Effect of peri-conceptional feed-intake on early embryo development and fetal growth in the Merino ewe

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With MATILDA the first sheep cloned in Australia

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ABSTRACT

In the studies reported in this thesis, feed intake was manipulated in adult Merino ewes during the course of a single reproductive cycle, from 18 days before until 6 days after ovulation. The effects of this relatively short-term exposure to high feed intake (H: 1.5 x maintenance ration), maintenance intake (M: 1 x maintenance ration) or low feed intake (L: 0.5 x maintenance ration) on embryo development, composition of oviductal fluid and pregnancy outcome in ewes that either ovulated naturally or were induced to superovulate were examined.

Live weight was significantly ($P<0.05$) reduced in sheep on a L compared with a M- or H-feed intake regime. The mean number of embryos collected, the mean number of corpora lutea (CL), and the proportion of embryos/CL recovered, was not influenced by differences in feed intake. However, embryos from sheep with a L feed intake contained significantly ($P<0.05$) more cells, due to a specific increase in the number of trophectoderm (TE) cells. Manipulating feed intake across the pre- vs. post-ovulatory periods revealed that exposure to a L feed intake in the 6-day post-ovulatory period caused this increase in the number of TE cells.

The effect of peri-conceptional changes in feed intake on oviductal fluid composition was then monitored in superovulated or naturally ovulating ewes fitted surgically with indwelling bi-lateral oviductal cannulae. Oviductal fluid volume, pH and osmolarity were monitored in daily collections of fluid from Day 0 to Day 6 after ovulation and these samples were also analysed for concentrations of ammonia, urea, amino acids, electrolytes, progesterone and insulin-like growth factor (IGF)-I. Low feed intake led to a significant ($P<0.05$) increase in fluid volume, a more rapid decline in fluid pH over time, a significant ($P<0.05$) lower fluid osmolarity and a significant ($P<0.05$) lower concentration of urea in oviductal fluid compared with animals fed the H diet. Ammonia concentrations tended to be higher in animals on H feed intake but the differences were not significant. Non-essential amino acids were found to comprise 82% of the total amino acids present, with glycine alone accounting for 66%. Electrolyte concentrations were affected by feed intake and ovulatory status with changes in bicarbonate and sodium concentrations the most significant. Sheep given a L feed intake had a significantly ($P<0.05$) higher mean concentration of progesterone and reduced levels of IGF-I in oviductal fluid. These

changes in the oviduct environment may to contribute to the differential development of embryos produced by sheep fed L and H diets.

In the final experiment, embryos were harvested from ewes given either the H or L feed from Day –18 to Day +6 post ovulation. Embryos were then transferred to recipient ewes and allowed to develop to Day 75 of pregnancy at which time uterine and fetal tissues were recovered. Pregnancy rates were not significantly different between the two groups. All fetal measurements were also comparable between the two groups except for significant ($P<0.05$) increases in the weight of the brain, pancreas and chest thymus in the H feed intake group.

Overall, these studies indicate that short-term changes in feed intake can significantly alter embryo development with potential consequences for subsequent fetal development. The way in which these short-term changes influence development is unclear but a number of avenues are presented for further investigation. The findings of this thesis are relevant both to animal husbandry and to improving our understanding of the mechanisms regulating early embryo development.

DECLARATION

I hereby declare that the material contained in this thesis in my own work, and contain no material, which has been accepted for the award of any other degree or diploma in any University. To the best of my knowledge and belief, this thesis contain no material previously published or written by another person, except where due reference is made in the text of the thesis.

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

M Azam Kakar
Date: 18th March 2003

This thesis is most affectionately DEDICATED
to my dear parents and to all my teachers in Pakistan,
Japan and here in Australia who have enriched my life
in ways too numerous to list

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ABBREVIATIONS

ABA	Amino butyric acid
ADP	Adenosine di-phosphate
Ad libitum	Without restraint
AI	Artificial insemination
ANOVA	Analysis of variance
BSA	Bovine serum albumin
^0C	Degree centigrade
CIDR	Control internal drug release
CL	Corpus luteum
CO_2	Carbon dioxide
CV	Coefficient of variation
DM	Dry matter
DNA	Deoxynucleic acid
DNP	Dinitrophenol
ECLIA	Electro chemiluminescence immunoassay
E_2	Oestradiol
e.g.	For example
EGF	Epidermal growth factor
EGP	Oestrus-associated glycoprotein
ELISA	Enzyme Linked Immuno Sorbent Assay
EPF	Early pregnancy factor
E_2R	Oestrogen receptor
et al.	*et alia* (and others)
etc	*et cetera* (and so on)
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FSH	Follicle stimulating hormone
g	Gram (s)
GF	Graafian follicle
GH	Growth hormone
GLDH	Glutamate dehydrogenase
GnRH	Gonadotrophin releasing hormone

GP	Glycoprotein
GVBD	Germinal vesicle breakdown
h	Hours (s)
HPLC	High performance liquid chromatography
HS	Human serum
ICM	Inner cell mass
IGF	Insulin like growth factor
i.m.	Intramuscular
IMT	Integrated multisensor technology
iu	International unit (s)
IVF	*In vitro* fertilization
IVM	*In vitro* maturation
i.v.	Intravenous
Kg	Kilogram (s)
L	Litre (s)
LH	Luteinizing hormone
LI	Laparoscopic insemination
ME	Metabolisable energy
Mg	Milligram (s)
Ml	Millilitre (s)
min	Minute (s)
Mr	Molecular weight
mRNA	Messenger ribonucleic acid
MTB	Methylation blue
N_2	Nitrogen
NGF	Nerve growth factor
NIRS	Near Infrared Spectroscopy
nmol	Nanomolar
ns	Non significant
NSW	New south Wales
OCPC	O-cresolphthalein complexone
O_2	Oxygen
OF	Oviductal fluid
P	Progesterone

PAF	Platelet activating factor
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
PGF_2	Prostaglandin $F_2\alpha$
PMAPS	P-methylaminophenol sulphate
PMSG	Pregnant mare serum gonadotrophin
PR	Progesterone receptor
PVP	Polyvinylpyrrolidone
RDP	Rumen degradable protein
REML	Restricted maximum likelihood
RNA	Ribonucleic acid
rpm	Revolution per minute
RUI	Real-time ultrasound imaging
SA	South Australia
SAS	Statistical analysis system
SE	Standard error of estimate
SEM	Standard error of least squares mean
SOF	Synthetic oviduct fluid
SS	Sheep serum
TE	Trophectoderm
TCM-199	Tissue culture medium-199
TGF	Transforming growth factor
TNBS	Trinitrobenzene sulfonic acid
UI	Uterine insemination
µl	Microlitre
µm	Micrometer
UV	Ultra violet
ZP	Zona pellucida

DEFINITION OF TERMS

Oogonium:	Female sex cell (gamete) before meiosis. Chromosome number 2N.
Oocyte, primary:	Female gamete after onset and during first maturation division (meiosis). Chromosome number 2N until extrusion of first polar body. Contained in primary and secondary follicles.
Oocyte, secondary:	Female gamete at onset and through second meiosis. Chromosome number 1N.
Ovum (ova):	(L. *ovum*, egg). A female gamete after completion of second meiotic division, but before union of male and female pronuclei. Chromosome number 1N.
Germinal vesicle:	Nuclear membrane (envelope) of the ovum.
Zygote (s):	(Gk. *zygote*, yoked). A fertilized ovum from the fusion of the male and female pronuclei until completion of the first cleavage. Chromosome number 2N.
Embryo (s):	(Gk. *embryon*, embryo). A zygote after cleavage; contains two or more blastomeres. Stage from a 2-cell embryo until cell migration and differentiation are largely completed. Only minor growth occurs at this stage, compared to the fetal stage.
Fetus (es):	(L. *fetus*, progeny). An embryo that has completed most of organogenesis and primarily is growing in size, taken in this study to be after implantation to day 75 of pregnancy (day 0 = day of oestrus).
Conceptus:	An embryo or fetus with all its membranes and accessory structures.
Corpus luteum (corpora lutea):	The glandular body developed from a Graafian follicle after extrusion of the ovum. For simplicity, corpus luteum is used synonymously with corpus haemorrhagicum (the body formed from the ruptured Graafian follicle and the blood clot).
Placenta (e):	(Gk. *flat cake*). The cotyledonary placenta of the sheep is formed by the attachment at about days 20-30 of pregnancy of the chorionic membrane to specific endometrial thickenings (caruncles) on the uterine wall. These points of attachment develop into bulb or round shaped cotyledons (also called placentomes).

Chapter 1

INTRODUCTION

1.1 INTRODUCTION

Photoperiod, temperature and nutrition are three well-studied environmental cues that influence reproduction in mammals. From a practical standpoint, the effects of nutrition on reproduction commands the greatest attention because livestock producers can easily manipulate the nutrition of domestic livestock, whereas alteration of photoperiod or temperature can only be accomplished in intensive and therefore expensive management situations (Boland *et al.*, 2001).

Effects of nutrition on reproductive performance have been recognised by shepherds for centuries and management practices have evolved which take account of this knowledge. Since the late 19th Century, animal husbandry has become a science and, beginning with the work of Heape (1899), Bell (1912) and Marshall and Potts (1921), the effects of nutrition on the animal's physiology have been described and quantified in ever increasing detail.

Our present inability to provide large numbers of viable embryos from selected females still restricts genetic improvement for livestock producers, whilst variability in ovarian response to hormones limits the present capacity for increasing reproductive efficiency. In addition, there is increasing evidence that even though *in vitro* fertilisation may be achieved, a significant percentage of resultant embryos are lost due to the poor quality of the oocytes at fertilisation (Webb, 1999). Females are born with a large number of primordial follicles, and follicles within this pool are routinely stimulated to undergo further development. The mechanisms that control this process are not known, and there is substantial potential to increase reproductive efficiency given that >99% of these follicles never reach the ovulatory stage.

In sheep, increased dietary intake for a relatively short time will increase the ovulation rate by increasing gonadotrophin secretion (Downing *et al.*, 1995). Dietary intake can affect steroids such as progesterone and also intra-follicular concentrations of factors such as IGF-1 and IGF-2 (Houseknecht *et al.*, 1988). The effects of altered dietary intake on the concentration of gonadotrophins and steroids in cattle are not as repeatable as those in sheep but the growth rate of follicles can often be altered in cattle. High nutrition has a negative effect on oocyte quality, with animals on *ad*

libitum high energy diets particularly at risk (O'Callaghan *et al.,* 2000). Overfeeding can decrease embryo quality in both sheep and cattle and it appears that this results from changes primarily at the level of the follicle or oocyte (Boland *et al.,* 2001). Restricted nutrition for a short time will enhance pregnancy rates in cattle; most of this benefit appears to occur if food is restricted before insemination. Thus feeding levels before mating may be particularly relevant to subsequent reproductive success (O'Callaghan and Boland, 1999).

Superovulation is one of the main methods used to provide embryos for the embryo transfer industry. However, with superovulation there has been relatively little progress achieved over the last 20 years in increasing the number of transferable embryos per donor animal. This is despite substantial progress being made in understanding factors that control follicular growth (Adams, 1994) and ovulation (Gong *et al.,* 1993). Deleterious effects on embryo development of excessive nutrition around mating are becoming evident both in non-superovulated (Dunne *et al.,* 1997) and superovulated cattle (Mantovani *et al., 1993;* Yaakub *et al.,* 1999; Nolan *et al.,* 1998). Mantovani *et al.* (1993) reported that the superovulatory responses tended to be lower and the yield of good quality embryos reduced in heifers that had been exposed to high levels of concentrates in association with reduced roughage.

The mechanisms by which nutrition influences follicle growth is not clear. It is known that follicle growth is influenced by FSH and Spicer *et al.* (1990) reported that FSH concentrations were influenced by over-feeding in heifers, while IGF-I concentrations can also be modified by the plane of nutrition (Houseknecht *et al.,* 1988). Maurasse *et al.* (1985) reported that high feed intake in heifers can influence ovarian function by reducing the number of large non-atretic follicles and increasing the number of small non-atretic follicles. Several studies have already indicated that excessive feeding is associated with a reduction in the yield and quality of embryos in cattle (Blanchard *et al.,* 1990; Mantovani, *et al.,* 1993; Nolan, *et al.,* 1997; Boland and Callaghan, 1999) and in sheep (Fahey *et al.*, 2001; McEvoy *et al.,* 1995a and 2001). This appears to be due to the decreased number of follicles that are unable to respond to exogenous FSH in over-fed animals or to inappropriate oocyte maturation with a consequent reduction in developmental competence. Assey *et al.* (1994) reported that

impaired developmental capacity of superovulated oocytes in the cow was associated with a number of subcellular deviations or abnormalities (structural changes in the degree of detachment of interchromatin-like granules within the oocyte nucleolus) compared with oocytes from untreated cows. Dietary intake may exacerbate these deviations.

Whilst the effects of an elevated plane of nutrition on embryo quality are evident in superovulated ewes maintained on improved diets for extended time periods (up to six months), it is not known if these effects are evident with short-term dietary changes. In addition, it is important to know if any effect of feed intake is mediated at the level of the oocyte/embryo or is caused by other pre- or postovulatory events, including changes in the oviduct/uterine environment. Thus, the objective of studies in this thesis was to determine the effect of short-term changes (i.e. within one oestrous cycle) in feed intake on follicle growth and embryo quality following both natural ovulation and superovulation in the ewe. The main hypothesis was that ewes on a restricted plane of feed intake have increased progesterone concentrations and produce oocytes and embryos of higher quality than ewes on a high plane of feed intake.

1.2 Aims and objectives

Embryo collection in the ewe is a procedure with highly variable results and anecdotal evidence implies that nutrition has a significant influence on outcome. Superovulation is being used increasingly in sheep with the commercial application of embryo transfer and for the developing biotechnologies of genetic manipulation and cloning. Oocyte quality is a major issue in the success of these programs and is equally important in normal reproductive processes. Nutrition is known to have both long-term and short-term effects on reproduction. In these studies, the effects of short-term feed intake on blastocyst quality and on the oviduct environment following both natural ovulation and superovulation were studied. The findings are expected to assist in improving embryo quality and survival in reproductive and genetic selection programs involving multiple ovulation, embryo transfer and *in vitro* fertilisation.

The main objectives of the research reported in this thesis were:

- To determine the effect of short-term changes in feed intake in the peri-conceptional period of the ewe on embryo quality and fetal development.

- To determine the time during nutritional signals influence the allocation of cells to the inner cell mass or trophectoderm within the blastocyst.

- To determine the effect of short-term feed intake on various aspects of the composition of oviduct fluid.

- To assess the hormone profiles in oviductal fluid during the nutritional treatment period with particular emphasis on progesterone and its effects on embryo quality.

The major long-term aim of this research is to assess how maternal feed intake prior to and during embryonic development might alter the phenotype of the developing conceptus.

1.3 Hypotheses

1. That a relatively low level of feed intake at specific time(s) of the oestrous cycle will result in a more efficient reproductive outcome compared with a high level of nutrition.

2. That feed intake alters the oviductal milieu resulting in changes in embryo development and, in particular, the structure of the blastocyst and its ability to survive.

3. That ewes on a restricted plane of feed intake for one estrous cycle have increased progesterone concentrations compared with ewes on a high plane of feed intake and that this difference impacts on the development of the blastocyst.

1.4 Potential benefits

The findings are expected to assist in improving embryo quality and survival in reproductive and genetic selection programs involving natural mating, multiple ovulations, *in vitro* maturation/fertilisation and embryo transfers. These benefits will accrue from the implementation of optimal feeding strategies before, during and after mating.

Chapter 2

LITERATURE REVIEW

The embryo transfer industry has undergone major changes in a relatively short time. Superovulation is a method used to increase the number of follicles grown with a subsequent increase in the number of ova released at a single oestrus. Most mammals regulate the number of ova ovulated. In sheep, this number (the ovulation rate) seldom exceeds three and it is a most important determinant of litter size (Hanrahan, 1980). Early studies (Heape, 1899; Bell, 1912; Marshall and Potts, 1921) have shown that an increase in live weight of ewes prior to mating results in a greater number of lambs per ewe. Coop (1966a,b) divided the flushing response into the effect of live weight *per se*, (static effect) and the effect of increased nutrient intake immediately prior to mating (dynamic effect). The dynamic effect is thought to increase secretion of pituitary gonadotrophins (Coop, 1966a).

The development of improved methods for inducing ovulation and for manipulating ovulation rate will depend to a large extent on a better understanding of the mechanisms responsible for follicular development and differentiation. Sheep are an excellent experimental model for this purpose, because ovulation rate is affected by genetic differences between breeds and by environmental factors such as photoperiod and nutrition (Scaramuzzi *et al.*, 1993). In the following section, the oestrous cycle of the ewe, the stages of oocyte development *in vivo*, fertilisation and embryonic development, the oviductal environment, oviductal transport and embryo - maternal interactions and nutrition and reproduction are reviewed.

2.1 CELL DIFFERENTIATION THROUGH DEVELOPMENT

2.1.1 Oogenesis and folliculogenesis

Primordial germ cells can first be seen very early in fetal development, in the epithelium of the dorsal endoderm of the yolk sac near the developing allantois. They migrate along the hindgut of the embryo by amoeboid like movement until they reach the tissue covering the ventral area of the primitive kidney (mesonephros) known as the genital ridge. At this stage, the germ cells are referred to as oogonia (Baker, 1982). During migration the number of oogonia increases rapidly by mitosis. Oogonia are then transformed into primary oocytes by the commencement of meiosis, which only proceeds to the prophase I stage (Fortune, 1994). Following this, the primary oocytes become surrounded by a layer of flat epithelial cells to form the

primordial follicle. By the time of birth, all oogonia have developed into primordial follicles but the primary oocytes remain arrested in meiosis and do not finish their first meiotic divisions until after puberty. In the sheep, some follicles at birth have reached the antral stage by 135d of pregnancy. Fig. 2.1 outlines the stages of germ cell differentiation in comparison with the developmental events that occur in the female reproductive life.

Mammalian ovaries have a pool of primordial follicles, each consisting of an oocyte arrested in prophase 1 of meiosis and a layer of flattened granulosa cells. This pool develops during fetal life in some species (e.g., primates, ruminants), but in others it develops during the early neonatal period (e.g., rodents, rabbit) (Hirshfield, 1991; Marion and Gier, 1971). The primary oocyte begins to increase in size with the surrounding follicle cells changing in shape to produce stratified layers of granulosa cells. At this stage the follicle is known as a primary follicle (Fig. 2.2). The development of a primary follicle into a secondary follicle is characterised by the following events: i) granulosa cells and the oocyte secrete a layer of glycoproteins onto the surface of the oocyte forming the zona pellucida, ii) the fibrous cells around the follicle become vascularized to form what is known as theca interna, which is in turn surrounded by theca externa and, iii) the follicle becomes fluid-filled to form an antrum. At the completion of these events, the secondary follicle enlarges further to form the tertiary (or Graafian) follicle, which then awaits the signal to ovulate (Fig. 2.2). During this time, the oocyte remains arrested in prophase I of meiosis. However, the nucleus does increase in size, due mainly to the production of large amounts of 'nuclear sap', so that the nuclei of advanced oocytes appear to be bloated with fluid and are usually referred to as germinal vesicles (Balinsky, 1970).

The growth of the follicle occurs under the influence of pituitary hormones follicle stimulating hormone (FSH) and luteinising hormone (LH). Not all follicles develop to the tertiary stage with the majority being eliminated by the degenerative process known as atresia. In the human, usually only one follicle per cycle is selected for ovulation. This number is greater in some species including mice and pigs. The

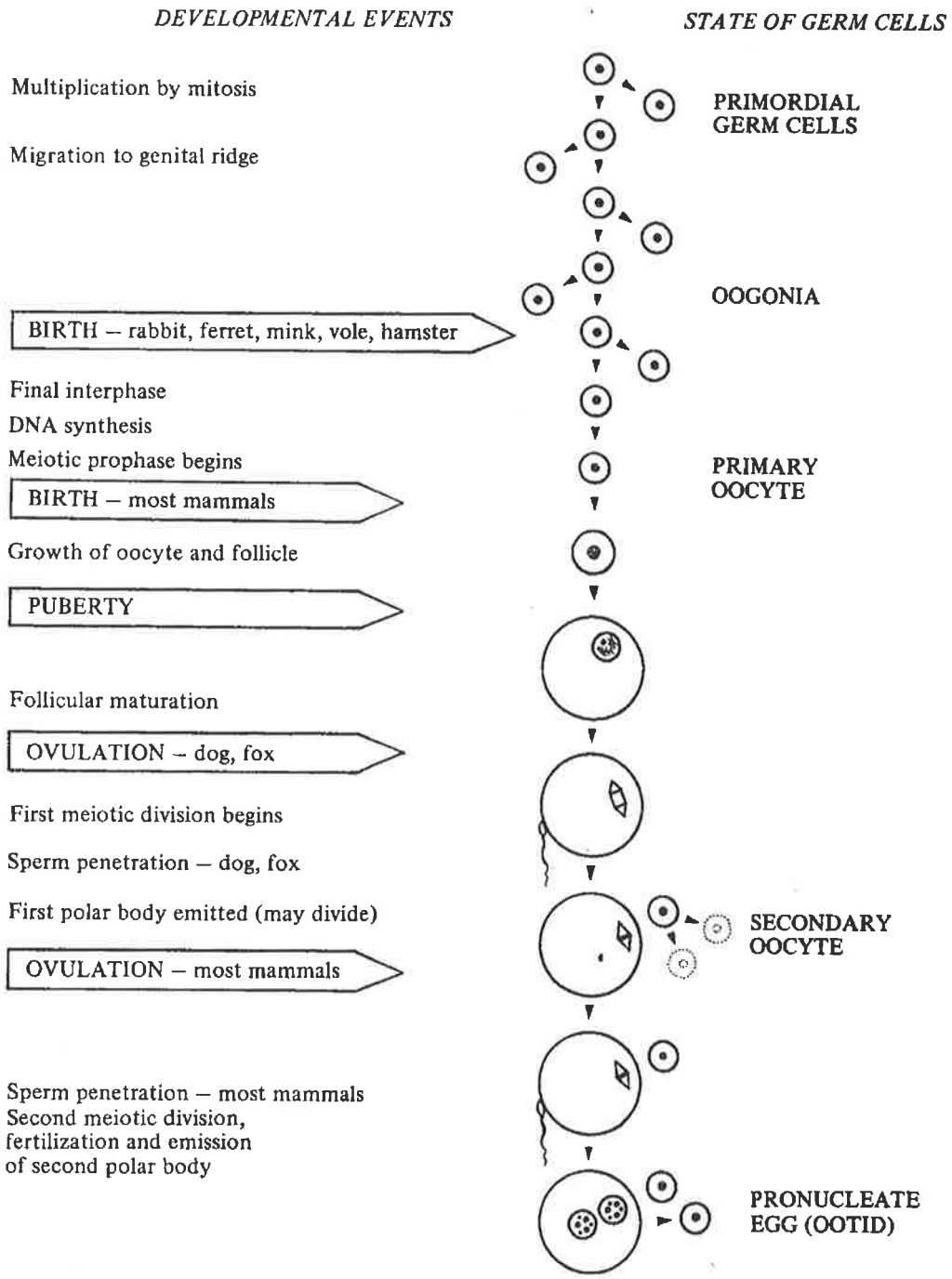


Fig. 2.1 Life history of female germ cells (Baker, 1982).

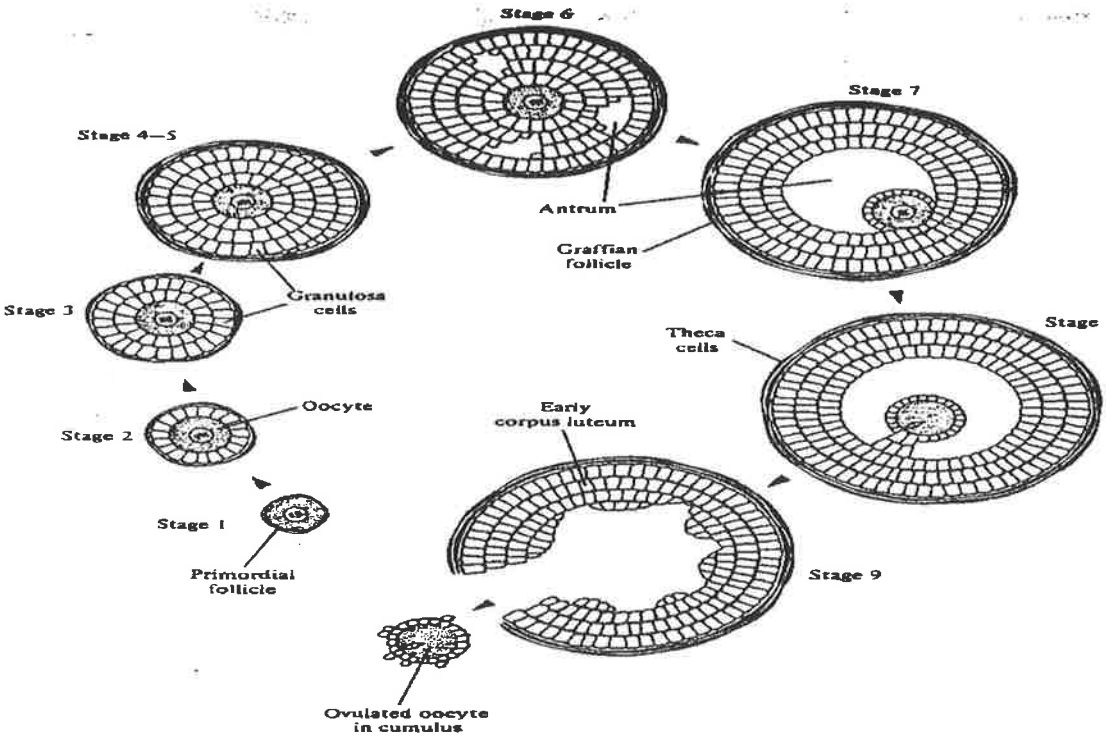


Fig. 2.2 Stages of follicular growth. In stage 1, the structure is called a primordial follicle, and in stages 7 and 8 a Graafian follicle. Stage 9 represents ovulation (Baker, 1982).

number of follicles that ovulate can be increased by increasing the concentration of FSH and LH in circulation and this is the basis of superovulation treatments that are routinely used in embryo transfer programs.

Oocyte meiosis resumes at the time of oestrus in response to the preovulatory surge of LH. Following the pre-ovulatory LH surge, the final stages of oocyte maturation take place, characterised by germinal vesicle breakdown (GVBD), chromosome condensation, formation of the meiotic spindle, completion of the first meiotic division, expulsion of the first polar body and arrest in metaphase of the second meiotic division.

Ovulation occurs under precisely controlled hormonal conditions. A surge in LH is thought to cause a final 'wave' of mitosis in granulosa cells so that an optimum number is reached for the tertiary follicle (Baker, 1982). The quantity of follicular fluid in the antrum also increases dramatically. At a reasonably precise time after this LH surge (24 h in the sheep) the oocyte is ovulated from the tertiary follicle.

2.1.2 Superovulation

Superovulation is a method of increasing the number of ova released by the female and thus is one method of accelerating genetic improvement in any species. Superovulation requires the administration of FSH over sufficient time to enable the follicles to grow and enable final maturation of the oocyte (Fig. 2.3). A better understanding of the endocrine and paracrine factors controlling the process of follicle selection, growth and atresia is required in order to develop more effective systems of superovulation. However, a variable and unpredictable superovulatory response remains one of the most limiting factors to successful embryo transfer (Gordon, 1982; Adams, 1994).

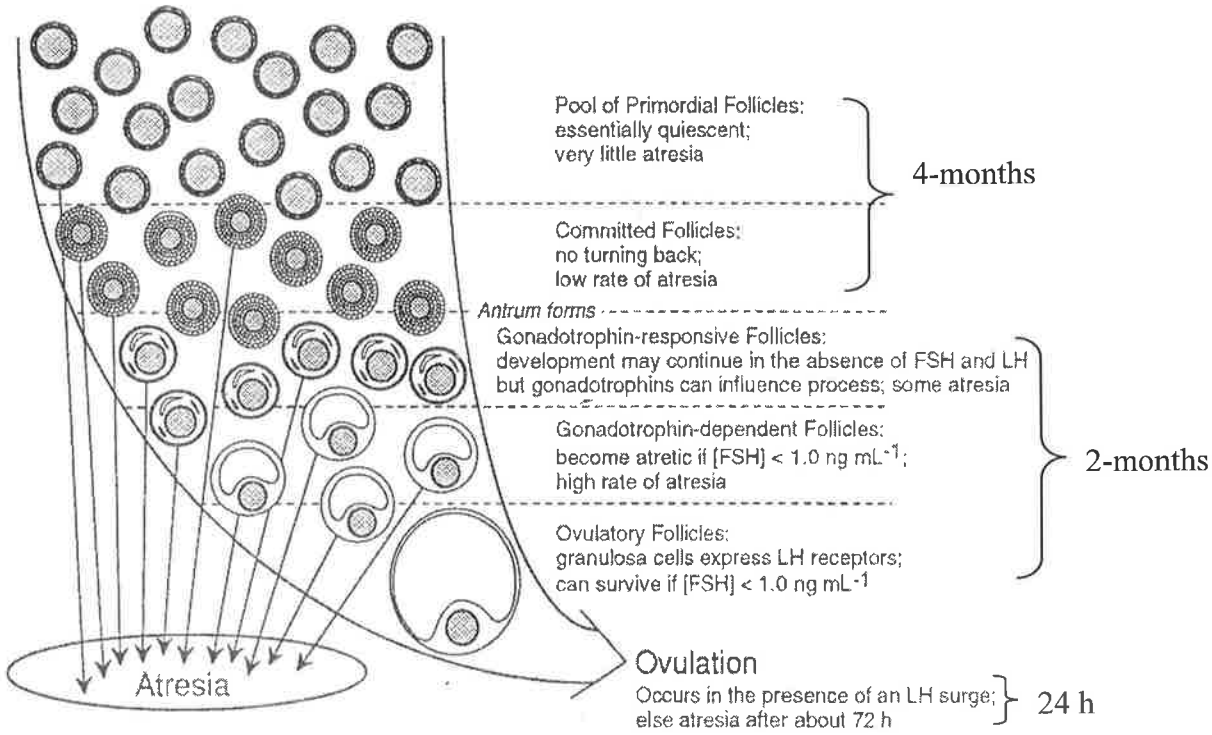


Fig. 2.3 A pictorial illustration of follicular growth in the ewe. The different types of follicles are not drawn to scale or in correct numerical proportions. The presence of an ovulatory follicle induces atresia in all gonadotrophin-dependent follicles because the oestradiol and inhibin it secretes blocks the supply of FSH and increases the amplitude of the LH pulses. It is not known if FSH and LH are obligatory for the formation and continued development of gonadotrophin-responsive follicles. Uncertainty also surrounds the concentrations of FSH and LH required at different stages of follicular development although their relativity is probably more important. The values selected should be regarded as approximate (Scaramuzzi *et al.*, 1993)

2.1.3 Hormonal patterns during the ovarian cycle

The patterns of secretion of reproductive hormones during the oestrous cycle and their concentrations in peripheral blood have been detailed more extensively in the sheep than in most other species (Baird and McNeilly, 1981; Goodman, 1988; Baird *et al.,* 1991). The ewe has a 17-day oestrous cycle and the day of oestrous (Day 0) generally coincides with the onset of the preovulatory surge of gonadotrophins (Fabre-Nys and Martin, 1991). Ovulation occurs approximately 24 h after the onset of the gonadotrophin surge. Luteolysis usually begins on Day 14 or 15 of the oestrous cycle (Scaramuzzi *et al.,* 1993).

2.1.3.1 Progesterone: Progesterone concentrations in peripheral blood begin to increase around Day 3-4 of the oestrous cycle, that is, 2-3 days after ovulation as the new corpus luteum becomes active. Maximum concentrations are observed by Day 10-12 and are maintained until luteolysis around Day 14-15. At luteolysis, progesterone concentration in the blood falls and within 24 h reaches the lowest values during the cycle. Concentrations remain low throughout the follicular phase until ovulation 2-3 days later.

2.1.3.2 Gonadotrophin-releasing hormone (GnRH): The pattern of GnRH secretion into hypophyseal-pituitary portal blood is pulsatile and has been described for several stages of the oestrous cycle in the ewe (Clarke, 1989; Thiery and Martin, 1991). The vast majority of GnRH pulses in portal blood are reflected by pulses of LH in jugular venous blood and in ovarian arterial blood.

2.1.3.3 Luteinising hormone (LH): The concentrations of LH are relatively low throughout most of the oestrous cycle and the pattern of secretion is characterised by infrequent pulses (Martin, 1984). During luteolysis, as the concentration of progesterone decreases, the secretion of LH increases owing to a higher frequency of pulses (Wallace *et al.,* 1988). This culminates in the preovulatory surge of LH about 4 days after luteolysis begins.

2.1.3.4 Follicle stimulating hormone (FSH): The secretion of FSH, although controlled by GnRH, appears to be continuous and is not acutely responsive to GnRH. As a result, FSH secretions are not pulsatile and during the luteal phase individual

ewes show 'waves' in their concentrations of FSH with peaks occurring about six days apart (Bister and Paquay, 1983; Campbell *et al.,* 1991). These are probably associated with the development and regression of large follicles in the ovary, as has been reported in cattle (Fortune *et al.,* 1991). A few hours after luteolysis begins, the concentration of FSH begins to decrease and reaches a nadir within 24-36 h; FSH then remains low until the preovulatory surges of LH. A second peak of FSH occurs 24-36 h after the preovulatory gonadotrophin surge that is associated with atresia of large non-ovulatory follicles. Further improvements are needed in the specificity of FSH assays with respect to individual isoforms of the hormone and their bioactivity (Combarnous, 1988), before the significance of fluctuations in the concentration of FSH can be interpreted with respect to follicular development.

2.1.3.5 Inhibin: There are significant technological difficulties in accurately determining levels of biologically active inhibin in peripheral and ovarian venous blood and current assay methods are of limited value (Knight, 1991; Knight *et al.,* 1998). Observations of inhibin concentrations during the oestrous cycle are not consistent between laboratories, probably as a result of these difficulties. A consensus could not be reached but there is broad agreement that inhibin concentrations throughout the luteal phase of the cycle remain relatively constant with some minor fluctuations (Campbell *et al.,* 1990b; Findlay *et al.,* 1991). During the interval from the onset of luteolysis until the preovulatory surge of gonadotrophins, it appears that the concentrations of inhibin either remain relatively constant or increase by up to two-fold (Tsonis *et al.,* 1988; Campbell *et al.,* 1990b; Findlay *et al.,* 1991). Immediately after the preovulatory surge, a decrease in the inhibin concentration coincides with the second peak of FSH (Findlay *et al.,* 1991).

2.1.3.6 Oestradiol: Knowledge is limited by the inability of currently available assays to measure peripheral concentrations during the oestrous cycle of the ewe with a degree of accuracy and precision sufficient to detect small fluctuations in oestradiol levels that may exert significant feedback on FSH and LH secretion (Scaramuzzi and Land, 1978; Webb *et al.,* 1985). Consequently, much of our understanding has been derived from studies of the rate of secretion by the ovary; this is a good indicator of follicular activity but does not necessarily reflect the degree of feedback because of effects associated with changes in the hepatic metabolism of oestradiol (Thomford

and Dziuk, 1988) and other factors which may also affect peripheral oestradiol concentrations (Atkinson and Adams, 1988).

Nevertheless, there is broad agreement about the overall pattern of oestradiol concentrations during the oestrous cycle of the ewe. At luteolysis, the secretion of oestradiol begins to increase in response to an increasing frequency of LH pulses, culminating in a peak of oestradiol secretion that triggers the preovulatory surges of GnRH and the gonadotrophins. Thereafter, oestradiol secretion decreases rapidly and reaches the lowest level for the cycle in the period immediately after the LH surge (Scaramuzzi *et al.,* 1993). There is a significant increase in oestradiol concentration around Day 3-4 of the cycle (Campbell *et al.,* 1990a) followed by regular fluctuations throughout the luteal phase. The consensus is that increases in oestradiol secretion coincide with waves of follicle growth as has been reported in the cow (Fortune *et al.,* 1991).

2.1.3.7 Feedback Control of the Ovarian Cycle: The ovarian cycle in sheep is regulated by numerous inter-relationships between the hormones from the hypothalamus (GnRH), pituitary gland (LH and FSH), follicle (oestradiol and inhibin), corpus luteum (progesterone and oxytocin) and uterus (prostaglandin $F_2\alpha$). These inter-relationships have been extensively studied in the ewe, so that the sequence of endocrine events controlling the oestrous cycle, ovulation and the establishment of pregnancy are now well-documented and presented in Fig. 2.5 (Scaramuzzi *et al.,* 1993). The oestrous cycle is controlled primarily by hypothalamic GnRH that stimulates gonadotrophin secretion from the anterior pituitary gland. The gonadotrophins stimulate follicular development (McNeilly *et al.,* 1991) leading eventually to ovulation and the formation of a corpus luteum. Follicle development is self-regulated by negative and positive feedback loops between the ovary and the hypothalamic-pituitary system (Baird *et al.,* 1991; McNeilly *et al.,* 1991). After ovulation, a corpus luteum is formed and secretes progesterone, which prevents further ovulation and facilitates the establishment of pregnancy. However, in the absence of pregnancy, oxytocin released from the corpus luteum induces luteolysis through the release of prostaglandin $F_2\alpha$ from the uterus (Flint *et al.,* 1989; Wathes, 1989; Bazer *et al.,* 1991) and a new ovulation follows.

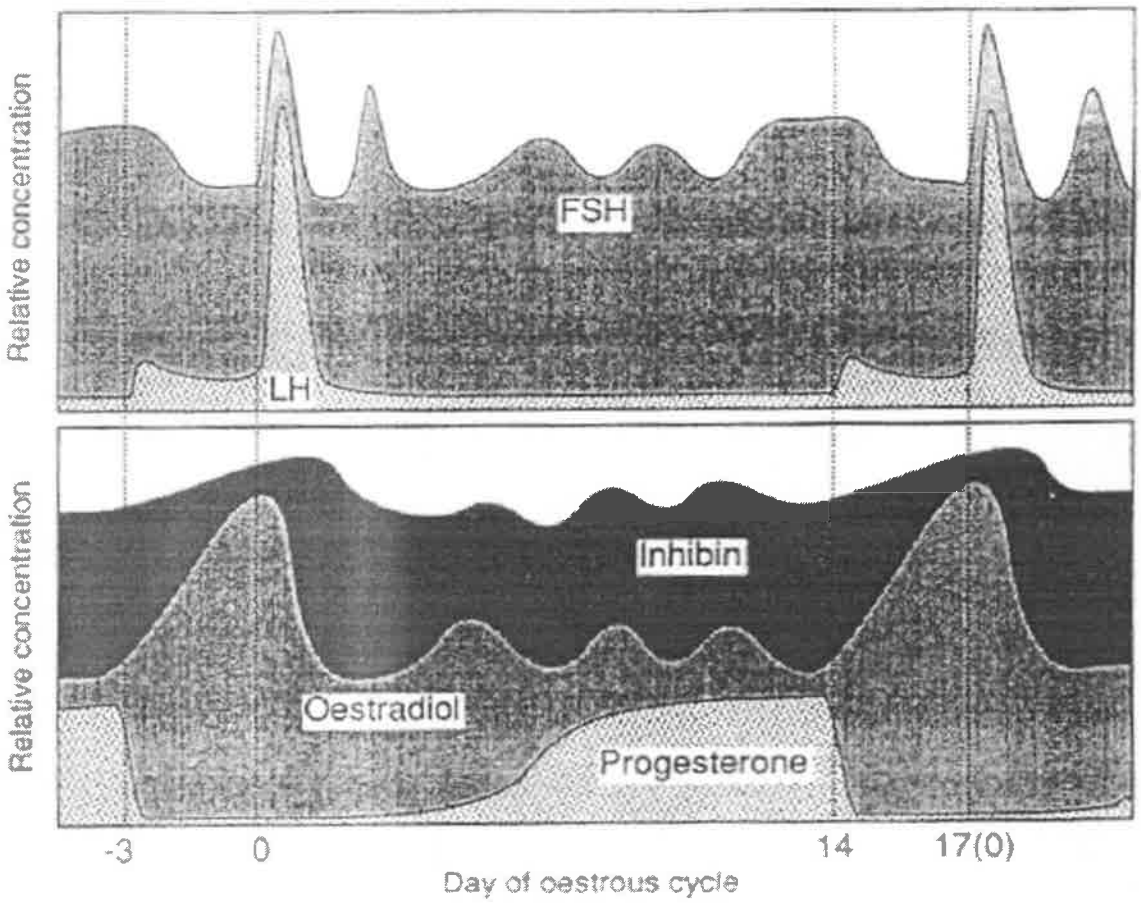


Fig. 2.4 Schematic representations of the circulating levels of the principal gonadal and pituitary hormones during the oestrous cycle of the ewe. Vertical axis: relative concentration of two pituitary hormones (upper panel) and three ovarian hormones (lower panel). Horizontal axis: day of the oestrous cycle. The idealised oestrous cycle is 17-days long; Day 0 is the day of oestrus and Day 14 is the beginning of luteolysis (Scaramuzzi *et al.*, 1993).

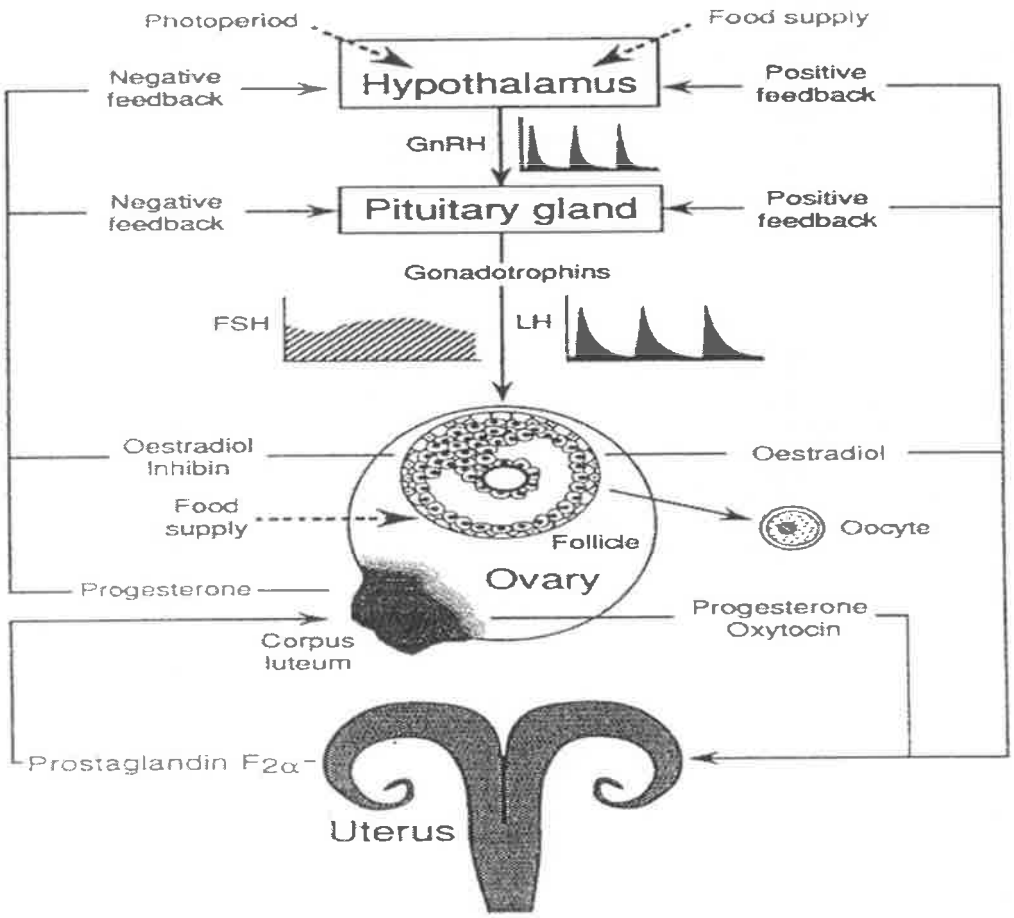


Fig. 2.5 The oestrous cycle is regulated by the inter-relationship between hypothalamus (GnRH), pituitary (LH and FSH), follicular (oestradiol and inhibin), luteal (progesterone and oxytocin) and uterine (prostaglandin $F_{2\alpha}$) hormones. The sequence of endocrine events that regulates the oestrous cycle and ovulation is controlled by GnRH of hypothalamic origin that stimulates LH and FSH secretion from the anterior pituitary gland. The gonadotrophins stimulate follicular development leading to ovulation and the development of a corpus luteum. Follicle development is self-regulated by oestradiol, progesterone and inhibin in a negative feedback loop between the ovary and the hypothalamus and the anterior pituitary gland. After ovulation, a corpus luteum is formed and secretes progesterone that prevents ovulation and is required for pregnancy. In the absence of pregnancy, oxytocin released from the corpus luteum causes luteolysis by releasing prostaglandin $F_{2\alpha}$ from the uterus and a new oestrous cycle follows. Photoperiod and nutrition, two of the principal external factors affecting the oestrous cycle and ovulation, are thought to act at the hypothalamus. Nutrition is also thought to act directly on the ovary (Scaramuzzi *et al.,* 1993).

2.1.4 Spermatogenesis

Spermatozoa are produced in the testis by a complex process known as spermatogenesis. This involves mitotic proliferation of spermatogonia to produce large numbers of spermatocytes, meiotic division to generate genetic diversity and halve the chromosome number in the spermatids and extensive cell modelling to package the chromosomes for transport as spermatozoa (reviewed in Setchell, 1982). Like oogenesis, the process begins in the developing fetus with the primordial germ cells. Mitotic proliferation recommences at puberty, at which time the germ cells are known as A0 spermatogonia. This occurs at the base of the seminiferous tubules and these cells proliferate slowly and act as a reservoir from which A1 spermatogonia are produced. A1 spermatogonia undergo a certain number of mitotic divisions to form primary spermatocytes. Following this initial proliferation are the meiotic divisions and the eventual production of four haploid round spermatids. Spermiogenesis occurs next with the maturation of spermatids to spermatozoa, ready to be carried out of the testis. Fully mature spermatozoa are stored in the epididymis awaiting ejaculation. For a more extensive review on spermatogenesis see Setchell (1982).

2.1.5 Fertilisation

Fertilisation is the process by which the male and female gametes fuse, and it usually occurs in the oviduct. Before fertilisation, the sperm must undergo two essential processes; capacitation and the acrosome reaction. Spermatozoa gain the ability to fertilise through the capacitation process. This process involves stripping the coat of glycoprotein molecules (acquired from the epididymis and seminal fluid) from the membrane of the spermatozoa. The so called acrosomal cap is part of the nuclear envelope at the tip of the spermatozoa and the process by which it breaks down and releases its enzyme contents is known as the acrosome reaction. Two main acrosomal enzymes that play important roles in fertilisation are hyularonidase, which digests hyularonic acid holding the cumulus cells together and acrosin, which is trypsin-like and digests the zona pellucida (Longo, 1997).

After attaching to the oocyte and proceeding through the cumulus cell layer, the spermatozoa bind to the zona pellucida and cross it in a curved pathway with the assistance of the acrosome enzymes. Once the spermatozoon has penetrated the zona,

it binds to the plasma membrane (oolemma). Fusion of the sperm head with the oolemma results in the sperm nucleus, inner acrosomal membrane and the sperm tail (in most species) being incorporated into the oocyte cytoplasm (Longo, 1997).

Sperm fusion also results in a series of biochemical and morphological events that together constitute what is known as activation of the oocyte. Although this process is not completely understood, particular events are recognised. The resting membrane potential shifts from –20mV to –40mV as a result of increasing potassium levels and a release of calcium takes place as a series of waves beginning at the site of spermatozoon attachment. This release in calcium is important for cortical granule release and polar body extrusion. Cortical granules are small cytoplasmic organelles located in the periphery of the oocyte cytoplasm. They contain hydrolytic enzymes, which are released into the perivitelline space after sperm fusion, causing the hardening of the zona and preventing polyspermy. Oocyte activation also induces changes in the oocyte cytoskeleton. The second meiotic division is completed and the second polar body extruded leaving the oocyte in a haploid state (Longo, 1997).

Once the sperm nucleus is incorporated into the oocyte cytoplasm its nuclear envelope disintegrates, disulphide bonds in the DNA are reduced, the chromatin decondenses and sperm specific protamines are replaced with histones. Both the maternal and paternal pronuclei are formed by the development of nuclear envelopes around the respective chromatin. Finally, the pronuclei migrate to the centre of the oocyte where syngamy occurs, consisting of the breakdown of pronuclear membranes and the assembly of chromosomes to form the metaphase plate in preparation for the first cleavage division. Formation of the metaphase plate depends on cytoskeletal activity involving microtubal formation and actin filaments (Longo, 1997).

2.1.6 Developmental stages of the preimplantation embryo

A one-cell embryo that forms immediately after fertilisation containing maternal and paternal pronuclei is called a **zygote**. Once the chromosomes have aligned on the spindle and separated to opposite poles, a furrow appears on the surface of the zygote and the cytoplasm divides to form a diploid two-cell embryo. Each cell of the embryo is referred to as a blastomere (Longo, 1997).

Blastomeres of the embryo multiply mitotically, although the rate of division is not uniform. For example, in the sheep the four cell stage exists for approximately 10-12 hours, whereas the embryo remains at the 8-16 cell stage for 24 hours or longer. Importantly, there is evidence that blastomeres are predestined very early in development to differentiate into certain tissues. For example, the earliest dividing blastomere in the 2-cell embryo continues to divide early and eventually contribute preferentially to the inner cell mass (ICM) (Moore and Kemler, 1997).

The **morula** stage generally refers to the 16- to 64-cell embryo in sheep. Around the formation of 32-cell stage, the blastomeres maximise their contact with each other by forming tight gap junctions. This process is known as compaction and the embryo is then referred to as a compact morula. At this stage of development, the outer blastomeres begin to develop polarity, leading to the development of a polar axis along which there is selective permeability to ions, an important feature in the formation of the blastocyst (Balinsky, 1970).

A **blastocyst** is characterised by the formation of a blastocoele, a fluid filled cavity that forms within the compacted blastomeres. The blastocoele develops as a consequence of fluid being released from polarised cells. The two groups of differentiated cells in the blastocyst are the trophectoderm cells and the ICM cells (Fig. 2.6). The trophectoderm cells develop from the polarised cells and form the periphery of the embryo and are involved in the formation of the placenta (Cross, 1998). The ICM cells, which develop from non-polar cells located on the inside of the embryo, ultimately develop into the foetus (Gardner *et al.,* 1973). Shortly after the formation of a blastocoele, the embryo sheds its zona pellucida in a process known as hatching. This occurs approximately seven days after fertilisation in sheep and the embryo remains free floating within the uterus until the process of implantation commences.

2.1.7 Activation of the embryonic genome

The survival of the embryo before the embryonic genome is activated depends upon the synthesis and accumulation of mRNA and proteins in the oocyte cytoplasm during oogenesis and cytoplasmic maturation. Most of the RNA is ribosomal and provides the translational machinery to perform the first polypeptide synthesis, whereas

proteins are involved in the general "housekeeping" of the early embryo as well as in gene expression (Raff, 1980). Some of these proteins are highly stable and can even be detected at the morula/blastocyst stage. However, the role of these proteins is poorly understood but they are likely to be involved in the activation of the embryonic genome (Latham and Solter, 1991).

The embryonic genome is not transcriptionally active at the zygote stage but becomes activated at a later stage that varies greatly between species. A major burst of embryonic genome activation occurs in the mouse at the late two-cell stage whereas in sheep it occurs at the 8-16 cell stage. The timing of genomic activation is not completely understood, although a biological event involving the depletion or accumulation of transcription activators or repressers is likely (Wassarman *et al.,* 1981). Initial transcription involves the synthesis of 70kD proteins known as 'heat shock proteins', which protect genome activity against non-lethal cellular stresses. Once the genome has been activated, the transcriptional activity of the embryo increases quickly producing proteins that are important in compaction and blastocoele formation. Also, there are a number of growth factor genes transcribed early in the embryo implying that they play an important role in early embryogenesis (Cross, 2001). For example, transforming growth factor beta (TGFβ) which is known to be involved in the control of cellular proliferation and migration, and could therefore play an important role in the allocation of cells to the trophectoderm and inner cell mass in the blastocyst (Marquant *et al.,* 1989; Larson *et al.,* 1990).

2.1.8 Differential expression of paternal and maternal genomes

Both paternal and maternal genomes are required for normal embryo development. However some genes are expressed differentially depending on whether the alleles are inherited from the mother or the father. This phenomenon is known as imprinting and is a consequence of the way in which the DNA is modified in the germ-line (Stewart, 1993). One recognised determinant of imprinting is the degree of DNA methylation. Methylation in general is recognised as influencing gene expression so that hypo-methylation results in upregulated and hyper-methylation in downregulated expression of an allele (Solter, 1988). One example of an imprinted gene is that of

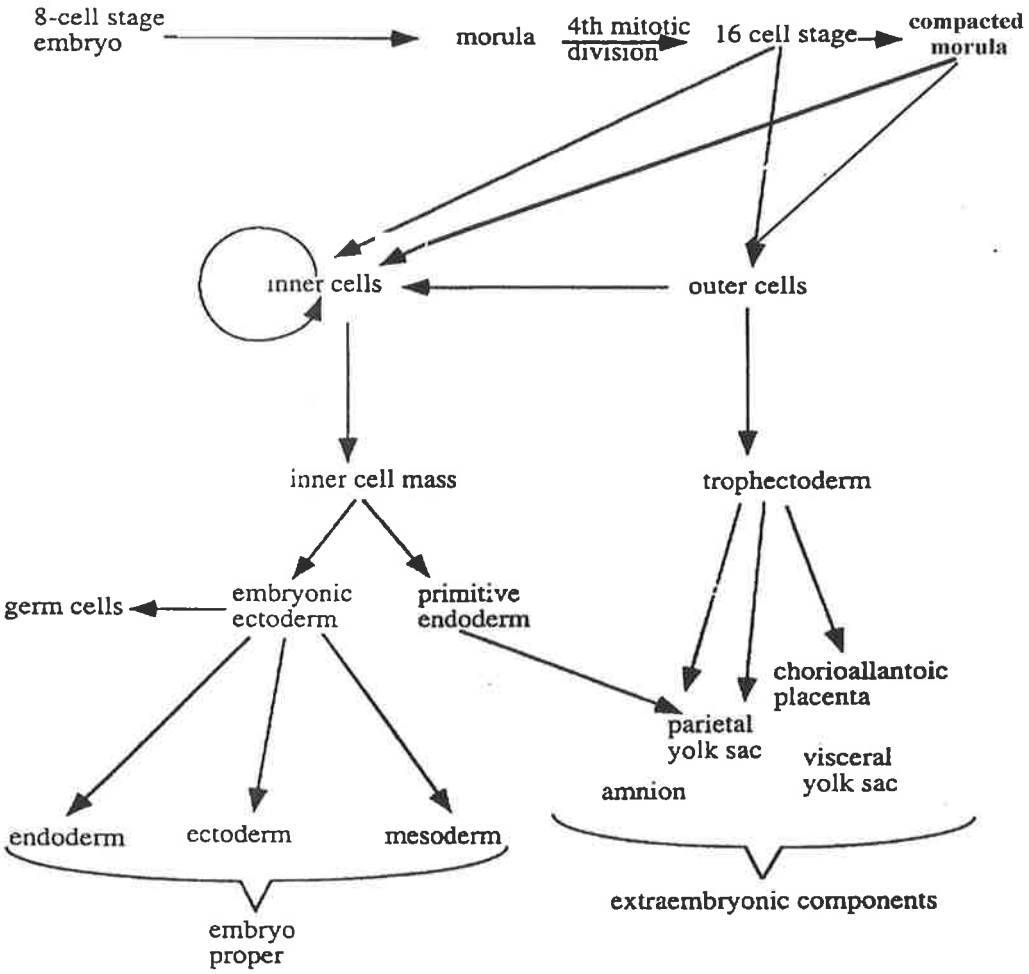


Fig. 2.6 Diagram showing the fate of embryonic cells in mouse. Adapted for sheep: the cell linages are the same (Moore and Kemler, 1997).

insulin-like growth factor 2 (IGF2). The IGF2 allele from the mother is hyper-methylated and therefore minimally transcribed while the opposite is the case for the paternal IGF2 allele (Heyner *et al.,* 1989). Imprinting contributes to epigenetic control of gene expression and can therefore influence phenotype. For example, the effect of in vitro culture environment on the embryo might contribute to the occurrence of large offspring through changes in the imprinting process (Walker *et al.,* 1998).

2.1.9 Embryonic cell lineage differentiation

The first contact between the embryo and the endometrium of the uterus is established by the trophectoderm. The polar trophectoderm lies above the embryonic disc and, depending on the species, it either disappears (ruminants, pigs and horses) allowing the embryonic disc to evert or continues to grow (mice and human) to play an important role in subsequent tissue formation (Handyside and Hunter, 1984). The parietal trophectoderm surrounds the blastocoel and in ruminants undergoes rapid elongation prior to implantation to form the elongated trophectoderm. In mice and humans it stops dividing after hatching but plays an important role in implantation (Fleming, 1987).

Soon after blastocyst hatching, the ICM develops into the endoderm, mesoderm, and ectoderm layers. The primitive endoderm originates from the blastocoele side of the ICM and differentiates further into the visceral endoderm and parietal endoderm, which migrate under the trophectoderm. The visceral endoderm develops into the viscera, which is responsible for the formation of organs such as the intestines, lungs and liver. The primitive ectoderm develops from the outer layer of the ICM and subsequently differentiates into the ectoderm and the mesoderm. The ectoderm develops into the epidermis and the nervous system while the mesoderm develops into nonvisceral organs and muscle tissue. The development of the mesoderm indicates the end of histiotrophic support and the commencement of haemotrophic support of the fetus during pregnancy (Van Soom *et al.,* 1997).

2.1.10 Metabolism of the early embryo

There is a considerable amount of inter- and intra-species variation in the metabolic requirements of the early embryo, which can be partially explained by the variation in

the activity of metabolic enzymes between species. Glucose is a key metabolite but is not required during the first two divisions (Takahashi and First, 1992; Robl *et al.*, 1991). Lipids are required for the synthesis of new membranes during cell multiplication. Some lipids can be obtained by *de novo* synthesis; however, embryos are generally capable of also incorporating saturated and unsaturated long chain fatty acids taken up from secretions from the female reproductive tract. Amino acids play a pivotal role in energy metabolism, particularly through the Krebs cycle, and are essential at the blastocyst and hatching blastocyst stages (Zhang and Armstrong, 1990).

2.1.11 Elongation of the embryo

The blastocyst undergoes expansion by extensive proliferation of the trophoblast shortly after hatching. The spherical fluid-filled sheep blastocyst proliferates to a much-extended conceptus (Rowson and Moor, 1966). During this phase the trophoblastic surface is wrinkled due to the rapid growth and relative lack of secretion into the trophoblastic cavity.

Sheep blastocysts formed on the fifth day after ovulation grow slowly to the seventh day and at this stage contain about 300 cells. By the ninth day however the blastocyst has about 3000 cells and two days later has achieved a length of 10-22 mm (Wintenberger-Torres and Flechon, 1974). Length of the tubular blastocyst on Day 13 and 15 measures about 10 and 15-19 cm respectively (Chang and Rowson, 1965). The embryos, therefore, undergo an extended free-living period in the uterus before the initiation of attachment.

2.1.12 Embryo migration

Intrauterine migration of the embryo is a common phenomenon in the pig (Dziuk *et al.*, 1964) but has a low incidence in sheep with a single ovulation. In ewes with two ovulations on the same ovary, one embryo was observed to migrate to the contralateral horn (Reimers *et al.*, 1973). In polyovular species such as the pig redistribution of embryos between the two horns occurs mainly during the ninth to eleventh days (Polge and Dziuk, 1970) and embryos are approximately evenly spaced by the time attachment is initiated on Day 13. Migration to the contralateral horn in sheep occurs mainly on Day 14 and is near completion by Day 15 (Cummins, 1979;

Nephew *et al.*, 1989a) prior to the beginning of attachment on Day 16 (Chang and Rowson, 1965; Short, 1969). Intrauterine migration in sheep was associated with an increase in oestradiol synthesis by the embryos, an event that also occurs in other species (Zavy *et al.*, 1979; Pope *et al.*, 1982). In pigs, peristaltic contractions of the myometrium (Cross and Ginther, 1988), which may be stimulated locally by the embryo (Pope *et al.*, 1982), were associated with migration. Synthesis of oestradiol by pig embryos appeared to stimulate contractions of uterine smooth muscle and intrauterine migration (Pope *et al.*, 1982). Moor and Rowson (1966) demonstrated that the signal to extend the life of the corpus luteum (CL) was given by the blastocyst by Day 12, indicating that maternal recognition of pregnancy precedes intrauterine migration in sheep.

2.1.13 Implantation

Implantation patterns vary greatly between species and can be described as either invasive or non-invasive. Invasive implantation occurs when the embryo has a free-living phase of one to two days (e.g. the human and mouse). In this case only a small number of trophectoderm cells make contact with the endometrium due to the small size of the embryo. The maternal response to the presence of a blastocyst occurs within hours and includes an increased vascular permeability in the stromal tissue underlying the endometrium that is in contact with the blastocyst (Roberts *et al.*, 1994). The stromal cells change in morphology and there is a multiplication and growth of capillaries. Collectively these changes are known as decidualisation, a process where the endometrial components of the placenta are prepared for implantation. The blastocyst signal that begins these changes is not known but it is thought that histamines and prostaglandins are implicated. Soon after decidualisation the surface of the epithelium associated with the conceptus becomes eroded, resulting in glandular and decidual tissue being destroyed to release large quantities of metabolic substrates, which serve to nourish the growing embryo (Bazer *et al.*, 1991).

Non-invasive implantation (eg. in sheep, cows, pigs and horses) is characterised by a much later attachment of the conceptus compared with invasive implantation (Figs 2.7; 2.8; 2.9). Another characteristic of the non-invasive implantation is the rapid elongation of the trophectoderm, resulting in a vast surface area for nutrient exchange

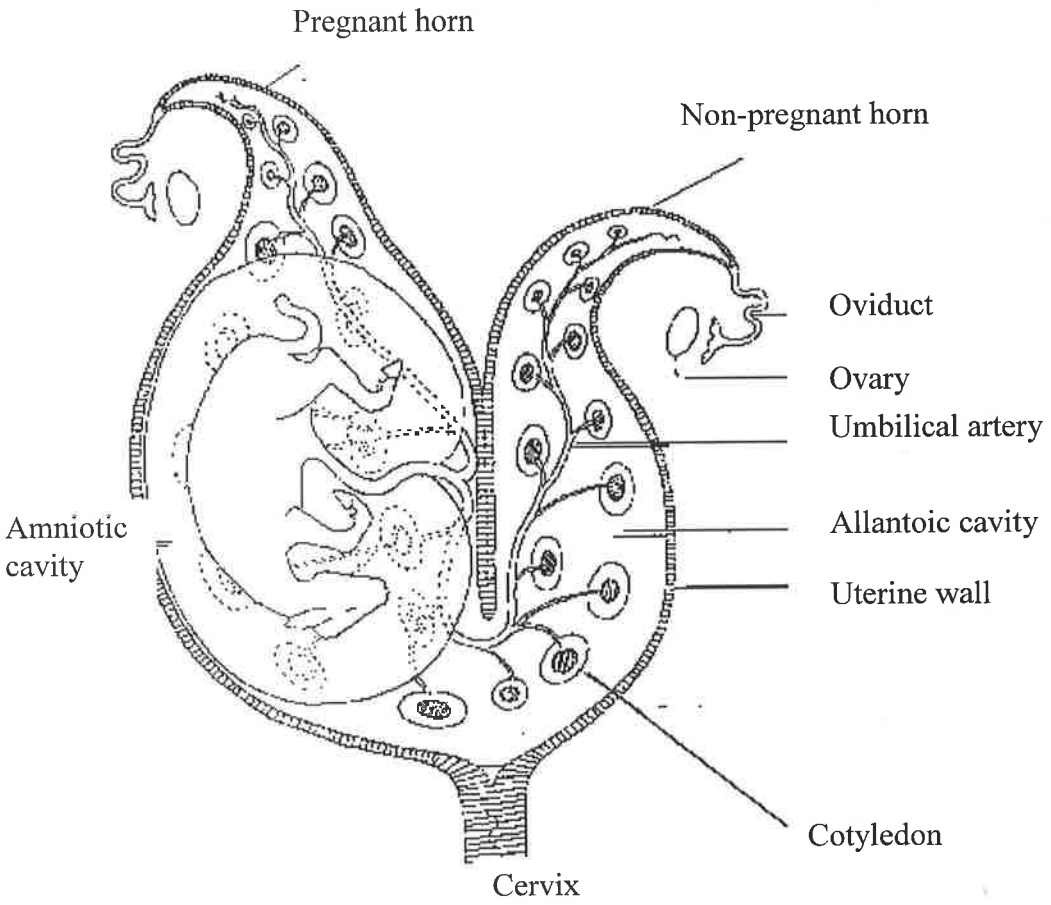


Fig. 2.7 Diagram of the cotyledonary placenta of sheep, illustrating the arrangement of placentomes and the fetus connected to the placental mass (Adapted from Steven, 1975).

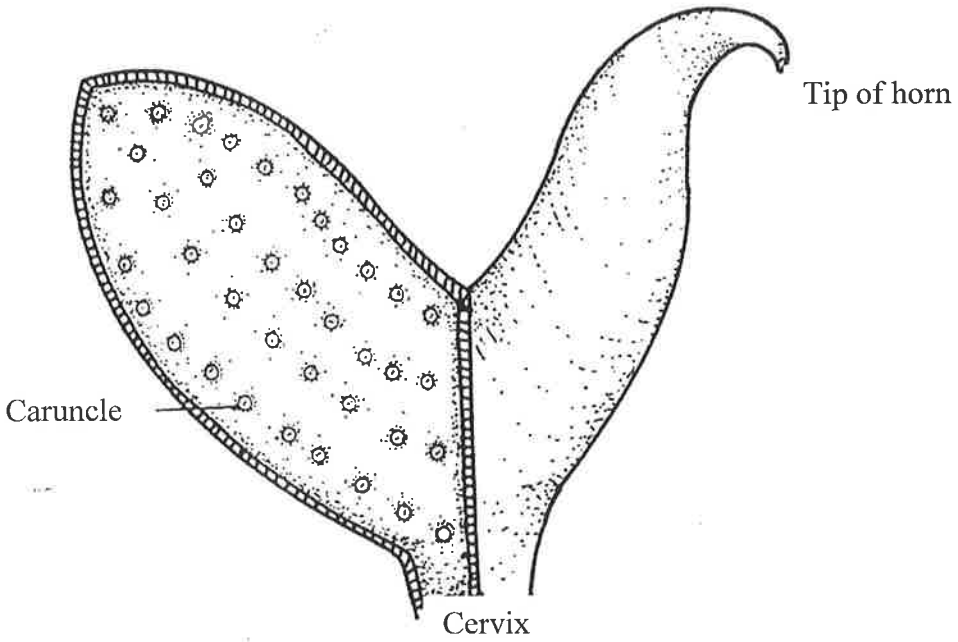


Fig. 2.8 Diagram of the non-gravid uterus of the sheep. One uterine horn is open to illustrate the arrangement of the implantation sites (endometrial caruncles) on the internal surface of the uterus (Adapted from Chidzanja, 1994).

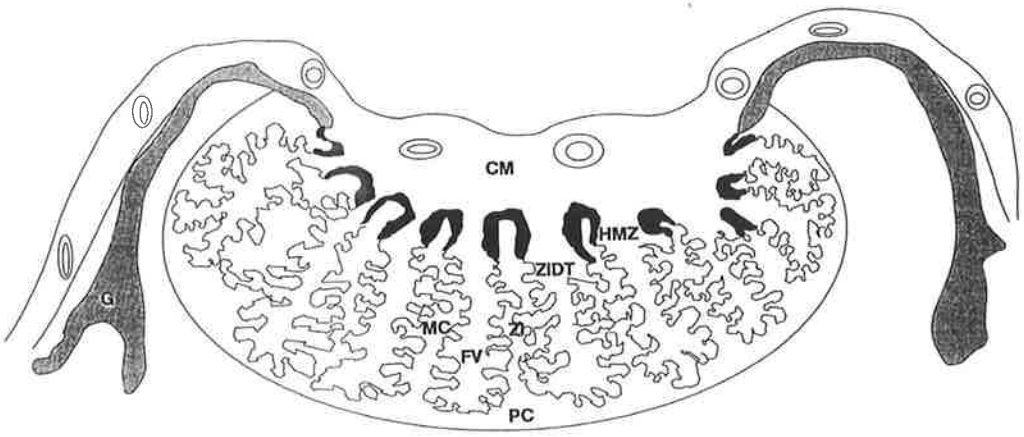


Fig. 2.9 Diagram of a vertical section through the centre of a placentome showing the chorionic membrane (CM) and large blood vessel within it, the haemophagous zone (HMZ); where the distances between fetal and maternal tissues are largest, then the zona intermedia (ZIDT) which merges with the zona intima (ZI); where there is intimate association between fetal villi (FV) and maternal crypts (MC). The MC stem from the placentome capsule (PC); the expanded caruncle. Extending from the placentome is uterine glandular tissue (G). (Adapted from Chidzanja, 1994).

(Sviatko *et al.,* 1993). In this case there is no decidual response but some stromal changes do occur indicating that the mother does recognise the presence of the embryo.

Following implantation, the scene is set for the fetus to proceed along its growth, differentiation and developmental trajectories at a species-specific pace until birth. Throughout, fetal development is characterised by differentiation of cells into complex, highly specialised tissues and organs in a carefully controlled spatial and temporal manner (Bazer and First, 1983). Control and regulation of these events is outside the scope of this literature review. However, at the completion of these differentiation processes, the somatic cells of an individual have reached their final differentiated status.

2.1.14 Early embryonic-maternal interactions

A few hours after fertilisation, the mouse zygote releases a factor that causes a transient change in maternal platelet numbers (O'Neil, 1985). The factor, platelet activating factor (PAF), is a chemical mediator produced in a number of tissues as well as being involved as an intercellular messenger and is a potential metabolic activator (Hanahan, 1986). It enhances embryonic implantation and viability (Ryan *et al.,* 1990). PAF represents the 'ovum factor' needed to induce synthesis of early pregnancy factor (EPF). PAF may act as an endogenous pulse generator for sheep of luteolytic $PGF_{2\alpha}$ release (Chami *et al.,* 1999). This PAF factor appears as a very early maternal response to pregnancy in all mammalian species including sheep (Clarke *et al.,* 1980) and although its function in regulating the maternal immune system is unclear (Whyte and Heap, 1983) it represents the earliest known embryo-maternal interaction, starting before embryonic transcription.

Embryos of several species including sheep and horses, synthesise prostaglandins of the E and F series. A clear functional role for prostaglandin E_2 (PGE_2) has been shown in the horse where oviductal transport to the uterus is signalled by high levels of PGE_2 secreted by 5 and 6-day old embryos (Weber *et al.,* 1991).

Very early mouse embryos (Werb, 1990) and sheep embryos (Watson *et al.,* 1994) produce mRNA transcripts for transforming growth factor (TGF) α, TGF β, insulin-like growth factor II (IGF-II) and platelet-derived growth factor A. A cooperative interaction among cultured mouse embryos increases their development (Paria and Dey, 1990) and could be associated with growth factor molecules. In fact, TGF β and epidermal growth factor (EGF) improve embryonic development in culture in mice (Paria and Dey, 1990) and cattle (Yang *et al.,* 1993). IGF-I and insulin increase cleavage, promote compaction and blastocyst formation and enhance protein synthesis in trophectoderm and inner cell mass cells in the mouse (Gardner and Kay, 1991; Harvey and Kaye, 1991).

Gandolfi and Moor, (1987) demonstrated that oviduct epithelial cells improved survival of embryos grown *in vitro* indicating a potential role for the oviduct in early embryonic development. A subsequent study indicated that products of oviduct epithelial cells might facilitate the initiation of embryonic transcription (Crosby *et al.,* 1988). Specific proteins from the oviductal epithelium become associated with the embryo. A glycoprotein (GP215) is selectively located in the perivitelline space of the mouse embryo and remains there until the zona pellucida is shed (Kapur and Johnson, 1988). In sheep, the oviduct-embryo interaction is even more pronounced since oviductal antigens are found in the perivitelline space, vitelline membrane and the embryonic cytoplasm (Gandolfi *et al.,* 1992). Oviductal proteins in intimate contact with the embryo have strong mitogenic activity in sheep (Gandolfi *et al.,* 1992; Hill, *et al.,* 1993) and these mitogens strongly synergise with insulin (Gandolfi *et al.,* 1992). As noted previously, this protein and the IGF family have an important role in early embryonic development; oviductal mitogens may also be involved in promoting insulin's action.

2.1.15 Maternal recognition of pregnancy

In order to establish pregnancy the conceptus needs to secrete products, such as steroids, proteins or prostaglandins that the affect maintenance of the CL. This can be done directly as a luteotrophic signal (e.g. chorionic gonadotrophin as in primates), as a luteal protective signal (e.g. PGE_2), or indirectly through antiluteolytic signals (e.g. trophoblast interferons or oestrogens, which inhibit uterine synthesis of luteolytic

quantities of $PGF_2\alpha$ (Bazer *et al.,* 1992)). Luteolytic pulses of $PGF_2\alpha$ occur in sheep after endometrial progesterone receptors decrease (Meyer *et al.,* 1988).

If embryonic signals act directly on the CL to stimulate progesterone production then increases in progesterone concentration should be noted during early pregnancy. Although Godkin *et al.* (1978) and Hansel *et al.* (1989) provide some evidence for a embryonic signal, Wiltbank *et al.* (1992a) found that serum progesterone concentrations were not different in pregnant and non-pregnant ewes between Days 1-15 indicating that no luteal stimulatory effect of the embryo was detectable in their study. However, there is evidence for a luteal protective effect caused by the developing embryo (Nancarrow *et al.,* 1982; Silvia and Niswender, 1984). The CL of early pregnancy is resistant to the effects of exogenous $PGF_2\alpha$, and the greater the number of embryos the greater the luteal resistance. The mechanism causing luteal protection is not clear; it might be related to a reduction in CL $PGF_2\alpha$ receptors (Wiepz *et al.,* 1991) or the responsiveness of small and large luteal cell progesterone production to $PGF_2\alpha$ (Wiltbank *et al.,* 1992b). Wiltbank *et al.* (1992a) suggested unknown substances secreted by the developing embryo act on the CL to cause luteal protection. These substances, other than ovine trophoblast protein-1 (oTP)-1 are known primarily to be responsible for reducing uterine $PGF_2\alpha$ secretion and inhibiting luteolysis.

2.1.16 Endocrine events during early pregnancy

Establishment of pregnancy in domestic species involves complex interactions between the developing embryo and embryonic membranes and the maternal environment. The amount and timing of progesterone release from the CL regulates uterine secretions essential for initiating and mediating changes in conceptus growth during early pregnancy. Endometrial secretion of growth factors, immunosuppressive agents, nutrients, enzymes, ions and steroids required for conceptus growth are mainly regulated by progesterone secreted by the CL. Regulation of the synthesis and secretion of prostaglandin $F_2\alpha$ ($PGF_2\alpha$) from the endometrium is also achieved through the release of progesterone from the CL and is necessary for luteolysis to occur in the absence of a conceptus.

2.1.17 The role of progesterone in early embryo survival

It is well established that appropriate changes in progesterone concentrations are essential for the maintenance of pregnancy in sheep (Wilmut *et al.,* 1985). Embryos appear particularly sensitive to changes in progesterone concentrations at critical stages of early pregnancy. In an experiment in which Epostane, an inhibitor of the enzyme 3 β-hydroxysteroid dehydrogenase, was administered to cause 48 h reductions in progesterone levels between Days 9 and 13 of pregnancy, embryo survival was significantly reduced only when progesterone levels were depressed on Day 11 and 12 (Parr, 1992). Changes in the timing and magnitude of peripheral progesterone concentrations affect the uterine environment, which in turn, affects embryo viability. Progesterone is believed to have a major role in controlling maternal secretion of nutrients, growth factors and enzymes required for successful embryo development. Indeed, the content of total protein and of several key metabolic enzymes in ovine uterine flushing follow the growth and regression of the corpus luteum (Ashworth *et al.,* 1989b). Furthermore, long-term ovariectomised ewes given a sequence of steroid implants designed to mimic changes in endogenous hormones during early pregnancy showed that several specific uterine fluid proteins were stimulated by progesterone (Ashworth, 1985). Subsequently, in vitro culture of endometrial tissue collected on Day 10 from intact pregnant ewes revealed that daily injections of 50 mg progesterone for the preceding six days had increased the secretion of 10 of the 30 endometrial proteins studied (Ashworth and Bazer, 1989a). Although the specific functions of these progesterone-induced proteins are not known at present, two appear to correspond to the retinol-binding proteins identified in the ovine endometrium (Dore *et al.,* 1992; Harney *et al.,* 1993).

2.1.18 Regulation of luteolysis by progesterone

Plasma progesterone concentrations are normally below 1 ng per ml during the first four days of the ovine oestrous cycle (Wiltbank *et al.,* 1992a) but thereafter the endometrium is under the influence of progesterone stimulation. Experiments have suggested that a 10-day exposure to progesterone is necessary for the initiation of luteolysis in the ewe (McCracken *et al.,* 1984; Vallet *et al.,* 1990) and that the length of timing of progesterone stimulation is possibly mediated through diminished

inhibition of endometrial oestrogen and oxytocin receptors (Cherny *et al.,* 1991; Lau *et al.,* 1993).

2.1.19 Regulation of uterine secretion and conceptus growth by progesterone

Ovarian steroids, particularly progesterone, have an important role in mediating changes in the uterus necessary for conceptus development and placental attachment (Bindon, 1971b; Roberts and Bazer, 1988; Ashworth and Bazer, 1989). Progesterone supplementation during the first few days following ovulation alters the nature of the uterine environment and has been associated with enhanced embryo development (Garrett *et al.,* 1988) and increased fetal weight in sheep by Day 74 of gestation (Kleemann *et al.,* 1994). Garrett *et al.* (1988) indicated that early administration of progesterone on Days 1-4 after oestrus increased bovine conceptus growth and bovine trophoblast protein-1 (bTP-1) at Day 14. This was associated with changed synthesis of polypeptides between Days 5 and 14. In sheep, conceptus growth to Day 13 was positively associated with progesterone concentration in plasma during CL formation (Day 2-6) and with oTP-1 production (Nephew *et al.,* 1989b) but was unrelated to progesterone concentrations during luteal phase (Sviatko *et al.,* 1993).

Endometrial factors involved with progesterone-mediated conceptus growth are not clearly defined. IGF-I and -II are present in the endometrium during the oestrous cycle but the time of rapid conceptus development is not associated with changes in these factors. However, IGF binding protein-2 (IGFBP-2) does appear to be regulated by progesterone between Days 15-18 in the cow (Geisert *et al.,* 1991). Granulocyte-macrophage colony stimulating factor (GM-CSF) may increase development of the murine trophoblast as early as the blastocyst stage, however synthesis of GM-CSF appears to be independent of progesterone concentration (Robertson and Seamark, 1992).

2.2 THE OVIDUCT

Both the physical and chemical environments provided by the oviduct are required to facilitate normal embryonic development (Buhi, 2002). There is a significant amount of literature describing the morphology and chemical environment of the oviduct, the relevant parts of which will be discussed in this section.

2.2.1 Oviduct morphology

The oviducts are a pair of convoluted tubes, which regulate the movement of ova from each ovary to the respective horn of the uterus and the movement of spermatozoa to the site of the fertilisation (Leese, 1983). In each case, movement is a consequence of combined ciliary and muscular action. The oviduct consists of three sections; the infundibulum, the ampulla and the isthmus (Fig. 2.10) each of which react specifically to hormonal changes of the oestrous cycle and to the passing ovum. The infundibulum is adjacent to the ovary and is extended to form a funnel-like structure. Ovulated ova are transported from the ovarian surface to the infundibulum by fringe-like fimbria and contractions of the oviductal musculature. These ova are subsequently transported through the mucosal folds of the ampulla to the site of fertilization at the ampullary-isthmic junction. Contractile activity of the oviduct, coordinated by the mesotubarium and mesosalpinx also aids the movement of spermatozoa through the uterotubal junction and isthmic folds to the site of fertilisation. Following fertilisation the cleaved embryos are transported to the uterus.

Extensive folding of the mucosa occurs along the length of the oviduct. For most of its length the oviduct has two muscle layers, an external longitudinal and an internal circular layer with the proportions of muscle and mucosa varying along the length of the oviduct. The best characterized species with respect to mucosal folding are the cow and the rabbit (El-Banna and Hafez, 1970; Leese, 1983). In both species there is a gradual decline in the proportion of mucosa, the degree of mucosal folding, the surface area of the lumen and an increase in the proportion of muscle, from the infundibulum to the uterotubal junction (Fig. 2.10 and 2.11).

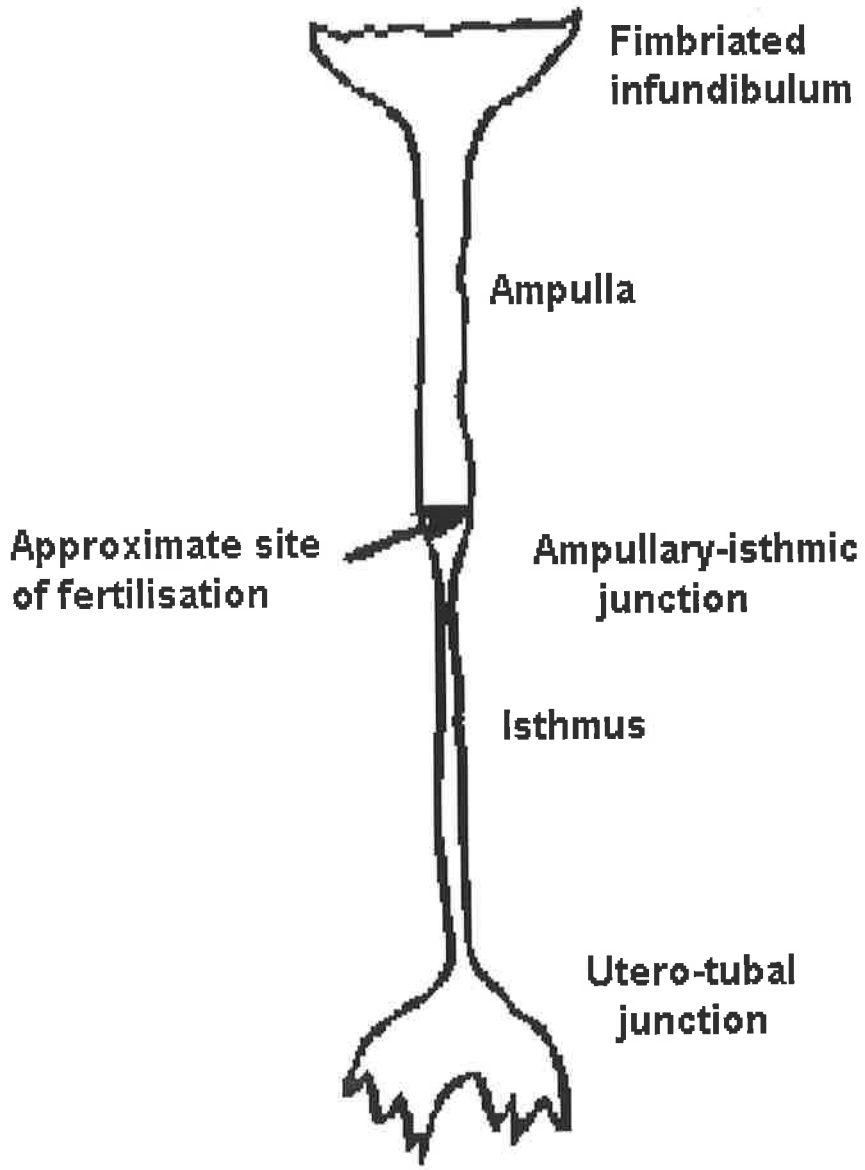


Fig. 2.10 A schematic representation of the oviduct, to indicate the infundibulum, ampulla and isthmus and the relative positions of the ampullary-isthmic and utero-tubal junctions. The embryo moves along the tract from the infundibulum to the uterus by ciliary and muscular movement. Fertilisation generally occurs at the ampullary-isthmic junction (Hill, 1994).

2.2.2 Cellular components

The oviduct epithelium is columnar and consists predominantly of ciliated cells and secretory cells. The proportion of these cell types in the three regions of the oviduct differs between species and in the some species varies during the oestrous cycle. In women, dense bunches of ciliated cells are present in the fimbria but are less numerous and more individual in the ampulla. In the isthmus, secretory cells become prominent and active with ciliated cells only being present on the upper surface of the folds (Ludwig and Metzger, 1976).

In addition to secretory and ciliated epithelial cells, a small population of cells associated with the basement membrane has been reported and given a variety of names including 'basal' cells (Carmichael and Jefferson, 1939, Rasweiler, 1972), 'indifferent' cells (Pauerstein and Woodruff, 1966), 'reserve' cells (Laguens *et al.,* 1967, Howard and Erikson, 1951) and 'clear' cells (Davies and Kusama, 1962-63). These cells have been described as small and oval or round with hyperchromatic nuclei, prominent nucleoli and scanty, poorly stained cytoplasm, and their resemblance to lymphocytes has been noted in women, cows, sows (Neller, 1965), rabbits (Odor, 1974), primates (Odor, 1982) and sheep (Hollis *et al.,* 1984). These basal cells appear to differ between species. In sheep (Hollis *et al.,* 1984) and primates (Odor, 1982) the presence of phagocytic bodies and lysosomes has been reported in these cells, suggesting a phagocytic role. Basal cells of the bat oviduct (Rasweiler, 1972) contain large granules typical of mast cells, however, this observation has not been reported in any other species examined. Further, basal cells in bovine oviducts have been reported to migrate through the epithelium into the lumen (DuBois *et al.,* 1980).

2.2.3 Secretory cells

These cells are characterised by the presence of secretory granules, numerous ribosomes, extensive endoplasmic reticulum, well developed supranuclear Golgi zones and a microvillous luminal surface. Nuclei are generally located in the mid to basal areas but, under progesterone domination, have been reported to be apically located in sheep (Hadek, 1955; Abdalla, 1968; Willemse, 1975a; Nayak, *et al.,* 1976) and cattle (McDaniel *et al.,* 1968; Nayak and Ellington, 1977). However, Hollis *et al.* (1984) also reported apically located nuclei at Day-1 of the follicular phase in sheep.

This was unusual as there have been several demonstrations of the rapid response of nuclei to oestradiol (Willemse, 1975a; Nayak *et al.,* 1976). It is possible that these samples were collected just prior to complete progesterone withdrawal. The same study demonstrated a greater density of secretory granules in the ampullary secretory cells than in isthmic cells at all stages of the oestrous cycle. Although apical protrusions of isthmic cells were evident prior to oestrus, no evidence of secretion was found (Hollis *et al.,* 1984).

The formation of secretory granules in these cells occurs in five stages (Hollis *et al.,* 1984). The secretory granules are closely associated with the Golgi membranes during Days 1-3 of the oestrous cycle and increase in size and electron density during stages 1-3. At stage 4, the granules become lamellated prior to the fifth stage when they disorganise and their contents are released into the oviduct lumen by exocytosis. These observations are similar to those of Willemse and Van Vorstenbosch (1975); Nayak *et al.* (1976) and Russe and Leibach (1979) in sheep; Nayak and Ellington (1977) in cattle; Odor *et al.* (1983) in monkeys. Reports concerning the ultrastructure of ovine ampullary secretory granules do not clearly define the precise mechanism by which the contents are secreted. Evidence for exocytotic (Brower and Anderson, 1969; Russe and Liebach, 1979; Odor *et al.,* 1983; Murray, 1992), apocrine (Nayak, 1977; Odor *et al.,* 1983; Brenner *et al.,* 1983) and holocrine (Clyman, 1966) secretion has been reported in various species.

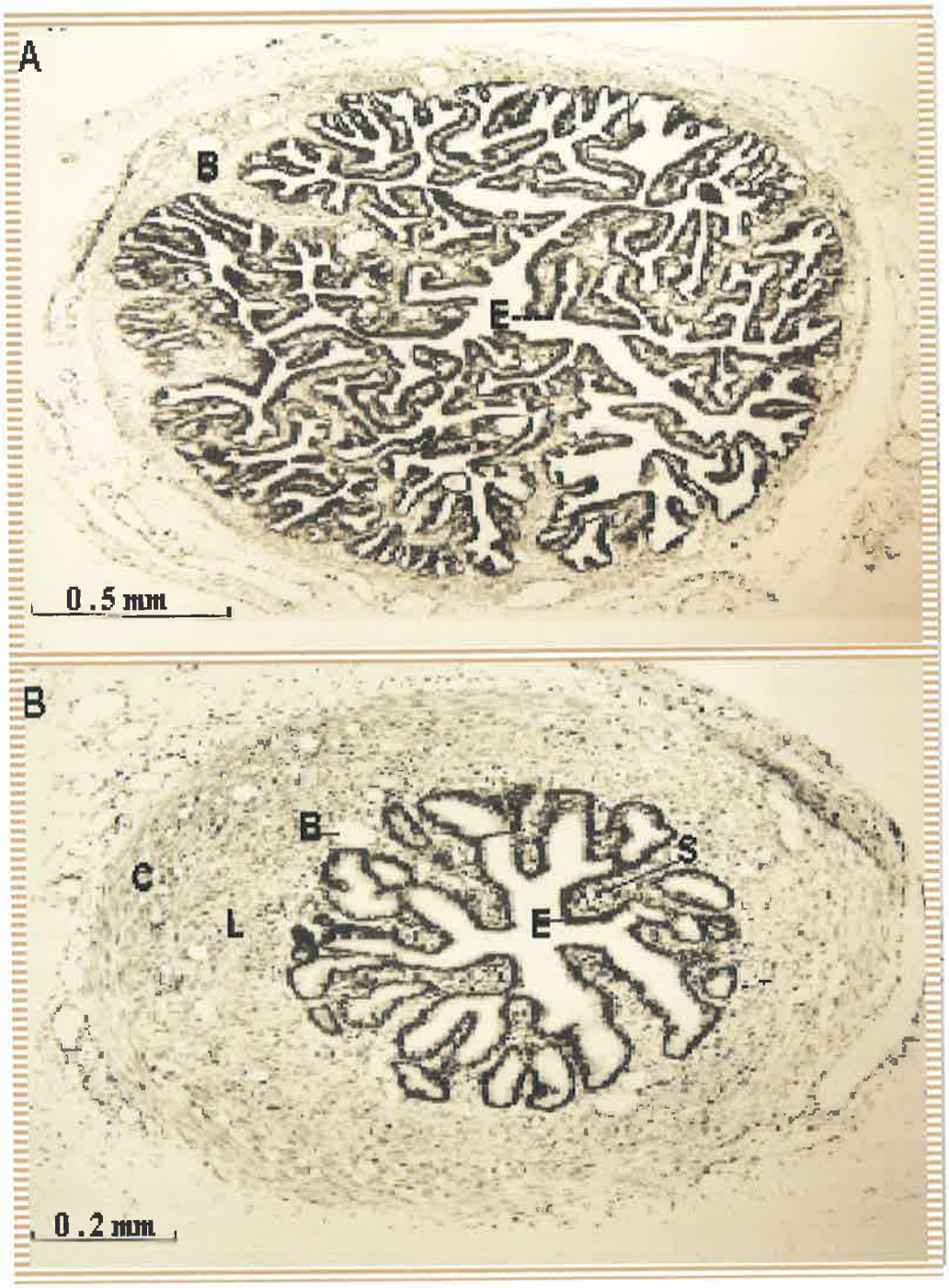


Fig. 2.11 Histology of the oviduct: Transverse sections of the ampulla (A) and isthmus (B) of the ovine oviduct showing the extensive musculature of the isthmus in relation to the ampulla, which has a greater degree of mucosal folding. E, epithelium; S, stroma; B, blood vessel; L, longitudinal smooth muscle; C, circular muscle (Hill, 1994).

2.2.4 Ciliated cells

The ciliated cells are columnar, have microvillae present on their luminal surface and are attached to the basal lamina (Fig. 2.12). The nuclei of these cells are elongated and medially or basally located. In primates the degree of ciliation changes during the oestrous cycle (Brenner, 1969; Verhage *et al.,* 1979; Odor *et al.,* 1980) with differentiation and ciliation being induced by oestradiol. This effect is attenuated or blocked by progesterone in human (Verhage *et al.,* 1979; Jansen, 1984), baboon (Verhage *et al.,* 1990) and cynomolgus macaques (Brenner *et al.,* 1983). However, in sheep, there is little or no change in the degree of ciliation during the cycle (Willemse, 1975b; Nayak, 1977; Hollis *et al.,* 1984). Mitochondria and Golgi are numerous in ciliated cells of the sheep oviduct, while ribosomes, rough endoplasmic reticulum, cytoplasmic filaments and microtubules are sparse (Hollis *et al.,* 1984). These features do not differ markedly during the oestrous cycle and are similar in both ampullary and isthmic ciliated cells. However, in isthmic ciliated cells, smooth endoplasmic reticulum is more extensive and apical vesicles containing filamentous material are more common than in cells of the ampulla. These apical vesicles coalesce and disperse part of their content into the cytoplasm during the cycle. At Day-1, the vesicles and dispersed material form prominent microvilli-free apical protrusions. The contents of these protrusions are released into the oviductal lumen in an apocrine fashion (Hollis *et al.,* 1984).

2.2.5 Steroid hormones and receptors

The physiology of the oviduct is responsive to hormones. The responsive tissues include the muscle layer (or myosalpinx), the vasculature and the epithelium (or endosalpinx) as well as the resultant fluids of the lumen. The most dominant humoral stimulation of the oviduct is provided by the fat-soluble hormones, oestradiol and progesterone. The ovarian steroids have a large impact on all segments of the oviduct and affect every aspect of growth, differentiation and function, including quantitative and qualitative responses to other hormones. Response to steroids is much slower (usually hours or days) than response to the water-soluble hormones.

The oviduct is able to concentrate oestradiol and progesterone to many times circulating plasma levels thereby indicating the presence of high affinity tissue

binding proteins or receptors (Batra *et al.,* 1980; Devoto *et al.,* 1980). Studies in monkeys have demonstrated that the concentrations of ovarian steroid hormones in tubal fluid before ovulation are approximately the same as those present in plasma, but after ovulation they are present in greater concentrations in tubal fluid. However, the concentration of steroid binding proteins is lower in tubal fluid than in plasma at all stages of the cycle. Therefore, free or diffusible steroids, including androstenedione, testosterone, oestrone, oestradiol and progesterone are all present at greater concentrations in tubal fluid than plasma both before and after ovulation (Wu *et al.,* 1977).

Cytosol oestrogen receptor and progesterone receptor concentrations are greater in the ampulla than in the isthmus (Pollow *et al.,* 1981; Robertson and Landgren, 1975; Flickinger *et al.,* 1977). Tubal steroid receptors are predominantly located in the epithelium (Jansen, 1984) and although cytosol E_2R and PR are greater in the ampulla than in the isthmus, the concentration of receptors in the epithelium becomes greater with increasing distance from the ovary. The fimbrial end of the oviduct has the highest epithelium:smooth muscle ratio of the entire tract but it has the lowest concentration of steroid receptors throughout the cycle (Pollow *et al.,* 1981; Flickinger *et al.,* 1977).

Pollow *et al.* (1982) have reported an asymmetry in oestrogen receptor concentration between the fimbrial ends of each oviduct after ovulation. These concentrations have been observed to be much lower on the side of the corpus luteum thereby indicating that ovarian steroids may have routes of access to the oviduct that are more direct than the peripheral circulation.

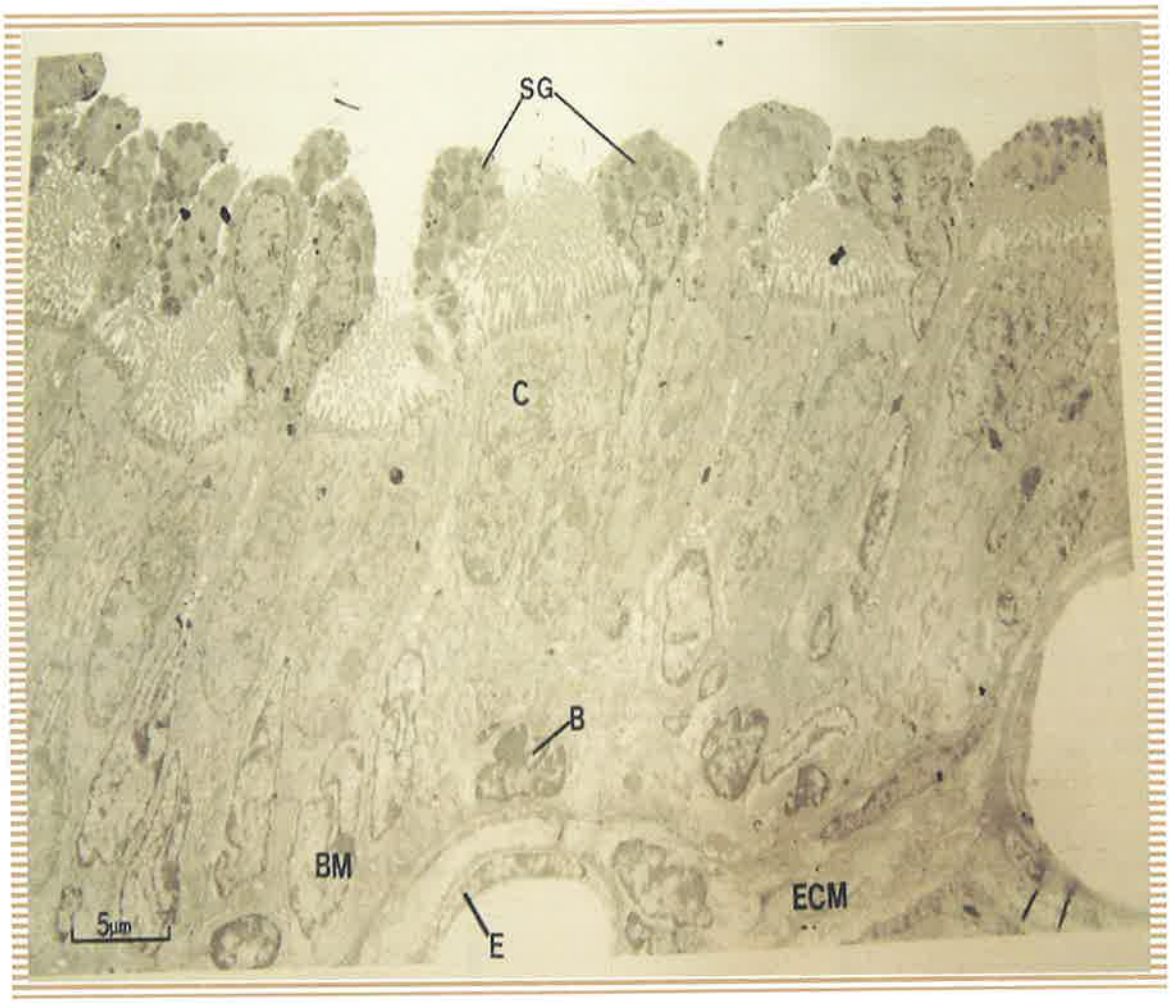


Fig. 2.12 Transmission electron micrograph of the ampulla: Transverse section through the ampullary epithelium of a sheep oviduct fixed by *in situ* perfusion via the tubal branch of the ovarian artery on Day 10 of the oestrous cycle. C, ciliated cells; SG, secretory granules in secretory cells; E, endothelial cell; B, basal cell; BM, basement membrane; ECM, extracellular matrix (Hollis *et al.,* 1984).

2.2.6 Tubal secretions

2.2.6.1 General

The bulk of oviduct secretions appear to be mucosal transudate with some oviduct-specific proteins being produced by the secretory cells. If the volume of fluid produced is proportional to the epithelial area (Figs. 2.11; 2.12) available for plasma filtration, then most tubal fluid comes from the ampulla. The volume of fluid produced is oestrogen dependent (Hammer and Larsson-Cohn, 1978) and is maximal around the time of ovulation (Mastroianni *et al.,* 1961; Lippes *et al.,* 1972; Sutton *et al.,* 1984a; 1986). Most serum components are identifiable in tubal fluid at considerably reduced concentrations, particularly the γ-globulins (Marcus, 1964). The chloride concentration decreases from the fimbria to the uterotubal junction whereas sodium, bicarbonate, inorganic phosphate, proteins and lactic acids concentrations increase to highest levels in the isthmus of the rabbit fallopian tube (David *et al.,* 1973). Oviduct epithelium actively transports the chloride ion into the lumen (Brunton and Brinster, 1971) resulting in a negative intraluminal potential. Studies in the rabbit have shown this to be considerably augmented 2-3 days after ovulation (Leese *et al.,* 1981). The bicarbonate ion, which is produced by epithelial carbonic anhydrase, is present at high concentrations at the time of oestrus and in monkeys this accompanies an increase in luminal pH from 7.1-7.3 in the follicular phase to 7.5-7.8 at the time of ovulation (Maas *et al.,* 1977). The high pH is maintained in the luteal phase. Bicarbonate is known to help disperse cumulus cells from around the ovulated oocyte and to stimulate sperm respiration (Hamner, 1973).

The primate oviduct is known to contain large quantities of glycogen at midcycle, which is presumably converted to glucose and pyruvate (Jansen, 1984). Glucose is a substrate for sperm metabolism and pyruvate is required for early embryonic development (Brackett, 1981).

The lymphatic system of the oviduct is extensive and greatly expands around oestrus (Anderson, 1927; Lombard *et al.,* 1950), associated with the increased volume of oviductal fluid. As the volume increases the osmolarity and dry content of the fluid increases.

The cyclicity of oviduct secretions has been demonstrated by numerous light and electron microscope observations and histological analyses. In most mammals the tissue of the follicular or early oestrous phase shows heightened synthetic apparatus-large, basal nuclei; extensive Golgi with dilated cisternae, well-developed endoplasmic reticulum and abundant secretory granules (Gupta *et al.,* 1970; Nayak and Ellington, 1977; Nilsson and Reinius, 1969) but is much reduced in the postovulatory or dioestrous animal.

2.2.6.2 Oviductal glycoproteins

The secretion of oviduct specific glycoproteins by epithelial cells around the time of oestrus has been reported in many species (Buhi, 2002). These glycoproteins are present when fertilisation and early embryo development occur in the oviducts. The nature of these polypeptides tends to differ between species.

The presence of an oviduct specific glycoprotein in ovine oviductal fluid was first described by Sutton *et al.* (1984a) and shown to be present for 3-4 days around the time of oestrus. The glycoprotein was reported to have a native molecular weight (Mr) of several million, a subunit of 70-90 kDa and an isoelectric point of 4.7. Lectin binding studies revealed the presence of galactose, N-acetylgalactosamine and fructose residues (Sutton *et al.,* 1985). This oestrus-associated glycoprotein is the major component of the sheep oviductal fluid void volume fraction on Sephacryl 300 columns (Mr> 1000 kDa) and appears as a broad band (Mr 70-90 kDa) on SDS gels (Sutton *et al.,* 1984a,b). Later studies of Gandolfi *et al.* (1989), which examined the secretory products of oviduct epithelial cells, demonstrated the presence of 2 proteins that also showed cyclic patterns of secretion. They had Mr of 92 and 46 kDa and were both detected during the first 4-5 days of the oestrous cycle. Immunocytochemical analysis has demonstrated that the 92 kDa glycoprotein is present only in the nonciliated secretory cells of the ampullary region of the ovine oviduct (Gandolfi *et al.,* 1991).

Buhi *et al.* (1991) have characterized *de novo* synthesis of ewe oviductal secretory proteins during the oestrous cycle to determine the synthesis of these proteins by the ampulla and isthmus under the influence of E_2 and P. Two major basic proteins (Mr 90-92 kDa) and 3 minor proteins were found to be present on Day one of the oestrous

cycle. These proteins were synthesized by the ampulla of oestrus and ovariectomized E_2-treated ewes. They were not detectable on days other than Day 1 or in untreated, ovariectomized and P treated animals. The results from this study differed from those of Sutton *et al.* (1984a) and Gandolfi *et al.* (1989) since the glycoproteins of Mr 90-92 kDa were found to be basic rather than acidic. Later studies of Murray (1992; 1993), similar to those of Buhi *et al.* (1991), have confirmed observations from all three groups of workers and have reported the pI of the 90-92 kDa glycoprotein to be acidic in agreement with Sutton *et al.* (1984a) and Gandolfi *et al.* (1989). Evidence from studies of all four groups of workers has shown that the oestrous-associated glycoprotein (EGP) is secreted by the ampullary region of the oviduct only and is induced by oestrogen alone. No role for progesterone in regulating the synthesis and secretion of this protein has been demonstrated.

Murray (1993) has also examined the secretion of this protein during early pregnancy. The glycoprotein was present in SDS gel and blots of oviductal flushings collected from animals on Days 4-6 of pregnancy but not in flushings from Day 16 pregnant animals or from ovariectomized untreated animals. Incorporation studies demonstrated that the intensity of ^{3}H-leu-labelled protein in ampullary tissue was reduced after Day 4 of pregnancy and ^{3}H-glcN was incorporated into the 90-92 kDa glycoprotein until Day three. Carbohydrate moieties are known to be important for glycoprotein secretion and biological function (Galway *et al.*, 1990; Catt and Dufau, 1991) and ovarian steroids have been shown to regulate the activity of enzymes involved in the attachment of O- and N-linked oligosaccharide chains to proteins in the rabbit endocervix (Chilton *et al.*, 1988). It is possible that pregnancy modifies the activity of enzymes involved in the glycosylation of oviductal steroid regulated secretory proteins thereby explaining why ^{3}H-glcN was not incorporated into protein after Day 3 of pregnancy. The studies of Murray (1993) also demonstrated that although the fimbria of the oviduct synthesized and released the same protein at oestrus as the ampulla and during the first few days of pregnancy, the temporal pattern differed from that in the oviduct. The glycoprotein incorporated ^{3}H-leu until Day 3-4 and ^{3}H-glcN until Day 1.5. The 90-92 kDa protein being secreted by the fimbria differed from the protein of the same molecular weight by its appearance on Western blots as two distinct bands rather than the diffuse bands observed for the ampullary protein. It is possible that this is the same protein with different patterns of

glycosylation. Alternatively, they may be two subtly different proteins with complementary roles.

2.3. NUTRITION AND REPRODUCTION

2.3.1 Introduction

There are a wide range of reproductive responses to nutrition, ranging from the time of puberty to the control of testicular growth and ovulation rate in adults (Lindsay *et al.*, 1993; Barker, 2000). Dietary nutrients promote the programming and expression of metabolic pathways that enable animals to achieve their genetic potential for reproduction. The effect of nutrition on reproductive performance has been the subject of many studies partly because nutrition is one of the major costs in the production and maintenance of livestock for sale. A better understanding of the mechanisms that control and modify ovulation rate in response to nutritional stimuli would be a powerful adjunct to management. Lindsay *et al.* (1993) speculated on some of the possible physiological mechanisms that might mediate the effects of nutrition on reproductive performance (Fig. 2.13) and most of these mechanisms involve the endocrine system.

In *Mechanism 1* (direct action on the gonads), nutrients are transported directly to the gonadal tissues where they supply substances for the cells (*Mechanism 1a*) and directly increase the production of ova or spermatozoa and completely circumvent the endocrine system. In *Mechanism 1b*, nutrients are transported directly to the gonadal tissues where they supply substances for the endocrine cells, suggesting that energy or protein affect the capacity of the endocrine cells to respond to the gonadotropins.

In *Mechanism 2* (hypothalamus and pituitary gland), nutrient supply affects the hypothalamus or the pituitary causing changes in the secretion of gonadotrophins. The effect could be exerted at the hypothalamus, where the neural mechanisms driving gonadotrophin secretion might be altered (2a) or on the pituitary gland to alter the response to the hypothalamic signal (2b). The relationship between the responses to nutrition and the patterns of secretion of LH and FSH are inconsistent.

In *Mechanism 3* (steroid inter-conversion), the activities of various enzymes controlling steroid metabolism in the liver are under the control of a pituitary 'feminising factor' (Denef, 1974), the secretion of which is controlled by regions of the hypothalamus adjacent to those controlling reproductive function (Gustafsson *et al.,* 1978). It has been shown that this factor is, in fact, growth hormone (Mode *et al.,* 1981; 1983).

In *Mechanism 4* (hormone clearance), dietary intake can alter the rate of blood flow, and the liver is a major site of metabolism of steroids. High dietary intake can reduce progesterone concentration probably due to an increase in the clearance rate from the liver (Parr *et al.*, 1978; 1993; McEvoy *et al.*, 1995).

In *Mechanism 5* (metabolic hormones), the nutritional status of an animal will reflect the patterns of secretion of the hormones that control metabolic systems. Information on the effects that these hormones have on reproductive tissue is relatively scarce at present but the field is developing rapidly. In the ewe, infusion of glucose to increase ovulation rate also increases the secretion of growth hormone (Downing *et al.,* 1989; 1995a; 1995b; 1995c), and growth hormone will increase steroid secretion when infused into the ovarian vein (Murray *et al.,* 1989). It is therefore possible that the effects of changes in nutrition on ovulation rate are mediated by growth hormone, independently of changes in the secretion of gonadotropins.

The results of many studies on the effect of nutrition on reproduction are not always repeatable depending on the source or combination of the diet and the duration or timing of feeding. This suggests that nutrition may be acting directly on the gonads, hypothalamus and pituitary gland, steroid interconversion, hormone clearance or metabolic hormone production individually or in combination (Barker, 2000). In this review, the effects of nutrition on each component of the reproductive process (progesterone and gonadotrophin secretion, ovulation, oocyte and embryo development) are considered separately. However, it should be remembered that these components are interdependent. For example, the effects of nutritional state on embryo survival or quality may depend on ovulation rate.

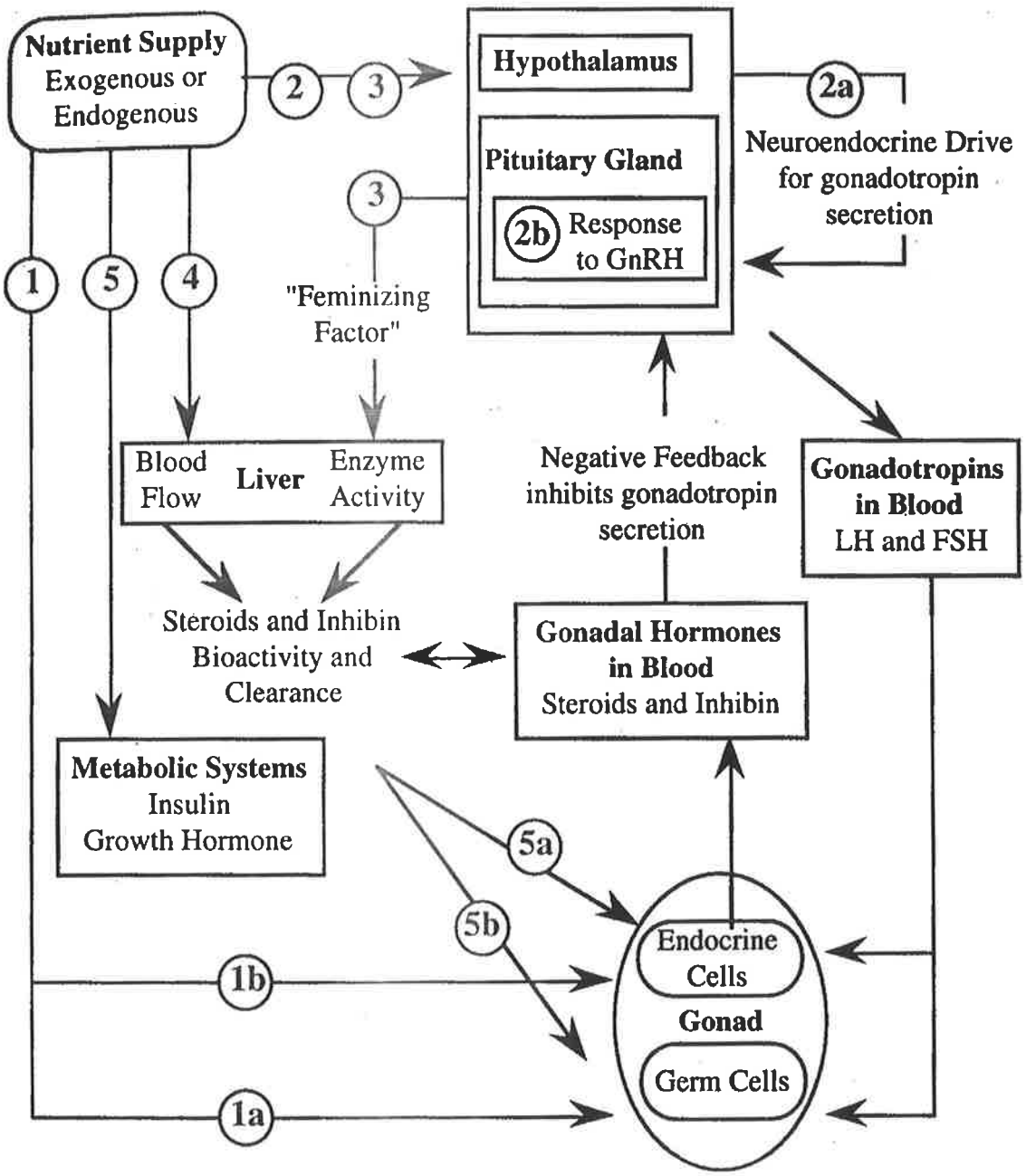


Fig. 2.13 A schematic representation of some of the possible physiological systems that might mediate the effects of nutrition on reproductive performance (Lindsay *et al.*, 1993).

2.3.2 The role of energy in reproductive function

Over the past 30 years considerable effort has been devoted to distinguishing between the relative importance of protein and of energy intake in stimulating the increases in ovulation rate arising from 'flushing'. In a study by Fletcher (1981), Merino ewes were fed diets containing different energy and protein levels. At the same low energy level, increasing the protein intake increased ovulation rate significantly. Conversely, at the same moderate protein level, increasing the energy intake increased the ovulation rate significantly. Smith (1985) also fed a range of diets varying in energy and protein levels for one oestrous cycle and observed an increase in ovulation rate as digestible energy intake increased and an independent stepwise increase in ovulation rate with the increased intake of crude protein. In a further study, ewes were fed high or low energy levels in diets containing low or high levels of protein and ovulation rate increases were obtained with the high energy level but the amount of protein had no effect (Memon *et al.*, 1969).

Rowe *et al.* (1983) found that the energy intake is more strongly related ($r^2 = 0.87$) to ovulation rate than is the amount of protein available in the small intestine ($r^2 = 0.40$). Studies of this type are confounded by the fact that ruminal degradation of dietary protein together with synthesis of microbial protein consequent to ruminal digestion of carbohydrate poses major problems in the interpretation of the effects of dietary protein and energy on reproductive function in ruminants.

If female ruminants are subjected to lengthy periods of food restriction, nutritional anoestrus will result (Wiltbank *et al.*, 1962; King and Williams, 1984; Imakawa *et al.*, 1986; Armstrong and Britt, 1987; Johnson *et al.*, 1987; Richards *et al.*, 1989). Feeding high energy or low energy levels to ewes from Day 10 of the oestrous cycle did not influence ovulation rate (Dufour and Matton, 1977). Wallace (1954) reported that the energy level fed to ewes during anoestrus did not appear to stimulate follicle activity or advance the breeding season. During the breeding season, the energy level and duration of exposure to the feeding regimes have been shown to affect ovulation rate (Bellows *et al.*, 1963). Therefore the effects of increasing energy intake may depend on when the diet is given and on the body condition of the animals (Hulet *et al.*, 1969; Dufour and Wolynetz, 1977).

Addition of energy to the diets of gilts for 8-10 days but not less than four days before oestrus will increase ovulation rate (Brooks and Cooper, 1972; Daily *et al.,* 1975). Increasing the caloric energy of the ration fed to gilts with glucose or lard stimulated an increase in ovulation rate (Zimmerman *et al.,* 1960; Rigor *et al.,* 1963). The pig is a monogastric species and for this reason it might not be comparable with what happens in ruminants. However, it could indicate that it is an increase in the available energy to the stomach, or abomasum in the case of the ruminant, which is the initial stimulus for the increase in ovulation rate.

2.3.3 The role of protein in reproductive function

Ovulation rate was increased in ewes offered greater pasture mass, less dead material and higher clover content, indicating the importance of diet quality (Rattray, 1980; 1981). Braden and Mattner (1970) found that differences in the protein content of diets offered to sheep promoted corresponding differences in the quantities of protein digested by the intestines but that this was not associated with changes in ovulation rate. Feeding ewes formaldehyde treated casein at 60 g/day significantly increased the apparent amino acid absorption in the small intestine compared with untreated casein but again had no significant effect on ovulation rate (Corbertt and Edey, 1977). In another study there was no response to 100 g formaldehyde-treated casein/day (Teleni *et al.,* 1989a). However, feeding 160 g formaldehyde-treated casein/day did increase ovulation rate and the response was similar to that obtained by feeding 500 g lupin grain/ day (Nottle *et al.,* 1988).

Smith (1985) reported a 20% increase in ewes with multiple ovulations when they were fed more than 125 g crude protein per day compared with ewes fed less than 125 g crude protein per day. Torell *et al.* (1972) reported that there is an inverse relationship between responses to protein and the amount of protein supplied in the basal ration. Feeding ewes a similar energy level and increasing the protein intake from 35-70 g/day increased ovulation rate but a further increase in the protein intake from 70-150 g/day had no additional effect. This suggests that protein levels only influence ovulation rate when the level previously fed was below maintenance requirements. Corbert and Edey (1977) considered that the lack of ovulation response to feeding formaldehyde-treated casein in their study could have been due to high levels (14.75%) of protein in the basal ration.

2.3.4 Relationship between dietary intake and nutrient availability in ruminants

The digestive system of ruminants is different from that of simple-stomached animals and thus the effect of short-term changes in nutrient intake is different between ruminants and monogastric animals. In simple-stomached animals, the diet consumed is digested in the stomach and small intestine, with animals being very sensitive to acute changes in dietary intake. In cycling primates, for example, metabolic cues transmitting information to the reproductive axis appear to be primarily dependent on energy intake and not body mass or composition and missing a single meal can dramatically alter LH pulse frequency (Cameron, 1996). In ruminants, however, the digestive process is more complex and is more analogous to a 'system within a system', with the majority of the diet acting as a nutrient supply for ruminal microflora, which in turn produces energy and protein components that can be digested and absorbed.

The rumen is populated by many types of micro-organisms, the relative amounts of which can be modified by changing diet (Orskov and Ryle, 1992). Some ruminal microflora digest complex carbohydrates, including cellulose-based carbohydrates that are unavailable to simple-stomached animals, to produce organic acids (volatile fatty acids) that can be subsequently used as an energy source. The main volatile fatty acids produced are acetate, propionate and butyrate. Propionate is the main energy substrate used by ruminants and is converted to glucose in the liver. The relative amounts of each volatile fatty acid produced is diet dependent, with roughage-type foods encouraging the production of acetate and cereal-based foods encouraging the production of propionate. Thus diet type can alter the energy available for productive purposes.

Animals require protein as a source of essential amino acids and, in ruminants, as a nitrogen source for rumen microflora. Essential amino acids must be supplied in the diet of simple-stomached animals but rumen microbes are themselves the main source of amino acids for ruminants. The dietary protein requirement of an individual animal is dependent on its physiological status and level of production. The quality of the protein in a feed is dependent on its amino acid profile and digestibility. Dietary protein is categorised as rumen degradable or undegradable (by-pass) on the basis of

the ability of rumen microbes to hydrolyse the protein in the rumen. The amount of protein that is degraded in the rumen is diet dependent. Thus, protein digestion is also influenced by dietary components.

Rumen microbes can utilise nitrogen from sources other than true protein. Urea is the predominant source of non-protein nitrogen used in animal foods and is readily hydrolysed in the rumen and results in the formation of ammonia. The ammonia, if not used by rumen microbes, is rapidly mobilised and requires immediate conversion to urea in the liver and subsequent excretion via the kidneys, to maintain animal health. Therefore intake of urea must be restricted as ammonia toxicity can occur if it is given in excess. Ruminants are also capable of reducing protein loss by re-cycling urea (O'Callaghan and Boland, 1999). Thus, high urea concentrations are generally indicative of excess protein intake and are likely to be associated with high levels of ammonia in the rumen and other body systems.

Energy is required for incorporation of nitrogenous materials into microbial protein and the synthesis or availability of energy and nitrogen affects the efficiency of protein anabolism. Because of this energy dependence, it is possible that high concentrations of urea in the circulation may occur due to inadequate energy in the diet resulting in reduced microbial protein synthesis, rather than as a direct consequence of surplus dietary crude protein. Thus, the ruminant has a requirement for energy and protein but the rumen microbes also have specific nutrient requirements. Rumen function will be most efficient when diets are formulated to satisfy both the requirements of the rumen microbes and the animal itself. These specific requirements of ruminants must be borne in mind when interpreting experiments where ruminants are exposed to extremes of nutrition and the implications of altering the nature of the diet offered may not be clear in terms of the effects of such a manipulation on nutrients availability for productive purposes. This is particularly true when short-term dietary alterations are imposed (O'Callaghan and Boland, 1999).

2.3.5 Nutrition and gonadotrophin secretion

Energy status is generally considered to be the major nutritional factor that influences reproductive processes, with prolonged low energy intake impairing fertility. In

sheep, poor nutrition that results in lower ovulation rate is associated with decreased LH pulse frequency. This reduced frequency is likely to be due to inadequate hypothalamic GnRH secretion (Rhind *et al.,* 1989a). In cattle, a strong correlation is evident between negative energy balance in early lactation and resumption of ovulation (Canfield and Butler, 1990). While ovulation may not occur in animals on low dietary intakes, follicle growth and atresia do occur. Follicle wave turnover without ovulation is often evident in post-partum cows in poor body condition (Stagg *et al.,* 1995). Long-term restriction of food intake has been shown to induce anoestrus in cattle (Rhodes *et al.,* 1995) due to insufficient circulating LH (Rhodes *et al.,* 1996), which is likely to suppress follicle growth and oocyte maturation. These effects, however, are not immediately evident and dietary restriction of several months may be required to prevent follicle growth and ovulation.

In contrast to the situation in monogastric animals such as pigs and monkeys, in ruminants it is more difficult to observe an effect of short-term dietary restriction on LH pulse patterns. In ewes, restricted dietary intake for approximately three weeks resulted in either no change in LH secretion (Abecia *et al.,* 1995) or only a relatively small reduction in LH pulse frequency (Rhind *et al.,* 1989a; 1989b). On the other hand, there is little evidence of an effect of nutrition on plasma FSH concentration, even though FSH is essential for follicular growth and ovulation (Findlay and Clarke, 1987). However, Mackey *et al.* (1997) have shown that short-term restriction of dietary intake to about 0.4 of maintenance energy requirements increased FSH in heifers compared with animals on double maintenance energy requirements. This trend was repeated in ovariectomised heifers on similar diets, suggesting that the effects are mediated, at least in part, by changes at the level of the pituitary and are not solely due to alteration in steroid feedback effects. In a recent study, O'Callaghan and Boland (1999) reported that LH pulse frequency, mean LH concentration and the proportion of pituitary cells producing FSH or LH were not significantly different in heifers kept on approximately two-thirds of maintenance compared with those kept on twice maintenance for three weeks. Thus, nutritional effects on gonadotrophin secretion in ruminants appear to be relatively minor unless dietary restrictions persist for extended periods of time.

2.3.6 Nutrition and ovarian function

The effect of nutrition on ovulation rate and lambing rates has long been known. A rapid improvement in body condition is usually associated with increased ovulation and lambing rates (Coop, 1966a); such effects of nutrition on ovulation rate have been reviewed by Smith *et al.* (1990). These alterations in ovulation rate may be related to the entry rate of glucose into cells in animals on a high plane of nutrition. Dietary supplements containing high energy and protein levels have been shown to increase ovulation rate in ewes (Downing *et al.,* 1995a). Similarly, an increase in ovulation rate was reported when glucose was infused directly into blood (Downing *et al.,* 1995b; Williams *et al.,* 1997). Thus, it is likely that short-term energy supply is directly involved in follicle growth.

In a practical attempt to boost glucose supply, Landau *et al.* (1995) found that the daily provision of 100 g of rumen undegradable starch increased mean ovulation rate (3.29 vs. 2.46) in carriers of the high fecundity Fec^B gene but not in non-carriers (1.44 vs. 1.36), suggesting that an important feature of responses in ovulation rate to nutrient supply may be the ovulation potential of individual animals. In an attempt to identify blood-borne metabolites that mediate the stimulatory effect of a lupin-grain supplemented diet on mean ovulation rate (2.5 vs. 1.9) in ewes, Downing *et al.* (1995a) suggested direct ovarian action through increased glucose availability. An accompanying sustained increase in plasma insulin also was observed when ovulation rate was increased by intravenous infusion of either glucose (Downing *et al.,* 1995b) or the branched chain amino acids, leucine, isoleucine and valine (Downing *et al.,* 1995c) for five days in the late luteal phase of the oestrous cycle. Using the auto-transplanted ovary model, Downing (1994) demonstrated that while infusion of glucose or insulin alone had no effect on ovarian steroid secretion, their combined infusion decreased the secretion of both androstenedione and oestradiol in response to a GnRH-stimulated LH pulse, again implying the involvement of altered steroid feedback in the ovulatory response.

Dietary restriction has been shown to alter follicle growth characteristics in cattle (Murphy *et al.,* 1991) and in superovulated sheep (Yaakub *et al.,* 1997b). Murphy *et al.* (1991) reported that feeding heifers on a low dietary intake reduced the size of the

dominant follicle and also reduced the persistence of the dominant follicle compared with follicle growth in animals offered higher energy intakes. Similar conclusions were drawn by Mackey *et al.* (1997b) who demonstrated that acute restriction in dietary intake 3-6 days pre-ovulation decreased the growth rate and maximum size of the first post-ovulatory dominant follicle, resulting subsequently in fewer follicles emerging in the second follicle wave of the oestrous cycle. In the case of superovulated heifers, Nolan *et al.* (1998b) reported an increase in follicle numbers after stimulation with exogenous FSH in heifers offered a low dietary intake compared with the number in heifers on a high dietary intake. This difference in response was predominantly due to an increase in the number of follicles in the 7-10 mm size range when measured around the time of the LH surge. However, this trend was not repeated when ovulations were recorded after superovulation of heifers on the same low and high diets (Nolan *et al.,* 1998b). In the case of superovulated ewes, a lower ovulation rate was recorded in ewes offered half maintenance energy requirements, compared with ewes offered twice maintenance energy requirements (Yaakub *et al.,* 1997b). In a later trial, ovulation rates of ewes superovulated on similar diets but using a different gonadotrophin were not different (Yaakub *et al.,* 1997c).

2.3.7 Nutrition, steroids and metabolic humoral agents

As diet influences the metabolites available for cell function, it is not surprising that changes in intake can alter substrates available for synthesis of both protein and steroid hormones. The effects on three classes of humoral agents regulating the reproductive system will be considered. Specifically the effects of dietary intake on changes in (1) progesterone (2) glucose and insulin and (3) insulin-like growth factors and their binding protein will be considered.

2.3.7.1 Progesterone: Food intake in sheep has been shown to affect the concentration of progesterone in the circulation, with a strong negative correlation between dietary intake and progesterone concentrations (Parr *et al.,* 1982; 1987; McEvoy *et al.,* 1995). This effect of dietary intake on circulating progesterone concentration is likely due to an increase in the rate of catabolism of progesterone in the liver at higher feeding levels. Liver weights can be substantially higher in animals on high compared with low diets, with proportionately about a 50% increase in liver

weight evident in ewes on a diet of twice maintenance energy requirements compared with those on half maintenance energy requirements for one month (O'Callaghan and Boland, 1999). Progesterone is believed have an important role in oocyte maturation and in early embryo development (Boatman, 1997). The effects of *ad libitum* feeding in sheep are to reduce progesterone concentrations compared with restricted feeding but the results in cattle indicate that the effect can be variable. *Ad libitum* feeding in heifers increased (McCann and Hansel, 1986), decreased (Villa-Godoy *et al.,* 1990) or had no effect (Spitzer *et al.,* 1978) on progesterone concentration when compared with restricted feeding. A negative correlation was reported between dietary crude protein intake in cows and serum progesterone (Jordan and Swanson, 1979a) and reproductive efficiency (Jordan and Swanson, 1979b). Low progesterone levels post-breeding can also reduce fertility (Larson *et al.,* 1997). Recently, the concentrations of progesterone and embryonic interferon tau have been reported to be positively correlated (Mann *et al.,* 1998). Thus, minor changes in maternal progesterone concentrations during the initial period of embryo development may alter the secretion of this antiluteolytic agent and may be critical to embryo survival.

In a recent study, Nolan *et al.* (1998b) reported progesterone concentrations in cattle to be proportionately higher in heifers on a low energy diet (40 MJ ME per day) compared with heifers on a high diet (120 MJ ME per day). It is possible that this difference was caused by changes in liver size and consequently steroid metabolism, as appears to be the case in sheep. However, as steroids are selectively stored in fat, any dietary regime that results in fat mobilisation will result in the release of stored progesterone. This may account for some of the increased progesterone evident in animals on low dietary intake. While animals on low dietary treatments are likely to have adequate progesterone to maintain the existing pregnancy, the significance of these observations may relate to the fact that high progesterone concentrations during an oestrous cycle prior to insemination can affect pregnancy rates in cattle (Meisterling and Dailey, 1987), suggesting that oocyte function may be compromised nutritionally. Similar trends have been reported for progesterone in follicular fluid, with higher progesterone concentrations in follicular fluid from large follicles in ewes on half maintenance diets compared with those on higher levels of nutrition (O'Callaghan *et al.,* 1998). No difference was evident in oestradiol concentrations, suggesting that differences in steroid concentrations in the circulation are not

exclusively due to metabolic clearance rate but may be affected by rates of production, at least in the pre-ovulatory phase, from luteinized granulosa cells within the follicle.

2.3.7.2 Glucose, insulin, growth hormone and insulin-like growth factors: Dietary changes cause an immediate and rapid change in a range of metabolic humoral agents. The most important of these from a nutritional point of view are glucose and insulin. Changes in insulin are closely related to changes in insulin-like growth factor concentrations (Pushpakumara *et al.,* 2002). The relevance of glucose concentrations in regulating ovulation rate in ewes is mentioned above. In an experimental model using lupin grains to increase ovulation rate in sheep, Downing *et al.* (1995a) suggested that increased ovulation rate resulted from a direct ovarian action of increased glucose availability. Direct intra venous infusion of glucose can increase ovulation rate (Downing *et al.,* 1995b) in association with a sustained increase in insulin concentrations. Thus, it has been suggested that nutritional effects on ovulation rate of sheep are specifically mediated through insulin-mediated glucose uptake. Indeed, where ewes were put on a diet at half of their energy maintenance requirements and offered an additional 1.5 times their maintenance energy requirements as food or in energy equivalent as direct intravenous infusion of glucose, the increase in ovulation rate achieved was similar in ewes given supplementary food or energy as a glucose infusion (Williams *et al.,* 1997). Similar effects of a glucogenic oral dose on ovulation rate have also been reported (Rodriguez Iglesias *et al.,* 1996), although glucose was only different from control animals for the first 6 h after treatment.

These results implicate glucose in the control of ovarian function. Since glucose is closely regulated by insulin, this also suggests a role for insulin in the regulation of follicle growth in the ewe. However, sustained infusion of insulin can reduce LH secretion (Downing and Scaramuzzi, 1997) implicating insulin, and in particular, the balance between insulin and glucose in the regulation of the hypothalamic-pituitary axis.

Growth factors, including IGF-1 and IGF-2 may also be involved in mediating the effects of nutrition on the reproductive system. These growth factors, their binding

proteins and receptors, are found in a wide range of tissues throughout the body, where they are involved in controlling the anabolic and catabolic processes that constitute the general responses to changes in the level of nutrition (Wathes *et al.*, 1998). The importance of growth factors in the maturation of oocytes and in early embryo development has been reviewed by Kane *et al.* (1997). The involvement of insulin-like growth factors in the interactions between nutrition and reproduction has been reviewed by Monget and Martin (1997). Increasing dietary intake has been shown to increase the development of small follicles in heifers (Gutierrez *et al.*, 1997). Similar observations were made by Gong *et al.* (1993) following treatment of heifers with exogenous growth hormone (GH). IGF-1 concentrations are affected by plane of nutrition and GH in circulation and IGF-1 has been shown to increase the sensitivity of granulosa cells to stimulation with FSH. Dietary intake has been shown to alter follicular fluid IGF-1 concentrations. Ryan *et al.* (1994) reported a relationship between serum IGF-1 concentrations and body condition score (which is an indicator of recent dietary intake); animals with extremely high or low body condition scores had lower IGF-1 concentration than animals with intermediate scores. However, other studies in heifers showed no effect of diet on plasma or follicular fluid IGF-1 concentrations (Spicer *et al.*, 1991). Yet, short-term fasting for 48 h decreased circulating IGF-1 concentrations without affecting follicular fluid concentrations of IGF-1 (Spicer *et al.*, 1992). Recently, O'Callaghan *et al.* (1998) reported a trend in the case of follicular fluid, with ewes on maintenance diets having higher IGF-1 concentrations in follicular fluid, compared with ewes on lower (half of maintenance requirement) or higher (twice maintenance requirement) dietary intake. In the same study, IGF-2 concentrations were lower in follicular fluid from ewes on a high (twice maintenance) dietary intake. These changes, however, were not evident with several IGF-binding proteins, ranging in weight from 95-20 kDa. Thus, it is likely that the insulin-GH-IGF-1 axis is, at least in part, responsible for dietary induced changes in gonadotrophin secretion and ultimately follicle growth and ovulation rate. Collectively, these results suggest that the environment within a follicle can be profoundly altered by nutrition. The importance of protein and steroid content of follicular fluid in the maturation of oocytes and resumption of meiosis was recently discussed by Sirard *et al.* (1998). Given the nutritionally induced changes that are evident in follicular fluid, it is possible (even quite likely) that intra-follicular

growth factors have a substantial effect on oocyte maturation and thus oocyte developmental competence.

2.3.8 Nutritional status and progesterone concentrations

It is well documented that there is an inverse relationship between post-mating nutritional status and peripheral progesterone concentrations in sheep (Williams and Cumming, 1982), with low progesterone concentrations following high plane feeding being associated with embryo loss. This relationship is believed to reflect an increase in the metabolic clearance of progesterone in the absence of concomitant changes in the entry rate of the hormone. The liver is the major site of steroid metabolism. Changes in nutrient intake could influence progesterone metabolism in at least three ways: by changes in liver mass, by changes in the rate of blood flow through the liver or by altering activity of the mixed function oxidase or *p-450* enzymes which catalyse steroid metabolism (Parr, 1992). These three mechanisms are clearly not necessarily mutually exclusive.

Parr (1992) proposed that increased feed intake during early pregnancy in sheep led to an elevation in blood flow through the gastrointestinal tract and liver. As over 95% of circulating progesterone is metabolised during one passage through the gut and liver, this results in a reduction in the concentration of peripheral plasma progesterone. Pregnant ewes consuming rations either above or below maintenance levels show no differences in the mass (Wallace *et al.,* 1994) or progesterone secretion (Abecia *et al.,* 1994) of corpora lutea on Days 16 or 14 respectively. These results suggest that corpora lutea are unable to alter progesterone secretion to maintain constant peripheral progesterone concentrations. Other groups demonstrated that both heavy ewes and ewes fed high protein diets had larger livers (Payne *et al.,* 1991) and greater than normal levels of hepatic microsomal protein, NADPH-cytochrome-c reductase and cytochrome b5 (Smith *et al.,* 1990). However, these increases in hepatic enzymes were not reflected in differences in the circulating concentrations of progesterone. The role of peripheral progesterone concentration in nutritional effects on embryo survival was clearly demonstrated by Parr *et al.* (1987); the 25-30% reduction in embryo survival arising from feeding 200% of maintenance rations from Day two after mating could be overcome by supplementary progesterone on day 8-14 after mating. Administration of progesterone to ewes fed 50 or 100% of maintenance

rations did not improve embryo survival, demonstrating that exogenous progesterone is remedial only when used to restore endogenous levels to those seen in control ewes.

Metabolic rate and the rate of blood flow through the liver increase with increasing energy intake, resulting in an increased hepatic clearance rate of progesterone (Parr *et al.,* 1993; Prime and Symonds, 1993). In the follicular phase or pre-insemination period, sub-optimal systemic progesterone concentration results in persistence of the dominant follicle with detrimental effects on fertility in cattle (Savio *et al.,* 1993; Mihm *et al.,* 1994). On the other hand, nutrition-induced reductions in systemic progesterone concentration after insemination have been associated with reduced early embryo survival rate in both sheep (Parr *et al.,* 1987) and pigs (Dyck and Strain, 1983; Jindal *et al.,* 1996). In this situation, progesterone may directly affect the growth and development rate of the conceptus (Garrett *et al.,* 1988). Progesterone also has a major role in controlling uterine secretion of protein and growth factors essential for early development of the embryo (Murray and Sower, 1992), suggesting a possible indirect effect of fluctuations in systemic progesterone.

Diet induced suppression of circulating progesterone during oocyte maturation in superovulated ewes primed with a single CIDR (control internal drug release) device (0.2 g progesterone) can induce developmental retardation that leads to decreased embryo survival (McEvoy *et al.,* 1995a). Again the causal mechanisms are the subject of speculation (McEvoy *et al.,* 1995b) but may involve abnormal oestradiol: progesterone ratios which could disrupt oocyte maturation by way of incomplete or late closure of gap junctions within the follicle. Alternatively, the expression of maternal mRNA required for maternally regulated development up to the blastocyst stage might be impaired by low progesterone.

2.3.9 Relationship between live weight and ovulation rate

Wallace (1958) found that the lambing percentage was closely related to the flock live weight at mating and he later confirmed that a substantial part of the differences in lambing percentage was related to differences in ewe live weight (Wallace, 1961). Analysis of a large volume of data, collected over many years led to the conclusion that live weight at mating has a considerable influence on the reproductive rate of ewes, especially the rate of twinning (Coop, 1962). From a between-flock analysis,

Coop (1962) estimated that the twinning rate rose by 5.3% for each additional 4.5 kg of live weight. Morley *et al.* (1978) made an extensive analysis of data from various published reports and concluded that there was a relationship between live weight and lambing rate. In most of the breeds studied, it was reported that for each additional kilogram in average live weight there was a 2.0-2.5% increase in lambs born per ewe. However, the relationship varied widely depending on whether it was measured within flocks of a similar genetic constitution or between flocks and breeds.

Studies of the interaction between live weight and reproduction were subsequently extended to include ovulation rate (Killeen, 1967; Allison, 1968; Cumming, 1977; Smith *et al.,* 1979; Smeaton *et al.,* 1981; Kelly and Johnstone, 1982; Kelly *et al.,* 1983; Oldham *et al.,* 1990). Cumming (1977) found that, within flocks, ovulation rate increases between 0.0-0.44 per ewe for each additional 10 kg of live weight with the increase in most flocks being 0.25 to 0.3. Various other studies also showed an increase in ovulation rate of approximately 0.03 per kilogram increase in live weight (Rattray *et al.,* 1980; 1981; Smeaton *et al.,* 1981; Kelly and Johnstone, 1982; Kelly *et al.,* 1983; Kleemann *et al.,* 1991).

2.3.10 Relationship between live weight change and ovulation rate

Short periods of improved nutrition before and during mating increase the proportion of ewes bearing twins (Underwood and Shier, 1941; Wallace, 1951; 1953; Coop, 1966; McInnes and Smith, 1966; Cumming, 1977; Rattray *et al.,* 1980; 1981; Torell *et al.,* 1972; Gunn *et al.,* 1984; Gunn and Maxwell, 1989). This practice has been termed 'flushing' as previously described. Coop (1962) calculated that half the response to 'flushing' could be accounted for by differences in absolute live weight and that the remainder could be related to a rapidly rising body condition. He introduced the term 'static' and 'dynamic' to distinguish between these two effects. When ewes were 'flushed' for one oestrous cycle the lambing percentage increased by 10% and included a 'static' component which when deducted left a 5-6 percent increase attributable to the 'dynamic' component (Coop, 1966).

Not until the 1960s was a large effort put into examining the relationship between live weight change and ovulation rate (Allen and Lamming, 1961; Killeen, 1967; Allison, 1968; Edey, 1968; Cumming, 1977; Gunn *et al.,* 1979; Smith *et al.,* 1979; Thompson

et al., 1985). In ewes, live weight at laparoscopy was found to be an effective predictor of the percentage of multiple ovulations, within the limitations imposed by genotype, age, season and nutritional treatments (Smith *et al.,* 1979). These workers also found that different nutritional treatments had a major effect on ovulation rate response above an effect solely due to live weight.

The effects of flushing are variable with no advantage being observed by a number of workers (Hart and Miller, 1937; Tribe and Seebeck, 1962; Gunn *et al.,* 1979; Gunn and Doney, 1975). Clark (1934) first suggested that the variability in response might be due to differences in body condition, with ewes in good body condition having a higher ovulation rate and therefore being closer to their genetic potential for this character. Continuous maintenance of ewes in good condition will maintain high ovulation rates and it is generally accepted that the greatest benefit from 'flushing' is from ewes in moderate body condition (Underwood and Shier, 1941; Wallace, 1953; Allen and Lamming, 1961; Gunn *et al.,* 1979; Gunn and Doney, 1975).

The period needed to obtain a suitable response to 'flushing' is usually 3-6 weeks (Rattray *et al.,* 1980; 1981; Mcinnes and Smith, 1966; Smith *et al.,* 1983; Thompson *et al.,* 1985). Wallace (1951) found an increase in lambing percentage after a one-week period of 'flushing'. However, greater responses were obtained when the increased level of feeding was extended for 3-4 weeks. 'Flushing' ewes for one cycle with oats and lucerne increased lambing percentage whereas an additional 17 days of supplementation gave no further increase (Hulet *et al.,* 1962).

2.3.11 Relationship between body condition and ovulation rate

Lindsay (1976) considered that live weight was "a crude inaccurate criterion which is incompatible, because it only describes long term changes in feeding, with studies on many components of the reproductive process that take place over a few days or even hours". In some grazing experiments, ewe live weight and premating live weight change accounted for only 42 and 18 percent, respectively, of the variations in ovulation rate (Smith *et al.,* 1982). Therefore, nutritional factors that influence live weight appear to have a major effect on ovulation rate. 'Flushing' includes two processes, firstly an increase in nutrient intake, either by an increase in the level of

feed intake and/or the intake of better quality feed and, secondly, a resultant increase in body condition.

The effects of improved nutrition on reproductive performance are largely associated with an improvement in body condition (Gunn *et al.,* 1979; Gunn and Doney, 1975). High levels of feeding need to be maintained until mating to ensure that ewes are in high body condition and have high ovulation rates (Gunn *et al.,* 1984). Often, alterations in body condition are measured as live weight change once gut fill has been taken into account. The live weight of a ewe is a combination of body size and body condition and consequently is not a good measure of body condition alone (Ducker and Boyd, 1977). However, because body size does not vary greatly for individual ewes, live weight change is a measure of changes in body condition. Ducker and Boyd (1977) found no difference in ovulation rate between ewes of large or small body size. Changes in body conditions in ewes of a similar body size do appear to be correlated with their ovulation rate.

2.3.12 Nett nutritional status of ewes and ovulation rate

Relationships between live weight, live weight change or body condition and ovulation rate are of limited value. In an effort to understand how nutritional influences can increase ovulation rate, Lindsay (1976) suggested that the effect of nutrition on reproductive processes should be thought of in terms of the ewe's 'nett nutritional status'. This encompasses both the endogenous and exogenous nutrient sources available to the ewe. Changes in the "nett nutritional status" are related to metabolic changes that take place in response to decreasing or increasing feed intake and the associated utilisation or storage of nutrients in body reserves.

2.3.13 Pre-mating feed intake and ovulation rate

Fasting ewes for 7 days before oestrus reduced the incidence of oestrus but had little effect on ovulation rate (Mackenzie *et al.,* 1975). Ovulation rate was found to be positively related to body condition but not significantly affected by the level of pre-mating nutrition when ewes were in good body condition (condition score 3.0; Gunn *et al.,* 1969; 1972). It was suggested by Gunn *et al.* (1969) that for ewes in poor body condition (condition score 1.5) at mating, ovulation rate might be positively related to the level of pre-mating nutrition. A later study however, failed to support this

suggestion (Gunn and Doney, 1979). A significant ovulatory response to a high level of premating nutrition has been demonstrated in ewes in moderately good body condition (condition score 2.5) but not in ewes in only moderate body condition (condition score 2.0) (Gunn and Doney, 1979; Gunn *et al.,* 1979). For any particular breed or strain of sheep, there is probably no consistent relationship between the level of pre-mating feed intake and ovulation rate over a range of body conditions. The body condition at mating has a greater influence on ovulation rate than does the level of feed intake at the time of mating.

2.3.14 Glucose and ovulation rate

At a cellular level, glucose is the primary energy source and reduced levels will have marked effects on the metabolically activity of ovarian follicles. On Day 16 of the oestrous cycle, intravenous infusions of glucose and amino acids in ewes failed to increase ovulation rate (Goerke and Dutt, 1978). When ewes were given abomasal infusions of glucose or soybean isolate over Days 8-17 of the oestrous cycle, the protein supplements gave a significant increase in the percentage of multiple ovulations whereas the slight increase in multiple ovulations in response to glucose was not significant (Cruickshank *et al.,* 1988). Short-term intravenous administration of the energy yielding substrates, glucose and acetate, produced ovulation rate increases of a similar magnitude to the feeding of lupin grain (Teleni *et al.,* 1984; 1989a). Glucose and acetate provided about 30% of the metabolisable energy provided by lupins. When ewes were fed a lupin grain supplement, the plasma glucose (130%) and acetate (73%) entry rates were increased compared with feeding a basal ration alone and the overall metabolic rate was increased by 56% (Teleni *et al.,* 1989b). There was a strong correlation between the glucose entry rate and the ovulation rate response. The increase in the entry rate of glucose observed in sheep on diets of lupin grain (Teleni *et al.,* 1989b), lasoloid (Casson *et al.,* 1986) and formaldehyde treated casein (Kuswandi and Teleni, 1987) can be explained by the increased availability of gluconeogenic substances. The increase in glucose entry rate when acetate is infused is less clear (Abdalla *et al.,* 1986). Acetate is likely to stimulate lipogenesis (Vernon, 1981) and the requirement of lipogenesis for NADPH and glycerol, which is derived from glucose, would increase glucose production using substrates mobilised from storage tissues. Acetate could increase ovulation rate in

ewes by increasing glucose availability and hence the glucose entry rate (Teleni *et al.*, 1989b).

2.3.15 Long-term effects of nutrition on ovulation rate

There are very few reports of studies into the effects of long-term nutrition on follicle development in the ewe. Fletcher (1974) found an effect of differential nutrition six months before mating on subsequent ovulation rate even though ewes were fed a common nutrient level in the intervening period. This effect may be related to events involved in initiation of follicle development, which occur about six months before ovulation (Moor *et al.*, 1980). The pattern of live weight recovery of the ewe from weaning to mating was of relatively little importance in determining ovulation rate compared with weight and body condition attained at mating (Gunn and Doney, 1973).

2.3.16 Nutrition and oocyte quality

Information reviewed above indicates that diet can profoundly alter endocrine signalling pathways. What is not so clear is the effect that these changes ultimately have on fertility. The relationship between the developing oocyte and the follicular environment is a sensitive balance of hormonal and receptor communication. The relationship between the oocyte and the follicular cells and endocrine environment has been reviewed recently by Driancourt and Thuel (1998).

While structural differences have been reported in oocytes from superovulated compared with unstimulated heifers (Assey *et al.*, 1994), very few studies have examined the effects of nutrition on oocyte quality. McEvoy *et al.* (1995b) reported that a higher proportion of ova from ewes on a low diet (10 days before ovulation) were considered more viable than of those produced by ewes on a high diet. In a recent study reported by Yaakub *et al.* (1997a), the effect of dietary intake on oocyte morphology was determined using electron microscopy. Structural changes were observed in the degree of detachment of interchromatin-like granules within the oocyte nucleolus in oocytes collected from ewes offered a low dietary intake (half of maintenance energy requirement) compared with a high diet (twice maintenance energy requirement). This suggested a diet-induced disruption in oocyte morphology.

More recently, Nolan *et al.* (1998a) suggested that restricting the dietary intake of heifers could enhance the *in vitro* blastocyst yield of oocytes collected over several weeks using trans-vaginal ovum aspiration. This indicates that the effect of nutrition on reproduction may occur at the level of the oocyte prior to ovulation.

2.3.17 Nutrition and embryo survival

Extremes in the level of feeding are detrimental to embryo survival as are extremes in the supply of specific dietary nutrients such as vitamins, trace elements and protein (Kwong *et al.,* 2000). For many of the vitamins and trace elements, the effects of a deficiency are understandable in view of their roles in metabolism. The retinols are the main metabolites of vitamin A and are involved in cell proliferation and differentiation, the expression of growth factors, gene transcription and steroidogenesis, all of which have important roles in embryo survival (Robinson, 1990; Ashworth, 1994; Cross, 2001). Folic acid, for which there is evidence of improved prenatal survival with supplementation, is essential for nucleic acid synthesis. Vitamin C appears to enhance luteal function, perhaps through its cofactor role in steroidogenesis, which again would explain its beneficial effect in maintaining early pregnancy in cattle. Correction of selenium deficiency in ewes reduces embryo mortality during implantation. Selenium also increases fertilisation rate, probably via its stimulating effect on uterine contractions and sperm transport, an effect which may be particularly important in combating the adverse influences on sperm transport and fertilization rate of the hostile uterine environment that occurs when animals are superovulated (Robinson, 1996).

Some attempts have been made to identify the mechanisms involved in the adverse effects of high protein diets on the fertility of dairy cows. Elrod and Butler (1993) found that high intakes of rumen degradable protein (RDP), leading to excess rumen ammonia production, were associated with a reduction in the pH of the uterine environment and Elrod (1992) reported that ammonia and urea differentially affected endometrial ion transport. For high RDP diets, which result in excessive ammonia production in the rumen, its hepatic detoxification is likely to impose an additional demand for amino acids (Lobley *et al.,* 1995). This implies that the provision of supplementary amino acids in the form of dietary proteins of low rumen degradable

status may avoid the adverse effects of excess RDP on the uterine environment and embryo survival.

2.3.18 Nutrition and gene function

In view of the detrimental effects of the diet-induced suppression of preovulatory progesterone on embryonic cell number and protein synthesis in the experiments of McEvoy *et al.* (1995b), some of the developmental retardation and reduced embryo survival may be due to either inadequate stimulation of 'early' response genes such as c-myc, which is progesterone-dependent or abnormal endometrial expression of erb-A, another 'early' response gene, which may modify the function of the progesterone receptor (Heap *et al.,* 1992).

Blastocyst formation is well recognized as a key developmental process in the growth of an embryo (Boland *et al.,* 2001). The blastocoele cavity forms as a consequence of fluid transport across the trophectoderm. This process is partially facilitated by Na/K-ATPase; messenger RNA for this enzyme has been identified in Day 7 bovine embryos (De Sousa *et al.,* 1997). Dietary intake and diet type can alter the expression of transcripts of genes involved in early embryo development, such as Na/K-ATPase and Cu/ZnSOD (Wrenzycki *et al.,* 1999).

2.3.19 Nutrition and embryo quality

Short-term restrictions in dietary intake have been shown to increase the subsequent pregnancy rate in cattle (Dunne *et al.,* 1997). Mantovani *et al.* (1993) reported that the yield of transferable embryos following superovulation in beef cattle was significantly reduced when heifers had *ad libitum* access to concentrates, compared with restricted levels of concentrates. Yaakub *et al.* (1996) demonstrated that concentrate type and level could affect the subsequent yield of transferable embryos following superovulation in beef cattle. Heifers on a restricted diet, where the predominant concentrate supplement was in the form of citrus and beet pulp, produced more transferable embryos compared with those where barley was the predominant concentrate or with those on *ad libitum* quantities of concentrates.

Severe restrictions in dietary intake have been shown to have beneficial effects on embryo development rate when heifers were superovulated and embryos were collected seven days after breeding and then cultured for 24 h (Nolan *et al.,* 1998b). This comparison of severe restriction vs. *ad libitum* feeding resulted in an increase in the number of blastocysts after culturing recovered embryos for 24 h. In the same study, an increase in the total blastocyst cell number was also noted. The effect of extremes of dietary intake on embryo development is evident but the point at which this change occurs is still unknown.

The contrasting situation where excess dietary intake reduces embryo quality is also consistent. When ewes were infused with high levels of glucose and superovulated, the yield of good quality embryos decreased (Yaakub *et al.,* 1997b). Glucose infusion has also been reported to reduce pregnancy rate (Rubio *et al.,* 1997). Thus, the optimum nutritional requirements for follicle growth and ovulation may be different from those required for optimum embryo development. While the reason for such an effect of glucose on embryo development is not clear, it may be due to unusually high plasma glucose concentrations interfering with cell signalling mechanisms during pre-ovulatory follicle growth, oocyte development or early embryo development. It is known that high glucose concentrations are deleterious to embryo development *in vitro* (Furnus *et al.,* 1996). There are also suggestions that hyperglycaemia in ewes is associated with embryopathy (Martin *et al.,* 1998), an observation consistent with the relatively high incidence of fetal developmental malformations occurring in diabetic pregnant women. Similar effects of excess glucose on pre-implantation embryo development are the basis for the practice in assisted reproduction programmes of administering insulin to diabetic mothers to reduce blood glucose before embryo recovery. Thus, nutrient requirements for optimum follicle growth and embryo development may be different. This highlights the importance of diet around the time of mating and in particular the significance of overfeeding, in regulating pregnancy rate.

2.4 Conclusion

The literature reviewed above has summarised the information that lead to the development of the hypotheses outlined in Chapter 1 (Section 1.2). The following

Chapters present the research procedures and results obtained from the experiments undertaken to address these hypotheses.

Chapter 3

MATERIALS AND METHODS

All procedures in these studies were conducted according to guidelines of the Australian Code of Practice for the Use of Animals for Scientific Purposes and received approval from the Adelaide University Animal Ethics Committee and the South Australian Research and Development Institute Animal Ethics Committee.

3.1 Source of animals

South Australian Merino ewes were obtained from commercial sources.

3.2 Care of animals

The ewes were normally kept under routine paddock conditions and managed by normal farm husbandry practices. Prior to experimental periods, ewes were moved indoors for 10-15 days and fed standard pelleted sheep rations (J.T. Johnson & Sons Pty. Ltd. Kapunda SA 5373) of standard composition (Table 3.1) at a rate of 600 g/day/ewe. During the experimental period, ewes were housed in single pens exposed to natural photoperiod. Each pen had an individual feed bin and water was provided *ad libitum*.

3.3 Feed intake

Daily energy need for maintenance was calculated from the Agricultural Research Council (1980) feeding standards using the Rumnut computer program (Pullman and Hughes, 1986). To determine individual feed intake, a weighed quantity of fresh feed (Table 3.1) was offered daily at 09:00 h. Any feed residues were collected every 2-3 days and weighed.

3.4 Superovulation

Superovulation (details provided in Table 3.2) was induced in ewes using a progestagen pessary containing 45 mg flugestone acetate (Intervet, Paris, France) for 12 days, followed by treatment with follicle stimulating hormone (FSH Ovagen, ICP, Auckland, New Zealand). The latter treatment consisted of 6 x 2.5 mg per ewe given (i.m.) twice daily and commencing 48 h before pessary removal. Pregnant mare serum gonadotrophin (Pregnecol, 500 IU, Horizon, New South Wales, Australia) was administered intramuscularly at the time of the first FSH injection. Synthetic gonadotrophin releasing hormone (Fertagyl, 50 μg per ewe; Intervet) was administered (i.m.) 27 h after pessary removal.

Table 3.1 Composition of feed used in all experiments

Test	Methods	Unit	Number of units
Moisture	NIR*	%	9.9
Dry matter	NIR	%	90.1
Ash	NIR	%	8.5
Crude protein	NIR	% of dry matter	19.1
Acid detergent fibre	NIR	% of dry matter	23.9
Neutral detergent fibre	NIR	% of dry matter	33.3
Digestibility	NIR	% digestible DM	69.3
Fat (ether extract)	Wet	% of dry matter	1.6
Metabolisable energy	calculated	MJ/Kg DM	10.1
Chloride	Wet	%	0.56
Sodium	Wet	%	0.20
Magnesium	Wet	%	0.20
Potassium	Wet	%	1.46
Calcium	Wet	%	1.06

*NIR (near infrared spectroscopy)

Table 3.2 Superovulation protocol used for all the experiments

Day	Time	Hormone/treatment
-14	am	Progestagen sponge inserted
-2	pm	FSH (2.0 ml) + PMSG (500 iu)
-1	am	FSH (1.5 ml)
	pm	FSH (1.0 ml)
0	am	FSH (1.0 ml)
	5 pm	FSH (0.6 ml) + sponge removed
+1	am	FSH (0.6 ml)
	8 pm	GnRH (30 μg)
+2	9 am	A.I.

3.5 Artificial insemination and embryo collection

Insemination occurred approximately 40 h after pessary removal. Semen was pooled from the same two rams of proven fertility in each experiment. Fresh semen was collected using an artificial vagina and diluted (1:1) with phosphate buffered saline (PBS) + 10% sheep serum. Laparoscopic insemination was performed as described by Killeen and Caffery (1982). Ewes were inseminated using laparoscopy with approximately 20 x 10^6 spermatozoa per uterine horn placed directly into the lumen.

Six days after insemination, embryos were collected under general anaesthesia by mid-ventral laparotomy (Walker *et al,*. 1989). The numbers of corpora lutea were recorded and each uterine horn was flushed with 20 ml of PBS containing 5% heat-inactivated sheep serum. Embryos were recovered from the flushing medium within 5 min of collection using a stereomicroscope.

3.6 Differential staining

Blastocysts were processed immediately whereas morulae and compact morulae were held in culture medium (SOF + BSA + amino acids; Appendix, 8.1; 8.2; 8.3) overnight in a humidified atmosphere of 5% CO_2: 5% O_2: 90% N_2 at 38.5 ^{0}C. The nuclei of the trophectoderm (TE) and inner cell mass (ICM) cells of the embryos were differentially labelled with polynucleotide-specific fluorochromes using a slight modification (Van Soom *et al.,* 1996) of the method of Hardy *et al.* (1989). Briefly, partial digestion of the zona pellucida was achieved by exposing the embryos, in groups of three to six, to prewarmed (37^{0}C) pronase solution (0.5% Protease; Sigma Chemical Co., St. Louis, MO) for 1-1½ min. Thereafter, the zona was completely removed which sometimes required mechanical assistance using a narrow-bored pipette. The zona-free blastocysts were washed in medium containing 10% (v/v) sheep serum in PBS (Appendix 8.2, Ca^{2+} and Mg^{2+} free) containing 4% (w/v) polyvinylpyrrolidone (PVP; ICN Biochemicals, Cleveland, Ohio). Blastocysts were then incubated in 10mM trinitrobenzenesulfonic acid (TNBS; Sigma) in PBS containing 4 mg/ml PVP (pH 7.4) on ice for 10 min. TNBS combines with N-terminal amines (Voet and Voet, 1990) and thus labels certain phospholipids or proteins in the embryonic membrane with covalently bound trinitrophenol groups. The embryos were washed three times in PBS/PVA and incubated in 0.1 mg/ml anti-DNP-BSA (specific antibody activity 1.3 mg/ml; Sigma) at 37^{0}C for 10 min. The

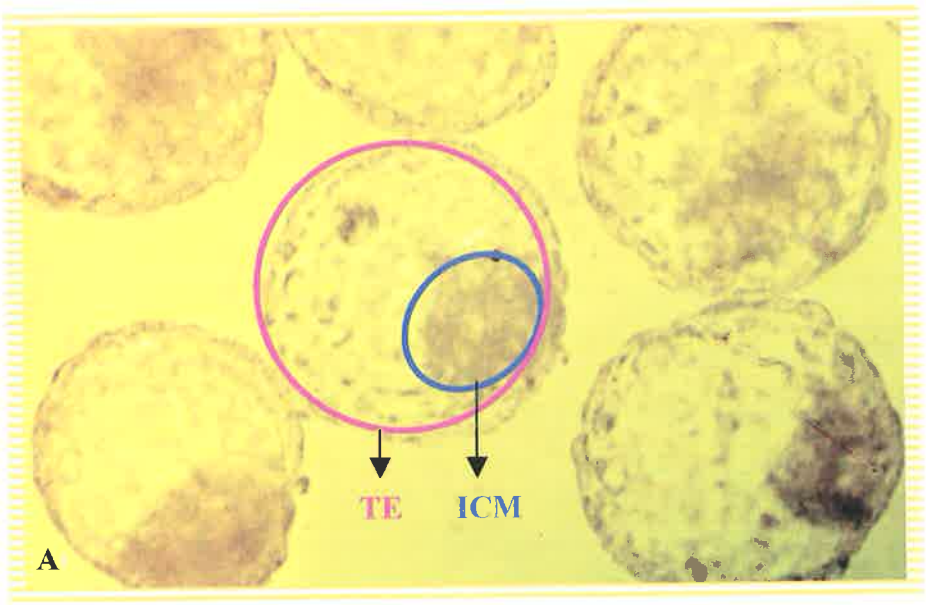
anti-DNP-BSA is a commercially available antiserum raised in rabbits against dinitrophenol groups (DNP), which also cross reacts with trinitrophenol groups. Excess antiserum was then washed away in PBS^-/PVA before exposure to complement, resulting in selective antibody-mediated complement lysis of the trophectoderm (TE). After a quick wash, the embryos were finally incubated in a 1:10 dilution of guinea pig complement (IMVS; Adelaide, Australia) in PBS^-, which included 10 µg/ml propidium iodide (PI; Sigma) for 15-30 min at 37^0C.

After the complement mediated cell lysis and the staining of the lysed cells by propidium iodide, the embryos were briefly washed in PBS to remove complement proteins, before being fixed with ice-cold absolute ethanol. After 5 min of fixation, the embryos were transferred into the second fluorochrome 10µg/ml bisbenzimide (Hoechst 33342; Sigma) in PBS^-, which penetrates both lysed and intact cell membranes and therefore stains all the nuclei of the embryo. The embryos were then transferred to absolute ethanol at room temperature or at 4^0C overnight. After incubation, the embryos were washed briefly in PBS^-/PVA and then transferred to a drop of glycerol containing Hoechst 33342 (1mg/ml) on a microscope slide and gently covered with a coverslip supported at each corner with vaseline: paraffin wax mixture (9:1). The embryos were carefully squashed and disaggregated using gentle pressure and then again examined in whole mount using a fluorescent microscope (Olympus BH2; Osaka, Japan). The number of ICM (blue) and TE nuclei (pink to red) were counted directly under the microscope (Fig. 3.1). Photomicrographs were taken on Fuji colour positive ASA 400 film corrected to ASA 800 for dark field using an Olympus BX 60 fluorescent microscope.

3.7 Collection of oviductal fluid

Merino ewes were used through out these studies and were housed in individual pens. They were fed each morning with a maintenance ration (standard sheep pellets) and given free access to water.

Ewes undergoing surgery for oviduct catheterisation were housed for at least two weeks prior to surgery and the abdominal areas were shorn. Ewes were fasted for 24 h prior to surgery. At the end of the study the reproductive tract was checked by a



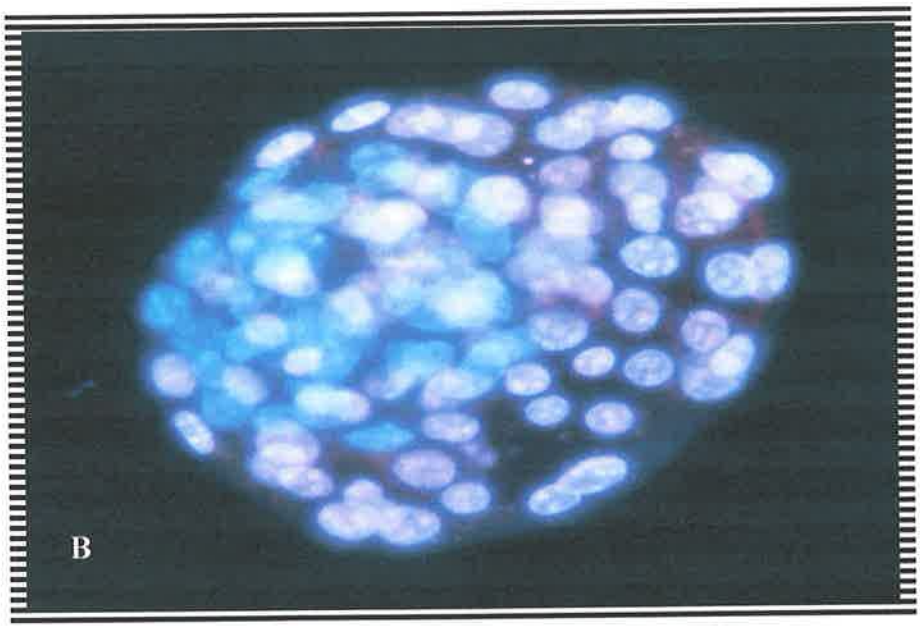


Fig. 3.1. (A) Stereoscope micrograph of expanded blastocysts (d 6 after ovulation). (B) Fluorescence (U.V.) micrograph of an expanded blastocyst (x 400) after completion of differential labelling. Trophectoderm (TE) nuclei labelled with propidium iodide and bisbenzimide (Hoechst 33342) appear pink; inner cell mass (ICM) nuclei labelled with bisbenzimide appear blue.

laparoscope. No discoloration or infection of the reproductive organs was apparent. All ewes behaved normally with the collection device in place and exhibited no signs of discomfort. In one ewe, the catheter remained functional for a period exceeding 70 days without apparent discomfort or harmful effects to the ewe.

3.7.1 Construction of catheters

The efferent oviduct catheters were about 40-45 cm in length and were prepared from Silicone tubing (1.0 mm ID; MAERSK Medical Sydney Australia). The catheters had a circular ridge of Silastic (0.062 in, ID x 0.125 in ID; Dow Corning Corporation Medical products Midland, Michigan, USA) placed 5 mm from one end or a sleeve of Silastic tubing placed at the same position. All items were packed and autoclaved prior to surgery.

3.7.2 Catheterization of ovine oviducts

Anaesthesia was induced with Pentothal (10-mg/kg body weight, Thiopentone sodium, Merial Australia Pty. Ltd., NSW, Australia) and maintained with fluothane. The reproductive tract was exposed by mid-ventral laparotomy. The ridged end of a catheter was inserted into the proximal end of the oviduct to a distance of 1-1.5 cm and fixed into position with two ligatures of 4/0 silk placed in front of and behind the ridge. The distal end of the oviduct was ligated with 4/0 Vicryl near the utero-tubal junction to ensure that no contamination with uterine secretions occurred. The free end of each catheter was then exteriorised through an incision in each flank and attached to the Eppendorf collecting tube. The abdomen was closed using Vicryl #1 and the sheep allowed to recover. The Eppendorf tubes were held in position using Setonet (Seton Products Ltd., Oldham, England, UK). Following recovery, the Eppendorf tubes were properly positioned and covered with Elastoplast (Elastic adhesive unstretched bandage 7.5 cm; BSN-Medical Pty. Ltd., Clayton, Australia. Three ml of Norocillin L.A. (Norbrook Laboratories Ltd., UK) was given (i.m.) post-operatively and repeated 48 h later.

3.7.3 Oviductal fluid collection tubes (pouches) and catheters

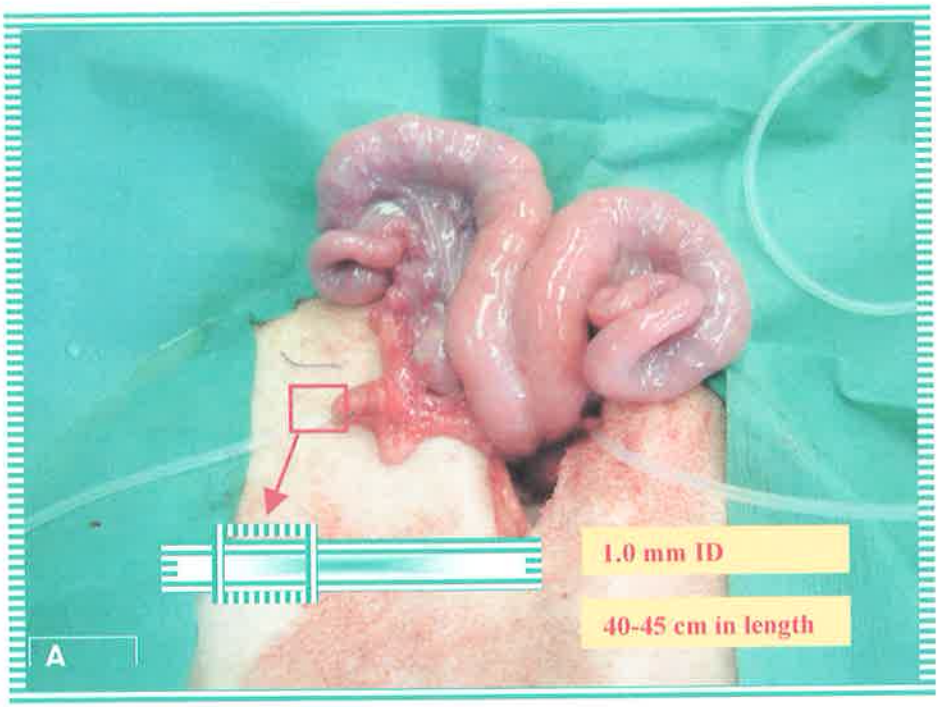
The collection tubes were attached to each side of the ewe (Fig. 3.2). An 18-gauge needle, attached to each catheter, was passed through a small hole into the lid of an Eppendorf tube. The needle was loosened to facilitate collection of oviductal fluid

and the tube was changed daily. Elastoplast was applied so that it could be folded to completely enclose the collecting tube.

Oviductal fluid was collected and analysed from both the left and right oviduct for the non-superovulated group but a pooled sample from the left and right oviduct was analysed for the superovulated sheep. Fluid volume, pH and osmolarity were measured immediately after collection in the oviductal fluid collected from 17 days prior to ovulation until 6 days thereafter. Samples from each animal were analysed independently and values reported are means of daily samples when volumes were sufficient for all the analyses. The volume of fluid was determined by weighing the tube. Osmolarity was measured using an advanced Micro-osmometer (Model 3MO plus, Advanced Instruments, Massachusetts, USA) and fluid pH recorded (Cyberscan 500, Eutech Cybernetics, Singapore) prior to centrifugation (MIKRO 12-24 Hettich Zentrifugen, HD Scientific Sydney). Appendices 8.4; 8.5 and 8.6 record examples of the daily variation in sample volumes, pH and osmolarity of the oviductal fluid collected during a typical oestrous cycle. The profiles of ammonia, urea, amino acids, progesterone, electrolytes and IGF-I of oviductal fluid were also measured but only during the peri-ovular period i.e. from Day 0 to Day 6.

3.7.4 Deproteinization of the sample

Oviductal fluid samples were collected daily and deproteinized immediately following collection. This was carried out by adding 50ul/ml of 4 mM Norleucine (Sigma) and then one part of 10% chilled sulphosalicylic acid (SSA; Sigma) solution to 4 parts of the sample. The resulting solution was subsequently cooled at 4^0C for about 30 min before centrifugation at 14000g for 10 minutes. Each supernatant was transferred to a sterile 1.5 ml Eppendorf tubes and stored at -80^0C until analysis.



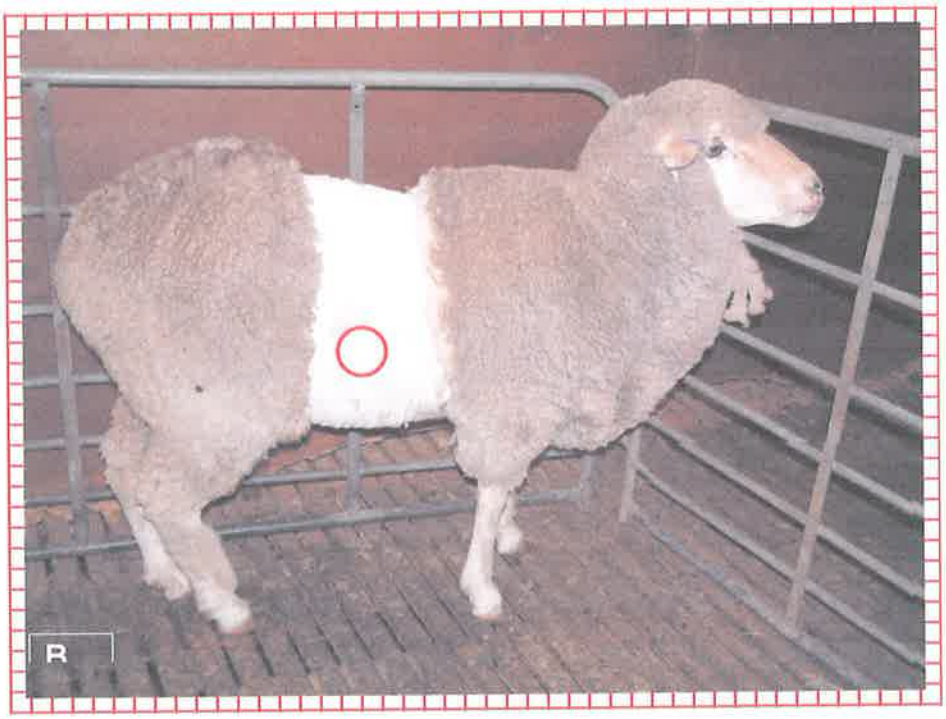


Fig. 3.2 (A) Oviductal catheters in position in both the oviducts. The efferent oviduct catheters (40-45 cm in length and 1 mm in diameter) were prepared from silicone tubing with a circular ridge of silastic placed 5 mm from one end. (B) A ewe 2-3 weeks after surgery indicating the position of the collecting tubes.

3.8 Determination of urea and ammonia

Urea and ammonia concentrations were determined in an autoanalyser (Dadebehring *Dimension* RxL Clinical Chemistry System, Newark, DE, USA). The urea nitrogen method employs an urease/glutamate dehydrogenase coupled enzymatic technique (Talke and Schubert, 1965). Urease specifically hydrolyses urea to form ammonia and carbon dioxide. The ammonia is used by the enzyme glutamate dehydrogenase (GLDH) to reductively aminate α-ketoglutarate (α-KG), with simultaneous oxidation of reduced nicotinamide adenine dinucleotide (NADH). The change in absorbance at 340 nm due to the disappearance of NADH is directly proportional to urea concentration in the sample and was measured using a bichromatic (340, 383 nm) rate technique.

$$\text{Urea} + H_2O \xrightarrow{\text{Urease}} 2NH_3 + CO_2$$

$$NH_3 + \alpha\text{-KG} + \text{NADH} \xrightarrow{\text{GLDH}} \text{L-glutamate} + \text{NAD}$$

Oviductal ammonia concentrations were determined using an adaptation of the glutamate dehydrogenase enzymatic method of Van-Anken and Schiphorst (1974) in which the substitution of NADPH for NADH eliminates interferences from other NADH-consuming reactions (da Fonesca-Wollheim, 1973). Glutamate dehydrogenase catalyses the condensation of ammonia and α-ketoglutarate with simultaneous oxidation of reduced nicotinamide adenine dinucleotide. The decrease in absorbance at 340 nm, due to the disappearance of NADH, is directly proportional to the ammonia concentration in the sample and was measured using a two cuvette (reagent blank) bichromatic (340, 383 nm) rate technique.

$$NH_4 + \alpha\text{-KG} + \text{NADH} \xrightarrow[\text{pH 7.0}]{\text{GLDH}} \text{L-glutamate} + NAD^+ + H_2O$$

Oviductal fluid sample levels of urea and ammonia were measured and the respective intra-assay coefficients of variations (CV), based on low, medium and high control

pools, were 5.1% and 9.8%. The sensitivities of the assays were 0.1 mmol l^{-1} and 4 μmol l^{-1}, respectively.

3.9 Determination of electrolytes

All of the electrolyte concentrations were determined in an autoanalyser (Dadebehring Dimension RxL Clinical Chemistry system, Newark, DE, USA). Concentrations of sodium, potassium, calcium, magnesium, phosphate, chloride, and bicarbonate were from Day o to Day 6 after ovulation. Samples from the superovulated and non-superovulated groups were analysed separately.

3.9.1 Sodium, potassium, chloride: The Na^+, K^+ and Cl^- methods use indirect sample sensing with the QuikLYTE Integrated Multisensor Technology (IMT) to develop an electrical potential proportional to the activity of each specific ion in the sample. Five electrodes were used to measure electrolytes on the *Dimension* system. Three of these electrodes were incorporated into the QuikLYTE Integrated Multisensor and were ion selective for sodium, potassium and chloride. A reference electrode was also incorporated in the multisensor. After each sample was positioned in the sensor, Na^+, K^+ or Cl^- ions established an equilibrium with the electrode surface and a potential was generated proportional to the logarithm of the analyte activity in the sample. The electrical potential generated by a sample was compared with electrical potential generated on a standard solution and the concentration of the desired ions was calculated by use of the Nernst equation (Eisenman *et al.,* 1957; Frant and Ross, 1970; Sternberg *et al.,* 1976).

3.9.2 Calcium: The calcium method is a modification of the calcium o-cresolphthalein complexone (OCPC) reaction originally reported by Sarker and Chauhan (1967). Calcium reacts with OCPC to form a purple complex. The amount of complex thus formed was proportional to the calcium concentration in the oviduct fluid and is measured using a bichromatic (577, 540 nm) endpoint technique. Magnesium ions, which also form a coloured complex with OCPC, were removed from the reaction by complexation with 8-quinolinol.

$$CA^{++} + OCPC \xrightarrow{pH\ 9.7} \text{Ca-OCPC complex (absorb at 577 nm)}$$

$$Mg^{++} + \text{8-quinolinol} \longrightarrow \text{Magnesium quinolinate}$$

(nonabsorbing at 577 nm)

3.9.3 Magnesium: The magnesium method is a modification of the methylene blue (MTB) complexometric procedure described by Connerty *et al.* (1971). MTB forms a blue complex with magnesium, which is proportional to the magnesium concentration and was measured using a bichromatic (600 and 510 nm) endpoint technique.

$$Mg^{++} + MTB \longrightarrow \text{Mg-MTB complex (Absorbs at 600 nm)}$$

3.9.4 Phosphorus: The phosphorus (PHOS) method is a modification by Gomori (1941) and Drewes (1972) of the classical phosphomolybdate method introduced by Fiske and Subbarow (1925). Organic phosphate combines with molybdate (MoO_4) in an acid solution to form a complex, which is reduced by p-methylaminophenol sulphate (PMAPS) and Na bisulphite. The 340 nm absorbance of the reduced phosphomolybdate solution is proportional to the inorganic phosphorus concentration and was measured using a bichromatic endpoint technique.

$$NaMoO_4\text{-}3 \longrightarrow \text{Phosphomolybdate}$$

$$\text{Phosphomolybdate} + \text{PMAPS} + NaHSO_3 \xrightarrow{\text{pH 1.6}} \text{Reduced Phosphomolybdate Complex (absorbs at 340 nm)}$$

3.10 Determination of progesterone

Progesterone was determined by *in vitro* quantitative electrochemiluminescence immunoassay (ECLIA) (Guillaume *et al.,* 1987; Johnson *et al.,* 1993), using a Roche Elecsys module immunoassay analyser (Roche Diagnostics GmbH, D-68298 Mannheim). The intra-assay coefficient of variation was 8.3%; the interassay coefficient of variation was 11.9% and the sensitivity (defined by the lower detection limit and the maximum of the master curve) of the assay was 0.095-191 nmol/l. Briefly, the assay consisted of two incubation steps, 1st incubation: 30 µl oviductal fluid sample - in the presence of a biotinylated monoclonal progesterone-specific antibody and a progesterone derivative labelled with ruthenium complex were incubated with Danzol to release progesterone which competes with the labelled progesterone derivatives for the antibody-binding site. 2nd incubation: after addition of streptavidin labelled microparticles, the complex produced is bound to the solid phase microparticles via biotin-streptavidin interaction. The proportion of the labelled

progesterone bound to the solid phase is inversely proportional to the progesterone content of the sample.

3.11 Determination of amino acids

Names, codes and classes of amino acids are given in Table 3.3. The profile of oviductal amino acids was determined using a Waters Alliance HPLC, fitted with a Waters AccQ.Tag Amino Acid Analysis column and a scanning fluorescence detector. The methodology was adapted from that used for pig plasma samples (Reverter *et al.*, 1997) and was developed in conjunction with the Pig and Poultry Production Institute at Roseworthy, South Australia. Samples were buffered and derivatised using the Waters AccQFluor Reagent Kit.

3.11.1 Chemicals: The derivatisation reagent AQC (Waters AccQ.Fluor Reagent), borate buffer (Waters AccQ.Fluor Borate Buffer), eluent A (Waters AccQ.Tag Eluent A, concentrate) and amino acid standards were obtained as a kit from Waters Assoc. (Mildford, MA, USA). Acetonitrile (HPLC grade), sulphosalicilic acid, DL-α-amino-*n*-butyric acid and sodium dihydrogen phosphate monohydrate were purchased from Sigma. High purity water was supplied by a Milli-QTM purification system (Millipore, Bedford, MA, USA).

3.11.2 Standard samples: Amino acid standard mixtures for calibration runs were prepared by diluting each stock solution 25 times with water and by adding the internal standard α- aminobutyric acid (ABA) (100 pmol μl^{-1} in 0.1 *M* HCL). The final concentration of ABA was 50 pmol μl^{-1} and for the other amino acids 45.45 pmol μl^{-1}. Before derivatization, 15 µl of each amino acid mixture was transferred to a vial containing 70 µl of the AccQ.Fluor borate buffer (pH 8.7).

3.11.3 Oviductal fluid samples: In order to obtain an optimal pH between 8.2 to 10.0 for derivatization, 120 µl of deproteinised oviductal fluid was diluted with 800 µl of the AccQ.Fluor borate buffer. Before derivatisation, 10 µl of internal standard (100 pmol μl^{-1}) were added.

3.11.4 Derivatisation reagent and procedure: 70 µl of deproteinised oviductal fluid diluted with borate buffer was pipetted into a 6×50 mm tube and mixed with 10 µl of

internal standard (ABA). The derivatisation reaction was then initiated by addition of 27 µl of AQC-reagent. After one min incubation at room temperature, the content was transferred to an autosampler vial and capped with a silicone-lined septum. The vial was placed in a heating block for 10 min at 55 ^{0}C in order to finish the derivatisation procedure. After that, the samples were ready for injection into the HPLC.

***3.11.5 Chromatographic analysis*:** Separations were carried out on a reversed-phase column (150×3.9 mm I.D. AccQ.Tag C_{18}) with a flow rate of 1.0 ml min^{-1} and performed at 37^{0}C. For regeneration, the column was washed for 10 min with 100% of eluent and then equilibrated for 9 min. The original eluent conditions were adjusted following the method described by Waters (1993). A fluorescence detector was used to quantify the content of all amino acids.

3.12 Measurement of Insulin-like Growth Factor-I (IGF-I)

Measurement of IGF-I was carried out in the *Gropep* Ltd. Laboratory Adelaide, South Australia. Oviductal fluid was acidified by diluting into 0.8M acetic acid, 0.2M trimethylamine, pH 2.8 (80% v/v), mixed and incubated at room temperature for 30 min to dissociate IGFs from IGF binding proteins. Following incubation, acidified oviduct fluid was centrifuged at 10 000g through a 0.1µm Micro-spin centrifuge filter (Alltech Associates Inc. Deerfield, IL, USA.) and the filtrate transferred to glass high performance liquid chromatography vials. IGFs and IGFBPs were separated from one another by high performance size exclusion liquid chromatography using a Protein-Pak 125 (Waters Corporation, Milford, MA, USA) and 0.2M acetic acid, 0.05M trimethylamine, pH 2.8 at a flow rate of 1ml/min, using a modification of the original procedure (Scott and Baxter, 1986), as described previously (Owens *et al.,* 1990). Oviductal fluid IGF-I was collected in a single 2ml fraction directly from the HPLC and its concentration measured by an IGF-I-specific enzyme linked immunosorbent assay (ELISA) using the manufacturer's instructions (Diagnostic Systems Laboratories, Inc. Webster, Texas, USA).

Table 3.3 Names, codes and classes of amino acids[†].

Code	Name	Class	Code	Name	Class
ARG	Arginine	E	THR	Threonine	E
CYS	Cysteine	E	TYR	Tyrosine	E
HIS	Histidine	E	ALA	Alanine	NE
ILE	Isoleucine	E	ASP	Aspartic acid	NE
LEU	Leucine	E	GLY	Glycine	NE
LYS	Lysine	E	GLU	Glutamic acid	NE
MET	Methionine	E	PRO	Proline	NE
PHE	Phenylalanine	E	SER	Serine	NE
VAL	Valine	E			

[†]According to Sigma's MEM essential (E) and non-essential (NE) amino acid classification for cell cultures (Sigma Chemicals, 1998).

Chapter 4

EFFECTS OF SHORT-TERM FEED INTAKE DURING THE PERI-CONCEPTIONAL PERIOD ON EMBRYO QUALITY IN THE SHEEP

4.1 INTRODUCTION

Whilst it has been established that dietary intake can influence reproductive function in ruminants, the relationship between nutrition and reproduction is complex and responses are often quite variable and inconsistent (O'Callaghan and Boland, 1999). In sheep, increased dietary intake for a relatively short time before mating can result in an increase in ovulation rate (Gunn *et al.,* 1979; Haresign, 1981; Rhind *et al.,* 1985), and acute restrictions in dietary intake can reduce ovulation rate (Yaakub *et al.,* 1997). The practice of 'flushing' has been used to increase the incidence of multiple ovulations. Ewes that are in good body condition already have a high ovulation rate, usually close to their genetic potential, so they respond poorly to flushing (Downing and Scaramuzzi, 1991). Investigations involving the feeding of high or low levels of ration to ewes during the first week of pregnancy have usually focused upon embryonic mortality. For example, Edey (1968), Foot and Russel (1979) and Parr and Williams (1982) failed to detect significant effects on embryonic growth by different levels of nutrition offered during the first week of pregnancy. Several studies indicate that excess energy intake reduces the response to superovulation and so decreases the yield of embryos, furthermore excess nutrition intake also alters expression of some gene constructs within the developing embryo (Boland *et al.,* 2001). Apart from changes in follicle growth, ovulation and embryonic development, dietary restriction can affect other components of the reproductive system; for example, plasma progesterone concentrations in sheep have been shown to be lower in animals on a high dietary intake (Parr, 1992; Yaakub *et al.,* 1997). This is probably due to an increase in the blood flow through the liver in ewes fed at above maintenance that results in increased metabolism of progesterone by hepatic tissue. Progesterone concentrations are fundamental to oocyte and early embryonic development (McKelvey *et al.,* 1986).

Nutrition has both short-term and long-term effects on various parameters of livestock reproduction (O'Callaghan and Boland, 1999). Dietary intake can affect plasma concentrations of steroids such as progesterone (see above) and also intra-follicular concentrations of factors such as IGF-1 and IGF-2 (Houseknecht *et al.,* 1988). High nutrition has a negative effect on oocyte quality (cumulus cover and cytoplasm granulation), with animals on *ad libitum* high-energy diets particularly at risk (O'Callaghan *et al.,* 2000). Furthermore, several studies have indicated that excessive

feeding is associated with a reduction in the yield and quality of embryos in cattle (Blanchard *et al.,* 1990; Mantovani, *et al.,* 1993; Nolan, *et al.,* 1998; Boland and Callaghan, 1999; Butler, 2000) and sheep (McEvoy *et al.,* 1995a). It has been suggested that these results are due to changes primarily at the level of the follicle or oocyte (Dunne *et al.,* 1997; Boland *et al.,* 2001). Deleterious effects of excessive nutrition around the time of mating on embryo development are also evident following superovulation (Mantovani *et al., 1993;* Yaakub *et al.,* 1999; Nolan *et al.,* 1998). Mantovani *et al.* (1993) reported that superovulatory responses in cattle were lower and the yield of good quality embryos reduced in heifers that had been exposed to high levels of nutritional concentrates in association with reduced roughage. Despite substantial progress being made in understanding factors that control follicle growth (Adams, 1994) and ovulation (Gong *et al.,* 1993), the mechanism by which nutrition influences embryo quality is not clear. Maurasse *et al.* (1985) reported that high feed intake in heifers can influence ovarian function by reducing the number of large non-atretic follicles and increasing the number of small non-atretic follicles. Assey *et al.* (1994) reported that impaired developmental capacity of superovulated oocytes was associated with a number of subcellular abnormalities (structural changes in the degree of detachment of interchromatin-like granules within the oocyte nucleolus) compared with oocytes from untreated cows; the question arises as to whether nutritional excess may exacerbate this effect.

Acute decreases in nutrient intake can alter embryo quality (Yaakub *et al.,* 1997). McEvoy *et al.* (1995) concluded that by restricting energy intake in ewes to 0.6 times maintenance requirements, *in vivo* and *in vitro* development of embryos could be enhanced compared with ewes that received 2.3 times daily maintenance requirement. Restricting dietary intake prior to insemination can also increase pregnancy rates in cattle (Dunne *et al.,* 1997). In superovulated beef cattle, Mantovani *et al.* (1993) reported that the yield of transferable embryos was significantly reduced when heifers had access to *ad libitum* concentrates compared with restricted concentrates for 100 days before superovulation.

While the effects of an elevated plane of nutrition on embryo quality are evident in superovulated ewes maintained on improved diets for extended time periods (up to six months) (Maurasse *et al.,* 1985; Sutton *et al.,* 1986; Dunn and Moss, 1992; Yaakub *et*

al., 1997; O'Callaghan and Boland, 1999; Boland *et al.,* 2001), less is known of the effects of short-term changes in feed intake. There is also little information, regarding the effects of the level of feed intake during the cycle of conception on embryo development. The primary objective of this study was thus to examine the effect of changes in feed intake (from 18 d before until 6 d after conception) on embryo production and quality in the superovulated ewe. The results of this study indicated that level of feed intake significantly influenced several parameters including blastocyst cell number and cell lineage differentiation. Consequently, a second experiment was conducted to determined if these changes resulted from nutritional influences during preovulatory or postovulatory periods.

4.2 MATERIALS AND METHODS

Chapter 3 provides information on the source of animals (3.1), the care of animals (3.2), the superovulation protocol (3.4) and the procedures of artificial insemination and embryo collection (3.5) and differential staining (3.6).

4.2.1 Feed intake

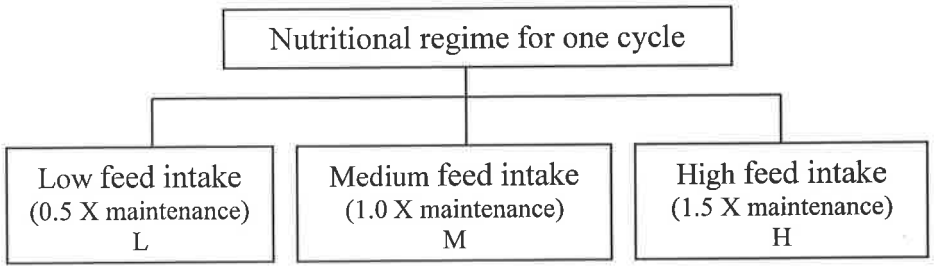
All ewes (4-5 years of age and of comparable liveweight weight (55-65 kg) and condition (3.0-3.5)) were weighed at the beginning and end of each experiment. All 48 ewes were kept for at least two weeks on a maintenance ration before the commencement of each experiment (Figs. 4.1 and 4.2). The feed intake treatments were imposed from 18 d before until 6 d after the expected time of ovulation. Daily energy needs for maintenance for individual sheep determined the net feed intakes (Table 3.1).

4.2.2 Experimental design

Experiment 1 aimed to examine the effect of short-term changes in feed intake (18 d before until 6 d after the expected time of ovulation) on blastocyst development and quality. This experiment was designed as a randomised complete block design with three blocks of 16 sheep (Fig. 4.1 and 4.2). Ewes were randomly allocated to one of three dietary treatments: high (1.5 x daily energy needs for maintenance), medium (1.0 x daily energy needs for maintenance) and low (0.5 x daily energy needs for maintenance). Daily energy need for maintenance was calculated using the computer

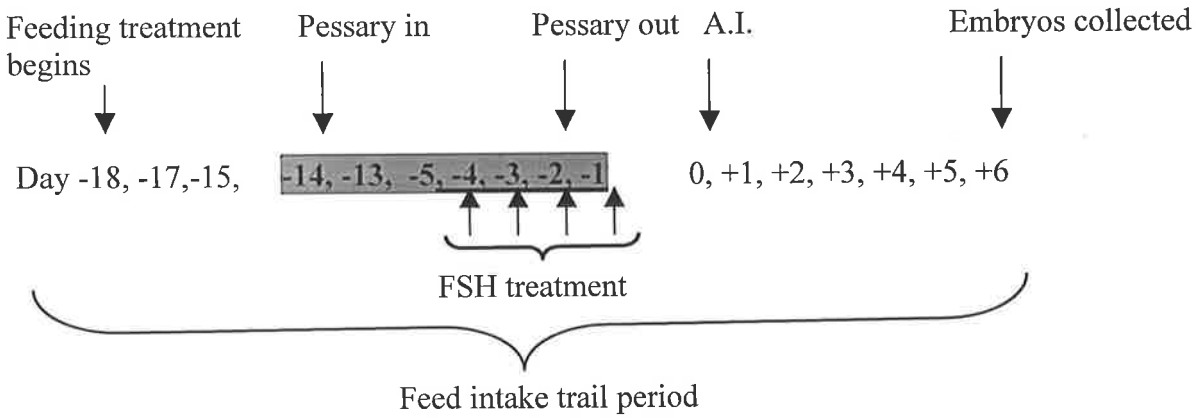
program Rumnut (Pullman and Hughes, 1986). These treatments are identified by the H, M and L notations respectively.

Fig. 4.1. Design of Experiment 1.



Regime	-18 Days	0 Day	+6 Days
1	H	H	H
2	M	M	M
3	L	L	L

Fig. 4.2 Experimental design for experiment 1 and 2 indicating the timing of synchronisation, superovulation, A.I. and embryo collection in sheep



Experiment 2 aimed to discriminate between the effects of feed intake influences during the preovulatory and postovulatory periods on embryo development. In this experiment, 76 ewes were randomly allocated to 12 randomised blocks on the basis of their initial weight. Within each block, between 6-8 sheep were randomly assigned to each of six nutrition regimes. These regimes consisted of a combination of the dietary treatments used in Experiment 1 but were for different lengths of time (Table 4.1 and Fig. 4.2). For example, sheep assigned to Treatment 1 were fed a diet with a high feed intake for the course of the experiment (HHH) and sheep assigned to Treatment 6 were fed a low feed intake for the course of the experiment (LLL). In comparison, sheep assigned to Treatments 2 to 5 were fed the M diet, for the first six days of the experiment (Day –12 to -6), before being randomly assigned to H or L diets at Day –6 and again randomly assigned to H or L diets after the expected time of ovulation (Day 0) until embryo collection at +6 days (Table 4.1 and Fig. 4.2). For the purpose of this study only the level of feed intake between –6 to 0 days and 0 to +6 days were analysed.

Table 4.1. The design for Experiment 2 indicating the periods of nutritional treatment relative to the day of ovulation (Day 0). Ewes were fed a high (H, 1.5 x maintenance), medium (1 x maintenance) or low (0.5 x maintenance) feed intake.

Regime	-18 to -6 Days	-6 to 0 Days	0 to +6 Days
1	H	H	H
2	M	H	H
3	M	H	L
4	M	L	H
5	M	L	L
6	L	L	L

4.2.3 Statistical analyses

Analyses of the two experiments were performed in GenStat for Windows, 5th Edition (Harding *et al.,* 2000). The results of Experiment 1 were analysed by Restricted Maximum Likelihood (REML) (Patterson and Thompson, 1971). REML is similar to analysis of variance (ANOVA) in that it accounts for more than one source of variation. However, REML has the added advantage of being able to handle unbalanced data. In a REML analysis the treatment effects are tested using a Wald test at the 5% level. This test statistic is distributed approximately as for Chi-square, with the appropriate degrees of freedom. The assumptions of REML analysis are that the data are normally distributed with constant variance. The validity of these assumptions can be examined using various diagnostic plots, such as the residuals versus fitted values plot. These plots were examined for each parameter and there was no indication that these assumptions were violated.

The null hypothesis was that there was no significant difference between the dietary treatments. However, for Experiment 2 this treatment allocation would not detect whether there was a significant difference between the High and Low feed intakes before, compared with after mating. Therefore an alternative treatment structure was considered so that the interaction between the pre-mating (-6 Days) diet allocation (H or L) and post-mating (after A.I) diet allocation (H or L) could be detected. Hence a nested model was considered, in which the factorial structure for pre-mating (with two levels, L and H) and post-mating (also with 2 levels) occurs within a factor treatment that distinguishes between the controls (either LLL or HHH) and the modified diets.

4.3 RESULTS

4.3.1 Experiment 1

Mean live weight was significantly ($P<0.05$) lower in the L group than in other groups at the end of the study period (Table 4.2). All groups lost weight during this period although the loss was not significantly different between the H and M treatments. The mean number of corpora lutea and the number of embryos collected as a proportion of the number of corpora lutea were not significantly influenced by diet. Of those embryos collected, not all had reached blastocyst and so an analysis was performed on the proportion of embryos that did not reach blastocyst. There were no statistically

significant differences between diets for this response (P=0.1). However, embryos from sheep fed the L diet had significantly (P< 0.05) more total cells (74.7 ± 1.45) compared with those from sheep fed the H diet (62.0 ± 0.84) (Fig. 3.1; Table 4.2). The TE:ICM ratio for sheep fed the L ration (0.73 ± 0.04) was also significantly (P < 0.05) higher than for the H (0.69 ± 0.03) or M (0.70 ± 0.04) ration (Table 4.2).

4.3.2 Experiment 2

Body weight was significantly (P<0.05) reduced and weight loss was significantly greater (P<0.05) in those sheep fed the L ration regardless of whether this treatment occurred before or after mating. The mean number of corpora lutea and the number of embryos collected as a proportion of the number of corpora lutea were not significantly influenced by diet (Table 4.3). Significantly (P<0.01) fewer embryos developed to the blastocyst stage in sheep fed the H diet after mating compared with the L diet (Table 4.4). The total number of cells per embryo was significantly (P<0.05) reduced for sheep receiving the H ration for the whole trial period or after mating compared with other rations (Table 4.5). However, this figure did not differ significantly when treatments were imposed before mating (P=0.488). The TE:ICM ratio differed significantly (P<0.001) between treatments only when treatments were applied after ovulation, with L sheep having more TE cells than H sheep (Table 4.6).

Table 4.2. The effect of feed intake in Experiment 1 (low, medium and high diets) on parameters of superovulation and on cell numbers in blastocysts collected on Day 6 (day 0 = day of ovulation). (Values are least square means ± SEM).

	Low	Medium	High
Final weight (kg)*	55.3±1.8^{a} (n=16)	61.9±1.8^{b} (n=15)	63.1±1.9^{c} (n=15)
Weight change (kg)	-7.8±0.7^{a} (n=16)	-1.3±0.4^{b} (n=15)	-0.1±0.6^{b} (n=15)
No. of corpora lutea	15.4±1.4 (n=16)	15.1±1.6 (n=15)	16.1±1.6 (n=14)
Proportion of embryos	0.34±0.1 (n=16)	0.31±0.1 (n=15)	0.47±0.1 (n=14)
Proportion not blastocyst	0.09± 0.0 (n=9)	0.04±0.0 (n=7)	0.18±0.0 (n=8)
Total cell no.	74.7±1.5^{a} (n=85)	66.4±1.4^{ab} (n=76)	62.0±0.8^{b} (n=85)
Proportion of TE	0.73±0.04^{a} (n=85)	0.70±0.0^{b} (n=76)	0.69±0.0^{b} (n=85)

* Final weight after adjusting for covariate (initial weight)

^{a,b} Values within rows with different superscripts are significantly different ($P<0.05$)

Fig. 4.3. Ewes were housed in single pens exposed to natural photoperiod. Each pen had an individual feed bin and water was provided *ad libitum*.

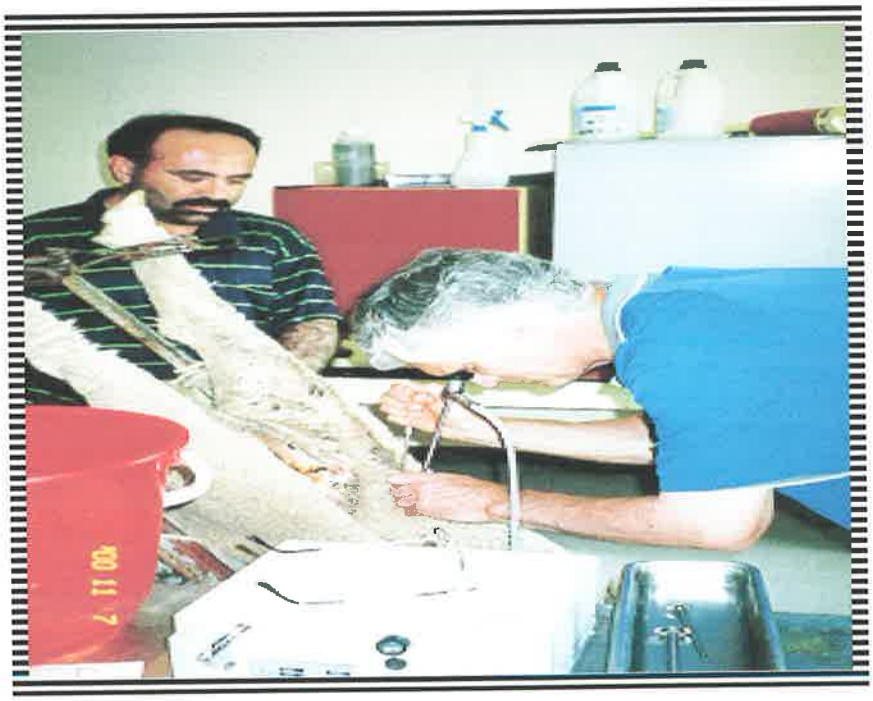


Fig. 4.4. Laparoscopic insemination of ewes with fresh collected semen. Approximately 20 x 10^6 spermatozoa were placed directly into the lumen of each uterine horn.

Table 4.3 Effect of feed intake on total corpora lutea, embryo number and embryo loss in Experiment 2.

Treatment	Corpora lutea			Embryos			Embryos lost	
	Left	Right	Total	L-Horn	R-Horn	Total	No.	%
HHH	127	107	234 (13)	52	57	109 (11)	125	53.4
MHH	97	116	213 (12)	62	60	122 (10)	91	42.7
MLH	103	88	191 (14)	43	39	82 (9)	109	57.1
MHL	106	114	220 (13)	44	52	96 (9)	124	56.3
MLL	95	102	197 (12)	44	34	78 (8)	119	60.1
LLL	81	91	172 (12)	46	45	91 (9)	81	47.1

Numbers of animals are shown in parentheses

Table 4.4 Effect of feed intake on number and stage of embryos collected on Day 6 in Experiment 2.

Treatments	No. ewes (Regressed CL No.)	No. embryos	Stage of embryos		Blastocysts (%)
			Blastocyst	Morula	
HHH	13 (2)	109	17	92	88 (80.1)[a]
MHH	12 (2	122	17	105	93 (76.2)[a]
MLH	14 (5)	82	13	69	63 (76.8)[a]
MHL	13 (4)	96	19	77	80 (83.3)[b]
MLL	12 (4)	78	12	66	70 (89.7)[b]
LLL	12 (3)	91	11	80	80 (87.9)[b]

[a,b] Values within columns with different superscripts differ significantly ($P<0.05$)

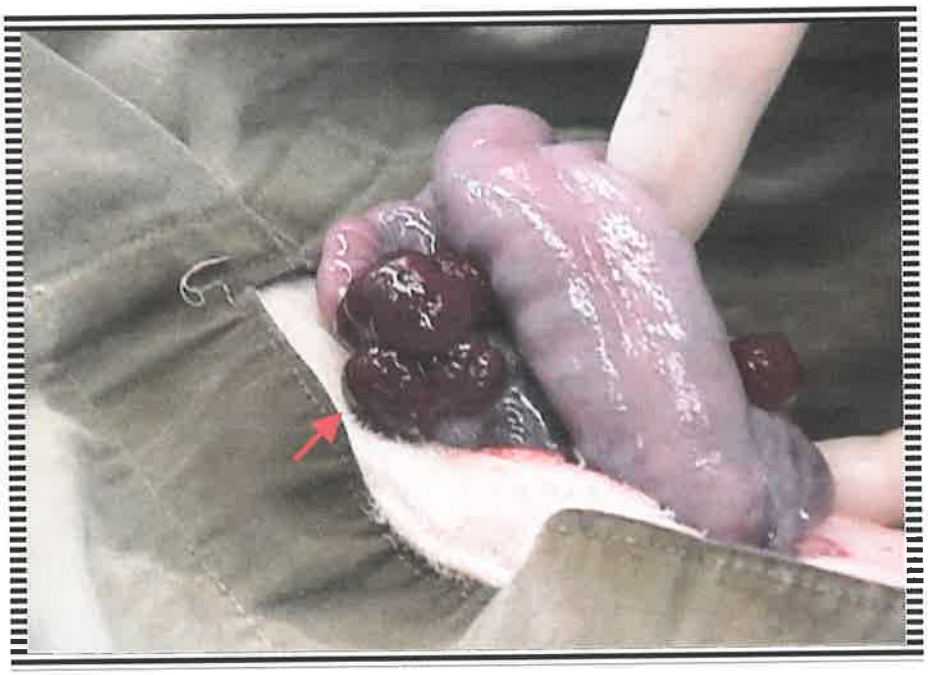


Fig. 4.5. Exposed uterus and ovary showing corpora lutea (arrow). Six days after insemination, embryos were collected under general anaesthesia by mid-ventral laparotomy and the numbers of corpora lutea were recorded on each ovary.

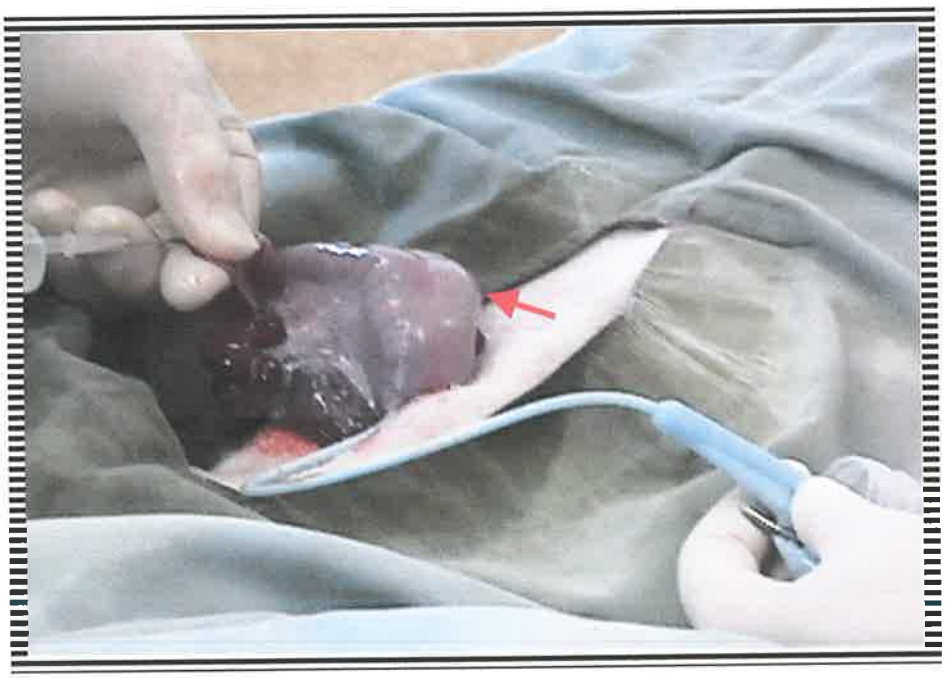


Fig. 4.6. Embryo collection procedure. Each uterine horn (arrow) was flushed with 20 ml of PBS containing 5% heat-inactivated sheep serum. Embryos were recovered from the flushing medium within 5 min of collection using a stereomicroscope.

Table 4.5 The effect of feed intake (low, medium or high) between 6-12 d before ovulation, 6 d before ovulation until the day of ovulation and between 0-6 after ovulation on body weight, superovulatory response and cell numbers in blastocysts collected on day 6 (day 0 = day of ovulation). (Values are least mean ± SEM) (Experiment 2)

Treatment	HHH	MHH	MHL	MLH	MLL	LLL
Final weight (kg)*	64.6 ± 2.42 (13)†	62.6±1.93 (12)	58.6±1.78 (13)	60.3± 2.66 (14)	57.7± 1.77 (12)	54.9±1.95 (12)
Change in wt. (kg)	1.00± 0.61^{a} (13)	-0.88± 0.63^{a} (12)	-4.82± 0.82^{b} (13)	-3.30± 0.84^{a} (14)	-5.79± 0.77^{b} (12)	-8.60± 1.06^{b} (12)
No. corpora lutea	18.1± 2.63 (11)	17.7± 1.84 (10)	14.8± 2.92 (7)	13.9± 2.69 (9)	16.42±1.98 (8)	14.3± 2.49 (9)
Blastocyst/CL	0.16± 0.04^{b} (129)‡	0.24± 0.03^{a} (121)	0.16± 0.04^{b} (96)	0.22± 0.05^{a} (82)	0.10± 0.03^{b} (78)	0.12± 0.03^{b} (91)
TE:ICM proportion	0.72± 0.04^{a} (88)	0.72±0.03^{a} (93)	0.74± 0.05^{b} (80)	0.72± 0.04^{a} (63)	0.76± 0.05^{b} (70)	0.76± 0.03^{b} (88)
Total cell no.	74.0± 1.20^{a} (88)	71.8± 1.11^{a} (93)	85.1±1.85^{b} (80)	70.0± 1.49^{a} (63)	88.0±1.89^{b} (70)	86.51±1.38^{b} (88)

* Final weight after adjusting for covariate (initial weight)

† ( ) Total number of animals

‡ [ ] Total number of embryos collected or stained

^{a,b} Values within rows with different superscripts differ significantly ($P<0.05$)

Table 4.6 Partitioning the effect of feed intake into comparisons with a single degree of freedom (Experiment 2).

	Final weight	Change in weight	No corpora lutea	Proportion of embryos	Blastocyst /corpora lutea	Total cell numbers	TE:ICM ratio
Diet (5 df) †	P < 0.001	P < 0.001	n.s	n.s	P = 0.039	P < 0.001	P < 0.001
Pre. X Post (1 df)‡	P = 0.173	P = 0.17	NA	NA	P = 0.559	P = 0.269	P = 0.147
Pre-mating (1 df) *	P = 0.003	P = 0.002	NA	NA	P = 0.285	P = 0.488	P = 0.210
Post-mating (1 df) •	P < 0.001	P < 0.001	NA	NA	P = 0.006	P < 0.001	P < 0.001

† Feed intake is partitioned into three comparisons of interest summarised below the dotted line.

‡ Interaction between pre-mating and post mating nutrition (i.e. MHH = MHL = MLH = MLL).

* MHH and MHL versus MLH and MLL.

• MHH and MLH versus MHL and MLL.

n.s – not significant at the 5% rejection level.

NA – partitioning not appropriate

4.4 DISCUSSION

This study has shown for the first time that short-term low feed intake imposed during the cycle of mating increased the total number of cells in blastocyst and that this increase was primarily due to an enhanced number of trophectoderm cells. In addition, the nutritional signals enhancing embryo cell numbers appeared to be manifested during the post mating period rather than the premating period. The lack of a difference between the total numbers of embryos produced by ewes fed low, medium and high feed intake suggests that the extra intake observed in ewes in the high diet group was partitioned towards improving ewe live weight and thus condition, rather than increasing ovulation or embryo growth. In contrast to spontaneously ovulating ewes where flushing (i.e. the increase of nutrition 4-6 week before the mating) may enhance ovulation rate (Rhind *et al.,* 1989), superovulatory responses were not compromised by restricted feeding in this study, presumably because individual potential was maximised as a result of gonadotrophic action. It must be stressed, however, that the results followed short-term feed restrictions applied to ewes, which were in good body condition. A similar gain could not be expected following either prolonged feed restriction or the imposition of the present regimen on already under-nourished animals. The lack of a significant increase in ovulation rate and number of embryos collected with an increase in food intake was unexpected and was at variance with previous reports (Smith, 1985; 1988). Lindsay (1976) described live weight as being 'a crude, inaccurate criterion which, because it describes only long-term changes in feeding, is incompatible with studies on many of the components of the reproductive process — which takes place over a few days or even hours'. This is supported by the finding of Smith *et al.* (1982) who, in summarizing a series of grazing experiments, found that ewe liveweight at mating and pre-mating liveweight change accounted for only 42.0 and 18.5% respectively, of the variation in ovulation rate. Thus nutritional factors over and above those influencing live weight appear to exert a major influence on ovulation rate as also found by Smith *et al.* (1979).

A high proportion of the embryos from ewes on the L feed intake progressed to the blastocyst stage and had higher total cell numbers than embryos from ewes on the H feed intake, although the ratio of inner cell mass to total cell number was not significantly different. The results support previously published data of Nolan *et al.*

(1998) and Negrao *et al.* (1997), who concluded that the yield of transferable embryos increased as the dietary energy intake decreased. In ruminants there is a risk that a rapid shift in rumen fermentation caused by abrupt changes in diet composition, and in the amount or pattern of feed intake may temporarily disrupt metabolic homeostasis with consequent adverse effects on the embryo (McEvoy *et al.,* 2001). Improved nutrition during the periovulatory period is generally regarded as beneficial to reproductive outcome for a naturally-occurring oestrus but this does not appear to be the case when superovulation is induced using exogenous gonadotrophins in ewes (McEvoy *et al.,* 1995a; 1995b; 2001; Lozano *et al.,* 2000; Boland *et al.,* 2001) or beef heifers (Nolan *et al.,* 1998; Yaakub *et al.,* 1999). These adverse effects of high feeding levels on embryo development are accentuated on barley-based diets that are rapidly fermented in the rumen (Yaakub *et al.,* 1999).

Total cell number has previously been reported to be a good indicator of embryo quality and development rate (Van Soom *et al.,* 1996). A sufficient proportion of inner cell mass cells are vital for normal embryonic development following transfer to recipients. The ratio of inner cell mass cells to total cells has been used as an indicator of embryo quality but it has been concluded that total number is of equal merit (Van Soom *et al.,* 1997). The results of the present study are in agreement with these data, where no difference was found in the percentage of inner cell mass cells contributing to total cell number but there was a higher total cell number recorded from embryos recovered from the ewes on a low nutrition diet compared with those on the high nutrition diet. Total cell number is consistent with changes in the developmental rate but there is no change in the differentiation of cells, which suggests that although embryo development may be retarded there is no evidence of a fundamental change in genetic programming. That embryos produced in animals fed the L feed intake had significantly higher cell numbers and developed quicker, suggests that these embryos may have been of better quality than those produced by sheep on the higher feed intake. The cell numbers were increased due to a greater proliferation of trophectoderm cells suggesting that events associated with implantation and placental development may also be affected by these early events.

Dyck and Strain (1983) suggested that the critical window for nutritional effects on embryonic survival was the early postcoital period. The present overall result shows

that the critical window for nutritional effects on embryo quality in ewes could occur in the period immediately after A.I. The results of this study show that the total number of cells within the embryo was increased in response to low feed intake after ovulation and this was due to a greater proliferation of trophectoderm cells indicating that implantation and placental development may also be affected by early dietary intake. In addition, culture conditions that caused a reduction in mouse ICM and total blastocyst cell number also reduced the embryo viability after transfer (Lane and Gardner, 1997), and a deficient ICM cell number has been identified as a potential causative component of fetal growth retardation and large placentae.

A number of peptide growth factors, expressed from either or both of the maternal and embryonic genomes, stimulate cell proliferation in the preimplantation embryo, which is mediated by signalling through their specific receptors (Kane *et al.,* 1997). In relation to our maternal nutritional data, insulin and IGF family growth factors have been shown in vitro to stimulate preferentially ICM proliferation in early blastocysts (Kaye *et al.,* 1992; Kane *et al.,* 1997). The preimplantation embryo does not express insulin and maternally derived insulin is delivered to the ICM by receptor-mediated transcytosis across the trophectoderm (Kwong *et al.,* 2000). Moreover, short-term exposure of preimplantation mouse embryos in vitro to insulin caused a long-term increase in foetal growth rate after transfer (Kaye and Gardner, 1999). In sheep, glucose metabolism is not critical for embryonic development but it is beneficial at low concentrations; conversely high concentrations can inhibit development, possibly by inhibiting the tricarboxylic acid (TCA) cycle (Thompson *et al.,* 1992). It can be concluded, that possibly the hyperglycaemic maternal environment generated by high feed intake provides the most likely mechanism to explain retarded preimplantation development and reduced total cell number, especially the reduced trophectoderm cell number.

Preimplantation embryos take up amino acids to enhance protein synthesis for growth of the embryo from the blastocyst stage (Lamb and Leese, 1994). Suboptimal exposure to amino acids might reduce the number of ICM cells and total cell number in the blastocyst as well as the rate of fetal development after embryo transfer (Lane and Gardner, 1997). The preimplantation embryo is particularly sensitive to epigenetic modifications that may have programming consequences (Reik *et al.,* 1993; Dean *et al.,*

1998), and the current data indicate that it is the preimplantation embryo itself that is programmed by nutrition in the early post ovulatory period. Some growth factors, when added to culture systems, can affect the ICM cells; for example, LIF in the cow (Margawati *et al.,* 1997), TGFβ in the cow (Marquant-Le Guienne *et al.,* 1989), insulin in the mouse (Harvey and Kaye, 1990), IGF-1 in the mouse (Harvey and Kaye, 1992) and glutathione in the cow (Kotaras and Seamark, 1997). CSF-1 in the mouse (Bhatnagar *et al.,* 1995) and IGF-1 in the buffalo (Narula *et al.,* 1996) stimulate development of TE cells. Further investigations need to determine the long-term outcome of changes in TE/ICM ratio.

Progesterone supplementation during the first few days following ovulation alters the nature of the uterine environment and has been associated with enhanced embryo development (Garrett *et al.,* 1988) and increased fetal weight in sheep by Day 74 of gestation (Kleemann *et al.,* 1994). Although fetal size was undoubtedly affected in these studies, these treatments do not consistently induce large offspring syndrome (Sinclair *et al.,* 2000c). Indeed, the temporary exposure of Day 3 ovine embryos to an advanced uterine environment failed to influence fetal growth in one study (Sinclair *et al.,* 1998), where fetuses were recovered at several time points during gestation, including close to term. Interestingly, despite the absence of differences in weight in that study, muscle fibre number and the ratio of secondary to primary muscle fibres were both significantly increased in fetuses derived from embryos temporarily exposed to an advanced uterine environment (Maxfield *et al.,* 1998). This was associated with a shift in the temporal expression of *Myf-5*, a member of the *MyoD* gene family and the gene responsible for myoblast proliferation. As there is a relationship between muscle fibre number and post-natal growth in pigs (Dwyer *et al.,* 1993), it has been speculated that the post-natal growth of lambs derived from embryos exposed to short-term nutrition changes around the time of mating may be enhanced. This hypothesis, however, remains to be tested.

The mechanism by which alterations in maternal nutrition affect embryo survival and development is not well understood. Wilmut *et al.* (1986) reviewed the literature relating circulating steroid hormone concentrations to embryo survival. They concluded that a sequence of four phases is essential: progesterone before mating, estradiol equivalent to that which induces estrous behaviour, a low (but not zero) level

of progesterone during the peri-ovulatory period, and a higher level of progesterone typical of luteal phase levels. Uterine changes essential to maintenance of pregnancy are induced by the timing of the change between peri-ovulatory levels of progesterone and luteal levels. Thus if this change was made prematurely, the uterine environment may be advanced to a stage that is not conducive to survival of the embryo.

An association between increased feed intake, decreased plasma progesterone, and increased metabolic clearance of progesterone has been shown by Parr *et al.* (1982). It has been speculated that high dietary intake induces suppression of circulating progesterone during oocyte maturation, which imparts a legacy of developmental retardation and decreased embryo survival (McEvoy *et al.,* 1995a; Robertson, 1995). Progesterone concentrations can be elevated by malnutrition and/or progesterone administration in the early stages (from Day 3-6) after fertilization, which in the sheep causes an increase in the weight of the fetus and its placenta (Kleemann, *et al.,* 1994). Early evidence indicates that progesterone treatment also changes cell lineage differentiation in the sheep, with differences in the proportion of ICM and TE (Hartwich *et al.,* 1995). The reproductive tracts of a variety of species are known sites of IGF production (Pushpakumara *et al.,* 2002). A particular feature of the IGF system is that the production of many of its components can be regulated by nutrition (McGuire *et al.,* 1992; Roberts *et al.,* 1997). This finding raises the possibility that metabolic status could alter oviductal activity via modulation of the IGF system. Oviductal secretory proteins have been identified in many species including baboons (Verhage *et al.,* 1989), cattle (Boice *et al.,* 1990), goats (Abe *et al.,* 1995), hamsters (Robitaille *et al.,* 1988), humans (Verhage *et al.,* 1988), mice (Kapur and Johnson, 1988), pigs (Buhi *et al.,* 1990), rhesus monkeys (Verhage *et al.,* 1997) and sheep (Sutton *et al.,* 1984). It has been suggested that these proteins may have some effects on early cleavage-stage development. Thus, there is a possibility that any change in post ovulatory progesterone concentration may affect early embryo quality or survival by modification of the oviductal environment and this possibility is the basis of the study reported in Chapter 5.

Chapter 5

EFFECTS OF SHORT-TERM CHANGES IN FEED-INTAKE DURING THE PERI-CONCEPTIONAL PERIOD OF THE EWE ON OVIDUCTAL FLUID COMPOSITION

5.1 INTRODUCTION

The oviduct maintains and modulates a dynamic fluid environment in which maturation of gametes is completed and the fertilization process occurs, followed by early embryonic development. Interestingly, the first cell division cycle is the stage potentially most sensitive to the influences of the oviductal environment (Boatmam, 1997). This unique milieu partly originates from a selective serum transudate and partly from specific secretions of the epithelium lining the oviduct wall (Gandolfi *et al.,* 1992). The oviduct is a most dynamic reproductive organ whose functions are yet to be fully determined (Leese *et al.*, 2001).

Diets that contain protein concentrations in excess of bodily requirements have been reported to reduce reproductive performance in both cattle and sheep (Elrod and Butler, 1993; McEvoy *et al.,* 1997). The mechanism whereby feeding high protein diets to ruminants compromises reproductive performance is not known, although a number of possible explanations have been proposed. It has been suggested that the reduction in fertility is associated with decreased embryo growth due to disruption in the uterine environment either just after insemination in dairy cows (Dawuda *et al.,* 2002) or as late as three days after insemination in sheep (McEvoy *et al.,* 1997). The reported effects of high protein diets on the uterine environment in the cow are reduced pH at seven days after insemination (Elrod and Butler, 1993) and changes in the composition of uterine secretions (Jordan *et al.,* 1983). A reduction in the number of transferable embryos in superovulated dairy cows (Blanchard *et al.,* 1990) and decreased luteal phase progesterone concentrations (Sonderman and Larson, 1989) have also been reported in response to high protein diets. It is clear from the literature that in ewes, progesterone concentrations are inversely related to dietary intake (Williams and Cumming, 1982; Parr *et al.,* 1987; 1993; McEvoy *et al.,* 1995a; Yaakub *et al.,* 1997). Adequate progesterone concentrations following ovulation are crucial for normal embryonic development within the oviduct and for the proper timing of embryo transport to the uterus (Boatman, 1997). Progesterone induces a membrane depolarisation and various ion fluxes (chloride efflux, sodium and calcium influxes), which are required for the sperm acrosome reaction (Patrat *et al.,* 2002). In most species, progesterone concentrations in the reproductive tract fluids from the time of capacitation to fertilisation are generally unknown.

High dietary protein leads to elevated systemic concentrations of ammonia and urea (Sinclair *et al.*, 2000) and these in turn have been associated with reduced fertility in cattle. Whereas evidence relating to the extent of the reduced fertility is equivocal, a possible mechanism by which elevated systemic ammonia or urea may reduce cow fertility has been proposed (Kenny *et al.*, 2002). Reports have suggested that elevated systemic concentrations of ammonia and/or urea may compromise early embryo development in the oviduct (McEvoy *et al.*, 1997; Fahey *et al.*, 2001; Dawuda *et al.*, 2002). Ammonia, applied in a time- and concentration-dependant manner during preimplantation development of mouse embryos, induced fetal retardation and exencephaly (Lane and Gardner, 1995). The oviductal fluid provides the nutritional environment for the early embryo, and therefore the metabolic and ionic composition of oviductal fluid is clearly important. However, to our knowledge, no data are available regarding the effect of feed intake on the concentrations of ammonia and urea in oviductal fluid.

Amino acids are essential ingredients in media used for *in vitro* culture of mammalian embryos; the media being originally designed for supporting growth of somatic cells in culture (Elhassan *et al.*, 2001). Apart from their use for protein synthesis, amino acids play important roles as osmolytes (Anbari and Schultz, 1993; Biggers *et al.*, 1993), intracellular buffers (Bavister and McKiernan., 1993; Edwards *et al.*, 1998), heavy metal chelators and energy sources (Bavister, 1995; Reiger, 1992). They also function as precursors for versatile physiological regulators, such as nitric oxide (NO) and polyamines (Wu and Morris, 1998). When preimplantation mouse embryos metabolise glutamine and presumably other amino acids *in vitro*, ammonium ions are produced that can adversely affect both pre-and post-implantation development (Gardner and Lane, 1993; Lane and Gardner, 1995). However, little information is available on amino acid concentrations in the ovine reproductive tract during the period of early development. Previous studies which have measured amino acid concentrations in the oviductal fluid of ewes have either only measured the concentrations on two days of the cycle (Menzo, 1973) or have combined samples from different animals (Nancarrow *et al.*, 1992; Hill, 1994) and from various stages of the oestrous cycle (Perkins and Goode, 1967). In view of this, the present study was designed to examine amino acid concentrations in the oviductal fluid of superovulated and non-superovulated ewes throughout the peri-ovulatory period with the aim of determining if feed intakes known to influence embryo development were having an associated effect on amino acid concentrations in the oviduct.

Several experiments in rodents and livestock species indicate that the growth and development of the pre-implantation embryos in the female reproductive tract is regulated by an array of cytokines and growth factors secreted from epithelial cells lining the oviduct and uterus (Pampfer *et al.,* 1991; Watson, *et al.,* 1994; Robertson *et al.,* 1994). Synthesis of growth factors and cytokines occurs in precise spatial and temporal patterns driven predominantly by ovarian steroid hormones (Sahlin *et al.,* 2001) but also by factors in seminal plasma and of maternal origin (Robertson *et al.* 1996; Tremellen *et al.,* 1998). A wide range of such factors, such as insulin (Harvey and Kaye, 1990), insulin-like growth factors (IGFs) (Gardner and Kaye, 1991), epidermal growth factor (EGF) (Wood and Kaye, 1989), colony stimulating factor-1 (CSF-1) and transforming growth factor beta (TGF β) (Paria and Dey, 1990) have all been shown to improve the secretory activity of embryonic and cultured cells and/or the proportion of mouse embryos that develop to the blastocyst stage and beyond. Exposure to insulin-like growth factor (IGF-1) *in vitro* has also been shown to benefit human (Lighten *et al.,* 1998) and bovine (Sakaguchi *et al.,* 2002; Moreira *et al.*, 2002; Mtango *et al.,* 2003) embryo development by increasing blastocyst cell number, due entirely to an increase of cells in the inner cell mass. Therefore, IGF-I increases embryonic metabolism and cell proliferation, influencing *in vitro* embryonic development (Herrler *et al.,* 1998). Furthermore, the expression of several components of the IGF system is regulated by nutrition (Wathes *et al.,* 1998). The oviductal mucosa produces IGF-1, which may influence oviductal secretions or act directly on, type 1 IGF receptors in the embryo. Thus, whilst it is reasonable to expect these growth factors (IGFs and others) to be present in oviductal fluid, there is little information regarding their concentration in oviductal fluid or how this is affected by feed intake, and there is a need to identify changes that might be implicated in causing changes in embryo development.

Given the ability of a short term change in the diet to influence embryo development (as outlined in Chapter 4), the aim of research outlined in this Chapter was to examine the way in which these dietary changes might influence the composition of oviduct fluid. The concentrations of ammonia, urea, amino acids, electrolytes, progesterone and IGF-1 were determined in oviductal fluid of both superovulated and non-superovulated (oestrus synchronised) ewes. Superovulated ewes were included in the study because of the potential value to embryo collection programs of the findings reported in Chapter 4.

5.2 MATERIALS AND METHODS

5.2.1 Collection of oviductal fluid: Merino ewes were used throughout this study and were housed in individual pens (details are given in Chapter 3: source of animals (3.1), care of animals (3.2). They were fed each morning with a maintenance ration (standard sheep pellets) and given access to water. Ewes undergoing surgery for oviduct catheterisation were housed for at least two weeks prior to surgery. Ewes were fasted for 24 h prior to surgery. All experiments involving the use of animals were carried out under approval of the Animal Care and Experimentation Committee (ACEEC) of the University of Adelaide and SARDI's committee. Experiments described in this chapter were carried out at Turretfield Research Centre, Rosedale, South Australia.

5.2.2 Feed intake: As in Chapter 4.2.1, the weights of all ewes were recorded, as was the net feed intake (Chapter 3).

5.2.3 Experimental design: This study examined the pH and osmolarity as well as the concentrations of ammonia, urea, amino acids, progesterone, electrolytes and insulin-like growth factor-I (IGF-I) in the oviductal fluid collected daily during the peri-ovular period. Twelve mature ewes (4-5 years, 58-67 kg) of comparable body condition were fed a standard diet for two weeks before the start of collection (Fig. 5.1). The experiment was designed in three randomised blocks on the basis of the initial weight of each sheep. Half of the ewes were superovulated using conventional treatment involving progestagen, FSH and GnRH treatment (Section 3.4). Oviducts were catheterised (Fig. 3.7) and after a recovery period of 18 days, oviductal fluid was collected at 9:00 am from each oviduct every 24h, for 24 days (-17 days before the time of ovulation (Day 0) and continuing until six days after ovulation). The responses considered for analysis were the initial weight, final weight and the number of corpora lutea (CLs) on each of the left and right ovary. The volume, pH and osmolarity were measured in the oviductal fluid collected from -17 days prior to ovulation and six days after the expected time of ovulation. The profiles of ammonia, urea (Section 3.8), electrolytes (Section 3.9), progesterone (Section 3.10), amino acids (Section 3.11; 3.7.4) and insulin-like growth factor-I (IGF-I) (Section 3.12) of oviductal fluid were also measured but only during the peri-ovular period i.e., from Day 0 to Day 6.

5.2.4 Statistical analysis

A split-plot structure was assumed with three replicates, where 12 sheep were randomly allocated to two feed intakes (high and low) and the day of the cycle was considered in the analysis. A repeated measure ANOVA (Crowder and Hand, 1990) was used which adjusts for the correlation that may have existed over time and corrects the degrees of freedom accordingly using a Greenhouse-Geiser estimate (Greenhouse and Geiser, 1959).

5.2.4.1 Analysis of live weight and CL: The data were analysed using analysis of variance for a randomised complete block design with three blocks and a factorial treatment structure for feed intake and superovulation. The responses of interest were the initial weight, final weight, change in weight and the number of corpora lutea (CL).

5.2.4.2 Volume of oviductal fluid, pH and osmolarity: A flexible statistical model, known as a mixed model was used to describe the relationship between each response and time for each combination of superovulation (S or NS) and feed intake (high or low). The model is considered "mixed" as it is made up of both fixed effects (treatment effects such as superovulation and feed intake) and random effects, which help explain the variation in the data (such as the variability between sheep). In order to model the pattern between each response and time for each treatment combination a smooth curve, known as a cubic smoothing spline was used. Essentially, a spline is made up of a straight line (considered as fixed) plus a special random component for the curvature. This approach is described in more detail by Verbyla *et al.* (1999).

From the exploratory data analysis, the following terms were included to accurately model the trend over time:

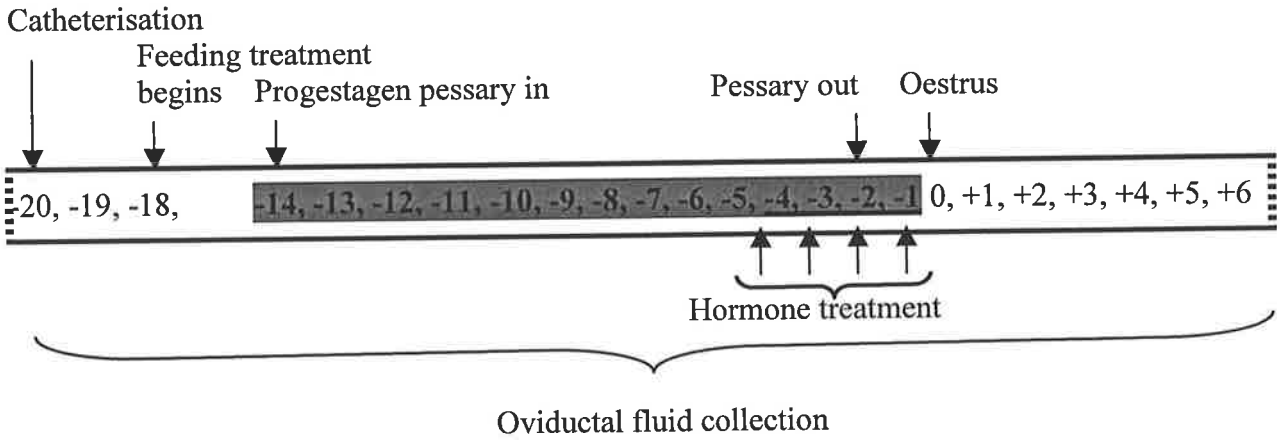
- A spline for days, corresponding to the 24 measurements. The spline is made up of the linear or fixed part (day) to account for any day-to-day variation plus a random component (spl(day)) to model the observed curvature over time.

Fig. 5.1 Experimental designs

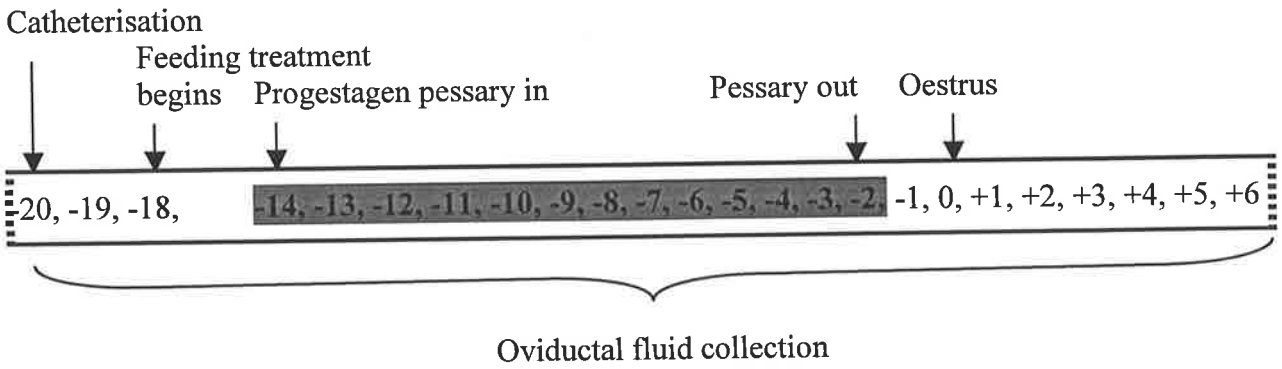
Group 1: HS-High feed intake (1.5 Maintenance ration) + superovulation (n=3)
LS-Low feed intake (0.5 Maintenance ration) + superovulation (n=3)

Group 2: HNS-High feed intake (1.5 Maintenance ration) cycling ewes (non-superovulation) (n= 3)
LNS-Low feed intake (0.5 Maintenance ration) cycling ewes (non-superovulation) (n=3)

Group-1 Superovulation



Group-2 Non-superovulation



- Due to the large unexplained variation over days, a lack of fit parameter was also modelled. If significant this would indicate that random deviations from the overall smooth cubic spline were present over days.
- A main effect for feed intake, which corresponds to fitting a separate intercept (or average effect) for each level of feed intake.
- A separate linear slope for each level of feed intake prior to Day –4
- A separate linear slope for each level of superovulation within each level of feed intake after Day -4
- Each level of feed intake and superovulation may have a different smooth non-linearity over the temporal component of the model. To ensure that this is modelled correctly a cubic smoothing spline is included for feed intake and for each level of superovulation within each level of feed intake.
- Similar to the overall smoothing spline over days, the smooth curve for each treatment may incorporate a lack of fit component. This ensures that any extra variation may be modelled appropriately.
- As each individual sheep may have a different response over time, it is important to allow for this variability. Therefore the model includes a separate variance for the intercepts and slopes and a correlation between them for sheep and a spline to model the curvature over time.
- A random effect was also included to allow for the variability between the left and right side within each sheep.

The contribution of each term for modelling time is assessed differently for fixed and random terms. For random terms, the change in log-likelihood when each term is removed separately from the model is examined and are used to determine the importance of the sources of variation in the model. Fixed terms were tested for significance using a Wald test compared with an asymptotic Chi-squared distribution. The assumptions for this method of analysis are that the joint distribution of the random components (which includes the residuals) is normally distributed. Diagnostic plots were used to check that the assumptions were satisfied. All analyses were performed using the Samm function in the SAMM (Spatial Analysis Mixed Models) library in SPLUS 2000 (Insightful Corporation).

5.2.4.3 Ammonia, urea, electrolytes, progesterone and IGF-I: A total of 12 sheep (three in each group) were included in this experiment. Six sheep were superovulated and six sheep were not superovulated. For the superovulated group, fluid samples from the left and right oviduct were pooled before testing because both sides had more than one ovulation, whereas for the non-superovulated group the fluid from each oviduct was tested to see the ovulation side effect on the fluid composition. The data were analysed by analysis of variance. A split-split-plot structure (with no blocks) has been assumed with three replicates, where there were 12 sheep randomly allocated to two feed intakes (H or L) and the day of cycle was considered in the analysis as sub-plots. Within each non-superovulated sheep, fluid from the right and left oviducts were collected and the responses were measured on a daily basis for seven days (Days 0-6). The assumptions of this analysis are that the data were normally distributed with constant variance and are examined by various diagnostic plots, such as the residuals versus fitted values. A repeated measure ANOVA (Crowder and Hand, 1990) was used which adjusts for the correlation that may exist over time and corrects the degrees of freedom accordingly, using a Greenhouse-Geiser estimate (Greenhouse and Geiser, 1959). If the estimate is one, the degrees of freedom are unchanged and it can interpret the results as an ordinary analysis of variance.

5.3 RESULTS

5.3.1 Sheep live weight: The mean initial live weights of the ewes were not significantly different between the treatment groups (Fig. 5.2) and therefore the data of the superovulation and non-superovulation groups have been combined within dietary treatments (Figs. 5.2 and 5.3). There was no significant interaction between the effects of feed intake and superovulation on ewe weight. There was however, a significant effect of feed intake on final ewe weight: the mean ewe weight adjusted for the initial weight as a covariate shows that sheep fed a high feed intake weighed significantly ($P<0.05$) more (H: 63.0±0.5 kg) at the end of the experiment than those fed a low feed intake (L: 56.7±0.4 kg).

There was no significant interaction between feed intake treatment and ovulatory status on change in sheep weight. There was also no significant effect of ovulatory status on change

in sheep weight. Sheep fed the low diet lost a significant ($P<0.05$) amount of weight whereas there was no significant change in ewes fed the high diet (Fig. 5.3).

5.3.2 Number of corpora lutea: The number of corpora lutea (CL) was analysed using analysis of variance with feed intake (high vs. low) and superovulation (S vs. NS) as the specified factors. Furthermore, there was no significant effect of feed intake or the side of collection on the number of corpora lutea. There was however, a significant effect of superovulation on number of corpora lutea ($P<0.05$) whereby sheep in the superovulated groups had significantly more corpora lutea compared to the non-superovulated groups.

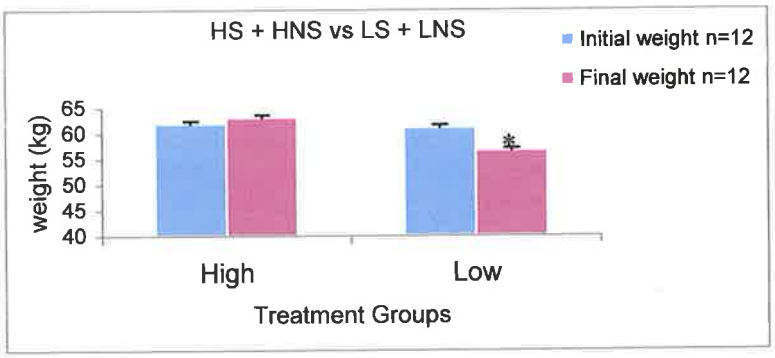


Fig. 5.2 Effect of feed intake (high vs. low) on sheep live weight (kg, mean±SEM). There was no significant difference in sheep weights between the high (data combined for high superovulation (HS) + high non-superovulation (HNS) and low (data combined for low superovulation (LS) + low non-superovulation (LNS) feed intake groups at the beginning of the experimental period. At the end of the 24-day period, sheep in the low feed intake group were significantly ($P<0.05$) lighter compared with the high intake group.

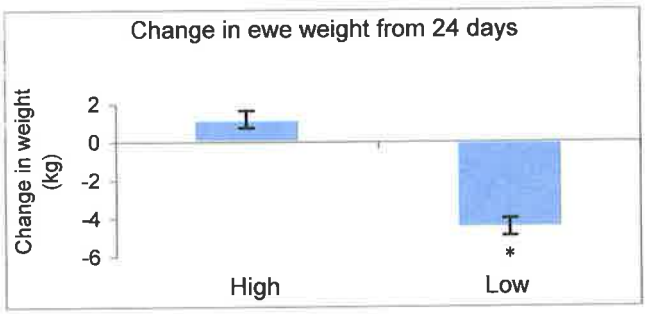


Fig. 5.3 Effect of feed intake on the change in sheep live weight (kg, mean±SEM). The change in live weight during the study period was significantly ($P<0.05$) different (data combined for the superovulated and non-superovulated groups).

5.3.3 Oviductal fluid volume: The volume of oviductal fluid collected followed a cyclic pattern. Individual secretion rates increased dramatically during estrus in each of the twelve ewes and reached a peak two days after estrus and then declined to levels characteristic of the luteal phase. The overall mean daily collection volume from Day 0 to ay 6 after ovulation was 1.37±0.13 ml.

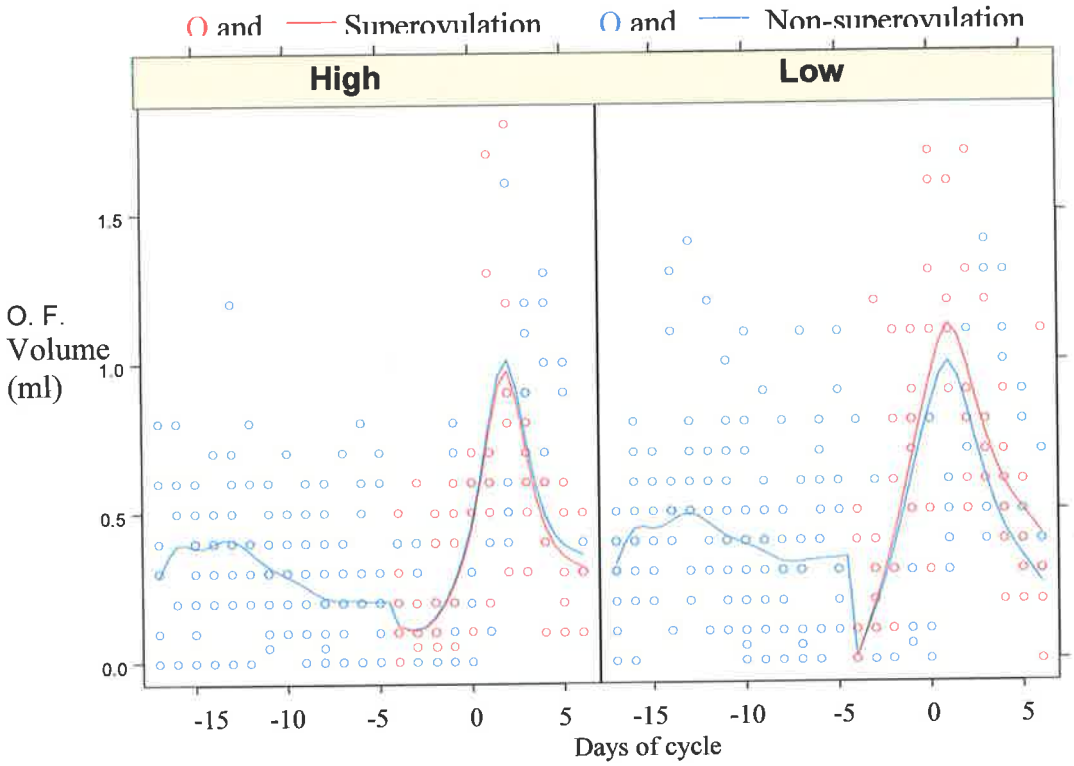


Fig. 5.4 Observed data and fitted curves for oviductal fluid (O.F.) volume over time for feed intake and superovulation

The data was transformed on the log scale, in order to satisfy normality assumptions. From the series of models fitted, it is evident that the pattern of oviduct fluid secretion for the superovulated ewes was significantly ($P<0.05$) different, within each level of feed intake, from that obtained in the non-superovulated ewes.

5.3.4 Oviductal fluid pH: A series of models was fitted to the data. The maximal model contained all the terms discussed in the Section 5.2.4.2. The only significant ($P<0.01$) treatment effect was a different cyclic pattern for the superovulated treatment, which was the same for both high and low feed intake groups (Fig. 5.5).

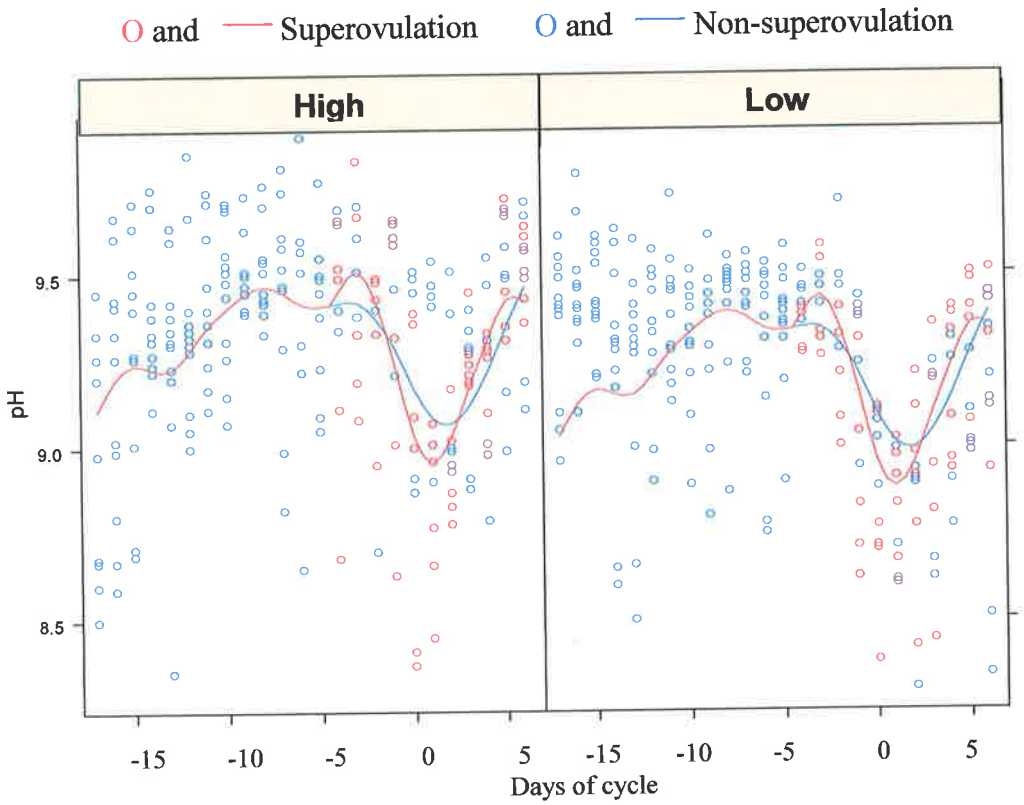


Fig. 5.5 Observed data and fitted curves for oviductal pH over time for feed intake and superovulation.

5.3.5 Oviductal fluid osmolarity

There were no significant differences between the treatments. From Fig. 5.6, there is some evidence that overall osmolarity was higher for sheep fed a high feed diet but this difference was not statistically significant.

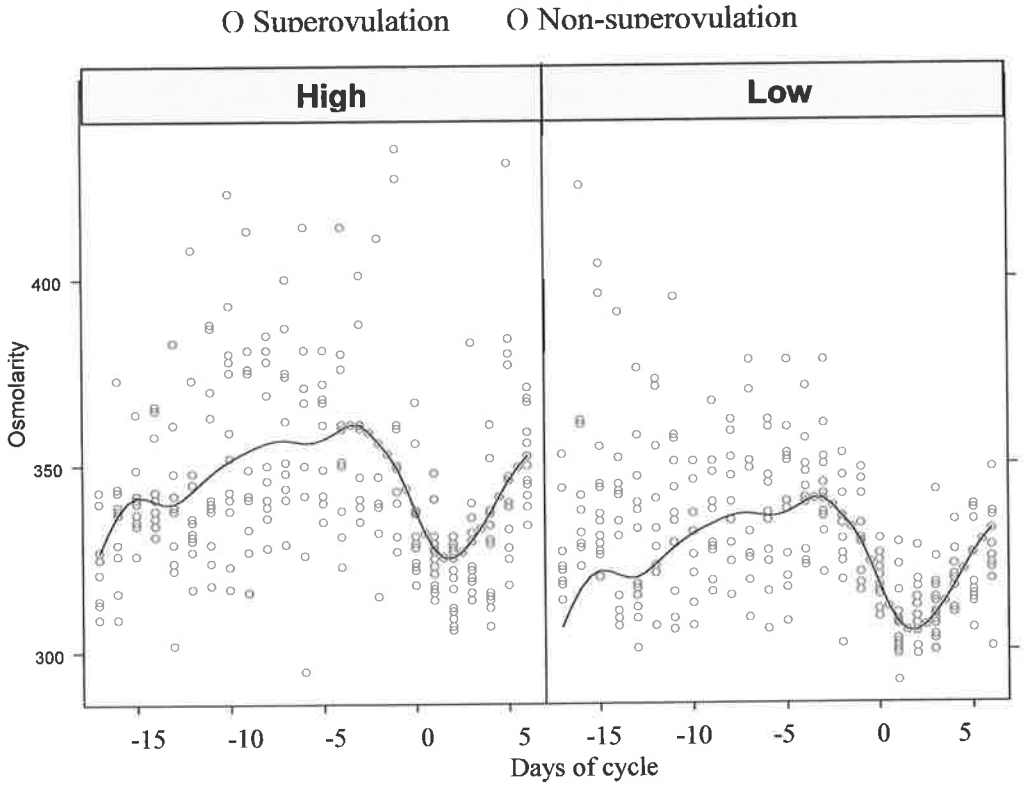


Fig.5.6 Observed data and fitted curves for oviductal osmolarity over time for feed intake and superovulation.

5.3.6 Ammonia: Differences in oviductal fluid ammonia concentrations approached significance (P=0.055) for feed intake treatment (H: 2369 ± 160.3 umol/l; vs. L: 1606 ± 143.3 umol/l) but were not significantly different for ovulatory status (S vs NS) or day of collection (Fig. 5.7; and Table 5.1).

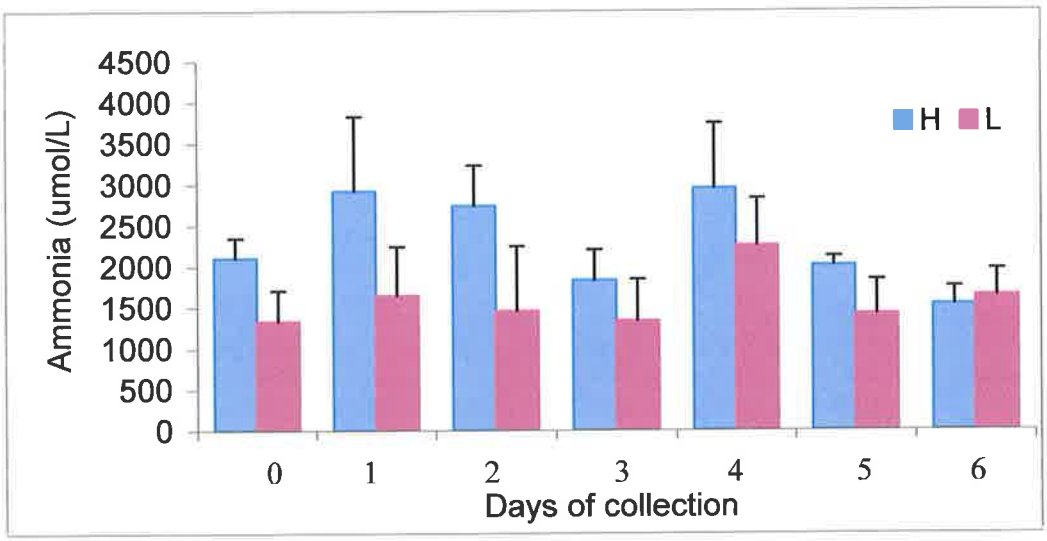


Fig. 5.7 Oviductal ammonia concentrations (mean±SEM, n=6) were not significantly different between sheep in the high feed intake (1.5 x maintenance) group compared with the low feed intake group (0.5 x maintenance) during the first six days after ovulation (Day 0= day of oestrus).

5.3.7 Urea: Oviductal urea concentrations were significantly (P=0.001) affected by feed intake treatment (H: 10.95 ± 0.59 mmol/l; vs. L: 7.26 ± 0.29 mmol/l) and day of collection (P<0.05, highest on Day 5) but were not affected by ovulatory status (Fig. 5.8; and Table 5.1).

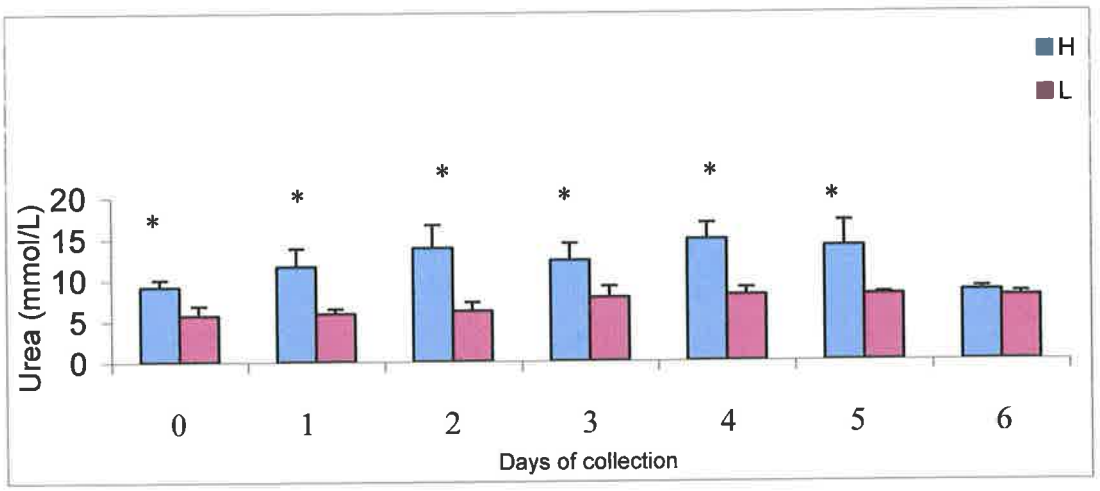


Fig. 5.8 Oviductal urea concentrations in the high (1.5 x maintenance) and low (0.5 x maintenance) intake groups during the first six days after ovulation (Day 0= day of oestrus).

Table 5.1 The effect of high (1.5 x maintenance) and low (0.5 x maintenance) feed intake and side (left vs. right) on the mean concentrations of oviductal ammonia and urea in non-superovulated sheep. (Day 0= day of ovulation).

		Day of cycle						
		0	1	2	3	4	5	6
Ammonia (umol/l):								
Left	HNS	3071	1542	2462	1397	1655	3498	2525
	LNS	1870	1852	2120	2021	2355	1798	1103
Right	HNS	3456	1661	2273	2244	2552	2936	3201
	LNS	1164	1879	749	783	833	1068	989
Urea (mmol/l):								
Left	HNS	10.34	6.74	8.50	7.40	13.83	14.17	12.63
	LNS	6.92	6.40	7.03	7.13	8.83	9.17	8.22
Right	HNS	11.13	8.90	9.40	7.73	8.03	14.59	11.47
	LNS	7.45	6.40	6.33	6.50	8.03	7.37	6.97

HNS: High non-superovulation

LNS: Low non-superovulation

5.3.8 Amino acids: Proline was excluded from the analysis because of a large number of missing values. Amino acids in the non-essential group were the most abundant in oviductal fluid and comprised 82% of the total amino acid concentration. In this group GLY alone represented 66% of the total amino acid concentration with ALA (13%), GLY and GLU (2%) present in relatively high concentrations. Concentrations of essential amino acids in oviductal fluid were relatively low and comprised <18% of total amino acids concentration (Fig. 5.9). The concentrations of HIS, LEU, LYS and VAL were relatively high compared with other essential animo acids in oviductal fluid.

5.3.8.1 Superovulation group: The number of corpora lutea per ewe (range 3-19) did not significantly influence the concentrations of the seventeen amino acids measured. From the repeated measures ANOVA, it can be concluded that the concentrations of ARG, ASP, CYS, MET, SER, THR and TYR did not change significantly over time. However, in animals fed the H feed intake, concentrations of GLY, ALA, LYS, HIS, ILE, PHE, MET, SER, and THR increased compared with L feed intake, while in the L feed intake, concentrations of LEU, VAL, GLU, ARG, TYR, ASP and CYS increased ($P<0.05$). However, for GLY, HIS, ILE, LEU, PHE and VAL, the effect of day was significant ($P<0.05$). GLY, ALA, LYS, LEU, HIS, VAL and GLU were present in higher concentrations (Table 5.2) than previously reported (Nancarrow *et al.*, 1992). These concentrations were significantly ($P<0.05$) decreased between Day 0 and Day 2 (range of decrease: 13 - 53%) and significantly ($P<0.05$) increased between Days 3-5 inclusively (range of increase: 32 - 159%), the latter period corresponding with the time by which embryos have migrated into the uterine horns.

5.3.8.2 Non-superovulation group: From the repeated measures ANOVA it can be concluded that there were no three way interactions, 2-way interactions or statistically significant main effects of feed intake, side or time of collection on concentrations of ALA, CYS, HIS, LYS, MET, PHE, SER, THR or TYR (Table 5.3). The concentrations of ARG, ASP, GLY, ILE, LEU and VAL differed significantly ($P<0.05$) over time. For GLU, there was a significant ($P<0.05$) interaction between feed intake and day. Note that due to the large number of missing values, the correlation over time has not been accounted for. From the table of means (Table 5.4), it appears that the concentration of GLU was

significantly higher in sheep fed a high diet compared with a low diet, except on Days 4 and 5.

5.3.8.3 Superovulation vs. non-superovulation: Samples from the left and right oviducts were pooled before testing, whereas for the non-superovulated group the fluid from each oviduct was tested to see if there was any effect of ovulation on the oviduct milieu. In order to test for differences between super and non-superovulated sheep, the amino acid response for the left and right sides in the non-superovulated group were also combined. From the ANOVA it can be concluded that there is no 3-way interaction, 2-way interactions or statistically significant main effects for ALA, ASP, SER or THR (Table 5.3). For ARG, HIS, ILE, LEU, LYS, MET, PHE, TYR and VAL the interaction between ovulatory status (S or NS) and day was significant (Table 5.5). For GLU, there was a significant ($P<0.05$) interaction between ovulatory status and day and the interaction between day and feed intake was statistically significant ($P<0.05$). For GLY, the interaction between ovulatory status and day was statistically significant and the main effect of feed intake was also significant ($P<0.05$). For CYS, the effect of ovulatory status was significant ($P<0.05$).

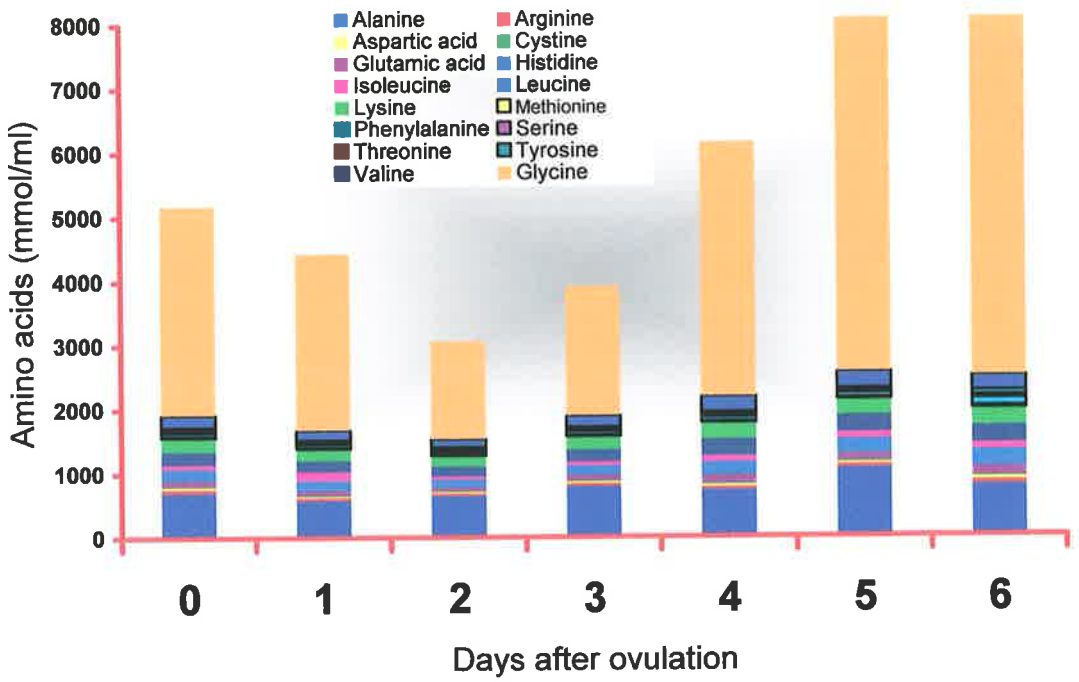


Fig. 5.9 Oviductal fluid amino acid concentrations from Days 0-6 after ovulation in ewes. Data for H (1.5 x maintenance) and L (0.5 x maintenance) feed intakes combined.

Table 5.2 Amino acids concentrations (μM; Mean ± SEM) in oviductal fluid collected from superovulated ewes between Day 0 to Day 6 after the expected time of ovulation (Day 0).

	Glycine	Histidine	Isoleucine	Leucine	Valine	Phenylalanine
Day -1	3261±512	170.0±7.1	78.3±9.5	200.8±19.5	185.7±15.4	52.4±6.6
Day 0	2752±454	147.7±11.4	66.7±9.8	174.4±23.4	154.7±12.1	49.2±12.1
Day 1	1528±280	118.3±11.4	54.3±6.2	157.4±13.8	125.7±14.3	45.6±12.8
Day 2	2021±327	136.1±18.6	69.6±9.6	188.6±24.2	180.3±24.2	54.0±18.1
Day 3	3960±860	197.0±18.3	106.0±14.7	263.0±33.1	245.0±24.1	60.3±13.1
Day 4	5769±1462	224.3±33.5	107.5±11.4	267.5±25.3	257.2±18.3	62.2±10.1
Day 5	6868±1786	261.4±44.1	107.1±14.7	272.0±31.9	240.9±27.7	116.1±19.9

Table 5.3 Mean oviductal fluid amino acid concentrations (μM) in non-superovulated ewes (n=6) fed high (1.5 x maintenance) or low (0.5 x maintenance) feed intake. Data are Mean of values pooled for 7 days (from ovulation at Day 0 until Day 6 after ovulation).

	High feed intake		Low feed intake	
	Left side	Right side	Left side	Right side
Alanine	521	797	679	352
Arginine	81.6	75.1	50.0	50.2
Aspartic acid	25.0	23.1	19.5	21.9
Cystine	9.7	16.6	19.4	11.6
Glutamic acid	120.2	100.8	55.2	103.3
Glycine	1265	1029	581	482
Histidine	110.8	98.3	80.6	47.5
Isoleucine	55.1	54.1	80.3	56.3
Leucine	127.3	123.9	207.5	153.7
Lysine	160.6	151.1	241.4	180.6
Methionine	13.5	11.2	15.3	9.6
Phenylalanine	46.0	43.2	57.6	40.8
Serine	14.8	8.70	8.70	6.56
Threonine	81.6	21.3	33.5	33.9
Tyrosine	41.3	31.3	40.8	31.4
Valine	130.1	119.1	165.7	127.0

There were no significant differences between values

Table 5.4 Mean concentrations of oviductal amino acids (μM) in superovulated and non-superovulated ewes fed high (1.5 x maintenance) versus low (0.5 x maintenance) feed intake. (Day 0= day of ovulation).

		Days of cycle						
		0	1	2	3	4	5	6
Alanine	HS	834	591	642	702	739	1204	984
	LS	561	559	649	878	710	913	590
	HNS	790	608	584	426	519	885	800
	LNS	728	494	380	467	431	511	597
Arginine	HS	55.6	47.1	32.8	40.4	50.0	60.4	75.1
	LS	69.7	53.6	46.2	42.5	53.6	59.2	65.7
	HNS	89.7	133.5	92.4	61.8	50.2	59.1	61.6
	LNS	75.8	58.1	48.7	39.0	43.4	47.1	38.7
Aspartic acid	HS	27.8	16.8	19.8	17.1	14.8	15.2	51.1
	LS	41.2	29.8	26.9	44.5	50.9	43.9	51.9
	HNS	23.8	44.3	24.1	14.2	14.7	20.2	27.1
	LNS	12.9	30.1	16.3	19.0	22.1	25.1	19.3
Cystine	HS	15.5	11.5	13.5	11.0	10.1	21.0	12.4
	LS	11.8	8.4	8.2	16.6	22.3	19.1	18.5
	HNS	12.6	22.6	14.0	9.2	13.7	9.4	10.7
	LNS	26.4	21.3	14.8	17.1	9.4	13.1	6.5
Glutamic acid	HS	77.7	46.2	51.4	40.2	25.2	28.4	68.9
	LS	105.5	107.1	71.2	146.4	232.7	204	216
	HNS	104.6	198.6	145.8	69.6	72.8	62.2	119.6
	LNS	32.2	79.8	66.6	73.7	112.8	88.2	101.5
Glycine	HS	3583	2744	1244	2303	5293	8138	9685

	LS	2938	2760	1812	1739	2626	3401	4050
	HNS	1615	1074	1137	1117	959	957	1167
	LNS	652	722	469	460	391	618	406
Histidine	HS	165.9	134.8	119.6	137.5	204.1	267.6	334.3
	LS	174.1	160.7	117.0	134.6	189.0	180.9	188.5
	HNS	110.4	153.0	109.8	85.7	92.5	88.9	91.6
	LNS	70.3	85.7	59.0	57.2	43.9	74.5	57.8
Isoleucine	HS	72.0	56.9	53.8	57.2	88.7	92.8	112.9
	LS	84.6	75.4	54.7	82.0	123.4	122.2	112.9
	HNS	52.6	86.4	57.6	37.5	43.1	49.8	55.3
	LNS	127.4	71.7	60.5	46.9	54.5	62.0	54.7
Leucine	HS	190.1	152.9	155.4	160.6	218.6	242.9	280.4
	LS	211.5	195.8	159.4	216.6	307.6	292.1	263.5
	HNS	139.3	195.1	131.6	86.9	89.5	115.2	121.6
	LNS	329.2	204.3	165.7	137.1	144.0	154.5	129.7
Lysine	HS	229.9	182.2	181.2	223.3	258.0	264.5	320.5
	LS	205.7	184.8	144.2	188.2	245.1	225.6	226.3
	HNS	152.2	234.9	169.4	108.9	122.3	143.1	159.6
	LNS	295.9	229.2	181.4	191.6	180.8	225.0	172.9
Methionine	HS	24.5	23.3	21.6	23.9	27.0	31.8	36.8
	LS	21.7	18.8	14.2	22.3	22.0	25.9	32.0
	HNS	11.9	23.6	14.4	6.9	8.1	8.4	13.0
	LNS	28.4	17.5	7.0	7.9	8.4	11.2	6.6
Phenylalanine	HS	52.9	50.4	54.3	55.8	54.1	61.3	138.1
	LS	51.8	48.1	37.0	52.1	66.5	63.1	94.1
	HNS	44.1	75.6	46.5	32.3	32.1	37.2	43.9
	LNS	89.3	57.7	48.5	45.1	34.9	36.9	31.8

Serine	HS	36.5	19.7	27.3	21.4	33.0	31.2	39.6
	LS	11.8	9.1	17.5	12.6	11.6	12.6	8.3
	HNS	10.15	13.40	10.43	11.42	12.33	10.17	14.51
	LNS	7.71	10.50	6.31	6.56	4.32	8.85	9.18
Threonine	HS	35.6	13.8	20.8	15.1	28.9	25.7	41.1
	LS	7.5	7.9	10.6	11.7	10.1	11.1	12.1
	HNS	56.0	67.1	59.4	38.9	39.4	38.7	60.7
	LNS	31.6	34.4	38.5	32.7	29.9	33.8	34.9
Tyrosine	HS	28.4	26.0	26.6	14.9	21.9	18.3	54.2
	LS	53.9	46.6	35.1	51.8	68.0	63.1	90.3
	HNS	37.9	70.3	35.5	22.8	25.2	29.1	33.3
	LNS	64.5	44.7	32.7	33.3	26.3	28.4	23.1
Valine	HS	187.5	143.2	128.3	179.9	226.0	249.0	269.0
	LS	183.9	166.2	123.1	180.6	163.6	265.5	212.1
	HNS	149.9	209.6	133.8	77.3	84.1	102.2	115.4
	LNS	274.0	169.3	128.1	108.4	115.3	136.5	93.1

HS: High superovulation

LS: Low superovulation

HNS: High non-superovulation

LNS: Low non-superovulation

Table 5.5 Mean concentrations of amino acids (μM), of pooled data for 7 days (from ovulation i.e., Day 0 until Day 6 after ovulation) of superovulation vs. non-superovulation group ewes fed High (1.5 x maintenance) and Low (0.5 x maintenance) feed intake.

	High feed intake		Low feed intake	
	NS	S	NS	S
Alanine	1304	814	783	694
Arginine	154.3	51.6	85.4	55.8
Aspartic acid	46.2	23.2	40.9	41.3
Cystine	25.6	13.6	24.1	15.0
Glutamic acid	214.0	48.3	148.3	154.9
Glycine	2782	4713	721	2761
Histidine	195.9	194.8	125.4	163.5
Isoleucine	107.5	76.3	129.8	92.0
Leucine	247.5	200.1	338.6	235.2
Lysine	292.9	237.1	382.5	202.8
Methionine	23.5	27.0	19.4	22.4
Phenylalanine	84.7	66.7	83.6	59.0
Serine	19.7	29.8	14.5	11.9
Threonine	95.2	25.9	57.1	10.1
Tyrosine	68.7	27.2	59.7	58.4
Valine	124.6	197.7	281.2	199.3

S: superovulation

NS: Non-superovulation

5.3.9 Electrolytes: Sodium (Na), potassium (K), calcium (Ca), magnesium (Mg), phosphorus (P), chloride (Cl), and bicarbonate concentrations were measured from Day 0 to Day 6 after ovulation for the H and L groups (Table 5.6). The superovulated and non-superovulated groups were analysed separately because of differences in sampling (Table 5.7).

5.3.9.1 Superovulation group

5.3.9.1.1 Bicarbonate: The H sheep had significantly ($P<0.05$) higher bicarbonate concentrations (29.8±4.8) compared with sheep fed a low feed intake (23.1±2.7), except at Day 1 (Table 5.6). There was a significant interaction between feed intake and day on the bicarbonate concentration ($P<0.001$).

5.3.9.1.2 Sodium: Feed intake but not day of collection significantly ($P<0.05$) influenced sodium concentration. Concentrations were higher in H sheep (176.8±4.3) compared with L sheep (161.2±5.5). There was no significant difference between feed intake and days (Table 5.6).

5.3.9.1.3 Calcium, Chloride, Potassium, Magnesium and Phosphorus: The concentrations of Ca, Cl, K, Mg and P (Table 5.6) were not significantly influenced by feed intake or day and nor were there any significant interactions.

5.3.9.2 Non-superovulation group

5.3.9.2.1 Bicarbonate: The effect of feed intake was not significant. There was a significant interaction between the effect of feed intake and day ($P<0.05$) on the oviductal bicarbonate concentrations (Tables 5.6 and 5.7). The interaction between feed intakes was most likely due to the higher concentrations of bicarbonate from Day 0 until Day 4 for sheep on the low feed intake, whereas on Day 5 the trend was reversed and sheep on a high feed intake had higher bicarbonate concentrations.

5.3.9.2.2 Chloride: The effect of feed intake was not significant. There was a significant interaction between the effect of feed intake and day on chloride levels ($P<0.05$). The interaction between feed intake and day was most likely due to the higher concentrations of chloride on Day 0 until Day 3 for sheep fed a low feed intake.

5.3.9.2.3 Potassium: The effect of feed intake was not significant. There was a significant interaction between the effect of feed intake and day ($P<0.05$). The interaction between feed intake and day was most likely due to the higher concentrations of potassium in sheep fed a low feed intake compared with a high feed intake on Days 2, 3, 4 and 5. There were no significant differences between the effect of feed intake on Day 1 or Day 6.

5.3.9.2.4 Sodium: The main effect of feed intake was not significant. There was a significant interaction between the effect of feed intake and day ($P<0.05$). The interaction between feed intake and day was most likely due to the higher concentrations of sodium on Day 0 until Day 4 for sheep fed a low feed intake.

5.3.9.2.5 Calcium, Magnesium and Phosphorus: The concentrations of Ca, Mg and phosphorus were not significantly influenced by feed intake or day and nor were there any significant interactions.

5.3.10 Progesterone: Oviductal fluid progesterone concentration in the superovulated and non-superovulated group were analysed separately because of differences in sampling.

5.3.10.1 Superovulation vs. non-superovulation: There was a significant interaction between feed intake and day ($P<0.05$) (Fig. 5.10). From Figs. 5.10 and 5.11, it is evident that the concentrations of progesterone increased significantly with time. Sheep fed a low feed intake had significantly ($P<0.05$) higher levels of progesterone at all time points measured. There were no significant three-way interactions between ovulatory status (S or NS), feed intake and day (Fig. 5.11). There were also no significant interactions between ovulatory status and day or ovulatory status and feed intake.

5.3.10.2 Superovulation group: There was a significant effect of day ($P<0.001$) (Figs. 5.10 and 5.11) with concentrations of progesterone increasing over time. There was a significant ($P<0.05$) interaction between the effect of feed intake and day. There was also a significant ($P<0.05$) effect of feed intake on progesterone concentrations.

5.3.10.3 Non-superovulation group: There was a significant effect of day on the oviductal fluid progesterone concentrations ($P<0.001$) (Fig. 5.11 and Table 5.8) such that

the concentration of progesterone in oviductal fluid increased over time. There was no significant three-way interaction between the effect of side (left or right), feed intake and day on the oviductal fluid progesterone concentrations (Table 5.8). There was also a significant effect of feed intake and day on the oviductal fluid progesterone concentrations.

5.3.11 Insulin-like growth factor-1: There was a significant ($P<0.05$) effect of feed intake on IGF-I concentration. The non-superovulation group had significantly ($P<0.05$) higher concentrations of oviductal IGF-I than the superovulation group, irrespective of feed intake treatments (Fig. 5.12). There were no significant interactions between the effect of feed intake and day ($P<0.05$) on the concentrations of IGF-I in oviductal fluid (Table 5.9). There was however, a significant effect of day on the oviductal fluid IGF-I concentration ($P<0.01$) (Table 5.9) such that the concentrations of IGF-I first increased for three days then decreased for the remaining four days. In the non-superovulation group, there was no significant three-way interaction between ovulation (S or NS), feed intake and day. The interaction between the effect of feed intake and day was also significant ($P<0.01$). It can be concluded that the levels of IGF-I increase over time and then decrease (Table 5.10). Overall, sheep fed a high feed intake had higher levels of IGF-I at all measurement times except Day 1.

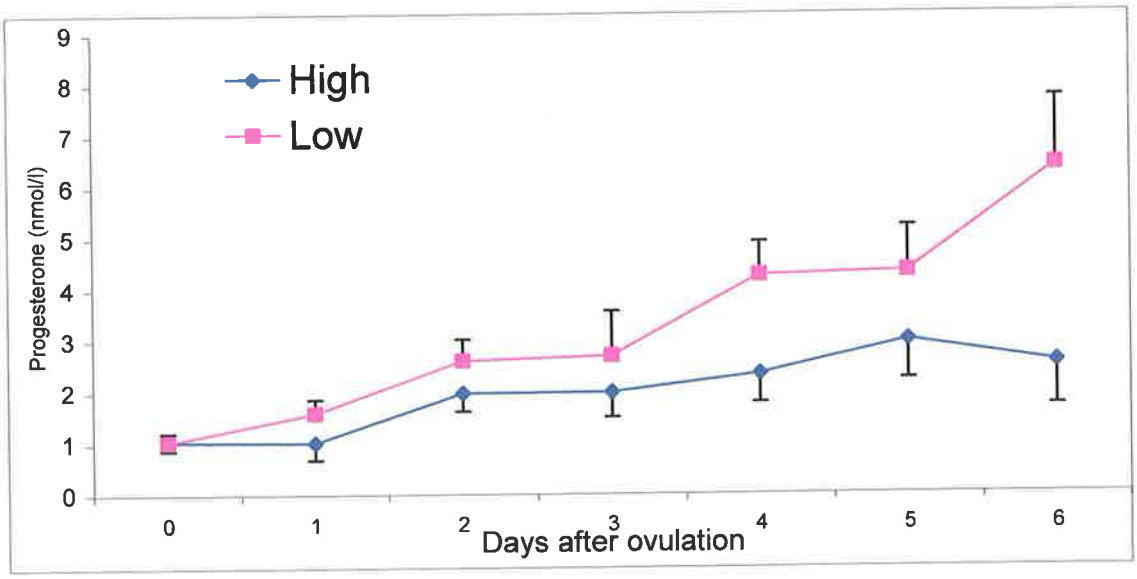


Fig. 5.10 Oviductal progesterone concentrations following ovulation in ewes fed H or L feed intakes. Data for superovulation and non-superovulation combined. Values are means ± SEM (n=12).

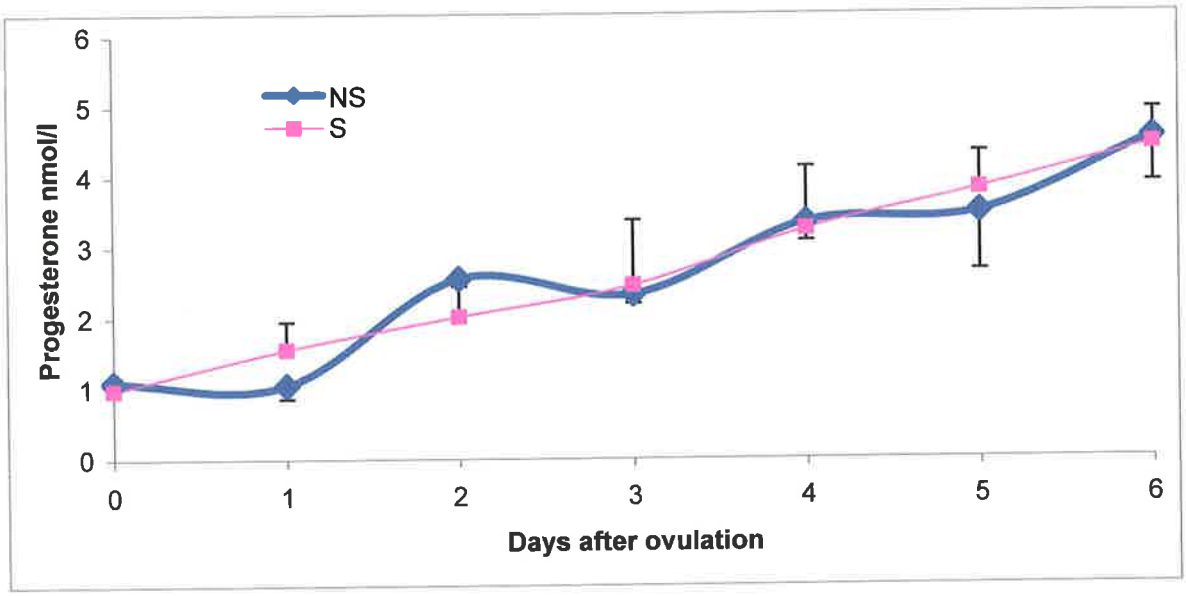


Fig. 5.11 Oviductal progesterone concentrations following ovulation in superovulated and non-superovulated ewes. Data for H (1.5 x maintenance) and L (0.5 x maintenance) feed intake combined. Values are means ± SEM (n=12).

Table 5.6 Concentrations of oviductal electrolytes (mmol/l) in superovulated and non-superovulated ewes fed high (1.5 x maintenance) versus low (0.5 x maintenance) feed intake (day 0= day of ovulation). Values are means ± SEM (n=12).

		Days of cycle						
Electrolytes	Treatment	0	1	2	3	4	5	6
Bicarbonate:	HS	33.7±4.8	26.00±13.1	31.21±2.3	30.00±2.4	31.33±5.7	30.67±15.3	25.92±6.2
	LS	20.7±2.3	26.33±2.7	27.83±0.5	28.00±2.5	20.67±1.8	20.33±0.3	18.01±2.5
	HNS	20.9±0.9	20.20±1.3	21.67±2.2	18.67±1.3	18.83±1.7	21.17±2.3	21.40±0.3
	LNS	21.4±1.3	22.25±3.7	23.00±3.5	26.50±4.1	20.67±3.4	15.50±2.3	16.79±1.4
Calcium:	HS	2.5±0.4	2.72±0.4	2.47±0.4	1.61±0.4	1.59±0.4	2.00±0.4	1.60±0.5
	LS	2.8±0.5	3.86±0.4	3.60±0.3	2.96±0.4	2.05±0.5	2.21±0.2	2.22±0.6
	HNS	2.3±0.7	1.89±0.4	1.98±0.4	2.02±0.2	2.42±0.5	2.46±0.3	2.67±0.8
	LNS	2.0±0.9	2.10±07	1.93±0.3	2.63±0.3	2.27±0.2	1.88±0.4	2.20±0.8
Chloride:	HS	152.3±16.1	169.7±16.1	160.2±10.1	153.0±16.1	145.7±9.1	153.7±11.1	136.5±19.7
	LS	145.0±4.1	154.3±7.5	157.0±9.8	149.3±6.3	144.7±4.1	130.7±5.9	125.6±5.0
	HNS	143.5±8.1	141.6±8.2	131.3±3.7	116.8±3.6	126.2±7.0	127.9±13.4	127.4±8.3
	LNS	139.4±5.9	141.2±12.6	144.2±8.8	154.3±13.1	136.0±6.2	120.8±8.0	127.2±2.3

Potassium:	HS	10.7±1.3	11.0±1.3	12.0±1.3	10.2±1.2	9.4±1.3	8.6±1.2	9.2±1.6
	LS	7.8±1.1	11.3±1.3	11.4±1.2	8.3±1.2	10.6±0.7	7.4±1.3	6.8±1.6
	HNS	7.8±0.7	7.3±0.8	6.5±0.6	6.3±0.8	6.1±0.9	6.2±0.8	6.6±0.7
	LNS	8.7±0.5	7.3±0.5	8.1±1.1	9.5±0.5	9.3±1.4	10.6±1.6	6.7±0.6
Magnesium:	HS	0.29±0.1	0.24±0.1	0.17±0.1	0.17±0.1	0.20±0.1	0.32±0.1	0.36±0.1
	LS	0.32±0.1	0.33±0.1	0.35±0.1	0.42±0.1	0.46±0.1	0.49±0.1	0.37±0.2
	HNS	0.52±0.3	0.58±0.1	0.52±0.2	0.55±0.2	0.59±0.1	0.66±0.2	0.59±0.3
	LNS	0.45±0.2	0.36±0.3	0.29±0.2	0.29±0.1	0.31±0.2	0.37±0.3	0.37±0.2
Sodium:	HS	188.3±3.0	197.3±8.1	174.5±6.2	174.7±5.3	172.3±4.1	164.7±9.1	166.1±5.9
	LS	165.3±5.5	164.7±5.8	166.3±6.7	168.0±6.1	163.7±7.3	155.0±5.5	145.6±6.7
	HNS	166.5±4.8	167.1±13.1	156.7±2.3	145.5±2.5	155.7±5.8	159.7±15.3	160.4±6.2
	LNS	155.7±5.2	151.8±3.5	156.2±6.6	162.8±8.3	160.0±6.7	145.7±8.4	152.5±4.2
Phosphorous:	HS	1.27±0.7	1.50±0.5	1.36±0.6	1.99±0.7	2.04±0.7	2.46±0.3	2.15±0.8
	LS	1.15±0.3	1.06±0.6	1.14±0.2	1.02±0.3	2.20±0.4	2.12±0.3	2.02±0.4
	HNS	2.41±0.1	2.16±0.4	2.12±0.7	2.70±0.3	2.13±0.2	2.20±0.2	2.71±0.5
	LNS	2.18±0.4	1.85±0.3	1.63±0.2	1.79±0.6	2.11±0.2	2.46±0.4	2.34±0.6

Table 5.7 Concentrations of oviductal electrolytes (mmol/L) from the non-superovulated group (HNS + LNS combined) separated for right and left sides. (Day 0= day of ovulation). Values are meanm ± SEM (n=12).

		Days of cycle						
Electrolytes	Collection side	0	1	2	3	4	5	6
Bicarbonate:	Left	20.7±6.2	20.2±5.6	22.3±9.8	22.5±7.8	21.3±5.8	16.8±3.6	19.7±2.8
	Right	21.6±9.6	22.1±7.8	22.3±5.8	22.6±4.9	18.1±6.3	19.8±6.2	18.4±7.8
Calcium:	Left	2.0±1.1	1.9±0.6	1.6±0.3	2.3±0.2	2.2±0.7	1.9±0.9	2.34±0.6
	Right	2.3±0.8	2.0±0.3	2.2±0.1	2.2±0.5	2.3±0.4	2.3±0.3	2.52±0.5
Chloride:	Left	140.6±5.4	142.3±9.3	133.7±9.2	130.5±18.0	137.2±9.5	113.3± 13.0	131.5±5.7
	Right	142.3±0.8	140.5±0.7	141.8±0.5	140.7±0.7	125.0±0.1	135.4±0.2	123.1±0.6
Potassium:	Left	7.7±1.1	7.4±0.8	7.5±1.0	8.2±0.9	7.2±1.1	7.9±1.2	6.9±1.1
	Right	8.8±2.7	7.2±1.1	7.1±0.7	7.5±0.8	8.2±0.6	8.9±0.8	6.5±1.3
Magnesium:	Left	0.43±0.1	0.47±0.1	0.38±0.1	0.43± 0.2	0.43±0.2	0.46±0.1	0.43±0.2
	Right	0.55±0.2	0.47±0.1	0.43±0.1	0.40±0.1	0.47±0.2	0.57±0.1	0.53±0.2
Sodium:	Left	158.9±10.0	167.7±6.6	159.7±4.9	151.8±5.8	162.8±4.7	139.5±7.5	156.1±4.3
	Right	163.3±7.7	151.2±9.1	153.2±9.6	156.5±11.1	152.8±6.7	165.9±8.2	156.8±6.8
Phosphorous:	Left	2.25±0.7	2.09±0.3	1.91±0.3	2.66±0.3	1.74±0.2	2.08±0.3	2.17±0.5
	Right	2.34±0.4	1.92±0.5	1.85±0.2	1.83±0.2	2.50±0.1	2.57±0.1	2.88±0.3

Table 5.8 Concentrations of oviductal progesterone (nmol/l) in non-superovulated ewes fed high (1.5 x maintenance) versus low (0.5 x maintenance) feed intake and the effect of right and left sides. (Day 0= day of ovulation; n=12).

		Days of cycle						
Collection side	Treatment	0	1	2	3	4	5	6
Left	HNS	0.61	0.79	0.99	1.43	1.14	1.86	2.19
	LNS	0.66	1.37	1.34	1.54	2.69	2.95	3.75
Right	HNS	0.79	0.65	1.80	2.23	2.19	3.57	3.80
	LNS	0.61	1.11	1.59	1.24	2.19	3.95	4.66

HNS: High non-superovulation

LNS: Low non-superovulation

Table 5.9 Concentrations of oviductal insulin-like growth factor-I (IGF-I; ng/ml) in ewes fed high (1.5 x maintenance) versus low (0.5 x maintenance) feed intake in both the superovulated and non-superovulated groups (Day 0= day of ovulation; n=12).

	Days of cycle						
Treatment	0	1	2	3	4	5	6
High superovulation	499	555	349	571	249	451	391
Low superovulation	666	838	649	690	731	583	427
High non-superovulation	1567	1128	1436	1508	1389	1367	856
Low non-superovulation	959	1413	1043	905	901	1087	508

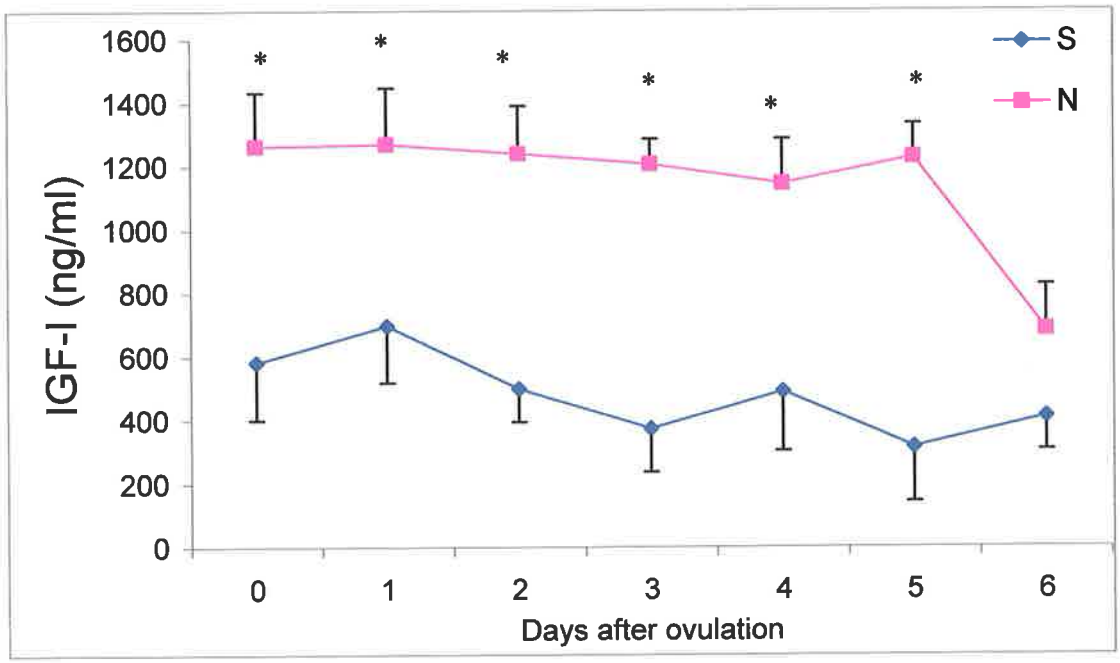


Fig. 5.12 Oviductal IGF-I concentrations following ovulation (day 0) in superovulated (S) and non-superovulated (NS) ewes. Data for H (1.5 x maintenance) and L (0.5 x maintenance) feed intake combined. (*P<0.05).

Table 5.10 Concentrations of oviductal insulin-like growth factor-I (IGF-I; ng/ml) in non-superovulated ewes fed high (1.5 x maintenance) versus low (0.5 x maintenance) feed intake and the effects of side of ovulation (Day 0 = day of ovulation; n=12).

		Days of cycle						
Side	Treatment	0	1	2	3	4	5	6
Left	HNS	1231	1308	1599	1682	1511	1463	923
	LNS	548	1054	1079	643	694	786	258
Right	HNS	1902	947	1274	1334	1267	1272	789
	LNS	1369	1772	1008	1167	1107	1388	757

HNS: High feed intake non-superovulated

LNS: Low feed intake non-superovulated

5.4 DISCUSSION

The oviduct provides a benign, optimised environment for fertilisation and for early embryonic development with the first cell cycle being the most sensitive to the influences of this environment (Boatman, 1997). The actual values reported for pH and osmolarity of oviductal fluids need to be interpreted with caution, and may be reported at maximal levels. In view of the fact that samples had to be retained in a collection tube for 0-24h prior to measurement of pH, an increase in pH due to escaping CO_2, and enzymatic digestion or bacterial breakdown of the fluid are possible. However, there were no significant changes in the pH and osmolarity of control PBS + 10% sheep serum samples, which were also kept in the animal house for comparison. Oviductal fluids of the high and low feed intake groups were handled similarly so the increased pH and osmolarity recorded in samples from the high nutrition group appear to be a real effect and may warrant further study.

Previous studies *in vitro* have reported, however, that changing the pH between 6.5 and 7.4 by adjusting $NaHCO_3$ concentrations from 6 to 50 mM, had no significant effect on embryo development in the hamster (Carney and Bavister, 1987). Furthermore, it was found in early studies by Whitten (1956) and Brinster (1965) that alterations in pH of the culture media had little to no detectable effect on the development of mouse embryos. On the other hand, altering intracellular pH has been reported to disrupt development and cellular organisation in preimplantation hamster embryos (Squirrell *et al.,* 2001). In this regard, data provided by Edwards *et al.,* (1998) indicate that amino acids act as buffers of intracellular pH in the early mouse embryo and play a key role in regulating cell physiology. It would be interesting therefore to determine if the changes we have demonstrated in the oviductal pH alters the intracellular pH of the sheep embryo. These changes in oviductal pH, might reflect alterations in the oviduct's secretory activity. How these alterations might relate to tissue urea, glutamine, or ammonia and directly to embryo survival cannot be determined from the present experiment.

The large differences in values for oviductal pH in the present study may reflect the extent to which cellular debris is included in the analysis. Because of the varying methods that have been used to recover oviductal fluids for analysis, comparisons of the present results with other published reports must be made cautiously. Tubal secretions invariably contain

debris such as sloughed off epithelial cells, and such material was found to be randomly present in some of the samples collected from catheterised oviducts. In the present study, all samples which had abnormally high cellular debris, were rejected from any analysis. Furthermore, catheterisation and ligation of the uterotubal junction may artificially elevate the levels of some constituents. A further limitation is imposed by the collection method since total secretion rate gives no indication of concentration gradients within the oviducts. The method of catheterisation used does not disturb the blood supply to the oviducts and catheterisation *per se* does not appear to disrupt the oviduct secretion process. Sloan and Johnson (1974) found no difference in secretions of cannulated and non-cannulated oviducts in the same individual (rabbit) and oviductal lipid and protein balances were not disturbed. Marked differences in composition between sheep oviduct fluid and sheep serum, as well as cyclic variations in concentrations of several constituents of sheep oviduct fluid, have been reported (Restall and Wales, 1966; Black *et al.*, 1970). No differences in concentrations of substances measured were found when oviduct fluid was held at 25^0C to 40^0C or -10^0C during collection (Black *et al.*, 1970).

It is known that, under the influence of oestrogen, secretion of oviductal fluid is increased by the oviductal epithelium around the time of ovulation (Edwards, 1980). Steroid hormones may influence the rate of fluid secretion by modulating the movement of ions across the oviductal epithelium (Leese *et al.*, 2001). It is also known that oestrogen causes an increase in capillary blood flow to the oviduct in sheep, whereby there is a 2 to 3-fold increase in blood flow around the time of oestrus with a peak at Day –1 and a secondary peak on Day 4 where ovulation occurs on Day 0 (Brown and Mattner, 1977). The peak in blood flow before oestrus may stimulate the rate of oviduct fluid production in some manner. The frequency and amplitude of muscular contractions along the oviductal wall is also increased at oestrus and over the subsequent 3 days (Bennett *et al.*, 1988). Furthermore, the directional flow of oviductal fluid is oriented towards the peritoneum during this period and reverses around the time of entry by the ovum or embryo into the uterus (Menezo and Guerin, 1997).

There is considerable evidence that moderately increased osmolarity is detrimental to the development of mouse embryos cultured *in vitro* (Collins and Baltz, 1999), particularly when they are cultured from the zygote stage (Davidson *et al.*, 1988). There appeared to be a sharp decrease in zygote development when the osmolarity of the culture medium

increased above 300 mOsm/kg (Van Winkle *et al.,* 1990), although the presence of a subset of amino acids that can function as intracellular organic osmolites does allow culture at higher osmolalities (Dawson and Baltz, 1997). Because of the very low volume of oviductal fluid, direct measurements of oviductal fluid osmolarity have not been published. However, measurements of the total content of K, Na, Cl, Ca and Mg in mouse oviductal fluid by electron probe x-ray microanalysis (Borland *et al.,* 1977) indicated that if these atoms were present entirely as ions, the sum of their concentrations would be in the range of 290 and 340 mM.. Assuming that the additional undetected ions needed to balance charge (e.g., HCO_3^-) are osmotically active, these measurements predict oviductal fluid osmolalities of 310 and 360 mOsm/kg, respectively for zygote and 2 cell embryos (Van Winkle *et al.,* 1990).

Strangely, oviductal fluid generally has a higher osmolarity than that which currently permits development of mouse zygotes *in vitro* (Collins and Baltz, 1999). A similarly high osmolarity is predicted for the human fallopian tube by electron probe measurements (Borland *et al.,* 1988). From the present study, the oviductal fluid from sheep also has a similar high osmolarity. Furthermore, high feed intake increased the osmolarity of the oviductal fluid compared with low feed intake in the non-superovulation group. In the superovulation group however there were no significant differences in the oviductal fluid between the nutritional treatments. Interestingly, superovulation decreased the osmolarity compared with non-superovulation irrespective of nutritional treatment. The present study appears to be the first to measure directly the oviductal fluid osmolarity in the ewe, but the question of how tonicity, total ion concentrations, pH and osmolarity are related in oviductal fluid cannot be directly answered. Amino acids that can act as organic osmolites, providing benign intracellular osmotic support, might allow development at moderately higher osmolarities than those reported to be optimal for *in vitro* culture (Collins and Baltz, 1999). The presence of high concentrations of glycine in oviductal fluid (Nancarrow and Hill, 1994; Van Winkle and Dickinson, 1995; Kakar *et al.,* 2003) can therefore be explained in part by the optimal osmoprotective effects it has at this osmolarity (Dawson *et al.,* 1998).

Hunter (1994) proposed that, by increasing the viscosity of luminal fluid, oviduct glycoproteins could stabilise the microenvironments immediately surrounding the gametes and embryo, preventing dispersal of essential nutrients and ions, particularly during ciliary

beating or muscular contraction. This increase in viscosity would buffer the embryo against osmotic changes and fluctuations in the constituents of luminal fluid such as those reported in the present study. Production of a more viscous fluid could also reduce the loss of luminal fluid into the peritoneal cavity. Epithelial cells increase in height at oestrus (Murray, 1995), when fluid production is maximal. This increase in height may increase the surface area of intercellular space available for localized accumulation of ions. Increased viscosity of luminal fluid, due to production and secretion of oestrus-associated glycoprotein, may also prevent rapid diffusion, thereby assisting accumulation of ions into microenvironments with high osmolarity (Hunter, 1994).

Bicarbonate ion is an integral part of the buffering system used in most *in vitro* embryo culture systems. Usually the bicarbonate concentration in the culture medium is set at a level appropriate to maintain optimal pH with the CO_2 concentration used. However, just as CO_2 has effects on embryo development via intracellular mechanisms, the bicarbonate concentrations can also alter cellular functions without necessarily affecting pH. Both utilisation of glutamate and lactate are increased as a function of bicarbonate concentration in cultured embryonic fibroblast cells (Miller *et al.,* 1981). However, for embryo development, a specific function for bicarbonate, separate from its role in buffering pH with CO_2 has not been reported. In the current study, oviductal bicarbonate concentrations were significantly higher in the high feed intake group compared with the low feed intake group. Boatman and Robbins (1991) concluded that maximal levels of spontaneous acrosome reaction occurred at much lower bicarbonate concentrations suggesting that the high oviductal bicarbonate concentrations may have a different role, possibly related to fertilisation and subsequent embryo development. It has been well documented that the concentrations of bicarbonate and CO_2 are much higher (2-3 times) in the oviduct and uterus than in blood plasma (Boatman and Robbins, 1991) but whether this has any functional significance for early development is unknown. To a degree, attempts to establish a specific role for bicarbonate may be moot because CO_2 and bicarbonate are freely interconvertable, especially in the presence of carbonic anhydrase, which is abundant in the oviduct (Bavister, 1995).

It has been documented that calcium ions are essential to the fertilization process in the mouse (Iwamatsu and Chang, 1971). Wales (1970) reported potassium and calcium ions to be essential for development of two cell murine embryos in culture. Interestingly Walker

et al., (1988) reported that varying the concentration of calcium in culture media from 1.3 to 5 mM had no effect on the *in vitro* development of ovine embryos. In the current study there was no significant difference in oviductal calcium concentrations between the two nutritional regimes. Furthermore, there was also no significant interaction between the effect of feed intake, ovulation and day on the oviductal calcium concentrations. There appear to be a range of calcium concentrations therefore that will support embryo growth and development.

In terms of sodium concentrations however, it appears that the optimal concentration is more tightly controlled. Van Winkle *et al.* (1985) considered that the small differences in Na^+ concentration in the ampulla compared with the isthmus of the mouse oviduct was sufficient to influence the uptake of amino acids by early embryos. In the present study, sodium concentration was significantly affected by the feed intake. In the high feed intake group, the concentrations of sodium in the oviductal fluid were significantly increased compared with low feed intake. The electrolyte concentrations (i.e., K, Mg, Cl etc) in ovine oviduct fluid reported in this thesis clearly fall within the range of concentrations determined to be functionally relevant for *in vitro* sperm capacitation and fertilisation in several species including ovine (Restall and Wales, 1966; Paisley *et al.,* 1979; Grippo *et al.,* 1992). This study has also demonstrated that cation concentrations in oviduct fluid are apparently regulated and that the changes in electrolytes concentration vary with day of the oestrous cycle.

Urea and ammonia have received much attention as biochemical compounds that may adversely affect mammalian embryo development. Based on *in vitro* and *in vivo* experiments, ammonia generated from cellular metabolism and the catabolism of amino acids in culture or *in vivo* has been found to be toxic to murine (Folman *et al.,* 1981), ovine (McEvoy *et al.,* 1997; Sinclair *et al.,* 1998; Fehay *et al.,* 2001) and bovine (Hammon *et al.,* 2000a; Dawuda *et al.,* 2002; Kenny *et al.,* 2002) gametes and embryos in cultures. Oocyte maturation, fertilisation, and early embryo development are all modulated by their nutritional micro-environment, which is, in turn influenced by dietary protein nitrogen intake (Hammon *et al.,* 2000b).

Urea is a small molecule, which readily diffuses across membranes and equilibrates within the body. Urea is one metabolite of dietary protein that is formed from detoxification of

ammonia by the liver. Plasma urea nitrogen concentrations have often been used as a correlate between dietary protein level and fertility (Canfield *et al.,* 1990; Elrod and Butler, 1993; McEvoy *et al.,* 1997; Sinclair *et al.,* 2000). Ammonia is also a protein metabolite, which escapes detoxification by the hepatic urea cycle system and is usually prevented from entering the general circulation by a very high affinity perivenous glutamine synthetase system (Haussinger, 1990). Glutamine, which then acts as the nitrogen carrier in the blood, is recognised as a readily available source of ammonia (Elrod and Butler, 1993). Urea thus curbs the damaging effect of ammonia (Ferguson and Chalpa, 1989), although urea itself also may contribute to some disruptive functions, notably alterations of follicular fluid pH and utero-oviductal pH (Elrod and Butler, 1993; Elrod *et al.,* 1993; Jordan *et al.,* 1983).

The present study strengthenes the hypothesis that high protein diets depress fertility by impacting on very early embryo development. Exposure of follicle-enclosed oocytes to high levels of ammonia and/or urea has previously been observed to compromise their capacity to develop to the blastocyst stage following a period of *in vitro* culture (Sinclair *et al.,* 2000). In this context, it is likely that in the present study high urea and (or) ammonia in the high feed intake may have had a debilitating effect on the oocyte, which reduced early embryo viability and total cell number (discussed in Chapter 4). Embryo retardation at Day 4, embryo degeneration after 3 days in culture and reduced pregnancy rates following embryo transfer have been reported as a consequence of excess dietary urea treatment in ewes (McEvoy *et al.,* 1997). In another study, urea-treated ewes produced embryos with lower cell numbers than untreated ewes and their subsequent rate of development during *in vitro* culture was poor (Bishonga, *et al.,* 1994; Bishonga, *et al.,* 1996). Furthermore, Fahey *et al.* (2001) concluded that the toxic effects of ammonia on the oocyte might have disrupted the maturation process, which in turn resulted in a reduced growth rate of embryos from urea-treated ewes. The reported link between reduced fertility and an altered uterine environment may misrepresent damaging effects that occur earlier at the oocyte maturation or early embryonic developmental stages (McEvoy *et al.,* 2001). Suggestions that the effects of urea on the embryo do not occur until after Day 8 are not consistent with experiments in sheep (Bishonga *et al.,* 1996; McEvoy *et al.,* 1997; Fahey *et al.,* 2001; Kakar *et al.,* 2002-present study) and cattle (Gath *et al.,* 1999; Kenny *et al.,* 1999). Kenny *et al.,* (2002) reported a significantly higher concentration of ammonia in bovine oviductal fluid than in blood. The concentration of ammonia reported in the

present study was higher in oviductal fluid than the reported systemic concentrations in ewe. A recent report of Hammon *et al.* (2000) indicates relatively high concentrations of ammonia in follicular fluid compared with blood. Consistent with this, Visek (1984) reported that concentrations of ammonia may vary by up to 10-fold or greater between different compartments of the human body.

In this study, the high feed intake was designed to be in excess of nitrogen requirements, and this appears to have resulted in increased oviductal urea concentrations in the high feed intake compared with low feed intake ewes. Urea concentrations greater than 6.8 mmol/l in plasma and milk (Butler *et al.,* 1996) have been associated with reduced fertility in dairy cattle. McEvoy *et al.,* (1997) measured urea and ammonia concentrations in media flushed through the utero-oviductal regions of control ewes and ewes fed high levels of urea. The concentrations of urea and ammonia in the microenvironment of the embryos were correlated with the concentrations in plasma. Because ammonia and urea can cause changes in ionic concentrations (Jordan *et al.,* 1983) and pH (Elrod *et al.,* 1993) of extracellular fluids, it is possible that follicular fluid prior to ovulation and the oviductal fluid environment after ovulation have been altered by the nutritional manipulations (Kakar *et al.,* 2002a; Kakar *et al.,* 2002b-present study). Nolan *et al.* (1998) suggested that nutrition during follicular development could affect reproductive function via alteration in follicle growth and by an effect on the quality of the resultant oocytes. However, in previous experiments (Chapter 4; Kakar *et al.,* 2002a), in the superovulated sheep, the total number of embryos collected were similar between the low and high feed intake groups. This suggests that there were no harmful effects of the low nutritional treatment on the pre-ovulatory follicle, at least in terms of ovulatory responses and the first seven days of embryo development. Whilst it is possible that embryos may produce ammonium via the utilisation of amino acids, this is likely to be absorbed or neutralised by the epithelial cells of the oviduct and uterus and this is very difficult to measure or quantify.

Amino acids support normal preimplantation development *in vitro* thus providing evidence for an important role *in vivo* (Van Winkle, 2001). The ovine oviductal concentrations of amino acids have been measured and certain amino acids found to be present in concentrations quite different to those in commercially prepared culture media mixtures (Nancarrow *et al.,* 1992; Walker *et al.,* 1996). Furthermore, when used *in vitro* these oviductal amino acid concentrations have been reported to improve ovine embryo

development significantly (Walker *et al.,* 1996). The current results demonstrate that the concentrations of amino acids present in the oviduct are quite different from those found in plasma (Nancarrow *et al.,* 1992) and are variable throughout the periovular period. Of note, the concentrations of amino acids reported in previous studies were lower than those found in the current study. The high concentrations of free amino acids in oviductal fluid suggest that they may provide a source of fixed nitrogen for the fertilised egg during its period of residence within the oviduct. The ruminant blastocyst is able to oxidise amino acids readily but has a limited capacity to oxidise carbohydrate, thereby implying that amino acids are a major source of energy for the preimplantation embryo (Hill *et al.,* 1997). Amino acids such as ARG, HIS, GLU, GLY, ILE, LEU, LYS, MET, TYR and VAL were shown to be increased after ovulation.

The findings of the present study indicate that short-term changes in feed intake can induce changes within the oviductal milieu that could provide a link between feed intake and embryo development and survival. Embryos at later stages may have different amino acid requirements compared with the early embryo. Indeed, the preimplantation embryo exhibits a changing nutrient requirement as development proceeds (Leese and Barton, 1984; Gardner and Leese, 1986), reflecting the changes in the energy metabolism of the embryo that occur between fertilisation and the blastocyst stage (Biggers *et al.,* 1989; Leese, 1992). The fact that significant differences between the feed intake groups (except GLY and GLU) have not been detected is possibly due to the large amount of variability between the experimental sheep over time. Therefore, peri-conceptional feed intake appears to influence components of the oviductal environment in ewes, and further studies using a larger number of ewes may be required to test whether fertility is also affected by such nutritional manipulations.

Essential amino acids have been reported to increase the cleavage rate of embryos *in vitro* after the eight-cell stage and also to promote development of blastocysts with more cells in their inner cell masses (review by Van Winkle, 2001). Such blastocysts more frequently give rise to viable fetuses upon transfer to surrogate mothers compared with blastocysts that do not develop in the presence of added essential amino acids (Lane and Gardner, 1997). Glycine is the major amino acid present in the oviductal fluid in all species studied but the relative abundance of other amino acids is species-dependent. Van Winkle *et al.* (1990) have demonstrated that glycine acts as an intracellular osmolyte by protecting the

developing embryo against a high ionic environment, such as that found in the oviductal lumen (Borland *et al.,* 1977; Borland *et al.,* 1988). In the current study, glycine was the only amino acid which was significantly increased by high feed intake and superovulation treatment. GLY, ALA, LYS and LEU are present as major components of oviductal fluid in all species but ARG and PRO make a relatively greater contribution to the amino acid pool in the cow and sow respectively. In the mouse, glutamine significantly increased blastocoel development and blastocyst hatching (Lane and Gardner, 1997). In the current study low feed intake and non-superovulation significantly increased the GLU concentration in oviductal fluid compared to high feed intake and superovulation treatment. It is possible that this may explain why there were more blastocysts in the low feed intake group of sheep compared with the high nutrition group.

Amino acids such as ARG, CYS, GLU, ILE, LEU, LYS, TYR and VAL were significantly increased in the oviductal fluid of the non-superovulation group compared with superovulated sheep irrespective of nutritional treatment. Gardner *et al.* (1989) have demonstrated that the uptake of GLU by single preimplantation mouse embryos increases with development, and that the uptake of this amino acid is affected by other energy sources during the first days of development. The interaction of GLU with other energy sources in other cell types has been well documented (Morgan and Faik, 1981). Conversely, HIS, GLY and MET concentrations in the oviductal fluid were increased by superovulation. Only GLY was significantly increased in the high feed intake group that were also superovulated. Although the significance of this high concentration is not clear, it has previously been suggested that GLY may be involved in regulating the internal osmolarity of the embryo. In some cells exposed to high osmolarity, GLY can act as a balancing intracellular osmolite; therefore, it is possible that GLY plays this role during the early development of embryos, protecting them from the relatively high ionic content and osmolarity of oviductal fluid as reported in this study and by Van Winkle *et al.,* (1990).

Adequate progesterone concentrations following ovulation are crucial for normal embryonic development within the oviduct, and for proper timing of embryo transport to the uterus (Boatman, 1997). Progesterone serves as an antidote to the previously unopposed oestrogenic action that leads to premature propulsion of some embryos into the uterine lumen (Vinijsanum and Martin, 1990). In a study that used lactating mice in order to differentiate the effects of progesterone from those of unopposed oestrogen dominance,

the anti-progestin RU486 administered on Day 1 and 2 of pregnancy caused a decrease in embryonic cell numbers at Day 4 of pregnancy (McRae, 1994). Although simultaneous administration of progestins with RU486 did not restore cell numbers to that of the control, progestins administered alone significantly increased embryonic cell numbers (McRae, 1994). The same author concluded that the growth rate of murine embryos in the oviduct is influenced by the maternal hormone milieu. It was noted in the present study that the variability in progesterone secretion during the early stages of pregnancy is associated with major differences in the concentrations of progesterone perfusing the oviductal environment in which early embryonic development takes place.

Changes in post-ovulatory progesterone concentrations are likely to affect early embryonal survival by modification of the oviductal environment as suggested by Jindal *et al.* (1996). It has been demonstrated that progesterone may modulate the expression of one or more components of the insulin-like growth factor system, the growth factors, their receptors and their binding proteins in the oviduct (Stevenson and Wathes, 1996); therefore it is possible that this is a mechanism whereby progesterone affects oviductal embryonic development. A positive relationship between embryo survival and progesterone concentrations on first 3-4 days of pregnancy lead to the conclusion that changes in progesterone concentrations in the immediate post-ovulatory period may be of greater importance in determining embryo survival than progesterone concentrations later in gestation (Pharazyn *et al.,* 1991). It is clear from the present study however that the persistence of high concentrations of oviductal progesterone during low feed intake at a time when embryos were resident in the oviduct influenced the tubal milieu or transportation of the embryos in such a way as to influence the change in ICM/TE ratio. It is important to note that many of the effects of steroids on functions of gametes in the oviduct may be mediated indirectly through actions on the vasculature, serosa, mucosa, and muscularis, affecting fluid and gamete transport, availability of metabolites, synthesis of growth factors and other biologically important molecules, such as the major class of oestrogen responsive glycoproteins (Battalia and Yanagimachi, 1979, 1980; Hunter, 1988).

Butler (2001) recently reported an inverse relationship between systemic urea and progesterone concentrations and postulated that high dietary crude protein or systemic urea may impair fertility by interfering with the normal inductive effects of progesterone on the microenvironment of the uterus, thereby leading to suboptimal conditions for the

maintenance of embryo growth and development (Butler, 1998). The present results also show that an increase in oviductal urea in the high feed intake group is associated with a decrease in oviductal progesterone concentrations.

Although nutritional status has been shown to regulate the expression of mRNA encoding components of the hepatic IGF system (Thissen *et al.,* 1994), the results presented here show, for the first time, a direct effect of feed intake and superovulation on oviductal fluid IGF-I concentrations. These results indicate that whilst IGF-I was always present in oviductal fluids during early embryo development the concentrations of IGF-I varied. It is evident that IGF-I concentrations increase and then decrease in oviductal fluid during estrus and the subsequent postovulatory period. Although oviductal fluid volume increased at oestrus, concentrations of IGF-I were diluted in the superovulated group of sheep compared with the non-superovulated group irrespective of feed intake treatment. These data indicate that differences in IGF-I response may not be due entirely to feed intake *per se*, but may reflect whether or not the sheep was superovulated. The non-superovulation group had significantly higher concentrations of oviductal IGF-I compared with the superovulation group irrespective of nutritional treatment. By way of confirmation, in the non-superovulation group, high feed intake resulted in higher IGF-I concentrations compared with the low feed intake group.

The growth factors travel to the oviduct via transudation from the blood and/or may have been synthesised by one of several cell types in the oviduct. If the main source of IGF-I is the blood, one could expect the plasma concentrations to be significantly higher during oestrus than during the rest of the cycle in order to deliver the 2- to 6 fold higher quantities required to maintain the oviductal fluid concentrations of IGF-I at oestrus (Wiseman *et al.,* 1992). An examination of the data in Tables 5.9 and 5.10 does not support this expectation; therefore the other likely source of the IGF-I is the oviductal cells. Wiseman *et al.* (1992) reported that culture for 48 h of a mixture of epithelial and stromal cells obtained from the oviduct on Day 2 after estrus showed that IGF-I and IGF-II were present at concentrations of 30-40 pg/ml of culture medium. Relative to this finding are the reports (Williams and Butler, 1991; Watson *et al.,* 1994) that ovine oviductal cells obtained at oestrus contain IGF-I mRNA. Together, these results support the conclusion that the IGFs in oviductal fluid can arise at least in part from oviductal cell synthesis. Previous studies showed that transcripts of IGF-I ligand and receptor genes are detected in oviductal cells

and early ovine embryos (Watson *et al.,* 1994) and that a increased level of IGF-I mRNA in the cells of the ovine oviduct is recognised at the time when the zygote passes through the oviduct (Stevenson and Wathes, 1996). Therefore, it is likely that IGF-I plays endocrine, autocrine and/or paracrine functions in providing an oviductal environment propitious to conception and early embryonic growth and metabolism *in vivo*.

Nutritional status also affects the circulating concentrations of IGF-I in ruminants. Circulating concentrations of IGF-I were reduced in growing cattle that were severely underfed (McGuire *et al.,* 1992), but differences were not apparent when steers were only moderately underfed (Breier *et al.,* 1986). Because circulating concentrations of IGF are dependent on IGF binding proteins (IGFBP), changes in IGF-I during nutrition restriction may reflect changes in IGFBP, which were not analysed in the current study. Thus nutrition may affect IGFBP in ruminants (McGuire *et al.,* 1992) but this cannot be concluded until the IGFBP types have been more rigorously examined. Subtle alterations in nutrition, typical of animal production systems, seem to have only minimal effects on circulating IGF.

IGF-I is an essential, direct regulator of ovine fetal growth (Owens, 1991; Gluckman *et al.,* 1992). It exerts this influence by a variety of actions in many fetal cell types and tissues *in vivo*, including stimulation of cell proliferation, protein synthesis, glycogen synthesis, and differentiation (Owens, 1991). In addition, it can be hypothesised that nutritionally induced changes in the oviductal fluid IGF system may play a key role in regulating embryo quality.

Finally, the overall data presented in this study indicate that future studies concerned with the formulation of diets designed to optimise fertility must take into account the possibility of unexpected divergent actions of nutrient supply on follicular growth and embryo quality.

Chapter 6

EFFECTS OF SHORT-TERM CHANGES IN FEED INTAKE DURING THE PERI-CONCEPTIONAL PERIOD OF THE EWE ON FETAL GROWTH AT MID-GESTATION

6.1 INTRODUCTION

Human epidemiological data linking altered fetal and placenta growth with hypertension and cardiovascular diseases during adult life (Barker *et al.,* 1990) has highlighted the need to understand the factors that regulate fetal and placental development. The "fetal origins of adult disease" hypothesis suggests that cardiovascular diseases, including hypertension, coronary heart disease and stroke are programmed by adaptations of the fetus to insults such as maternal undernutrition at critical or sensitive periods of fetal development (Barker, 1997). The placenta is thought to play a major role in these adaptive mechanisms, as emphasised by several epidemiological studies, which have revealed a strong link between changes in placental mass and maternal nutritional deficiency (Godfrey *et al.,* 1991). Furthermore, a high placental/birth-weight ratio has also been reported to be associated with cardiovascular disease in the later life (Barker *et al.,* 1992). The placenta is an important determinant of fetal growth rate and it has been suggested that the survival of the fetus is jeopardized more by a small placenta than by maternal underfeeding (Mellor, 1983). It has been reported in sheep that placental weights were increased in ewes that received reduced nutrition in early and mid gestation (McCrabb *et al.,* 1991) compared with control animals. The increase in placental weight associated with undernutrition during pregnancy is possibly an attempt to compensate for the reduction in nutrient supply from the mother (Barker *et al.,* 1990). However, most aspects of the placental response to maternal nutritional imbalance are still unclear.

Perturbations in the environment of the pre-implantation embryo can act to influence subsequent development. For example, subjecting sheep zygotes to *in vitro* culture for 3-5 days (Walker *et al.,* 1992) or transferring bovine nuclei to host oocytes (Willadsen *et al.,* 1991) can result in progeny of excessive birth weight. In the cow (Garrett *et al.,* 1988) and sheep (Kleemann *et al.,* 1994; 2001), progesterone administered during the first few days of pregnancy can enhance fetal growth. These alterations to the embryo or its environment occurred within the first few days of development, highlighting the pivotal role that this period has on normal development. There is limited information on the growth characteristics of fetuses that develop from ovine embryos subjected to such treatments. Kleemann *et al.*

(1994) observed that enhanced fetal growth was associated with a significant increase in crown-rump length. In cattle, Farin and Farin (1995) examined the development of fetuses obtained from embryos produced either *in vivo* or *in vitro*. Fetuses in the *in vitro* group were 17% heavier than their *in vivo* contemporaries and this was reflected in both greater skeletal and internal organ measurements.

There are changes in fetal growth associated with a number of factors including breed, sex, number of fetuses, maternal nutrition, placental size and function, the growth characteristics of which have been studied (Robinson *et al.,* 1977; Mellor, 1983; Bell, 1984; McCrabb *et al.,* 1991; Kelly, 1992). However, growth characteristics of fetuses have not been defined in the situation where the embryo or its environment has been altered through differential nutrition during the peri-conceptional period. Similarly, the consequences of perturbation of the embryonic environment for placental development, which may also contribute to changes in fetal growth, have not been described.

Poor nutrition early in life results in delayed puberty, aberrant oestrous cycles, lowered conception rates, and reduced birth weight (Armstrong *et al.,* 2001). Short-term changes in the plane of nutrition have been shown to affect ovarian follicular dynamics in cattle without any change in the circulating concentrations of gonadotrophins (Gutierrez *et al.,* 1997). In addition, short-term decrease in dietary energy intake influences both oocyte morphology and developmental potential (McEvoy *et al.,* 1995a; O'Callaghan *et al.,* 2000).

In contrast, feeding excess degradable protein can affect early embryonic development and survivability and reduce reproductive efficiency in ruminants (Ferguson and Chalupa, 1989; Butler, 1998). In addition, the intake of high levels of highly degradable protein increases plasma ammonia concentrations and can also increase the concentrations of ammonia in bovine follicular fluid (Sinclair *et al.,* 2000) as well as increasing the ammonia and urea concentrations in ovine oviductal fluid (as discussed in Chapter 5). This high level of dietary protein has been associated with altered follicular growth patterns and a reduction in both the number of ova that cleave following insemination and the proportion that develop to the blastocyst stage (Armstrong *et al.,* 2001). Clearly there are a number of mechanisms

through which nutrition can act to influence both follicle dynamics and the developmental competence of oocytes. One of the mechanisms for this effect may be related to the ability of the liver to accommodate the challenge of excess ammonia production, leading to uremia or some other product of protein metabolism, which in turn affects reproductive tissues and processes (Stalheim and Gallagher, 1977; Bishonga *et al.,* 1994; Butler, 2001). The effects of excess feed intake on embryonic survival in sheep may occur early (Day 1 to 11) during embryonic development (Bishonga *et al.,* 1996; McEvoy *et al.,* 1997; Chapter, 4). However, these effects may occur sooner, during the initial stages of development in the oviduct (Chapter, 5). The sheep represents an important model for the investigation of fetal development and extensive research has indicated that growth factors such as IGF-I play an essential role in regulating ovine fetal growth (Owens, 1991). It is therefore of interest to investigate whether this growth factor mediated influence is initiated during the preimplantation period (as was noted in Chapter, 5). The effect (s) on the early developing embryo may be carried over into later stages of development or cause a change in the ability of the embryo to synchronise its development with uterine changes necessary to sustain it. Presently, there are limited data in ruminants regarding the effect of feed intake on oviductal embryonic development or the associated steroid environment. Therefore, the objective of this study was to determine if peri-conceptional feed intake during the 18 days before insemination and 6 days after ovulation alters fetal and placental growth at mid-gestation (Day 75).

6.2 MATERIALS AND METHODS

6.2.1 Feed intake and body weights

The net feed intake was recorded, as was the weight of ewes, as outlined in Section 4.2.1.

6.2.2 Experimental design

In this experiment, 24 sheep (details have been provided in Chapter 3 on the source of animals, Section 3.1), were randomly allocated to two randomised blocks on the basis of their initial weight. Within each block, ewes were randomly allocated to either a high feed intake (1.5 x daily energy needs for maintenance) or a low feed intake (0.5 x daily energy needs for maintenance), identified by H and L notations respectively

(Fig. 6.1). All ewes were kept for at least two weeks on a maintenance ration before the commencement of the trial (Section 3.2). The nutritional treatments were imposed from 18 d before until 6 d after the expected time of ovulation.

6.2.3 Preparation of recipient ewes

Recipient ewes were synchronized using progestagen pessary containing 45 mg flugestone acetate (Intervet, Paris, France) for 12 days according to the schedule of donor ewes as described previously by Walker *et al.* (1989).

6.2.4 Embryo transfer

Six days after artificial insemination (Section 3.4 and 3.5), embryos were collected under general anaesthesia by mid-ventral laparotomy as described previously by Walker *et al.* (1989). The number of corpora lutea (CL) were recorded and each uterine horn was flushed with 20 ml phosphate-buffered saline containing 5% heat inactivated sheep serum. Embryos were recovered from the flushing medium within 5 min of collection using a stereomicroscope. Prior to being transferred to the recipient ewes embryos were held in culture media for up to 2 h. Within each treatment group (H or L), one or two embryos were laparoscopically transferred to the uterine horn ipsilateral to an ovary with at least one CL. After transfer, recipient ewes were maintained on pasture with free access to feed with lucerne hay supplements and water. In addition, approximately 400 g sheep pelleted ration (Section 3.2) were given for about two weeks after embryo transfer. Pregnancy and fetal number were confirmed at Day 45 by ultrasonography.

6.2.5 Measurements

On Day 75, blood samples were collected via jugular venipuncture from all the recipient ewes, centrifuged at 1500 g for 20 min and the plasma stored at –20 ^{0}C until analysed. All ewes were then killed by captive bolt. The gravid uterus was removed post-mortem through a midline incision in the abdomen. Fetal blood was collected from the umbilical cord and placed on ice for no more than 20 min before being centrifuged at 1500 g for 15 min and the plasma was subsequently stored at –20 ^{0}C until analysed. The fetus was removed from the uterus and blotted dry with tissue paper, weighed and sexed (Fig. 6.2). The crown-rump length (CRL), fetal girth at the fore leg (thoracic girth) and at umbilicus (abdominal girth) was measured and the fetal

head was detached and its length and width was noted. The internal organs were dissected out and weighed with the fluid contents of the fetal gut and heart being removed before weighing. Tissue samples were collected in plastic bags, rapidly frozen in liquid nitrogen and stored at –80 ^{0}C until required for sectioning. From each placenta, an average medium size placentome was randomly selected, frozen in liquid nitrogen and then stored at –80 ^{0}C until required for sectioning. The remaining placentomes from the uterine wall were dissected and trimmed of the membranes, blotted with tissue paper and then counted and weighed individually (Fig. 6.3). The aggregate weight of all placentomes was recorded as total placental weight. After dissection of the placentomes, the remaining fetal membranes were also weighed.

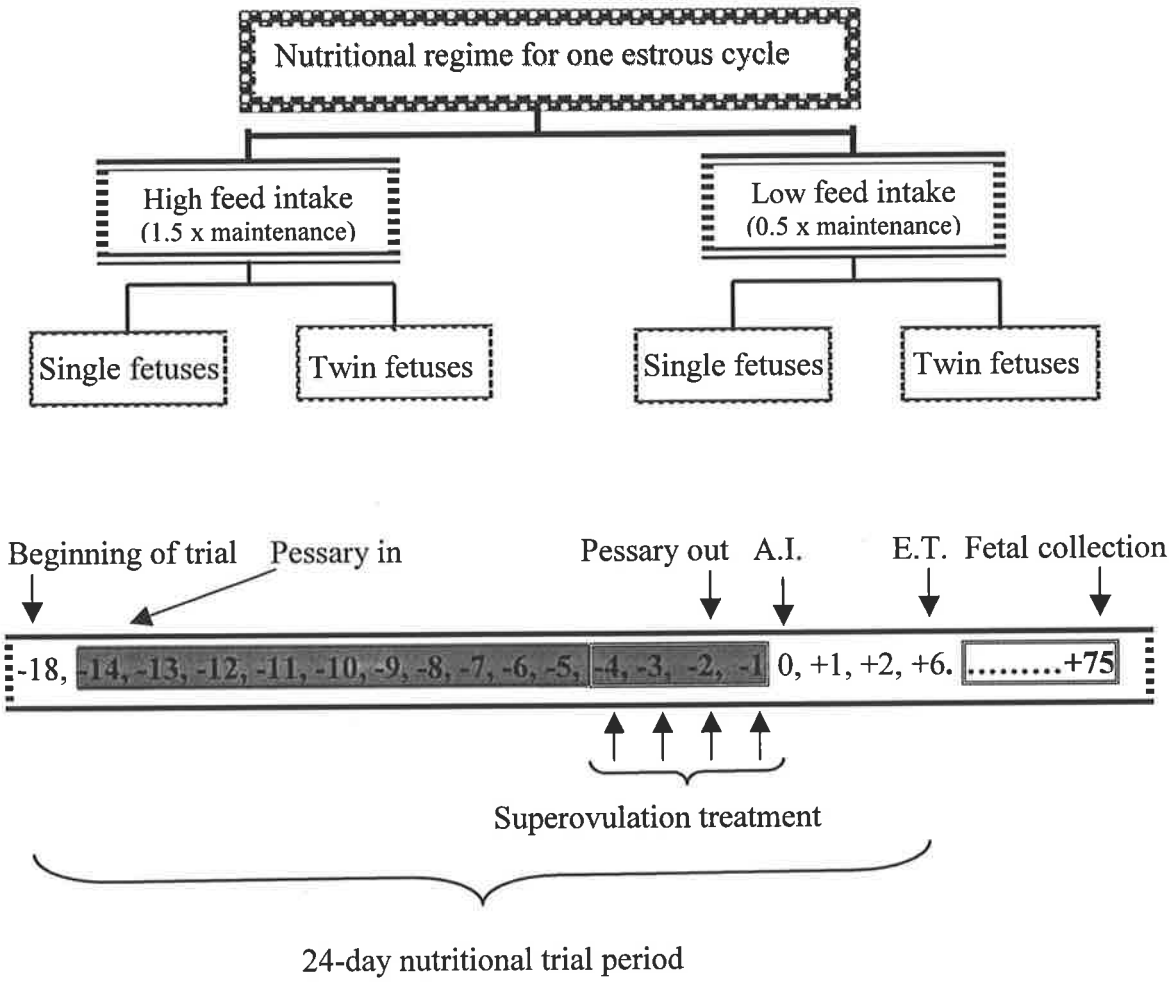
6.2.6 Statistical analyses

A total of 24 donor ewes were included in this experiment. For management purposes, the experiment was designed in two randomised blocks according to initial weight. The start of the trial was then staggered over a two-week period. Within each block, high or low feed intakes were randomly allocated to 24 sheep. All sheep were superovulated and embryos from these sheep were transferred to 49 recipient ewes as single or twin embryos. In the analysis of final weight, initial weight was used as a covariate to minimize experimental error (there were no significant differences between treatments for initial weight).

For donor ewes, the data was analysed using analysis of variance techniques, assuming a randomised complete block design with two blocks. The effect of feed intake (high or low) on initial weight, final weight, number of embryos, number of CL and the proportion of embryos lost were analysed. The assumptions of ANOVA are that the data is normally distributed with constant variance. Various diagnostics, such as residuals versus fitted values, examine these assumptions.

Parameters of fetal development were analysed by ANOVA or by regression analysis techniques. All analyses were performed in GenStat – 6th Edition.

Fig. 6.1 Experimental design:



6.3 RESULTS

6.3.1 Donor ewes

6.3.1.1 ***Initial weight:*** There were no significant differences in ewes weight between treatment groups at the beginning of the experiment.

6.3.1.2 ***Final weight:*** The covariate initial weight was highly significant ($P< 0.001$). Sheep fed a H diet were significantly ($P<0.05$) heavier at the end of the experiment (63.2±1.4 kg) than those fed a L diet (56.5±1.9 kg).

6.3.1.3 ***Change in weight:*** There was a highly significant effect of treatment on the change in live weight ($P<0.001$). Sheep fed a L feed intake lost a significant ($P<0.05$)

amount of weight (-4.5±0.4 kg) and those sheep fed a H feed intake gained a significant (P<0.05) amount of weight (2.3±0.4 kg).

6.3.1.4 Number of corpora leutea: Feed intake did not significantly influence the number of CL (H, 12.7±2.7 and L, 13.7±2.2) in donor ewes.

6.3.1.5 ***Number of embryos:*** The feed intake treatment had no significant effect on the number of embryos collected (H = 3.6±1.0and L = 4.9±1.3).

6.3.1.6 ***Proportion of embryos lost:*** The effect of feed intake on the proportion embryos lost as a proportion of CL counted approached significance (P = 0.063) with the loss being greater in H ewes compared with L ewes. Overall, a significantly (P<0.05) higher proportion of embryos were lost from the left side (0.8±0.06) compared with the right side (0.6±0.08).

6.3.2 Fetal development

Table 6.1 summarises the number of recipient ewes that received either single or twin embryos from each nutritional treatment.

Table 6.1 Number of embryos transferred to recipient ewes within each treatment group (High 1.5 x maintenance or Low 0.5 x maintenance).

	Embryo transfer			Pregnancy confirmed		
	Single	Twin	Total	Single (S)	Twin (T)	Total
High	7	11	18	3	3T and 4S	10
Low	10	21	31	5	1T and 9S	15

Of the 7 recipient ewes receiving a single embryo transfer from donor ewes on the high feed intake, 3 ewes were confirmed to be pregnant (or 43%). Of the 11 recipient ewes with twin embryo transfer from the high feed intake group only 3 of the 7 pregnant ewes were actually carrying twins. Of the 10 recipient ewes receiving a

single embryo transfer from donor ewes on the low feed intake, 5 ewes were confirmed to be pregnant (or 50%). Of the 21 recipient ewes with twin embryo transfer from the low feed intake group only 1 of the 10 pregnant ewes was actually carrying twins.

As there were only four ewes carrying twins (2 female/female, 1 male/female and 1 male/male) it was not possible to determine if feed intake or gender influenced the development of twin fetuses.

6.3.2.1 *Fetal organ weight*

Tables 6.2, 6.3, 6.4 and 6.5 summarise the distribution of fetal organ weights in response to low and high maternal feed intake. All measurements were similar between the two feed intake groups except for a significant increase in the weight of the brain, pancreas and chest thymus in the high feed intake group. In male fetuses, the brain and left ventricle weights were significantly increased compared with their female counterparts (Tables 6.3; 6.6). In the case of twins vs. singles, only ovarian weight was significantly decreased in twins compared with singletons (Table 6.4).

Table 6.2 Fetal and placental weights (means ± SEM) at 75 days of gestation for embryos derived from a H feed intake (1.5 x maintenance ration) vs a L feed intake (0.5 x maintenance ration) donor ewes (Values are). There are no significant differences between treatments.

	High (n=13)	Low (n=16)
Fetal weight (g)	219.1±4.5	213.5±8.4
Crown rump length (cm)	22.1±0.1	21.4±0.4
Abdominal circumference (cm)	13.6±0.2	13.7±0.2
Thoracic circumference (cm)	13.3±0.1	13.3±0.1
Head width (mm)	33.3±0.5	31.9±0.6
Head length (mm)	59.8±0.6	58.3±0.6
Placentome weight (g)	799.4±65.8	829.6±57.5
Placentome number	63.6±7.6	71.9±2.7
Average placentome wt. (g)	11.9±0.3	11.5±0.2
Placental membranes (g)	402.0±60.7	444.0±23.9
Fetus:placenta weight ratio	0.21	0.20

Table 6.3 Fetal and placental weights (means ± SEM) at 75 days of gestation in female and male fetuses.

	Female (n=15)	Male (n=14)
Fetal weight (g)	209.6±5.9	222.9±8.1
Crown rump length (cm)	21.7±0.3	21.7±0.3
Abdominal circumference (cm)	13.5±0.1	13.7±0.2
Thoracic circumference (cm)	13.2±0.1	13.3±0.1
Head width (mm)	32.1±0.5	32.9±0.7
Head length (mm)	58.4±0.5	59.6±0.7
Placentome weight (g)	785.7±58.3	850.0±59.5
Placentome number	73.1±4.6	65.6±4.8
Average placentome wt. (g)	10.6±0.3[a]	12.6±0.3[b]
Placental membranes (g)	411.0± 39.9	446.0± 30.5
Fetus:placenta weight ratio	0.21	0.21

Values within rows with different superscripts differ significantly ($P<0.05$)

Table 6.4 Fetal and placental weights (means ± SEM) at 75 days of gestation in single and twin fetuses.

	Single (n=21)	Twin (n=8)
Fetal weight (g)	217.6±6.3	211.7±8.0
Crown rump length (cm)	21.5±0.3	22.1±0.2
Abdominal circumference (cm)	13.8±0.1	13.2±0.1
Thoracic circumference (cm)	13.4±0.1	13.1±0.1
Head width (mm)	32.5±0.4	32.6±0.9
Head length (mm)	59.2±0.5	58.8±0.8
Placentome weight (g)	835.2±42.5	840.1±58.0
Placentome number	69.6±2.8[a]	94.3±10.8[b]
Average placentome wt. (g)	11.9±0.2	10.9±0.3
Placental membranes (g)	417.0± 24.6[a]	539.0± 33.4[b]

Values within rows with different superscripts differ significantly ($P<0.05$)

Table 6.5 Fetal organ weights (mean ± SEM) and per cent organ to body weight at 75 days of gestation from embryos derived from high feed intake (1.5 x maintenance ration) vs. low feed intake (0.5 x maintenance ration) donor ewes.

	High (n=13)		Low (n=16)	
	Weight	% body weight	Weight	% body weight
Adrenal glands	0.08±0.02	0.03±0.001	0.1±0.03	0.03±0.001
Kidney left	1.7±0.06	0.8±0.03	1.6±0.10	0.7±0.02
Kidney right	1.7±0.07	0.8±0.03	1.5±0.09	0.7±0.02
Brain	7.7±0.13[a]	3.5±0.08	7.0±0.26[b]	3.3±0.08
Liver	14.1±0.52	6.4±0.20	13.9±0.64	6.5±0.18
Lungs	11.9±0.37	5.5±0.12	11.2±0.56	5.3±0.11
Gastro intestinal tract	8.8±0.49	3.9±0.21	7.9±0.54	3.7±0.19
Pancreas	0.3±0.02[a]	0.1±0.006[a]	0.2±0.01[b]	0.1±0.006[b]
Spleen	0.3±0.01	0.1±0.09	0.3±0.02	0.1±0.08
Heart	2.5±0.06	1.1±0.04[a]	2.2±0.15	1.0±0.04[b]
Left ventricle	1.5±0.10	0.7±0.04	1.3±0.09	0.6±0.04
Right ventricle	0.7±0.05	0.3±0.03	0.6±0.06	0.3±0.03
Thymus chest	0.3±0.03[a]	0.8±0.56[a]	0.2±0.01[b]	0.2±0.51[b]
Thymus neck	0.4±0.03	1.6±1.07	0.4±0.04	1.0±0.96
Thyroid glands	0.1±0.01	0.3±0.38	0.1±0.01	0.2±0.34
Ovaries	0.03±0.005[a]	0.01±0.002[a]	0.04±0.003[b]	0.02±0.002[b]
Testes	0.1±0.01	0.05±0.005	0.1±0.01	0.05±0.005

Values within rows with different superscripts differ significantly ($P<0.05$)

Table 6.6 Fetal organ weights (mean ± SEM) at 75 days of gestation in female and male fetuses.

	Female (n=15)		Male (n=14)	
	Weight	% body weight	Weight	% body weight
Adrenal glands	0.1±0.03	0.03±0.001	0.1± 0.03	0.03±0.002
Kidney left	1.6±0.07	0.7±0.02	1.7±0.11	0.7±0.02
Kidney right	1.5±0.08	0.7±0.02	1.6±0.10	0.7±0.02
Brain	7.0±0.23[a]	3.3±0.08	7.7±0.20[b]	3.5±0.09
Liver	13.5±0.48	6.5±0.19	14.6±0.69	6.5±0.20
Lungs	11.1±0.47	5.3±0.12	12.1±0.51	5.4±0.12
Gastro intestinal tract	8.2±0.53	3.9±0.19	8.4±0.55	3.7±0.20
Pancreas	0.2±0.02	0.1±0.06	0.3±0.02	0.1±0.06
Spleen	0.2±0.02	0.1±0.08	0.3±0.02	0.1±0.08
Heart	2.2±0.11	1.1±0.04	2.5±0.15	1.1±0.04
Left ventricle	1.2±0.10[a]	0.6±0.04[a]	1.5±0.08[b]	0.7±0.04[b]
Right ventricle	0.7±0.07	0.3±0.03	0.6±0.04	0.3±0.03
Thymus chest	0.2±0.01	0.1±0.09	0.2±0.03	0.1±0.09
Thymus neck	0.4±0.03	0.2±0.02	0.4±0.05	0.2±0.02
Thyroid glands	0.1±0.01	0.07±0.006	0.1±0.02	0.06±0.006

Values within rows with different superscripts differ significantly ($P<0.05$)

6.3.2.2 Weight distribution of placentomes

The number of placentomes and the percentage of the total number of placentomes across the weight distribution range are illustrated in Fig. 6.4a and b respectively. There was no significance difference in these distributions between the high and low feed intake groups. While the data suggests an interesting difference between the high and low feed intake groups, these differences were not significant.

6.3.2.3 Total placental weight

There was no significant effect of fetal sex or feed intake on total placental weight. There was also no significant interaction between feed intake and fetal sex on placental weights.

6.3.2.4 Number of placentomes

The effect of fetal sex and feed intake did not significantly affect the number of placentomes. There was also no significant interaction between feed intake and fetal sex on the number of placentomes.

6.3.2.5 Fetal weight as a proportion of total weight (fetus + placenta)

The main effects of fetal sex and feed intake on fetal weight as a proportion of total weight (fetus + placenta) were also not statistically significant. There was no significant interaction between feed intake and fetal sex on this proportion. Due to the small number of sheep within each combination of feed intake and sex it was not possible to test the strength of the correlation between placental weight and fetal weight and placental weight versus organ weight. The relationship between fetal and placental weights for the combined groups and sex had a correlation coefficient of r=0.345, P= 0.124. However, for liver (P=0.007), right kidney (P=0.044), spleen (P=0.047), left ventricle (P=0.028) and placental membranes (P=0.024) the correlations with placental weight were significant. The data are presented graphically for fetal organ weight and placental weight for liver (Fig. 6.5), right kidney (Fig. 6.6), spleen (Fig. 6.7), left ventricle (Fig. 6.8) and placental membranes (Fig. 6.9). The correlation coefficients for organ weights are tabulated in Table 6.7.

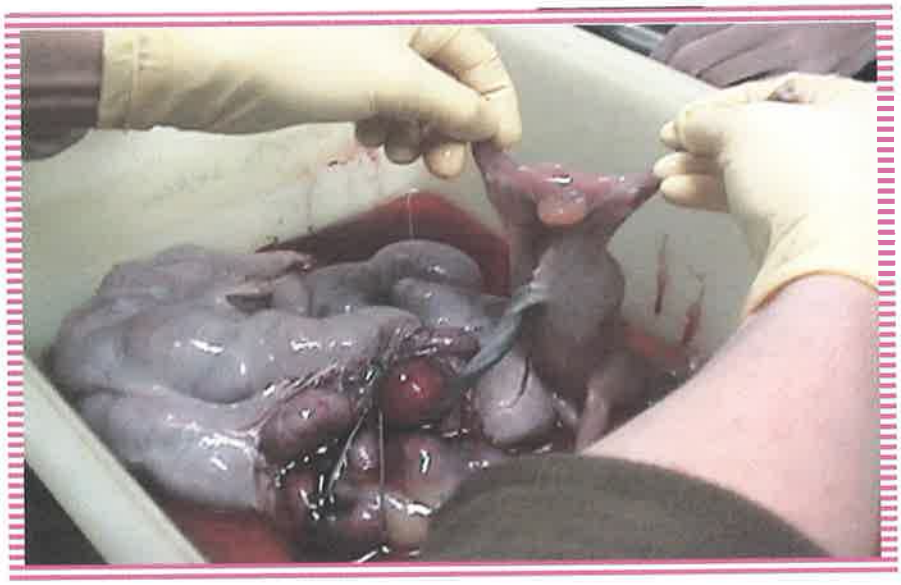


Fig. 6.2 Fetus and fetal membranes with attached umbilical cord.

Fig. 6.3 Variation in placentomes size within one placenta.

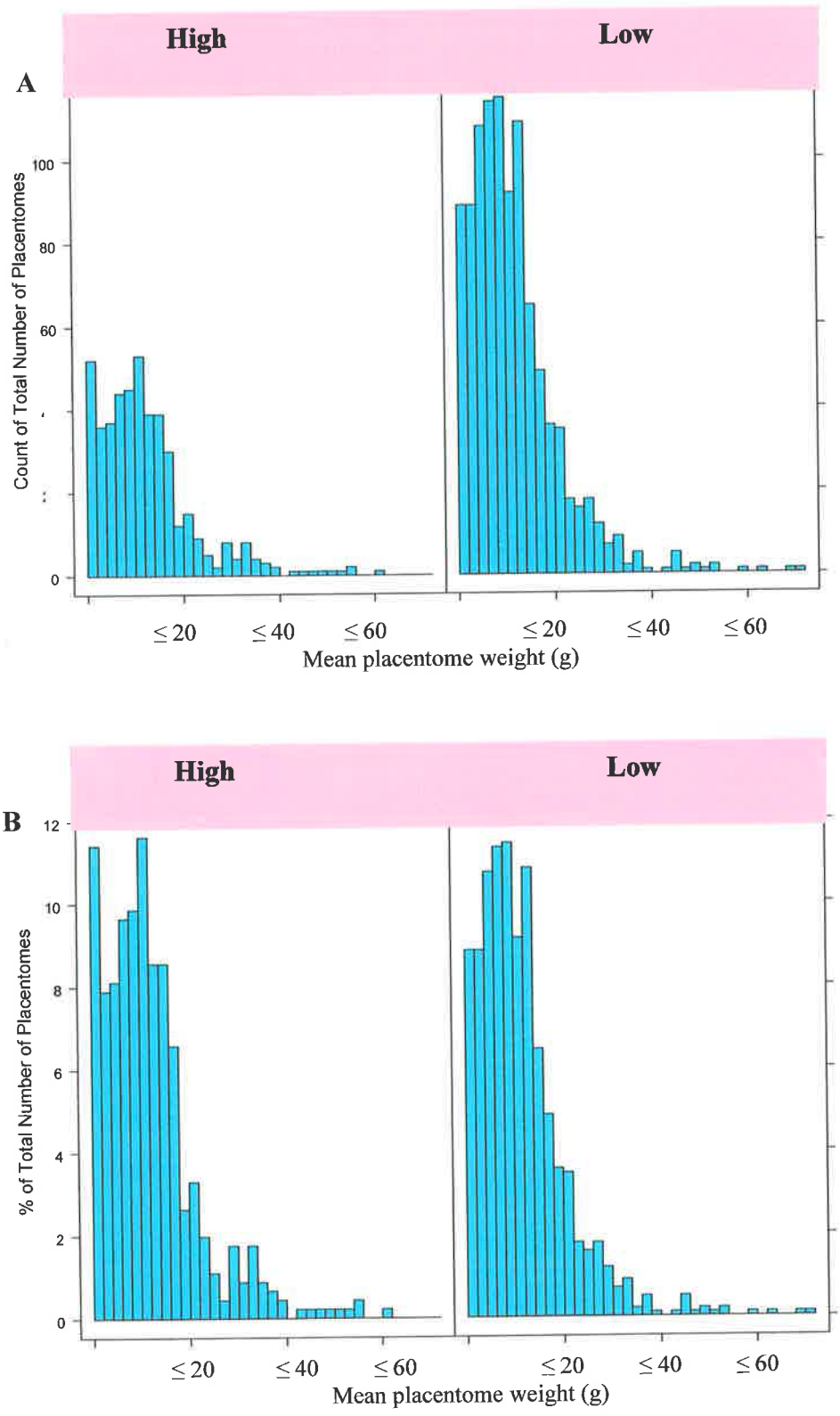


Fig. 6.4 The effect of high (n=7) and low (n=14) maternal feed intake on the weight distribution of (A) placentome number and (B) percentage of total number of placentomes.

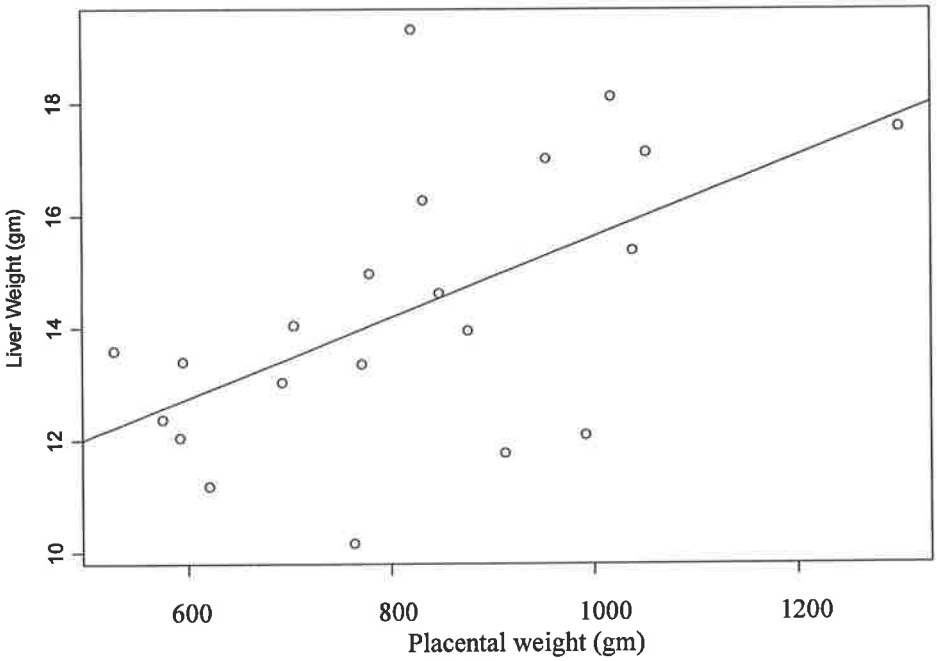


Fig. 6.5 Relationship between liver and placental weights for the combined groups and sex (r=0.567, P= 0.007).

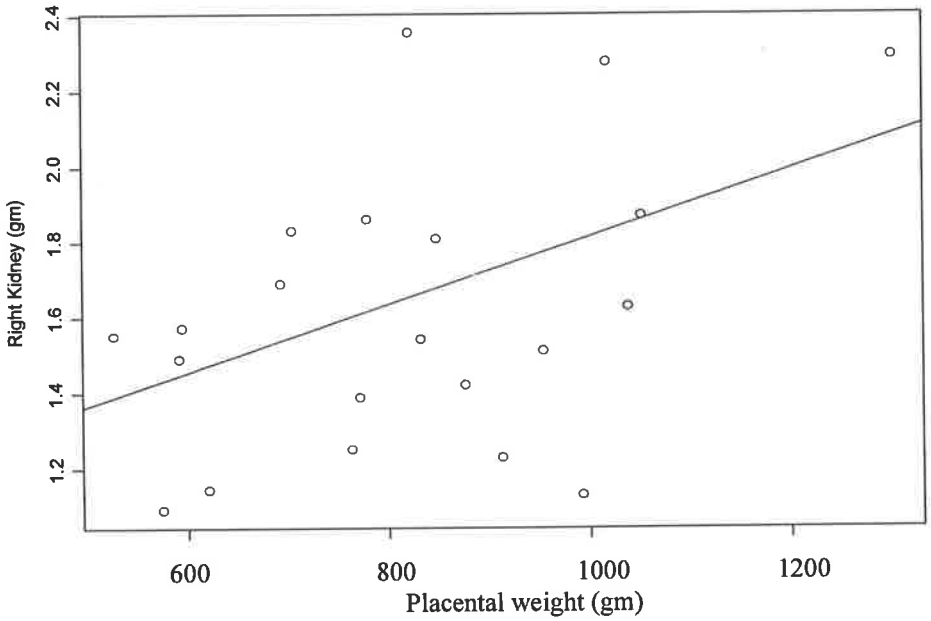


Fig. 6.6 Relationship between right kidney and placental weights for the combined groups and sex (r=0.444, P= 0.043).

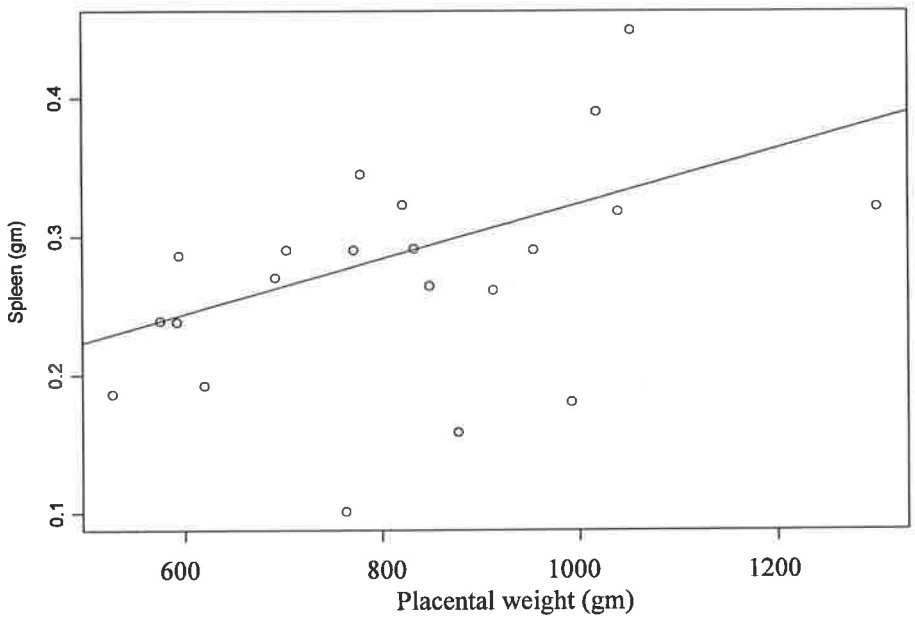


Fig. 6.7 Relationship between spleen and placental weights for the combined groups and sex (r=0.437, P= 0.047).

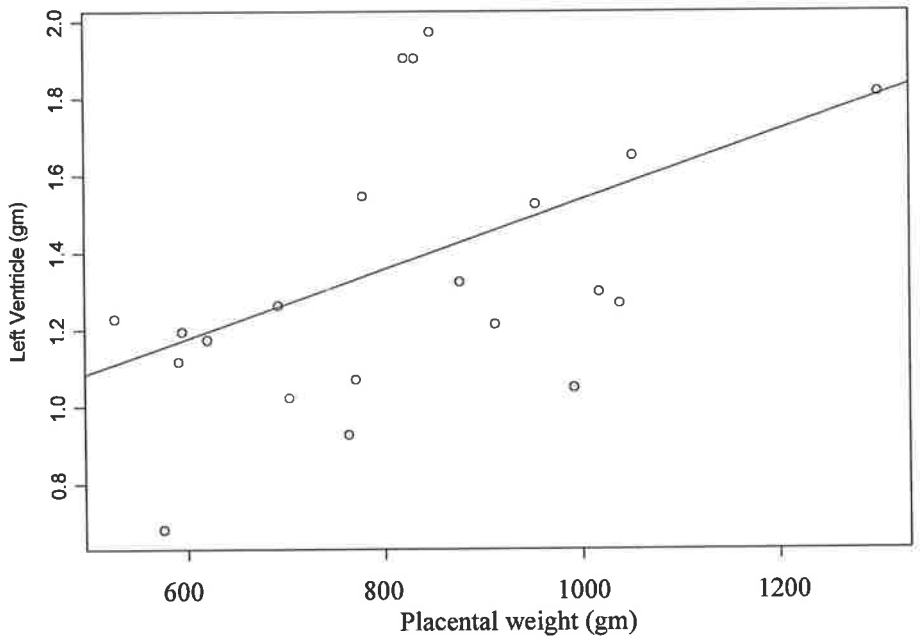


Fig. 6.8 Relationship between left ventricle and placental weights for the combined groups and sex (r=0.477, P= 0.028).

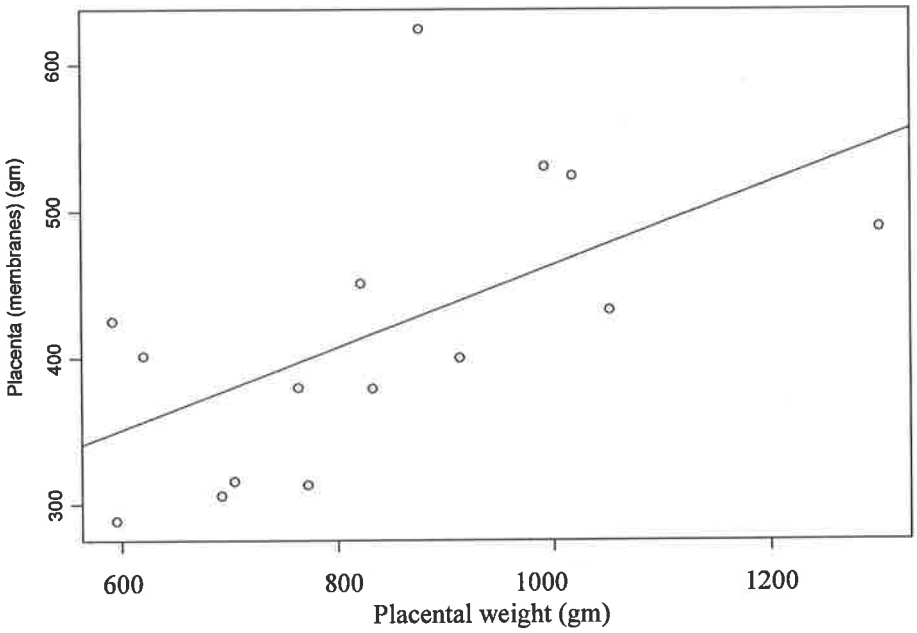


Fig. 6.9 Relationship between placental membranes and placental weights for the combined groups and sex (r=0.579, P= 0.023).

Table 6.7 Correlation coefficients (*r*) between placental weight with fetal weight and fetal organ weights at 75 days of gestation.

	r-value
Fetal weight	0.345
Adrenal	0.331
Kidney left	0.396
Kidney right	0.444*
Brain	6.405
Liver	0.567*
Lungs	0.246
GIT	0.248
Pancreas	2.136
Spleen	0.437*
Heart	0.343
Left ventricle	0.477*
Right ventricle	9.170
Thymus chest	0.236
Thymus neck	0.377
Thyroids	0.362
Ovary	0.291
Testis	0.326
Placental membranes	0.579*

* Denotes a significant correlation, $P<0.05$

6.4 DISCUSSION

The present study demonstrates that there was a tendency of increased placental development at Day 75 of gestation with low peri-conceptional maternal feed intake of the donor ewes. Such alterations may have important functional consequences. Interestingly in the present study, fetal body growth was maintained, suggesting that, in so far as fetal growth is concerned, the changes that occurred in placental development were apparently adaptive responses, providing compensation for the effects of a low feed intake environment during the peri-conceptional period. A greater understanding of the mechanisms involved in adapting to sub-optimal nutrition may allow the development of methods to modify the intensity of inputs from nutritional stimuli to the hypothalamus thereby increasing the ability to regulate reproduction. Although it is not known whether deficiencies of nutrients limit reproduction through common or discrete mechanisms, provision of appropriate quantities of nutrients is required for optimal reproduction. Less information is available on the effect of excesses of nutrients on reproduction. By manipulation of nutrient intake, livestock producers might strive to maintain herds or flocks in moderate body condition in order to maximise reproductive outcome.

Similar fetal weights at mid pregnancy between the high and low feed intake groups suggest that the process of adaptive fetoplacental angiogenesis was successful in maintaining fetal growth. This is consistent with the suggestion of Faichney and White (1987) and Harding and Johnston (1995) that increased placental size reflects an attempt by the fetus to compensate for the reduction in nutrient supply. In fact, in these studies, they found that not only was placental weight increased but also fetal weight was increased in response to undernutrition between 50 and 100 days of gestation. Although fetal growth, (other than that of the brain, pancreas and chest thymus) was unaffected in the present study it is possible that there were other effects of differential feed intake on fetal development. Battaglia (1997) concluded that amino acid exchange and metabolism clearly demonstrates that the fetal liver and placenta function as an integrated system of organs. In the present study there was no significant correlation between placental weight and fetal weight at Day 75 of the gestation.

More importantly, the present study has shown that the effects of feed intake on early embryo development are reflected in an increase in the weight and dimensions of fetal organs. For example, the fetal brain, pancreas and chest thymus weight were increased in fetuses exposed to high peri-conceptional feed intake. When expressed as a proportion of fetal weight, only the pancreas and chest thymus were significantly increased by high feed intake. On the other hand, the weight of fetal ovaries was significantly increased in response to low feed intake compared with high feed intake and when expressed as percentage of fetal body weight, the proportional ovarian weight was also significantly increased. Osgerby *et al.,* (2002) and Da Silva *et al.,* (2002) have also reported a reduction in ovarian weight at Day 90 in response to low nutrition. Maternal dietary restriction in sheep has previously been shown to reduce the fetal brain weight (Harding and Johnston, 1995; Clarke *et al.,* 1998) disrupt ovarian development (Borwick *et al.,* 1998) and compromise lifetime reproductive performance in subsequent female offspring (Gunn *et al.,* 1995; Rhind *et al.,* 2001). It could be speculated that these disproportionate changes in fetal organ growth are linked to the effect of low feed intake increasing progesterone levels during early pregnancy since Kleemann *et al.* (2001) have indicated that exogenous progesterone administered to ewes during the first three days of pregnancy significantly altered growth of fetal heart and brain tissue relative to fetal mass. Despite these alterations in fetal organ growth, the fetal weight did not alter significantly, confirming the insensitivity of fetal weight as an indicator of fetal growth as proposed by Harding and Johnston (1995).

Kalache *et al.* (2001) reported that maternal nutrient restriction in early gestation did not affect fetal weight but resulted in adaptive changes in placental morphology and function. In the current study, it is evident that manipulation of feed intake for a relatively short period of time can affect the fetus and its placenta at mid-pregnancy. The present study also supports the idea that differential peri-conceptional maternal feed intake is capable of producing subtle changes in gross placental morphology. Whether these changes are present near term in nutritionally restricted fetuses remains to be determined. Changes developed during a critical period of pregnancy may permanently modify physiological functions, such that they cannot be restored by a normalisation of the environment later in pregnancy. The trend towards a change in placentome distribution in the present study appears to compensate for external

modulating influences such as maternal feed intake. It is possible that in the different morphology of placentomes, one might observe differences in vascular structure or architecture, which might influence the efficiency of transplacental exchange. The physiological mechanisms by which these changes occur and the biological significance of such response remains to be determined.

There is now strong evidence to suggest that sexual dimorphism in mammalian embryos begins shortly after conception (Sinclair *et al.,* 2000). Male embryos develop more quickly to the blastocyst stage than female embryos in *in vitro* culture (Ray *et al.,* 1995), resulting in a higher proportion normally being transferred to recipients thus leading to a skewed sex ratio in favour of males at birth in both ruminants (Behboodi *et al.,* 1995) and humans (Menezo *et al.,* 1999). In the present study, the brain and left ventricle weights in male fetuses were significantly increased compared with their female counterparts. In addition, the proportional weight of the left ventricle in relation to body weight was also significant increased in male fetuses. Despite these alterations in organ growth, the overall fetal weight did not alter significantly. This sexual dimorphism could be due to the parental origin of the X chromosome (Thornhill and Burgoyne, 1993) or the presence of the Y chromosome. Transcripts of the Y-linked *Zfy* gene have been detected as early as the two-cell stage in both murine and ovine embryos and together with other genes (e.g., *Sry* and *Smcy)*, could serve as candidates for the Growth Factor Y effect (Erickson, 1997) where effects are seen only in male fetuses. Interestingly, in the placenta from the male fetus pregnancies, the average placentome weight was significantly increased compared with placentas from female pregnancies. There is also evidence suggesting that the fetus can influence the placenta, through both effects of fetal sex (Alexander, 1974) and fetal nutrient supply to the placenta (Owens, 1991). Clearly the coordination between the placenta and fetus is important for optimal fetal well-being.

Female embryos are known to differ metabolically from their male counterparts and to be less tolerant of the presence of glucose in culture (Gutierrez-Adan *et al.,* 1998). This has been attributed to the higher concentrations and relative activities of X-linked enzymes, e.g., glucose-6 phosphate dehydrogenase and hypoxanthine phosphoribosyl transferase (Rieger, 1992), which prevail in the female embryo during the period before and during X chromosome inactivation (Kay, 1998). Although it is

possible that aberrations in the imprinted expression of the X-inactive specific transcript (*Xist*) gene in the mouse may lead to a loss or partial loss of X inactivation, so contributing to some of the sex-differentiated phenotypes associated with large offspring syndrome (LOS), this is not consistent with the observation that both sexes are affected by LOS (Wilson *et al.,* 1995).

There is also a link between post-natal malnutrition and sex as severe malnutrition affects seven times more female than male infants in the developing world (World Development Report, 1993). Vitamin A, iodine or iron deficiency during pregnancy and early life each have a specific and immense impact on the offspring's health, contributing 11.7%, 7.2% and 14%, respectively, to the total human disease burden throughout the developing world (World Development Report, 1993). It is now clear from a range of human and animal studies that poor maternal health and/or nutritional deficiencies affect key tissues during fetal development, and can be responsible for pathological changes in the offspring.

The factors regulating feto-placental growth and angiogenesis and thus placentome differentiation in the sheep, are not well understood. Growth factors, such as the insulin-like growth factors (IGFs) and vascular endothelial cell growth factor (VEGF), may play an important role (Dunk and Ahmad, 2000). There synthesis or that of there receptors or binding proteins may be affected by nutrition. It is clear from this study that short-term nutritional restrictions during the peri-conceptional period results in significant alterations in fetal and placental development. The mechanisms involved are as yet not understood. Investigation of various growth factors in the different placentome types may help elucidate the mechanisms involved in the generation of different placentome types observed in the sheep.

Variations in fetal growth may be associated with differences in placental growth, but they usually only become evident in the second half of gestation, as fetal and substrate demand increases substantially and begin to be restrained by placental functional capacity (Alexander, 1974; Mellor, 1983; Bell 1984; Owens, 1991). In the current study, placental weight did not differ significantly with different feed intakes. However this does not exclude the possibility that changes to the functional development of the placenta may be a contributing factor responsible for enhanced

fetal organ growth. However, there was a tendency of increased weight with low feed intake compared with high feed intake in total placentome weight (high: 799.36 grams vs. low: 829 grams), placentome number (high: 63.6 vs. low: 71.9) and placental membranes weight (high: 402 grams vs. low: 444 grams) but these did not reach a level of statistical significance. While low placental weights are usually associated with small numbers of placentomes (Mellor, 1983), it is not clear from the present study whether it was the low feed intake that increased the placental weight or if it was the high feed intake that actually decreased the total placental weight. Interestingly in the placentas from male fetus pregnancies the average placentome weight was significantly increased compared with placentas from female pregnancies. There was also a significant increase in both placentome number and placental membrane weight in twins compared to singletons.

It has been previously shown that short-term changes in feed intake during the peri-conceptional period of the ewe affect blastocyst cell number (Chapter 4). The findings of the current study suggest that the altered development of the trophectoderm is translated into enhanced development of the placenta, in addition to the enhanced growth of certain fetal organs by mid gestation. In general the liver, right kidney, spleen, left ventricle and placental membranes weight were significantly correlated with placental weight. Because of the different development patterns of these fetal organs, this may be more likely to reflect an enhancement of placental function following early high progesterone exposure through low feed intake, which then promotes growth of fetal tissues. Support for this contention of improved placental function mediated through the effects of progesterone during early pregnancy is given by Kleemann *et al.* (2001). Concomitant and independent enhancement of placental development and growth of specific fetal tissue by early high levels of progesterone through low feed intake cannot be excluded however.

There is a negative relationship between progesterone concentrations and the level of nutrition, with low levels of nutrition associated with higher concentrations of progesterone (Chesworth and Easdon, 1983). This relationship was valid whether the progesterone was derived from endogenous sources (ovary) (Chapter 5) or from injections administered to ovariectomized ewes (Parr *et al.*, 1982). In underfed ewes, concentrations of progesterone have been reported to increase more rapidly between

Day 2 and Day 6 after ovulation than in ewes that received adequate feed (Rhind *et al.,* 1989). On the other hand, overfeeding has been associated with embryo loss in sheep (Rhind *et al.,* 1989; McEvoy *et al.,* 1995; Yaakub *et al.,* 1997; Boland *et al.,* 2001). Because of the inverse relationship between level of feeding and progesterone concentration, Parr *et al.* (1987) suggested that progesterone concentrations in some well-fed ewes were insufficient to support embryo development.

Walker *et al.* (1996) have advanced a number of possible physiological and molecular mechanisms that may explain the increase in fetal growth observed after manipulations of the embryo or its environment. They hypothesised that manipulation before or at the 8-16 cell stage could influence transcription of genes associated with early embryo development, change inherited cytoplasmic factors essential for early embryo development or disrupt sequential signalling that occurs between the embryo and the maternal environment. Clearly, an understanding of how enhanced fetal organ growth and alterations in placental development result from perturbation of the embryo's environment via early maternal nutrition requires further investigation. These perturbations in fetal development may have significant implications on adult health and body conformation, raising important health and economic issues in medical and agricultural sectors.

Chapter 7

GENERAL DISCUSSION

7.1 Introduction

This chapter describes the implications of the observations made throughout this thesis and suggests possible underlying mechanisms. In addition, the limitations of the work undertaken and possible future directions are outlined.

Although dietary intake can clearly influence reproductive function, the relationship between nutrition and reproduction is complex and, despite much research over a number of decades, the reproductive responses to different levels of nutrition are often variable and inconsistent. A high dietary intake exerts a negative effect on the developmental capacity of embryos (McEvoy *et al.*, 1995). Experimental diets providing below maintenance requirements resulted in improved embryo development compared with animals on *ad libitum* diets in several experiments (Boland *et al.*, 2001). These effects of nutrition are exerted in the early stages of oocyte development, possibly before fertilisation, during the acquisition of developmental competence by the oocyte. Pre-mating nutritional strategies have, to date, been inadequate and need to be extended to cover the entire period between the previous lactation and mating. It is during this period that ovarian follicles grow and develop in preparation for ovulation but despite this, most nutritional studies have focussed on the period immediately before mating. It is likely that much of the variability observed in artificial insemination and embryo transfer programs are sourced during this period and that potential exists to develop management strategies to overcome this problem. A key issue in improving reproductive efficiency and embryo production may be to supply the nutritional needs of the animal in a physiological way and avoid abnormal or unbalanced amounts of any one component in the diet. Thus, nutritional balancing is critical for high-production animals. The challenge remains to modify nutritional and management strategies in such animal production systems to maintain the level of production that can now be achieved as a consequence of genetic selection and still maintain an acceptable level of fertility.

It is becoming evident that "high" feed intake is not uniformly desirable and that there are stages where subtle changes towards "low" feed intake are necessary to maximise reproductive performance. This hypothesis encompasses a strategy that is contrary to conventional thinking and practise. However there is growing evidence (Chapter 4; 5)

that subtle reductions in feed intake at specific times enhance reproductive performance. Low feed intake during the mating period (i.e. following a high plane of feed intake immediately before mating) appears to be beneficial to embryo quality. This has been demonstrated in both sheep and cattle (O'Callaghan and Boland, 1999). Data preseneted in Chapter 4 indicate "low" feed intake during the mating period results in the production of embryos with an increased number of trophectoderm cells. Such allocation of cells could result in enhanced rates of implantation and fetal survival by virtue of changing the structure and function of the placenta. It is important that relevant research be conducted to assess the significance of judicious application of a "low" and "high" feed intake model on reproductive outcome.

The mechanisms by which nutrition affects reproductive outcome are still not fully understood, because of the complexity of the neuro-hormonal dialogue and the equally complex nutrient partitioning involved. Nevertheless, some advances have been made and interesting possibilities put forward. Some of these nutrient-mediated effects that act directly on the gonads and other reproductive organs, while others that produce similar effects act indirectly via the hypothalamo-pituitary-gonadal axis. It is worth noting that although the emphasis in the literature is mainly on the negative impacts of nutrient deficiencies, excesses and/or imbalances of different nutrients may also result in reproductive disorders (Smith and Akinbamijo, 2000).

7.2 Peri-conceptional feed intake and embryo quality: This study has shown for the first time that short-term low feed intake imposed on superovulated ewes prior to ovulation increased the total number of cells in the embryo and that this increase was primarily due to an enhanced number of trophectoderm cells. The lack of a difference between the total numbers of embryos produced by ewes fed low, medium and high feed intakes suggest that the extra intake observed in ewes in the high feed intake group was partitioned towards improving ewe body weight and thus condition, rather than increasing ovulation or embryo growth. In contrast to spontaneously ovulating ewes where flushing (i.e., the increase of nutrition 4-6 week before the mating) may enhance ovulation rate (Rhind *et al.,* 1989), superovulatory responses were not compromised by restricted feeding in this study, presumably because individual potential was maximised as a result of gonadotrophic action. It must be stressed, however, that the results followed short-term feed restrictions applied to ewes which

were in good body condition. A similar gain could not be expected following either prolonged feed restriction or the imposition of the present regimen on already under-nourished animals. The lack of a significant increase in ovulation rate and number of embryos collected with increase in food intake was unexpected and is at variance with previous reports (Smith 1985, 1988) and possibly superovulation maximally stimulated the ewes, not allowing for any further increase. Lindsay (1976) has described live weight as being 'a crude, inaccurate criterion which, because it describes only long-term changes in feeding, is incompatible with studies on many of the components of the reproductive process — which takes place over a few days or even hours'. This is supported by the finding of Smith *et al.* (1982) who, in summarising a series of grazing experiments, found that ewe liveweight at mating and pre-mating liveweight change accounted for only 42.0 and 18.5%, respectively, of the variation in ovulation rate. Thus nutritional factors over and above those influencing live weight, fat reserve and condition score appear to exert a major influence on ovulation rate as also found by Smith *et al.* (1979).

Following short-term culture of morula stage embryos in the present study, a high proportion of the embryos from ewes on the low feed intake progressed to the blastocyst stage and had higher total cell numbers than embryos from ewes on high feed intake, although the ratio of inner cell mass to total cell number was not different following different dietary intakes. The results support previously published data of Nolan *et al.* (1998) and Negrao *et al.* (1997), who concluded that the yield of transferable embryos increased as the dietary energy intake decreased. In ruminants there is a risk that a rapid shift in rumen fermentation caused by abrupt changes in diet composition, and in the amount or pattern of feed intake may temporarily disrupt metabolic homeostasis with consequent adverse effects on the embryo (McEvoy *et al.*, 2001). Improved nutrition during the periovulatory period is generally regarded as beneficial to reproductive outcome for a naturally-occurring oestrus but this does not appear to be the case when superovulation is induced using exogenous gonadotrophins in ewes (McEvoy *et al.*, 1995a; b; 2001; Lozano *et al.*, 2000; Boland *et al.*, 2001) or beef heifers (Nolan *et al.*, 1998; Yaakub *et al.*, 1999).

7.3 Feed intake signals before and after mating: The results of the first experiment, described in Chapter 4 indicate that the level of feed intake significantly influences

several parameters including blastocyst cell number and cell lineage differentiation. It was subsequently determined that the nutritional signals enhancing embryo cell numbers appeared to be manifested during the post-mating period rather than the pre-mating period. Dyck and Strain (1983) suggested that the critical window for nutritional effects on embryonal survival was the early postcoital period. Overall, these results show that the critical window for nutritional effects on embryo quality in ewes could occur in the period immediately after mating.

Evidence that the timing of changes in feed intake in the immediate post-mating period affects early embryo survival and quality may indicate why there are inconsistencies in the literature. For example, if flushing is used to increase ovulation rates in ewes, this nutritional management practice should be discontinued immediately after mating to avoid negative effects of increased feed intake on embryo survival and quality. This conclusion confirms the importance of nutrition during the immediate post-mating period to the developing embryo. Ultimately, a better understanding of the complex mechanisms of maternal-embryo dialogue and their role in setting the stage for the first differentiation of the developing embryo is required to optimise reproductive outcome.

The presence of serum in the culture medium from the 2-cell stage was recently shown to reduce the ICM:TE ratio by increasing the level of programmed cell death (apoptosis) within the ICM of bovine embryos (Byrne *et al.,* 1998). Some authors (e.g., Walker *et al.,* 1996; Leese *et al.,* 1998) have speculated that an alteration in the allocation of cells between the ICM and TE may also explain some of the features of the large "offspring syndrome". They argue that a disproportionate number of cells allocated to the TE may increase placental size, which in turn could support a larger fetus. A direct association between cell number and allocation between the ICM and TE and aberrant increases in fetal size, however, has yet to be proven, and such indirect evidence on this subject is inconclusive. In the present study, low feed intake increased total cell number and especially TE cells, in the embryo at Day-6 although no increase in fetal growth parameters were recorded at Day 75 of gestation.

From a general perspective, there seems to be increasing evidence that a number of the effects of nutrition and metabolic state on embryonic survival are mediated by

progesterone. However, the precise mechanisms involved need to be elucidated and a concerted effort is needed to determine the timing and the location of the progesterone-mediated effects. A high plane of nutrition in the immediate postmating period has been reported to result in a delayed rise in peripheral plasma progesterone concentrations (Pharazyn, 1991; Jindal *et al.,* 1997). This might also result in an earlier difference in progesterone concentrations in the oviduct thus providing a potential mechanism by which nutrition can exert a critical effect on oviductal function in the periovulatory period. If oviductal secretions affect the development of the embryo, nutrition could exert a direct effect via changes in the steroid environment of the oviduct (Foxcroft, 1997). Alternatively, nutritionally dependent changes in the steroid environment could affect the rate of tubal transport of the embryo and hence the precise synchrony between the development of the uterus and the preimplantation embryo. In this context, there is a need to define clearly the stage of development at which embryo quality might be affected by nutrition. When changes in feed intake are made in the immediate post-mating period, it is important to realise that there is the possibility that both the oviductal and uterine stages of development are affected. The experiments described in this thesis provide convincing evidence for nutritionally mediated effects on the embryo during the oviductal stage of development. From a mechanistic viewpoint, this may help to explain why differences in the timing of nutritional treatments have produced such inconsistent effects in previous reports on embryo survival.

7.4 Feed intake and oviductal fluid concentrations: As part of an examination of the environment influencing the embryo, the present study determined the effect of peri-conceptional feed intake on concentrations of ammonia, urea, amino acids, electrolytes progesterone and IGF-1, in oviductal fluid present at the time of fertilisation and early embryo development, in both superovulated and naturally ovulated ewes.

The mammalian oviduct provides the milieu for several important reproductive events including transport of ova and spermatozoa, conditioning of the gametes, fertilisation and early development of the resulting embryo. Fluid found within the normal oviductal lumen contains numerous constituents, many of which vary in concentration in response to endocrine influences. The experiments in Chapter 5 were a

continuation of efforts to understand the factors that might influence the ICM:TE ratio in embryos. Further understanding of these physiological conditions should provide greater insight into the possibility of manipulating or controlling reproductive processes at the oviductal level and thus improving embryo development and outcome.

7.4.1 Oviductal fluid volume, pH and osmolarity: The oviductal fluid volume collected in the studies reported here followed a definite cyclical pattern and a significantly larger volume was collected during the follicular phase. Individual secretion rates increased dramatically during oestrus in each of the twelve ewes, reached a peak two days after oestrus and then declined to levels characteristic of the luteal phase. The oviductal fluid in sheep fed a high feed intake had a significantly higher pH compared with sheep fed a low feed intake. Both levels of feed intake exhibited a decrease in pH over time, but the fluid collected from sheep fed a low feed intake decreased at a much faster rate compared to sheep fed a high feed intake. Superovulated sheep appeared to have a slight increase in oviductal pH regardless of the level of feed intake whereas the pH of oviductal fluid of non-superovulated ewes did not change. Strangely, oviductal fluid generally had a higher osmolarity than that which is thought to facilitate development of mouse embryos *in vitro* (Collins and Baltz, 1999). A similarly high osmolarity is predicted for human fallopian tube fluid by electron probe measurements (Borland *et al.,* 1988). Furthermore, the high level of feed intake increased the osmolarity of the oviductal fluid compared with low feed intake in the non-superovulation group. In the superovulation group, however, there were no significant differences in oviductal fluid osmolarity between the nutritional treatments. Interestingly, superovulation treatment decreased the osmolarity in both oviducts irrespective of nutritional treatment compared with non-superovulation and this may be due to increased fluid production in the superovulation group. The present study appears to be the first to measure directly the oviductal fluid osmolarity in the sheep but the question of how tonicity, total ion concentrations, pH and osmolality are related in oviductal fluid cannot be directly answered. In general, preimplantation embryos appear to be very adaptive to a wide range of osmotic pressures. Bavister (1995) indicated that there is little point in trying to establish osmotic pressure optima for embryo development, within a fairly broad range, and it may not be necessary to adjust osmotic pressure too precisely after making changes in

the culture medium formulation. It seems a good precaution however to include amino acids in the medium to serve as osmolites in order to help embryos cope with variations in osmotic pressure.

7.4.2 Oviductal fluid electrolyte concentration: No difference was observed in the concentrations of metabolites or ions measured in the non-superovulated oviductal fluid ipsilateral or contralateral to the corpus luteum. To my knowledge, no equivalent data have been published that compare the concentrations of ions and metabolites between opposite oviducts, although one recent study (Elhassan *et al.,* 2001) reported no difference in the amino acid concentration of bovine oviductal fluid harvested from the ipsilateral or contralateral oviduct. Many of the constituents that have been identified in oviductal fluids of mammalian species have been shown to be important for early reproductive events. Bicarbonate has been postulated to be a major component in the secretions of the female reproductive tract, especially the oviduct, which is beneficial for the completion of fertilisation (Brackett and Mastroianni, 1974). In the current study, oviductal bicarbonate concentrations were significantly higher in the high feed intake group compared with the low feed intake group. Interestingly, Boatman and Robbins (1991) concluded that maximal levels of spontaneous acrosome reaction occurred at much lower bicarbonate concentrations. It is well known that the concentrations of bicarbonate are much higher (2-3 times) in the oviduct and uterus than in blood plasma (Boatman and Robbins, 1991) but whether this has any functional significance for early development is not known. A high bicarbonate concentration has been implicated in facilitating fertilisation *in vivo* by at least three mechanisms: (i) as a dispersing factor for cumulus and corona cells thereby weakening a cellular barrier to sperm penetration (Stambaugh *et al.,* 1969); (ii) as a sperm motility stimulating substance (Boatman and Robbins, 1991) and (iii) as a cofactor for spontaneous and ligand mediated acrosome reactions (Hyne, 1984; Boatman and Robbins, 1991). There was no significant difference in oviductal calcium concentrations between the two nutritional regimes. Furthermore, there was also no significant interaction between feed intake, ovulatory status and day for the oviductal calcium concentrations. There appears to be a range of calcium concentrations that will support embryo growth and development. Sodium was significantly affected by the feed intake such that in the high feed intake group the concentrations of sodium in oviductal fluid were increased compared with the low

feed intake group. The electrolyte concentrations of other ions (i.e., K, Mg, Cl etc) in oviduct fluid reported in this thesis clearly fall within the range of concentrations determined to be functionally relevant for *in vitro* sperm capacitation and fertilisation in several species including ovine (Restall and Wales, 1966; Paisley *et al.,* 1979; Grippo *et al.,* 1992). This study has also demonstrated that cation concentrations in oviduct fluid varies with the stage of oestrous cycle.

7.4.3 Oviductal fluid urea and ammonia concentrations: High systemic concentrations of urea have been associated with a reduction in uterine pH, (Elrod and Butler, 1993) and an alteration in the ionic composition of uterine fluid (Jordan *et al.,* 1983) both of which have been suggested as possible causes of embryo death. The present study supports this conclusion as there were higher blastocyst rates in the low feed intake group compared to high feed intake group. However, irrespective of the concentrations of systemic urea or indeed ammonia, either these variables were not significantly affected by feed intake or the embryos can survive wide variations in oviduct/uterine pH or in ionic composition of oviductal and/or uterine fluid. In Experiments 1, 2 and 4, embryo survival rate was unaffected by presumably high concentrations of urea and and/or ammonia.

Elevations in systemic ammonia have also been suggested as a putative cause of reduced reproductive performance but there is little direct evidence to support this (Kenny *et al.,* 2002). The relationship between systemic ammonia and urea makes it difficult to attribute exclusively the cause of reduced conception rate to either metabolite. In the current study, ewes were exposed to a high or low feed intake from 18 days before until 6 days after insemination; however, there was no relationship between oviductal ammonia concentrations and embryo survival rate. The weaker relationship between the level of dietary protein and systemic ammonia (Laven and Drew, 1999; Sinclair *et al.,* 2000b), as opposed to urea, has resulted in a lack of information on the relationship between systemic ammonia and embryo survival (Kenny *et al.,* 2002). No threshold systemic concentration of ammonia has been reported above which embryo survival is compromised. In the current study, the ewes on the high feed intake experienced mean oviductal concentrations of ammonia of 1968 μmol/l with a maximum value of 3456 μmol/l with no detrimental effect on embryo survival. Some studies have reported reduced oocyte and embryo viability in

the presence of high concentrations of ammonia. Sinclair *et al.* (2000b) reported that, following oocyte recovery from beef heifers that experienced concentrations of systemic ammonia of up to 330 µmol/l, *in vitro* fertilisation and blastocyst production rates were compromised. McEvoy *et al.* (1997) reported poor embryo development and survival *in vitro* and *in vivo* following embryo transfer to ewes that had peak systemic concentrations of ammonia of approximately 150 µmol/l. In these latter studies however, there were also concomitant elevations in systemic urea and the reduction in embryo viability cannot be attributed specifically to the concentration of ammonia generated. In contrast, Hammon *et al.* (2000a) demonstrated that bovine oocytes are typically exposed to follicular concentrations of ammonia up to 366 µmol/l during their normal *in vivo* development and that *in vitro* fertilisation rate and subsequent embryo development of bovine oocytes were not impaired by exposure to ammonia concentrations of up to 356 µmol/l in culture media. Subsequently, Hammon *et al.* (2000b) reported that the effect of ammonia on the *in vitro* viability of bovine oocytes and early embryos was dependent on the timing and duration of exposure and the concentration of ammonia used.

7.4.4 Oviductal fluid amino acids: The results of the present study demonstrate that the concentrations of amino acids present in the oviduct are quite different from those found in plasma (Nancarrow *et al.,* 1992) and are variable throughout the peri-conceptional period. The concentrations of amino acids reported in previous studies (Nancarrow *et al.,* 1992; Hill, 1994; Hill *et al.,* 1997) were lower than those found in the current study. This difference is probably due, at least in part, to concentration variations associated with the stage of the cycle, pooling of samples or dilution effect of flushing fluids. Furthermore, pathological conditions precipitated either by tubal ligation or the introduction of catheters during sampling or possibly more importantly the nutrition status and body condition and age of the ewe may also alter concentrations in the oviduct. Although maximum care was exercised during the handling and preparation of fluid samples in this study, one has to acknowledge some of the limitations associated with these cautionary measures. Such limitations involve the accuracy of estimating the time of ovulation, the impact of the time between collection and sample freezing, the absence of follicular fluid in oviductal fluid at ovulation, as well as the effect of the absence of an embryo in the reproductive tract. Amino acids such as ARG, HIS, GLU, GLY, ILE, LEU, LYS, MET, TYR and VAL

were shown to be increased in concentration after ovulation. The fact that significant differences between the feed intake groups (except GLY and GLU) have not been detected is possibly due to the large amount of variability between the experimental sheep over time. Glycine was the only amino acid whose concentration was significantly increased by high feed intake and superovulation treatment. GLY, ALA, LYS and LEU are present as major components of oviductal fluid in all species but ARG and PRO make a relatively greater contribution to the amino acid pool in the cow. Low feed intake and non-superovulation significantly increased the GLU concentration in oviductal fluid compared with high feed intake and superovulation treatment. It is possible that this may explain why there were more blastocysts in the low feed intake group of sheep compared with the high feed intake group. Amino acids such as ARG, CYS, GLU, ILE, LEU, LYS, TYR and VAL were significantly increased in the oviductal fluid of the non-superovulation group compared with superovulated sheep irrespective of nutritional treatment.

The results presented in this thesis show that non-essential amino acids (e.g. GLY, ALA, GLU) are more abundant in oviductal fluid than essential amino acids (e.g. HIS, LEU, LYS, VAL) (ratio approximately 4:1). Ratios of amino acids are critical for protein synthesis in living cells (Elhassan *et al.,* 2001). Thus it is of value to investigate whether the ratios or relative amino acid concentrations rather than the absolute concentrations of amino acid are more important for embryo development. This notion is supported by reports of improved embryo development with supplementation of non-essential amino acids alone (Liu and Foote, 1995; Pinyopummintr and Bavister, 1996) or in combination with lower concentrations or later addition of essential amino acids (Lane and Gardner, 1997; Liu and Foote, 1995; Liu *et al.,* 1996; Steeves and Gardner, 1999) during the first 2-3 embryonic cleavages. Other studies indicated that some amino acids are present in very low concentrations in ovine oviductal fluid suggesting that they might be embryotoxic at normal plasma concentrations (Nancarrow and Hill, 1994).

Deficiency of certain amino acids and/or their transport systems may well result in abnormal expression of proteins involved in key developmental processes such as uvomorulin, Na/K ATPase, gap and tight junction proteins, which are all essential for normal compaction and blastocoele formation (Hill *et al.,* 1997). In addition, amino

acids are important as energy substrates. Some amino acids such as aspartate, glutamate, and glycine may also be utilized in the synthesis of purines and pyrimidines (Hill *et al.,* 1997) and therefore amino acid deficiencies in embryos may limit RNA synthesis as well as protein synthesis. Conversely, GLY concentration was strikingly high (66% of the free amino acid pool). GLY concentration was also significantly increased by high nutrition. Similar findings were previously reported for cows (Moore and Bondioli, 1993; Elhassan *et al.,* 2001), sheep (Hill *et al.,* 1997; Moses *et al.,* 1971), rabbits (Miller and Schultz, 1987), horses (Engle *et al.*, 1984) and mice (Menezo *et al.,* 1988). Leese and Gray (1985) reported that the secretion rate of GLY into the rabbit oviduct lumen was the highest of the amino acids and it comprised 70% of the amino acid pool in blastocysts. This high concentration of GLY suggests an unusual role(s) for this amino acid in the reproductive tract. Aside from being a constituent of proteins, GLY is one of the most effective organic osmoregulators in the mouse embryo (Dawson and Baltz, 1997; Dawson *et al.,* 1998; Van Winkle *et al.,* 1990). It may play a similar role during preimplantation development of the ovine embryo, providing protection from the high osmolarity in oviduct and uterine fluids (350 to 370 mOsM) (Anbari and Schultz, 1993; Biggers *et al.,* 1993). In light of these finding, the concentrations and/or proportions of amino acids in defined culture media need to be re-evaluated in order to improve the development, quality and viability of embryos produced *in vitro*. In view of the rationale that the concentrations or proportions of amino acids measured in oviductal fluid are indicative of the optimal requirements for embryo development, it is important to test this using a two step embryo culture in a defined medium with amino acids initially at oviductal concentrations followed by uterine concentrations.

7.4.5 Oviductal fluid progesterone concentration: The persistently high concentrations of progesterone in oviductal fluid during low feed intake might have influenced the tubal milieu and/or transportation of the embryos in such a way as to influence the ICM/TE ratio. It is important to note that many of the effects of steroids on gametes in the oviduct may be mediated indirectly through actions on the vasculature, serosa, mucosa, and muscularis, affecting fluid and gamete transport (Battalia and Yanagimachi, 1979; 1980; Hunter, 1988), availability of metabolites, synthesis of growth factors and other biologically important molecules, such as the major class of oviduct glycoproteins.

In vitro, progesterone induces capacitation, hyperactivated motility, and acrosome reactions in sperm, possibly acting through a non-genomic plasma membrane receptor, but neither progesterone functions nor concentration within the mammalian oviduct during the peri-ovulatory period are well characterized (Boatman, 1997). Progesterone-regulated processes in reproduction are diverse (Graham and Clake, 1997) and in the oviduct and uterine endometrium, these, in conjunction with the distinct effects of embryonic and/or ovarian-derived estrogens, result in the synthesis and/or secretion of a variety of signalling molecules that include growth factors, cytokines, proteases, and protease inhibitors (Simmen and Simmen, 1990). These regulatory molecules most likely mediate the integration of multiple signalling pathways that underline the complex processes involved in embryo development, although the mechanisms by which these occur remain poorly understood.

It is well documented that there is an inverse relationship between postmating nutritional status and peripheral progesterone concentrations in sheep (Williams and Cumming, 1982). Circulating levels of progesterone, particularly during the pre- and peri-implantation period, influence the development of embryos and fetuses. The mechanisms by which this occurs are unclear but probably do not involve a classic, direct effect of progesterone on the developing embryo. Previous studies in ewes have suggested that the level of priming of progesterone, modulated by pre-ovulatory nutrition, influences embryo survival through direct effects on the developing oocyte (McEvoy *et al.,* 1995a). In subsequent studies, McEvoy *et al.* (1995b) reported that the provision of supplementary progesterone to ewes on a high plane of feeding during the pre-ovulatory priming phase elevated plasma progesterone levels and enhanced subsequent ovum development. It seems likely that progesterone also acts indirectly and that the mechanism involves alterations in the timing or synthesis of oviductal and uterine secretory products (Garrett *et al.,* 1988). Early embryonic development seems to be particularly susceptible to the effects of progesterone; administration of supplemental progesterone to female cows between Days 1 and 6 after breeding resulted in conceptuses that were significantly longer compared to controls (Garrett *et al.,* 1988). Nephew *et al.* (1991) demonstrated that this phenomenon could occur naturally, as ewes with elevated levels of progesterone between Days 2 and 4 after breeding also had conceptuses that were more advanced in

development on Day 13. This is important because it indicates that differences in progesterone levels induced by nutrition are large enough to be physiologically significant. In sheep, progesterone administration during embryo development can also affect subsequent development as fetuses resulting from embryos exposed to exogenous progesterone between Day 1-3 or 1-6 of gestation were significantly larger at midgestation than untreated controls (Kleemann *et al.,* 1994). Progesterone treatment between Day 1-3 of gestation was also associated with an increase in the proportion of embryos with ≥16 blastomeres on Day 3 of gestation. Differences in the distribution of cells to the inner cell mass vs. trophectoderm in embryos may also occur as a result of progesterone treatment (reviewed by Walker *et al.,* 1996).

The cause(s) of the increased progesterone concentrations during low nutrition is (are) yet to be identified but a number of propositions have been put forward: (1) Activation of pituitary-adrenal axis with increased cortisol, where Kawate *et al.* (1993) demonstrated using *in vitro* studies, that cortisol induced increases in progesterone secretion in bovine granulosa cells, (2) the adrenal gland may also play a contributory role in progesterone elevation during low nutrition. Treatment with adrenocorticotrophic hormone (ACTH) causes an increase in plasma progesterone in pregnant ewes and wethers (van Lier *et al.,* 1998), sows (Tsuma *et al.,* 1998), zebu cows (Bolanos *et al.,* 1997) and deer (Asher *et al.,* 1989), (3) there are also possibilities, as Parr *et al.* (1987) contended, that metabolic clearance of progesterone is affected by reduced blood flow to the liver through the hepatic portal circulation during low nutrition and (4) a possibility that either all or some of the luteal cells are somehow affected by 'nutritional stress' leading to an increase in the production of progesterone.

7.4.6 Oviductal fluid insulin-like growth factor-I (IGF-I) concentration: It has been demonstrated that IGF-I concentrations in oviductal fluid increase during oestrus and then decrease during the subsequent postovulatory period. In the present study, in normally cycling ewes, there was a significant effect of feed intake, such that high feed intake resulted in higher IGF-I concentrations compared with the low feed intake group. It has been hypothesised that secretions of the oviduct and uterus are involved in stimulating cell proliferation in preimplantation embryos and promotion of early differentiation events that lead to successful implantation (Schultz and Heyner, 1993).

Taken together with the factors present in oviduct and uterine secretions, it is clear that preimplantation embryos reside in an environmental milieu in which they are exposed to growth factors and cytokines of many kinds and that regulatory pathways at the autocrine, juxtacrine, and paracrine levels may all be operating (Schultz and Heyner, 1993). The exact circuit or pathway and mechanism through which they exert their effects remain, for the most part, to be elucidated. None-the-less, the results in this thesis are not entirely conclusive, as the possible presence of several other growth factors in oviductal fluid has not yet been investigated. The results clearly suggest, however, that these molecules are present in the oviduct milieu and may act on early embryonic development. It is possible that cooperation exists with those oviduct specific glycoproteins, which, in several species, become associated with developing embryos and show localization in the oviduct epithelium and oviductal fluid similar to that described for IGF-I ((Wathes *et al.,* 1998). It is, therefore, tempting to speculate that the oviduct may provide the developing embryo with other growth factors (other than IGF-I) or their binding proteins. Even though the present study provides evidence that oviductal IGF-I concentrations change in association with reproductive performance, any direct actions that this factor may have in mediating nutritional effects on the hypothalamic:pituitary:ovarian axis remain to be determined. It is possible and also as speculated by Roberts *et al.* (1997) that the circulating and (or) locally produced components of the IGF system may provide a mechanism by which changes in the metabolic status of animals may be perceived at the hypothalamic:pituitary:ovarian axis to regulate reproductive function.

In sheep and cattle, expression of IGF-I mRNA and protein in the oviduct occurs in response to high concentrations of oestrogen associated with oestrus (Schmidt *et al.,* 1994; Stevenson and Wathes, 1996; Wathes *et al.,* 1998). In addition, elevated levels of IGF-I expression by oviductal mucosa continue for approximately 2-4 days following oestrus. During this period, the early cleavage-stage embryo is present within the oviduct and expresses mRNA for the IGF Type-1 receptor (Watson *et al.,* 1992; Watson *et al.,* 1994). Thus, it is likely that interactions with IGF-I could influence embryonic growth and development during this period. Based on the localization of IGF-II mRNA and protein principally to the oviductal muscularis, with relatively little expression present in the oviductal mucosa *in vivo*, it has been suggested that IGF-II plays a minor role, if any, in regulating the development of

ovine embryos while they are resident in the oviduct (Wathes *et al.,* 1998). Differences in the expression of IGF ligands by oviductal epithelial cells *in vivo* and *in vitro* may contribute to differences in developmental characteristics of embryos produced either *in vivo* or *in vitro*. Because the IGF system is known to have important effects regulating embryonic and fetal growth, it is logical to suggest that alterations in the production and secretion of components of the IGF system by reproductive tract tissues, either *in vivo* or when maintained *in vitro*, may influence embryonic and subsequent fetal development. If the basic underlying mechanism involves disruption of imprinting patterns for IGF related genes, the type of imprinting disruption could differ in ICM-derived fetal tissue compared with trophoblast-derived placental tissues. Alternatively, imprinting disruptions could occur to a greater extent in one-tissue type vs. the other (i.e., ICM vs. TE). Furthermore, the severity or specific type of misimprint occurring during the methylation process could vary with specific conditions used for embryo production, resulting in phenotypes that may exhibit only fetal abnormalities, only placental abnormalities, or both fetal and placental abnormalities. Because *in vitro* embryo production underlies the successful application of several advanced livestock technologies, a better understanding is needed of efficient systems for producing embryos *in vitro* that will result in normal pregnancies and offspring. Conversely, understanding how perturbations of the embryonic environment can alter growth phenotypes may lead to alternative methods (i.e. non-genetic) for selecting and manipulating the birth weight and growth characteristics of livestock. It can be anticipated from the rapid progress of the past decade that substantial improvements will be forthcoming, so that the information gained can be incorporated into new culture media that will benefit human clinical efforts, toxicology studies, the production of food animals, and attempts to conserve endangered species.

7.5 Peri-conceptional feed intake and fetal/placental development at mid gestation: The present study demonstrates that at Day 75 of gestation, fetal development was altered as a result of alteration in the peri-conceptional maternal feed intake of donor sheep. Such alterations in development may have important functional consequences. Interestingly, fetal body growth was maintained, suggesting that the tendency for change that occurred in placental development was an adaptive

response that was successful in compensating for the effects of low feed intake during the peri-conceptional period.

Earlier evidence from the present studies indicated that low peri-conceptional maternal feed intake changes the cell lineage differentiation in the sheep with differences in the proportion of inner cell mass and trophectoderm (Chapter, 4). In the final experiment, fetal tissues examined were representative of the three embryonic cell lines derived from primitive ectoderm of the inner cell mass (Tortora and Anagnostakos, 1990; Guillomont *et al.,* 1993): the ectoderm (nervous system, skin, and sense organ), endoderm (digestive, respiratory and urinary tracts) and mesoderm (muscle, blood, kidney and reproductive organs). The results suggest that altered fetal growth was not specific to any one of the three embryonic cell germ layers as development of most fetal tissues examined were approximately proportionately to fetal weight.

The findings of the current study suggest that perturbation of the embryonic environment may also promote fetal growth indirectly, by altering placental development so as to enhance subsequent function, possibly through similar early cellular and molecular mechanisms. One of the striking features of this study is that the manipulations of the embryos were imposed very early in development (before implantation). The indications are that cell programming can be altered by events that occur even before apparent embryonic cell determination and commitment events are entertained. This has implications not only for the implementation of embryo transfer technologies but also for human reproductive technologies. It is hoped that this work will encourage further investigation of the mechanisms involved in the control of placental growth and development.

7.6 Concluding remarks: Results presented in this thesis contribute to a better understanding of the interactions between nutrition and reproduction and to the nutritional management of breeding ewes. A better understanding of the mechanisms that control ovulation rate and modify the response to nutritional stimuli would be a powerful adjunct to the management of both nutrition and reproduction. There are many steps involved in the production of viable embryos. All are affected by nutrition; this thesis shows that peri-ovulatory feed intake is of utmost importance.

Results from this thesis help understand part of the physiological processes that are involved. An increase in ovulation rate alone is not enough, the oocytes that are released from the follicles must be competent and develop to the blastocyst stage with the appropriate ratio of inner cell mass: trophoblast component of cells. In order to study the direct effect of feed intake on oocyte or embryo quality, a complex working model is needed. It should be possible to differentiate between the events occurring before and after ovulation. Understanding the effect of feed intake on oocyte maturation will give a clear indication of subsequent embryo development capability. The critical time when nutrition has an effect on the oocyte quality also needs to be determined. An important point is that neither artificial insemination nor embryo transfer programs can be "spur of the moment" events. In fact, considerable forward planning, particularly in relation to nutrition, is essential. Despite all of the research done on the effects of nutrition of reproductive performance, there remains a lot to be discovered. However, it is fair to say that the notion that "fat sheep are good" from a reproductive perspective is likely to be incorrect. Evolutionary forces have ensured that lean animals are best at reproducing – i.e., they are at risk; therefore from an evolutionary perspective they must reproduce and reproduce efficiently. Modern husbandry should make the most of this evolutionary quirk.

7.7 Limitations of the work undertaken: Limitations of the work presented in this thesis were largely related to sampling, at various levels of the sampling hierarchy. Firstly, the numbers of catheterised ewes used in different treatment groups were generally low. In those ewes that were catheterised, one oviductal catheter was lost and five catheters become blocked before the completion of the protocol. The direct cause of this problem was unknown; hence they were removed from the data analyses. Both the pH and osmolarity of oviductal fluids found in this study must be considered to be at a maximum. In view of the fact that samples had to be retained in a collection tube for 0-24h prior to measurement of pH, an increase in pH due to escaping CO_2, and enzymatic digestion or bacterial breakdown of the fluid cannot be ruled out. Furthermore, in the experiments involving twins, only three ewes were available to study the effect of feed intake and to compare twins with the singletons. Thus the results referring to twin pregnancies may only be regarded as preliminary. Other limitations of this work are concerned with the fact that there are other important parameters of the tissue that were not analysed due to time constraints. These include,

general histology of all the organs, the vascularity of the fetal and maternal components of the placenta, the abundance of binucleate cells and/or some measure of their function and the harmonic mean barrier thickness of the trophectoderm and feto-maternal syncytium, which measure the variability of the membrane thickness that is more directly related to membrane diffusibility to substrates than arithmetic mean barrier thickness (Jackson *et al.,* 1988). Thus it has not been possible to relate the results of this work to the more direct measure of placental function. In addition, factors that determine placentome size and morphology need to be determined. This may be carried out by the analysis of the uterine position of the placentomes in relation to blood flow patterns and concentrations of hormonal substances and metabolites within the fetal and maternal blood.

7.8 Future directions: Research reported in this thesis should stimulate ideas for new experiments and provoke discussion. By way of summary, after surveying a large body of published literature on nutrition and embryo development, there are several areas of experimental strategy and technique that I feel need to be emphasized. Some of these are as follows.

1. No studies have been conducted to determine the preferred nutritional requirements during the premating six-month period, which is characterised by a four-month period of pre-antral follicle development and a two-month period of antral follicle development (Cahill and Mauleon, 1980). However, it is anticipated that good nutrition during the period of pre-antral follicle growth will increase the number of follicles that enter the antral pool and be available for ovulation.

2. There is no doubt that a rising plane of nutrition during the second half of the period in which antral follicles develop is beneficial. This is the well-known "flushing response" (Coop, 1966). However, it is important to realise that the preferred nutritional conditions for follicle growth are not the same as for embryo development.

3. In this study, it was our intention not to collect blood samples through the peri-conceptional period in order to minimise stress to the animals. It remains an

interest to relate the observed changes in embryo development and oviductal fluid components to that of the systemic circulation.

4. Less detailed information is available with respect to the enzymes present in oviductal fluids. Various enzyme activities have been reported in oviductal fluids. These include amylase, alkaline phosphatase, catalase, diesterase, lysozyme and lactate dehydrogenase (LDH). It will be of interest to study the effects of nutrition on these enzymes.

5. It is also of interest to investigate the different nutritional effects on the level of oviductal carbohydrate, protein, non-protein nitrogen, pyruvate, lactate, glucose, citrate, lipids and dry matter present in the oviduct fluid of all species so far examined (Leese *et al.*, 2001).

6. Both free amino acids and protein have been suggested as potential sources of fixed nitrogen during early development of fertilised oocytes. Unfortunately we were unable to detect amino acids such as taurine, hypotaurine. We used the Waters method (Reverter *et al.*, 1997) and these amino acids were not shown on the chromatographs. Since taurine is known to have strong reducing properties, if present in significant amounts in oviductal fluids it may function as an antioxidant with potentially incumbent biochemical or physiological significance (Van Winkle, 2001). It is highly recommended therefore to see if it is affected by feed intake.

7. The effects of single amino acids on hamster embryos in culture have been reported (Bavister and Arlotto, 1990; Bavister and McKiernan, 1993), for example, asparagine, aspartate, glycine, histidine, serine and taurine were all found to be stimulatory, while cysteine, isoleucine, leucine, phenylalanine, threonine, and valine were inhibitory to development. In the current study, changes in feed intake also significantly changed the oviductal fluid concentrations of amino acids such as glutamic acid and glycine. It would be useful to test these *in vivo* results on *in vitro* ovine embryo development.

8. It would also be interesting to determine the mechanisms by which genes are regulated in cases where amino acid regulation underlies the dramatic changes in transport system expression that occur during preimplantation development. Similarly, it remains to be determined how the components of the oviductal and uterine environments regulate transport system activities in blastocysts.

9. The oviduct, residing in the peritoneum, is bathed in peritoneal fluid. The exchange of components between peritoneal and oviductal fluids is therefore possible. Also, in normal circumstances, the preampulla may be open to the peritoneum, allowing peritoneal fluid to enter the oviduct. Therefore, in further studies the oviductal fluid components could be compared with the peritoneal fluids in order to identify any differences among the constituents that may influence oviduct fluid composition. In addition, further studies are now needed to evaluate the specific roles of these components on ovine gamete function, particularly the effects of electrolyte concentrations on sperm capacitation and fertilisation.

10. It is worth noting that although the emphasis in the literature is mainly on the negative impacts of vitamin deficiencies, excess and/or imbalances of micronutrients (vitamins) may also result in reproductive disorders. Interestingly, reported reproductive disorders such as reduced conception rate and anoestrus (Smith and Akinbamijo, 2000) have, however, usually been linked to high dietary intakes rather than to a deficient intake. For sheep embryos, the inclusion of vitamins in the culture media does not affect embryo morphology *in vitro* but significantly increases glucose uptake and lactate production per cell (Gardner *et al.*, 1994). To date the physiological levels and the effect of feed intake on vitamins in oviductal fluid is unknown. Insights into the effect of feed intake on oviductal fluid vitamin levels have the potential to increase success rates in embryo culture and increase fertility in livestock species.

11. Circulating leptin regulates energy availability. In a variety of species, short-term feed restriction leads to rapid changes in the reproductive axis and reduced serum concentrations of leptin (Amstalden *et al.*, 2000; Almeida *et al.*, 2001). The secretion of leptin by human cytotrophoblast is thought to be regulated by IL-1β

and oestradiol (Chardonnes *et al.,* 1999). In this scenario, short-term changes in feed intake during the preimplantation period might have some effects on the oviductal fluid leptin concentrations and its effects on the early stage embryo, which have not been mentioned in the literature so far.

12. The presence of serum in the culture from the 2-cell stage was recently shown to reduce the ICM: TE ratios by increasing the level of programmed cell death (apoptosis) within the ICM of *in vitro* cultured bovine embryos (Byrne *et al.,* 1998). Insulin and insulin-like growth factor-I promote embryonic development by preventing apoptosis and by increasing cell proliferation (Herrler *et al.,* 1998). It is not known whether feed intake may also affect the level of apoptosis in preimplantation embryos.

13. The effect of sperm or seminal components on the composition of oviductal fluid also need consideration. In the present study it was not possible to determine the sperm effects on oviductal fluid composition because both ends of the oviducts were ligated. However, it will be of interest to catheterise the oviduct around the time of insemination to determine the effect of seminal/sperm factors on oviductal fluid composition. Observations from these experiments have the potential to add to existing proposed molecular mechanisms that may operate to facilitate communication via a novel semen-uterine-ovarian axis (O'Leary *et al.,* 2002).

14. Dawuda *et al.* (2002) suggested that the cow is able to adapt within 10 days to the toxic effects of excess urea. The most likely explanation for such adaptation is altered hepatic metabolism or increased rumen production of methane from the utilisation of ammonia by ruminal microflora. In ewes, adaptation of the rumen micro-flora and micro-fauna to a high level of urea/ammonia could possibly lead to increased methane production and reduced concentrations of blood/oviductal urea and ammonia. A fuller analysis of the time course of plasma/oviductal urea and ammonia concentrations in response to high feed intake is required before definitive conclusions can be made from the present results.

15. Epidermal growth factor (EGF) has been shown to increase protein synthesis in the mouse blastocyst. Its effects however are limited to the TE only with no effect

on the ICM (Wood and Kaye, 1989). Thus, different growth factors affect different cell populations of the blastocyst and there exist specific transport mechanisms permitting transcytosis of selective ligands through the TE to affect the ICM (Harvey and Kaye, 1990). As in the current study, low feed intake significantly increased the number of TE cells in the blastocyst; it would be of interest to determine the concentrations of EGF in oviductal fluid with special reference to low peri-conceptional feed intake.

16. It is now clear that members of the IGF family (IGF-I, IGF-II, insulin), EGF family (EGF, TGFα) and TGFβ family collectively are involved in influencing a number of events during early murine development; among these events are RNA and protein synthesis, cleavage rates and embryo cell number, blastocoel expansion and the proportion of blastocysts hatching from zona pellucida in vitro (Mattson *et al.,* 1988; Watson *et al.,* 1994; Dardik and Schultz, 1991; Harvey and Kaye, 1991). The expression of growth factor transcripts very early in mammalian development suggest that these molecules fulfil a necessary role(s) in supporting the progression of early embryos throughout the preimplantation interval (Watson *et al.,* 1994). It would be of interest to study all the above-mentioned factors *in vivo* in sheep. In addition, the necessity of a specific intrauterine cytokine profile during different stages of the preimplantation period should be investigated in sheep, as has been done in humans (Schafer-Somi, 2003).

17. Growth factors may be multifunctional in directing developmental events. They might promote cell proliferation in one instance and act in combination with another growth factor to induce a differentiation event at a later point in the developmental programme (Schultz and Heyner, 1993). It would be of interest to determine if the peri-conceptional nutritional effects influence subsequently development including the reproductive performance of resultant offspring. Such knowledge would be valuable for practical applications such as artificial insemination, embryo transfer, IVF and the invention of new contraceptive methods, which minimise side effects.

18. Although cortisol was not measured in this study, it is known that maternal undernutrition can elevate cortisol in the late-gestation fetal sheep (Binienda *et al.*, 1990) and that cortisol exposure can increase blood pressure (Tangalakis *et al.*, 1992; Edwards *et al.*, 1999; Edwards and McMillen, 2001; 2002) in the fetal sheep. Corticosteroid-induced growth retardation of the fetal rat also lowers plasma IGF-I (Price *et al.*, 1992). Whether altered cortisol levels are causally related to reprogramming of the fetal IGF axis awaits further investigation.

19. It has been reported that the importance of the placenta as a determinant of fetal growth rate has been greatly underestimated and that the survival of the fetus is jeopardized more by a small placenta than by maternal underfeeding (Mellor, 1983). The determinants of fetal growth rate and their influences on lamb viability and post-natal growth are therefore of considerable practical importance.

20. Trophoblast cells produce a number of hormones and growth factors including progesterone (Reimers *et al.*, 1985), placental lactogen (PL) (Duello *et al.*, 1986), pregnancy associated glycoproteins (PAG-1,-2,-3) (Roberts *et al.*, 1995), and TGFß (Munson *et al.*, 1996). These hormones are associated with fetal growth and development and the maintenance of pregnancy. Progesterone receptors are present within nuclei of many different tissues of the placentome, including trophoblast cells, suggesting paracrine function (Shular *et al.*, 1999). Whilst it is clear from the results presented in this thesis that low periconceptional feed intake increases the number of trophoblast cells, its not known how this under-nutrition affects the trophoblast secreted hormones and growth factors.

Chapter 8

APPENDICES

Appendix 8.1 Preparation of culture media (Synthetic Oviduct Fluid Medium, SOFM)

Stock A (lasts three months)

NaCl	6.29g
KCL	0.534g
KH_2PO_4	0.162g
$MgSO_4.7H_2O$	0.182g
Penicillin	0.06g
Milli Q	99.4ml
Then Na lactate	0.6ml

Stock B (lasts two weeks)

$NaHCO_3$	0.210g
Phenol Red	2/3 grains
Milli Q	10ml

Stock C (lasts two weeks)

Sodium pyruvate	0.051g
Milli Q	10 ml

Stock D (lasts three months)

$CaCl_2.2H_2O$	0.262g
Milli Q	10ml

BICARB + SOFM + AMINO ACIDS

Milli Q	22.79ml
Stock A	2.91ml
Stock B	2.91ml
Stock C	0.20ml
Stock D	0,29ml
Check osmolarity – adjust to 265-285 mOsm	
Add amino acids	0.99ml
Add PVP/PVA	30mg(1mg/ml)
Filter	

Appendix 8.2 Flushing medium for embryo collection (Dulbicco's Complete Formula PBS)

Reagent	x1/1L	x1/5L	x10/2L#
$CaCl_2 2H_2O$*	0.133	0.663	-
KCL	0.2	1.0	4
KH_2PO_4	0.2	1.0	4
$MgCl_2.6H_2O$*	0.1	0.5	-
NaCl	8.0	40.0	160
Na_2HPO_4	1.15	5.75	23

* Omit these reagents for Ca^{2+} and Mg^{2+} free PBS

Ca and Mg salts will not dissolve if x10 conc. Add to diluted solution.

-Add the above ingredients, one at a time, in the order listed.

-Adjust pH and then osmolarity ~280 mOsm

-Filter

-Add 5% of heat inactivated sheep serum

Appendix 8.3 Differential Staining of Ovine Blastocysts - Solutions

Ca^{2+}/Mg^{2+} free PBS (PBS^-)

KCl	0.1 g
KH_2PO_4	0.1 g
NaCl	4.0 g
Na_2HPO_4	0.575 g
Milli-Q	500ml

Adjust pH to 7.4

Adjust osmolarity to 280 mOsm

Filter

PBS^-/PVA

PVA	40 mg (4 mg/ml)
PBS^-	10 ml

SOFaa

SOF	9.265 ml
TRCaa mix	0.735 ml

Filter

SOF Hepes-BSA

SOF-Hepes	10 ml
BSA	40 mg (4 mg/ml)

Filter

Pronase

Stock solution: 5% pronase

Weigh 50 mg of pronase and dissolve into 1 ml of SOF-Hepes (no protein)

Filter

Aliquot into 50 µl volumes

Store at –20°C

Working solution: 0.5% pronase

Add 450 µl SOF Hepes-BSA (4 mg/ml) to stock solution

Store in fridge for up to 2 weeks

2,4,6 Trinitrobenzenesulfonic acid (TNBS)

Stock solution: 5% TNBS (w/v)

Obtained from Sigma - liquid

Working solution: 10 mM TNBS

To 25 ul of stock solution add 475 ul of PBS^-/PVA

Anti-DNP solution

Stock solution:

Anti-DNP-BSA (Sigma)

Specific antibody activity 1.3 mg/ml

Aliquot into 30 ul volumes and store at –80°C

Avoid repeat freeze/thaw of this reagent

Working solution: 0.1 mg/ml anti-DNP-BSA

Thaw a 30 ul aliquot of Anti-DNP-BSA

Add 360 ul of SOFaa

Guinea pig complement

Stock solution:

Guinea pig complement

Aliquot into 100 µl volumes

Store at –80°C

Propidium Iodide (PI) stock: 1mg/ml

Weigh out 1 mg of PI

Add 1 ml of PBS^-

Aliquot into 25 µl volumes

Store at –80°C

Working solution: 1:10 dilution of complement

Thaw 100 µl of complement

Dilute with 890 µl PBS^-

Add 10 µl of PI stock (final PI concentration 10 µg/ml)

Hoechst stain (bisBenzimide)

Stock solution: (1 mg/ml)

Weight out 1 mg Hoechst

Dissolve in 1 ml SOF Hepes

Aliquot 10 µl per tube

Store at –20°C

Working solution: (10 µl/ml)

Thaw a tube of stock solution

Add 990 µl of absolute ethanol

Vortex and use on same day

Keep out of light

Glycerol-Hoechst

Stock solution: (1 mg.ml)

Dissolve 1 mg Hoechst in 1 ml of Milli-Q

Aliquot into 100 µl volumes

Store at –20°C

Working solution: (10 µg/ml)

Add 1 ml of PBS to one tube of stock solution

Vortex for 1 min

Add 9 ml of glycerol and vortex for 3 min

Store in cold room for up to 1 month

Protect from light

Vaseline:paraffin mounting mixture

Vaseline	18 g
Paraffin wax (hard)	2 g

Heat until liquid, mix

Pour into 5 ml syringes and allow to cool

Differential staining of ovine blastocysts - protocol

Step 1: set-up

Using the Solution recipe sheet prepare the following:

-pronase, antibody (drops in petri dish, under oil, warming tray)

-complement/PI (drops in petri dish, under oil, warming tray, protect from light)

-TNBS (drops in 4-well plate, under oil, place on ice, protect from light)

-ethanol/Hoechst stain (4-well plate, place on ice, protect from light)

-washes - 3 x PBS^-/PVA (large drop in petri dish, under oil, place on warming tray)

- 1 x PBS^-/PVA (in petri dish, no oil)
- 1 x PBS + 10% sheep serum (large drop in petri dish, under oil, warming tray)

Step 2: Removal of zona

-Place 3 x 100 µl drops of 0.5% pronase solution in a 35mm petri dish, cover with oil, place on 38°C warming tray

-Wash blastocysts in Ca/Mg free-PBS (PBS^-)

-Incubate blastocysts in pronase until the zona is gone (should occur in < 1 min)

-IT IS VERY IMPORTANT THAT THE ZONA IS COMPLETELY REMOVED

Mechanical assistance using a narrow bored pipette may be required

-Once the zona has been removed wash blastocysts (x 1) in media containing 4 mg/ml of BSA or 10% sheep serum then wash (x 2) in PBS^-/PVA.

Step 3: Labelling cell surface proteins with TNP groups

-Place 50 µl of TNBS solution in each well of a 4-well plate, cover with oil and place directly on ice out of direct light

-Incubate blastocysts in ice cold 10 mM TNBS for 10 min. then wash (x 3) in PBS^-/PVA

Step 4: Incubation with antibody

-Place 75 µl volumes of antibody in a 35mm petri dish and cover with oil, place on warming tray

-Incubate blastocysts in 0.1 mg/ml anti-DNP-BSA at 37°C for 10 min

-Wash (x 3) in PBS^-/PVA

Step 5: Complement mediated lysis

-Place 75 µl volumes of complement/PI in a 35 mm petri dish, cover with oil, keep out of light, place on warming tray

-Incubate blastocysts in complement at 37°C for 15-30 min

-Wash briefly in PBS^-

Step 6: Staining of the ICM

-Fix blastocysts in ice-cold absolute ethanol for 5 min

-Transfer blastocysts to Hoechst stain

-Leave overnight at 4°C or at room temperature for 5-10 min.

Step 7: Mounting stained blastocysts for viewing

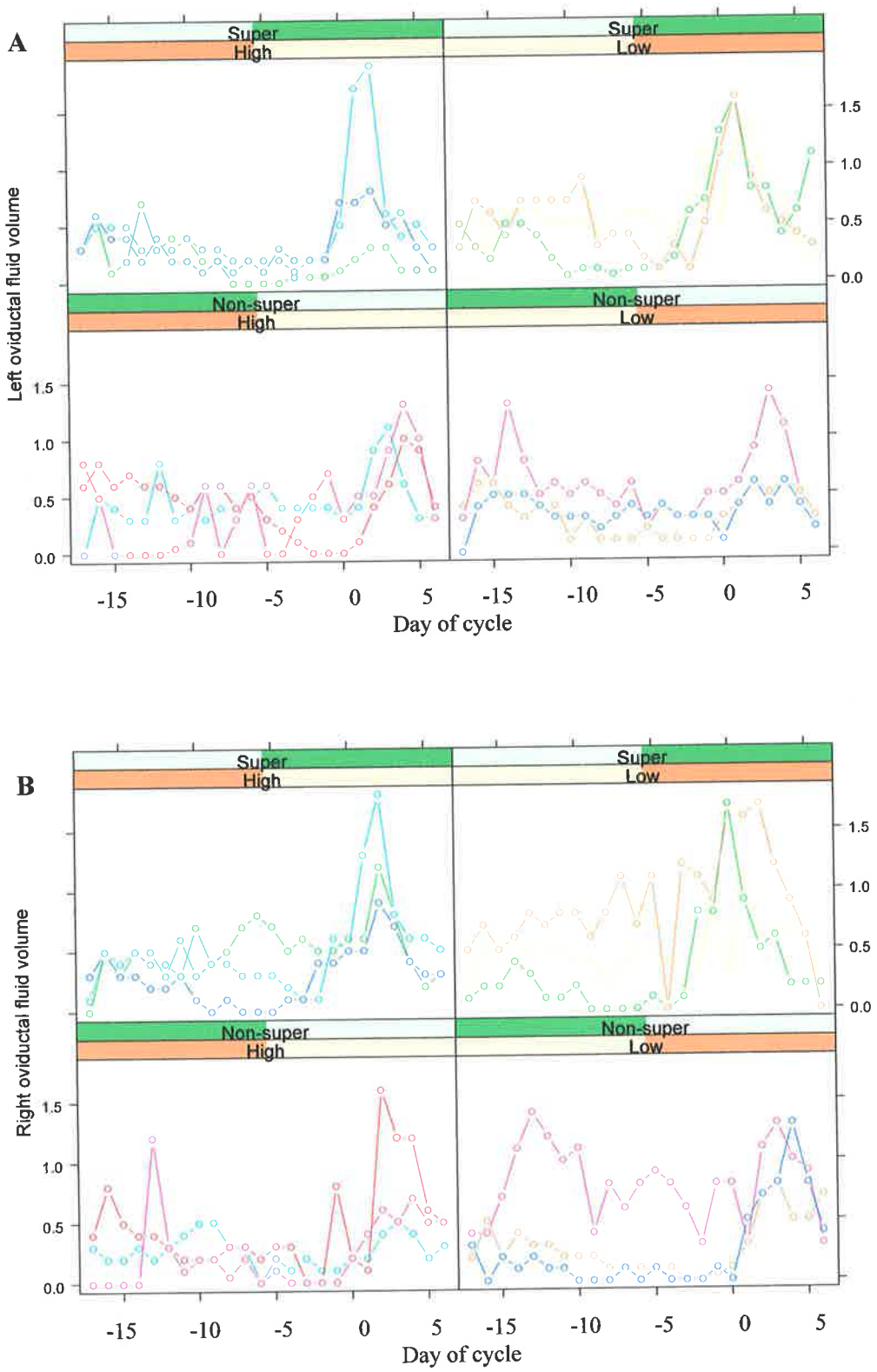
-Briefly wash blastocysts in a large volume of PBS^-/PVA (not under oil)

-Mount blastocysts in a drop of glycerol/Hoechst

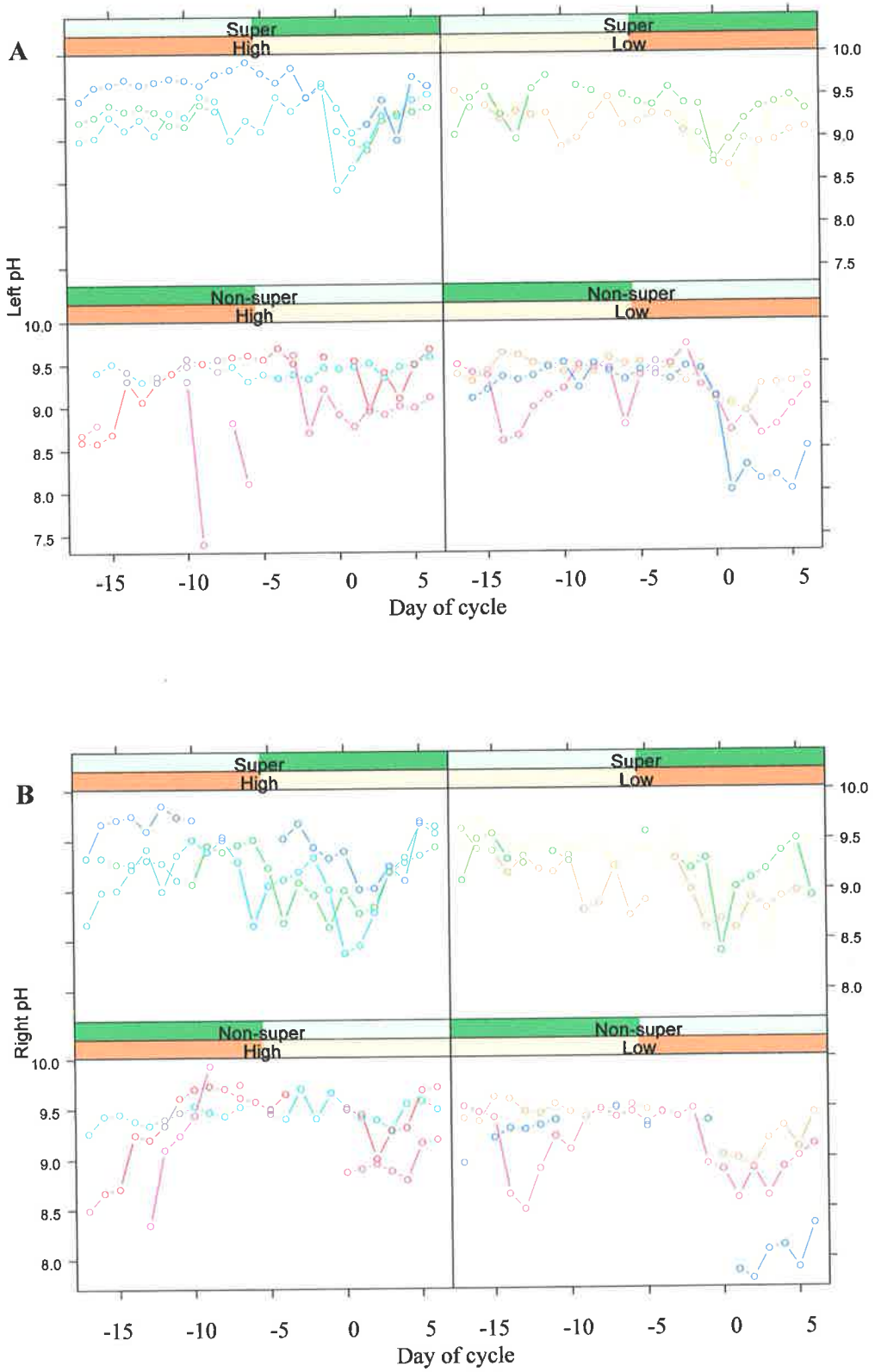
-Gently cover with a coverslip supported at each corner with 9:1 Vaseline: paraffin wax mixture

-Dual fluorochromes can be visualised with UV excitation filters

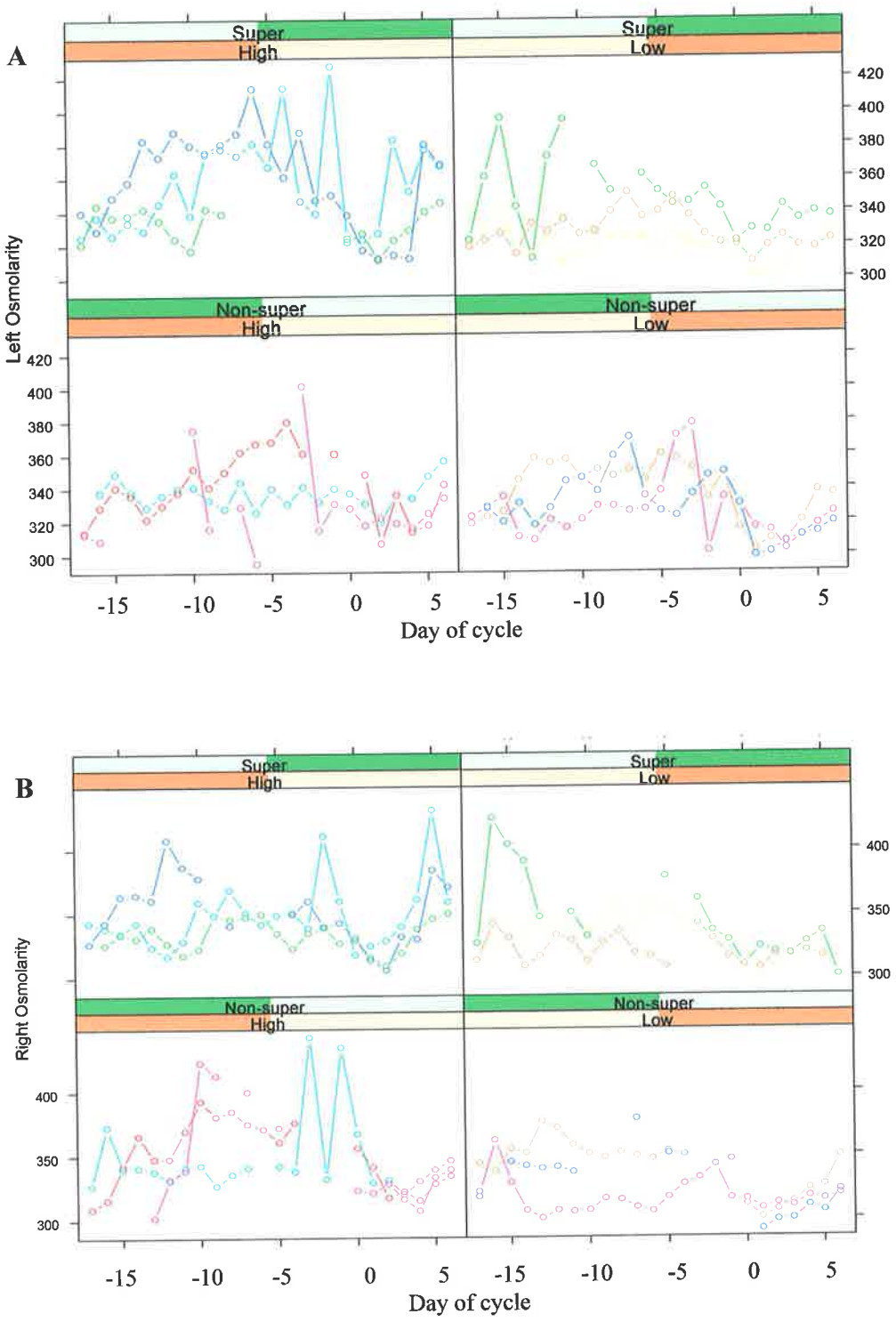
-The ICM (stained with Hoechst) will fluoresce blue whereas the TE cells (dual stained) will be pink/light red.



Appendix 8.4 Left oviductal fluid volume (A) and right oviductal fluid volume (B) over time.



Appendix 8.5 Left oviductal fluid pH (A) and right oviductal fluid pH (B) over time.



Appendix 8.6 Left oviductal fluid osmolarity (A) and right oviductal fluid osmolarity (B) over time.

Appendix 8.7 Time of selected events in ovine embryonic and fetal development (adapted from Bazer *et al.,* 1987, and Jainudeen and Hafez, 1987)

Embryonic stage	Days (post fertilization)
Two-cell	1.0
Four-cell	1.3
Eight-cell	1.5
Genome activation*	1.5-2.0
Transport to uterus	2.7-3.0
Blastocyst	6-7
Hatching	7-8
Blastocyst elongation	11-16
Maternal recognition	12
Initial placentation	15
Primitive-streak formation	14
Open neural tube	15-21
Differentiation of first somite	17
Apparent heart beat	20
Closed neural tube	21-28
Visible forelimb and hind limb buds	28-35
Differentiation of digits	35-42
Differentiation of nostril and eyes	42-49
Hair follicle appear	42-49
Eyelids close	49-56
Tooth eruption	98-105
Hair covering body	119-126
Birth	145-155

*Occurs during the 8-16-cell stage.

Appendix 8.8 Receptor expression and bioactive responses of pre-implantation embryos to cytokines/growth factors synthesised in the female reproductive tract

Cytokine/ Groth factor	Bioactivity Species	Receptor expression Species	Reference
CSF-1	+ Rodent	+ Rodent	Pampfer *et al.*, 1991; Arceci *et al.*, 1992
GM-CSF	+ Rodent	+ Rodent	Robertson *et al.*, 2001
	Human	Human	Sjoblom *et al.*, 1999
	Bovine		de Moraes and Hansen, 1997
SCF		+ Rodent	Arceci *et al.*, 1992
IL-1	- Human		Hill *et al.*, 1987; Haimovici *et al.*, 1991
	+ Rodent		Schneider *et al.*, 1989
IL-2	- Rodent		Hill *et al.*, 1987
IL-6	+ Rodent		Jacobs *et al.*, 1992
IFNγ	+ Rodent	+ Rodent	Hill *et al.*, 1987; Haimovici *et al.*, 1991
TNFα	+ Rodent	+ Rodent	Pampfer *et al.*, 1994; Pampfer *et al.*, 1995
TGFβ	+ Rodent	+ Rodent	Paria and Day, 1990
TGFα	+ Rodent	+ Rodent	Brison and Schultz, 1996; Brison and Schultz, 1998
	Bovine		Larson *et al.*, 1992
LIF	+ Rodent	+ Rodent	Robertson *et al.*, 1991
	Human	Human	Dunglison *et al.*, 1996; van Eijk *et al.*, 1996
	Bovine		Thibier, 1993
	Ovine		Fry *et al.*, 1992
HB-EGF	+ Human		Martin *et al.*, 1998
PDGF	+ Bovine		Larson *et al.*, 1992
IGF-1	+ Rodent	+ Rodent	Gardner and Kaye, 1995; Lighten *et al.*, 1997
	Human	Human	Lighten *et al.*, 1997; Lighten *et al.*, 1998
Insulin	+ Rodent	+ Rodent	Harvey and Kaye, 1990
EGF	+ Rodent		Paria and Day, 1990
bFGF	+ Bovine		Larson *et al.*, 1992

Receptor expression and bioactive responses of pre-implantation embryos to cytokines and growth factors synthesised by uterine cells. (+) Cytokine/growth factor shown to have embryotrophic effect. (-) Factor shown to have no effect on embryos.

Appendix 8.9 Fetal organ weights (means ± SEM) at 75 days of gestation in high feed intake (1.5 x maintenance ration) vs low feed intake (0.5 x maintenance ration) groups in single males and females.

	High		Low	
	Female (n=7)	Male (n=6)	Female (n=8)	Male (n=8)
Adrenal glands	0.1±0.04	0.1±0.05	0.1±0.04	0.1±0.05
Kidney left	1.8±0.13	1.6±0.14	1.4±0.12	1.7±0.14
Kidney right	1.8±0.12	1.6±0.13	1.4±0.11	1.7±0.13
Brain	7.5±0.30	7.8±0.33	6.5±0.28	7.7±0.32
Liver	14.4±0.87	14.6± 0.95	13.0±0.79	15.1±0.94
Lungs	12.2±0.71	12.2±0.78	10.3±0.65	12.3±0.76
Gastro intestinal tract	9.5±0.75	8.7±0.82	7.3±0.68	7.9±0.81
Pancreas	0.3±0.02	0.3±0.03	0.2±0.02	0.2±0.02
Spleen	0.3±0.03	0.3±0.03	0.2±0.03	0.3±.03
Heart	2.5±0.19	2.6±0.21	2.0±0.17	2.3±0.21
Left ventricle	1.4±0.13	1.5±0.15	1.0±0.12	1.5±0.14
Right ventricle	0.7±0.09	0.6±0.10	0.6±0.08	0.5±0.10
Thymus chest	0.3±0.03	0.3±0.03	0.2±0.03	0.2±0.03
Thymus neck	0.4±0.05	0.5±0.05	0.4±0.05	0.4±0.05
Thyroid glands	0.1±0.02	0.1±0.03	0.1±0.02	0.2±0.02
Ovaries	0.03±0.004	*	0.04± 0.004	*
Testes	*	0.1±0.02	*	0.1±0.01
Placental membranes	415.2±56.7	322.0±84.0	419.2±49.4	461.7±35.8

There were no significant differences in organ and placental weights.

Appendix 8.10 Fetal organ weights (means ± SEM) at 75 days of gestation in high feed intake (1.5 x maintenance ration) vs low feed intake (0.5 x maintenance ration) groups in single and twins.

	High		Low	
	Single (n=7)	Twins (n=6)	Single (n=14)	Twins (n=2)
Adrenal glands	0.1±0.04	0.1±0.05	0.1±0.03	0.1±0.08
Kidney left	1.8±0.13	1.6±0.14	1.6±0.09	1.5±0.25
Kidney right	1.8±0.12	1.5±0.13	1.5±0.08	1.7±0.23
Brain	7.7±0.29	7.7±0.32	7.0±0.21	7.4±0.59
Liver	15.0±0.85	13.1±0.91	13.8±0.61	14.4±1.68
Lungs	12.6±0.69	11.3 0.74	11.2±0.49	11.4±1.37
Gastro intestinal tract	9.6±0.73	7.8±0.79	8.0±0.52	6.4±1.46
Pancreas	0.3±0.02	0.3±0.02	0.2±0.02	0.3±0.04
Spleen	0.3±0.03	0.2±0.03	0.3±0.02	0.3±0.05
Heart	2.6±0.19	2.4±0.20	2.2±0.133	2.0±0.37
Left ventricle	1.4±0.13	1.6±0.14	1.3±0.09	1.2±0.26
Right ventricle	0.7±0.09	0.6±0.09	0.6±0.06	0.4±.17
Thymus chest	0.3±0.03	0.2±0.03	0.2±0.02	0.2±0.06
Thymus neck	0.4±0.05	0.5±0.05	0.4±0.05	0.4±0.05
Thyroid glands	0.1±0.02	0.2±0.02	0.1±0.01	0.1±0.04
Ovaries	0.04±0.005	0.02±0.006	0.04±0.004	0.03±0.007
Testes	0.1±0.02	0.1±0.02	0.1±0.01	*
Placental membranes	326.9±59.9	611.3±104.5	432.6±29.8	531.0±101.6

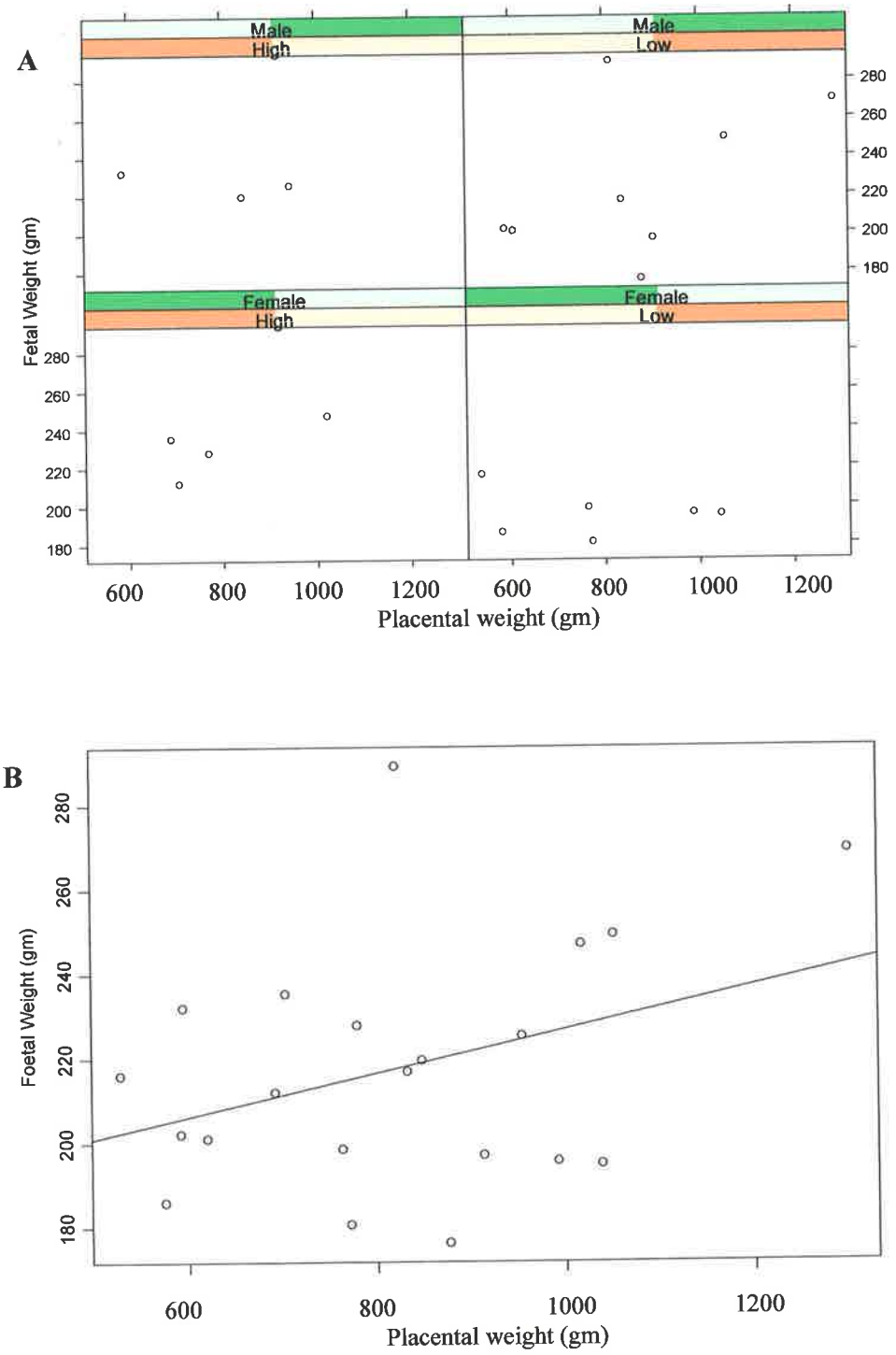
* No twin male was available in low nutrition group.

There were no significant differences in organ and placental weights.

Appendix 8.11 Fetal organ weights (means ± SEM) at 75 days of gestation in single and twin fetuses irrespective of the level of feed intake.

	Single (n=21)	Twin (n=8)
Adrenal glands	0.1±0.03	0.1±0.03
Kidney left	1.7±0.08	1.5±0.06
Kidney right	1.6±0.08	1.5±0.05
Brain	7.4±0.21	7.3±0.23
Liver	14.4±0.54	13.0±0.47
Lungs	11.8±0.46	10.7±0.44
Gastro intestinal tract	8.6±0.46	6.9±0.56
Pancreas	0.3±0.01	0.3±0.03
Spleen	0.3±0.02	0.2±0.02
Heart	2.4±0.12	2.2±0.13
Left ventricle	1.3±0.08	1.4±0.17
Right ventricle	0.7±0.06	0.6±0.06
Thymus chest	0.2±0.02	0.2±0.01
Thymus neck	0.4±0.03	0.4±0.04
Thyroid glands	0.1±0.01	0.1±0.02
Ovaries	0.04 ± 0.003^{a}	0.02 ± 0.003^{b}
Testes	0.1±0.07	0.1±0.03

Values within rows with different superscripts differ significantly ($P<0.05$)



Appendix 8.12 (A) High feed intake: fetal weight vs. placental weight for male fetuses (n=3) and female fetuses (n=4). Low feed intake: fetal weight vs. placental weight for male fetuses (n=8) and female fetuses (n=6). (B) Relationship between fetal and placental weights for the combined groups and sex (r=0.345, p= 0.124).

Chapter 9

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