---|
| Treatment | no. of ewes | interval from CIDR device removal to oestrous onset, mean ±SEM (range) | ovulation rate, mean ±SEM, (range) | No. of embryos recovered/ewe mean ±SEM (range) | Percentage embryos recovered/e ±SEM (ran |
| Control | 10 | 28 ±1.0 (24-32) | 4.1 ±0.92 (1-10) | 2.2 ±0.71 (0-8) | 50.1 ±10.9 (0-100) |
| low urea | 10 | 27 ±1.1 (26-34) | 2.9 ±0.5 (1-5) | 1.1 ±0.31 (0-3) | 40 ±12.4 (0-100) |
| High urea | 10 | 30 ±1.4 (24-38) | 2.4 ±0.42 (1-5) | 0.8 ±0.42 (0-4) | 24.7 ±10.4 (0-80) |
| | Within columns none of the values are significantly different ($p<0.10$). | | | | |

Table 9 provides data on the effect of treatment and day of recovery on percentage of corpora lutea represented by recovered embryos. At day 4 there were no significant differences among the groups ($p < 0.10$). The low urea group had the lowest recovery rate. The high percent recovery rate in the control group at day 4 was attributed to one ewe which showed a typical superovulatory response.

At day 11 the low percent recovery rate in the high urea group was not significantly different ($p < 0.10$). The recovery percent in the control group at day 4 was significantly different from the percent recovery at day 11 in the high urea group ($p < 0.05$).

| Table 9 | Treatment mean values for embryo recoveries on Day 4 and 11 after inseminatio(in percentage) | | | | |
|---|---|---|---|---|---|
| | | | Treatment | | |
| Day of recovery | No of ewes per group | Control (±SEM) | low urea (±SEM) | high urea (±SEM) | Combined low and high urea (±SEM) |
| 4 | 5 | 56a±14.2 | 29±14.4 | 39±16.7 | 34±10.5 |
| 11 | 5 | 44±17.8 | 51±20.8 | 10a±10 | 30±12.8 |
| | Values with the same letter (a) are significantly different (p<0.05) | | | | |

## 3.9 Periovulatory events

Table 10 gives the across-treatment correlation coefficients for a number of periovulatory parameters. The time interval between LH surge onset and oestrous onset had a positive correlation ($r = 0.74$; $p < 0.001$). There was a positive correlation between the plasma concentraton of urea and ammonia ($r = 0.57$; $p<0.002$).

Table 10. Across treatment correlation coefficients (r) for a number of periovulatory parameters. n =30

| Periovulatory parameter | r |
|---|---|
| P4* conc. before CIDR out vs Time of LH surge onset | 0.17 |
| P4* conc. before CIDR out vs The LH surge peak height | 0.15 |
| Time of LH surge onset vs Time of oestrous onset | 0.74 |
| Time of LH surge onset vs Ovulation rate | 0.11 |
| The LH surge peak amplitude vs Ovulation rate | 0.02 |
| Plasma urea conc vs Plasma ammonia conc | 0.57 |

P4* Plasma progesterone
Conc.- concentration

## 3.10 Embryo development stages

The stages of embryo development at time 0 (time = 0 is time of recovery, in this case day 4) and after 72 hours in culture are given in Table 11.

Although the numbers of embryos are small, in general the embryos of ewes receiving urea, particularly at the high level, were less well developed than those from the control ewes. The same picture emerged following 72-hour *in vitro* culture.

| Table 11 | The numbers of Day 4 embryos and their stages of development at recovery (time=0) and following 72 hours in vitro culture. | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | stages of development | | | | | | |
| Time of culture (hrs) | Treatment | <16 cells | 16-32 cells | Early morula | mid morula | Early blastocyst | Late blastocyst |
| At recovery time time=o | control | 2 | 3 | 3 | 2 | | |
| | low urea | 1 | | 1 | 1 | | |
| | high urea | 3 | 1 | | | | |
| Following 72 hrs in vitro culture | control | | 3 | 1 | | 4 | 2 |
| | low urea | 1 | | | | 1 | 1 |
| | high urea | 2 | 2 | | | | |

## 3.11 Rates of protein synthesis

(a) Day 4 recovered embryos.

Table 12 gives data on the embryo grade and the corresponding values for protein synthesis in ß disintegrations per minute (DPM). These reflect the incorporation of $^{3}H$- phenylalanine during a 2-hour incubation that followed the 72-hour period of *in vitro* culture. The embryo grading is based on a scale of 1-5 where 1 = excellent, 2 = good, 3 = fair, 4 = poor and 5 = dead.

| Table 12 | Embryo grades after 72 hours of culture in vitro and corresponding values for protein synthesis (in DPM), based on the incorporation of ph | | | | | |
|---|---|---|---|---|---|---|
| Control | | low urea | | high urea | |
| Embryo grade | DPM | embryo grade | DPM | Embryo grade | DPM |
| 4.00 | 64.0 | 4.25 | 35.6 | 5.00 | 12.8 |
| 4.00 | 13.4 | 2.25 | 207.4 | 3.00 | 74.8 |
| 3.75 | 22.6 | 3.00 | 217.2 | 5.00 | 15.0 |
| 2.50 | 370.2 | | | 3.75 | 53.4 |
| 2.50 | 38.2 | | | | |
| 2.50 | 47.6 | | | | |
| 2.50 | 96.8 | | | | |
| 3.00 | 150.0 | | | | |
| 3.25 | 40.0 | | | | |
| 3.50 | 28.2 | | | | |

These parameters were then correlated and the results are shown in the *Table 13*

| *Table 13* | *Correlation coefficients (r) for the relatioships b* | | | |
|---|---|---|---|---|
| | stage and egg grade and protein synthesis base | | | |
| | incorporation (Day 4 recovered embryos) | | | |
| | n=17 | | | |
| | | | | |
| | Parameter | | | r |
| | | | | |
| | Treatment vs DPM | | | -0.37 |
| | | | | |
| | Egg stage vs DPM | | | 0.75 |
| | | | | |
| | Egg grade vs DPM | | | -0.44 |
| | | | | |
| | | | | |
| DPM; Disintegrations per minute, i.e. number of beta parti | | | | |
| minute. | | | | |

There was a positive correlation ($r = 0.75$; $p<0.02$) between embryo stage and rate of protein synthesis. There was a small negative correlation $r = -0.44$ between embryo grade and rate of protein synthesis which implied that the rate of protein synthesis declined with increase in the grade number from 1 to 5. There was a wide variation in the protein synthesis rates within the same grade.

(b) Day 11 recovered embryos

Table 14 gives data on the stage of development of the Day 11 recovered embryos at time of recovery, their sizes in terms of length and width, and the corresponding values for protein synthesis in DPM based on the incorporation of $^{3}$H-phenylalanine after 2-hour incubations. One ovum from the control group was developmentally retarded; the rest of the embryos were at the blastocyst stage. The larger embryos as measured by length and width had higher rates of $^{3}$H-phenylalanine incorporation. The rates of protein synthesis in DPM were higher for Day 11 recovered embryos than Day 4 recovered embryos following 72 hours of *in vitro* culture.

| Table 14 | Embryo development stage and size and the corresponding values for protein synthesis (in DPM) based on laballed phenylalanine incorpo after a 2-hour period of incubation of Day 11 recovered embryos. | | |
|---|---|---|---|
| Treatment | Stage of development | Size (length x width;microns) | Rate of protein synthesis (DPM) |
| low urea | Blastocyst | 3894 | 1546.4 |
| control | Blastocyst | 4108 | 1720.0 |
| control | Blastocyst | 8512 | 3104.8 |
| control | Blastocyst | 2392 | 564.0 |
| Control | One cell ovum with cumclus cells | | |
| Control | Blastocyst | 2162 | 108.8 |

## 3.12 Embryo viability *in vitro*

(a) Day 4 recovered embryos

The determination of viable and non-viable embryos tabulated in Table 15 was based only on embryo stage and grade . Where a poor grade did not coincide with stage of development, a stage of development above 16 cells was taken as viable at time zero (time = 0 is time of recovery) and above 16-32 cells at 72 hours after culture . Of the Day 4 recovered embryos that were cultured for 72 hours, 7/10 embryos in the control group were viable 2/3 were viable in the low urea group and 0/4 embryos in the high urea group.

| Table 15 | Number of viable and non-viable embryos at time = 0 (i.e. time of recovery) and after 72 hours of in vitro culture. | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | | | Treatment | | |
| Time (hrs) | viability | | control | | low urea | | high urea |
| 0 | viable | | 8 | | 2 | | 1 |
| | non-viable | | 2 | | 1 | | 3 |
| 72 | viable | | 7 | | 2 | | 0 |
| | non-viable | | 3 | | 1 | | 4 |

(b) Day 11 recovered embryos.

All embryos recovered on day 11 were considered viable *in vivo*. Of the viable embryos that were not put back to the donor ewes and were analysed for protein snythesis rate, 4 came from the control group and 1 from the low urea group; no embryo came from the high urea group (see Table 14).

### 3.13 Pregnancy rates

Table 16 provides data on the number of pregnancies sustained in each treatment according to the time when auto-transfer was carried out. Embryos transferred back to the donor (autotransfers) were considered viable if the ewe was diagnosed pregnant in Day 18 from the time of IUAI. Pregnancy was diagnosed by determining the plasma progesterone concentration on Day 18 blood samples. Plasma progesterone concentration > 1 ng/ml was considered a positive indicator of pregnancy. One ewe from the high group with no embryo recovery at Day 11 was found pregnant on Day 18 and this was confirmed by ultrasound scanning on Day 45. The control group sustained the highest number of pregnancies followed by the low-urea group and the high-urea group had the least with only 1.

| Table 16 | Number of pregnancies sustained by Day 18 after IUAI for each treatment according to time of autotransfer. | | |
|---|---|---|---|
| Time of autotrasfer | control | low urea | high urea |
| Day 4 | 3 | 2 | 0 |
| Day 11 | 3 | 3 | 1 |
| Total transferred | 8 | 7 | 3 |
| Pregnancy rate | 75% | 71% | 33% |

## DISCUSSION

The experiment was designed to investigate whether or not excess dietary rumen degradable protein (RDP) had an effect on ovulation rate, fertilization and embryo viability *in vivo* and *in vitro*.

Fertile ewes with a recent lambing were used and a sufficient period for uterine involution of 60 days postpartum was allowed. Twenty of the ewes were then challenged with high rumen degradable protein provided by two levels of urea in the period before, during and after progesterone priming, and up to 30 days after IUAI.

### 4.1 The Diet

All ewes consumed the allocated amount of diet (1600 g daily). To estimate the amount of rumen degradable protein that was available to the ewes, the amount of protein derived from microbial fermentation of hay was calculated as follows:-

The hay had 89.62% dry matter (DM) and 0.834% nitrogen (N) on fresh basis. Assuming that 6.25 g of protein = 1 g nitrogen then protein supply from the hay was:
(0.834/89.62) x 100 x 6.25 = 5.82 g/100 g DM or 58.2 g/kg DM.

The degradability of protein in the hay was taken to be 0.8.

The degradable protein therefore was:
58.2 x 0.8 = 46.6 g/kg DM.

The ewes received 1600 g of feed/day which is equivalent to 1434 g of DM. Therefore the total degradable protein intake was 46.6 x 1.434 = 66.8 g/d. Energy requirements for body weight maintenance were: 0.42 MJ of ME/kg of body weight $(BWT)^{0.75}$ (Robinson *et al*, 1980) ; 0.42 x $65.5^{0.75}$ = 9.6 MJ of ME/day.

Therefore, in their daily feed of 1600 g the ewes received 9.6 MJ of ME which maintained their weight.

In order to meet the requirements of the rumen microbes for degradable protein, the diet requires 8 g degradable protein/MJ of ME i.e. 9.6 x 8.0 = 76.8 g of degradable protein/day.

*Since their actual intake of degradable protein was 66.8 g/d, the deficit in RDP was 76.8 - 66.8 = 10 g.*

To supply an extra 10 g/day of degradable protein from urea, on the assumption that urea is 100% degradable and its nitrogen is incorporated into microbial protein with an efficiency of 80 % (ARC, 1980), would required: 10/0.8 = 12.5 g degradable protein.

Thus the hay used in this study was deficient in degradable protein to the extent of 12.5 g/day.

In contrast the low urea diet supplied 24 g urea/day = 24 x 0.46 = 11 g nitrogen or 69 g of degradable protein. The excess degradable protein in the diet was therefore 69 - 12.5 = 56.5 g/day and on the high urea diet 56.5 + 69 = 125.5 g/day.

| Table 17 | The degradable protein status of the three the requirement of the rumen microbes. | | | |
|---|---|---|---|---|
| | | Diet | | RDP (g/kg) |
| | | Control | | -12.5 |
| | | Low urea | | 56.5 |
| | | High urea | | 125.5 |

## 4.2 Body weight

The lack of a treatment effect on the final body weight of the ewes was as expected as the diet was calculated to supply the ewes maintenance energy requirements and the small deficit in the rumen degradable protein on the control diet,(12.5 g/day) (see Table 17), was unlikely to influence weight changes. Both of the supplemented diets had an excess of RDP ; low urea

to the extent of 50 to 60 g/day and the high urea to the extent of over 120 g/day.

### 4.3 Plasma urea and ammonia

Plasma urea concentrations largely reflect protein intakes (Harmeyer and Martens, 1980). Plasma urea is a product of ammonia metabolism by the liver. The plasma urea and ammmonia in this study was positively correlated, r = 0.75. The deficiency in degradable protein on the control diet was reflected in the low plasma urea profile, its steady decline following the morning feeding and continued decline over the 8-hour period following the afternoon feeding, only to start rising again in the 6-8 hour period preceding the next morning's feed. These post-feeding decreases in plasma urea imply that due to a slight inadequancy of rumen degradable nitrogen (see Table 17), urea was recylced into the rumen immediately after feeding thereby enhancing the amount of nitrogen available to the rumen bacteria for synthesis into microbial protein. In contrast, rapid but transitory elevations in plasma ammonia after feeding were observed in the low urea and high urea treatments. This reflected the temporary, particularly in the ewes on the high urea diet, inability of the liver to rapidly convert to urea the large amount of ammonia released from the rumen and entering the blood. The plasma urea concentrations observed in the low and high urea supplemented groups were comparable to or slightly higher than those concentrations reported by Jordan *et al* (1983), Blauwiekel *et al* (1986), Howard *et al* (1987) and Canfield *et al* (1990) in dairy cows and Elrod et al (1993a and 1993b) in heifers fed high levels of dietary protein that resulted in a reduction in fertility.

Elrod and Butler (1993) observed that when heifers were fed once per day at (10:00 hours) on a diet that was high in rumen degradable protein and that exceeded the requirement of rumen degradable protein by 50 %, plasma urea levels rose from 14.8 ± 0.19 mg/dl prefeeding and peaked at 23.6 ± 0.23 mg/dl some 8 to 12 hours after feeding and were elevated at all times during the 24-hour sampling period. Plasma ammonia levels were variable and did not differ with either treatment or day of the oestrous cycle.

In the present study plasma urea levels ranged from 1.3 to 3.2 mmol/l in the control, 3.8 to 6.4 mmol/l in the low urea and 5.7 to 8.2 mmol/l in the high urea treatment over the 24 hour sampling period. For comparison of these values with those of Elrod and Butler (1993), their observations, when expressed in mmol/l, range from 2.5 to 3.9. These are only about 50 % of those observed on the high urea diet in the present study.

Since dry matter intake (DMI) was constant, feeding times were standardized, and no refusals were recorded, it was assumed that the daily plasma profiles for urea and ammonia were consistently the same throughout the experimental period. Thus the treatments generated major differences in plasma concentrations of urea that were sustained throughout the experimental period and, in the case of the high urea supplemented diet, transitory elevations occurred in plasma ammonia for periods of up to 5 to 6 hours after each feed.

In studies in which high dietary protein was fed to cows, the reduction in fertility observed was ascribed to ammonia (Jordan and Swanson, 1979; Elrod *et al*, 1993). Although the mechanism of action is not known, ammonia is a toxic substance to mammalian tissues (Visek, 1982). It has been suggested that ammmonia, through some unknown mechanisms, alters the uterine secretory activity thereby causing changes in the ionic concentrations (Jordan *et al*, 1983) and consequently pH in the uterine environment during the luteal phase of the oestrous cycle (Elrod *et al*, 1993b).

Since the present study is the first to investigate the effect, if any, of high levels of rumen degradable protein on ovulation rate, fertilization and embryo survival in ewes, the aim was firstly to test if a detrimental effect of high rumen degradable protein on embryo survival occurred in the ewe. A second aim of the experiment was to monitor the effects of alterations in rumen degradable protein, and therefore in plasma urea and plasma ammonia, on the circulating concentrations of hormones (progesterone and lutenizing hormone) that are critical to the success or otherwise of ovulation, fertilization and embryo survival. None of the previous studies in cattle investigated whether the detrimental effects on fertility occurred as a result of fertilization failure; their only index of reproductive performance was calving rate in relation to dietary protein intake.

Therefore, an important feature of the present study was the detailed information that it provided on fertilization rate and early embryo development.

### 4.4.0 Effects of high urea supplementation on:

### 4.4.1 Hormone levels

a) Progesterone

Adequate circulating concentrations of progesterone prior to ovulation are required for subsequent normal development of the embryo (Ashworth, 1992), and there is evidence that these concentrations can be influenced by dietary factors. For example McEvoy *et al* (1993) showed that a high level of feeding prior to ovulation could suppress the circulating concentrations of progesterone in ewes receiving exogenous progesterone via a controlled internal drug release device (CIDR-0.3 g progesterone) as a method of administering exogenous progesterone. The same CIDR device was used to supply progesterone during the preovulatory progesterone priming phase in the present study and clearly in view of McEvoy *et al's* (1993) findings, it was important to test if the present dietary treatments altered the ability to sustain circulating progesterone concentration during the 12-day priming phase. Actual progesterone profiles were only studied on the extreme treatments (control versus high urea). There was no suggestion that urea supplementation influenced circulating progesterone concentrations. Similarly progesterone concentrations immediately before CIDR withdrawal, a time when they have a critical effect on the subsequent viability of the embryo (Ashworth, 1992), were not different between treatments, although they were on average lower for the ewes receiving the urea supplements (Control 1.9 ± 0.19; low urea 1.5 ± 0.14, high urea 1.6 ± 0.12 ng/ml). However, it was considered that this variation between treatments was unlikely to play a major role in altering fertility in the present study in that across treatment correlations between the progesterone concentrations immediately prior to CIDR withdrawal and ovulation rates did not show any significant association. Elrod *et al* (1993a) and Blauwiekel *et al* (1986) found no effect of dietary protein on circulating progesterone concentrations in cattle but an earlier study by Jordan and Swanson (1979), working with cattle, did observe a small but significant

depression in cows on 19.3 as opposed to 12.7 percent crude protein in the diet.

b) Luteinizing hormone

There was no evidence that the treatments influenced the mean interval to the LH surge onset from the time of CIDR device withdrawal (34 ± 0.7 hr, n = 30) or the interval between LH surge and IUAI (19 ± 0.7 hr, n = 30). There was no correlation between LH surge onset, the amplitude of the LH surge or the area under surge and ovulation rate. Thus the sustained high circulating concentrations of urea and the corresponding high concentrations of ammonia in plasma, albeit of a much more transitory nature than the urea concentrations, had no effect on either the timing of the activation of the gonodotrophin releasing hormone (GnRH) pulse generator or perhaps more importantly, on the amounts of LH released from the pituitary. To the author's knowledge these are the first observations to test whether or not blood ammonia concentrations influence pituitary function in ewes Jordan and Swanson (1979) challenged cows with a standard injection of GnRH and found a much higher LH surge in those on a 19.3 % crude protein diet compared with those on either 16.3 or 12.7 % . They did not however record the effect of the dietary protein intakes on blood ammonia.

Correlations between the various parameters of the LH surge and ovulation rate were not significant in the present study. These correlations span a much wider range of parameters in terms of ovulation rate, than that normally encountered (Rhind, 1992) and therefore add a further degree of generality to the hypothesis that the parameters of the LH surge play little if any role in controlling the number of follicles that ovulate (Rhind 1992).

In the present study the exact time of ovulation was not determined but it can be assumed that it occurred at a fairly constant time in relation to the LH surge. Since there was no significant difference between treatments in the time of the LH surge onset, it is argued that major differences in the stage of development of the embryos when recovered from the ewes on Day 4 following insemination, were unlikely to be attributed to differences in the time of ovulation and therefore of the age of the oocytes at fertilization. Rather, treatment differences in embryo development on Day 4 were

considered to reflect either an effect on oocyte maturation, brought about by the high blood urea and ammonia concentrations, but not mediated through an alteration in preovulatory progesterone concentrations, or alternatively, a direct effect of the urea and/or ammonia on the oviductal environment during the early cell division stages of the embryo. Clearly, the nature of the present study is such that it is difficult to separate pre- and post-ovulatory influences on embryo development . Nonetheless the finding that two ova from a ewe on the high urea treatment were unfertilized and were without spermatozoa on their zona pellucida, despite the excessive number of sperm ($100 \times 10^6$) deposited close to the site of fertilization, implies that the high urea treatment may have led to a hostile uterine and oviductal environment that compromised sperm viability.

### 4.4.2 Effect of treatment on embryo recovery rate

Embryo recovery rates were evaluated in the light of the following:
That all semen used in IUAI came from the same ram ; all inseminations were performed by one experienced inseminator and the embryo collection was performed by two experienced operators. A chi-square test on whether or not day of recovery (hence method of recovery) had an effect on the number of embryos recovered did not show any significant difference ($x^2 = 1.04$).

The following can be observed with regards to ovulation rate and embryo recovery the low and high urea groups had ovulations which were 30 % and 42 % respectively lower than in the control group. However, much of this difference was due to one control ewe that exhibited a superovulatory response and there is no reason to believe that this was anything other than a chance result. If her data are removed from the mean ovulation rate for the control group was $3.4 \pm 0.72$ compared with $2.9 \pm 0.50$ for the low urea and $2.4 \pm 0.42$ for the high urea. Nonetheless the percent of ovulations represented by recovered ova on the low and high urea diets was 20 % and 50 % lower than for the controls, implying a detrimental effect of the urea treatment on the successful capture of the ova by the fimbriated infundibulum at the time of ovulation. This phenomenon can be accentuated by the intra-uterine insemination technique if it is carried out around the time of ovulation (Robinson *et al*, 1989) and in the absence of a suitable tranquilizer to prevent the technique causing stress on the

ewes (Scudamore, 1992). However, in the present study acepromazine maleate was used to traquilize the ewes at the time of IUAI and there was no reason to suspect treatment differences in the time of ovulation in the present study (see earlier part of discussion). All the evidence would therefore point to the low embryo recovery on the urea treatments being a true treatment effect on the oocytes reaching the oviducts. The progressive decline in embryo recovery rates with increasing dietary urea therefore implies that the associated high blood urea and ammonia concentrations interfered with the natural functioning of the fimbriated infundibulum at the time of ovulation. This would again support the view that the urea treatments were impinging on uterine or oviductal function which is in keeping with the hypothesis on sperm transport and oviduct function (see earlier part of discussion).

Of course, the lower embryo recovery rate on the urea diets as compared to the control diet is an overall phenomenan based on a combination of the Day 4 and Day 11 data. For Day 11 the effect was extreme with only 10% of the corpora lutea resulting in recovered embryos in ewes on the high urea treatment, thereby implying a further detrimental effect of the urea on embryo survival between Day 4 and 11. Indeed the data for the autotransfers bear out this suggestion in that only 1 of the 3 embryos returned to the donor ewes on the high urea diet resulted in a pregnancy on Day 18 as confirmed by elevated progesterone in plasma. This contrasts with to 6 out of 8 in the control ewes.

### 4.4.3 Embryo quality

Atlhough embryo grading based on a 1-5 scale, as described by Lindner and Wright (1983) in bovine embryos, has been used in assessing ovine embryo viability *in vivo* after recovery from donor ewes (Scudamore 1991), it is a very subjective measurement.

In the present study it was considered that the rate of protein synthesis could give a more quantitative measure of predicting embryo viability if it was considered in the context of the grade assigned to the embryo and the stage of development. The rate of protein synthesis was correlated with the embryo stage of development and the grade on the 1 - 5 scale (see Table 13). There was a positive correlation between the stage of development and rate of protein synthesis ($r = 0.75$). The wide variation in the rates of protein synthesis within stages of development and grades did not allow the simultaneous use of all three parameters to assess embryo viability. However, in the absence of a standard, and in view of the small number of embryos subjected to $^{3}H$ phenylalanine incorporation, the predicted embryo viability *in vitro* was based only on stage of development and the assigned grade on the 1-5 scale.

Evidence of further development during culture and the ability to hatch are both regarded to be good signs of initial viability of the embryo at recovery (Renard *et al*, 1976). In the present study all embryos from the control ewes continued to develop during the period of *in vitro* culture. In contrast only two-thirds of those from the low urea treatment showed further development. None developed beyond the 32-cell stage in the high urea group. The culture medium TCM-199 supplemented with foetal calf serum (FCS), which was used in the present study, is able to support development of ova from the 16-cell stage to hatching. Short term *in vitro* culture of embryos does not provide a definitive indication of embryo viability but the ability to hatch from the zona pellucida is considered a good indicator that an embryo is viable. The nature of the present study was such that the Day 4 embryos would not have been expected to reach hatching stage by the time the *in vitro* culture was terminated. That indeed was the case. During *in vitro* culture more embryos from the control group continued to develop to the expected stages of development than from the high urea treatment. Indeed the fact that all embryos from the

## II Conclusion

Based on the experimental data generated in this thesis it can be concluded that high dietary urea supplementation in ewes ;-

1. reduced embryo recovery rate.
2. reduced both the quality of embryos recovered on Day 4 following insemination and their ability to continue to develop in an *in vitro* culture system.
3. reduced the viability of Day 4 embryos that were returned to their *in vivo* enviroment immediately after visual assessement.

These detrimental effects on the early embryo were not the result of any abnormal influences on either the preovulatory progesterone concentrations or the timing, duration and magnitude of the LH surge. The data suggest that the elevations in plasma ammonia following feeding may adversely affect the normal functioning of the fimbriated infundibulum at oocyte capture and the enviroment in the oviducts both at fertilization and during the early cleavage stages of the embryo.

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