THE NUTRITIONAL REGULATION OF OOCYTE QUALITY AND EARLY EMBRYO DEVELOPMENT IN CATTLE

by

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ABSTRACT

The importance of nutrition on reproductive performance is well recognised. However, the mechanisms and specific factors regulating their interaction are still poorly understood. Interest has recently intensified, as fertility in dairy cows has declined. There is a body of evidence on the negative effects of reduced body condition, the high levels of energy and protein intake, however, the results are inconsistent between studies and the role of specific factors, such as insulin and leptin, in the regulation of ovarian function in cattle remains unclear. The main objective of this thesis, therefore, was to test the effects of animal body composition, planes of nutrition and diet composition with emphasis on endocrine and metabolic factors, including progesterone, oestradiol, insulin, IGF-I and leptin, on oocyte quality and early embryo development in cattle.

This thesis has demonstrated that alterations in planes of nutrition (Maintenance (M) vs twice maintenance (2M)) affected oocyte quality, and that the effects were dependent on animal body condition score (BCS) (Low vs Moderate). Whilst high (2M) levels of feeding were beneficial for oocytes from animals in Low BCS, they were detrimental to oocyte quality for animals in Moderate BCS. Furthermore, the combination of feeding level and animal BCS created low (10 μIU/ml), moderate (20 μIU/ml) and high (40 μIU/ml) plasma insulin concentrations. Inevitably, however, it also led to the creation of three corresponding levels of plasma leptin concentrations (low: 2 ng/ml, moderate: 4 ng/ml, and high: 7 ng/ml), indicating that the effects of these metabolic hormones on ovarian function cannot be separated *in vivo*. However, there was no correlation between changes in metabolic hormones and oocyte quality, suggesting that other endocrine or metabolic factors, not recorded in this study, may be involved in determining the post-fertilisation developmental competence of oocytes.

Animal body composition also played an important role in the dietary-induced modifications to oocyte developmental competence both *in vivo* and *in vitro*. Indicated by post-fertilisation development *in vitro*, oocyte quality was enhanced in Low BCS heifers offered the Fibre than the Starch based diets (43.6 vs 24.6% blastocysts for Fibre vs Starch diet), whereas inclusion of protected lipid reduced oocyte developmental competence (27.7 vs 42.9% blastocysts for presence vs absence of Megalac). However, in Moderate BCS heifers, diet composition had little effect on oocyte quality. Although

dietary treatments had little effect on endocrine profile, elevated plasma leptin concentrations were consistently correlated ($P<0.001$) with increased animal body fatness. The effects of dietary treatments on oocyte maturation *in vivo*, however, varied from those observed when sera from these animals were used during the final stages of oocyte maturation *in vitro*, and during early embryo development. These findings suggest that while some diets may be beneficial to oocyte development, they could have a negative effect on embryo quality and subsequent survival. Interestingly, serum harvested from Low BCS heifers enhanced blastocyst formation *in vitro* compared to serum from heifers in Moderate BCS (24.2 vs 19.0%). This indicates that animal BCS is an important factor involved in the regulation of not only oocyte quality, but also early embryo development. The reasons for these discrepancies between BCS groups, however, remain unknown.

Finally, this thesis produced an interesting and novel insight into the nutritional regulation of the lipid content and fatty acid composition of bovine plasma, granulosa cells (GCs) and cumulus-oocyte complexes (COCs), and also the effects of serum from contrasting nutritional backgrounds on the lipid content and fatty acid composition of embryos. In general, the inclusion of supplemental fatty acids in the diet significantly increased the lipid content of all tissues tested. The fatty acid composition of COCs and embryos, however, was not correlated with their quality. Nevertheless, differences in the fatty acid composition of plasma, GCs and COCs indicated that there is a selective mechanism of fatty acid uptake by the follicle compartment, which has a preference for saturated fatty acids. The importance and function of specific fatty acids in determining oocyte and embryo quality remain to be elucidated.

This thesis has therefore demonstrated that animal body composition is an important factor determining the effects of feeding level and diet composition on oocyte and early embryo development. In summary, high levels of feeding and the Fibre based diets can enhance oocyte quality in thin, but not in moderately fat animals. Alterations in plasma insulin and leptin concentrations did not appear to be correlated with oocyte quality in the present studies, indicating that other factors, not monitored in this project, are possibly involved in the regulation of oocyte developmental competence.

PUBLICATIONS

Some of the data presented in this thesis has been published in preliminary form.

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Robinson, R.S., Hammond, A.J., Sinclair, K.D., Adamiak, S.J., Hunter, M.G. and Mann G.E. (2003) Angiogenic factors in the developing bovine corpus luteum. *Reproduction*, Abstract Series No **30**: 46.

Adamiak, S.J., Mackie, K., Ewen, M., Powell, K.A., Watt, R.G., Rooke, J.A., Webb, R., and Sinclair, K.D. (2004) Dietary carbohydrates and lipids affect *in vitro* embryo production following OPU in heifers. *Reproduction, Fertility and Development*, **16**: 193-194 [Abstract].

Adamiak, S.J., Ewen, M., Powell, K.A., Rooke, J.A., Webb, R. and Sinclair, K.D. (2004) Inclusion of bovine serum from different dietary backgrounds influences embryo viability *in vitro*. *Reproduction*, Abstract Series No **31**: 19.

Adamiak, S.J., Ewen, M., Rooke, J.A., Webb, R. and Sinclair, K.D. (2005) Diet and fatty acid composition of bovine plasma, granulosa cells and cumulus-oocyte complexes. *Reproduction, Fertility and Development*, **17**: 200-201 [Abstract].

Adamiak, S.J., Mackie, K., Watt, R., Webb, R. and Sinclair, K.D. (2005) Impact of nutrition on oocyte quality: Cumulative effects of body composition and diet leading to hyperinsulinaemia in cattle. *Biology of Reproduction*, **73**: 918-926.

Adamiak, S.J., Powell, K., Rooke, J.A., Webb, R. and Sinclair, K.D. (2006) Carbohydrate and fatty acid metabolism in bovine oocyte donors determines post-fertilization development *in vitro*. *Reproduction*, **131**: 247-258.

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ABBREVIATIONS

Acetyl-CoA	Acetyl-Coenzyme A
ADP	Adenosine diphosphate
AFRC	Agricultural and Food Research Council
AHEE	Acid Hydrolysed Ether Extract
ANOVA	Analysis of variance
ANS	8-anilino-1 napthalene sulphonic acid
ATP	Adenosine triphosphate
AU	Absorbance units
BC	Body condition
BCS	Body condition score
BMPs	Bone morphogenic proteins
Bo	Zero standard
BSA	Bovine serum albumin
cAMP	cyclic adenosine monophosphate
CIDR	Controlled internal drug-release dispenser
CL	Corpus luteum
COCs	Cumulus-oocyte complexes
cpm	Counts per minute
CSP	Charcoal stripped plasma
DF	Dominant follicle
DM	Dry matter
DNA	Deoxyribunucleic acid
dpm	Disintegrations per minute
ED	Estimated dose
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
EMP	Embden-Meyerhof pathway
F	Fibre
$FADH_2$	Flavin adenine dinucleotide, reduced
FAF	Fatty acid free
FCS	Foetal calf serum
FFA	Free fatty acids

FGF-2	Fibroblast growth factor 2
FSH	Follicle stimulating hormone
g	G force
g	Gram
GCs	Granulosa cells
GDF-9	Growth differentiation factor 9
GH	Growth hormone
GLUTs	Facilitative glucose transporters
GnRH	Gonadotrophin-releasing hormone
GTT	Glucose tolerance test
GV	Germinal vesicle
GVBD	Germinal vesicle breakdown
h	Hours
HDL	High density lipoprotein
i.m.	Intra-muscularly
i.v.	Intra-venously
ICM	Inner cell mass
IGFBPs	Insulin-like growth factor binding proteins
IGF-I	Insulin-like growth factor I
IGF-II	Insulin-like growth factor II
IGF-IR	Insulin-like growth factor I receptor
IGF-IIR	Insulin-like growth factor II receptor
IGFs	Insulin-like growth factors
Ins-R	Insulin receptor
IRS	Insulin receptor substrate
IU	International units
IVC	*In vitro* embryo culture
IVF	*In vitro* fertilisation
IVM	*In vitro* maturation
IVP	*In vitro* embryo production
kBq	Kilobecquerels
kg	Kilogram
LH	Luteinizing hormone
M	Maintenance

MAPK	Mitogen-activated protein kinase
MBq	Megabecquerels
mCi	Millicuries
ME	Metabolisable energy
mECM	Modified embryo culture medium
mg	Milligram
mIU	Milliinternational units
ml	Millilitre
mm	Millimetre
mM	Millimolar
mmol	Millimoles
mOsmo	Milliosmolarity
MPF	Maturation Promoting Factor
mRNA	Messenger ribonucleic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenytetrazolium bromide
NADH	Nicotinamide adenine dinucleotide, reduced form
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
ND	Not detected
NDF	Neutral Detergent Fibre
NEBAL	Negative energy balance
NEFA	Non-esterified fatty acids
NES	Normal emu serum
ng	Nanogram
NMP	Neutralised mobile phosphate buffer
NS	Not significant
NSB	Non-specific binding
OB-R	Leptin receptor
OB-RL	Long form of leptin receptor
OB-RS	Short form of leptin receptor
OPU	Ovum pick-up (transvaginal aspiration of follicles)
P450arom	Cytochrome P450 aromatase
P450scc	Cytochrome P450 side chain cleavage
PBS	Phosphate buffered saline

PCBG	Phosphate citrate buffer with gelatin
PFA	Paraformaldehyde
pg	Picogram
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
pH	Log of reciprocal hydrogen ion concentration
PI	Propidium iodide
PI-3 kinase	Phosphatidylinositol-3 kinase
PKB	Protein kinase B
PPP	Pentose-phosphate pathway
PTPases	Protein-tyrosine phosphatases
PUFA	Polyunsaturated fatty acids
PVA	Polyvinyl alcohol
PVP	Polyvinylpyrrolidone
QC	Quality controls
Ras	GTP-binding switch protein
RIA	Radioimmunoassay
ROS	Reactive oxygen species
rpm	Revolution per minute
RTK	Receptor tyrosine kinase
S	Starch
SAES	Sheep anti-emu immunoglobulin serum
sec	Seconds
SED	Standard error of difference
SEM	Standard error of mean
SH2	*src* homology 2 domain
Shc	Adaptor protein
SOF	Synthetic oviductal fluid
StAR	Steroidogenic acute regulatory protein
STAT	Signal transducers and activators of transcription
TCM199	Tissue Culture Medium 199
TCs	Thecal cells
TE	Trophectoderm
TFA	Total fatty acids
TGFβ	Transforming growth factor β

TG-rich LP	Triglyceride rich lipoprotein
TZPs	Transzonal projections
v/v	Volume to volume
VFA	Volatile fatty acids
w/v	Weight to volume
μCi	Microcuries
μg	Microgram
μIU	Microinternational units
μl	Microlitre
17β-HSD	17β-hydroxysteroid dehydrogenase
2M	Twice maintenance

CHAPTER 1

Literature review

1.1. Introduction

1.1.1. Declining fertility in dairy cattle

For the past 20 years the dairy industry in the UK and many other developing countries have been dealing with the problem of declining fertility in high yielding dairy cows. According to recent reports more than two inseminations per cow are required in order to establish pregnancy, and conception rates to first service are now less than 40% and are still declining at around 1% per annum (Smith and Wallace, 1998; Beam and Butler, 1999; Royal *et al.*, 2000; Lucy, 2001; Gong *et al.*, 2002).

The most effective method to reduce the cost of milk production is to increase annual milk production and/or to reduce the length of the dry period. In order to achieve this, pregnancy needs to be established within 12 weeks of parturition, reducing the calving interval to less than a year. Longer intervals between calving and first ovulation, increased services per cow and/or an inability to conceive are estimated to cost the UK dairy industry around £1 billion per annum (£4 per day/cow). Hence, improvements in conception rates would result in a higher annual milk production and eventually higher incomes per cow (Royal *et. al.*, 2000).

There are three main hypotheses for this decline in fertility: (i) genetic, (ii) environmental and/or (iii) nutritional.

(i) According to some, the introduction of North American Holstein genes (now constituting around 80% of British dairy herd (Royal *et. al.*, 2000)) and/or the intensive selection for high producing dairy cows, contribute to poor quality oocytes (Snijders *et al.*, 1999) and hence reduced fertility (Nebel and McGilliard, 1993; Royal *et. al.*, 2000; Snijders *et al.*, 2000; Gong *et al.*, 2002). However, studies conducted in the UK and the USA reported high pregnancy rates in dairy heifers (Royal *et al.*, 2000), indicating no inherent genetic defect at least in nulliparous heifers.

(ii) Changes in the management of dairy cattle in the UK such as increased average herd size (approximately 200 cows/herd) and a reduction in man-hours spent on each cow lead to reduced heat detection and hence pregnancy rates (Royal *et al.*, 2000).

(iii) As mentioned earlier, ability to establish pregnancy within 12 weeks from parturition is essential to reduce calving interval to less than a year. However, the post-partum period is associated with negative energy balance (NEBAL) when cow nutritional requirements increase rapidly in support of lactation. This in turn, is negatively correlated with various parameters of fertility (Butler, 1998; Beam and Butler, 1999; Kendrick *et al.*, 1999; Rukkwamsuk *et al.*, 1999; Butler, 2000), and this will be the main subject of this review.

1.1.2. Nutrition and reproduction

The influence of nutrition on reproduction is well established. However, the mechanisms by which nutrition influences fertility are still poorly understood (Lucy, 2003). Conventional wisdom states that changes in dietary intake affect the functioning of the hypothalamo-pituitary-ovarian axis, but the evidence suggests that gonadotrophin secretion is generally unaffected by dietary treatments (Gutierrez *et al.*, 1997a; Boland *et al.*, 2001; Armstrong *et al.*, 2002a), indicating that other factors acting directly at the ovarian level must have an effect on ovarian function and hence fertility (Gong, 2002).

Negative energy balance begins a few days before calving and reaches its most negative level (nadir) about 2 weeks later. It is associated with many hormonal changes, including a rise in circulating growth hormone (GH) and a decrease in metabolic hormones and growth factors such as insulin, insulin-like growth factor I (IGF-I) (McGuire *et al.*, 1995; Yung *et al.*, 1996; Prunier and Quesnel, 2000b; Walters *et al.*, 2002; Lucy, 2003) and leptin (Block *et al.*, 2001; Holtenius *et al.*, 2003). All these changes can lead to a prolonged interval to first ovulation *post partum*, possibly through an inhibition of luteinizing hormone (LH) pulse frequency (Butler, 2000). Food deprivation can induce similar alterations in hormonal secretion observed during NEBAL, especially post-ovulation, which can have a negative effect on the oviductal environment and in turn have a detrimental effect on embryo development (Mburu *et al.*, 1998). Furthermore, nutritional restrictions and/or NEBAL lead to increased mobilisation of the adipose tissue and consequently increased concentrations of non-esterified fatty acids (NEFA) (Olsson *et al.*, 1998; Rukkwamsuk *et al.*, 1999; Mackey *et al.*, 2000; Jorritsma *et al.*, 2003), which can further suppress insulin and oestradiol secretion (Comin *et al.*, 2002) and contribute to insulin resistance (Sivan *et al.*, 1998; Mason *et al.*,

1999). Since increased GH, insulin and IGF-I concentrations have been associated with increased number of small-sized follicles (Gutierrez *et al.*, 1997a) and increased aromatase activity within these follicles (Armstrong *et al.*, 2002a), it is believed that changes in these metabolic hormones and leptin (Barash *et al.*, 1996; Cunningham *et al.*, 1999; Clarke *et al.*, 1999) are responsible for impaired ovarian function in ruminants (Robinson, 1996; Beam and Butler, 1999; Sinclair *et al.*, 2000a; Miyoshi *et al.*, 2001; Lucy, 2003) and pigs (Prunier and Quesnel, 2000b). Recent studies support this hypothesis, demonstrating that nutritionally induced high levels of insulin in dairy cows can reduce the interval from calving to first ovulation (Gong *et al.*, 2002) and that a high insulin:glucagon ratio enhances early embryo development (Mann *et al.*, 2003). However, the timing and the mechanisms of the nutritionally induced changes in follicular development are still not fully understood. So the challenge remains to design diets that would maintain both high levels of milk production and acceptable levels of fertility (Boland *et al.*, 2001).

1.1.3. Objectives

This review will consider nutritionally-induced alterations to fertility in cattle and other species, with emphasis on metabolic hormones such as insulin, leptin and the insulin-like growth factors (IGFs) together with their effects on oocyte maturation and embryo development *in vivo* and *in vitro*.

1.2. Physiology of reproduction

1.2.1. Hypothalamo-pituitary-ovarian axis

Gonadotrophin-releasing hormone (GnRH) secreted by the hypothalamus and transported to the anterior pituitary via the hypophyseal portal blood system controls secretion of the gonadotrophic hormones (follicle stimulating hormone (FSH) and LH) from the anterior pituitary. In contrast to LH, FSH is not stored in the pituitary gland in large amounts. Once synthesised, it is slowly released into the bloodstream (Farnworth, 1995; Anderson, 1996, McNeilly *et al.*, 2003). The main function of FSH is stimulation of follicular emergence, followed by increased secretion of oestradiol by growing follicles (Badinga *et al.*, 1992; Roche *et al.*, 1999). Oestradiol binding to its

receptors present within granulosa, luteal and also thecal cells triggers proliferation and steroidogenesis of GCs (Rosenfeld *et al.*, 2001). Elevated concentrations of oestradiol and decreased progesterone in plasma induce release of GnRH from the hypothalamus and subsequent LH release from the pituitary gland (Wettemann *et al.*, 1972; Chenault *et al.*, 1975), which at its peak induces ovulation and subsequent luteinization of follicular cells. This is associated with a sharp decline in oestradiol, followed by a steady increase in progesterone levels which have an inhibitory (negative feedback) effect on GnRH release; in this way completing the hormonal loop between the hypothalamus and the gonads (Bevers *et al.*, 1997; Rosenfeld *et al.*, 2001, McNeilly *et al.*, 2003) (Figure 1.1).

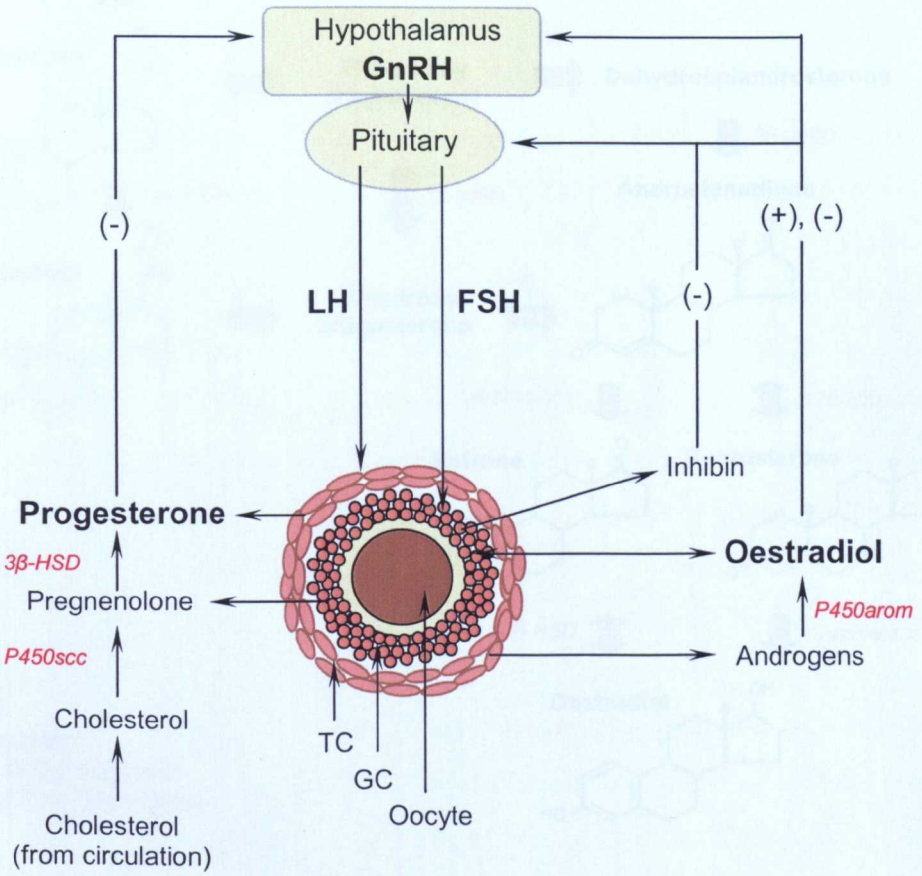

Figure 1.1. Schematic representation of hypothalamo-pituitary-ovarian axis and steroid hormones synthesis by follicular cells, during the oestrous cycle. Thecal cells – TCs; granulosa cells – GCs; hydroxysteroid dehydrogenase – HSD; cytochrome P450 side chain cleavage – P450scc; cytochrome P450 aromatase – P450arom, steroidogenic acute regulatory protein – StAR.

Synthesis of steroid hormones by follicular cells is initiated by translocation of the cholesterol, the main precursor of steroid hormones, from the circulation to the inner

mitochondrial membranes, catalysed by StAR (Stocco and Clarke, 1996; Devoto *et al.*, 1999). Once within the mitochondria, cholesterol is converted into pregnenolone and eventually into progesterone and oestradiol (Bao and Garverick, 1998; Devoto *et al.*, 1999; Bao *et al.*, 2000; Erickson and Shimasaki, 2001). Major pathways of steroid hormones biosynthesis are presented in Figure 1.2.

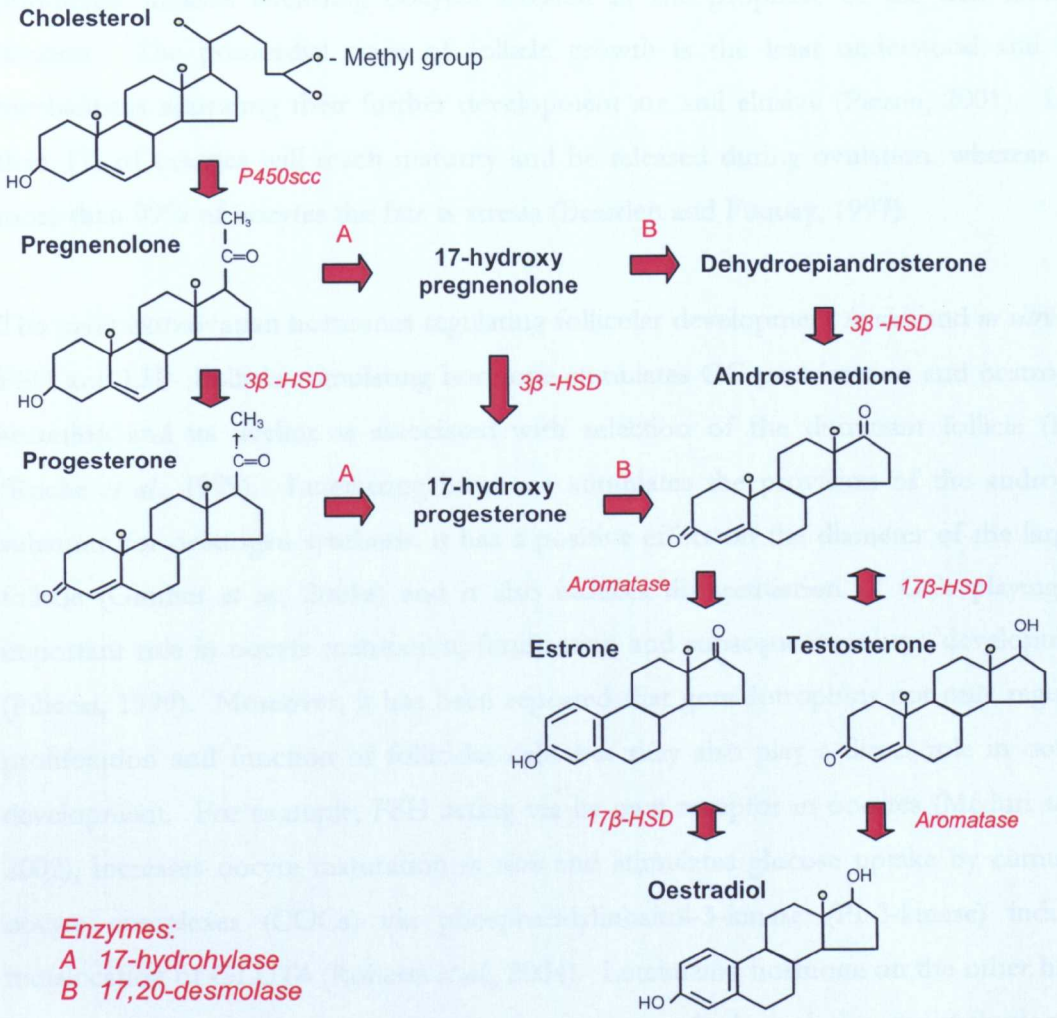

Figure 1.2. Major pathways of steroid hormone synthesis. HSD – hydroxysteroid dehydrogenase. Adapted from Gibbons *et al.* (1982) and Berg *et al.* (2002).

If fertilisation does not occur, prostaglandin ($PGF_{2\alpha}$) secreted by the uterus will inhibit progesterone production by the corpus luteum (CL) and allow the whole cycle to resume. Whereas, when the oocyte is fertilised and pregnancy is established, protein signals produced by the embryo (interferon τ) inhibit $PGF_{2\alpha}$ release, hence sustaining the CL and progesterone secretion throughout the pregnancy.

1.2.2. Endocrine regulation of follicle development

Ovarian follicular growth is a complex process during which follicles acquire a number of characteristics, each of which is essential for development and establishment of successful pregnancy. Oogenesis begins in the pre-natal period following initial formation and mitotic divisions of oogonia. Ovaries at birth contain a fixed number of primordial follicles enclosing oocytes arrested in late prophase of the first meiotic division. The primordial stage of follicle growth is the least understood and the mechanisms activating their further development are still elusive (Picton, 2001). Less than 1% of oocytes will reach maturity and be released during ovulation, whereas for more than 99% of oocytes the fate is atresia (Bearden and Fuquay, 1997).

The main extraovarian hormones regulating follicular development *in vivo* and *in vitro* are FSH and LH. Follicle stimulating hormone stimulates GC proliferation and oestrogen secretion and its decline is associated with selection of the dominant follicle (DF) (Roche *et al.*, 1999). Luteinizing hormone stimulates the provision of the androgen substrate for oestrogen synthesis, it has a positive effect on the diameter of the largest follicle (Ginther *et al.*, 2001a) and it also induces differentiation of GCs playing an important role in oocyte maturation, fertilization and subsequent embryo development (Filicori, 1999). Moreover, it has been reported that gonadotrophins not only regulate proliferation and function of follicular cells but they also play a direct role in oocyte development. For example, FSH acting via its own receptor in oocytes (Méduri *et al.*, 2002), increases oocyte maturation *in vitro* and stimulates glucose uptake by cumulus-oocyte complexes (COCs) via phosphatidylinositol-3-kinase (PI-3-kinase) induced translocation of GLUT4 (Roberts *et al.*, 2004). Luteinizing hormone on the other hand, plays a part in oocyte cytoplasmic maturation, which includes redistribution of microfilaments, acceleration of Golgi apparatus development and generation of lipid droplets (Sutovsky *et al.*, 1994). In addition, LH has been demonstrated to control oviductal contractions essential during the fertilisation process (Gawronska *et al.*, 2000). In contrast, studies of Choi *et al.* (2001) and Eppig *et al.* (1998, 2000) indicated that FSH and LH did not improve the development of bovine oocytes *in vitro*, suggesting that growth factors and/or other growth hormones control ovarian function in cattle. These contradictory reports suggest that our knowledge of the mechanisms and factors regulating ovarian function is still limited and requires further investigation.

According to the majority of reports, the earliest stages of follicular development in cattle are independent of gonadotrophins (Roche, 1996; Webb *et al.*, 1999; Drummond and Findlay, 1999), but are influenced by other extraovarian signals such as metabolic hormones (e.g. insulin, leptin) and intraovarian factors; including the IGFs, follistatin, fibroblast growth factor 2 (FGF-2), epidermal growth factor (EGF), transforming growth factor β (TGFβ) (McNatty *et al.*, 1999, 2000), growth differentiation factor 9 (GDF-9) and some of the bone morphogenic proteins (BMPs) (Braw-Tal, 2002; Picton *et al.*, 2003; Fair, 2003). In rat ovarian tissue it has been demonstrated that insulin, but not IGF-I, acting via its own receptor can influence the primordial to primary follicle transition (Kezele *et al.*, 2002). In sheep, EGF and TGFα have been reported to stimulate FSH-induced granulosa cell proliferation and simultaneously inhibit oestradiol secretion *in vitro* whereas, *in vivo*, they induced atresia in large antral follicles (Campbell *et al.*, 1994). However, in many species the roles of these numerous intraovarian growth factors still remain to be elucidated. Recent studies by Campbell *et al.* (2004), using ovarian autografts in sheep (Campbell *et al.*, 2000), confirmed the existence of a gonadotrophin-independent intraovarian feedback loop regulating the initiation of primordial follicles and also primary and secondary follicle development. Interestingly, the same study also demonstrated for the first time that the preantral follicle development *in vivo* can be influenced by gonadotrophins.

In cattle, ovarian follicular development during the 21–day oestrous cycle is characterised by two or three waves of growth (Fortune, 1993; Fortune *et al.*, 2001). Each wave is defined as the recruitment of a group of follicles 4 to 5 mm in diameter, but only one wave results in ovulation of the dominant follicle (McGee and Hsueh, 2000) (Figure 1.3). Each follicular wave is initiated by transient rises of FSH, concentrations of which peak in conjunction with the pre-ovulatory LH surge (Badinga *et al.*, 1992; Roche, 1996; Ireland *et al.*, 2000; Mihm and Austin, 2002) (Figure 1.4). Follicular progress from early antral to later antral (predominant) stages has been associated with increasing number of gonadotrophic receptors acquired by each follicle (Driancourt, 2001; Fair, 2003). Mural granulosa cells of antral follicles 3 to 4 mm in diameter develop FSH receptors and when follicles reach 8 mm in diameter they develop LH receptors (Bevers *et al.*, 1997). As a result, enhanced responsiveness to gonadotrophins initiates oestradiol synthesis by GCs, which is correlated with increased mRNA expression of the key steroidogenic enzymes, P450scc and P450arom (Guttierez

et al., 1997b; Bao and Garverick, 1998; Webb *et al.*, 1999; Fortune *et al.*, 2001), and also elevated inhibin production (Lévy *et al.*, 2000), which in synergy with oestradiol act as the main FSH suppressants (Martin *et al.*, 1988; Ginther *et al.*, 2001b). Following the initial transient rise in FSH concentrations gradually decline (Figure 1.4), and this coincides with selection of the first dominant follicle, which switches its gonadotrophic dependency from FSH to LH (Dobson *et al.*, 1997; Campbell *et al.*, 1999). Aromatase activity, and consequently oestrogen synthesis, by the dominant follicle is also increased (Badinga *et al.*, 1992; Bao *et al.*, 1998; Mihm and Austin, 2002).

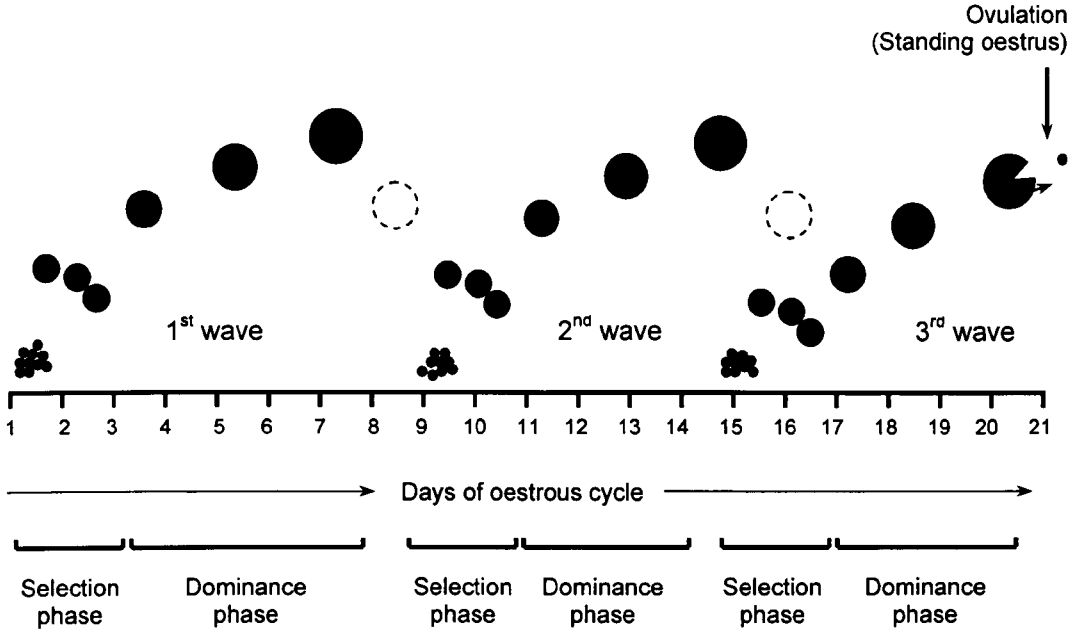

Figure 1.3. Diagram of follicular waves during oestrous cycle in cattle. Adapted from Roche (1996).

The combination of declining levels of FSH and also the direct effect of dominant follicle on coexisting follicles induces atresia in subordinate follicles (Ginther *et al.*, 1999; McGee and Hsueh, 2000; Prunier and Quesnel, 2000a; Baker *et al.*, 2001; Driancourt, 2001). However, the dominant follicle from the first follicular wave (on day 5 – 8 of oestrous cycle) also becomes oestrogen-inactive (ratio of oestradiol to progesterone in follicular fluid of <1 (Ireland *et al.*, 2000; Mihm and Austin, 2002)), looses its FSH and LH receptors and therefore its capacity to produce oestradiol and inhibin precursors which leads to atresia (Roche, 1996).

This loss of dominance is associated with reduced frequency of LH pulses induced by negative effects of luteal progesterone secretion (Stock and Fortune, 1993; Savio *et al.*, 1993; Sunderland *et al.*, 1994; Ireland *et al.*, 2000; Mihm *et al.*, 2002). Between days 7 and 9 of the oestrous cycle, reduced oestradiol concentrations and another transient FSH rise initiate a new follicular wave while the old dominant follicle regresses (Sunderland *et al.*, 1994) (Figure 1.4). The second dominant follicle will only ovulate if luteolysis occurs and progesterone does not suppress LH pulse frequency (Cooke *et al.*, 1997), otherwise the second dominant follicle will also undergo atresia and a third follicular wave will emerge giving rise to a third dominant follicle (Mihm *et al.*, 2002). Reduced progesterone secretion leads to high frequency and very high amplitude LH pulses observed during the late luteal phase, which are necessary to maintain dominance and eventually lead to ovulation (Rahe *et al.*, 1980; Cupp *et al.*, 1995) (Figure 1.4).

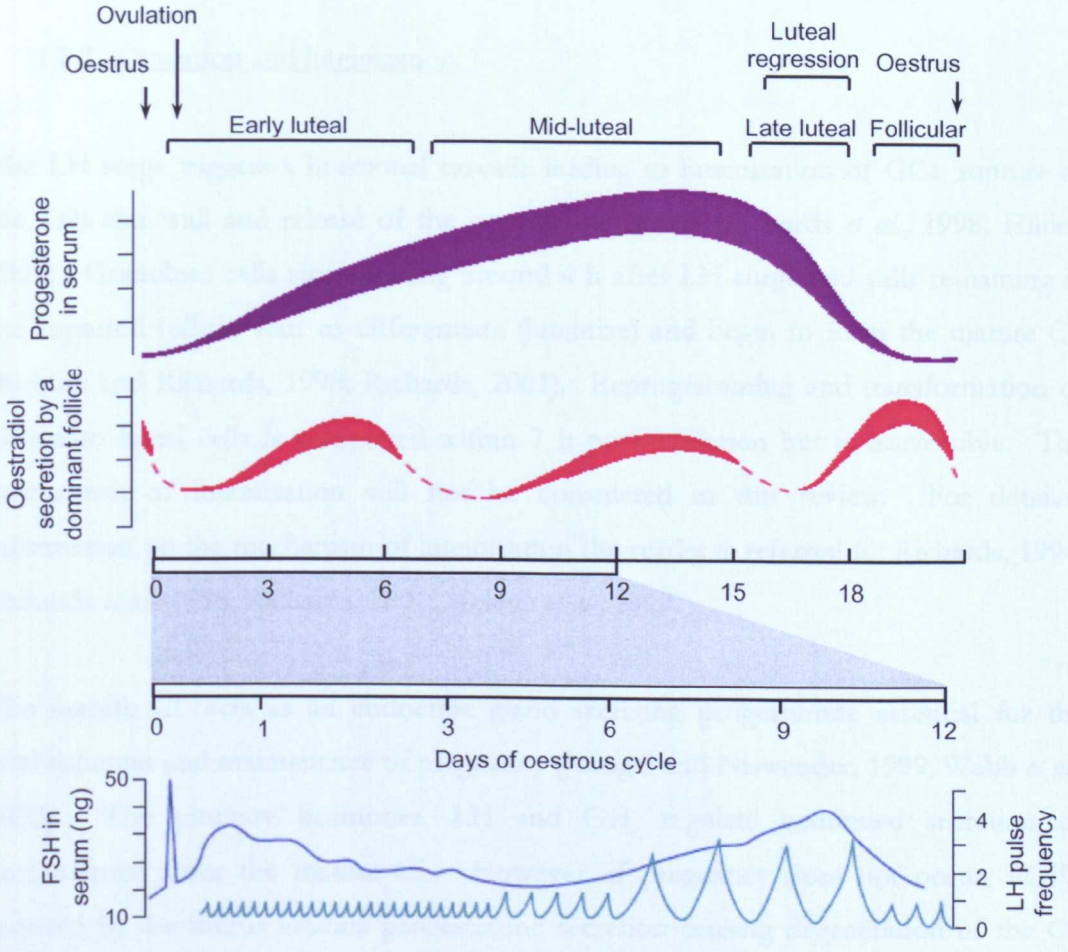

Figure 1.4. Schematic diagram of progesterone, oestradiol, FSH and LH secretion during oestrous cycle in cattle. Adapted from Roche (1996).

In addition to FSH and LH, intrafollicular factors such as inhibins and activins, IGFs and their binding proteins also play an important role in determination of follicular fate (Roche, 1996; Campbell, 1999; Ireland *et al.*, 2000). Higher concentrations of activin than inhibin in oestrogen-active follicles lead to dominance, whereas higher concentrations of inhibin and IGFBPs in oestrogen-inactive (subordinate) follicles lead to atresia (Roche, 1996; Austin *et al.*, 2001; Mihm and Austin, 2002). In addition, follicles destined to ovulate attain aromatase activity (e.g. Tian *et al.*, 1995; Fortune *et al.*, 2001). The ability to synthesise large amounts of oestradiol is possibly maintained by cell-to-cell contact via gap junctions and further facilitated by reduced levels of insulin-like growth factor binding protein –2 and –4 (IGFBP–2 and –4) leading to an increase in IGF bioavailability (Stewart *et al.*, 1995, 1996; Campbell *et al.*, 1996; Funston *et al.*, 1996; Monniaux *et al.*, 1997; Guttierez *et al.*, 2000; Mihm *et al.*, 2000; Austin *et al.*, 2001; Rhodes *et al.*, 2001).

1.2.3. Ovulation and luteinization

The LH surge triggers a hormonal cascade leading to luteinization of GCs, rupture of the follicular wall and release of the oocyte (ovulation) (Richards *et al.*, 1998; Hillier, 2001). Granulosa cells stop dividing around 4 h after LH surge and cells remaining in the ruptured follicle start to differentiate (luteinize) and begin to form the mature CL (Robker and Richards, 1998; Richards, 2001). Reprogramming and transformation of GCs into luteal cells is completed within 7 h post-ovulation but is irreversible. The mechanism of luteinization will not be considered in this review. For detailed information on the mechanism of luteinization the reader is referred to: Richards, 1994; Richards *et al.*, 1998; Richards, 2001; Meidan *et al.*, 1999.

The mature CL acts as an endocrine gland secreting progesterone essential for the establishment and maintenance of pregnancy (Juengel and Niswender, 1999; Webb *et al.*, 2002). The pituitary hormones, LH and GH, regulate continued secretion of progesterone from the mature CL. However, if pregnancy does not occur, $PGF_{2\alpha}$ secreted by the uterus inhibits progesterone secretion causing degeneration of the CL (Juengel and Niswender, 1999). Abnormalities in CL function can lead to embryonic loss, and this is recognised as one of the factors contributing to sub-fertility (Webb *et al.*, 2002).

1.2.4. Follicular development - Studies *in vitro*

Novel experiments on sustaining development of preantral follicles *in vitro* indicate that follicles can be maintained in culture for up to 28 days and their growth and antrum formation can be stimulated by FSH, EGF and IGF-I (Gutierrez *et al.*, 2000). These results confirm the findings of previous studies conducted *in vitro*, indicating that factors such as insulin and IGFs can interact with gonadotrophins and increase sensitivity of ovarian tissue to their actions. It has been demonstrated that insulin, in synergy with FSH, stimulates proliferation, differentiation and steroidogenesis of follicular cells (Gutierrez *et al.*, 1997b; Amsterdam *et al.*, 1998; Spicer *et al.*, 2002), possibly by increasing the number of gap junctions and enhancing development of the mitochondria, endoplasmic reticulum and Golgi complexes (Amsterdam *et al.*, 1998). Studies by McCaffery *et al.* (2000) suggest that IGF-I has a negative effect on the oocyte and GC proliferation in bovine preantral follicles but, according to Mao *et al.* (2004), IGF-I has a positive effect on porcine GC proliferation and follicular antrum formation. The existence of contradictory reports regarding interactions between insulin, IGFs and their binding proteins (IGFBPs) with FSH (Latham *et al.*, 1999; Ingman *et al.*, 2000; Bhatia and Price, 2001; Greisen *et al.*, 2001) and with LH (Sekar *et al.*, 2000) suggests that further studies are required in order to establish the exact relationship between these factors and their effects on follicular development *in vivo* and *in vitro*.

1.2.5. Oocyte maturation *in vivo*

Despite our limited knowledge of the mechanisms involved during oocyte maturation *in vivo*, completion of this developmental stage is believed to be the key to successful embryo development (Blondin and Sirard, 1995; Krisher and Bavister, 1998; Blondin *et al.*, 2002; Lonergan *et al.*, 2001, 2003a; Dieleman *et al.*, 2002; Rizos *et al.*, 2002a; Krisher, 2004; Rodriguez and Farin, 2004). *In vivo*, follicle enclosed oocytes have to complete certain stages of nuclear and cytoplasmic maturation which involve a range of metabolic and structural modifications necessary for successful fertilisation and later embryo development (Assey *et al.*, 1994; Fulka *et al.*, 1998; Telfer, 1998; Fair, 2003). The general ultrastructure and transcriptional activity of the bovine oocyte during development has been extensively reviewed by: Fair *et al.*, 1997a, b; Hyttel *et al.*, 1997, 2001.

Within the follicle, the oocyte and surrounding granulosa and thecal cells communicate with one another by extensive bidirectional gap junctions (Carabatsos *et al.*, 2000) formed mostly by protein connexin 43 between GCs (Ackert *et al.*, 2001; Vozzi *et al.*, 2001). The presence of this protein is increased by FSH (Sommersberg *et al.*, 2000) and oestrogen (Rosenfeld *et al.*, 2001). Connexin 37 is the main component of gap junctions formed between oocytes and GCs (Kidder and Mhawi, 2002). The major function of gap junctions is nutrient supply and transport of regulatory molecules to the oocyte (Eppig *et al.*, 1996; Kidder and Mhawi, 2002; Fair, 2003; Sutton *et al.*, 2003). Gap junctions are also associated with LH-dependent follicular maturation (Patino *et al.*, 2001) and coordination of oocyte cytoplasmic and nuclear maturation (Carabatsos *et al.*, 2000; Vozzi *et al.*, 2001; Rodriguez and Farin, 2004). In addition, oocyte and GCs communicate with each other through transzonal projections (TZPs), trafficking various paracrine factors such as IGFs, GDF-9 and Kit-ligand (Albertini *et al.*, 2001; Albertini and Barrett, 2003).

The discovery of maturation promoting factor (MPF) gave a new perspective on oocyte development, indicating that the oocyte is in control of its own fate (Vanderhyden and Macdonald, 1998; Li *et al.*, 2000). Even though gonadotrophins are necessary for GC differentiation, the oocyte by secreting soluble paracrine factors regulates follicle development by influencing GC proliferation, morphology and steroid synthesis (Vanderhyden, 1996; Vanderhyden and Macdonald, 1998; Li *et al.*, 2000; Eppig, 2001; Matzuk *et al.*, 2002; Brankin *et al.*, 2003; Gilchrist *et al.*, 2001, 2004). As oocyte growth and maturation progresses so does its ability to regulate granulosa cell functions (Gilchrist *et al.*, 2001). In antral follicles, a major role of the oocyte is to retain the distinct cumulus cell phenotype, by promoting growth (Vanderhyden *et al.*, 1992; Lanuza *et al.*, 1998; Li *et al.*, 2000), preventing luteinization by regulating steroidogenesis and inhibin synthesis (Vanderhyden and Macdonald, 1998; Lanuza *et al.*, 1999), and suppressing LH receptor (Eppig *et al.*, 1997) and Kit-ligand expression (Joyce *et al.*, 1999).

Final oocyte maturation is triggered by the gonadotrophin surge prior to ovulation and coincides with the alteration in GC steroidogenesis from oestrogen to mainly progesterone synthesis, and production of hyaluronic acid, which initiates mucification and expansion of cumulus GCs, blocking of gap junction contact between the oocyte

and surrounding it cells (Picton *et al.*, 2003). In the ovulating follicle the oocyte enables cumulus expansion thus facilitating release of the oocyte into the oviductal fimbria and subsequent fertilisation (Salustri *et al.*, 1990; Fulop *et al.*, 2003; Gilchrist *et al.*, 2004; Dragovic *et al.*, 2005). In species such as the cow (Li *et al.*, 2000), pig (Prochazka *et al.*, 1998; Nagyova *et al.*, 2000) and mouse (Salustri *et al.*, 1990; Tirone *et al.*, 1997) production of hyaluronic acid, mucification and expansion of cumulus cells is initiated by oocyte-secreted factor called cumulus expansion-enabling factor (CEEF). In addition, in mice factors such as TGFβ and GDF-9 can facilitate FSH-induced cumulus expansion (Vanderhyden *et al.*, 2003; Dragovic *et al.*, 2005). However, the identity of some of the oocyte-derived factors remains elusive, and so establishing their exact role in oocyte and follicle development is proving somewhat difficult. To date, most of the identified oocyte-secreted factors belong to TGFβ superfamily, namely GDF-9, GDF-9B (also called BMP-15) and BMP-6, since these proteins have been reported to affect follicular cells in a similar way to the oocyte (McGrath *et al.*, 1995; Eppig, 2001; Gilchrist *et al.*, 2001, 2004; Juengel *et al.*, 2004). Furthermore, both GDF-9 and BMP-15 are essential for normal follicle development, since mutations in their genes have been associated with impaired follicular development and infertility in sheep (Galloway *et al.*, 2000; Hanrahan *et al.*, 2004) and mice (Dong *et al.*, 1996). While sheep require both GDF-9 and BMP-15 genes for fertility (Galloway *et al.*, 2000; Juengel *et al.*, 2002; 2004; Hanrahan *et al.*, 2004), mice only require GDF-9, as BMP-15 knock-out mice are fertile (Yan *et al.*, 2001). In addition, studies *in vitro* demonstrated that while BMP-15 mRNA is not expressed in ovine and GDF-9 mRNA is not expressed in murine follicles until they begin to grow (Juengel *et al.*, 2004), GDF-9 mRNA and protein in sheep ovaries is expressed as early as day 56 of foetal development before follicle formation (Mandon-Pepin *et al.*, 2003). In bovine ovaries GDF-9 protein is expressed from the beginning of primordial follicle stage (Bodensteiner *et al.*, 1999). Even though each new report sheds more light on the regulation of oocyte growth and maturation there is still a clear deficiency in our understanding of the fundamental mechanisms determining its development and hence fertility.

1.2.5.1. Nuclear maturation

Due to insufficient regulatory factors, oocytes enclosed within preantral follicles are unable to progress beyond the diplotene stage of first meiotic division, and thus are

meiotically incompetent (Eppig, 2001). Oocytes are maintained in meiotic arrest by cumulus-derived cyclic adenosine monophosphate (cAMP), which minimizes the availability of pre-MPF by repressing synthesis of pre-MPF component cyclinB1 and by inhibiting MPF activation (Dekel *et al.*, 1981, 1988; Josefsberg *et al.*, 2003). Whereas antral follicles 2 to 3 mm in diameter contain mostly meiotically competent oocytes around 110 μm in diameter (excluding zona pellucida) (Hyttel *et al.*, 1997; Driancourt and Thuel, 1998; Yang *et al.*, 1998; Gandolfi and Gandolfi, 2001; Hyttel *et al.*, 2001), with a characteristic large, pale nucleus (the 'germinal vesicle' (GV); Picton *et al.*, 1998; Dekel, 2005). LH-induced oocyte maturation involving breakdown of gap junctions, is followed by the reduction in intraoocyte cAMP levels, since the oocyte is unable to produce sufficient amounts of cAMP, and thus meiosis is resumed (Dekel *et al.*, 1981; Eppig, 1985; Dekel, 2005). In addition, oocyte nuclear maturation is mediated by the activation of pre-MPF, catalysed by the cdc25B phosphatase, what also leads to an increase in mitogen-activated protein kinase (MAPK) activity (Fissore *et al.*, 1996; Bevers *et al.*, 1997; Wehrend and Meinecke, 2001; Picton *et al.*, 2003). Oocyte progression from the prophase of the first meiotic division to the metaphase II of the second meiotic division (Sutton *et al.*, 2003; Rodriguez and Farin, 2004), involves condensation of the chromosomes and dissolution of the nuclear membrane (so called GV breakdown 'GVBD'), formation of the chromosomal spindle and finally extrusion of the first polar body (Van den Hurk *et al.*, 2000). Due to MAPK action oocyte maturation is arrested for the second time at the metaphase II until fertilisation occurs, which is followed by extrusion of the second polar body and completion of oocyte nuclear maturation (Josefsberg *et al.*, 2003; Dekel, 2005). More recent studies of Tomek *et al.* (2002) have attempted to elucidate some of the molecular mechanisms occurring during nuclear maturation, namely the relationship between transcription, translation and polyadenylation of mRNA. The results indicate that protein synthesis is altered during meiotic maturation and significantly increased with the onset of GVBD.

1.2.5.2. Cytoplasmic maturation

Completion of cytoplasmic maturation is necessary for the successful fertilisation of the oocyte and subsequent development to the blastocyst stage (Bearden and Fuquay, 1997). Cytoplasmic maturation involves the synthesis and presence of specific factors, reorganisation of cytoplasmic organelles (Smith, 2001), including the movement of

mitochondria and cortical granules from a peripheral location to more spatial distribution (Hartshorne, 1997; Driancourt and Thuel, 1998; Fulka *et al.*, 1998). It is also associated with redistribution of endoplasmic reticulum and an increase in lipid droplets (Fair, 2003) and glycogen granules forming 'yolk' (Picton *et al.*, 1998). According to Krisher and Bavister (1998) the cytoplasm of the oocyte takes part in establishing metabolic pathways for energy production, essential for maturation and subsequent cleavage and blastocyst formation. However, the initiation and mechanisms of those changes are still not fully understood.

1.2.6. Oocyte maturation *in vitro*

Fully grown oocytes removed from the follicle spontaneously resume meiosis (Pincus and Enzmann, 1935; Eppig and Downs, 1984; Eppig, 2001; Tomek *et al.*, 2002), suggesting that granulosa and thecal cells secrete factors (e.g. cAMP) that maintain oocytes in meiotic arrest (Sirard and Blondin, 1996; Richard and Sirard, 1996a, b; 1998; Sirard, 2001). During 24 h of maturation *in vitro*, the oocyte has to complete the same stages of cytoplasmic and nuclear maturation as it does *in vivo* in order to undergo successful fertilisation and subsequent embryo development. Cumulus cells surrounding the oocyte are necessary for oocyte maturation and subsequent blastocyst formation. Studies conducted *in vitro* have shown that removal of cumulus cells from the oocyte before maturation can result in decreased cytoplasmic maturation (Kim *et al.*, 1997), fertilisation and impaired embryo development (Bevers *et al.*, 1997; Hashimoto *et al.*, 1998; Familiari *et al.*, 1998; Fatehi *et al.*, 2002; Kidder and Mhawi, 2002; Mingoti *et al.*, 2002; Sutton *et al.*, 2003). Even disruption in cell to cell contact has been correlated with increased incidence of apoptosis (Luciano *et al.*, 2000). However, according to Geshi *et al.* (2000) inclusion of sodium pyruvate during *in vitro* maturation of denuded oocytes can compensate for the absence of cumulus cells.

Oocyte maturation *in vitro* is not only stimulated by gonadotrophins (Zuelke and Brackett, 1992), but also by a range of other factors, such as oestradiol, progesterone, testosterone, EGF, inhibin and IGF-I and -II (Lorenzo *et al.*, 1994; Driancourt and Thuel, 1998; Rieger *et al.*, 1998; De la Fuente *et al.*, 1999; Silva and Knight, 2000; Sirotkin *et al.*, 2000; Dode and Graves, 2002; Sakaguchi *et al.*, 2002). Most of these factors enhance oocyte maturation by stimulating cumulus expansion (EGF, IGF-I) and

regulating their physiological functions. The exact mechanisms and the key factors involved in the oocyte maturation, however, are still not known.

Recent studies suggest that oocyte developmental competence is acquired during a lengthy process of folliculogenesis (Blondin *et al.*, 1997; Mermillod *et al.*, 1999; Sutton *et al.*, 2003) and the final stages of oocyte maturation *in vitro* cannot improve oocyte quality. Furthermore, studies in cattle indicate that biological factors such as oocyte donor age (Yang *et al.*, 1998), follicular diameter (Arlotto *et al.*, 1996; Yang *et al.*, 1998; Hagemann, 1999; Vassena *et al.*, 2003) and the phase of the oestrous cycle (Sirard and Blondin, 1996; Salamone *et al.*, 1999; Hendriksen *et al.*, 2004) have a profound effect on oocyte developmental competence, which determine later embryo development and final *in vitro* embryo production (IVP) outcome. Despite improved *in vitro* maturation (IVM) conditions, oocytes matured *in vivo* are more competent than those matured *in vitro* (Lonergan *et al.*, 2001, 2003a).

1.2.7. Fertilisation

In most mammalian species fertilisation occurs in the ampullary-isthmic junction of the oviduct. The main function of the oviductal epithelial cells is to maintain sperm viability, synchronise oocyte and sperm transport within the oviduct and facilitate sperm capacitation, which involves removal of glycoproteins from the sperm cell surface (Cunningham, 1997). Carbohydrate-protein interactions between the sperm and the oocyte enable capacitated sperm to recognise the oocyte and respond to its signals in an appropriate manner (Töpfer-Petersen *et al.*, 2000). Sperm binding to the zona pellucida initiates a signalling cascade leading to activation of the acrosome reaction, which involves release of the enzymes: hyaluronidase (which causes breakdown of hyaluronic acid, the main component of the intercellular matrix of GCs surrounding the oocyte) and acrosin (which digests the acellular coating around the oocyte) from the acrosomal cap, thus allowing the sperm to penetrate and fuse with the oocyte (Cunningham, 1997). After penetration by the sperm, cortical granules within the oocyte are released, thus preventing the oocyte from polyspermy (Bearden Fuquay, 1997; Cunningham, 1997). Finally, successful fertilisation results in the formation of a diploid zygote containing the genetic code for a new individual. Early development is further supported by an adequate nutritional environment within the oviduct (Nieder and Corder, 1983).

1.2.8. Cleavage and embryo development *in vivo* and *in vitro*

After fertilisation, whilst being transported along the oviduct towards the uterus, the zygote divides without any increase in cytoplasm content. At around the 8 – 16 cell stage the bovine embryo enters the uterus (Day 3 – 4 of development). By the morulae stage (32 – 64 cell stage) the embryo begins to compact due to the formation of gap and tight junctions between blastomers, a necessary step in blastocyst formation (Bearden and Fuquay, 1997). In cattle, by Day 7 of development most embryos will have reached the blastocyst stage. Blastocysts consist of (i) trophectoderm cells (TE) which will later form placenta and (ii) a small group of inner cell mass (ICM) cells, which will give rise to the embryo (Bearden and Fuquay, 1997; Devreker and Englert, 2000). As the blastocyst expands, the zona pellucida eventually cracks and the cells within are released. Hatched blastocysts begin to elongate (in cattle by Day 14 – 16) in preparation for attachment (Bearden and Fuquay, 1997).

Despite no reported differences in cleavage rates, blastocyst yields are significantly higher from oocytes fertilised *in vivo* than those fertilised *in vitro* (Lonergan *et al.*, 2001). The evidence from studies *in vitro* suggest that pre-implantation embryo development is not only associated with high mitogenic activity, but also many other morphological changes including embryonic metabolism. The early stages of embryo development are characterised by low metabolism and an inability to utilise glucose. In bovine embryos the embryonic genome is activated around the 8 – 16 cell stage (Frei *et al.*, 1989; Lim and Hansel, 2000), and this period is associated with increased energy requirements and hence increased embryonic metabolism and the ability to utilise glucose (Lane, 2001). Genome activation within the embryo is characterised by a shift from maternal to embryonic control (Devreker and Englert, 2000). Under *in vitro* conditions, this stage of bovine embryo development is often associated with a developmental block, which can be overcome by supplementation of culture media with embryotrophic substances such as growth factors (Lim and Hansel, 2000). Furthermore, formulation of defined and semi-defined embryo culture systems allow scientists to study mechanisms involved during pre-implantation embryo development (Thompson *et al.*, 1996; Thompson, 1997, 2000), which will be discussed in more detail in later paragraphs. It is generally accepted that *in vitro*-produced embryos have a lower developmental capacity compared to *in vivo*-derived viable embryos (Lonergan *et al.*, 2001, 2003a; Leese, 2002), what can be linked

with morphological and physiological differences between them (Wright and Ellington, 1995; Thompson, 1997; Abe *et al.*, 1999a, b, 2002; Khurana and Niemann, 2000a; Crosier *et al.*, 2000, 2001; Rizos *et al.*, 2002b) and also differences in the pattern of gene expression (Rizos *et al.*, 2002c).

1.3. Animal metabolism

1.3.1. Carbohydrate and glucose metabolism

In the lactating dairy cow, the rumen, liver and mammary gland are the major organs involved in the metabolism of carbohydrates. Microorganisms within the rumen ferment carbohydrates to produce energy, gases (e.g. methane and carbon dioxide), heat, and organic acids. Volatile fatty acids (VFA: acetic, propionic and butyric acid) produced in the rumen are absorbed through the rumen wall and transported via the portal blood system to the liver, where most of the propionate is converted to glucose (O'Callaghan and Boland, 1999; Boland *et al.*, 2001).

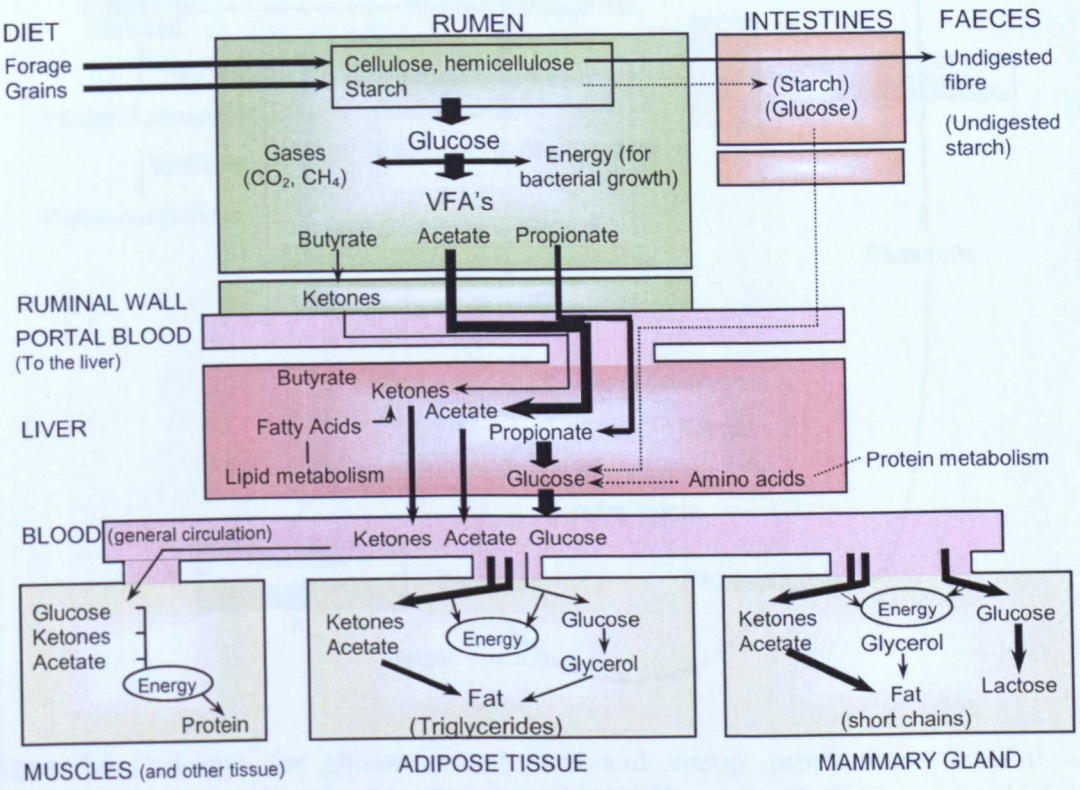

Figure 1.5. Overview of carbohydrate metabolism in dairy cows. Adapted from Wattiaux and Armentang, (2002).

Glucose formed during intestinal digestion is also transported to the liver and contributes to its own supply (O'Callaghan and Boland, 1999) (Figure 1.5).

Within body cells, under complete aerobic conditions glucose is converted into CO_2 and H_2O with release of 36 molecules of adenosine triphosphate (ATP). Glycolysis, also called the Embden-Meyerhof pathway (EMP), is the initial stage of glucose metabolism and does not involve oxygen. The final products of glucose metabolism are CO_2 and H_2O (Cunningham, 1997; Lodish *et al.*, 2000) (Figure 1.6). Energy produced during glucose metabolism, in the form of ATP, contributes to other cellular processes.

In embryonic cells, glucose-6-phosphate can either enter the EMP, with the end product of two molecules of pyruvate and H_2O, or the pentose-phosphate pathway (PPP) resulting in one molecule of ribose and CO_2. Ribose is the main substrate for synthesis of nucleotides (Gardner, 1998) (Figure 1.6).

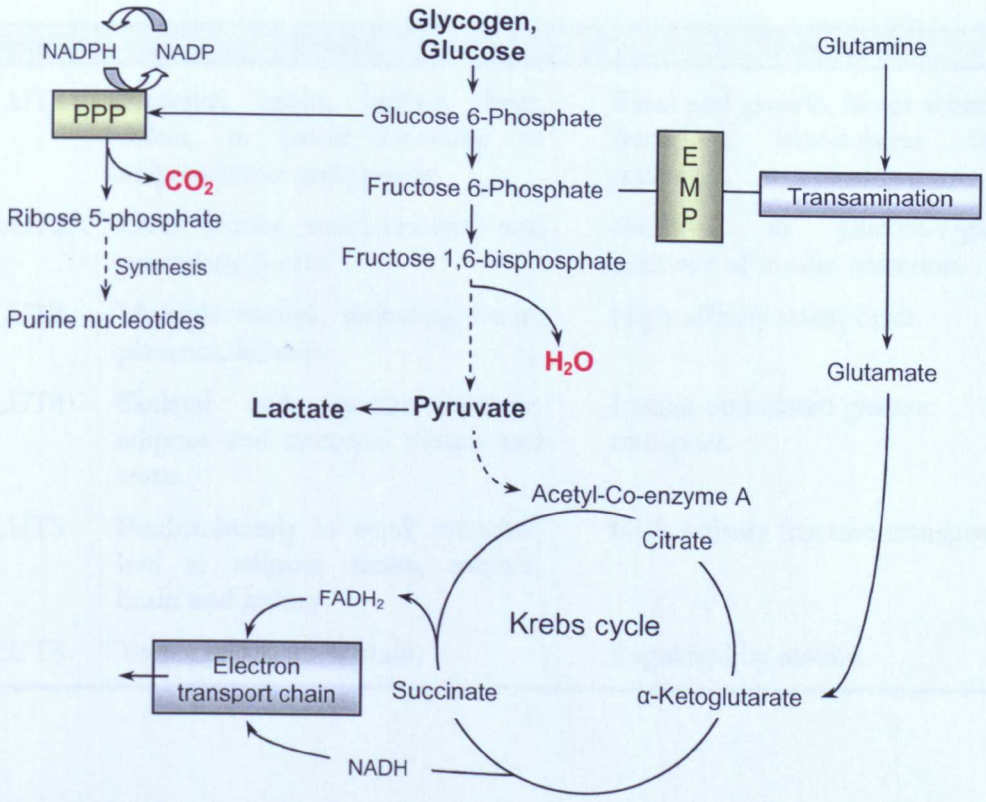

Figure 1.6. Pathways for glucose metabolism and energy production. NADP – nicotinamide adenine dinucleotide phosphate; NADPH – reduced nicotinamide adenine dinucleotide phosphate; NADH – nicotinamide adenine dinucleotide; $FADH_2$ – reduced flavin adenine dinucleotide. Adapted from Rieger *et al.* (1992) and Gardner *et al.* (1993).

Glucose uptake into various tissues is attained by two types of glucose transporters:

(i) Na^+- dependent (not regulated by insulin; present in the intestinal tract and kidney)

(ii) Facilitative glucose transporters known as GLUTs (Pantaleon and Kaye, 1998; Lodish *et al.*, 2000; Augustin *et al.*, 2001), of which there are estimated to be around a dozen.

The presence of specific GLUTs within body tissues is listed in Table 1.1. In the insulin sensitive tissues, insulin acting via PI-3-kinase causes a rapid translocation of the intracellular GLUT4 to the plasma membrane (Martin *et al.*, 2000; Lizcano and Alessi, 2002) resulting in a 20 to 30-fold increase in the rate of glucose uptake (Cheatham and Khan, 1995).

Table 1.1. Glucose transporters and their presence and function within body tissues (Pantaleon and Kaye, 1998).

Name	Tissue	Function
GLUT1	Placenta, brain, kidney, liver, colon, in lower amounts in adipose tissue and muscle.	Basal and growth factor stimulated transport; blood-tissue barrier transport.
GLUT2	Liver, kidney, small intestine and pancreatic β-cells	Involved in glucose-regulated pathway of insulin secretion.
GLUT3	Multiple tissues, including brain, placenta, kidney.	High affinity transporter.
GLUT4	Skeletal and cardiac muscle, adipose and neuronal tissues and testis.	Insulin-stimulated glucose transport.
GLUT5	Predominantly in small intestine, less in adipose tissue, muscle, brain and kidney.	High affinity fructose transporter.
GLUT8	Testis, blastocysts, brain.	Regulated by insulin.

Glucose availability *in vivo* has been associated with alterations in LH secretion, possibly by acting on the hypothalamus and regulating GnRH release (Bucholtz *et al.*, 1996). The study of Rabiee and Lean (2000) suggested that glucose can stimulate cholesterol uptake by ovarian cells and that cholesterol can also stimulate glucose uptake by the same cells. This may be of great importance in the future studies on the regulation of ovarian

function. Studies in sheep have demonstrated that administration of glucose *in vivo* results in elevated concentrations of insulin, which in turn can lead to alterations in ovarian function and increased luteal progesterone secretion (Rubio *et al.*, 1997). However, studies *in vivo* on glucose effects alone are difficult to conduct and interpret because they will always be linked with alterations in plasma insulin concentrations.

1.3.2. Proteins

Proteins provide amino acids essential for growth, reproduction and lactation. In contrast to other animals, ruminants are able to synthesise amino acids by utilising non-protein nitrogen sources, such as urea, which can also be re-cycled to reduce protein loss. Dietary protein is categorised as rumen degradable, which is hydrolysed to ammonia by rumen microbes and with dietary energy form microbial protein digested in the intestine; and undegradable protein, which by-passes rumen and is digested and absorbed in the intestine (O'Callaghan and Boland, 1999) (Figure 1.7).

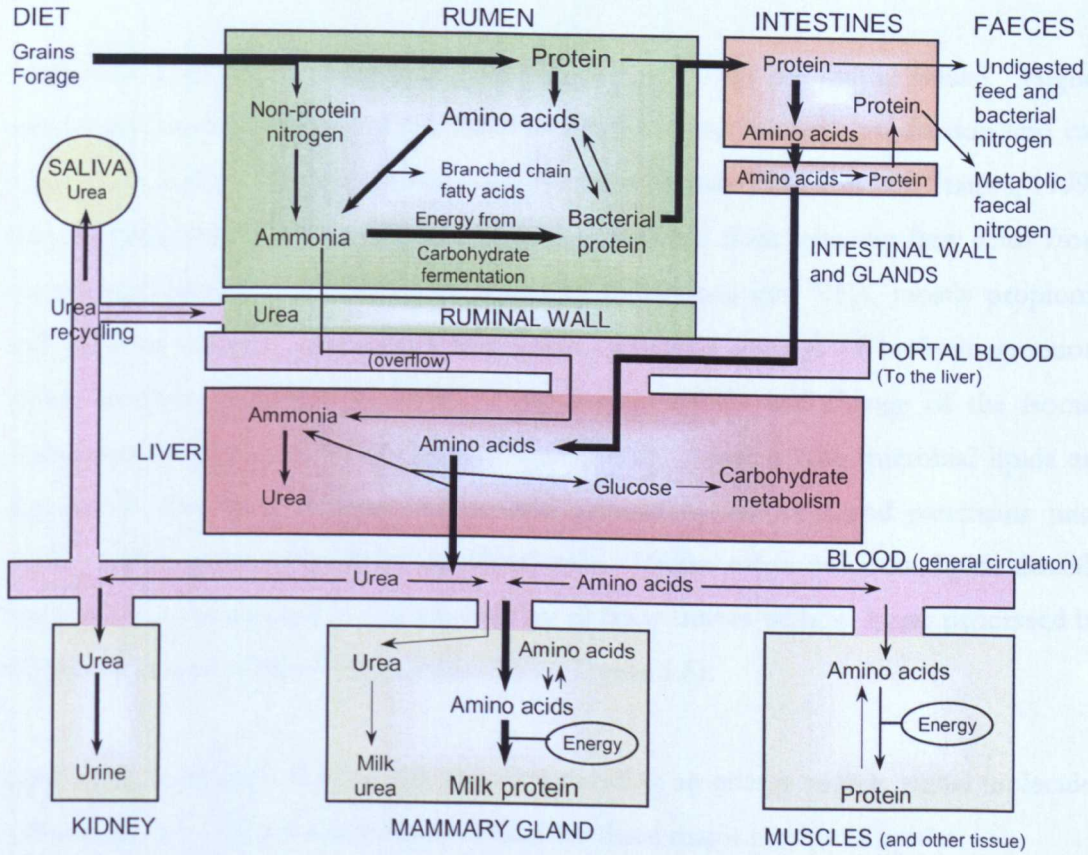

Figure 1.7. Overview of protein metabolism in dairy cows. Adapted from Wattiaux (2002).

Lactating dairy cows fed high energy diets were reported to have an increased number of good quality oocytes compared to animals fed low energy diets (Kendrick *et al.*, 1999). However, according to Boland *et al.* (2001) increased numbers of follicles induced by high energy diets are not necessarily associated with an increased number of good quality oocytes. In fact, high energy and crude protein diets in ruminants can lead to increased concentrations of urea and ammonia in plasma and follicular fluid, which are negatively correlated with oocyte quality, follicular diameter and aromatase activity of follicular cells (Butler, 1998; O'Callaghan and Boland, 1999; Butler, 2000; Boland *et al.*, 2001; Sinclair *et al.*, 2000a; Armstrong *et al.*, 2001). According to De Wit *et al.* (2001) high urea concentrations do not have an effect on embryonic development. In contrast to the De Wit study, others have reported that high urea plasma concentrations can alter the intrauterine environment, which in turn may have a detrimental effect on embryo development (McEvoy *et al.*, 1997; Butler, 1998; Kendrick *et al.*, 1999; Sreenan *et al.*, 1999) and hence increase early embryo mortality (Butler, 2000; Boland *et al.*, 2001).

1.3.3. Lipids

Unprotected lipid constitutes only 2 to 4% of cow's total dry matter intake. Higher concentrations of dietary lipid can have a negative effect on milk production and can significantly reduce fibre digestibility and dry matter intake (Williams and Stanko, 1999). Dietary lipids within the rumen are digested by ruminal flora releasing fatty acids from the glycerol backbone. Glycerol is fermented by bacteria into VFA, mostly propionic acid whereas released unsaturated fatty acids undergo a process of biohydrogenetion, which involves reduction of some of the double bonds and change of the isomer orientation (Ashes *et al.*, 1992; Mattos *et al.*, 2000). Most of the microbial lipids are digested in the small intestine, where bile secreted by the liver and pancreatic juice facilitate absorption of lipids by intestinal cells. Unlike other nutrients, lipids directly enter the general circulation and are used by all body tissues without being processed by the liver (Christie, 1981; Cunningham, 1997) (Figure 1.8).

Lipids play a number of roles that include providing an energy source, signal molecules and components of cell membranes. There are three major groups of lipids:

(i) Cholesterol – a component of the cellular membranes and also precursor of steroid hormones;

(ii) Phospholipids – main components of the cellular membranes and source of fatty acids necessary for the synthesis of eicosanoids, prostaglandins, thromboxanes and leukotrienes;

(iii) Triacylglycerols – also called neutral fats, which are highly concentrated stores of metabolic energy (Mattos *et al.*, 2000; Berg *et al.*, 2002).

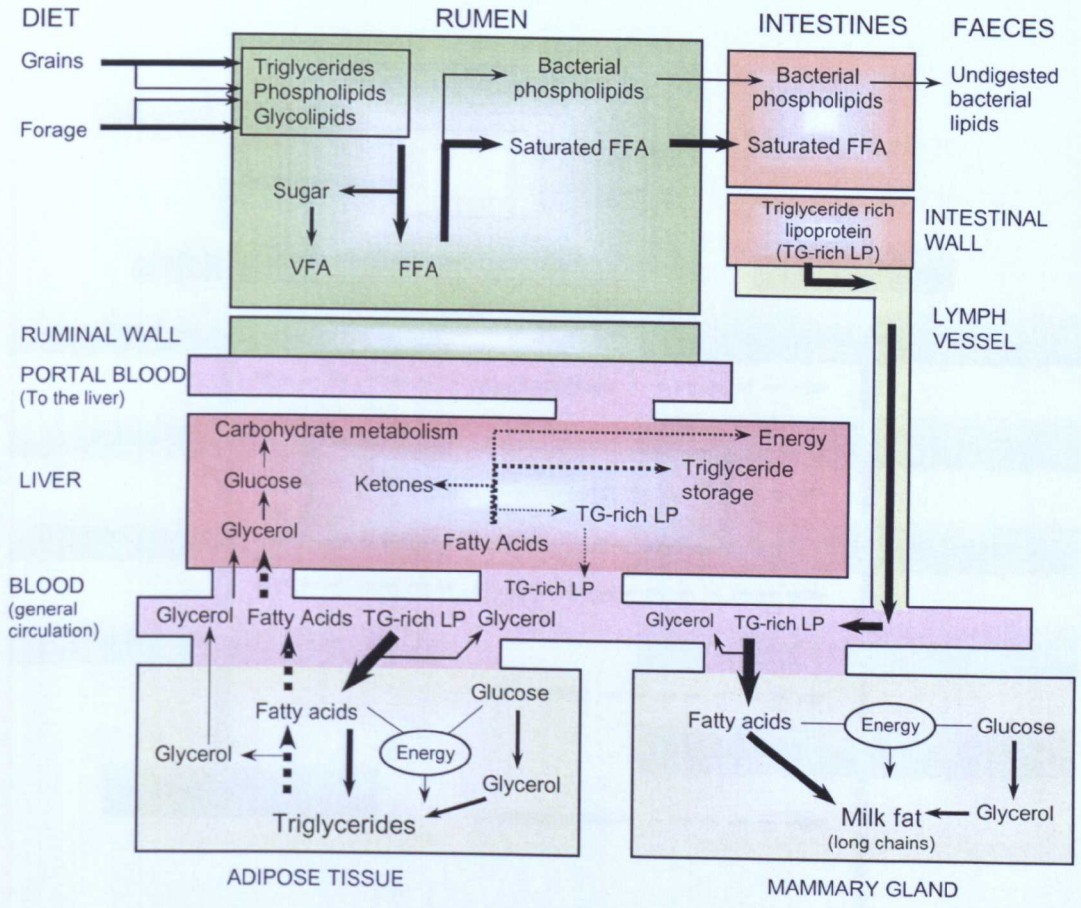

Figure 1.8. Overview of lipid metabolism in dairy cows. Adapted from Wattiaux and Grummer (2002).

The properties and function of specific fatty acids is largely dependent on their structure, which includes the length of the hydrocarbon chain and a degree of saturation (Mattos *et al.*, 2000; Berg *et al.*, 2002). Saturated fatty acids do not contain any double bonds within the chain, whereas unsaturated fatty acids containing double bonds and can be further divided into monounsaturated (one double bond) and polyunsaturated (two or more double bonds) fatty acids depending on the number of double bonds

within the hydrocarbon chain. Unsaturated fatty acids are classified into different families according to the position and number of double bonds, with the two main families being n-3 and n-6 polyunsaturated fatty acids. The process of elongation (addition of two carbon units to the chain, catalysed by elongase) and desaturation (insertion of a double bond into the chain, catalysed by desaturase enzymes) of fatty acids in one family can only generate fatty acids of the same family (Figure 1.9) (Mattos *et al.*, 2000).

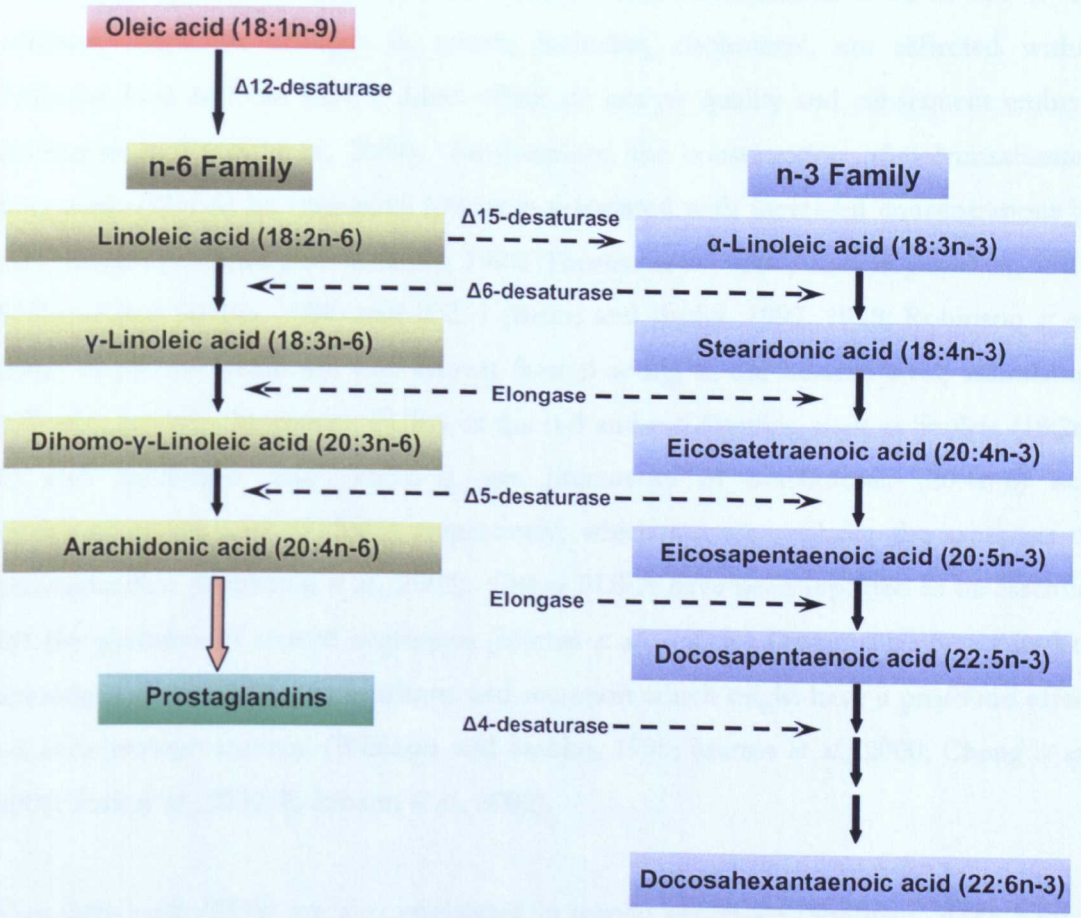

Figure 1.9. Outline of the metabolic transformations of the n-3 and n-6 families of polyunsaturated fatty acids. Adapted from Calder (2003).

In the past, dietary lipid supplements were only considered as a concentrated source of energy ideal for supporting the high energy requirements of lactating dairy cows. However, more recent studies suggest that dietary lipids affect reproductive parameters as indicated by increased concentrations of progesterone in plasma and lifespan of

induced CL (Hawkins *et al.*, 1995; Williams and Stanko, 1999), increased number and diameter of ovarian follicles (Lucy *et al.*, 1991; Lammoglia *et al.*, 1997; Thomas *et al.*, 1997), increased conception rates to first service (McNamara *et al.*, 2003) and alterations in the uterine environment (Staples *et al.*, 1998; Mattos *et al.*, 2000). However, according to Butler (2000) diets supplemented with additional fat, in spite of increasing follicle diameter, do not have any effect on the onset of ovulation.

The studies of Comin *et al.* (2002) demonstrated that NEFA can increase concentrations of IGFBPs within follicular fluid, but do not affect intrafollicular levels of IGF-I. In addition, metabolic changes in serum, including cholesterol, are reflected within follicular fluid and can have a direct effect on oocyte quality and subsequent embryo development (Leroy *et al.*, 2004). Furthermore, the consumption of polyunsaturated fatty acids (PUFA) by ruminants has been correlated with increased concentrations of GH, insulin (Thomas and Williams, 1996; Thomas *et al.*, 1997; Lammoglia *et al.*, 1997; Williams and Stanko, 1999) and IGF-I (Beam and Butler, 1997, 1998; Robinson *et al.*, 2002) in plasma which are well known factors acting at the ovarian level, stimulating follicular growth. Moreover, PUFA of the n-3 and n-6 families, such as linoleic (18:2n-6) and linolenic-α acid (18:3n-3), are precursors of arachidonic (20:4n-6) and eicosapentaenoic acid (20:5n-3) respectively, which are essential for the synthesis of prostaglandins (Robinson *et al.*, 2002). Other PUFA have been reported to be essential for the synthesis of steroid hormones (Mattos *et al.*, 2000). Dietary PUFA can lead to alterations in prostaglandin synthesis and secretion which might have a profound effect on early embryo survival (Williams and Stanko, 1999; Mattos *et al.*, 2000; Cheng *et al.*, 2001; Petit *et al.*, 2002; Robinson *et al.*, 2002).

Free fatty acids (FFA) are also implicated in insulin resistance (Shulman, 2000; Kim *et al.*, 2001a; Zick, 2001), usually occurring in obese or diabetic subjects and associated with alterations in the insulin signalling cascade (Dresner *et al.*, 1996; Griffin *et al.*, 1999) and inhibition of GLUT4 facilitated glucose uptake (Roden *et al.*, 1996; Peraldi and Spiegelman, 1998; Shulman, 2000). A reduction in FFA can restore insulin sensitivity (Ahrén, 2001).

1.4. Nutritional requirements and embryo metabolism

The developing embryo passing along the oviduct is exposed to a continually changing environment and hence its metabolic requirements for nutrients also change (Gardner and Leese, 1990). In the early stages of embryo development, pyruvate, lactate and amino acids are preferable sources of energy since the embryo's ability to utilise glucose is limited (Gardner, 1998; Moley *et al.*, 1998a; Boland *et al.*, 2001; Lane and Gardner, 2000; Devreker and Englert, 2000; Lane, 2001; Pantaleon *et al.*, 2001). This inability to metabolise glucose has been attributed to a block in the regulatory glycolytic enzyme, phosphofructokinase (Thompson, 2000). Progression through compaction and blastulation is associated with a rapid increase in energy requirements and a switch from the oxidation of pyruvate to the use of glucose as a main substrate, which can be metabolised both oxidatively and by glycolysis (Thompson *et al.*, 1996; Thompson, 2000; Chi *et al.*, 2002). The increase in glycolytic activity has also been related with embryo preparation for implantation.

Recent advances in *in vitro* embryo production lead to the formulation of defined and semi-defined culture systems which enabled scientists to study and elucidate some of the physiological and metabolic mechanisms activated during embryo development. Due to difficulties in assessing embryo metabolism *in vivo*, most of our knowledge on this subject is based on studies conducted with *in vitro* produced embryos (Leese, 1991) and a few comparative reports between embryos derived *in vivo* and those produced *in vitro* (Thompson, 1997; Lonergan *et al.*, 2001). Apart from morphological differences, there are also variations in activity of metabolic pathways. This include increased glycolytic activity (cow: Khurana and Niemann, 2000a; pig: Swain *et al.*, 2002) and reduced amino acid uptake (cow: Partridge *et al.*, 1996) between embryos cultured *in vitro* and their *in vivo* counterparts (Thompson, 1997). Therefore, metabolic activity has been recognised as a valuable marker of embryo development and viability (Gardner, 1998, 1999; Devreker and Englert, 2000; Gardner *et al.*, 2001; Houghton *et al.*, 2002). As concluded by Leese (2002), the most viable embryos produced *in vitro* are characterised with having low metabolic activity, low glycolytic rates and low amino acid turnover.

1.4.1. Glucose uptake and metabolism

It has recently been suggested that glucose, and not lipids, is the major source of energy for the ovary, mainly involved in the development and functioning of the CL (Chase *et al.*, 1992; Rabiee *et al.*, 1997; Rabiee and Lean, 2000). Glucose supplementation is essential for oocyte maturation and fertilisation *in vitro* (Krisher *et al.*, 1999; Lim *et al.*, 1999; Downs and Utecht, 1999) and higher glycolytic rates have been related to increased oocyte developmental competence (Krisher and Bavister, 1999; Krisher *et al.*, 1999). Our knowledge on glucose transporters within bovine oocytes is very limited. It has been demonstrated that transcripts for GLUT1 are present within human and mouse oocytes, however its function is triggered only post-fertilisation (Morita *et al.*, 1992; Dan-Goor *et al.*, 1997; Pantaleon *et al.*, 2001). This was confirmed by studies of Khurana and Niemann (2000b) and Boland *et al.* (2001), who reported that only a small quantity of glucose was incorporated by immature oocytes and glucose incorporation increased steadily between the 1-cell and blastocyst stage embryos. Cumulus cells surrounding the oocyte are responsible for most of glucose uptake by the COC (Downs and Utecht, 1999; Sutton *et al.*, 2003). Studies in sheep support this observation, reporting the presence of GLUT1 and -4 in follicular cells (GC and TC). Concentrations of these transporters are unaffected by nutritional treatments and are not correlated with follicular diameter (Williams *et al.*, 2001).

Unlike for oocytes, glucose transporters within developing embryos have been the subject of numerous investigations. Results from these studies demonstrated that most of the identified GLUTs are present throughout embryo development and they play a significant role in normal development. GLUT1 is present throughout pre-implantation embryo development, whereas GLUT2 is detectable from the 8-cell stage onwards. Its role in glucose transport, however, is uncertain. GLUT3 is present from the 4-cell stage and is essential for blastocyst formation in mice (Pantaleon *et al.*, 1997; Pantaleon and Kaye, 1998; Pantaleon *et al.*, 2001). GLUT8 has been detected in pre-implantation mouse blastocysts and shown to be responsible for increased insulin-mediated glucose uptake and is believed to play a critical role in embryo survival (Carayannopoulos *et al.*, 2000; Pinto *et al.*, 2002a). Within bovine embryos GLUT1, 3 and 8 are present from the earliest stages of development, whereas GLUT2 and 4 are present only from the blastocyst stage onwards (Santos *et al.*, 2000; Augustin *et al.*, 2001).

As mentioned previously, glucose function changes from being inhibitory in the early stages of development to stimulatory during the latter stages of development, following genome activation (Gardner and Leese, 1990; Rieger *et al.*, 1992; Gardner *et al.*, 1993; Lim *et al.*, 1999; Martin and Leese, 1999; Pantaleon *et al.*, 2001). Most studies report that supplementation of embryo culture media with glucose has a stimulatory effect on blastocyst formation *in vitro*, both in presence and absence of serum (Kim *et al.*, 1993; Gomez and Diez, 2000). It is believed that glucose is not only required for energy production, but also for the synthesis of triacylglycerols and phospholipids and to provide precursors for complex sugars (Leese, 1991). In contrast, others report that high glucose concentrations may impair oocyte development and subsequent embryo development (Furnus *et al.*, 1997), possibly by increasing levels of reactive oxygen species (ROS) and reducing the content of glutathione within oocytes (Hashimoto *et al.*, 2000; Boland *et al.*, 2001). However, according to Biggers *et al.* (1997) negative effects of high glucose concentrations on blastocyst formation can be counteracted by addition of amino acids.

Some interesting studies with hyperglycemic mice demonstrated that high glucose levels can be responsible for decreased glucose uptake by pre-implantation embryos cultured *in vitro*. This coincides with down-regulation of facilitative glucose transporters GLUT1, 2 and 3 (Moley *et al.*, 1998a; Moley and Mueckler, 2000). The mechanisms for this down-regulation are not fully understood but are thought to involve a complex series of interactions with the IGF system. In addition, decrease in glucose transport has been associated with increased incidence of apoptosis within the ICM and TE cells which can result in foetal deformations and/or miscarriages (Moley *et al.*, 1998a, b; Chi *et al.*, 2000a; Moley and Mueckler, 2000).

1.4.2. Amino acid uptake and metabolism

Amino acids along with carbohydrates are essential for the oocyte and later for embryo development. Apart from being a major source of energy (Lane and Gardner, 1998; Houghton *et al.*, 2002), amino acids also act as osmolytes (Van Winkle and Campione, 1996; Dawson *et al.*, 1998), pH regulators (Edwards *et al.*, 1998; Lane, 2001), protectors of cell viability (Jung *et al.*, 1998; Lane, 2001) and substrates for proteins and nucleic acid synthesis (Gardner, 1998; Jung *et al.*, 1998).

Amino acids added to the maturation medium can enhance cytoplasmic maturation of oocytes *in vitro* (Lim *et al.*, 1999) and can stimulate attachment, outgrowth and hatching rate of mouse blastocysts (Gardner, 1998). Moreover, as reported by Lane (2001) addition of non-esssential amino acids protects developing embryos *in vitro* from pH, osmotic and metabolic stress.

In general amino acids are known to have a positive effect on embryo development *in vitro* in different species (rat: Zhang and Armstrong, 1990; cow: Moore and Bondioli, 1993; Lee and Fukui, 1996; Lim and Hansel, 2000; mouse: Van Winkle and Campione, 1996; Gardner, 1998; human: Devreker and Englert, 2000; Van Winkle, 2001). It is also known that pre-implantation embryos possess carriers of specific amino acids which maintain their endogenous pool (Gardner, 1998). However, excessive concentrations of essential amino acids have been reported to have a negative effect on bovine embryo development, indicated by reduced cleavage rates. These findings suggest that requirements for amino acids alter during embryo development, resembling conditions *in vivo* and the switch from maternal to embryonic regulation (Steeves and Gardner, 1999). Recent investigations indicate that embryos cultured *in vitro* use most of the amino acids available to them, with the exception of alanine which in turn is produced by the embryo and released into the culture medium (Lee and Fukui, 1996; Donnay and Leese, 1999; Houghton *et al.*, 2002; Kuran *et al.*, 2002). However, some studies suggest that amino acid metabolism can be altered by the addition of different protein supplements which can compromise embryo development *in vitro* (Orsi and Leese, 2004).

1.4.3. Fatty acid uptake and metabolism

Fatty acids have been the most neglected group of nutrients studied to date and only recently they have been the subject of more detailed investigations. It is now known that fatty acid concentrations decrease with increasing follicular size. However, there is no information on lipid function during oocyte maturation (Sutton *et al.*, 2003). The only available reports focus on quantitive lipid content within oocytes, including phospholipids, cholesterol, triglycerides and FFA. Within the follicle, lipids in the form of triglyceride constitute a source of energy to the developing oocyte; the content prior to maturation has been estimated to be around 59 ng. During maturation this level

decreases to around 34 ng (Ferguson and Leese, 1999). Triglycerides metabolised into fatty acids are oxidised via the Krebs cycle and this can be stimulated by growth factors such as IGF-I, EGF and insulin (Ferguson and Leese, 1999). This indicates that lipids are used as an energy source for protein synthesis, which is essential for meiosis and cytoplasmic maturation (Sturmey and Leese, 2003).

The fatty acid content of follicular fluid is dependent on follicular diameter, with linoleic acid being the most abundant fatty acid. This fatty acid can significantly inhibit oocyte nuclear maturation *in vitro* (Homa and Brown, 1992). Within immature oocytes, saturated fatty acids are most abundant, comprising 45 – 55% of total fatty acids together with mono- (27 – 34%) and polyunsaturated (11 – 21%) fatty acids (McEvoy *et al.*, 2000). Palmitic, oleic and stearic acids are the most abundant fatty acids within immature cattle, sheep and pig oocytes, with remaining fatty acids not exceeding 5% of total lipid content (McEvoy *et al.*, 2000; Kim *et al.*, 2001b). The total lipid content of oocytes comprise 25 – 29% of phospholipids and 28 – 46% of triglycerides, depending on the species studied (McEvoy *et al.*, 2000).

Fatty acid composition is an important indicator of oocyte quality in cryopreservation studies (Kim *et al.*, 2001b; Zeron *et al.*, 2001). Studies *in vitro* indicate that inclusion of NEFA can delay oocyte maturation and impede subsequent fertilisation and embryo development (Jorritsma *et al.*, 2004). Addition of serum, a widely used supplement known to enhance oocyte and embryo development *in vitro*, can increase the total lipid content and can alter fatty acid composition of the embryo (Sata *et al.*, 1999; Reis *et al.*, 2003). This indicates that results obtained during culture with serum supplements should be interpreted with caution.

1.5. Metabolic hormones and reproduction

1.5.1. Insulin

Insulin is a pancreatic peptide hormone produced by the β-cells of the islets of Langerhans and its release is stimulated by postprandial increase in plasma glucose concentrations. The main target organs for insulin action are muscle, adipose tissue, and liver (Kaye, 1997; Poretsky *et al.*, 1999). Insulin plays a major role in the regulation

of carbohydrate, lipid and protein metabolism (White and Kahn, 1994; Berg *et al.*, 2002), but it also promotes a number of other cellular events including the regulation of ion and amino acid transport, glycogen synthesis (Navarro *et al.*, 1999), gene transcription and mRNA turnover, protein synthesis and degradation, and DNA synthesis (Cheatham and Kahn, 1995).

Insulin binding to its own receptor (Ins-R), a dimeric tyrosine kinase receptor (RTK) consisting of two α- and two β-subunits, initiates the cascade of its growth promoting and metabolic actions (White and Kahn, 1994; Cheatham and Kahn, 1995; Navarro *et al.*, 1999; Poretsky *et al.*, 1999; Lodish *et al.*, 2000), followed by internalisation of the receptor itself (Guglielmo *et al.*, 1998).

There are two recognised pathways of insulin action:

(i) Ras-independent pathway – involving phosphoinositides leading to activation of protein kinase B (PKB); PKB/Akt is believed to induce survival signals and a number of other metabolic effects of insulin (Downward, 1998).

(ii) Ras-dependent pathway – leading to activation of mitogen-activated protein kinase (MAPK), responsible for the metabolic and growth-promoting effects of insulin (Scrimgeour *et al.*, 1997; Navarro *et al.*, 1999; Lodish *et al.*, 2000; Zick, 2001).

Insulin signalling pathways are initiated by auto-phosphorylation of the tyrosine residues within β-subunits of the receptor followed by the translocation of PI-3-kinase to the plasma membrane (Lizcano and Alessi, 2002) and interaction with other signalling molecules such as insulin receptor substrates (IRS 1 – 4), which occur via regions known as *src* homology-2 (SH2) domains (White and Kahn, 1994; Cheatham and Kahn, 1995; Ogawa *et al.*, 1998; Kido *et al.*, 2001). Insulin receptor substrates-1 and -2 are the main mediators of insulin effects on the regulation of carbohydrate and lipid metabolism *in vivo*, where IRS-1 is more effective in muscle cells alone and IRS-2 in liver, muscle and adipose tissue (Previs *et al.*, 2000). The best studied substrate and the one essential for nearly all of the insulin biological responses is IRS-1 (White and Kahn, 1994), which binds PI-3-kinase involved in the initiation of glucose transport (Scrimgeour *et al.*, 1997; Ogawa *et al.*, 1998; Poretsky *et al.*, 1999; Zick, 2001), amino acid transport, mitogenesis and glycogen synthesis (Scrimgeour *et al.*, 1997) (Figure 1.10).

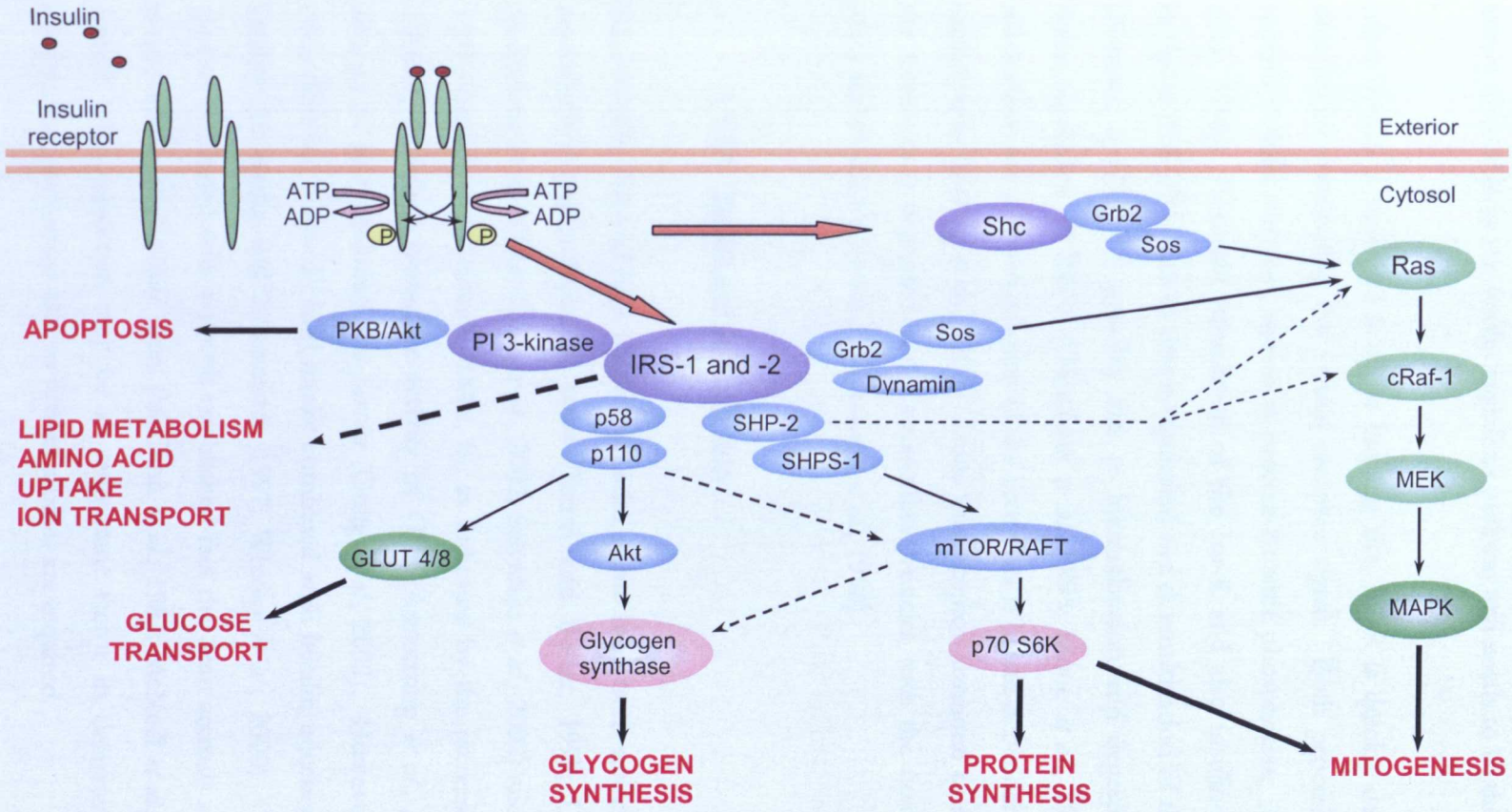

Figure 1.10. Insulin signalling pathway. Adapted from Ogawa *et al.* (1998) and Kido *et al.* (2001). ATP – Adenosine triphosphate; ADP – Adenosine diphosphate; Akt – a product of the akt protooncogene; P – phosphorylation; IRS – insulin receptor substrate; MEK - a dual-specificity tyrosine/ threonine kinase; mTOR – mammalian target of rapamycin; p58 – subunit of PI-3-kinase, p110 – catalytic PI-3-kinase subunit; PKB – protein kinase B; Ras – a low-molecular-weight, monomeric G protein; Raf – a serine/ threonine kinase; Grb2, SHP-2 – adaptor proteins; Sos – son-of-sevenless, exchange factor; Shc – adaptor protein.

Although the function of IRS-2 is not well understood, it has been demonstrated that female mice lacking IRS-2 are infertile (Burks *et al.*, 2000). Even though IRS-3 (Lavan *et al.*, 1997a) and IRS-4 (Lavan *et al.*1997b) have been identified as members of the IRS family, their role in the insulin signalling pathway still needs to be determined.

After insulin is removed from its binding site, Ins-R is quickly dephosphorylated and deactivated terminating the insulin receptor signal. Both processes are catalysed by specific cellular enzymes known as protein-tyrosine phosphatases (PTPases) (Goldstein *et al.*, 1998). Constant stimulation of the Ins-R and also insulin-like growth factor-I receptor (IGF-IR) leads to down-regulation and desensitization of their action pathways (Navarro *et al.*, 1999) possibly due to internalization and degradation of Ins-R and down-regulation of IRS-1 (Cheatham *et al.*, 1993; Inoue *et al.*, 1996). The principal mechanism for down-regulation of the receptors is ligand-dependent receptor-mediated endocytosis (Lodish *et al.*, 2000). Once the receptor-hormone complex is internalised, the hormone is degraded in the endocytotic vesicles with the final degradation taking place within the lysosomes (Duckworth *et al.*, 1998).

1.5.1.1. Insulin and reproduction

Nutritionally induced high levels of insulin *in vivo* have been associated with improved reproductive performance in cows (Beam and Butler, 1999; Landau *et al.*, 2000; Armstrong *et al.* 2002a; Gong *et al.*, 2002; Selvaraju *et al.*, 2002) and pigs (Whitley *et al.*, 1998; Prunier and Quesnel, 2000a, b), as indicated by the increased number of small follicles, increased aromatase activity of GCs (Armstrong *et al.*, 2002a) and reduced interval to first ovulation *post partum* (Gong *et al.*, 2002). However, in well-nourished ewes different levels of food intake combined with insulin injections failed to improve fertility (Downing and Scaramuzzi, 1997; Whitley *et al.*, 2000). These contradictory reports combined with the well established fact that lean animals are more sensitive to insulin action than obese ones (McCann *et al.*, 1987; McNeill *et al.*, 1997), suggest that animal body condition may be an important factor in determining insulin-induced effects on reproduction and so further studies are required.

On the other hand, numerous experiments *in vitro* demonstrated that insulin alone or in synergy with gonadotrophins stimulates follicular development *in vivo* (e.g. Yu *et al.*,

1999), but most of all enhances proliferation and production of androstenedione, oestradiol and progesterone by granulosa and thecal cells from different species (Spicer and Echternkamp, 1995; Poretsky *et al.*, 1999; cow: Gong *et al.*, 1993, 1994; Spicer and Stewart *et al.*, 1996; Spicer, 1998; Silva and Price, 2002; Spicer *et al.*, 2002; human: Willis *et al.*, 1996; Devoto *et al.*, 1999; Greisen *et al.*, 2001; pig: Sekar *et al.*, 2000). Furthermore, treatment with insulin and IGF-I has been reported to promote StAR mRNA and gene expression in human (Devoto *et al.*, 1999), bovine (Mamluk *et al.*, 1999) and porcine granulosa luteal cells (Sekar *et al.*, 2000). However, in the presence of high doses of insulin, FSH can have a negative effect on steroidogenesis indicated by reduced aromatase activity of bovine GCs (Bhatia and Price, 2001). Moreover, studies conducted on mice showed that addition of FSH or insulin did not improve oocyte developmental competence and more strikingly FSH and insulin together had a deleterious effect on oocyte quality (Eppig *et al.*, 1998) and blastocyst development *in vitro*, indicated by altered protein synthesis (Latham *et al.*, 1999).

During embryo development, insulin acts as a mitogenic and anti-apoptotic factor (Herrler *et al.*, 1998; Augustin *et al.*, 2003), stimulating mouse (Harvey and Kaye, 1992; Kaye *et al.*, 1992; Kaye and Gardner, 1999), rat (Zhang and Armstrong, 1990), rabbit (Herrler *et al.*, 1998), cow (Matsui *et al.*, 1995a, b; 1997; Augustin *et al.*, 2003) and pig (Lewis *et al.*, 1992; Koo *et al.*, 1997) pre-implantation embryo growth *in vitro*, possibly acting via IGF-IR (Matsui *et al.*, 1997). It should be emphasised that almost all of the studies *in vitro* apply super-physiological concentrations of insulin and hence comparisons between insulin-induced effects *in vivo* and *in vitro* are very difficult to interpret.

1.5.2. Insulin-like growth factors

Insulin-like growth factors-I and -II (IGF-I and -II) are single-chain polypeptides, sharing 70% sequence homology with each other and 50% with pro-insulin (Thissen *et al.*, 1994; Poretsky *et al.*, 1999). The liver is the major source of circulating IGF-I, which acts as an endocrine factor and is essential for normal postnatal growth (Yakar *et al.*, 1999). Insulin-like growth factors are also produced by other body tissues including the ovary (Spicer and Echternkamp, 1995). Within the ovary, mainly luteinized granulosa cells (Guttierez *et al.*, 1997c; Poretsky *et al.*, 1999; Spicer and Chamberlain, 2000), but

also thecal and luteal cells produce IGFs. These act as the intraovarian autocrine and paracrine factors (Spicer and Echternkamp, 1995; Mazerbourg *et al.*, 2003).

Dietary energy and protein intake play a critical role in the regulation of IGF-I concentrations in plasma. Fasting significantly reduces circulating levels of IGF-I which can be restored by optimal dietary intake (Thissen *et al.*, 1994). In addition, IGF-I acting on the hypothalamo-pituitary-ovarian axis stimulates GnRH-induced LH secretion and, by interacting with oestradiol, increases GnRH responsiveness (Hashizume *et al.*, 2002).

In contrast to insulin, IGFs act mainly as mitogens, even though 50% of their receptor structure is identical with Ins-R (Prager and Melmed, 1993). Similarly to Ins-R, IGF-IR consists of two α- and two β-subunits, where activation results in tyrosine phosphorylation of β-subunits followed by interaction with the PI-3-kinase and activation of MAPK (LeRoith *et al.*, 1995; Scrimgeour *et al.*, 1997; Poretsky *et al.*, 1999; Lodish *et al.*, 2000; Mazerbourg *et al.*, 2003). The mitogenic effects of IGF-I can involve more than one signalling pathway which are distinct from the MAPK and PI-3-kinase signalling pathways (Scrimgeour *et al.*, 1997). Nevertheless, inhibition of PI-3 and MAPK pathways results in decreased cell proliferation, accompanied by an increase in apoptosis (Scrimgeour *et al.*, 1997). The IGF-IR can also bind IGF-II and insulin, but with lower affinity (Thissen *et al.*, 1994; Matsui *et al.*, 1997; Poretsky *et al.*, 1999). Whereas, the type II IGF receptor (IGF-IIR) is identical to mannose-6-phosphate receptor, which main function is internalization and degradation of IGF-II, although it is not clear if it initiates IGF-II signalling (Poretsky *et al.*, 1999). Most of metabolic and growth-promoting actions of IGF-II are mediated via IGF-IR (Willis *et al.*, 1998; Poretsky *et al.*, 1999) or Ins-R (Morrione *et al.*, 1997; Frasca *et al.*, 1999; Poretsky *et al.*, 1999; Louhio *et al.*, 2000). Both InsR and IGF-IR are present in GCs and TCs (Spicer *et al.*, 1994).

In addition, actions of IGF-I and -II are controlled by a group of IGF binding proteins (IGFBP 1-6) (Thissen *et al.*, 1994; Hwa *et al.*, 1999), carried by blood or synthesized locally within the follicle (Funston *et al.*, 1996). The main function of IGFBPs is to inhibit IGF action by limiting their bioavailability in circulation and hence reducing binding with their receptors (Poretsky *et al.*, 1999; Mazerbourg *et al.*, 2003). Insulin and IGFs can also inhibit IGFBP-1 production by GCs, with IGFs reported to be more

potent inhibitors than insulin (Poretsky *et al.*, 1996a, b). During follicular development, intrafollicular levels of IGF-I (Spicer and Echternkamp, 1995; Poretsky *et al.*, 1999) and IGFBPs (De la Sota *et al.*, 1996; Funston *et al.*, 1996; Stewart *et al.*, 1996) increase with increasing concentrations of oestradiol and follicular diameter. The granulosa and thecal cells are the main source of intrafollicular IGFBP-2, -4 and -5 (Chamberlain and Spicer, 2001) and their concentrations increase during progression from subordinate to the dominant follicle stage. In contrast, IGFBP-3 is mainly synthesised by TCs (Chamberlain and Spicer, 2001) and its levels remain unchanged throughout follicular growth (de al Sota *et al.*, 1996; Funston *et al.*, 1996; Stewart *et al.*, 1996).

1.5.2.1. Insulin-like growth factors and reproduction

As reported by Armstrong *et al* (2002b) IGFs are not present in preantral follicles, only mRNA encoding IGF-IR and IGFBP-2 and -3 have been detected. Absence of endogenous IGF-I and –II mRNA expression indicates an endocrine role for IGFs in follicular development (Armstrong *et al.*, 2000). Perks *et al.* (1999) reported that IGF-II is produced locally and is the main intrafollicular IGF ligand, regulating bovine antral follicle development, with IGF-I mostly derived from the circulation. In contrast, others demonstrated that IGF-IR does not fully develop until the antral stages of follicular growth, indicating a more paracrine than autocrine function of IGF-I (Funston *et al.*, 1996; Guttierez *et al.*, 2000).

Analogous to insulin, IGFs regulate ovarian (Armstrong and Webb, 1997; Adashi, 1998; Schams *et al.*, 1999; Lucy, 2000) and luteal function (Woad *et al.*, 2000) in the bovine (Spicer and Echternkamp, 1995; Glister *et al.*, 2001; Silva and Price, 2002; Spicer *et al.*, 2002), porcine (Sekar *et al.*, 2000; Shores *et al.*, 2000), ovine (Monniaux and Pisselet, 1992; Monniaux *et al.*, 1994), human (Wang and Chard, 1999; Giudice, 2001), murine (Zhou *et al.*, 1997) and rat (Adashi *et al.*, 1988; Bley *et al.*, 1992; Adashi, 1995; Khamsi and Roberge, 2001). They achieve this by increasing proliferation and gonadotrophin-stimulated steroidogenesis of follicular and luteal cells. In contrast, McCaffery *et al.* (2000) reported that IGF-I had a negative effect on oocyte growth and granulosa cell proliferation. Insulin-like growth factor II has been reported to stimulate progesterone biosynthesis, by acting on P450scc (Garmey *et al.*, 1993) and, as reported by Mason *et al.* (1994), IGF-II in synergy with insulin can enhance oestradiol synthesis by human GCs.

Furthermore, IGF-I and -II can enhance FSH-stimulated cumulus expansion (Singh and Armstrong, 1997) and induce nuclear maturation of pig (Sirotkin *et al.*, 1998, 2000), but not sheep (Guler *et al.*, 2000) and mouse (Demeestere *et al.*, 2004) oocytes. However, supplementation of oocyte maturation medium with IGF-I can enhance subsequent blastocyst development *in vitro* and increase total cell numbers (Demeestere *et al.*, 2004). Both IGF-I and –II are necessary for normal embryo development (Allan *et al.*, 2001) and mRNA encoding Ins-R, IGF-I and –II and their receptors can be detected at all stages of embryo development (Kaye, 1997).

Numerous studies have demonstrated that IGF-I enhances embryo development (Watson *et al.*, 1999) by stimulating cell proliferation and reducing apoptosis in mouse (Harvey and Kaye, 1992; Kaye *et al.*, 1992; Kaye and Gardner, 1999; Lin *et al.*, 2003; Fabian *et al.*, 2004), rabbit (Herrler *et al.*, 1998), human (Spanos *et al.*, 2000) and bovine (Makarevich and Markkula, 2002; Sirisathien and Brackett, 2003) blastocysts. The fact that embryos from the 2-cell stage onwards are capable of producing IGF-I (Carayannopoulos *et al.*, 2000), IGF-II, IGF-IIR and IGF-IR, suggests a predominantly autocrine mechanism of their action (Yaseen *et al.*, 2001).

1.5.3. Apoptosis – insulin and IGFs effects

Apoptosis is a self-programmed cell death mechanism that characteristically affects single cells and is required for the normal development and maintenance of tissue homeostasis. Apoptotic signalling pathways involve activation of Bax, Bcl-2, apoptotic protease activating factor 1 (Apaf-1) and caspase family members, where activation is controlled by a release of cytochrome *c* from the mitochondria (Desagher and Martinou, 2000). Cells affected by apoptosis morphologically shrink, become denser with cytoplasmic and chromatin condensation, and DNA fragmentation, organising into discrete masses against the nuclear membrane. The final phases of the apoptotic process are characterised by formation of so called apoptotic bodies containing pyknotic nuclear fragments and other cellular organelles (Hardy, 1999; Van Wezel *et al.*, 1999; Betts and King, 2001). These apoptotic bodies may be either phagocytised by macrophages of neighbouring cells or extruded into the lumen (Betts and King, 2001).

Within the ovary apoptosis is responsible for controlled deletion of ovarian cells during follicular atresia (Tilly, 1996; Yang and Rajamahendran, 2000) and degeneration of an old CL in each oestrous cycle, maintaining normal cyclicity (Amsterdam and Selvaraj, 1997; Amsterdam *et al.*, 1999). During early embryo development cell death can eliminate abnormal cells (Hardy, 1999). However, above a certain threshold apoptosis can have a detrimental effect on subsequent embryo development (Jurisicova *et al.*, 1998; Hardy, 1999). Apoptosis may be inhibited by maternal survival factors that are present in abundance *in vivo* or due to the absence of environmental upstream induction proteins (Betts and King, 2001). The incidence of apoptosis can also be an indicator of embryo quality (Makarevich and Markkula, 2002), since blastocysts with a higher number of cells are more likely to implant and give rise to a live offspring (Van Soom *et al.*, 1997). Moreover, blastocysts produced *in vitro* are of lower quality and viability compared to their *in vivo* counterparts, indicated by lower cell numbers and higher incidences of DNA fragmentation (Jurisicova *et al.*, 1998; Byrne *et al.*, 1999; Hardy, 1999).

Insulin and IGFs are recognised survival factors, preventing follicular (Luciano *et al.*, 2000) and embryonic cells from apoptosis (Chun *et al.*, 1994; Butt *et al.*, 1999; Louhio *et al.*, 2000; Byrne *et al.*, 2002; Makarevich and Markkula, 2002). The anti-apoptotic effects of these growth factors are probably mediated by the IGF-IR (Butt *et al.*, 1999), and IGF-I is believed to have a stronger anti-apoptotic effect than insulin (Markström *et al.*, 2002), possibly because the activation of IGF-IR initiates multi-apoptotic pathways (Peruzzi *et al.*, 1999). However, high concentrations of insulin and IGF-I can trigger apoptosis, probably by down-regulating the IGF-IR (Chi *et al.*, 2000b). Moreover, high concentrations of IGF-I *in vivo* and *in vitro* have been associated with increased embryo resorption rates in mice (Pinto *et al.*, 2002b).

1.5.4. Leptin

Leptin, a peptide hormone from the cytokine family, is a product of the obese gene and is synthesised by adipose tissue. It can regulate food intake and body weight (Cohen *et al.*, 1996; Mantzoros and Moschos, 1998; Keisler *et al.*, 1999; González *et al.*, 2000; Smith *et al.*, 2002; Chelikani *et al.*, 2004). Once released into the blood stream, leptin binds to a family of binding proteins (Hossner, 1998; González *et al.*, 2000; Ahima and Flier, 2000).

In most mammalian species, leptin is synthesised according to overall adiposity (Friedman and Halaas, 1998; Ahima and Flier, 2000; Delavaud *et al.*, 2000; Ehrhardt *et al.*, 2000). By acting on the central nervous system it reduces voluntary feed intake (Friedman and Halaas, 1998; Ahima and Flier, 2000). In addition, it has been proposed that leptin controls the homeostatic system for intracellular storage of triglycerides in adipocytes and protects non-adipocytes from lipotoxicity (Unger *et al.*, 1999).

Leptin and its receptor were first detected in the brain and hypothalamus and most recently their presence has been reported within the ovary, mature oocyte and also during early stage embryos (González *et al.*, 2000).

There are two major forms of the leptin receptor (OB-R):

(i) the short form (OB-RS) detected in many peripheral organs such as the liver, pancreas, skeletal muscle. It is capable of activating the MAPK signalling pathway;

(ii) the long form (OB-RL) detected mainly in the hypothalamus and in lower amounts in other tissues. It activates the signal transducers and activators of transcription (STAT) proteins (Hossner, 1998; Keisler *et al.*, 1999; González *et al.*, 2000; Smith *et al.*, 2002; Hegyi *et al.*, 2004).

Leptin concentrations within follicular fluid are similar to those observed in serum and are independent of follicular size (Zhao *et al.*, 1998; Agarwal *et al.*, 1999). In dairy cattle plasma leptin can be reduced by short term fasting, but returns to normal levels after refeeding (Chelikani *et al.*, 2004). In humans, high circulating concentrations of leptin are associated with adiposity and reduced ovarian responsiveness to gonadotrophins which can result in impaired fertility (Bützow *et al.*, 1999). Nutritional regulation of leptin expression in humans is possibly mediated by insulin, where peak insulin concentrations are followed by an increase in leptin expression. In the presence of low insulin concentrations leptin levels are reduced (Ahima and Flier, 2000). However, in sheep plasma levels of leptin are not related to changes in glucose and insulin concentrations (Kauter *et al.*, 2000).

Most recent reports suggest that leptin may provide a possible link between nutrition and reproduction (Barash *et al.*, 1996; Clarke and Henry, 1999; Cunningham *et al.*, 1999;

Keisler *et al.*, 1999; González *et al.*, 2000; Boland *et al.*, 2001; Smith *et al.*, 2002). Studies in mice have shown that leptin can accelerate the onset of puberty indicated by vaginal opening, fertility, oestrous cyclicity and increased secretion of GnRH and LH (Chehab *et al.*, 1997; Chehab, 2000). However, in rats leptin reduces the number of ovulations (Duggal *et al.*, 2000).

A number of *in vitro* experiments have reported that leptin has a direct inhibitory effect on insulin- (cow: Spicer and Francisco, 1997, 1998; human: Brannian *et al.*, 1999) and IGF-I-induced (rat: Zachow and Magoffin, 1997; human: Agarwal *et al.*, 1999) progesterone, oestradiol and androstenedione production by granulosa and thecal cells. Most recent studies conducted in mice revealed that leptin can induce meiotic maturation in preovulatory follicle-enclosed oocytes (Ryan *et al.*, 2002) and that it can promote pre-implantation embryo development indicated by an increased number of cells mainly within the trophectoderm (Kawamura *et al.*, 2002).

1.6. Working hypothesis and experimental objectives

From the foregoing discussion one can hypothesise that nutrition has a major influence on ovarian function by affecting not only the hypothalamo-ovarian axis but also by regulating the intra-follicular environment. Alterations in dietary composition can induce changes in hormonal secretion, including metabolic hormones such as insulin, insulin-like growth factors and leptin, which regulate growth factor activity of follicular cells and, in turn, may improve oocyte developmental competence (Figure 1.11). Furthermore, mitogenic and anti-apoptotic actions of insulin and IGFs may enhance embryo development, thereby leading to the successful establishment of pregnancy. Studies to date, however, report mainly on the effects of nutrition at systemic (endocrine) and/or follicular levels, and it remains to be determined to what extent dietary composition can directly influence the oocyte. The main objective of this project, therefore, was to investigate the effects of alterations to dietary energy intake and diet composition on oocyte quality and subsequent embryo development *in vitro*.

Body condition score (BCS) of dairy cows (and other animals) is an indicator of animal body fatness. Fluctuations in body fatness and inadequate dietary intake during

lactation can affect the resumption of oestrous cycles and reproductive performance during the post-partum period (e.g. Short *et al.*, 1990; O'Callaghan and Boland, 1999; Roche *et al.*, 2000). In non-lactating animals, whilst high energy and protein diets can increase ovulation rates in ewes (Downing *et al.*, 1995), and follicle numbers in cattle (Nolan *et al.*, 1998; Armstrong *et al.*, 2001), they may also impair oocyte viability (McEvoy *et al.*, 1995; Armstrong *et al.*, 2001). The results are inconsistent between studies, however, and in most cases experimental animals were in moderately high BCS. The objective of the first study (Chapter 2), therefore, was to determine the effects of animal BCS and feeding level, and simultaneously the influence of low, moderate and high plasma insulin concentrations on oocyte quality and subsequent embryo development.

Dietary carbohydrates and lipids not only can counteract energy losses during early lactation, but may also influence oocyte developmental competence and embryo survival. However, there is little information on the direct effects of carbohydrates and lipids on ovarian function and oocyte quality. The next step, therefore, was to investigate the impact of dietary carbohydrates (fibre vs starch) and lipids (calcium soaps of palm oil fatty acids) on oocyte quality, again in heifers of either Low or Moderate BCS (Chapter 3). Chapter 4 extended these investigations to the final stages of oocyte maturation and early embryo development. To achieve this, sera from oocyte donors from Chapter 3 were used as media supplements during oocyte maturation and post-fertilisation development *in vitro*. Because, there is a dearth of information on the importance of fatty acids during oocyte development and their possible role in determining oocyte quality, so, Chapter 5 focused on the alteration in the lipid content and the fatty acid composition of plasma and follicular cells induced by the dietary treatments offered in Chapter 3.

The outcomes of this project should provide a novel insight to the nutritional regulation of oocyte and early embryo development in cattle, and produce dietary treatments which improve fertility in cattle.

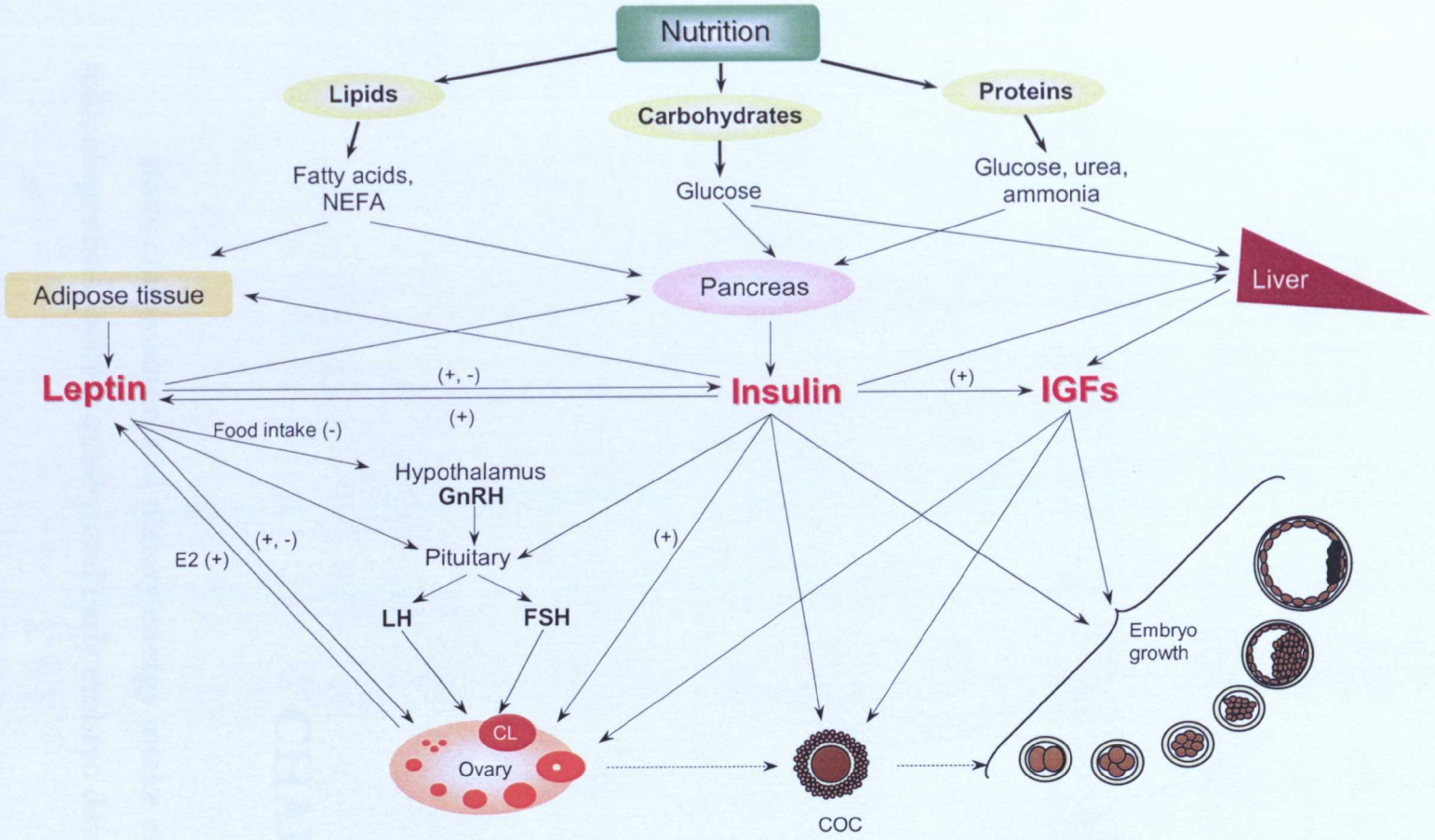

Figure 1.11. Schematic representation of nutritionally induced alterations on reproduction via the metabolic hormones insulin, leptin and IGFs.

CHAPTER 2

Body composition and dietary energy intake affect ovarian folliculogenesis, oocyte quality and early embryo development in cattle.

2.1. Introduction

Nutrition has a profound influence on reproductive performance in ruminants and other species, but the timing and the mechanisms of its action are still poorly understood. In dairy cows, inadequate dietary intake is believed to be one of the factors contributing to the decline in fertility (Robinson, 1996; Beam and Butler, 1999; Butler, 2000, Boland *et al.*, 2001). Early lactation is a critical period correlated with NEBAL, associated with a number of hormonal changes including increased GH secretion and reduced plasma concentrations of insulin, leptin and IGF-I (McGuire *et al.*, 1995; Yung *et al.*, 1996; Olsson *et al.*, 1998; Prunier and Quesnel, 2000b; Block *et al.*, 2001; Walters *et al.*, 2002; Holtenius *et al.*, 2003; Lucy, 2003) which may result in impaired DF growth and ovulation (Mackey *et al.*, 2000). However, interactions between nutritional status and reproduction are complex and often variable, and conflicting responses are difficult to interpret.

Recent investigations have shown that high dietary energy intakes can increase concentrations of plasma insulin and IGF-I, which are associated with increased numbers of small (<4 mm) and medium (4 – 8 mm) sized follicles (Gutierrez *et al.*, 1997a), enhanced aromatase activity of GCs within small follicles (Armstrong *et al.*, 2002a), increased dominant follicle growth rates (Armstrong *et al.*, 2001) and reduced intervals to first ovulation *post partum* (Gong *et al.*, 2002). Numerous studies conducted *in vitro* confirm that insulin and IGF-I and -II alone, acting via the Ins-R and/or IGF-IR, or in synergy with gonadotrophins, stimulate proliferation and steroidogenesis of bovine GCs and TCs (Gong *et al.*, 1993, 1994; Spicer and Stewart, 1996; Spicer, 1998; Silva and Price, 2002; Spicer *et al.*, 2002). According to Diskin *et al* (2003) the effects of undernutrition on ovarian function are induced through the hepatic regulation of IGF-I and alterations in plasma concentration of insulin, IGFs and their binding proteins. As a result, follicular responsiveness to LH is reduced and oestradiol secretion is inhibited.

Increased number of follicles induced by high energy diets does not necessarily coincide with improved oocyte developmental capacity (McEvoy *et al.*, 1995; Boland *et al.*, 2001) which may in fact be reduced (Armstrong *et al.*, 2001). Alterations in the systemic concentrations of metabolites (e.g. NEFA), metabolic hormones (e.g. insulin, leptin) and growth factors (e.g. IGF-I, IGFBPs) induced by modifying dietary intake and/or energy

status, are usually reflected in the composition of follicular fluid (Bützow *et al.*, 1999; Kendrick *et al.*, 1999; O'Callaghan *et al.*, 2000; Comin *et al.*, 2002; Walters *et al.*, 2002; Jorritsma *et al.*, 2003) which may in turn affect oocyte developmental competence. There is evidence that poor quality oocytes are less likely to become fertilised and to successfully complete blastocyst formation (Lonergan *et al.*, 2003a; Krisher, 2004), leading to increased early embryo mortality, which is recognised as the main cause of reproductive failure (Butler, 2000; Snijders *et al.*, 2000; Boland *et al.*, 2001). It has therefore been hypothesised that improvements in dietary composition and energy intake can enhance ovarian function by regulating the secretion of metabolic hormones and growth factors, thus enhancing oocyte developmental competence (Robinson, 1996; Beam and Butler, 1999; Kendrick *et al.*, 1999; Boland *et al.*, 2001; Miyoshi *et al.*, 2001; Armstrong *et al.*, 2002a), thereby leading to improved pregnancy rates. However, some studies report that, in the presence of high concentrations of insulin, FSH can reduce aromatase activity of bovine GCs (Bhatia and Price, 2001) and this may be detrimental to the oocyte and post-fertilisation development (Eppig *et al.*, 1998; Latham *et al.*, 1999). Hence, the aim of this experiment was to determine the effects of body condition and feeding level on systemic concentrations of insulin, IGF-I, and leptin and oocyte quality, as indicated by post-fertilisation development *in vitro*. Ultimately, the aim of this experiment was to establish the optimal peripheral concentrations of insulin for enhanced oocyte quality.

2.2. Materials and Methods

2.2.1. Animals and dietary treatments

Twenty four beef x dairy heifers of approximately 20 months of age and with an average weight (±SEM) of 432.7±14.6 kg were used in this experiment. All animals were accommodated in an individual pens on a slatted floor. Four months prior to the start of the experiment, the live weight and body condition score (BCS) of each heifer was recorded. Body condition was scored on the six-point scale of Lowman *et al.* (1976) where 0 = lean and 5 = obese. In order to create homogeneous groups of animals of both Low and Moderate BCS at the beginning of experimental period, heifers were allocated to one of four levels of feeding based on AFRC (1993) values for the energy requirements of cattle: (A) 350 kJ ME/day/$kg^{0.75}$, (B) 450 kJ ME/day/$kg^{0.75}$, (C) 700 kJ

ME/day/kg$^{0.75}$ and (D) 900 kJ ME/day/kg$^{0.75}$. Feeding levels A and B were respectively offered to 6 moderately fat (~3.5 units) and 6 lean heifers (~2.0 units) and were designed to create a homogeneous group of Low BCS heifers at the beginning of the experimental period. Feeding levels C and D were also respectively offered to 6 moderately fat and 6 lean heifers but these levels of feeding were designed to create a homogeneous group of Moderate BCS heifers at the beginning of the experimental period. Once this had been achieved, animals were allocated at random within each of the two groups to one of two levels of feeding, either M (500) or 2M (1000 kJ ME/kg Wt$^{0.75}$/day), resulting in a 2 x 2 factorial design, where the factors were Low vs Moderate BCS and M vs 2M level of feeding. Details of diet composition and the chemical analysis of the diets are presented in Table 2.1. The experiment was conducted over 3 successive oestrous cycles and repeated two months later (denoted as Periods 1 and 2). In order to reduce excessive gain of live weight throughout the study, animals were re-aligned to the same levels of BCS between the end of the first and beginning of the second periods of study. Furthermore, the levels of feeding within BCS group during Period 2 were changed from M to 2M and *vice versa.*

Table 2.1. Composition and chemical analysis of feeds.

(a) Ingredient g/kg	
Barley	180
Wheat	400
Maize grain	90
Sugar beet pulp	90
Maize gluten	100
Rapeseed meal	70
Molasses	60
Urea	10
Total	1000

(b) Chemical analysis ‡		**Concentrate**	**Straw**
Dry matter	g/kg	873	846
ME	MJ/kg DM	13.2	4.6
Crude protein	g/kg DM	163	30
NDF	g/kg DM	157	832
Starch	g/kg DM	456	11
AHEE	g/kg DM	43	14

‡DM – Dry matter; ME - Metabolisable energy; NDF – Neutral Detergent Fibre; AHEE – Acid Hydrolysed Ether Extract.

2.2.2. Oestrous synchronisation and follicular development

To establish a reference oestrus all heifers had their oestrous cycles synchronised using a 10-day progesterone-releasing device CIDR (controlled internal drug-release dispenser; SmithKline Beecham, Tadworth, Surrey, UK) containing 1.9 g of progesterone. Prostaglandin $F_{2\alpha}$ analogue (Luprositol 15 mg; Prosolvin, Intervet, Cambridge, UK) was administered intramuscularly 8 days after CIDR insertion. Heifers were predicted to be in oestrus two days after CIDR removal (Day 0 = Reference oestrus) (Figure 2.1).

Ovarian follicular growth was monitored daily for a six-day period leading up to oestrus using transrectal real-time ultrasonography (Aloka SSD-500V scanner, Japan). The total number of visible (≥ 2 mm) follicles and their diameters were recorded. Blood samples were collected daily, prior to the early morning meal, by jugular venipuncture during a six-day period leading up to oestrus and on the predicted day of oestrus into ethylenediaminetetraacetic acid (EDTA) treated vaccutainers. Additional blood samples were collected on the day before, the day of and the day after each ultrasound guided transvaginal follicular aspiration (ovum pick up, OPU) session, in order to confirm cyclicity and to establish endocrine profiles. Harvested plasma was stored at –20°C until analysis.

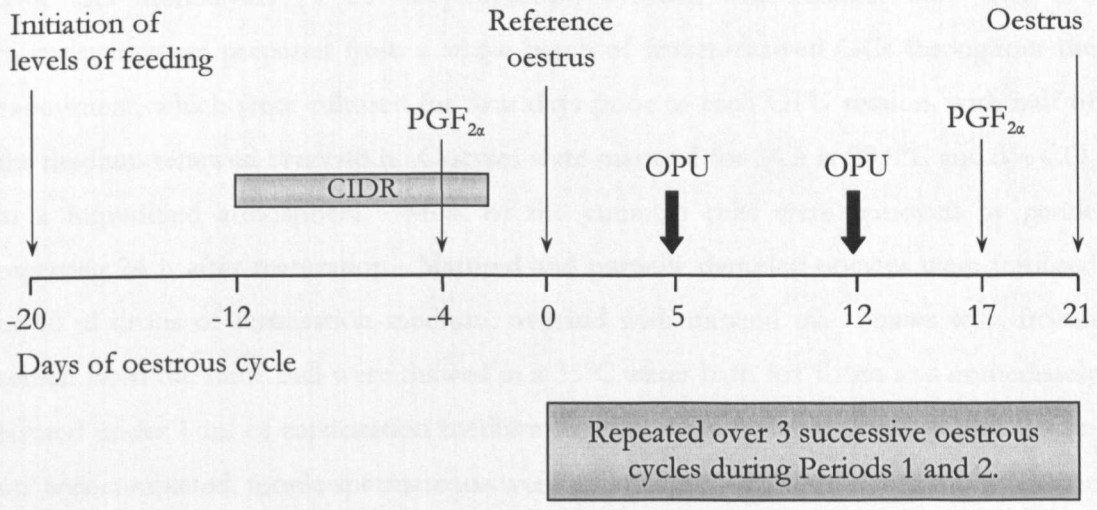

Figure 2.1. Graphic representation of the experimental protocol within a single period, indicating initiation of dietary treatments, outline of the oestrous synchronisation and OPU procedures (see text for details).

2.2.3. Oocyte collection and embryo culture *in vitro*

All reagents and media used in the experiment were obtained from Sigma Chemical Co. (Dorset, UK) unless otherwise stated (Appendix 5). Details of media composition and protocols of IVP procedures are included in Appendix 1. Animal identity was retained throughout. On Days 5 and 12 of each of three successive oestrous cycles (Figure 2.1), animals were subjected to OPU, as described by Goodhand *et al.* (1999) and Reis *et al.* (2002) (Appendix 1). In brief, prior to OPU each heifer was sedated and each received an epidural anaesthetic. An ultrasound probe equipped with a 5 MHz multi angle transducer (Aloka SSD-500V; BCF, Livingstone, UK) was inserted vaginally and both ovaries visualized via rectal manipulation. Prior to aspiration the number and diameter of all follicles were recorded. Subsequently, all visible follicles were punctured and follicular fluid aspirated into a conical tube containing 5 ml of warm PBS supplemented with 0.1% w/v bovine serum albumin (BSA) (Fraction V) and antibiotics, using a 19 gauge single lumen needle. Tubes containing aspirants were transported in a polystyrene box (kept at 37°C) to the laboratory where the aspirants were filtered through sterile 70 μm nylon strainer (BD Falcon, USA). The filtered media was then searched for COCs. Recovered COCs were graded according to the number of compact cumulus cell layers and granulation of the oocyte cytoplasm (Appendix 1). Only grade 1 – 3 COCs were selected for maturation. Selected COCs were washed twice in Maturation medium and transferred into 50 μl drops of Maturation medium with GC monolayers (≤ 20 oocytes/drop), overlaid with mineral oil. The GC monolayers were prepared from a single batch of frozen-thawed GCs throughout the experiment, which were cultured for four days prior to each OPU session, with half of the medium renewed every 48 h. Oocytes were matured for 24 h at 38.8°C and 5% CO_2 in a humidified atmosphere. Most of the cumulus cells were removed by gentle pipetting 24 h after maturation. Matured and partially denuded oocytes were fertilised in 46 μl drops of fertilisation medium, overlaid with mineral oil. Straws with frozen semen from the same bull were thawed in a 35°C water bath for 1 min and immediately layered under 1 ml of capacitation medium for the 'swim-up' procedure. Once 'swim-up' was completed, motile spermatozoa were counted and added in a maximum volume of 4 μl to each fertilisation drop, giving a final concentration of 10^6sperm/ml. At 20 – 22 h after fertilisation, presumptive zygotes were washed twice in synthetic oviductal fluid (SOF) (Tervit *et al.*, 1972) supplemented with 0.3% w/v BSA Fatty Acid Free

(FAF), 1% v/v non-essential amino acids and 2% v/v essential amino acids and antibiotics (SOF–). Zygotes were denuded from remaining cumulus cells and attached sperm by gentle pipetting before transfer into 20 µl drops of SOF– overlaid with mineral oil. Groups of up to 20 zygotes per drop were incubated at 38.8°C in 5% CO_2 and 5% O_2 in a humidified air, until Day 8 of development. Cleavage rates were recorded within 48 h of fertilisation. Embryos were transferred into fresh drops of SOF– every 48 h. Blastocyst yields were recorded on Day 8 of culture.

2.2.4. Total cell counts

Once assessed, Day 8 blastocysts were fixed individually in 1 ml of 3:1 acetic acid:ethanol solution per well and stored in a sealed 24-well plates in a cool room until total cell counts were conducted. On the day of cell counts, fixed blastocysts were stained with 10 µg/ml Hoechst 33342 solution (Appendix 2) and immediately visualised by fluorescence microscopy. The total number of cells per blastocyst was recorded.

2.2.5. Glucose tolerance test

This was conducted within 3 weeks of the end of Period 2 during an induced oestrus. Oestrus was induced on Day 12 of the oestrous cycle following administration (intramuscular) of Luprositol (15 mg) on Day 9. On the day prior to the glucose tolerance test, flexible catheters (18 gauge) were placed into the jugular vein of each heifer. The glucose tolerance test (GTT) commenced 5 h after the animal's morning meal with an intravenous infusion of sterile glucose solution (Glucose 40% w/v, Arnolds Veterinary Products, Ltd, Shrewsbury, UK) at 0.3 g/kg of body weight. Three blood samples collected at 20 minute intervals before glucose infusion, established glucose and insulin baselines. Glucose infusion was completed within 5 minutes and this was followed by the collection of blood samples at 5, 10, 20, 30, 40, 60, 80, 100 and 120 minutes post-infusion. All blood samples were collected into EDTA coated vacuum tubes and stored on ice until centrifugation (15 min at 600 x *g*). Plasma was transferred into auto-analyser cups and stored at –20°C until analysed. Plasma samples were analysed for glucose using a BMD/Hitachi 705 auto-analyser. The kit for glucose was supplied by Randox Laboratories Ltd (Glucose GOD PAP; Cat. No. GL2623). Plasma insulin concentrations were determined by RIA (Appendix 3).

2.2.6. Animal slaughter and granulosa cell culture

At the end of the experiment all heifers were slaughtered in a local abattoir and ovaries from each animal were recovered. On arrival at the laboratory ovaries were washed with warm (37°C) PBS followed by follicular aspiration of small and medium-sized follicles using a 21 gauge needle attached to a 5 ml syringe. Follicular fluid was transferred into a conical tube containing 1 ml of warm Search medium (Appendix 1) and centrifuged for 5 min at 300 x *g*. Harvested GCs from heifers within treatment were pooled together to obtain a sufficient number of cells for *in vitro* culture and were washed three times by centrifugation. The final cell pellet was resuspended in 1 ml of Search medium and a sample taken to establish cell number by trypan blue exclusion. Granulosa cells were then cultured in 24-well plates coated with collagen, at a seeding density of 4 x 10^5 live cells/ml in 500 µl of Cell Culture medium per well. This medium consisted of 90% v/v TCM199 and 10% v/v of double distilled water supplemented with 0.1% w/v BSA (Fraction V), 1 ng/ml FSH, 10 ng/ml of insulin, 500 ng/ml of testosterone, 0.5 µg/ml bovine transferrin, 5.0 ng/ml sodium selenite, 50 IU/ml penicillin, 50 µg/ml streptomycin, 20 ng/ml tetracycline and 2.5 µg/ml amphotericine. Granulosa cells were incubated for 96 h at 38.8°C and 5% CO_2 in a humidified atmosphere with 80% of the medium renewed at 48 h. Spent media collected at 48 h and 96 h was transferred into auto-analyser cups and stored at –20°C until analysis for progesterone and oestradiol. At the end of the incubation period, the number of live cells was assessed by trypan blue exclusion and cell viability was assessed by MTT assay (Appendix 1).

2.2.7. Hormone assays

Detailed protocols of all hormone radioimmunoassay (RIAs) are to be found in Appendix 3 but assay statistics for this experiment are presented here.

The minimum detection limit for the oestradiol assay at 85% binding (ED85) was 0.3 pg/ml. The inter-assay and intra-assay coefficients of variation for low, medium and high controls were 22.3%, 24.5% and 23.7%; and 15.0%, 10.8%, and 10.9%, respectively. The sensitivity of the progesterone assay at ED80 was 0.24 ng/ml. The inter-assay coefficients of variation for low, medium and high controls were 15.8%,

14.2% and 10.1%, respectively, and corresponding intra-assay coefficients of variation were 11.2%, 9.4% and 7.2%, respectively. The minimum detection limit for the plasma insulin assay (also at ED85) was 2.6 μIU/ml. The inter-assay coefficients of variation for low, medium and high controls were 8.2%, 9.0% and 10.0%, respectively and corresponding intra-assay coefficient of variation were 6.7%, 7.9% and 7.1%. All samples analysed for IGF-I concentrations were processed in a single assay with a detection limit of 12.5 ng/ml. The intra-assay coefficients of variation for low and high controls were 11.7% and 8.4%, respectively. The leptin assay was based on that of Blache *et al.* (2000). The detection limit for leptin assay was 0.2 ng/ml and the inter-assay coefficients of variation for low, medium and high controls were 13.0%, 9.7% and 10.5%, respectively. Corresponding intra-assay coefficients of variation were 12.8%, 9.9% and 10.3%, respectively.

2.2.8. Statistical analyses

Changes in animal live weight, body condition and plasma hormone concentrations during the three successive oestrous cycles were analysed by repeated measures ANOVA within GenStat 6 (Genstat, 2002), where the main effects of body condition (Low vs Moderate), level of feeding (M vs 2M), Period (1 vs 2), Week within Period (OPU sessions 1 to 6) and all interactions were tested. Residual variation was partitioned into between Animal variation, Period within Animal variation and Week within Period variation. The within Animal degrees of freedom were adjusted using the calculated Greenhouse-Geiser epsilon to account for non-independence of measurements between weeks in the same animal. The growth rate of the largest follicle during each of the six oestrous cycles was calculated for each animal by regressing follicle diameter against day, and then analysing the regression coefficients by ANOVA. Data presented are predicted means with either appropriate standard errors of differences (SED) or standard errors of means (SEM).

Oocyte recovery and *in vitro* embryo production data (i.e. oocytes cleaved and Day 8 blastocyst yields) were analysed using generalised linear models assuming binomial errors with logit link functions within GenStat (Genstat, 2002). Terms fitted to the model were Animal, Period, Body Condition, Level of Feeding and OPU session within Period. Comparisons between means were conducted by analysis of deviance. Data are

presented as predicted means from these models. Changes in blastocyst yields during the nine-week experimental period (i.e. for weeks 1, 2, 4, 5, 7 and 8 during which OPU was conducted) are presented as the change in logit proportions with time (t) expressed as weeks.

Glucose tolerance was calculated according to the method described by Opsomer *et al.* (1999) and was determined as fractional glucose turnover rate (k) and the half life ($T½$), starting at 5 min after the infusion (T5). The k value represents the clearance of infused glucose and it was calculated as:

$$k = ((\text{glucose T10} - \text{glucose T40})/(\text{T40} - \text{T10}))*100 = \%\text{min}$$

The glucose half life is the time required for the glucose concentration to fall by one-half. Using k-value, the glucose $T½$ was calculated as:

$$T½ = (0.693/k)*100 = \text{min}$$

The insulin response following glucose infusion was demonstrated as the insulin peak concentration and the insulin increment (ΔMax = insulin peak level – insulin basal level). All parameters of the glucose tolerance test were tested for normality using the Ryan-Joiner test statistics and then analysed by ANOVA. A P value of 0.05 or less was considered significant.

2.3. Results

2.3.1. Live weight and body condition

Mean (±SEM) live weight and BCS were lower ($P<0.001$) at the initiation of the two experimental periods for heifers allocated to the Low than the Moderate BCS groups (Figure 2.2). Heifers in the Low BCS group weighed on average 391.9±6.4 kg and were ascribed a BCS of 2.0±0.06 units. The mean live weight of heifers in Moderate BCS was 517±7.9 kg and a BCS of 3.7±0.05 units was ascribed. As anticipated, during the experimental period animals fed at M retained their live weight and BCS. In contrast, for heifers fed at 2M, live weight and BCS increased ($P<0.001$) during the experimental period (Figure 2.2).

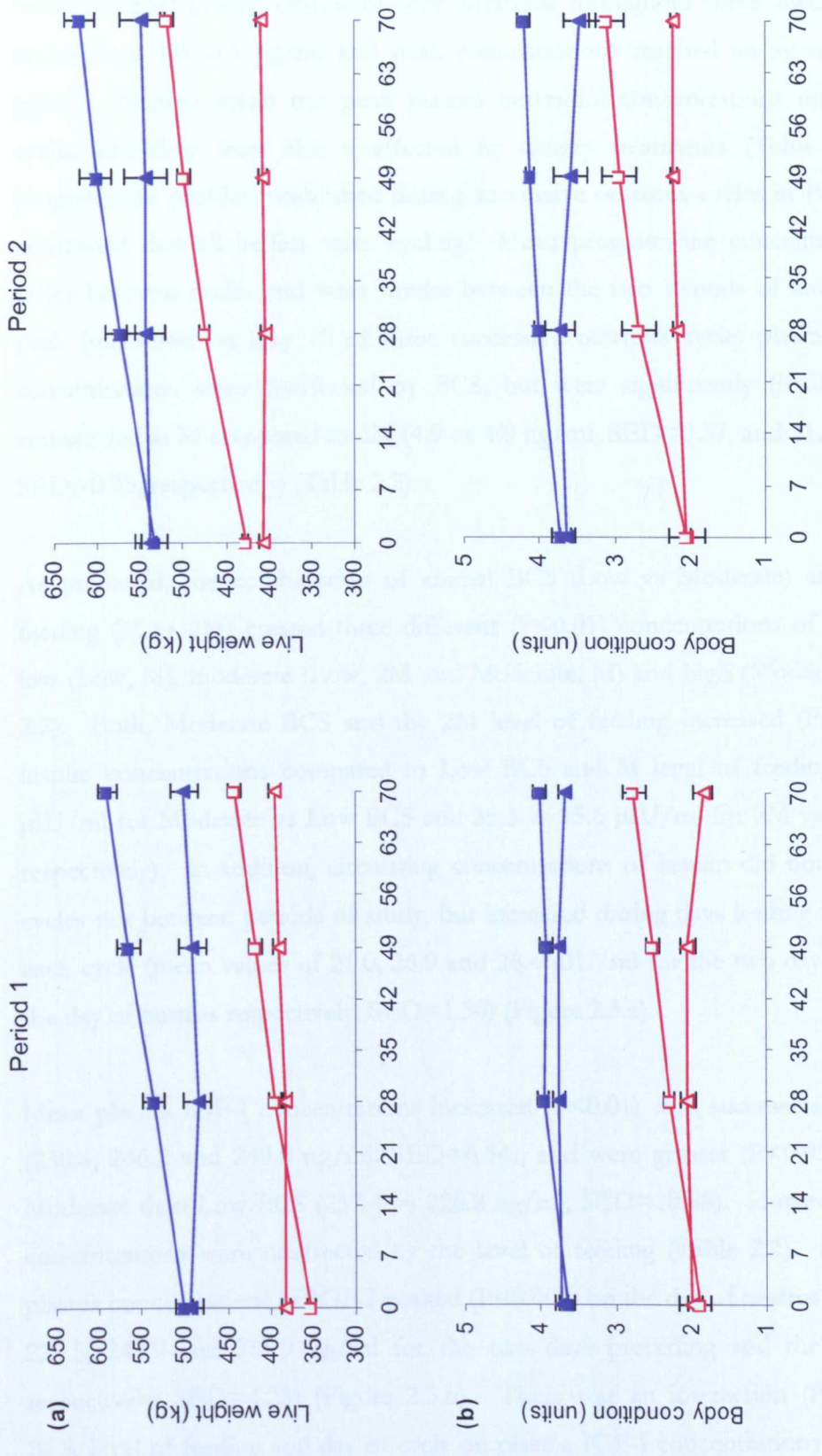

Figure 2.2. Changes in the mean live weight (a) and BCS (b) during experimental Periods 1 and 2. Treatments are delineated as follows: Low BCS, M (△) and 2M (□), Moderate BCS, M (▲) and 2M (■).

2.3.2. Plasma hormone concentrations

Mean (±SEM) plasma oestradiol concentrations throughout three successive oestrous cycles were 1.6±0.1 pg/ml and peak concentrations reached an average of 3.4±0.3 pg/ml. Neither mean nor peak plasma oestradiol concentrations differed between cycles and they were also unaffected by dietary treatments (Table 2.2). Plasma progesterone profiles established during successive oestrous cycles in Periods 1 and 2, confirmed that all heifers were cycling. Mean progesterone concentrations did not differ between cycles and were similar between the two Periods of study. Mean and peak (measured on Day 12 of three successive oestrous cycle) plasma progesterone concentrations were unaffected by BCS, but were significantly ($P<0.05$) greater in animals fed at M compared to 2M (4.9 vs 4.0 ng/ml, SED=0.37, and 11.2 vs 9.1 ng/ml, SED=0.95, respectively) (Table 2.2).

As predicted, the combination of animal BCS (Low vs Moderate) and the level of feeding (M vs 2M) created three different ($P<0.01$) concentrations of plasma insulin: low (Low, M), moderate (Low, 2M and Moderate, M) and high (Moderate, 2M) (Table 2.2). Both, Moderate BCS and the 2M level of feeding increased ($P<0.001$) plasma insulin concentrations compared to Low BCS and M level of feeding (33.2 vs 17.8 μIU/ml for Moderate vs Low BCS and 35.3 vs 15.6 μIU/ml for 2M vs M, SED=3.33, respectively). In addition, circulating concentrations of insulin did not differ between cycles nor between periods of study, but increased during days leading up to oestrus in each cycle (mean values of 21.0, 26.9 and 28.4 μIU/ml for the two days preceding and the day of oestrus respectively; SED=1.50) (Figure 2.3.a).

Mean plasma IGF-I concentrations increased ($P<0.01$) with successive oestrous cycles (230.4, 246.2 and 249.8 ng/ml; SED=6.34), and were greater ($P<0.05$) for heifers in Moderate than Low BCS (257.4 vs 226.8 ng/ml; SED=10.88). However, mean IGF-I concentrations were unaffected by the level of feeding (Table 2.2). As with insulin, plasma concentrations of IGF-I peaked ($P<0.001$) on the day of oestrus (mean values of 227.5, 242.9 and 255.9 ng/ml for the two days preceding and the day of oestrus respectively; SED=4.23) (Figure 2.3.b). There was an interaction ($P<0.01$) between BCS, level of feeding and day of cycle on plasma IGF-I concentrations which indicated

that the increase in plasma IGF-I concentrations in the days leading up to oestrus was greatest in Low BCS heifers fed at 2M (207.3, 226.9 and 253.9 ng/ml; SED=12.68).

In keeping with insulin, mean plasma leptin concentrations were greater (P<0.001) in animals of Moderate compared to Low BCS (5.7 vs 2.5 ng/ml, SED=0.47) and were further increased (P=0.001) by the level of feeding (5.0 vs 3.2 ng/ml; for 2M vs M, respectively, SED=0.47) (Table 2.2). Mean plasma leptin concentrations increased (P<0.001) with time (cycle number) on experiment (3.8, 4.0 and 4.3 ng/ml for cycles 1, 2 and 3; SED=0.09) (Figure 2.3.c) and there was an interaction (P<0.001) between level of feeding and cycle number indicating that plasma leptin concentrations increased with time for heifers on 2M (4.5, 4.9 and 5.4 ng/ml; SED=0.25), but not for heifers on M (3.1, 3.0 and 3.2 ng/ml; SED=0.25) diets. In contrast to insulin and IGF-I, plasma leptin concentrations did not vary over the six-day period leading up to oestrus in any of the three cycles averaged over both Periods.

Table 2.2. Peak plasma oestradiol and progesterone concentrations; and mean plasma insulin, IGF-I and leptin concentrations during the experimental period.

Body condition score	**Low**		**Moderate**		**SED**
Level of feeding	**M**	**2M**	**M**	**2M**	
No. of heifers	6	6	6	6	
Oestradiol † (pg/ml)	3.1	2.8	4.5	3.1	0.83
Progesterone ‡ (ng/ml)	11.0	8.5	11.5	9.8	1.34
Insulin (μIU/ml)	13.3^{a}	22.2^{b}	18.0^{b}	48.3^{c}	4.71
IGF-I (ng/ml)	223.9^{a}	228.6^{a}	266.4^{b}	246.7^{ab}	15.32
Leptin (ng/ml)	1.8^{a}	3.1^{b}	4.5^{b}	6.9^{c}	0.67

Means within a row with different superscripts are significantly different (P<0.05).
† Peak oestradiol measured for 3 successive oestrous cycles.
‡ Peak progesterone measured on Day 12 of the oestrous cycle, over 3 successive cycles.

Regression analysis revealed that there was a positive relationship between plasma leptin concentrations and animal BCS (Figure 2.4.a). There was also a positive but weaker correlation between plasma insulin concentrations and animal BCS (Figure 2.4.b). Plasma leptin concentrations were also positively correlated to plasma concentrations of insulin and this relationship is presented in Figure 2.4.c. In contrast, plasma leptin and plasma IGF-I concentrations were not correlated ($y=0.0168x - 0.2595$, $R^2=0.04$, n=24; P>0.05).

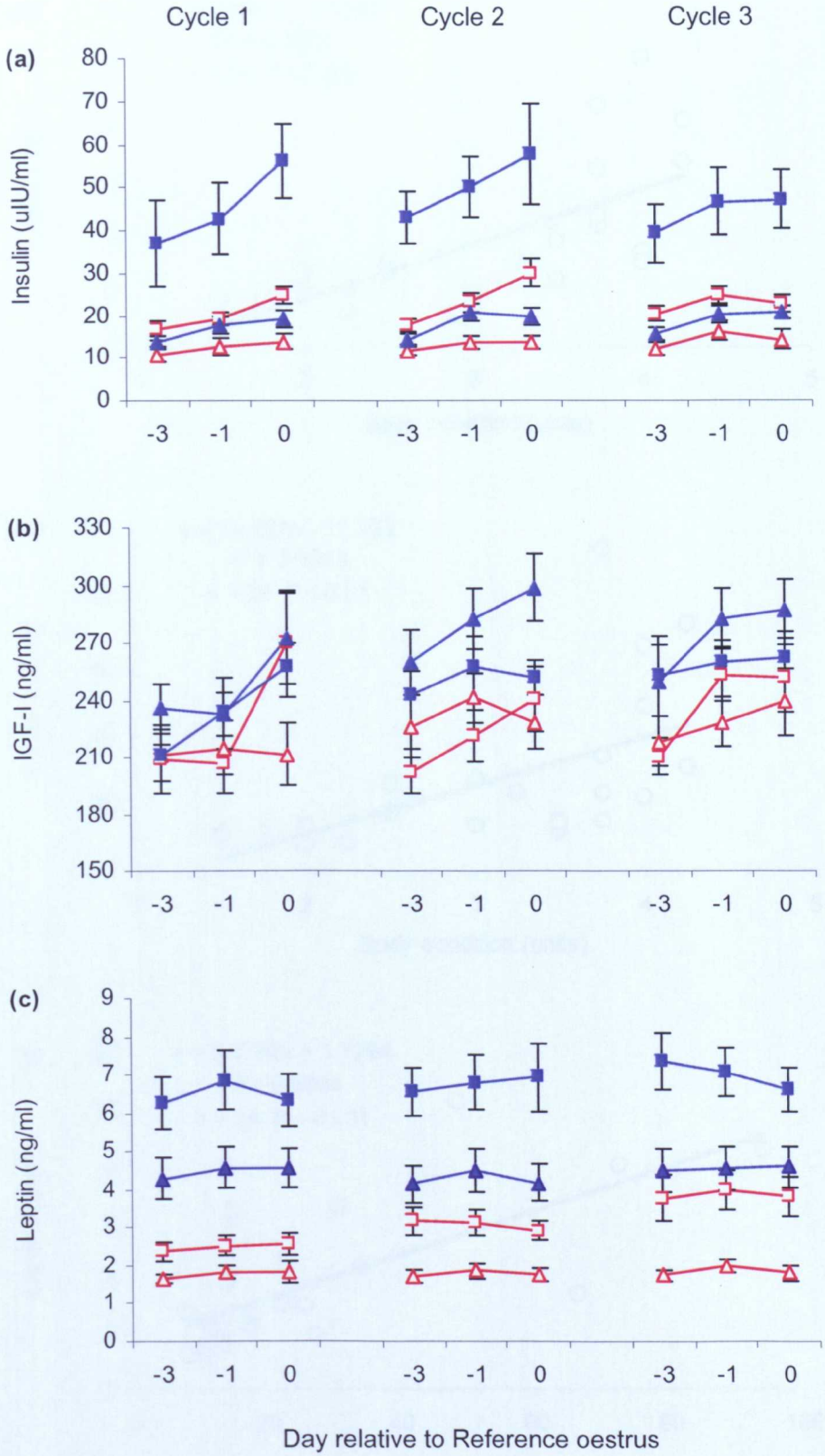

Figure 2.3. Mean (±SEM) concentrations of plasma insulin (a), IGF-I (b) and leptin (c) measured three days prior to (-3), one day prior to (-1) and on the day of oestrus (0), over three successive oestrous cycles. Treatments are marked as follows: Low BCS, M (Δ) and 2M (□), Moderate BCS, M (▲) and 2M (■).

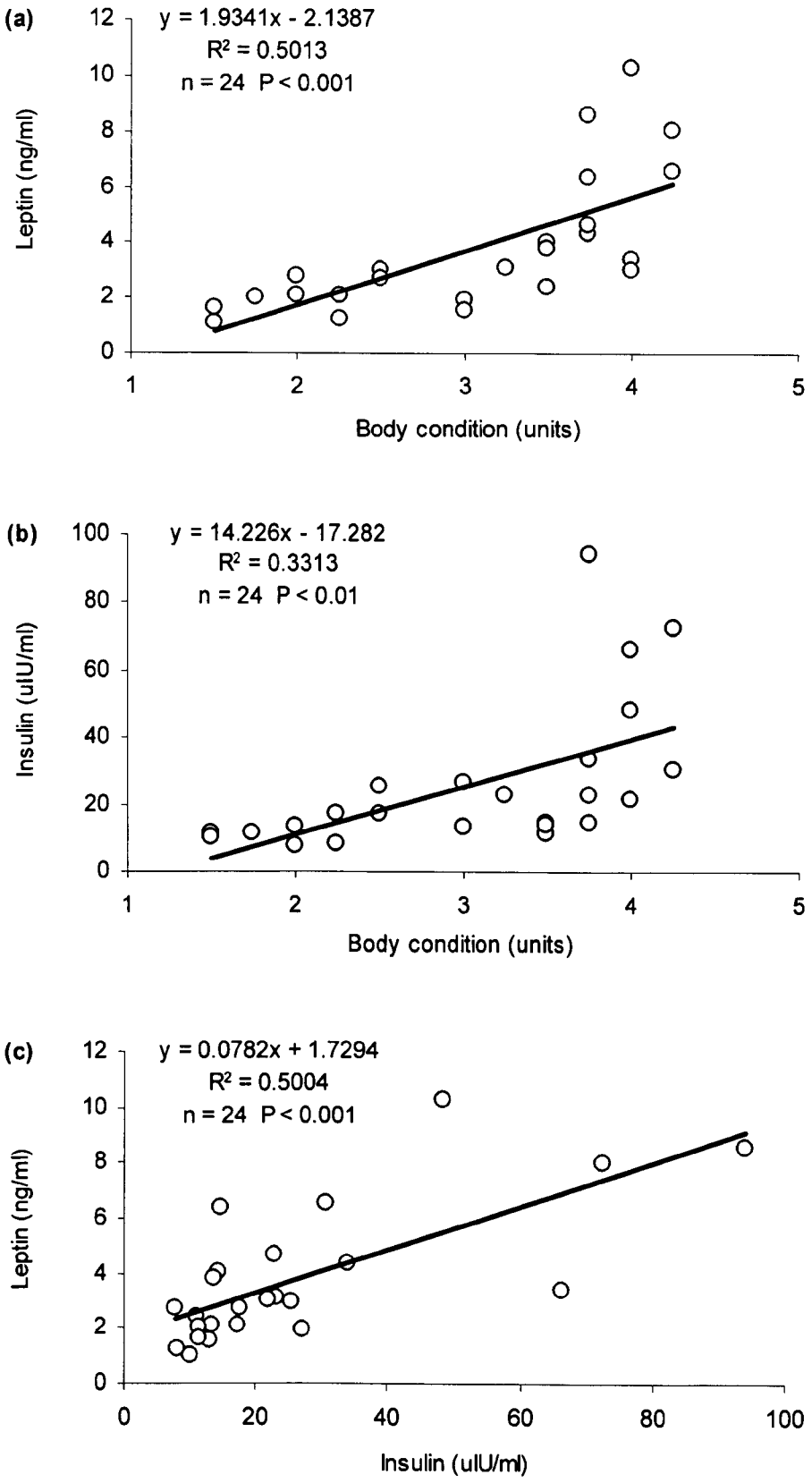

Figure 2.4. Relationships between plasma leptin (ng/ml) and animal BCS (units) (a), plasma insulin (μIU/ml) and animal BCS (b), and the relationship between plasma concentrations of leptin and insulin (c).

2.3.3. Glucose tolerance test

Baseline glucose concentrations were greater (P<0.01) for heifers fed at 2M compared to M (4.0 vs 3.7 μmol/ml, SED=0.10), but did not differ between animal BCS groups. Following glucose infusion, peak glucose concentrations were greater for heifers in Moderate than in Low BCS (18.3 vs 17.1, SED=0.60; P=0.063) and for heifers fed at M compared to 2M (18.3 vs 17.0, SED=0.60; P=0.053) although differences did not reach statistical significance (Figure 2.5.a). Glucose clearance rate (*k*) was greater and the half life (*T½*) lower for animals in Moderate than in Low BCS (24.4 vs 21.1%/min, SED=1.23, P<0.05; and 2.9 vs 3.3 min, SED=0.16, P<0.05, respectively). Level of feeding did not affect glucose clearance rate or half life (Table 2.3).

Prior to glucose infusion, baseline insulin concentrations were greater for heifers in Moderate than in Low BCS (42.8 vs 29.0 μIU/ml, SED=6.27; P<0.05) and for animals fed at 2M compared to M (46.6 vs 25.2 μIU/ml, SED=6.27; P<0.01). However, following glucose infusion, peak insulin concentrations and the increase in insulin concentrations (ΔMax) were not significantly different between treatments (Table 2.3). Although, significant differences in insulin response occurred during the first 20 minutes post-infusion, when mean insulin concentrations for Moderate BCS, 2M heifers were greater (P<0.05) compared to Low BCS, M heifers, thereafter, differences were not statistically significant (Figure 2.5.b).

Table 2.3. Plasma glucose and insulin responses following the intravenous infusion of glucose (0.3 g/kg body weight).

Body condition score	**Low**		**Moderate**		**SED**
Level of feeding	**M**	**2M**	**M**	**2M**	
No. of heifers	6	6	6	6	
Basal glucose (μmol/ml)	3.7^{a}	4.0^{b}	3.8^{ab}	4.0^{b}	0.15
Peak glucose (μmol/ml)	17.5^{a}	16.7^{a}	19.1^{b}	17.4^{a}	0.85
k (%/min)	21.2^{a}	21.0^{a}	26.7^{b}	22.1^{a}	1.73
T½ (min)	3.3^{a}	3.3^{a}	2.6^{b}	3.2^{a}	0.23
Basal insulin (μIU/ml)	20.1^{a}	37.9^{ab}	30.3^{a}	55.4^{b}	9.71
Insulin peak (μIU/ml)	178.3^{a}	239.5^{ab}	237.2^{ab}	367.3^{b}	79.67
ΔMax (μIU/ml)	158.2^{a}	201.6^{ab}	206.9^{ab}	311.9^{b}	71.56

Means within a row with different superscripts are significantly different (P<0.05).

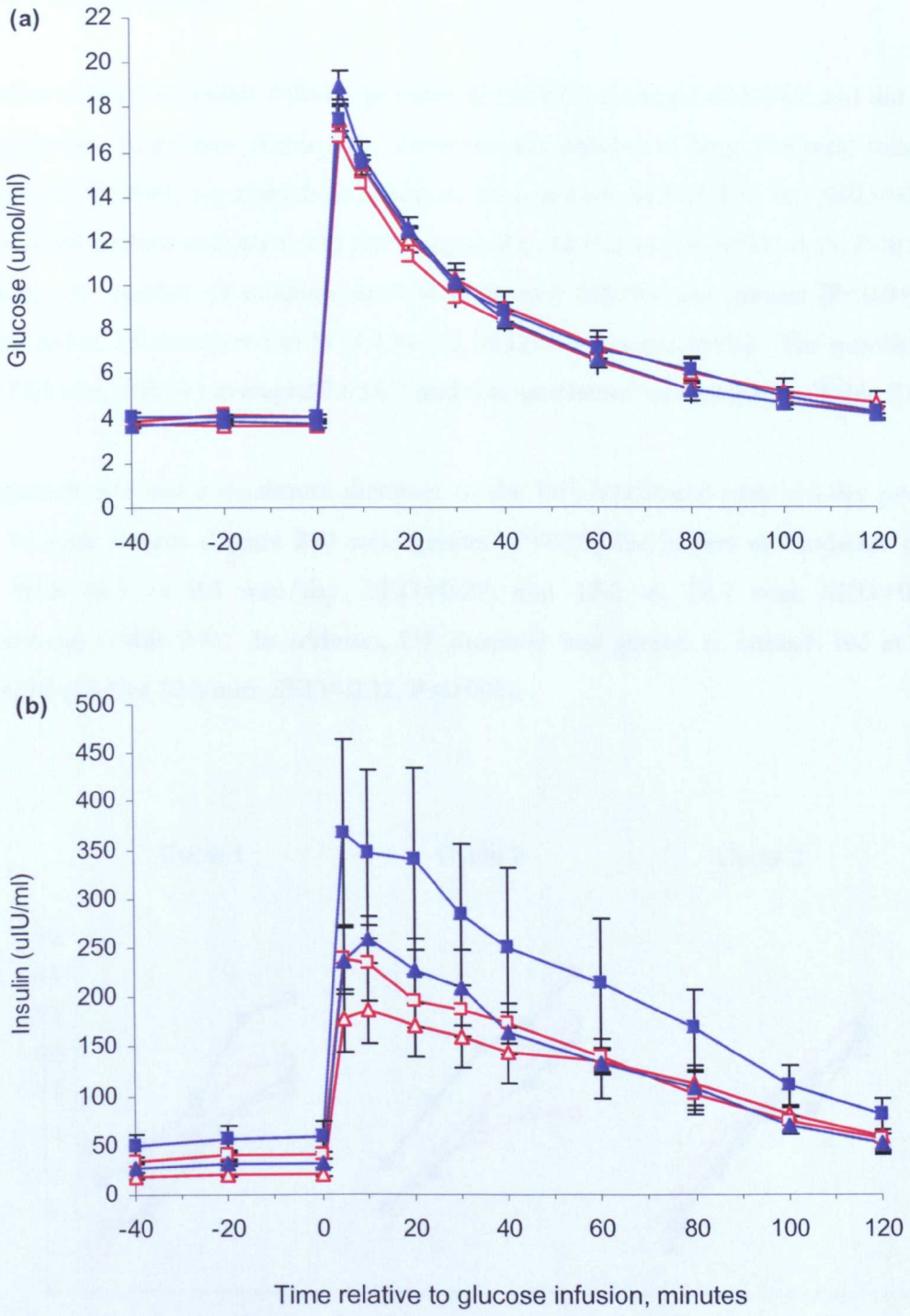

Figure 2.5. Response in plasma concentrations (±SEM) of glucose (a) and insulin (b) following the infusion of 0.3 g/kg of Glucose 40% (Arnolds Veterinary Products, Ltd, Shrewsbury, UK). Blood samples were collected over a 40 minute period prior to glucose infusion (0) and for 120 minutes after infusion. Treatments are indicated as follows: Low BCS, M (△) and 2M (□), Moderate BCS, M (▲) and 2M (■).

2.3.4. Follicular growth

The total number of visible follicles per animal (±SEM) averaged 12.8±0.2 and did not differ between treatments (Table 2.4). However, the number of large (>8 mm) follicles was greater (P<0.01) for animals in Moderate than in Low BCS (1.3 vs 0.9, SED=0.15, respectively) and for animals fed at 2M compared to M (1.3 vs 0.9, SED=0.15; P<0.01). Similarly, the number of medium-sized (4 to 8 mm) follicles was greater (P<0.01) in animals fed at 2M compared to M (4.4 vs 3.8, SED=0.19, respectively). The number of small (<4 mm) follicles averaged 7.6±0.7 and was unaffected by treatments (Table 2.4).

The growth rate and a maximum diameter of the DF, monitored over a 6-day period prior to each oestrus (Figure 2.6) were greater (P<0.05) for heifers in Moderate than Low BCS (0.8 vs 0.3 mm/day, SED=0.22; and 13.2 vs 12.4 mm, SED=0.32, respectively) (Table 2.4). In addition, DF diameter was greater in animals fed at 2M than at M (13.4 vs 12.2 mm; SED=0.32, P<0.001).

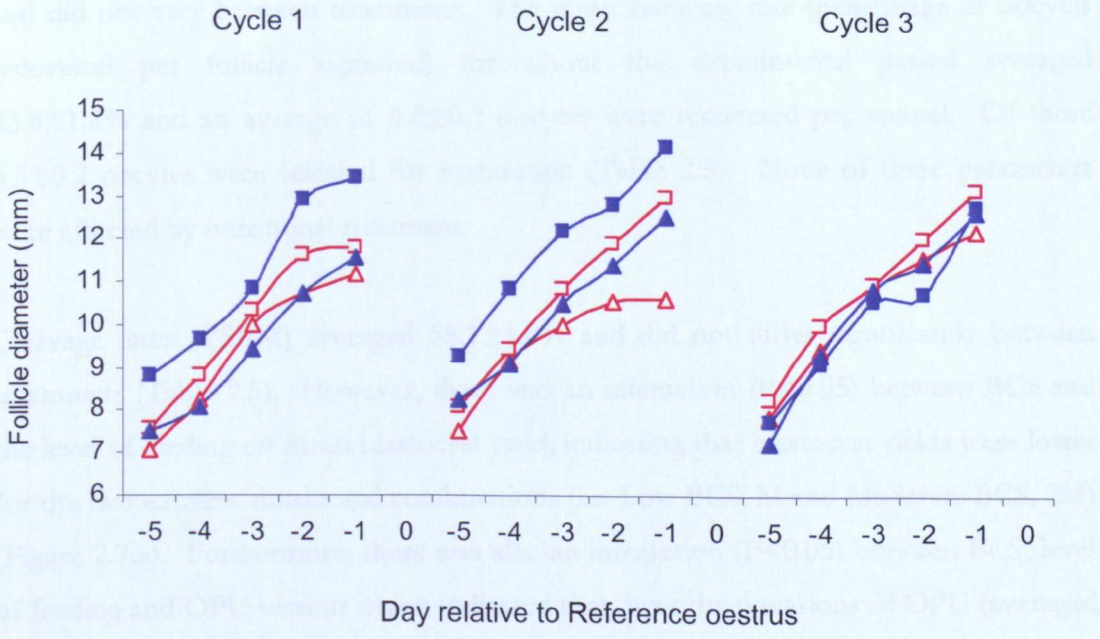

Figure 2.6. Mean diameter of the ovulatory follicle monitored for 6 days immediately prior to oestrus, during three successive oestrous cycles. Treatments are indicated as follows: Low BCS, M (Δ) and 2M (□), Moderate BCS, M (▲) and 2M (■).

Table 2.4. Mean total follicle number and number of small (<4 mm), medium (4 to 8 mm) and large sized (>8 mm) follicles per heifer. Mean maximum diameter of the dominant follicle (DF) during a 6-day period prior to ovulation and the growth rate of this DF.

Body condition score	**Low**		**Moderate**		**SED**
Level of feeding	**M**	**2M**	**M**	**2M**	
No. of heifers	6	6	6	6	
Total follicle number (n)	13.0	12.7	12.2	13.3	0.64
Small follicles (n)	8.3	7.3	7.4	7.3	0.65
Medium follicles (n)	3.9	4.4	3.8	4.5	0.41
Large follicles (n)	0.7^{a}	1.1^{b}	1.0^{b}	1.5^{c}	0.21
Max. diameter of DF (mm)	11.8^{a}	12.9^{b}	12.6^{ab}	13.8^{c}	0.45
Growth rate of DF (mm/day)	1.3	1.4	1.3	1.2	0.11

Means within a row with different superscripts are significantly different (P<0.05).

2.3.5. Oocyte and embryo development *in vitro*

The number (±SEM) of follicles observed prior to OPU averaged 11.9±0.2 per animal and did not vary between treatments. The mean recovery rate (percentage of oocytes recovered per follicle aspirated) throughout the experimental period averaged 53.6±1.2% and an average of 5.8±0.2 oocytes were recovered per animal. Of these 5.3±0.2 oocytes were selected for maturation (Table 2.5). None of these parameters were affected by nutritional treatment.

Cleavage rates (±SEM) averaged 58.1±1.8% and did not differ significantly between treatments (Table 2.5). However, there was an interaction (P<0.05) between BCS and the level of feeding on mean blastocyst yield, indicating that blastocyst yields were lower for the two extreme nutritional combinations (i.e. Low BCS, M and Moderate BCS, 2M) (Figure 2.7.a). Furthermore, there was also an interaction (P<0.05) between BCS, level of feeding and OPU session which indicated that, over the 6 sessions of OPU (averaged over both Periods) blastocyst yields for heifers of Moderate BCS fed at 2M decreased relative to heifers of Low BCS fed at 2M (Figure 2.7.b). Although numerically greater for heifers of Moderate BCS, total cell number per Day 8 blastocyst did not differ significantly between treatments (Table 2.5).

Table 2.5. Mean number of follicles aspirated and the mean number of oocytes recovered by OPU. Data from *in vitro* embryo culture: mean number of oocytes matured, cleaved and produced blastocysts following fertilisation.

Body condition score	Low		Moderate		SED
Level of feeding	M	2M	M	2M	
No. of heifers	6	6	6	6	
Follicle number (n)	11.9	11.6	11.7	12.2	0.54
Follicles aspirated (n)	10.6	10.4	10.4	10.5	0.52
Oocytes recovered (n)	6.0	5.7	5.3	5.5	0.48
Oocytes matured (n)	5.3	5.1	5.0	5.4	0.40
Oocytes cleaved (n)	3.1	2.8	2.7	3.5	0.36
Cleavage rate (%)	57.1	55.2	54.7	63.8	5.50
Blastocyst number (n)	0.4	0.7	0.6	0.6	0.15
Total cell counts (n)	97.4	99.0	107.2	116.8	11.17

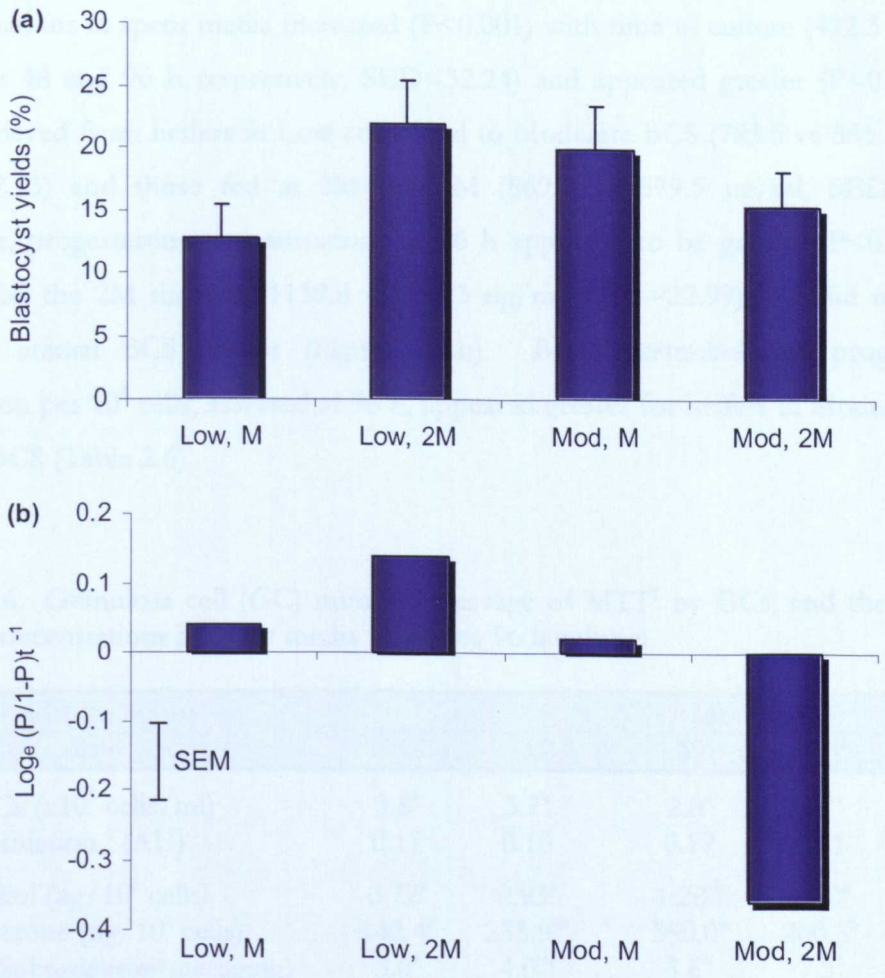

Figure 2.7. (a) Mean (±SEM) blastocyst yields (%) and (b) change in blastocyst yields (change in logit proportion with time) during the 9 week experimental period.

2.3.6. Granulosa cell number, viability and steroidogenesis

The number of live GCs assessed following 96 h of culture appeared greater for cells harvested from heifers in Low than in Moderate BCS (4.7 vs 3.2 $x10^5$ cells/ml, SED=0.33) and from animals fed at 2M compared to M (4.7 vs 3.3 $x10^5$ cells/ml, SED=0.33). In contrast, viability of cultured GCs averaged 0.17±0.06 absorbance units (AU) and did not differ between treatments. When adjusted for cell number the quantity of MTT cleaved appeared numerically greater for cells from the Moderate than Low BCS group, but this difference was not statistically significant (Table 2.6). Oestradiol concentrations in spent media declined (P<0.001) with time in culture (15.1 vs 4.4 ng/ml at 48 and 96 h respectively, SED=1.64). At both 48 h and 96 h, oestradiol concentrations were greater (P<0.01) for GCs harvested from animals fed at 2M than M (19.4 vs 10.9 ng/ml, SED=2.96 and 6.1 vs 3.1 ng/ml, SED=0.50, respectively), but did not differ between animal BCS groups (Figure 2.8.a). In contrast, progesterone concentrations in spent media increased (P<0.001) with time in culture (492.3 vs 956.7 ng/ml at 48 and 96 h respectively, SED=32.24) and appeared greater (P<0.001) for GCs retrieved from heifers in Low compared to Moderate BCS (783.5 vs 665.5 ng/ml, SED=32.13) and those fed at 2M than M (869.5 vs 579.5 ng/ml, SED=32.13). However, progesterone concentrations at 96 h appeared to be greater (P<0.001) for heifers fed the 2M than M (1139.8 vs 758.3 ng/ml, SED=22.99), but did not differ between animal BCS groups (Figure 2.8.b). Both, oestradiol and progesterone production per 10^5 cells, assessed at 96 h, appeared greater for heifers in Moderate than in Low BCS (Table 2.6).

Table 2.6. Granulosa cell (GC) number, cleavage of MTT† by GCs, and the ratio of steroid concentrations in spent media following 96 h culture.

Body condition score	**Low**		**Moderate**		**SED**
Level of feeding	**M**	**2M**	**M**	**2M**	
Live GCs ($x10^5$ cells/ml)	3.8^a	5.7^b	2.8^a	3.7^a	0.47
MTT oxidation † (AU)	0.11	0.18	0.17	0.21	0.066
Oestradiol (ng/10^5 cells)	0.72^a	0.93^a	1.28^{ab}	1.90^b	0.30
Progesterone (ng/10^5 cells)	142.4^a	233.9^{ab}	350.0^b	260.3^b	40.78
Oestradiol:progesterone(pg:ng)	5.0^a	4.0^{ab}	3.6^b	7.3^c	0.62

Means within a row with different superscripts are significantly different (P<0.05).
† Increase in absorbance at 550 nm due to formation of MTT-formazan during incubation of cells with MTT for 4 h at 38.8°C.

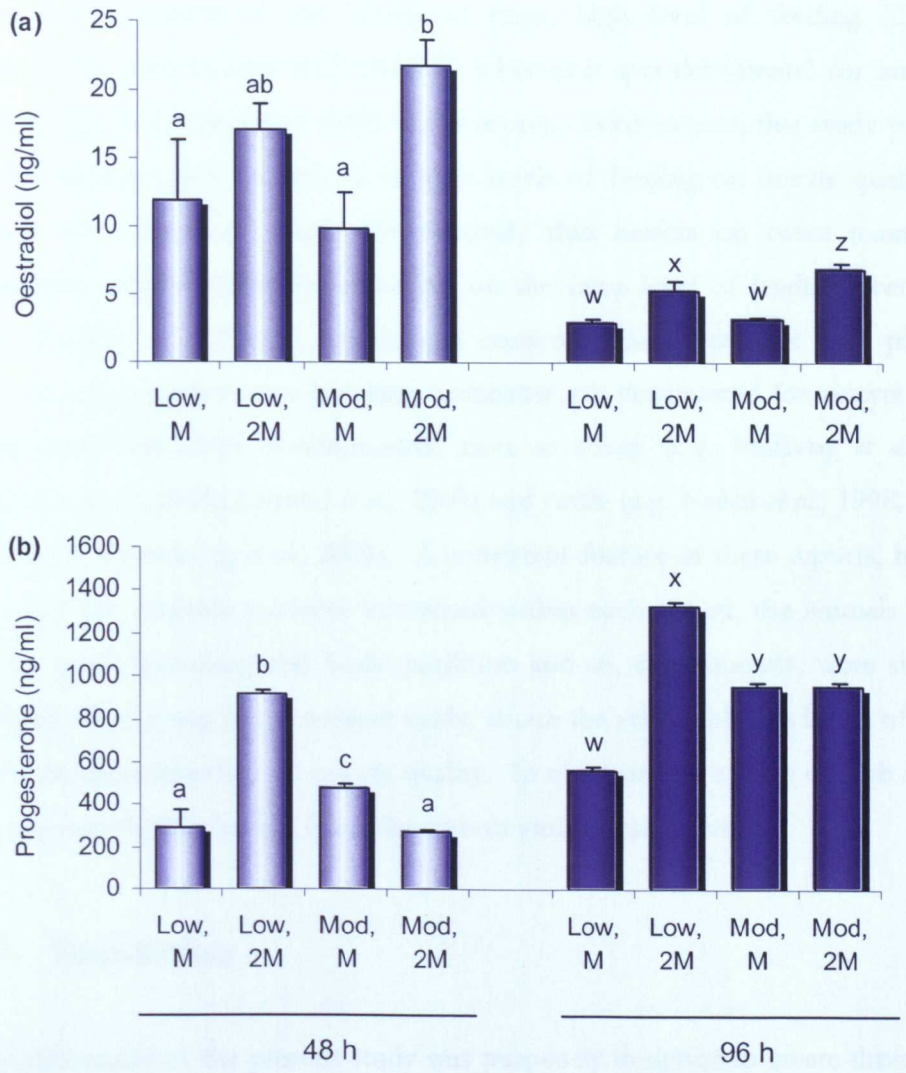

Figure 2.8. Mean (±SEM) oestradiol (a) and progesterone (b) concentrations in spent media from GCs culture, collected at 48 and 96 h.

2.4. Discussion

As an initial step to properly understand dietary nutrient effects on oocyte quality, the present study sought to purposely separate the effects of body composition (BCS) from level of dietary energy intake (level of feeding). This study also employed OPU procedures to recover oocytes on six separate occasions spanning three oestrous cycles and seven weeks, so that the cumulative effects of nutritional status on oocyte quality could be determined. One of the main findings from this study was that the effects of level of feeding on oocyte quality were dependent on the initial BCS of the heifer. Collectively, the evidence presented in Figure 2.7 indicates that, in terms of post-

fertilisation development to the blastocyst stage, high level of feeding (2M) was beneficial for animals in Low BCS (BCS 2) whereas it was detrimental for animals in moderately-high body condition (BCS 3.5 or more). Furthermore, this study produced evidence to indicate that the effects of high levels of feeding on oocyte quality were cumulative, with blastocyst yields for relatively thin heifers on twice maintenance diverging from that of relatively fat heifers on the same level of feeding over the six sessions of OPU. Until now, the general consensus has been that high planes of nutrition in non-pregnant, non-lactating ruminants are detrimental for oocyte quality, with low post-fertilisation developmental rates in sheep (e.g. McEvoy *et al.*, 1995; Papadopoulos *et al.*, 2001; Lozano *et al.*, 2003) and cattle (e.g. Nolan *et al.*, 1998; Yaakub *et al.*, 1999a, b; Armstrong *et al.*, 2001). A consistent feature of these reports, however, is that, from the available evidence contained within each report, the animals were of moderately good (pre-slaughter) body condition and so, consequently, were similar to the moderate BCS group in the present study, where the effects of high levels of feeding also had a detrimental effect on oocyte quality. In contrast, the effects of high levels of feeding in relatively thin heifers from the current study were beneficial.

2.4.1. Endocrinology

The factorial nature of the present study was purposely designed to create three groups of animals with low (~10 μIU/ml), moderate (~20 μIU/ml) and high (~40 μIU/ml) plasma insulin concentrations (Table 2.2), representative of the broad range encountered in commercial practice, with the lower values representative of those (2 to 20 μIU/ml) recorded with lactating cattle (Gong *et al.*, 2002; Andersen *et al.*, 2004). Inevitably, the design also led to the creation of three corresponding groups of animals with low (~2 ng/ml), moderate (~4 ng/ml) and high (~7 ng/ml) plasma leptin concentrations, so that the effects of these two metabolic hormones on follicular growth and oocyte quality cannot be easily separated. Indeed, across treatments, plasma leptin concentrations were positively ($P<0.001$) correlated with both BCS and plasma insulin (Figure 2.4) confirming previous findings in rodents, humans and lactating dairy cows (Kamoda *et al.*, 1998; Saad *et al.*, 1998; Fernandez-Real *et al.*, 2000; Sonnenberg *et al.*, 2001; Block *et al.*, 2003). As with the other mammalian species examined, plasma leptin concentrations in ruminants are related to body fatness and level of feeding (Ehrhardt *et al.*, 2000; Delavaud *et al.*, 2000, 2002; Thomas *et al.*, 2001; León *et al.*, 2004). This latter effect can

be attributed, at least in part, to the combined actions of insulin on leptin gene expression (Mizuno *et al.*, 1996; Sonnenberg *et al.*, 2001) and insulin-stimulated glucose uptake by adipocytes (Mueller *et al.*, 1998). Surprisingly, this study detected only a very modest effect of BCS on plasma IGF-I concentrations. A significant effect of feeding level on plasma concentrations of this growth factor, paralleling that of insulin was previously observed, with the same assay (Armstrong *et al.*, 2001).

2.4.2. Ovarian folliculogenesis and granulosa cell culture

In the present study, the high (2M) level of feeding increased the number of medium- and large-sized follicles, and the growth rate and maximum size of the pre-ovulatory follicle independently of the effects of BCS (Table 2.4). The high level of feeding also increased granulosa cell proliferation *in vitro*, which was also greater for cells derived from Low than Moderate BCS heifers (Table 2.6). Working with a similar genotype of heifers, Gong *et al.* (2002) reported a significant increase in the number of small (2 – 4 mm) follicles leading to an increase in the number of large (> 9 mm) follicles following FSH treatment in heifers fed at 2M compared to M. The population of medium-sized follicles was unaffected by level of feeding in that study. This apparent discrepancy between studies may be due to differences in the duration of exposure to the levels of feeding (3 weeks in the study of Gong *et al.* (2002) compared to the mean of approximately six, nine and 12 weeks in the present study). Similarly, Armstrong *et al.* (2002a) failed to observe any effect of level of feeding (M vs 2M) on granulosa cell proliferation *in vitro*, but the BCS of their animals was closer to that of the Moderate BCS group of heifers in the present study, where differences in GC proliferation were small and statistically non-significant. Indeed, in the present study the number of live GCs following 96 h culture appeared to be lower for Moderate BCS heifers than for Low BCS heifers (Table 2.6).

Both BCS and level of feeding increased oestradiol secretion from cultured granulosa cells in the present study, but the effects of level of feeding was greatest for cells derived from Moderate BCS heifers (Figure 2.8.a). Similarly, progesterone secretion was greatest for GCs from Moderate BCS heifers. However, whilst level of feeding increased progesterone secretion from GCs from Low BCS heifers it decreased progesterone secretion from GCs recovered from Moderate BCS heifers (Figure 2.8.b).

These observations are in broad agreement with those of Armstrong *et al.* (2002a) who cultured GCs from heifers offered either a maintenance or twice maintenance level of feeding for up to 144 h *in vitro*. In accord with the observations of the current study, oestradiol secretion from GCs at 96 h appeared to be greater for those cells recovered from heifers fed at 2M compared to M. These authors also observed that level of feeding failed to affect progesterone secretion by GCs. Although, as a single factor, it seemed that level of feeding failed to influence progesterone secretion by GCs in the current study, the actual effect of feeding level on progesterone secretion appeared dependant on heifer BCS; with the high level of feeding leading to increased progesterone secretion from GCs from Low BCS heifers, but decreased progesterone secretion from GCs from Moderate BCS heifers (Figure 2.8.b).

Body condition and level of feeding are known to influence a number of factors that regulate ovarian follicular development, granulosa cell proliferation and steroidogenesis. In this regard, the effects of insulin are particularly well documented. Insulin is known to stimulate bovine granulosa (e.g. Gutierrez *et al.*, 1997c) and thecal (e.g. Stewart *et al.*, 1995) cell proliferation, and steroidogenesis *in vitro* (e.g. Gong *et al.*, 1993, 1994). Similarly, IGF-I is also known to stimulate GC proliferation and steroidogenesis *in vitro* (Silva and Price, 2002; Spicer *et al.*, 2002). In contrast, leptin is known to inhibit gonadotrophin and/or insulin-induced steroidogenesis by granulosa and thecal cells *in vitro*, although it does not seem to be able to affect GC proliferation at physiological levels (Spicer and Francisco, 1997, 1998; Armstrong *et al.*, 2003). The fact that GC number assessed at 96 h appeared greater and steroidogenesis (particularly progesterone production) lower for GCs from Low than Moderate BCS heifers, indicates that these cells may have been less differentiated by 96 h (Table 2.6). In fact the effects of BCS on bovine GC proliferation *in vitro* are not well documented, but clearly they operate, at least in part, independently of the effects of the three metabolic hormones studied here.

2.4.3. Post-fertilisation embryo development

Blastocyst yields in this study were somewhat lower than we have come to expect in our laboratory and there are a number of possible reasons for this. Firstly, in order to retain animal identity, oocytes were matured, fertilised and cultured in small numbers, the latter under serum and somatic-cell free conditions (i.e. in SOF plus BSA with essential

and non-essential amino acids). Individually, these conditions are known to lead to lower blastocyst yields following *in vitro* culture (Canseco *et al.*, 1992; Lane and Gardner, 1992; O'Doherty *et al.*, 1997; Ward *et al.*, 2000; Kane, 2003). The highest blastocyst yields in the current study were observed for the treatment combinations Low BCS, 2M and Moderate BCS, M (Figure 2.7.a). These treatment combinations were associated with moderate concentrations of both plasma insulin and leptin (Table 2.6). It is tempting to speculate that moderate plasma concentrations of these two metabolic hormones are associated with improved post-fertilisation embryo development whereas either low or high concentrations are associated with impaired post-fertilisation embryo development. The high plasma leptin concentrations have previously been related to altered activity of insulin signalling pathways, what to some extent can elucidate changes in steroidogenic activity of GCs and reduced oocyte quality (Cohen *et al.*, 1996; Nowak *et al.*, 1998; Brannian *et al.*, 1999; Smith *et al.*, 2002). Improved post-fertilisation development may also be linked to the steroidogenic potential of GCs of follicles from which the oocytes came. Granulosa cells from Low, 2M and Moderate, M heifers secreted the greatest quantities of progesterone following both 48 and 96 h of culture (Figure 2.8.b), intermediate quantities of oestradiol and had the lowest E2:P4 ratio (Table 2.6). The effects of ovarian steroids on oocyte maturation, fertilisation and pre-implantation development, however, are equivocal and depend on additional factors such as follicle health, gonadotrophin background, and timing and duration of steroid exposure (e.g. Kobayashi *et al.*, 1991; Oussaid *et al.*, 2000). Direct comparisons with the present study are problematic given that steroid secretions were from GCs cultured *in vitro* for 4 days, and were based on granulosa cell populations pooled across animals from each treatment. However, it is known that GCs from hyperinsulinaemic and anovulatory polycystic ovarian syndrome subjects are less prolific and have a greater capacity to produce oestradiol *in vitro* (Franks *et al.*, 1996).

2.4.4. Hyperinsulinaemia and oocyte quality

The formal assessment of glucose tolerance and insulin resistance conducted approximately two weeks after the completion of Period 2 failed to confirm that the 'high-insulin' animals on treatment Moderate BCS, 2M were intolerant to glucose or resistant to insulin to any significant extent (Figure 2.5). Closer inspection of individual animal data, however, revealed that a single animal from this treatment group with very

high (~100 μIU/ml) basal plasma insulin concentrations produced a hyperinsulinaemic response to infused glucose (Figure 2.9). Visual inspection of the plasma insulin data for the 12 sessions of OPU identified eight animals from this treatment group (five during Period 1 and three during Period 2) with similarly high basal concentrations of plasma insulin. Fasting plasma insulin concentrations for the eight hyperinsulinaemic animals identified in the current study (58 μIU/ml) were greater than those previously reported for obese cattle and sheep (24 to 33 μIU/ml; McCann *et al.*, 1986a, b), but comparable to the extreme values (40 to 52 μIU/ml) reported in women with polycystic ovary syndrome by Dunaif *et al.* (1997). It is reasonable to assume that infused glucose would have evoked a similar hyperinsulinaemic response in these animals had the GTT been conducted during either of these two periods. A hyperinsulinaemic state was formally designated, therefore, as plasma insulin concentrations more than three standard deviations above the combined mean of animals on the 'Low BCS 2M' and 'Moderate BCS M' treatment groups; animals in these groups had moderate concentrations of plasma insulin (Table 2.7). This formal designation confirmed that eight out of the 12 heifers on the Moderate, 2M treatment group were hyperinsulinaemic. From Table 2.7 it is apparent that neither plasma IGF-I nor plasma leptin concentrations differed between the hyperinsulinaemic and 'normal' animals within this treatment group. However, the hyperinsulinaemic animals had fewer follicles, particularly medium-sized follicles; an observation consistent with that for well fed (~120 MJ ME/day) heifers of good body condition from the study of Nolan *et al.*, (1998). Furthermore, these hyperinsulinaemic animals produced significantly fewer oocytes and had lower blastocyst yields following *in vitro* fertilisation and embryo culture (Table 2.7). Large (14 to 20 mm) persistent follicles were observed in four of these animals on separate occasions following OPU during the six oestrous cycles. Large, persistent follicles are known to yield oocytes with lower post-fertilisation developmental potential (e.g. Mihm *et al.*, 1999) but, as these follicles were not present at the time of follicular aspiration, so this mechanism cannot explain the low blastocyst yields following OPU and IVF in this study. Instead the data suggest that elevated concentrations of insulin in these animals had a more direct, negative impact on the follicle-enclosed oocyte, so impairing post-fertilisation development. A causal mechanism for insulin is suggested because the negative association of elevated insulin concentrations with post-fertilisation development occurred in the absence of differences in circulating IGF-I and leptin (Table 2.7).

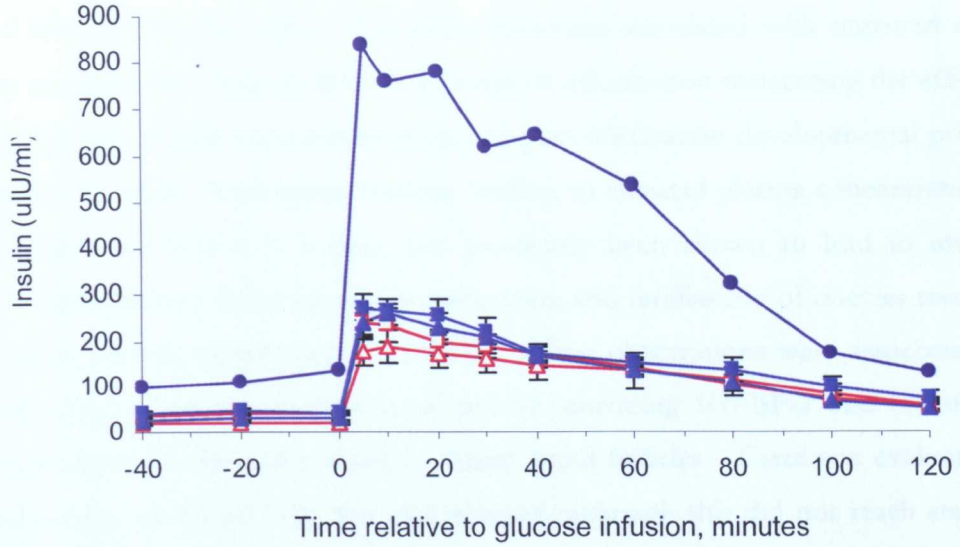

Figure 2.9. Response in mean (±SEM) plasma insulin following glucose infusion with indication of hyperinsulinaemic animal (●) compared to other animals within treatment.

Table 2.7. Differences in plasma hormone concentrations, follicular development and in vitro embryo development between heifers in the Moderate BCS, 2M treatment group with plasma insulin concentrations either within the normal range (Normal) or >3 standard deviations above mean of heifers in Low BCS, 2M and Moderate BCS, M treatment groups (Hyperinsulinaemic).

Parameter	Normal (< 37.2 μIU/ml)	Hyperinsulinaemic (> 37.2 μIU/ml)	P value
No. of heifers	4	8	
(a) Metabolic hormones			
Insulin (μIU/ml)	29.0 ± 5.16	58.0 ± 3.65	<0.01
IGF-I (ng/ml)	255.1 ± 7.56	244.1 ± 5.34	NS
Leptin (ng/ml)	7.1 ± 0.41	6.5 ± 0.28	NS
(b) Follicular development			
Total follicle number (n)	16.0 ± 0.93	12.0 ± 0.58	<0.05
Large follicles (n)	1.3 ± 0.22	1.6 ± 0.26	<0.05
Medium follicles (n)	6.3 ± 0.59	3.5 ± 0.33	0.08
Small follicles (n)	8.3 ± 0.50	6.8 ± 0.60	0.07
Max. diameter of DF (mm)	13.4 ± 0.77	14.0 ± 0.67	NS
Growth rate of DF (mm/day)	1.3 ± 0.04	1.4 ± 0.10	NS
(c) Oocyte and early embryo development *in vitro*			
Oocytes matured (n)	7.1 ± 0.67	4.9 ± 0.50	<0.01
Cleavage rates (%)	65.9 ± 4.17	62.3 ± 4.13	NS
Blastocyst yields (%)	26.2 ± 4.53	13.4 ± 3.56	<0.05

This is the first known report of hyperinsulinaemia associated with impaired oocyte quality in ruminants. Indeed, there is a dearth of information concerning the effects of elevated plasma insulin concentrations on the post-fertilisation developmental potential of oocytes in cattle. High-plane feeding, leading to elevated plasma concentrations of both insulin and IGF-I in heifers, has previously been shown to lead to impaired embryo development following *in vitro* maturation and fertilisation of oocytes recovered from these animals (Armstrong *et al.*, 2001). These observations were associated with reduced steady-state concentrations of mRNA encoding IGFBP-2 and -4, and the insulin receptor locally within small (< 4mm) antral follicles. There was evidence that the expression of the IGF-IR was also reduced, although this did not reach statistical significance. Although it was hypothesised that the expected increase in bioavailability of intra-follicular IGF-I and -II may have been detrimental to the developing oocyte, the precise mechanisms of such an effect are not understood, and it is not known if similar effects were operating in the current study. However, peripheral concentrations of insulin for the eight hyperinsulinaemic animals in the present study were significantly greater than those reported earlier.

Previous studies with cattle have assessed the maturation potential of oocytes from small antral follicles that coexisted with cystic follicles (Takagi *et al.*, 1998a), and the ability of follicular fluid from cystic follicles to support the *in vitro* maturation and fertilisation of oocytes (Takagi *et al.*, 1998b). Oocyte maturation and fertilisation was not affected in either study. Neither study presented any information on the underlying metabolic profiles of these animals, nor on metabolic hormone concentrations within follicular fluid. Furthermore, the post-fertilisation developmental potential of these oocytes was not assessed. At present the detrimental effects of hyperinsulinaemia on the follicle enclosed oocyte are not understood. However, Eppig *et al.* (1998) showed that the co-incubation of insulin (~126 mIU/ml) and FSH with murine oocyte-granulosa cell complexes derived from pre-antral follicles reduced the percentage of fertilised oocytes developing into blastocysts, and this observation is broadly in keeping with that reported here.

2.5. Conclusions

Results from the present study show that the effects of level of feeding on oocyte quality are dependent on the body condition of the animal. High levels of feeding improve post-fertilisation development for animals in low body condition but reduce post-fertilisation development for animals in good body condition. Furthermore, the effects of high levels of feeding are cumulative, with blastocyst yields for heifers of moderately good body condition deteriorating over time relative to heifers of low body condition. This study also presents, for the first time, evidence of impaired oocyte quality associated with hyperinsulinaemia in ruminants and establishes a nutritional framework to enable quantifiable assessments of future dietary effects on oocyte quality in heifers to be made.

CHAPTER 3

Dietary carbohydrates and lipids affect the developmental competence of oocytes.

3.1. Introduction

The terminal stages of oocyte growth (during antral follicle development) and the mechanisms controlling oocyte maturation *in vivo* are poorly understood, but by many are believed to be the key to successful post-fertilisation development (Krisher and Bavister, 1998; Blondin *et al.*, 1995, 2002; Rizos *et al.*, 2002a). While some laboratories have tried to improve the composition of *in vitro* maturation media others have sought to promote oocyte maturation *in vivo*. Recent studies by Blondin *et al.* (2002) reported that their ovarian stimulation protocol produced a very high proportion of developmentally competent oocytes, more representative of those collected following spontaneous ovulation, as indicated by a high (80%) proportion of COCs reaching blastocyst stage during *in vitro* culture.

Nutrition can influence fertility by targeting key processes at a number of levels within the hypothalamo-pituitary-ovarian axis. However, identifying the specific factors involved in the nutritional regulation of oocyte and embryo development *in vivo* has proved difficult. Recent studies (e.g. Wrenzycki *et al.*, 2000) have focused on gene expression patterns within oocytes and embryos, but have shed little light on dietary factors contributing to oocyte quality. The previous study reported in Chapter 2 demonstrated that oocyte and subsequent embryo development *in vitro* are influenced by both the BCS of the donor animal and level of feeding. Both high BCS and high levels of feeding increased circulating concentrations of insulin and leptin. Although the higher level of feeding improved oocyte quality in Low BCS heifers, it impaired oocyte competence in heifers of Moderate BCS. Having tested the effects of level of feeding on oocyte quality, the main objective of the present study was to test the effects of alterations in dietary composition by feeding different sources of carbohydrates either with or without a source of rumen protected lipids. In ruminants, the principle sources of energy are provided by volatile fatty acids (VFA) from the rumen. The amounts of specific VFA produced are dependent on the source of concentrate (Moloney *et al.*, 1994). Starch based diets lead to the preferential production of propionate, which is the main precursor for glucose synthesis in the liver, and the more slowly fermented fibre based diets favour acetate production (Sloan *et al.*, 1988). Glucose can act directly on the hypothalamo-pituitary-ovarian axis (Bucholtz *et al.*, 1996), but also leads to elevated concentrations of insulin, which have been linked to reproductive function in cows (e.g.

Beam and Butler, 1999; Landau *et al.*, 2000; Armstrong *et al.* 2002a; Gong *et al.*, 2002; Selvaraju *et al.*, 2002). On the other hand, dietary lipids are the least investigated group of nutrients but unlike other nutrients, directly enter the general circulation without being processed by the liver (e.g. Christie, 1981; Cunningham, 1997). Dietary lipids can affect various reproductive parameters such as CL function (Hawkins *et al.*, 1995; Williams and Stanko, 1999) and folliculogenesis (Lammoglia *et al.*, 1997; Thomas *et al.*, 1997; Beam and Butler, 1998). However, it is still unclear how the inclusion of protected lipid affects follicle and oocyte development. Therefore, the aim of the current study was to assess the effects of alterations in oocyte donor carbohydrate and lipid metabolism during ovarian stimulation indicated by the production and viability of blastocysts *in vitro*.

3.2. Materials and Methods

3.2.1. Animals and dietary treatments

Thirty-two beef x dairy heifers of approximately 20 months of age and with an average initial weight (mean±SEM) of 396.9±4.1 kg were used. All animals were accommodated in individual pens on slatted floors. Prior to the experiment, their BCS was determined (six-point scale of Lowman *et al.* 1976) and they were allocated accordingly to either a Low (2.5±0.09 units) or Moderate (3.4±0.06 units) BCS group. Animals within each BCS group were then ranked again by BCS and allocated to one of four dietary treatments: High Fibre (F) either with or without 6% rumen-protected fatty acids (calcium soaps of fatty acids; Megalac®, Volac International Ltd, Herts, UK), or High Starch (S) again either with or without 6% rumen protected fatty acids. This resulted in a 2 x 2 factorial arrangement with diets containing predominantly one of two types of carbohydrate (Fibre vs Starch), either with or without Megalac, (6% vs 0%) replicated four times within each of the Low and Moderate BCS groups. Alterations in the source of fermentable carbohydrate and the lipid content of the diets inevitably led to differences in energy density (MJ metabolizable energy (ME)/kg dry matter) (Table 3.1). Concentrate levels for each of the four dietary treatments, therefore, were adjusted to ensure that each animal received 1000 kJ ME/kg liveweight$^{0.75}$/day, equivalent to approximately twice the maintenance energy requirements for these animals (AFRC, 1993). This feeding level also accounted for the intake of straw, which was fixed at 2 kg

fresh material daily. Animals were weighed every two weeks and feeding levels adjusted accordingly. The experimental diets were introduced one week prior to the initial period of oestrous synchronization (Figure 3.1).

Table 3.1. Composition of concentrates and chemical analysis of feeds.

Principle Carbohydrate	**Fibre**		**Starch**	
Megalac (%)	**0**	**6**	**0**	**6**
(a) Ingredient (g/kg)				
Wheat	-	-	450	500
Maize	-	-	200	200
Malt culms	-	62	14	146
Wheat Feed	219	250	250	-
Oat Feed	200	200	28	18
Sugar beet pulp	489	307	-	-
Rapeseed Meal	-	33	-	18
Urea	12	8	8	8
Molasses	80	80	50	50
Megalac	-	60	-	60
	1000	1000	1000	1000
(b) Chemical analysis – Concentrate ‡				
Dry matter, g/kg	864	863	857	866
ME, MJ/kg DM	11.3	12.1	13.1	14.2
Crude protein, g/kg DM	139	140	153	149
NDF, g/kg DM	361	380	170	143
Starch, g/kg DM	210	187	512	495
AHEE, g/kg DM	24	67	35	71
(c) Chemical analysis – Straw ‡				
Dry matter, g/kg		860		
ME, MJ/kg DM		5.6		
Crude protein, g/kg DM		31		
NDF, g/kg DM		855		
Starch, g/kg DM		11		
AHEE, g/kg DM		18		

‡DM – Dry matter; ME - Metabolisable energy; NDF – Neutral Detergent Fibre; AHEE – Acid Hydrolysed Ether Extract.

3.2.2. Ovarian stimulation and follicular growth

All heifers had their oestrous cycles synchronised using a 10 day progesterone-releasing device CIDR (SmithKline Beecham, Tadworth, Surrey, UK) containing 1.9 g of progesterone, and prostaglandin $F_{2\alpha}$ analogue (Luprositol 15 mg; Prosolvin, Intervet,

Cambridge, UK) administered intramuscularly 8 days after CIDR insertion. On Day 5 of the oestrous cycle all visible follicles were aspirated by OPU, primarily to induce a new follicular wave according to the protocol of Blondin *et al.* (2002). Aspirants were collected into conical tubes containing warm PBS supplemented with 0.1% w/v BSA (FAF) and antibiotics (Appendix 1) and transported in a polystyrene box (kept at 37°C) to the laboratory, where they were filtered and COCs retrieved. The COCs were assessed according to the number of compact cumulus cell layers and cytoplasmic appearance (Appendix 1), prior to fatty acid analysis (Chapter 5). The ovarian stimulation commenced 48 h after the first OPU session (Day 5 of the oestrous cycle; Figure 3.1) and was based on the method of Blondin *et al.* (2002), but modified as follows. Four constant doses of ovine FSH (total amount 9 mg of NIADDK-oFSH-17; Ovagen, ICP, New Zealand) were administered intramuscularly at 12 h intervals over 48 h. There then followed a 40 h "coasting" period prior to OPU, during which no further FSH was administered. A GnRH analogue (0.012mg Buserelin; Receptal, Hoechst Animal Health, Milton Keynes, UK) was administered intravenously six hours prior to OPU. A second similar period of ovarian stimulation and OPU (during the same oestrous cycle) followed (Figure 3.1).

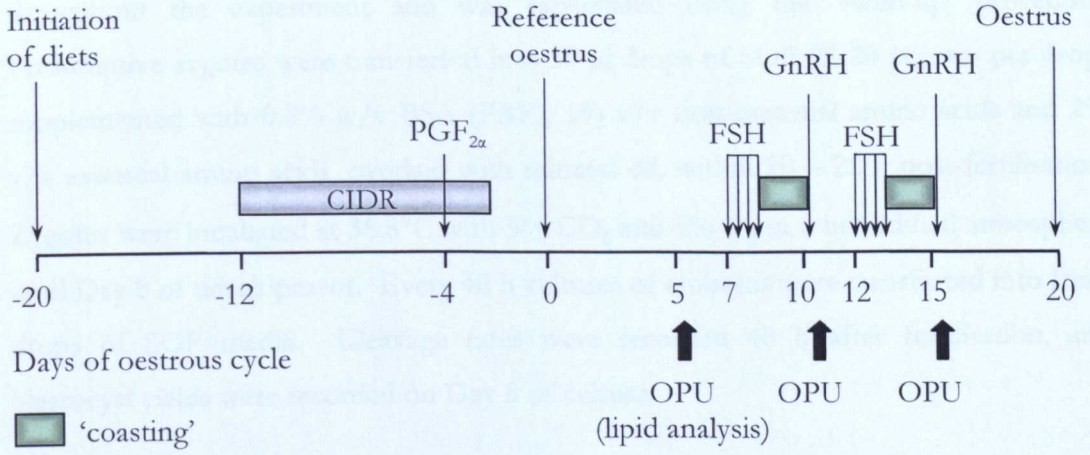

Figure 3.1. Schematic representation of oestrus synchronisation using CIDR inserts and a prostaglandin ($PGF_{2\alpha}$) injection. Ovarian stimulation involved 4 doses of FSH administered over a 48 h period and GnRH was administered 6 h prior to OPU. Day 0 – reference oestrus.

Ovarian follicular growth was monitored daily for a four-day period commencing three days prior to oestrus. Ovarian follicles were visualised by transrectal real-time

ultrasonography using a 5 MHz transducer (Aloka SSD-500 V; BCF, Livingstone, UK). The total number of visible (> 2mm) follicles and their diameters were recorded.

3.2.3. Oocyte recovery and embryo culture *in vitro*

Details of media composition and IVP procedures are provided in Appendix 1. Animal identity was retained throughout. All heifers were subjected to OPU on Days 10 and 15 of the oestrous cycle. Follicular fluid was collected into conical tubes containing warm PBS supplemented with 0.2% w/v BSA (FAF) and transported to the laboratory where aspirants were filtered and searched for COCs. These were assessed as described previously, washed twice in fresh PBS/BSA and then twice in Maturation medium. Finally, selected grade 1 – 3 COCs were matured for 24 h in 50 µl drops (≤ 20 COCs per drop) of Maturation medium with GC monolayers, overlaid with mineral oil and incubated at 38.8°C and 5% CO_2 in a humidified atmosphere. Granulosa cell monolayers were prepared from a single batch of frozen-thawed GCs throughout the experiment and were cultured for 4 days prior to each OPU session, with half the medium renewed every 48 h. Matured COCs were fertilised in 46 µl drops of Fertilisation medium. Semen from the same bull (Beechpike Energy) was used throughout the experiment and was capacitated using the 'swim-up' procedure. Presumptive zygotes were transferred into 30 µl drops of SOF (≤ 20 zygotes per drop) supplemented with 0.3% w/v BSA (FAF), 1% v/v non-essential amino acids and 2% v/v essential amino acids, overlaid with mineral oil, within 20 – 22 h post-fertilisation. Zygotes were incubated at 38.8°C with 5% CO_2 and 5% O_2 in a humidified atmosphere until Day 8 of development. Every 48 h cultures of embryos were transferred into fresh drops of SOF media. Cleavage rates were recorded 48 h after fertilisation, and blastocyst yields were recorded on Day 8 of culture.

3.2.4. Animal slaughter and GC culture

All heifers were slaughtered at a local abattoir at the end of the experiment and ovaries from each animal recovered. On arrival at the laboratory ovaries were washed with warm (37°C) PBS followed by follicular aspiration of all visible follicles using a 21 gauge needle attached to a 5 ml syringe. Aspirants were collected into conical tubes containing 1 ml of warm Search medium (Appendix 1) and then searched for COCs. Selected

(Grades 1 – 3) COCs were matured, fertilised and zygotes cultured in the same conditions as for OPU (described earlier and in Appendix 1). The remaining follicular fluid containing GCs was centrifuged for 5 min at 300 x *g*. Granulosa cells harvested from two heifers within treatment were pooled together to obtain a sufficient number of cells for *in vitro* culture. Cell pellets were resuspended in 1 ml of Search medium and gently transferred onto 2 ml of histopaque and centrifuged for 20 min at 600 x *g*. After 20 min, the top 2 layers containing live GCs were transferred into a centrifuge tube and washed twice (centrifugation for 5 min at 300 x *g*) with Cell Culture medium. This medium consisted of 90% v/v TCM199 and 10% v/v of double distilled water supplemented with 0.1% w/v BSA (Fraction V), 1 ng/ml FSH, 10 ng/ml of insulin, 0.5 μg/ml bovine transferrin, 5.0 ng/ml sodium selenite, 50 IU/ml penicillin, 50 μg/ml streptomycin, 20 ng/ml tetracycline and 2.5 μg/ml amphotericine. The final cell pellet was resuspended in 1 ml of Cell Culture medium and a sample taken to establish cell number by trypan blue exclusion. Granulosa cells were cultured in 24-well plates coated with collagen (one treatment per plate), at a seeding density of 2 – 3.5 x 10^5 cells/ml in 500 μl of Cell Culture medium per well, incubated for 96 h at 38.8°C and 5% CO_2 in a humidified atmosphere with 80% of the medium renewed at 48 h. Cell proliferation and viability were assessed at the end of the incubation period by trypan blue exclusion and by MTT assay, respectively (Appendix 1).

3.2.5. Total cell counts and TUNEL assay

Day 8 blastocysts were fixed in 4% w/v paraformaldehyde solution and stored in a cool room until cell number and the percentage of TUNEL positive cells were established. Fixed blastocysts were subjected to the sequence of staining procedures for cell counts (Hoechst 33342) and TUNEL positive cells based on a modified ApopTag® staining kit protocol (Appendix 2, section A.2.2). Stained blastocysts were visualised by fluorescence microscopy and cell number and the number of TUNEL positive cells per embryo recorded.

3.2.6. Hormone assays

Blood samples were collected by jugular venipuncture into EDTA treated vacuum tubes. Retrieved plasma was transferred in duplicates into auto-analyser cups and stored

at –20°C until hormone analyses were conducted. Detailed protocols for the RIAs are to be found in Appendix 3. Plasma insulin, IGF-I and leptin concentrations were measured on Day 3 prior oestrus (Day -3), the day before oestrus (Day -1) and on the day of oestrus (Day 0).

The sensitivity of the plasma progesterone assay at ED80 was 0.62 ng/ml. The inter-assay coefficients of variation for low-, medium- and high quality controls were 13.0, 11.3 and 12.0%, respectively, and corresponding intra-assay coefficients of variation were 12.7, 9.8 and 6.2%, respectively. The detection limit for the insulin assay was 2.6 μIU/ml. The inter-assay coefficient of variation for low-, medium- and high quality controls were 10.0, 7.9 and 10.3%, respectively, and corresponding intra-assay coefficient of variation were 10.0, 7.9 and 10.3%. All samples for measurement of IGF-I concentrations were processed in a single assay with a detection limit of 12.5 ng/ml and the intra-assay coefficient of variation for low- and high quality controls were 11.7 and 8.4%, respectively. The sensitivity of the leptin assay was 0.2 ng/ml and the inter-assay coefficient of variation for low-, medium- and high quality controls were 13.0, 9.7 and 10.5%, respectively and corresponding intra-assay coefficients of variation were 12.8, 9.9 and 10.3%, respectively.

3.2.7. Statistical analyses

Endocrine data was analyzed by repeated measures ANOVA within GenStat 6 (Genstat, 2002), where BCS (Low vs Moderate), carbohydrate source (Fibre vs Starch), fatty acid inclusion (0 vs 6%) and interactions between these factors formed the 'between animal stratum'; sample day, BCS, carbohydrate source and fatty acid inclusion formed the 'within animal x day stratum'. Oocyte recovery and *in vitro* embryo production data (i.e. oocytes cleaved and Day 8 blastocyst yields) were analyzed using generalized linear models assuming binomial errors with logit link functions within the same statistical package. Terms fitted to the model were animal, BCS, carbohydrate source, fatty acid inclusion and OPU session. Comparisons between means were conducted by analysis of deviance. Data presented are predicted means with either appropriate SED or SEM. A P value of 0.05 or less was considered to be significant.

3.3. Results

3.3.1. Animal live weight and body condition

Heifers at the beginning of the experiment were divided into two groups depending on animal BCS. The average live weight and BCS (±SEM) of heifers in the Low BCS group were 382.2±4.9 kg and 2.6±0.1 units respectively, and were lower (P<0.001) than that of the Moderate BCS heifers (411.7±4.2 kg and 3.0±0.1 units, respectively). At the end of the experiment both overall mean animal live weight and BCS were greater (P<0.001) than at the beginning (473.2 vs 396.9 kg, SED=5.5; and 3.06 vs 2.8 units, SED=0.07, respectively). Growth rates, however, were greater (P<0.05) for heifers in Moderate than in Low BCS (1.5 vs 1.3 kg/day; SED=0.07) and for animals fed without than with Megalac (1.5 vs 1.3 kg/day, SED=0.07, respectively). Carbohydrate source did not affect animal growth rate.

3.3.2. Endocrinology

Mean plasma progesterone concentrations were greater (P=0.063) for heifers fed the diets with than without protected lipid (7.1 vs 5.9 ng/ml, SED=0.59) even though differences did not reach statistical significance. Progesterone concentrations in plasma did not vary between the F and the S diets or between animal BCS groups. However, analysis by body condition indicated that mean plasma progesterone concentrations in Low BCS heifers were greater (P=0.05) for those heifers fed the F than the S diet (7.1 vs 6.2 ng/ml, SED=0.40) and were further increased by the inclusion of Megalac (8.1 vs 5.1 ng/ml for 6 vs 0% respectively, SED=0.40; P<0.001) (Figure 3.2). In addition, peak plasma progesterone concentrations (Day 15 of the oestrous cycle) were greater (P<0.05) for heifers fed with than without protected lipid (14.4 vs 8.6 ng/ml, SED=1.56). There was also an interaction (P<0.05) between principle carbohydrate source and the inclusion or exclusion of Megalac (Table 3.2). In contrast, mean and peak plasma progesterone concentrations for animals in Moderate BCS averaged 6.4±0.4 ng/ml and 12.9±1.0 ng/ml respectively, and were unaffected by nutritional treatments (Figure 3.2).

Mean plasma insulin concentrations were greater (P<0.01) for heifers fed the S than the F diet (16.8 vs 12.4 μIU/ml, SED=1.16), but the inclusion of Megalac and animal BCS

did not affect plasma insulin concentrations (Table 3.2; Figure 3.3.a). Plasma concentrations of IGF-I were also greater ($P<0.05$) for the S than the F diet (255.7 vs 226.9 ng/ml, SED=11.0), but only for animals of Moderate BCS. Mean plasma IGF-I concentrations in Low BCS heifers were unaffected by dietary treatments. Numerically the inclusion of Megalac reduced plasma concentrations of IGF-I compared to diets without lipid supplementation (231.4 vs 251.8 ng/ml for 6 and 0% respectively, SED=13.87), although this difference was not statistically significant (Table 3.2).

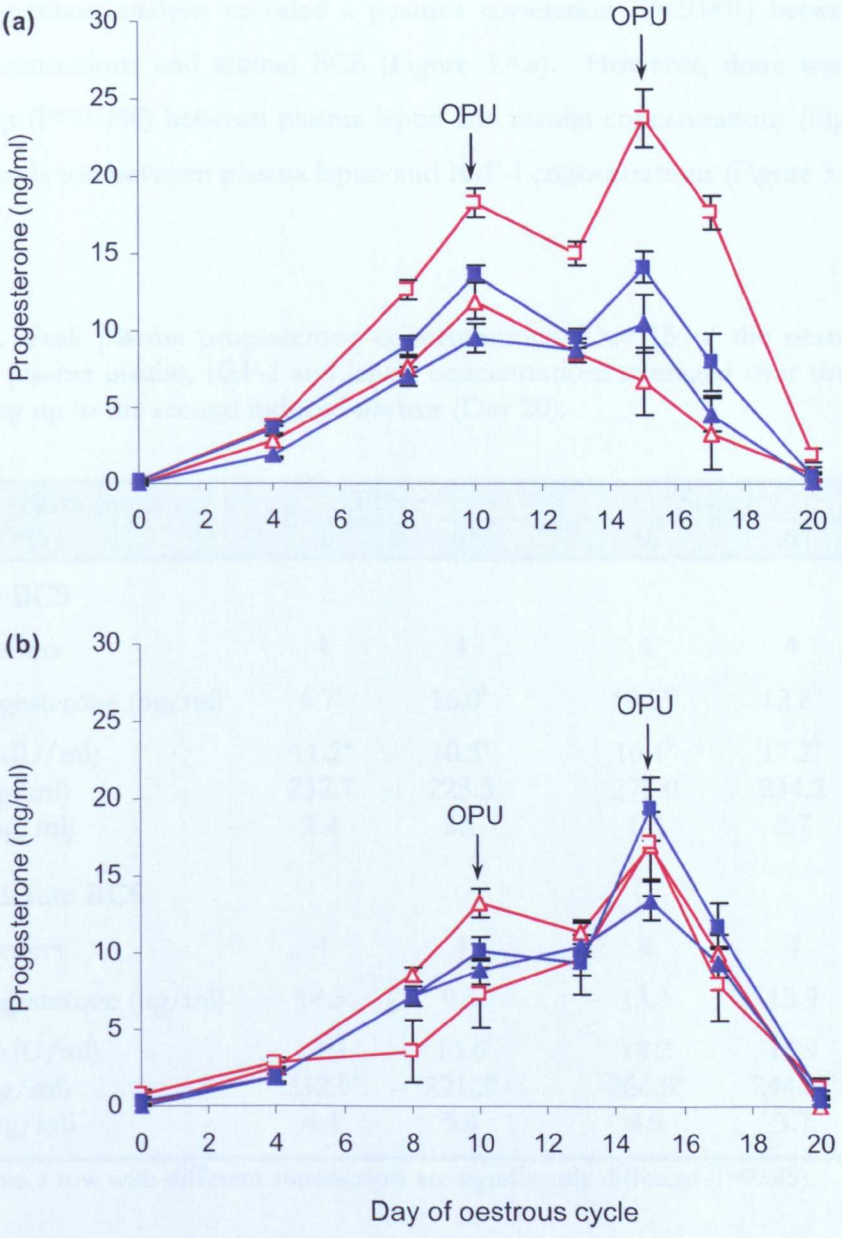

Figure 3.2. Mean (±SEM) concentrations of plasma progesterone measured during a single oestrous cycle for animals in Low (a) and Moderate BCS (b). Dietary treatments are marked as follows: (Δ) F0, (□) F6, (▲) S0 and (■) S6.

In contrast to insulin and IGF-I, mean plasma leptin concentrations were unaffected by nutritional treatments (average of 3.44±0.3 ng/ml), but they were greater (P<0.001) for heifers in Moderate compared to Low BCS (4.63 vs 2.25 ng/ml; SED=0.39) (Figure 3.3.c). Furthermore, for animals in Low BCS, there was an interaction (P=0.053) between the carbohydrate source and the presence or absence of Megalac, which indicated that the increase in plasma leptin concentrations was greatest for treatments F0 and S6 than F6 and S0 (Table 3.2), but differences were not statistically significant.

Finally, regression analysis revealed a positive correlation (P<0.001) between plasma leptin concentrations and animal BCS (Figure 3.4.a). However, there was a weaker relationship (P=0.088) between plasma leptin and insulin concentrations (Figure 3.4.b), and no correlation between plasma leptin and IGF-I concentrations (Figure 3.4.c).

Table 3.2. Peak plasma progesterone concentrations (Day 15 of the oestrous cycle) and mean plasma insulin, IGF-I and leptin concentrations averaged over the last three days leading up to the second induced oestrus (Day 20).

Principle carbohydrate	**Fibre**		**Starch**		**SED**
Megalac (%)	**0**	**6**	**0**	**6**	
(a) Low BCS					
No. of heifers	4	4	4	4	
Peak progesterone (ng/ml)	6.7^{a}	16.0^{b}	10.5^{ab}	12.8^{b}	2.21
Insulin (μIU/ml)	11.2^{a}	10.5^{a}	16.1^{b}	17.2^{b}	2.08
IGF-I (ng/ml)	232.7	225.5	275.0	234.2	24.81
Leptin (ng/ml)	2.4	2.1	1.8	2.7	0.45
(b) Moderate BCS					
No. of heifers	4	4	4	4	
Peak progesterone (ng/ml)	14.5	9.5	13.1	13.9	2.98
Insulin (μIU/ml)	14.3	13.6	18.2	15.9	2.60
IGF-I (ng/ml)	232.5^{a}	221.2^{a}	266.8^{b}	244.6^{ab}	15.56
Leptin (ng/ml)	4.4	5.4	4.9	3.7	0.93

Means within a row with different superscripts are significantly different (P<0.05).

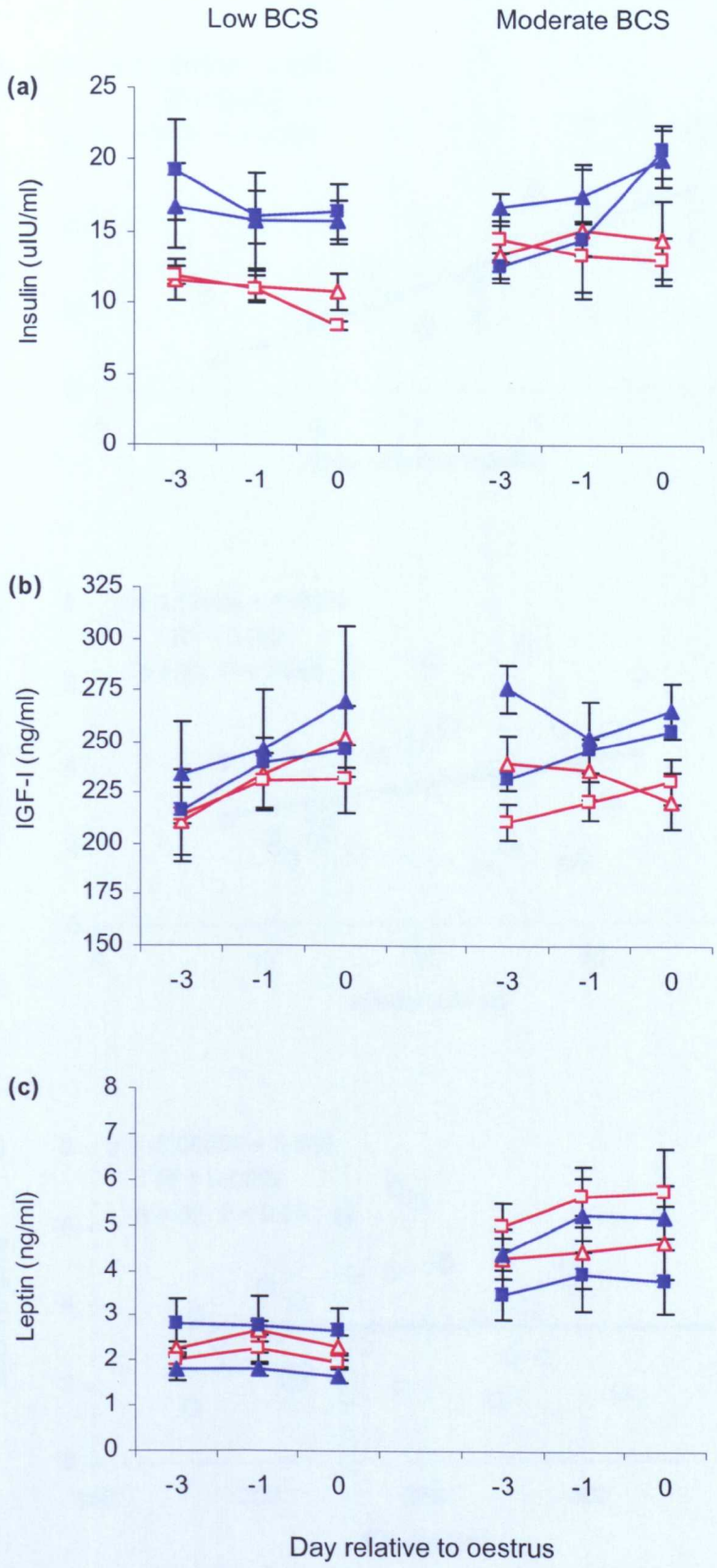

Figure 3.3. Mean (±SEM) plasma concentrations of insulin (a), IGF-I (b) and leptin (c) measured during the three day period prior to oestrus (0) for heifers in Low and in Moderate BCS. Dietary treatments are marked as follows: F0 (△), F6 (□), S0 (▲) and S6 (■).

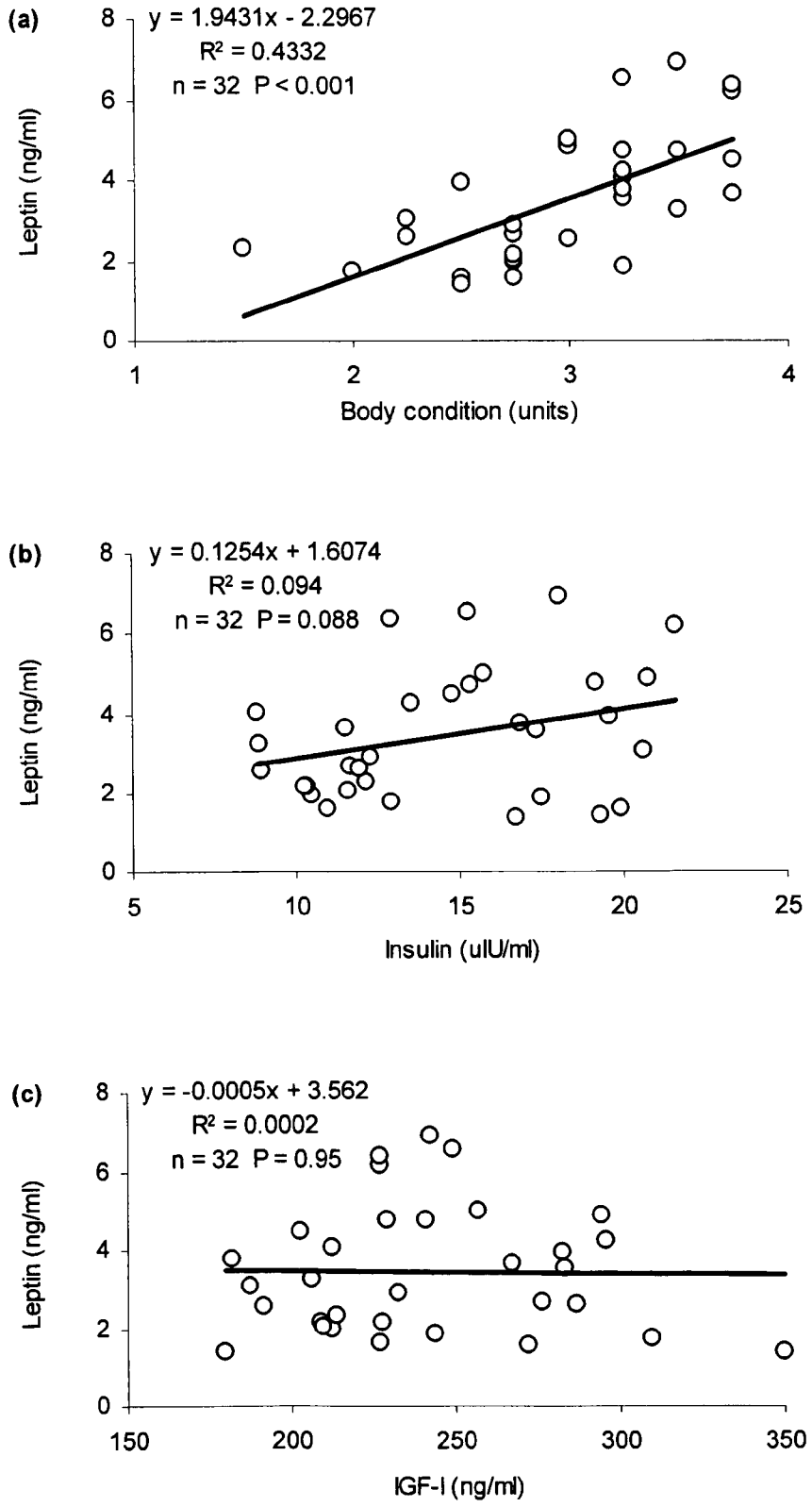

Figure 3.4. Relationships between plasma leptin (ng/ml) and animal BCS (units) (a); plasma leptin and insulin (μIU/ml) (b); and plasma leptin and IGF-I (ng/ml) (c).

3.3.3. Follicular development

The number of follicles aspirated on Day 5 of the oestrous cycle prior to ovarian stimulation were fewer ($P<0.05$) than the number aspirated following ovarian stimulation. However, the number of oocytes recovered did not vary between non-stimulated and stimulated OPU sessions (Figure 3.5.a). Nevertheless, the proportion of good (Grades 1 and 2) and poor (Grades 3 and 4) quality oocytes differed ($P<0.001$) between un-stimulated and stimulated animals, with ovarian stimulation increasing the proportion of good whilst reducing the proportion of poor quality oocytes (Figure 3.5.b).

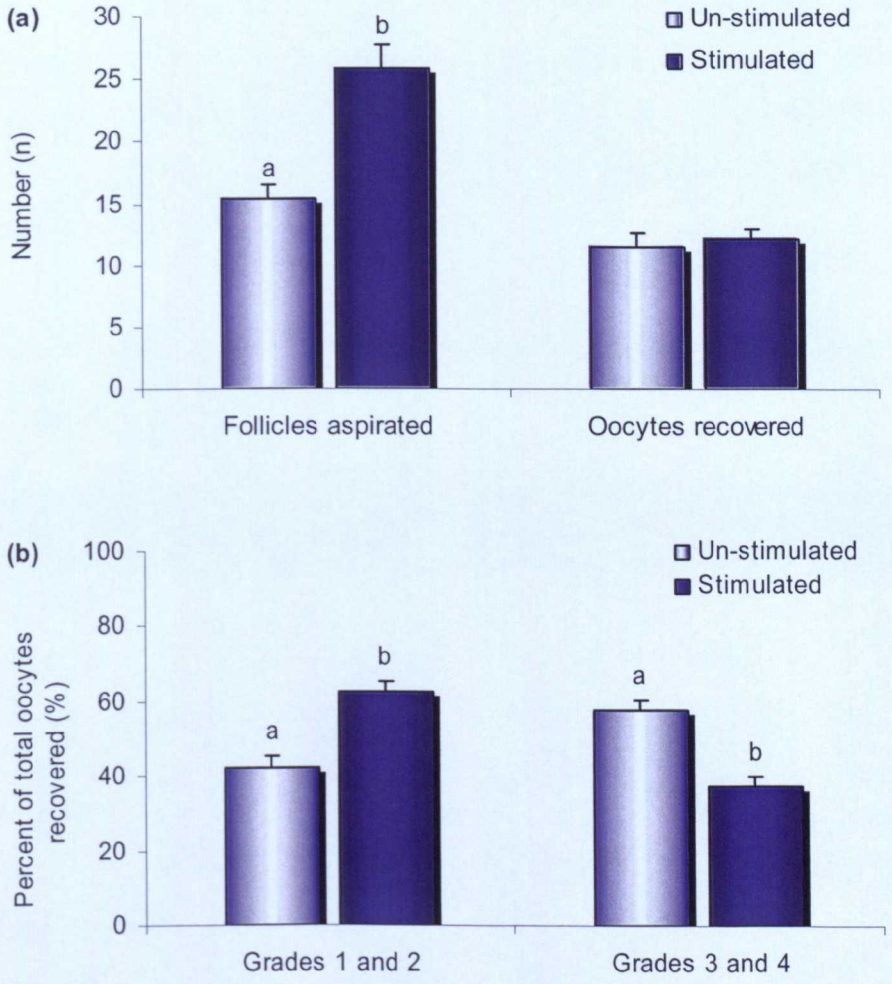

Figure 3.5. Comparisons between un-stimulated and gonadotrophin stimulated heifers in terms of follicle number and oocytes recovered (±SEM) (a) and percentage (±SEM) of oocytes of Grades 1 and 2, and 3 and 4 (b). Different letters indicate significant ($P<0.001$) difference between un-stimulated and stimulated animals within parameter measured.

The total number of follicles observed during scanning (four-day period commencing three days prior to oestrus) averaged 14.6±1.2 and was unaffected by nutritional treatments and did not differ between animal BCS groups. The number of large- (> 8 mm) and small-sized (< 4 mm) follicles also did not vary between nutritional treatments and between BCS groups, but the number of medium-sized (4 to 8 mm) follicles was greater (P=0.053) for heifers fed the F than the S diet (3.9 vs 2.9, SED=0.48). The maximum diameter of the dominant follicle averaged 13.3±0.8 mm and did not differ between dietary treatments nor between animal BCS groups. Analysis by BCS, however, indicated that nutritional treatments had a more significant effect on follicular growth in Low than in Moderate BCS heifers (Table 3.3). The number of medium-sized follicles was greater (P=0.09) and the number of small follicles less (P=0.07) for heifers fed the F compared to the S diet (4.1 vs 2.7, SED=0.75 and 7.4 vs 11.3, SED=1.93, respectively), but these differences were not statistically significant. There was also an interaction (P<0.05) between carbohydrate source and the inclusion or exclusion of Megalac on the maximum diameter of the DF, which was greatest for treatments F0 and S6 compared to F6 and S0 (Table 3.3).

Table 3.3. The number of small (<4 mm), medium (4 to 8 mm) and large (>8 mm) follicles per heifer and the maximum diameter of the dominant follicle (DF) during the four-day period leading up to ovulation.

Principle carbohydrate	**Fibre**		**Starch**		**SED**
Megalac (%)	**0**	**6**	**0**	**6**	
(a) Low BCS					
No. of heifers	4	4	4	4	
Total follicle number (n)	14.2	12.6	16.8	14.4	3.15
Large follicles (n)	2.4	1.5	1.4	1.6	0.44
Medium follicles (n)	5.0	3.3	2.9	2.6	1.05
Small follicles (n)	6.9	7.9	12.5	10.0	2.73
Max. diameter of DF (mm)	15.5^{a}	11.3^{b}	12.8^{ab}	14.5^{ab}	1.77
(b) Moderate BCS					
No. of heifers	4	4	4	4	
Total follicle number (n)	14.6	13.8	13.4	15.0	3.69
Large follicles (n)	1.6	2.1	1.6	1.8	0.45
Medium follicles (n)	3.8	3.8	3.5	2.9	0.92
Small follicles (n)	9.2	7.9	8.2	9.2	3.77
Max. diameter of DF (mm)	12.3	14.5	12.3	13.8	2.67

Means within a row with different superscripts are significantly different (P<0.05).

3.3.4. Oocyte and embryo development *in vitro*

The mean (±SEM) number of follicles aspirated (25.9±1.9), oocytes recovered (12.1±0.9) and oocytes matured (12.0±0.9) per animal were unaffected by nutritional treatments and did not vary between animal BCS groups (Table 3.2). However, heifers fed the F diet had a greater (P=0.057) proportion of good (Grades 1 and 2) quality oocytes (67.5 vs 57.1% for treatments F and S respectively, SED=5.36) and a lower (P=0.057) proportion of poor (Grades 3 and 4) quality oocytes (32.5 vs 42.9% for the respective treatments, SED=5.36). Furthermore, heifers in Moderate BCS had a greater (P=0.065) proportion of good quality oocytes compared to Low BCS heifers (67.4 vs 57.3%, SED=5.36), but these differences did not reach statistical significance. As expected, the increased proportion of good quality oocytes correlated with greater (P<0.05) cleavage rates for heifers fed the F compared to the S diet (78.4 vs 68.6%, SED=4.79) and there was also an interaction (P=0.076) between carbohydrate source and the presence or absence of Megalac, which indicated that cleavage rates were greater for treatments F6 and S0 compared to F0 and S6 (72.0, 84.7, 70.9 and 66.3% for treatments F0, F6, S0 and S6, respectively, SED=6.77). Although nutritional treatments did not have an effect on the number of Day 8 blastocysts and Day 8 blastocyst yields, the number of blastocysts was somewhat greater (P=0.057) for heifers in Low than in Moderate BCS (3.41 vs 2.16, SED=0.64). Analysis by animal BCS revealed, that for heifers in Low BCS, there was an interaction (P=0.086) between source of carbohydrate and the inclusion of Megalac on the number of follicles aspirated, which indicated that for treatments F6 and S0 follicle number was increased compared to F0 and S6 treatments (Table 3.4). Blastocyst yields were also greater (P=0.05) for animals fed the F compared to the S diet (43.6 vs 24.6%, SED=7.65), but were reduced (P<0.05) when Megalac was included in the diet (27.7 vs 42.9%, SED=7.65) (Figure 3.6.b). In contrast, for heifers in Moderate BCS only cleavage rates were greater (P<0.05) for animals fed the F than the S diet (79.0 vs 65.5%, SED=6.41) (Figure 3.6.a). All other parameters did not vary between dietary treatments. There was, however, an indication that the number of Day 8 blastocysts was greater for animals fed diets with than without Megalac (2.8 vs 1.5 for 6 and 0% respectively, SED=0.75), but the difference was not statistically significant (P=0.09) (Table 3.4).

The total number of cells (±SEM) per Day 8 blastocyst averaged 123.8±4.6 and was unaffected by nutritional treatment. There was, however, an interaction ($P<0.05$) between carbohydrate source and the inclusion or absence of Megalac, which indicated that for treatments F6 and S0 total cell number was greater than for F0 and S6 treatments (107.8, 132.2, 138.3 and 114.8 cells/embryo for treatments F0, F6, S0 and S6 respectively, SED=12.2). Analysis by animal BCS demonstrated that the same interaction existed only for animals in Low BCS, whereas for heifers in Moderate BCS total cell number averaged 116.5±6.7 and was unaffected by nutritional treatment (Table 3.4). The percentage of TUNEL positive cells averaged 4.2±0.5% and did not differ between dietary treatments nor between animal BCS groups (Table 3.4).

Table 3.4. Mean number of follicles aspirated per animal during the two FSH stimulated sessions of OPU and the number of oocytes recovered. Results following IVP are reported as numbers of oocytes cleaved, the number of zygotes reaching the blastocyst stage by Day 8, total cell number of cells and percentage TUNEL positive cells per blastocyst.

Principle carbohydrate	Fibre		Starch		SED
Megalac (%)	0	6	0	6	
(a) Low BCS					
No. of heifers	4	4	4	4	
Follicles aspirated (n)	25.6	35.3	32.9	22.0	8.15
Oocytes recovered (n)	11.3	14.4	15.4	10.5	3.97
Oocytes matured (n)	11.3	14.4	15.3	10.1	3.94
Oocytes cleaved (n)	8.6	11.5	10.9	7.5	3.06
Blastocyst number (n)	4.6	3.9	3.5	1.6	1.43
Total cell count (n)	111.5^{a}	131.8^{ab}	154.2^{b}	122.8^{ab}	14.5
TUNEL (%)	4.4	4.9	3.7	3.1	1.8
(b) Moderate BCS					
No. of heifers	4	4	4	4	
Follicles aspirated (n)	21.1	24.4	22.6	23.4	6.73
Oocytes recovered (n)	12.3	13.4	10.4	9.6	3.52
Oocytes matured (n)	11.9	13.1	10.3	9.5	3.49
Oocytes cleaved (n)	7.6	10.3	6.6	6.1	2.39
Blastocyst number (n)	1.0	3.5	2.0	2.1	1.06
Total cell count (n)	109.4	130.3	118.8	106.2	20.2
TUNEL (%)	4.0	3.5	3.2	6.2	2.4

Means within a row with different superscripts are significantly different ($P<0.05$).

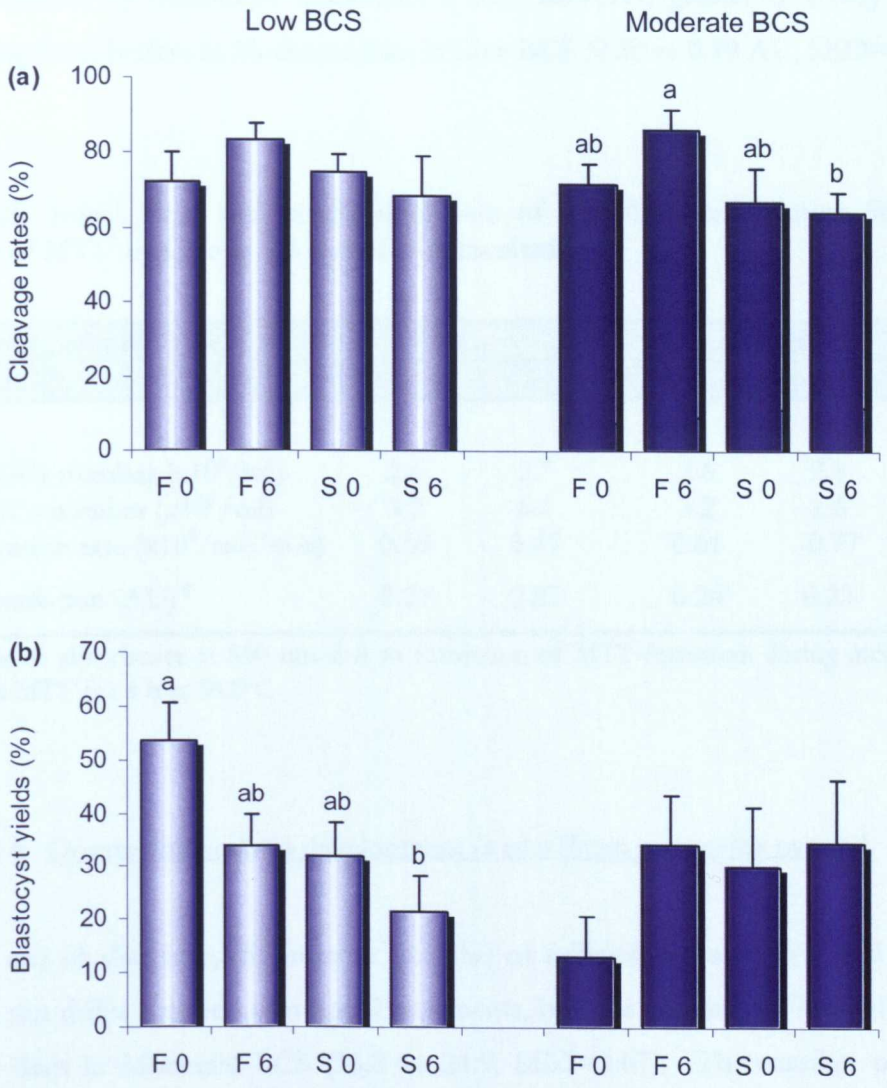

Figure 3.6. Mean (±SEM) cleavage rates (%) (a), and blastocyst yields (%) (b) for animals in Low compared to Moderate BCS. Different letters indicate significant ($P<0.05$) difference between treatments within animal BCS group.

3.3.5. Granulosa cell culture

Seeding density varied between nutritional treatments at the initiation of granulosa cell culture (Table 3.5), therefore cell proliferation assessed after 96 h in culture is presented as cell proliferation rate calculated by subtracting the initial seeding density from the final cell number at 96 h (Table 3.5). Granulosa cell numbers after 96 h in culture and their proliferation rate during that time did not differ between nutritional treatments nor between animal BCS groups (Table 3.5). Although GC viability (cleavage of MTT) was

also unaffected by nutritional treatments it was, however, greater (P<0.01) for cells originating from heifers in Moderate than in Low BCS (0.28 vs 0.19 AU, SED=0.023).

Table 3.5. Initial, final and proliferation rate of granulosa cells during 96 h; and cleavage of MTT† by GCs at the end of 96 h incubation.

Principle carbohydrate	Fibre		Starch		SED
Megalac (%)	0	6	0	6	
Initial GCs number (x10^5/ml)	2.6	2.7	2.6	2.4	
Final GCs number (x10^5/ml)	3.2	6.1	3.2	1.6	2.07
Proliferation rate (x10^5/ml/96 h)	0.59	3.47	0.61	-0.77	2.18
MTT oxidation (AU)†	0.21	0.27	0.24	0.23	0.03

† Increase in absorbance at 550 nm due to formation of MTT-formazan during incubation of cells with MTT for 4 h at 38.8°C.

3.3.6. Oocyte and embryo development *in vitro* (from *post mortem* ovaries)

On the day of slaughter, the number (±SEM) of follicles aspirated averaged 30.3±2.9 and did not differ between nutritional treatments, but was greater (P=0.067) for heifers in Low than in Moderate BCS (35.8 vs 24.9, SED=1.67). The number of oocytes recovered, matured and cleaved averaged 24.1±2.8, 22.7±2.6 and 13.5±1.5 respectively, and did not vary between dietary treatments nor between animal BCS groups. Similarly, mean (±SEM) cleavage rates (66.3±2.9%) and blastocyst yields (15.1±2.8%) (Figure 3.7) were unaffected by nutritional treatments and by animal BCS (Table 3.6).

Total cell number per embryo averaged 108.6±6.2 and did not vary between nutritional treatments (Table 3.6), but there was an interaction (P=0.074) indicating that cell number per embryo was greater for treatments F0 and S6 than for treatments F6 and S0 (119.2, 91.0, 106.3 and 122.9 cells/embryo for treatments F0, F6, S0 and S6 respectively, SED=24.31). In addition, embryos from Low BCS heifers had more (P<0.05) cells compared to embryos from Moderate BCS heifers (124.5 vs 95.2 cells/embryo, SED=11.2).

Table 3.6. Mean number of follicles aspirated and oocytes recovered from *post mortem* ovaries. Results of IVP are reported as numbers of oocytes matured, cleaved, cleavage rates, number of Day 8 blastocysts and the total number of cells per embryo.

Principle carbohydrate	Fibre		Starch		SED
Megalac (%)	0	6	0	6	
(a) Low BCS					
No. of heifers	4	4	4	4	
Follicles aspirated (n)	34.0	40.0	37.5	31.8	10.79
Oocytes recovered (n)	22.8	35.5	28.5	23.5	10.71
Oocytes matured (n)	21.8	33.8	26.0	22.5	9.91
Oocytes cleaved (n)	14.0	19.5	15.3	12.5	5.53
Cleavage rates (%)	66.3	63.5	69.1	65.3	7.49
Blastocyst number (n)	2.3	2.5	4.0	2.5	2.07
Total cell count (n)	128.0	109.4	122.8	137.0	35.99
(b) Moderate BCS					
No. of heifers	4	4	4	4	
Follicles aspirated (n)	29.3	25.0	27.5	17.8	13.15
Oocytes recovered (n)	22.5	20.8	25.8	13.8	12.94
Oocytes matured (n)	21.5	20.3	23.0	12.8	11.66
Oocytes cleaved (n)	13.8	14.0	12.5	6.3	7.14
Cleavage rates (%)	76.0	61.0	65.0	64.5	16.12
Blastocyst number (n)	2.3	3.5	1.5	0.5	1.91
Total cell count (n)	109.7	74.1	88.2	113.0	45.55

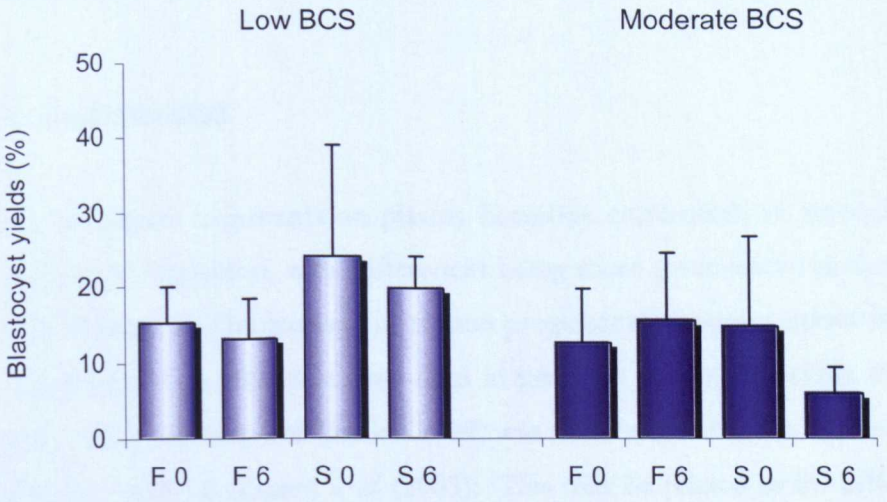

Figure 3.7. Mean (±SEM) blastocyst yields (%) obtained from oocytes recovered from *post mortem* ovaries, for animals in Low compared to Moderate BCS.

3.4. Discussion

The previous study demonstrated that both level of feeding and animal body composition determined oocyte developmental potential. To further elucidate the influence of nutritional status on oocyte quality, the current study investigated the effects of different sources of carbohydrate (Fibre vs Starch) and levels of fatty acids (0% vs 6% Megalac) on oocyte developmental competence. A key difference between the experiments was that the modified ovarian stimulation protocol of Blondin *et al.* (2002) facilitated the assessment of dietary treatments on more developmentally competent oocytes, representative of pre-ovulatory oocytes. One of the main findings of the present study was that the effects of nutritional treatments on follicular growth and oocyte quality, as indicated by post-fertilisation development *in vitro*, were dependent on animal BCS, which is consistent with the previous study (Chapter 2). Based on post-fertilisation development *in vitro*, Fibre based diets enhanced oocyte quality in Low BCS heifers, but impaired oocyte quality in Moderate BCS heifers. Similarly, the inclusion of protected lipid had a negative ($P<0.05$) effect on oocyte developmental potential in Low BCS heifers whereas in Moderate BCS animals, it had little or no effect on post-fertilisation development *in vitro*. Overall, the outcomes of the present study highlighted the importance of animal body composition in nutritional regulation of ovarian function, what to our knowledge has not been taken into consideration in the earlier studies in sheep (e.g. McEvoy *et al.*, 1995; Papadopoulos *et al.*, 2001; Lozano *et al.*, 2003) and cattle (e.g. Nolan *et al.*, 1998; Yaakub *et al.*, 1999a, b; Armstrong *et al.*, 2001).

3.4.1. Endocrinology

The effects of dietary treatments on plasma hormone concentrations were dependent on animal body composition, with differences being more pronounced in Low than in Moderate BCS heifers. The increase in plasma progesterone concentrations induced by dietary supplementation with protected lipid in previous studies (Hawkins *et al.*, 1995; Staples *et al.*, 1998; Williams and Stanko, 1999) was not observed in the current study, in accord with the study of Lessard *et al.* (2003). This may be related to the differences in the lipid source. Only for the Low BCS group were plasma progesterone concentrations greater ($P<0.05$) for heifers fed the high Fibre diet with Megalac

compared to other treatment groups, whereas in Moderate BCS heifers plasma progesterone concentrations were unaffected by nutritional treatments (Figure 3.2). This finding indicates that the diet and animal body composition may possibly alter cholesterol availability for steroid synthesis and/or reduce clearance rate of progesterone from the circulation (Hawkins *et al.*, 1995). As expected, compared to the Fibre diets, the high Starch diets significantly increased plasma insulin concentrations, but only in Low BCS heifers, with little effect in Moderate BCS heifers. These discrepancies might be associated with altered metabolism in Moderate BCS heifers and/or the effects of elevated leptin concentrations on insulin secretion (Spicer and Francisco, 1997, 1998; Brannian *et al.*, 1999).

The inclusion of Megalac did not have any statistically significant effect on plasma insulin or IGF-I concentrations. These observations are in accord with the earlier findings of Salado *et al.* (2004) and Selberg *et al.* (2004). Consistent with the previous study (Chapter 2) and those of others (Ehrhardt *et al.*, 2000; Delavaud *et al.*, 2000, 2002; Thomas *et al.*, 2001; León *et al.*, 2004) mean plasma leptin concentrations were positively correlated with animal body fatness. However, in the present experiment plasma leptin concentrations were less well correlated with plasma insulin concentrations (Figure 3.4.b).

3.4.2. Ovarian folliculogenesis and GC culture

Not-surprisingly ovarian stimulation significantly increased follicular development but at the same time oocyte recovery rates were reduced compared to un-stimulated animals (Figure 3.5.a). In general, heifers in Low BCS fed the Fibre based diets had an increased number of medium-sized follicles, which has previously been associated with increased oocyte competence (e.g. Lonergan *et al.*, 1994; Hagemann, 1999) but, for heifers in Moderate BCS, differences in follicle number were not statistically significant (Table 3.3). Increases in follicle number and diameter induced by the inclusion of protected lipid (Lammoglia *et al.*, 1997; Thomas *et al.*, 1997) were not observed in the current study. Considering that insulin is a well known factor stimulating proliferation and steroidogenesis of bovine granulosa cells *in vitro* (e.g. Gong *et al.*, 1993, 1994; Gutierrez *et al.*, 1997c), it was surprising that the increased concentrations of insulin induced by feeding Starch based diets in Low BCS heifers, did not seem to have any effect on

follicular growth and proliferative capacity of granulosa cells recovered post-mortem, contradicting the previous findings (Chapter 2). However, high leptin concentrations have previously been reported to have an inhibitory effect on insulin activity within the follicular compartment (Cohen *et al.*, 1996; Nowak *et al.*, 1998; Brannian *et al.*, 1999; Smith *et al.*, 2002), providing a possible explanation why in Moderate BCS heifers there was no observation of any nutritional effects on plasma insulin concentrations and any differences in follicular development. At present, however, due to the limited evidence in the literature, this hypothesis requires further investigation in order to establish the exact interactions between insulin, leptin and gonadotrophins, and their impact on follicle and oocyte development *in vivo*.

3.4.3. Post-fertilisation development *in vitro*

As for follicular development, ovarian stimulation with four doses of FSH and GnRH administered 6 h prior to OPU significantly increased oocyte developmental competence, as indicated by the increased proportion of Grades 1 and 2 oocytes (Figure 3.5.b) and the greater cleavage rates and number of embryos reaching the blastocyst stage *in vitro*, compared to un-stimulated animals (Figure 3.8).

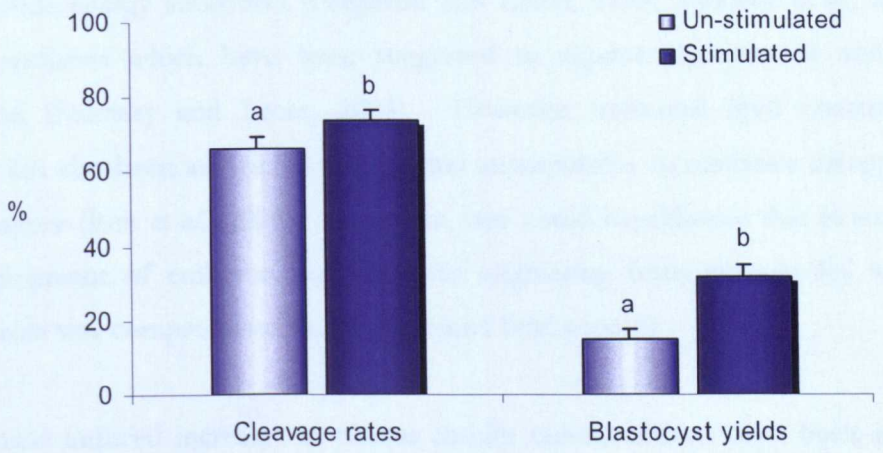

Figure 3.8. Mean (±SEM) cleavage rates and blastocyst yields between un-stimulated (*post mortem* IVP) and stimulated animals from the present study. Different letters indicate significant ($P<0.05$) difference between un-stimulated and stimulated animals within measured parameter.

Although blastocyst yields were not as high as those reported by Blondin *et al.* (2002), it should be noted that, unlike the study of Blondin *et al.* (2002), oocytes in the present study were matured, fertilised and zygotes cultured in small numbers, in serum and in somatic cell-free conditions. These conditions are known to contribute to lower blastocyst yields *in vitro* (e.g. Canseco *et al.*, 1992; O'Doherty *et al.*, 1997; Ward *et al.*, 2000; Kane, 2003). Nevertheless, blastocyst yields in this study were significantly greater than those in the previous experiment (Chapter 2). As suggested by Blondin *et al.* (2002), ovarian stimulation with FSH and LH (in our case GnRH), more closely resembles the pre-ovulatory follicular environment, initiates COC's cytoplasmic maturation and hence increases oocyte capacity to mature *in vitro*. This fact further emphasizes the importance of completion of both cytoplasmic and nuclear maturation in order to produce developmentally competent oocytes.

The current study provides evidence that oocyte developmental competence is dependent on the oocyte donor's nutritional background and body composition, even when undergoing ovarian stimulation. In general, Fibre based diets improved oocyte quality compared to Starch based diets, as indicated by the greater blastocyst yields, but only in Low BCS heifers. In contrast, they were detrimental in Moderate BCS heifers (Figure 3.6.b). Furthermore, the inclusion of protected lipid reduced ($P<0.05$) blastocyst yields in Low BCS but did not appear to have any effect in Moderate BCS heifers. It has previously been suggested that the main function of lipids (triglycerides) is to provide energy substrates (Ferguson and Leese, 1999; McEvoy *et al.*, 2000) for protein synthesis which have been suggested to support cytoplasmic and nuclear maturation (Sturmey and Leese, 2003). However, increased lipid content within embryos has also been associated with greater susceptibility to oxidative damage during *in vitro* culture (Reis *et al.*, 2003). Therefore, one could hypothesise that in some cases the development of embryos from oocytes originating from animals fed with lipid supplements was compromised due to increased lipid content.

Nutritionally induced increases in plasma insulin concentrations have been associated with improved reproductive performance in cattle (e.g. Beam and Butler, 1999; Landau *et al.*, 2000; Armstrong *et al.* 2002a; Gong *et al.*, 2002; Selvaraju *et al.*, 2002). Accordingly, the previous experiment (Chapter 2) demonstrated that moderate (~ 20 μIU/ml) plasma insulin concentrations were correlated with improved follicular and oocyte

development. However, the improvement in blastocyst yields induced by the Fibre based diets in the current study was not associated with greater plasma insulin concentrations. Indeed, among Low BCS heifers the Starch based diets, which increased plasma insulin concentrations, appeared to have a negative effect on oocyte quality indicated by reduced blastocyst yields. The difference between the previous and the present study was that herein heifers were undergoing ovarian stimulation with FSH. This ovarian stimulation protocol has been previously demonstrated to significantly improve oocyte developmental competence (Blondin *et al.*, 2002). The nutritional background of oocyte donors used in the study of Blondin *et al.* (2002), however, was unknown. From the available evidence it appears that, at high concentrations, FSH can inhibit oocyte maturation and alter COC's energy metabolism (e.g. Moor *et al.*, 1985; Roberts *et al.*, 2004). Furthermore, in a mouse model, FSH in association with elevated insulin concentrations (in the present study induced by the swap from Fibre to Starch based diet) can reduce aromatase activity of GCs (Bhatia and Price, 2001), and this may have a detrimental effect on the oocyte leading to impaired post-fertilisation development in *vitro* (e.g. Eppig *et al.*, 1998; Latham *et al.*, 1999). Therefore, it is tempting to speculate that oocyte development was compromised in conditions of ovarian stimulation with super-physiological concentrations of FSH and elevated plasma insulin concentrations, induced by Starch based diets but only in Low BCS heifers. In moderately fat heifers, plasma leptin concentrations were significantly greater than in thin animals, and this may have blunted the negative effects of FSH and high insulin concentrations (Cohen *et al.*, 1996), since leptin has previously been reported to have an antagonising effect on insulin activity (Spicer and Francisco, 1997, 1998; Brannian *et al.*, 1999). To further support this hypothesis, the outcomes of the IVP following animal slaughter suggested that the Starch based diet fed to un-stimulated heifers might have a positive effect on oocyte quality. This was indicated by greater blastocyst yields although differences were not statistically significant. It also appears that nutritionally induced effects on oocyte developmental competence varied between stimulated and un-stimulated animals. However, it must be emphasized that these comparisons between stimulated and un-stimulated animals be made with caution, in the knowledge that the IVP outcomes following animal slaughter are not as statistically powerful. Only further studies designed specifically to test differences of nutritional effects in stimulated and non-stimulated animals will provide answers to these questions.

3.5. Conclusions

The current study provides further evidence that the nutritional regulation of fertility (e.g. follicular and oocyte development) and the secretion of key metabolic hormones such as insulin and leptin are dependent on animal body composition. While Fibre based diets enhanced oocyte quality and improved post-fertilisation development *in vitro* in thin heifers they, at best, had no effect on oocyte quality and subsequent embryo development in moderately fat animals. Furthermore, the modified protocol of ovarian stimulation (Blondin *et al.*, 2002) significantly improved oocyte developmental competence when cultured in semi-defined conditions, indicating that ovarian stimulation created a more favourable follicular environment that enabled oocytes to more fully complete the process of cytoplasmic maturation. However, the improvement in oocyte competence was not related to changes in any of the metabolic hormones investigated, suggesting that other undetermined factors were involved.

CHAPTER 4

Serum from different dietary backgrounds affects oocyte maturation and post-fertilisation development *in vitro*.

4.1. Introduction

The intrinsic quality of the oocyte is the main factor determining post-fertilisation development both *in vivo* and *in vitro* (e.g. Sirard and Blondin, 1996; Rizos *et al.*, 2002a). Oocyte developmental competence has been associated with follicular diameter (Lonergan *et al.*, 1994; Arlotto *et al.*, 1996; Yang *et al.*, 1998; Hagemann, 1999; Vassena *et al.*, 2003), oocyte donor age (e.g. Yang *et al.*, 1998) and also the stage of the oestrous cycle (Sirard and Blondin, 1996; Salamone *et al.*, 1999; Hendriksen *et al.*, 2004). In addition, as demonstrated in the previous studies (Chapters 2 and 3), the nutritional background and body composition of oocyte donors also play an important role in regulating follicle and oocyte development. These earlier findings suggested that high levels of feeding had either a positive or negative effect on oocyte quality depending on animal body condition (Chapter 2). Furthermore, the effects of diet composition (Fibre vs Starch and inclusion vs absence of protected lipid) were more pronounced in Low compared to Moderate BCS heifers. In general, the Fibre based diets and the absence of protected lipid supplement were beneficial to oocyte quality in Low BCS heifers, but not in heifers of Moderate BCS.

Dietary treatments in these *in vivo* studies affected oocyte competence by probably influencing both early antral and antral follicular environment. However, many critical stages of oocyte and embryo development have to be completed prior to the successful establishment of pregnancy resulting in a birth of viable offspring. In addition, the same dietary factors may have a different effect on the intrafollicular than the uterine environment, therefore their impact on oocyte maturation following ovulation, and during early embryo development could vary from those observed in the early stages of oocyte growth. For example, studies of Sinclair *et al.* (2000b) demonstrated that exposure of oocytes in antral follicles to high concentrations of ammonia impaired subsequent cleavage and post-fertilisation development, whereas studies of Hammon *et al.* (2000) showed that the effects of ammonia on pre-implantation bovine embryos was dependent not only on ammonia concentrations but also the stage of development when exposure occurred. As mentioned earlier, in the previous chapter it was demonstrated that oocyte developmental competence, prior to ovulation, was influenced by diet composition, as indicated by subsequent embryo development *in vitro*. However, it remains unclear what effects the same diet may have on the oocyte

following ovulation and on early embryo development. Therefore, the objectives of the present experiments were to compare the effects of serum harvested from oocyte donors used in Chapter 3 during *in vitro* maturation (IVM) and embryo culture (IVC), on the development of pre-implantation embryos. The outcome of these experiments should provide a full spectrum of dietary induced alterations during all stages of oocyte and early embryo development, and provide evidence on the stages that are most influenced by dietary treatment.

In vitro embryo production (IVP) systems have their limitations, however. Most importantly they do not mimic exactly the oviductal and uterine environments. Not-surprisingly, therefore, the majority (60 – 70%) of immature oocytes fail to develop to the blastocyst stage following IVP. In fact, *in vitro* maturation and culture systems have been reported to induce ultrastructural and molecular abnormalities in the oocyte (e.g. Hyttel *et al.*, 1989; 1997) and in the embryo (e.g. Rizos *et al.*, 2002a; 2003; Lonergan *et al.*, 2003a, b). Nevertheless, current IVP systems allow the assessment of factors that determine the final stages of oocyte maturation, fertilisation and embryo metabolism and quality.

4.2. Materials and Methods

4.2.1. Serum collection and preparation

Sera were harvested from the group of 32 beef x diary heifers used in Chapter 3. These heifers participated in a 2 x 2 factorial experiment, where they were offered either a high fibre (F) or a high starch (S) diet alone (0) or supplemented with 6% w/w of Ca soaps of fatty acids (Megalac, Volac International Ltd, Herts, UK). Half of the heifers in that study were in Low and half in Moderate BCS. Blood samples were collected from each heifer on Day 4 of the oestrous cycle by jugular venipuncture into vacuum tubes and allowed to clot at room temperature for at least 30 minutes. After that time all tubes were centrifuged at 600 x *g* for 15 minutes. Serum was transferred into 25 ml sterile universal containers and stored at –20°C until required. Before use, the sera were heat-inactivated in a water bath at 56°C for 30 minutes. Sera from four heifers in the Low and four in the Moderate BCS groups within each dietary treatment (F0, F6, S0 and S6) were pooled together to give a representative sample for each experimental replicate.

Finally, the sera was filtered through a sterile 0.2 μm fast flow filter then aliquoted into 1.5 ml microcentrifuge tubes and stored at –20°C until use.

4.2.2. Oocyte maturation in the presence of sera

Details of media composition and IVP procedures are included in Appendix 1. Briefly, ovaries collected from a local abattoir were transported in a flask containing warm PBS, once in the laboratory where they were washed in warm (37°C) PBS before being subjected to aspiration. Only small and medium-sized follicles were aspirated using a 19 gauge single lumen needle attached to a 5 ml syringe. Follicular fluid was searched for COCs and only grade 1 and 2 COCs were selected for maturation. A total of 1,603 abattoir derived COCs (4 replicates) were matured in the presence of 10% v/v sera derived from Low and Moderate BCS heifers on the F0, F6, S0 and S6 treatments offered in Chapter 3. Groups of 50 – 60 COCs per treatment (200 – 250 COCs per replicate) were matured in 4-well plates containing 500 μl of Maturation medium per well and incubated for 24 h at 39°C and 5% CO_2 in a humidified atmosphere. Matured COCs were fertilised using the same procedures as described previously (Chapter 3). Briefly, following 24 h maturation COCs were partially denuded in Oocyte wash medium by gentle pipetting before being transferred into wells containing 460 μl of Fertilisation medium and 50 – 60 COCs per well. Straws with frozen semen of one bull (Eugene) were used throughout the experiment. These were thawed in a water bath (35°C) for 1 min before being layered under 1 ml of Capacitation medium and allowed to 'swim-up' for 30 min at 39°C and 5% CO_2 in a humidified air. Gametes were co-incubated for 20 – 22 h at 39°C and 5% CO_2 in a humidified atmosphere. Within 20 – 22 h after fertilisation a total of 1,151 presumptive zygotes were transferred into 20 μl drops (10 zygotes per drop) of SOF supplemented with 0.3% w/v BSA (FAF), 1% v/v non-essential amino acids and 2% v/v essential amino acids and antibiotics, overlaid with mineral oil. Zygotes were cultured at 39°C in a humidified atmosphere of 5% CO_2 and 5% O_2 until Day 8 of development. Embryos were transferred into fresh drops of SOF medium every 48 h. Cleavage rates were recorded 48 h post-fertilisation and blastocyst yields were recorded on Day 7 and Day 8 of culture. Day 8 blastocysts were subjected to differential staining and TUNEL assay.

4.2.3. Zygote culture in the presence of sera

Ovaries collected from a local abattoir were processed as described previously. A total of 2,099 abattoir derived COCs (5 replicates) were matured and fertilised using IVP procedures (Appendix 1). In brief, selected grade 1 and 2 COCs were matured in groups of 200 – 250 COCs per batch (replicate), in a small Petri dish containing 3 ml of Maturation medium supplemented with 10% v/v FCS and incubated for 24 h at 38.8°C and 5% CO_2 in a humidified atmosphere. Matured COCs were partially denuded by gentle pipetting prior to fertilisation. Semen from a single bull (Beechpike Energy) was used throughout the experiment and prepared by the 'swim-up' procedure (Appendix 1). Gametes were co-incubated for 20 – 22 h in 500 µl of Fertilisation medium (average group size of 53 COCs per well), as described previously. The following day, presumptive zygotes (n=1,480) were washed twice in SOF supplemented with 10% v/v of treatment sera (F0, F6, S0 and S6 treatments from heifers in Low and Moderate BCS) before being transferred into 20 µl drops of SOF supplemented with 10% v/v treatment sera (10 zygotes/drop), overlaid with mineral oil. Zygotes were cultured at 38.8°C in a humidified atmosphere of 5% CO_2 and 5% O_2 until Day 8 of development. Embryos were transferred every 48 h into fresh drops of SOF supplemented with sera. Cleavage rates were recorded 48 h post-fertilisation and blastocyst yields recorded on Days 7 and 8 of development. Day 8 blastocysts were subjected to total cell counts and TUNEL analysis, and a pyruvate metabolism assay. Blastocysts obtained from two replicates in the current experiment were subjected to fatty acid analysis (Chapter 5).

4.2.4. Differential staining and TUNEL assay

On Day 8 of development individual blastocysts were assessed and then immediately subjected to the triple differential staining as described in Appendix 2 (section A.2.3). Once staining was completed, blastocysts were washed four times in PBS/BSA and then mounted onto glass slides using Vectashield Mounting medium and allowed to dry for 1 to 3 minutes. These blastocysts were immediately visualised by fluorescence microscopy and the number of cells within inner cell mass (ICM), trophectoderm (TE) and TUNEL positive cells recorded.

4.2.5. Total cell counts and TUNEL assay

Day 8 blastocysts were fixed in 4% w/v paraformaldehyde solution and stored in a cool room until total cell counts and TUNEL assay were conducted. Fixed blastocysts were subjected to the sequence of staining procedures based on a modified apoptosis detection kit protocol (ApopTag® Red Kit, Intergen, NY, USA) (Appendix 2; A.2.2). Once staining procedures were completed, blastocysts were mounted onto a glass slides using Citifluor mounting medium (Agar Scientific, Essex, UK), overlaid with a glass cover slips and left overnight to dry. The next day blastocysts were visualised by fluorescence microscopy and the total number of cells and number of TUNEL positive cells recorded.

4.2.6. Pyruvate metabolism assay

Modified from McEvoy *et al.* (1997) the "hanging drop" incubation technique with [2-^{14}C]-pyruvic acid (American Radiolabeled Chemicals, St Loise, MO, USA) as a marker was used to measure pyruvate metabolism by individual embryos (Appendix 2; A.2.4). Briefly, Day 8 blastocysts were assessed and then washed three times in mECM. Each embryo was incubated in a 6 µl drop of HOT ECM (mECM with [2-^{14}C]-pyruvic acid). Additional drops without embryos (sham controls) were also prepared to provide non-specific background reference data. Each drop was suspended in a sealed 1.5 ml eppendorf tube containing 1.0 ml of 0.1M NaOH as a trap for $^{14}CO_2$. All tubes were incubated for exactly 3 h at 38.5°C and 5% CO_2 in a humidified atmosphere during which time scintillation vials were prepared by adding 4 ml of scintillation fluid (Emulsifier safe, Packard BioScience, Berkshire, UK) into each vial. After 3 h incubation, the contents of each tube were transferred into two scintillation vials (0.5 ml of $^{14}CO_2$ enriched NaOH) and 105 µl of 0.5M HCl added to avoid chemifluorescence. Each vial was counted for 20 minutes on a liquid scintillation analyser (Tri-Carb 2300TR, Packard BioSciences, Berkshire, UK). Pyruvate metabolism was expressed as pmoles per cell per 3 h, taking account of blank sample readings and the specific activity of [2-^{14}C]-pyruvic acid in the hanging drop. Capture efficiency of $^{14}CO_2$ in our laboratory has been estimated at 79%.

4.2.7. Statistical analyses

Data were analysed using generalised linear models within Minitab, Release 13.1. Terms fitted to the models were carbohydrate source (Fibre vs Starch), Megalac (0% vs 6%) and body condition (Low vs Moderate), and interactions between these factors. All parameters were tested for normality using the Ryan-Joiner test statistic prior to ANOVA analysis. Data are presented as means with either appropriate SEM or SED.

4.3. Results

4.3.1. *In vitro* maturation in the presence of sera

The mean (±SEM) number of oocytes matured and number of cleaved oocytes per treatment averaged 57.3±1.8 and 41.1±1.2 respectively, and did not vary between treatments. Cleavage rates (78.2±1.3%) were also unaffected by serum source (Table 4.1). There was an interaction (P=0.074), however, between animal BCS and carbohydrate source. This indicated that oocytes matured in the presence of serum from heifers in Moderate BCS offered the Fibre based diets improved cleavage rates, but the serum from Low BCS heifers fed the same carbohydrate source reduced cleavage rates (83.4 vs 74.3% and 77.9 and 78.5%, SED=3.58, for Fibre: Moderate BCS vs Low BCS and Starch: Moderate BCS vs Low BCS, respectively), however, differences did not reach statistical significance. In terms of blastocyst yields on Day 7 or Day 8, there were no differences between serum sources (Table 4.1; Figure 4.1.a). However, blastocyst yields on Day 8 of development were greater (P<0.001) than on Day 7 (30.2±1.3 vs 21.4±1.5%). Although there was no statistically significant differences in embryo development between sera originating from Low BCS heifers fed either with or without Megalac, there was a suggestion that oocytes matured in the presence of serum harvested from Low BCS heifers fed with the protected lipid supplement were more competent to develop to blastocyst stage *in vitro* compared to diets without lipid supplement (32.1 vs 26.7%, SED=5.03) (Figure 4.1.a). In contrast, sera from Moderate BCS heifers fed with Megalac had little or no effect on oocyte maturation and subsequent post-fertilisation development (Figure 4.1.a).

Mean (±SEM) total cell number per Day 8 blastocyst averaged 134.1±3.6 and did not vary between treatments (Table 4.1). However, there was a suggestion (P=0.093) that embryos derived from oocytes matured in the presence of serum originating from heifers fed the Fibre compared to the Starch based diets had more cells per embryo (140.6 vs 128.4 cells/embryo for F and S diet respectively, SED=10.06). Furthermore, the number of cells within the ICM was greater (P<0.01) when oocytes were matured with serum from heifers in Moderate than in Low BCS (48.7 vs 38.5 cells/ICM, SED=2.97), but the number of cells within the TE was unaffected by treatment (Table 4.1). In contrast, the proportion of TUNEL positive cells was greater (P<0.01) in embryos originating from oocytes matured with serum from the Starch than the Fibre fed heifers (4.8 vs 3.3%, SED=0.47). Analysis by animal BCS demonstrated that differences between nutritional treatments were more pronounced with serum from Low than from Moderate BCS heifers. With serum from Low BCS heifers, although total cell number per embryo did not vary between treatments (mean of 128.4±5.2 cells/embryo), the number of cells within the ICM was greater (P<0.05) for serum from Fibre compared to Starch fed heifers (42.9 vs 34.2 cells/ICM, SED=3.42). There was also an interaction (P<0.01) between carbohydrate source and the presence or absence of protected lipid indicating that the number of cells per embryo was increased for treatments F0 and S6 compared to F6 and S0 treatments (Figure 4.1.b). Total cell number and distribution of cells within ICM and TE were unaffected by nutritional treatments from heifers in Moderate BCS.

Table 4.1. Mean number of oocytes matured and cleaved, cleavage rates (%), blastocyst number and yields on Days 7 and 8 of development. Mean number of total cells, cells within inner cell mass (ICM) and trophectoderm (TE) on Day 8.

Principle Carbohydrate	Fibre		Starch		SED
Megalac (%)	0	6	0	6	
Oocytes matured (n)	56.6	58.6	56.7	57.1	5.16
Oocytes cleaved (n)	39.1	42.7	40.0	42.6	3.44
Cleavage rates (%)	77.1	79.3	77.9	78.7	3.77
Day 7 blastocyst number (n)	7.3	10.8	8.7	9.0	1.92
Day 7 blastocyst yields (%)	18.2	25.0	21.7	21.6	3.57
Day 8 blastocyst number (n)	11.4	13.3	12.1	13.6	2.43
Total cell count (n)	143.7	137.4	124.3	132.5	10.06
ICM cell number (n)	46.5	43.8	40.7	43.4	4.21
TE cell number (n)	97.3	100.8	88.9	91.7	11.46

In addition, the percentage of TUNEL positive cells increased in embryos originating from oocytes matured with serum from heifers fed the Starch compared to the Fibre based diet for Low (4.3 vs 3.1%, SED=0.59; P=0.059) and Moderate (5.2 vs 3.6%, SED=0.72; P<0.05) BCS heifers (Figure 4.1.c).

4.3.2. *In vitro* culture in the presence of sera

The number of oocytes matured and oocytes cleaved per treatment averaged 52.5±0.7 and 37.0±0.8 respectively and did not vary between replicates (Table 4.2). Cleavage rates averaged 70.8±1.3% and were unaffected by serum source (Table 4.2). Although the number and percentage of Day 7 blastocysts were also unaffected by serum source, they were greater for embryos cultured in the presence of serum harvested from Low compared to Moderate BCS heifers (8.0 vs 5.6, SED=1.05, P<0.05; and 22.1 vs 15.2%, SED=2.44, P<0.01, respectively for number and percentage Day 7 blastocysts for Low and Moderate BCS). Similarly, the number and the percentage of Day 8 blastocysts were greater when embryos were cultured with serum from Low than Moderate BCS heifers (9.1 vs 7.0, SED=1.07, P=0.058; and 24.2 vs 19.0%, SED=2.36, P<0.05, respectively), but did not differ between nutritional treatments (Figure 4.2.a). As expected, the number and the percentage of blastocysts on Day 8 of development were greater (P<0.01) compared to Day 7 (8.0±0.6 vs 6.7±0.5 and 21.8±1.3% vs 18.3±1.28% for Day 8 vs Day 7, respectively). Although sera from each of the dietary backgrounds did not have any overall effect on blastocyst yields, there was an interaction (P=0.09) suggesting that serum from heifers fed the Fibre based diets supplemented with protected lipid reduced blastocyst yields but serum from heifers fed the Starch diets with the same lipid supplement increased blastocyst yields (Figure 4.2.a). Analysis by animal BCS did not show any effect of this factor.

Cell number per embryo averaged 109.5±3.4 and did not differ between treatments. The percentage of TUNEL positive cells (5.6±0.3%) was also unaffected by serum source, but there was an interaction (P<0.01) between carbohydrate source and the presence or absence of Megalac which indicated that for treatments F0 and S6 the percentage of TUNEL positive cells was reduced compared to treatments F6 and S0 (Figure 4.2.b).

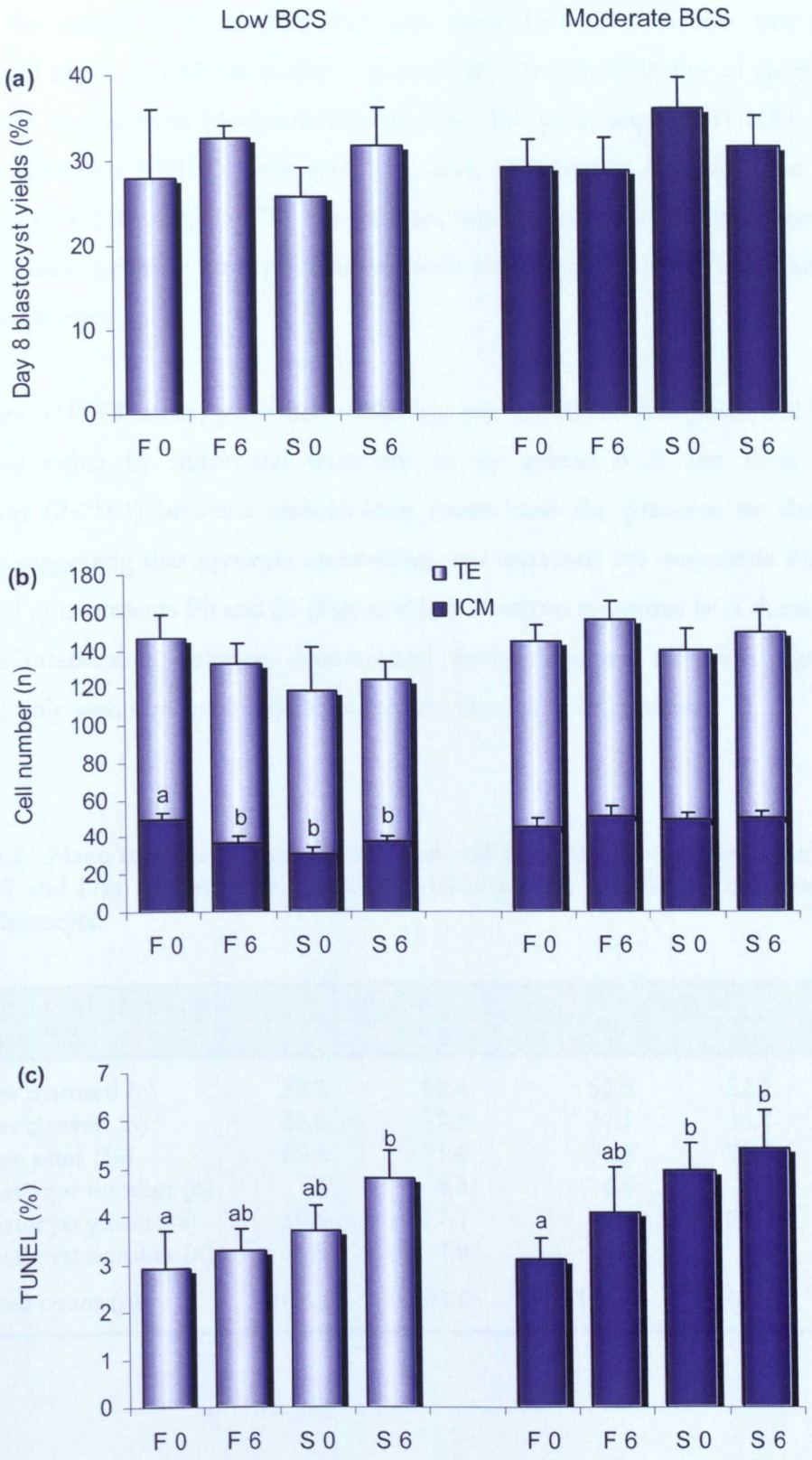

Figure 4.1. Mean (±SEM) blastocyst yields on Day 8 (a), the number of cells within inner cell mass (ICM) and trophectoderm (TE) (b), and the percentage of TUNEL positive cells per embryo (c) for heifers in Low compared to Moderate BCS, following *in vitro* maturation with different serum source.

Analysis by animal BCS revealed that sera from Low BCS heifers had a more pronounced effect on embryo quality (e.g. total cell number, incidence of apoptosis) in comparison to sera from Moderate BCS heifers. The percentage of TUNEL positive cells was increased ($P<0.05$) with sera from Low BCS heifers fed with than without Megalac (7.0 vs 5.0%, SED=0.78). In contrast, total cell number and the percentage of TUNEL positive cells in embryos cultured with sera from Moderate BCS heifers was unaffected by diet.

As for the TUNEL assay, pyruvate metabolism per cell (0.2±70.03 pmol/cell/3h) was unaffected either by nutritional treatment or by animal BCS, but there was an interaction ($P<0.01$) between carbohydrate source and the presence or absence of Megalac, suggesting that pyruvate metabolism was increased for treatments F6 and S0 compared to treatments F0 and S6 (Figure 4.2.c). Analysis by animal BCS demonstrated that the interactions between experimental factors reached statistical significance ($P<0.01$) only with sera from Moderate, but not from Low BCS heifers.

Table 4.2. Mean number of oocytes matured and cleaved, cleavage rates, the number of Day 7 and Day 8 blastocysts, blastocyst yields on Day 7 and total cell number per Day 8 blastocyst.

Principle Carbohydrate	**Fibre**		**Starch**		**SED**
Megalac (%)	**0**	**6**	**0**	**6**	
Oocytes matured (n)	52.8	52.4	52.5	52.2	1.9
Oocytes cleaved (n)	36.6	37.5	37.1	36.7	2.4
Cleavage rates (%)	69.6	71.6	71.3	70.6	3.8
D7 blastocyst number (n)	7.1	6.4	6.0	7.7	1.48
D7 blastocyst yields (%)	19.5	17.7	16.5	21.0	3.46
D8 blastocyst number (n)	8.9	7.9	6.8	8.6	1.5
Total cell count (n)	109.8	103.6	116.4	106.2	9.8

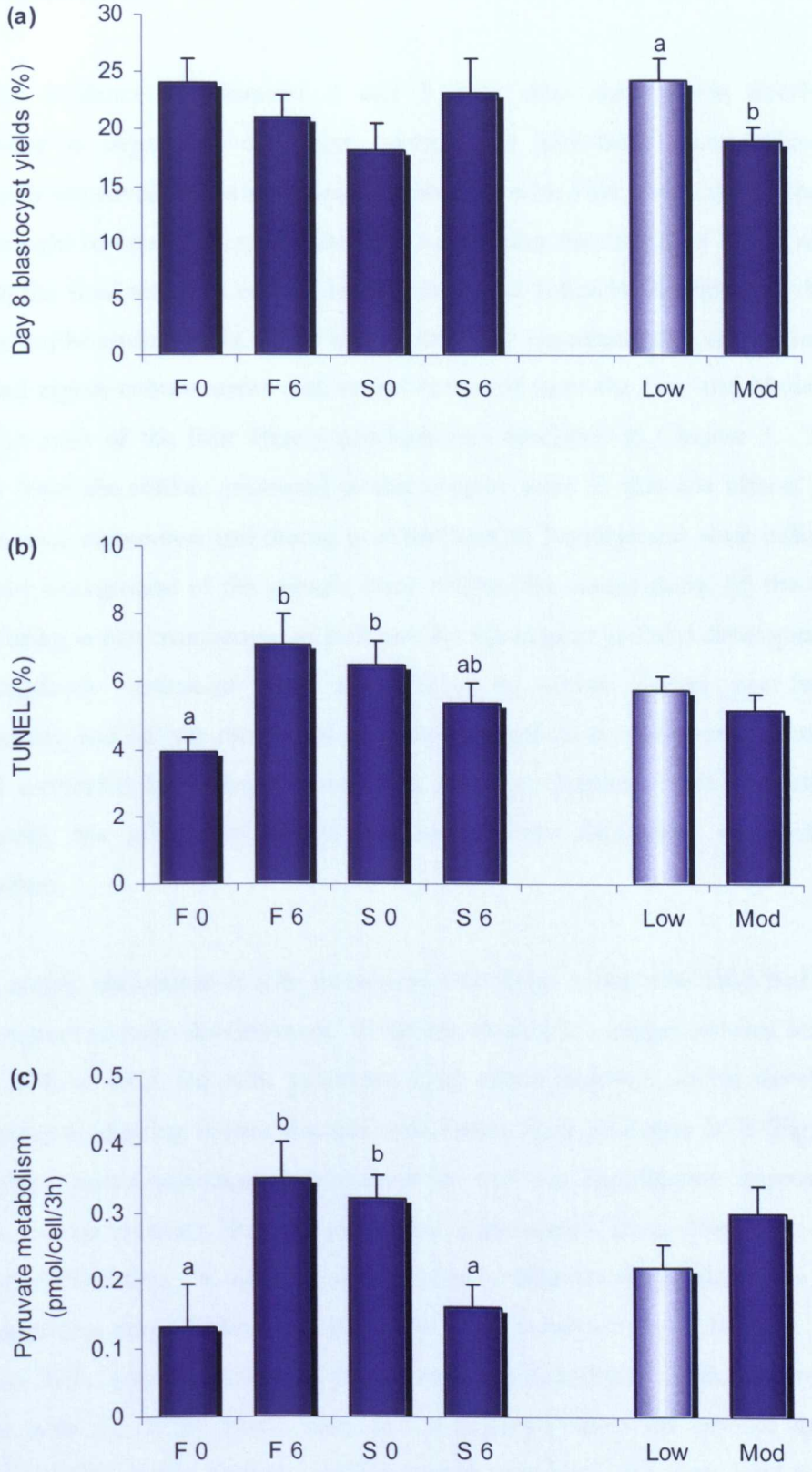

Figure 4.2. Mean (±SEM) blastocyst yields on Day 8 (%) (a), the percent of TUNEL positive cells (b) and pyruvate metabolism expressed in pmol/cell/3h (c) on Day 8 of development, following embryo culture in the presence of sera.

4.4. Discussion

From the evidence of Chapters 2 and 3 it is clear that oocyte developmental competence is dependent on oocyte donor diet and body composition and is significantly improved following ovarian stimulation with FSH and GnRH. The present studies sought to extend these observations to consider the effects of donor nutritional status on the final stages of oocyte development and, following fertilisation, during the early pre-implantation stages. This was achieved by supplementing oocyte maturation media and zygote culture media with serum harvested from the Low and Moderate BCS heifers on each of the four dietary combinations described in Chapter 3. The main findings from the studies presented in this chapter were (i) that the effects of serum during oocyte maturation and during post-fertilisation development were influenced by the dietary background of the animals from whence the serum came, (ii) the effects of serum during *in vitro* maturation, as indicated by subsequent embryo development, were not completely consistent with the effects of serum during post-fertilisation development, and (iii) the nutritionally induced alterations in oocyte competence *in vitro* differed somewhat from these observed *in vivo* (see Chapter 3) but consistently and importantly, the effects of dietary treatments were dependent on animal body composition.

During oocyte maturation *in vitro*, nutritional treatments and animal BCS had no effect on subsequent embryo development. However, there was a suggestion that serum from heifers in Low BCS fed with protected lipid might improve oocyte developmental competence to develop *in vitro*, but not with serum from Moderate BCS (Figure 4.1.a). In contrast, post-fertilisation development *in vitro* was significantly improved when embryo culture medium was supplemented with serum from Low, but not from Moderate BCS heifers. In addition, during post-fertilisation development the effects of sera originating from heifers offered diets with protected lipid, both in Low and Moderate BCS, were dependent on the source of carbohydrate. Whilst the inclusion of Megalac with the Fibre based diets had a negative effect on embryo quality (i.e. increased incidence of apoptosis and pyruvate metabolism), the same lipid supplement had a positive effect on embryo development when animals were fed with the Starch based diets (Figure 4.2). However, it still remains unclear what factors underlie the differences between animal BCS groups.

The main disadvantage of this *in vitro* approach, is firstly that abattoir derived oocytes were used and these are mostly from moderately fat animals and, as we demonstrated earlier, that can have an effect on oocyte quality, although with the random selection of oocytes the differences between animal BCS should be nullified. Secondly, current *in vitro* embryo production systems do not exactly mimic the intrafollicular and/or oviductal environment and hence the addition of serum cannot be modified by either follicular cells or oviductal cells before it has an effect on oocyte and/or embryo development. Given these limitations, however, the available *in vitro* culture systems do allow the assessment of post-fertilisation development to the blastocyst stage, an important milestone in early mammalian embryo development.

4.4.1. Oocyte maturation

In vivo matured oocytes are reported to be more competent than those matured *in vitro* (e.g. Greve *et al.*, 1987; Leibfried–Rutledge *et al.*, 1987; van de Leemput *et al.*, 1999; Dieleman *et al.*, 2002; Rizos *et al.*, 2002a) and oocyte developmental competence is still considered to be the key factor to successful post-fertilisation development (Lonergan *et al.*, 2001, 2003a; Dieleman *et al.*, 2002; Rizos *et al.*, 2002a, 2003). Despite numerous studies on oocyte maturation *in vitro* it remains unclear what triggers and regulates completion of nuclear and cytoplasmic maturation and hence determines oocyte developmental potential. It is generally agreed that the presence of gonadotrophins (e.g. Zuelke and Brackett, 1992; Choi *et al.*, 2001; Eppig *et al.*, 2000) and also protein supplementation e.g. BSA or serum (e.g. Kim *et al.*, 1993; Holm *et al.*, 1999; Chanson *et al.*, 2001) are required for successful fertilisation and subsequent embryo development *in vitro*. Various other growth factors, including EGF, IGF-I and insulin, have also been the subject of numerous investigations (Lorenzo *et al.*, 1994; Rieger *et al.*, 1998; de la Fuente *et al.*, 1999; Eppig *et al.*, 2000; Sakaguchi *et al.*, 2002) in attempt to improve developmental competence of *in vitro* matured oocytes, compared to their *in vivo* counterparts. However, most of these factors were able to enhance nuclear maturation, but failed to trigger cytoplasmic maturation.

By using serum from animals of known nutritional background, the present study sought to determine if the effects of the dietary treatments offered during antral follicle development in Chapter 3 could be recapitulated by the inclusion of serum during *in*

vitro maturation. It appears that, in the previous study (Chapter 3) whilst oocyte quality in Low BCS heifers fed with protected lipid was impaired, serum from the same dietary treatment/BCS combination applied during *in vitro* maturation enhanced oocyte developmental competence (Figure 4.1.a), although differences were not statistically significant. Studies conducted with pigs may provide a partial explanation for this apparent contradiction. According to Sturmey and Leese (2003) increased lipid content provides energy source for protein synthesis and hence may improve oocyte potential to develop *in vitro*. However, consistently with the earlier findings serum from Moderate BCS heifers seemed to nullify the effects of nutritional treatments therefore making final conclusions difficult to make. The only identified difference between Low and Moderate BCS was the increased plasma concentrations of leptin in the latter group. However, as reported by Swain *et al.* (2004) even superphysiological concentrations of leptin (100 ng/ml), which are 50 fold greater than plasma leptin observed in Moderate BCS heifers, did not have any effect on mouse oocyte maturation, suggesting that other factors may play an important role in determining oocyte competence both *in vivo* and *in vitro*. Therefore, it remains unclear what factors are responsible for these discrepancies between animal BCS groups. In terms of embryo quality, total cell number and number of cells within the ICM and the TE were unaffected by nutritional treatments and did not vary between animal BCS groups (Figure 4.1.b). However, serum from heifers fed the Starch based diets gave rise to an increased incidence of apoptosis compared to serum from heifers fed the Fibre diets (Figure 4.1.c), but it is unclear what factor(s) within serum may be responsible for this effect. According to Watson *et al.* (2000), even though *in vitro* produced embryos undergo apoptosis, they are not greatly influenced by the maturation conditions. Nevertheless, alterations in abundance of certain oocyte transcripts induced by the maturation media are associated with reduced oocyte developmental competence (De Sousa *et al.*, 1998; Watson *et al.*, 2000; Gandolfi and Gandolfi, 2001; Pocar *et al.*, 2001; Lonergan *et al.*, 2003b). However, little is known of the oocyte gene transcripts that contribute to oocyte quality both *in vivo* and *in vitro*.

Differences between nutritionally induced alterations in oocyte quality *in vivo* and *in vitro* indicate that oocyte competence is mainly determined prior to *in vitro* maturation, prior to the LH surge (Assey *et al.*, 1994). This suggests that studies conducted *in vitro* do not provide the full temporal spectrum to assess the full effects of maternal nutrition on oocyte developmental potential. These findings further emphasise the problem of the

inefficiency of *in vitro* conditions to trigger cytoplasmic maturation. Attempts have been made to improve embryo development *in vitro* by artificially maintaining oocytes in meiotic arrest (Kubelka *et al.*, 2000; Lonergan *et al.*, 2000; Sirard, 2001), hence allowing cytoplasmic maturation to progress. However, although blastocyst formation was improved, there was no evidence of improvement in oocyte competence *per se*.

4.4.2. Post fertilisation development

Serum source had contrasting effects on oocyte maturation and post-fertilisation development *in vitro*. As mentioned earlier, serum from heifers in Low BCS fed with protected lipid had a stimulatory effect on oocyte maturation indicated by a greater number of zygotes reaching blastocyst stage, although differences were not of statistical significance. During post-fertilisation development serum harvested from heifers fed protected lipid and the Starch based diets slightly enhanced blastocyst formation and, most importantly, improved embryo quality by reducing the incidence of apoptosis and pyruvate metabolism. In contrast, serum from heifers fed protected lipid and the Fibre based diets had a negative effect on embryo quality (Figure 4.2). The indices of apoptosis and energy metabolism used in the current study have previously been shown to be good indicators of embryo quality (Gardner and Leese, 1988; Rieger, 1992, 1998; Gardner, 1998; Chi *et al.*, 2002). It is generally known that glucose transport and metabolism are important for blastocyst formation and further development (Gardner and Leese, 1988; Martin and Leese, 1999). Also, due to the switch from the oxidation of pyruvate via the tricarboxylic acid cycle to the use of glucose as the main substrate via glycolysis (Gardner, 1998), blastocysts are very sensitive to any glucose deprivation. According to Leese (2002), the most viable embryos are those with the lowest overall metabolism and the lowest glycolytic rates. Increased pyruvate uptake has previously been associated with reduced developmental capacity of bovine embryos *in vitro* (Eckert *et al.*, 1998) and *in vivo* (Wrenzycki *et al.*, 2000). Furthermore, the studies of Chi *et al.* (2002) with mouse embryos suggest that altered glucose uptake and metabolism induced by an inadequate embryo culture environment (e.g. maternal diabetes or *in vitro* hyperglycemia) can result in increased pyruvate uptake leading to severe changes in mitochondrial physiology that can trigger the apoptotic cascade. Therefore, one can hypothesise that current dietary treatments (i.e. F6 and S0 treatments), which increase the incidence of apoptosis and pyruvate uptake are detrimental to embryo development

in vitro. These findings also suggest that the same nutritional treatments might possibly lead to higher rates of embryo mortality or foetal malformations *in vivo*. To further support this hypothesis, recent studies demonstrated that dietary composition might have led to alterations in the oviductal environment *in vivo*, which in turn affected embryo energy metabolism, gene expression and consequently embryo survival (Nolan *et al.*, 1998; Yaakub *et al.*, 1999a, b; Wrenzycki *et al.*, 2000). More surprisingly, serum harvested from Low BCS heifers in the current study significantly improved blastocyst formation compared to Moderate BCS heifers. However, embryo quality was not affected to the same extent. As mentioned earlier, the physiological difference, known to us, between sera from Low and Moderate BCS heifers was the concentration of plasma leptin, which was significantly greater in Moderate compared to Low BCS heifers (4.63 vs 2.25 ng/ml). Therefore, leptin might be a possible factor impairing embryo development *in vitro*. However, studies conducted in mice (Swain *et al.*, 2004) demonstrated that even superphysiological concentrations of leptin (up to 100 ng/ml) did not have any effect on blastocyst formation.

There is growing evidence in the literature to indicate that *in vitro* embryo culture conditions have a dramatic impact not only on embryo ultrastructure, morphology (e.g. Abd El Razek *et al.*, 2000; Crosier *et al.*, 2000; 2001; Rizos *et al.*, 2002a, b; Reis *et al.*, 2003), and metabolism (Khurana and Niemann, 2000a; Thompson, 2000; Leese, 1991, 2002), but also on the pattern of gene expression, which taken together has a major implication for the normality of *in vitro* produced blastocysts (e.g. Wrenzycki *et al.*, 1999, 2000; Rief *et al.*, 2002; Rizos *et al.*, 2002b, c; 2003; Lonergan *et al.*, 2003b, 2004). Furthermore, *in vitro* culture conditions have also been associated with the large offspring syndrome (e.g. Young *et al.*, 1998; Sinclair *et al.*, 2000c; Walker *et al.*, 2000), proof of an inadequate environment for normal embryo growth. Therefore, it is difficult to conclude if the same nutritional treatments, used to create the sera used in these experiments, when extended to embrace the early post-fertilisation period would have had a similar effect on embryo development *in vivo*.

4.5. Conclusions

The inclusion of sera from known nutritional origins had differential effects on oocyte maturation and post-fertilisation development *in vitro*. While the inclusion of protected lipid (of the sort used in the current series of studies) in the diet can be detrimental to oocyte development *in vivo*, it may be beneficial when sera harvested from animals fed these diets are used to supplement oocyte maturation media. Furthermore, maternal diet and body composition influenced embryo quality. However, it remains unclear whether the same factors in serum impacted on embryo development *in vivo* compared with *in vitro*.

CHAPTER 5

Lipid content and fatty acid composition of plasma, granulosa cells and cumulus-oocyte complexes derived *in vivo* and embryos cultured *in vitro.*

5.1. Introduction

Chapters 3 and 4 detailed the effects of diet composition (Fibre vs Starch and the presence and absence of rumen protected fatty acids) on the developmental competence of oocytes *in vivo* and *in vitro*, and post-fertilisation development *in vitro*. This chapter presents data on the lipid content and fatty acid composition of plasma, granulosa cells (GCs) and cumulus-oocyte complexes (COCs) harvested from the oocyte donors used in Chapter 3 and also the fatty acid composition of *in vitro* produced embryos cultured in the presence of serum harvested from heifers from Chapter 3.

In the past, dietary lipid supplements were only considered as a concentrated source of energy, ideal for supporting high energy requirements of lactating dairy cows. Recent studies, however, indicate that dietary lipids can also increase conception rates at first service (McNamara *et al.*, 2003), possibly by influencing ovarian function and the uterine environment (Bao *et al.*, 1997; Staples *et al.*, 1998). Furthermore, dietary polyunsaturated fatty acids (PUFA) have been associated with increased plasma concentrations of metabolic hormones such as GH and insulin, which at the ovarian level can stimulate follicular development (Thomas and Williams, 1996; Beam and Butler, 1997; Mattos *et al.*, 2000; Petit *et al.*, 2002). In addition, PUFA of the n-6 and n-3 families such as linoleic (18:2n-6) and α-linolenic (18:3n-3) acids act as precursors of arachidonic (20:4n-6) and eicosapentaenoic (20:5n-3) acids respectively, which are essential for the synthesis of prostaglandins (Robinson *et al.*, 2002). Other PUFA have been reported to be essential for the synthesis of steroid hormones (Mattos *et al.*, 2000). Collectively, these findings indicate that changes in dietary fatty acids can lead to alterations in endocrine profiles with implication for ovarian function and early embryo survival (Abayasekara and Wathes, 1999; Williams and Stanko, 1999; Mattos *et al.*, 2000; Petit *et al.*, 2002; Robinson *et al.*, 2002). However, our knowledge of the uptake and metabolism of fatty acids during oocyte and early embryo development is still very limited. There are only a few reports on fatty acid composition of bovine oocytes and embryos (Ferguson and Leese, 1999; McEvoy *et al.*, 2000; Kim *et al.*, 2001b). According to Ferguson and Leese (1999) and Kim *et al.* (2001b), lipids not only provide a source of energy, but are also a major component of the cell membranes, precursors of second messenger molecules (Calder, 2003) and are associated with oocyte developmental competence and cryotolerance (Kim *et al.*, 2001b). It has also been suggested that environmental factors,

such as seasonal fluctuations in temperature, can affect the composition of cell membranes of oocytes, leading to impaired developmental competence and fertility (Zeron *et al.*, 2001). Furthermore, routinely used serum supplements during oocyte and embryo development *in vitro*, have also been associated with the major alterations in the lipid content of oocytes and pre-implantation embryos (Sata *et al.*, 1999; Abd El Razek *et al.*, 2000; Reis *et al.*, 2003; Leroy *et al.*, 2005) and as a result lead to altered gene expression, increased sensitivity to oxidative damage and reduced cryotolerance (Rizos *et al.*, 2003; Lonergan *et al.*, 2004). In addition, high non-esterified fatty acids (NEFA) concentrations have been reported to inhibit granulosa cell proliferation (Vanholder *et al.*, 2005) and impair oocyte maturation and post-fertilisation development *in vitro* (Jorritsma *et al.*, 2004).

In summary, there is currently a dearth of information regarding the nutritional regulation of fatty acid composition of granulosa cells (GCs) and cumulus-oocyte complexes (COCs) originating from donors of known nutritional background. Little is also known on the effects of serum harvested from such donors on the fatty acid composition of embryos. Hence the outcomes of this study should help to better understand nutritionally regulated lipid uptake by ovarian tissues *in vivo* and embryos produced *in vitro*.

5.2. Materials and Methods

5.2.1. Animals and dietary treatments

As described previously in Chapter 3. Briefly, a group of 32 cross beef x dairy heifers of approximately 20 months of age were offered either a high fibre (F) or a high starch (S) diet fed alone (0) or supplemented with 6% w/w of calcium soaps of fatty acids (Megalac®, Volac International Ltd, Herts, UK). The composition (g/kg) of Megalac® as stated by manufacturer was oil, 790; methionine, 30; Ca, 90; and moisture, 50. The fatty acid profile (g/kg of total fatty acids) of the oil component was palmitic acid, 440; oleic acid, 400; linoleic acid, 95; stearic acid, 50 and myristic acid, 15 (McNamara *et al.*, 2003). Due to the fact that half of the heifers were in significantly ($P<0.001$) higher BCS than others, the experimental group of 32 animals was divided into two blocks of heifers in either Low (2.5±0.09 units) or Moderate BCS (3.4±0.06 units) with 16 animals

per block. However, it must be emphasised that BCS was not a design factor in this experiment. All heifers were fed at twice maintenance (AFRC, 1993) and daily allowance was adjusted for metabolic live weight and for differences in energy density (MJ ME/kg DM). Dietary treatments were initiated 20 days before the established reference oestrus (Day 0) and fed for a total period of 6 weeks.

5.2.2. Collection of biological material – plasma, GCs and oocytes and embryos

Blood samples were collected daily by jugular venipuncture into EDTA treated vacuum tubes. Immediately after collection samples were stored on ice until all the samples were collected and then centrifuged for 15 minutes at 300 x g. Serum was transferred into auto-analyser cups and stored at –20°C until use. For lipid analysis 1 ml of fresh plasma was used.

On Day 5 of the oestrous cycle all animals were subjected to OPU (Appendix 1). All accessible follicles >2 mm in diameter were aspirated and follicular fluid was collected into 50 ml conical tubes containing 5 ml of warm (37°C) PBS supplemented with 0.2% w/v BSA (FAF) and antibiotics (Appendix 1). Harvested GCs were washed twice in PBS/BSA by centrifugation before being transferred into a glass tubes containing 25 ml of chloroform:methanol (2:1 v/v solution) and 137.5 ng of internal standard (IS, Pentadecanoic acid, C15:0) for subsequent lipid analysis (Appendix 4). At the same time, retrieved COCs were assessed (Appendix 1) and allocated to groups of either good (Grades 1 and 2) or poor quality (Grades 3 and 4) oocytes. Due to the limited number of GCs and COCs anticipated from each heifer and the minimum number required for lipid analysis, GCs and COCs from 2 heifers within each treatment were pooled. Groups containing a minimum of 5 good and 5 poor quality oocytes within each treatment were transferred in a minimum volume of media into glass tubes containing chloroform:methanol solution.

Day 8 blastocysts produced in the experiment described in Chapter 4 (section 4.2.3) were assessed and then in groups of 5 or more blastocysts per treatment were transferred in a minimum volume of media into glass tubes for subsequent fatty acid analysis.

5.2.3. Fatty acid analysis

Plasma, GCs, COCs and blastocysts, were transferred into glass tubes containing 25 ml of 2:1 v/v chloroform:methanol solution and 137.5 ng of internal standard (IS, Pentadecanoic acid, C15:0). The content of each tube was subjected to a total lipid extraction as described by Christie (1982). Samples of Search and SOF media (100 µl) were also analysed to provide a reference fatty acid profile of the media. The esterified lipid fractions were subjected to transmethylation by refluxing with 20:10:1 v/v methanol:toluene:sulfuric acid solution, extracted into a final volume of 50 µl hexane and assayed immediately in duplicate. Using a splitless method, 2 µl of the fatty acid methyl esters were injected via a CP8200 autosampler (Varian Analytical Instruments, Walton-on-Thames, UK) into HP-225 capillary column (30 mm long, 0.25 mm internal diameter, 0.25 µm film thickness; Hewlett Packard Ltd, Stockport, Cheshire, UK). Details of lipid extraction and analysis are included in Appendix 4. Peaks of specific fatty acids were identified and quantified using a Varian Star Chromatography Workstation (Version 5.5; Varian Analytical Instruments) with reference to the internal standard (15:0) and a known standard (Supelco 37 Component mix; Supelco, Poole, Dorset, UK) (Figure 5.1). The content of specific fatty acids is expressed as percentages (w/w) of methyl esters of total lipid content.

5.2.4. Statistical analyses

Data were analysed by Generalised Linear Models within ANOVA using Genstat 6 (Release 6.1). Terms fitted to the model were BCS (Low vs Moderate), source of carbohydrate (Fibre vs Starch), inclusion of protected lipid supplement (0 vs 6%) and the interactions between all these factors. Differences in fatty acid composition between all biological materials analysed, that is plasma, GCs, COCs and embryos, were tested using a simple Paired t-test within Minitab, Release 13.1. Data are presented as means with either appropriate SED or SEM.

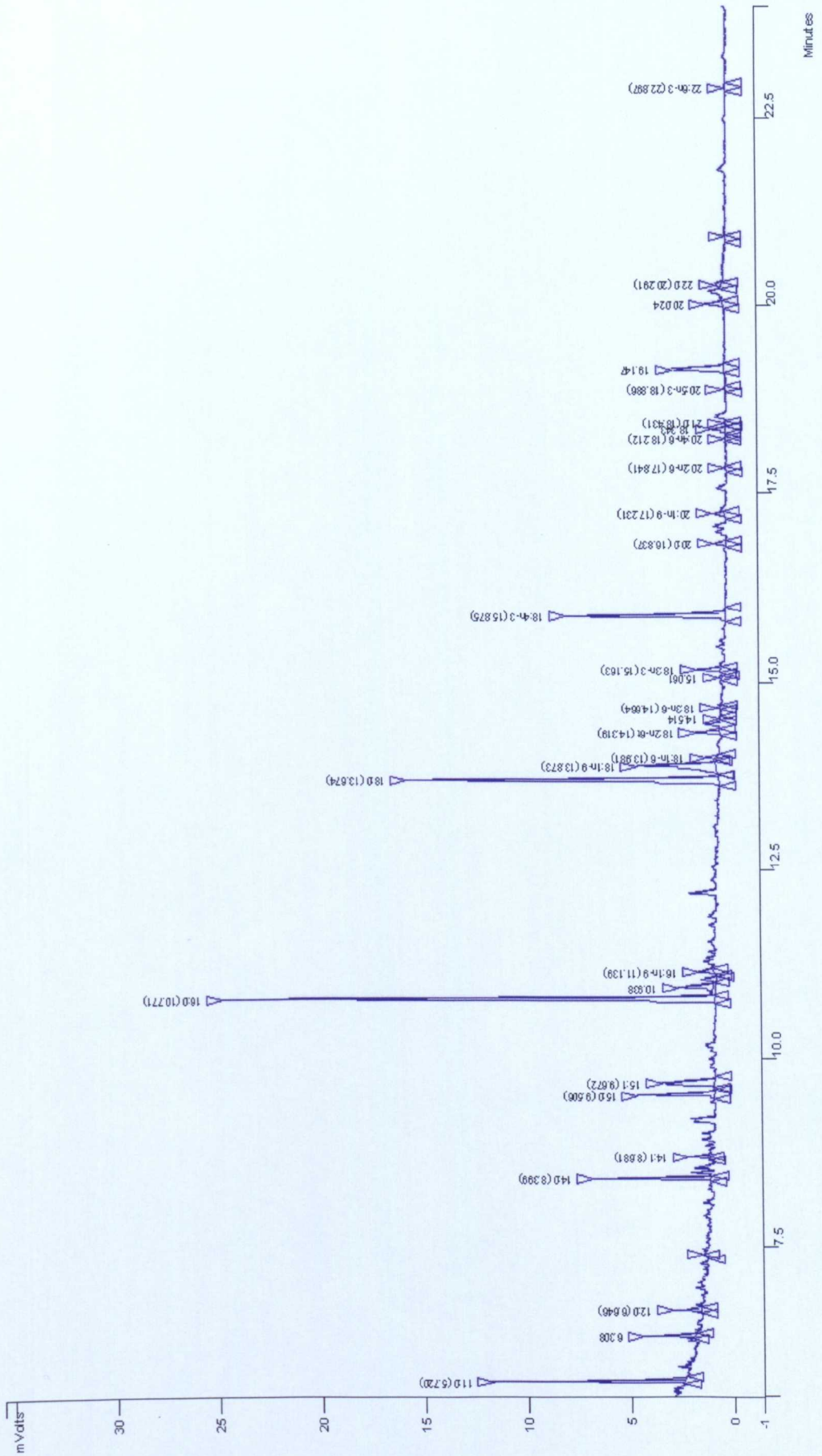

Figure 5.1. A typical gas chromatogram of immature oocytes from oocyte donors used in Chapter 3.

5.3. Results

5.3.1. Fatty acid composition of plasma

The total fatty acid (TFA) content of plasma was greater (P<0.001) for the F than the S diets and increased with the inclusion of Megalac with similar patterns observed in Low and Moderate BCS heifers (Table 5.1). However, the mean TFA content did not vary between animal BCS groups. Furthermore, heifers fed the F diet had a greater (P<0.001) proportion of PUFA and reduced (P<0.001) monounsaturates compared to animals fed the S diet (31.2 vs 25.3%; SED=1.61 and 18.8 vs 21.7%; SED=0.73, respectively). The inclusion of Megalac in the diet increased the percentages of mono- (21.0 vs 19.5% for 6 vs 0; SED=0.73; P=0.051) and polyunsaturated (30.0 vs 26.4% for 6 vs 0; SED=1.61, P<0.05) fatty acids and at the same time reduced (P<0.01) saturated fatty acids (48.9 vs 54.2% for 6 vs 0, SED=1.54). Plasma from heifers in Low BCS had more (P<0.01) saturated (54.0 vs 49.1%, SED=1.54) and monounsaturated (22.4 vs 18.2%, SED=0.73) fatty acids but less (P<0.001) PUFA (23.7 vs 32.8%, SED=1.61), mostly due to changes in linoleic acid (18.7 vs 25.4%; SED=1.58; P<0.05), compared to heifers in Moderate BCS. The n-3 family of PUFA was on average 1.77±0.1% and was unaffected by nutritional treatments or animal BCS. Although the ratio of n-6/n-3 PUFA was greater (P<0.05) for the F than the S diet (18.6 vs 14.5, SED=2.01), it was unaffected by the inclusion of Megalac or animal BCS (Table 5.1).

Analysis by animal BCS indicated that, in Low BCS heifers dietary composition had little effect on fatty acid composition. However, saturates were reduced (P<0.05) by the inclusion of Megalac (50.7 vs 57.2% for 6 vs 0, SED=2.22) and monounsaturates were also reduced (P<0.05) in the F compared to the S diet (20.6 vs 24.1%, respectively; SED=1.31). In contrast, plasma from heifers in Moderate BCS was more affected by dietary composition, where the S diets significantly (P<0.01) increased the percentage of saturated (52.1 vs 46.2%, SED=1.25) and monounsaturated (19.3 vs 17.1%, SED=0.71) fatty acids, but reduced (P<0.001) PUFA (28.6 vs 36.9%, SED=1.29) mainly the members of the n-6 family (26.8 vs 35.1%, SED=1.20). Furthermore, the inclusion of Megalac reduced (P<0.01) saturates (47.2 vs 51.1% for 6 vs 0, SED=1.25) and at the same time increased (P<0.05) PUFA (34.3 vs 31.2% for 6 vs 0, SED=1.29) mainly members of the n-6 family (32.3 vs 29.6%, SED=1.20). There was also an interaction (P<0.01) between the source of carbohydrate and the presence or absence of Megalac

indicating that the proportion of saturated (45.9, 46.4, 56.4 and 47.9% for F0, F6, S0 and S6 respectively, SED=1.8) and monounsaturated fatty acids (15.6, 18.6, 20.1 and 18.5% for F0, F6, S0 and S6 respectively, SED=1.0) were increased whereas PUFA (38.6, 35.1, 23.7 and 33.6% for F0, F6, S0 and S6 respectively, SED=1.8) and n-6 PUFA in particular (37.0, 33.1, 22.1 and 31.6% for F0, F6, S0 and S6 respectively, SED=1.7) were reduced for the S0 treatment compared to the other treatments.

The abundance of individual fatty acids in plasma, expressed as percentages (w/w) of methyl esters of TFA, are presented in Table 5.2. Stearic acid (18:0) was the most abundant out of 22 detected fatty acids, comprising 27.4±0.7% of TFA. Its content was greater ($P<0.01$) for animals in Low compared to Moderate BCS (28.9 vs 25.9%, SED=0.95), but was reduced ($P<0.001$) by the inclusion of Megalac (29.8 vs 25.0%, SED=0.95 for 0 and 6, respectively). Palmitic (16:0) and linoleic (18:2n-6) acids were the second and the third most abundant (22.5±0.6% and 22.0±1.1%, respectively). The presence of palmitic acid was reduced ($P<0.05$) in the F compared with the S diet (21.3 vs 23.8%, SED=0.99) and for animals in Moderate than in Low BCS (21.4 vs 23.6%, SED=0.99). In contrast, linoleic acid was elevated by the same treatments (24.7 vs 19.3% for F vs S diet and 25.4 vs 18.7%, for Moderate vs Low BCS, SED=1.58). Apart from oleic acid (18:1n-9), comprising on average 17.3±0.54% of TFA and representing ~85% of monounsaturates, all remaining fatty acids comprised less than 2% of TFA. In addition, the percentages of most of the fatty acids in plasma, except for erucic (22:1n-9) and docosadienoic (22:2n-6) acids, were greater ($P<0.01$) for heifers in Low compared with Moderate BCS. There was a suggestion ($P=0.051$) that the proportion of arachidonic acid was greater for the F than the S diet (1.0 vs 0.6%, SED=0.17), but only in Low BCS heifers.

The ratio of linoleic (18:2n-6) to arachidonic (20:4n-6) acid was on average 20.2±1.9 and did not differ between nutritional treatments, but was greater ($P<0.001$) in plasma from heifers in Low than in Moderate BCS (26.4 vs 13.9, SED=3.00).

5.3.2. Fatty acid composition of GCs

Granulosa cells harvested from Low BCS heifers contained less ($P<0.01$) saturated (49.3 vs 63.8%, SED=1.47) and more ($P<0.001$) PUFA (18.2 vs 3.1%, SED=1.64) of both

the n-6 and n-3 families (17.0 vs 2.5%, SED=1.61 and 1.2 vs 0.6%, SED=0.16, respectively) compared to Moderate BCS heifers. However, the fatty acid composition of GCs was unaffected by nutritional treatments. In contrast, the percentage of monounsaturated fatty acids did not differ between animal BCS groups, but it was greater ($P<0.01$) for heifers fed without than with Megalac (34.5 vs 31.1% for 0 vs 6, SED=1.03). The ratio of n-6/n-3 PUFA was on average 10.9±1.8 and was greater ($P<0.001$) for animals in Low than in Moderate BCS and was further increased ($P<0.01$) by the inclusion of Megalac (Table 5.1). Analysis by animal BCS indicated that, unlike plasma, nutritional treatments had a more pronounced effect on the fatty acid composition of GCs from heifers in Low than in Moderate BCS. In Low BCS heifers, there were interactions ($P<0.05$) between the dietary factors for saturated, PUFA and n-6 PUFA suggesting that the Starch based diet fed without Megalac (S0) increased saturates (47.3, 48.9, 53.6 and 47.3% for F0, F6, S0 and S6 respectively, SED=1.6) and reduced PUFA (18.7, 20.5, 11.1 and 22.3% for F0, F6, S0 and S6 respectively, SED=2.0), mainly members of the n-6 family (17.2, 19.2, 10.0 and 21.4% for F0, F6, S0 and S6 respectively, SED=1.9), compared to the other treatments. Furthermore, the inclusion of Megalac increased ($P<0.01$) the percentage of PUFA (21.4 vs 14.9%, SED=1.39), n-6 PUFA (20.3 vs 13.6%, SED=1.31) and the ratio of n-6/n-3 PUFA (19.4 vs 10.3, SED=1.68) compared to diets fed without Megalac. Fatty acid composition of GCs from Moderate BCS heifers was unaffected by nutritional treatments.

The fatty acid profile of GCs is presented in Table 5.3. Unlike plasma, the most abundant fatty acid within GCs was palmitic acid (16:0) comprising on average 34.2±2.2% of TFA. Its content was greater ($P<0.001$) for animals in Moderate compared to Low BCS (42.6 vs 26.7%, SED=1.29). Oleic (18:1n-9) and stearic acids (18:0) were the second and the third most abundant fatty acids (27.9±0.9% and 17.5±0.6% of TFA, respectively). As for palmitic acid, the percentage of stearic acid was greater ($P<0.05$) for animals in Moderate than Low BCS (18.8 vs 16.3%, SED=0.84). Only arachidonic (20:4n-6), linoleic (18:2n-6) and myristic (14:0) acids comprised more than 2% of TFA (4.3±1.1, 3.8±0.6 and 2.1±0.2%, respectively). Arachidonic acid was increased ($P<0.05$) by the inclusion of Megalac (9.7 vs 5.7%, SED=1.04), but only in Low BCS heifers. As for plasma, the percentage of most of these fatty acids was increased ($P<0.01$) for animals in Low compared to Moderate BCS

(Table 5.3). The ratio of linoleic (18:2 n-6) to arachidonic (20:4 n-6) acid was on average 1.7±0.4 and did not vary between dietary treatments, but was greater (P<0.001) in GCs from heifers in Moderate than in Low BCS (3.6 vs 0.8, SED=0.19).

5.3.3. Fatty acid composition of COCs

An increase in plasma TFA induced by dietary treatments was reflected within COCs, but only the increase in TFA induced by the inclusion of Megalac reached statistical significance (P<0.05) (Table 5.1). Furthermore, TFA content was greater (P<0.001) within COCs from animals in Low compared with Moderate BCS (78.4 vs 69.2 ng/COC, SED=1.78) and in good (Grades 1 and 2) than in poor (Grades 3 and 4) quality oocytes (79.2 vs 68.4 ng/COC respectively, SED=1.78). Saturated, mono- and polyunsaturated fatty acids comprised on average 87.3±0.9%, 11.0±0.8% and 2.2±0.30% of TFA, respectively, and did not vary between dietary treatments. However, saturates were lower (84.4 vs 90.1%; SED=1.13; P<0.01) and PUFA greater (3.2 vs 1.1%, SED=0.51; P<0.01) for animals in Low compared to Moderate BCS. This increase was for both the n-6 and n-3 families of PUFA (2.4 vs 0.9%, SED=0.22 and 0.9 vs 0.2%, SED=0.20, respectively). In addition, the proportion of saturated fatty acids was lower and monounsaturated greater in good compared to poor quality COCs (85.5 vs 89.0%, SED=1.13 and 13.0 vs 8.9%, SED=0.88, respectively). The ratio of n-6/n-3 PUFA averaged 4.1±0.7 and was unaffected by dietary treatments and did not differ between animal BCS groups. Analysis by BCS indicated that TFA content in COCs from Low BCS heifers was greater (P<0.05) in good than in poor quality COCs (81.4 vs 74.4 ng/COC, SED=2.07) and that the n-6/n-3 ratio was greater (P<0.01) for animals fed the S than the F diet (8.2 vs 3.9, SED=0.71) and in poor compared to good quality COCs (7.4 vs 4.7, SED=0.71). However, specific classes of fatty acids were unaffected by nutritional treatments. In contrast, TFA content in COCs from Moderate BCS heifers was greater (P<0.05) for the S than the F diet (73.5 vs 64.9 ng/COC, SED=2.74) and for heifers fed with than without Megalac (73.0 vs 65.4 ng/COC, SED=2.74). Moreover, TFA and monounsaturated fatty acids were increased in good compared to poor quality COCs (77.1 vs 61.3 ng/COC, SED=2.74, P<0.001; and 12.4 vs 7.5%, respectively, SED=2.03, P<0.05).

In keeping with GCs, palmitic acid (16:0) was the most abundant fatty acid within COCs, comprising 41.1±0.6% of TFA, with stearic acid (18:0) in second place at 36.4±0.7% of TFA. The remaining fatty acids, except for oleic (18:1n-9), myristic (14:0) and behenic (22:0) acids comprising 6.7±0.7%, 4.4±0.2% and 3.0±0.4% of TFA respectively, did not exceed 2% and were unaffected by nutritional treatments. However, their abundance tended to be greater for animals in Low compared with Moderate BCS (Table 5.4). The percentage of arachidonic acid was greater ($P<0.05$) in COCs from Low BCS heifers fed the S than the F diet (0.7 vs 0.2%, SED=0.17) and in good compared to poor quality oocytes (0.7 vs 0.2%, SED=0.17). There was also an interaction ($P<0.01$) between carbohydrate source and the presence or absence of Megalac, suggesting that Megalac increased the abundance of arachidonic acid in the F diets, but reduced the abundance of arachidonic acid in the S diets (0.07, 0.41, 1.17, 0.16% for F0, F6, S0 and S6 respectively, SED=0.25). In contrast, the percentage of arachidonic acid in COCs from Moderate BCS heifers was unaffected by nutritional treatments. The ratio of linoleic (18:2n-6) to arachidonic (20:4n-6) acid averaged 3.7±0.5 and was greater ($P<0.05$) in oocytes from the F than the S fed heifers (4.3 vs 1.4, SED=1.04), but was not affected by Megalac nor animal BCS groups.

5.3.4. Fatty acid composition of embryos

The total fatty acid content per embryo reflected that of plasma, where the F diets and Megalac supplemented diets increased ($P<0.001$) the TFA content compared to the S diets and un-supplemented diets, respectively in both Low and Moderate BCS heifers (Table 5.1). The percentage of saturated fatty acids was greater ($P<0.01$) for embryos cultured with serum from animals fed the S than the F diet (81.4 vs 77.0%, SED=1.08) and was further increased ($P<0.05$) by the inclusion of Megalac (80.8 vs 77.7% for 6 vs 0, SED=1.08). Interestingly, embryos cultured in the presence of serum harvested from animals in Low BCS had reduced ($P<0.01$) saturates (77.6 vs 80.9%, SED=1.08) and increased ($P<0.001$) PUFA (10.4 vs 6.3%, SED=0.64) of both the n-6 and n-3 families (4.2 vs 2.5%, SED=0.35 and 6.2 vs 3.8%, SED=0.69, respectively) compared to serum from Moderate BCS heifers. Furthermore, the percentage of monounsaturated fatty acids was greater ($P<0.01$) in embryos from the F than the S diet (14.3 vs 10.6%, SED=1.11), but was unaffected by the inclusion of Megalac and did not differ between animal BCS groups. Also, the n-6 family of PUFA were increased ($P<0.05$) in the

embryos cultured with serum from the F than the S diet (3.8 vs 2.9%, SED=0.35). There was a suggestion also that the ratio of n-6/n-3 PUFA was increased in embryos cultured with serum from the F than the S diet, but the difference did not reach statistical significance (0.8 vs 0.6, SED=0.13, P=0.083). Analysis by animal BCS indicated that embryos cultured in the presence of serum from Low BCS heifers fed the F diet had more (P<0.05) monounsaturates (13.8 vs 10.3%, SED=1.11), but less (P<0.05) saturates (75.7 vs 79.5%, SED=1.31) compared to the S diet. In addition, the inclusion of Megalac increased (P<0.05) the proportion of saturated fatty acids within embryos (79.8 vs 75.3%, SED=1.31). Other classes of fatty acids were unaffected by nutritional treatments. Embryos cultured with serum from Moderate BCS heifers fed the F diet had a reduced (P=0.052) percentage of saturated (78.4 vs 83.4%, SED=1.82) and increased (P<0.05) the n-6 PUFA (3.1 vs 1.9%, SED=0.37). The ratio of n-6/n-3 PUFA (0.9 vs 0.5, SED=0.12) was also increased (P<0.05).

As for GCs and COCs, palmitic acid (16:0) was the most abundant (44.0±0.9%) fatty acid within embryos, followed by stearic acid (18:0) comprising 26.0±0.5% of TFA. Only oleic (18:1n-9), myristic (14:0), α-linolenic (18:3n-3) and palmitoleic (16:1n-7) acids comprised more than 2% of TFA, which comprised 6.1±0.5%, 4.6±0.3%, 4.4±0.5% and 2.9±0.4% of TFA, respectively. In general, the F diet and the absence of Megalac supplement increased the level of specific fatty acids compared to the S diet and the presence of Megalac in the diet (Table 5.5). Furthermore, differences in fatty acid composition induced by nutritional treatments were more pronounced for embryos cultured in the presence of serum from animals in Moderate compared to Low BCS. The abundance of arachidonic acid in embryos cultured with serum from Low BCS heifers was unaffected by nutritional treatments but, with serum from Moderate BCS, there was a suggestion (P=0.076) that the inclusion of Megalac reduced its abundance (0.35 vs 0.03%, SED=0.14). The ratio of linoleic (18:2n-6) to arachidonic (20:4n-6) acid averaged 7.2±1.7 and was greater (P=0.05) in embryos cultured with serum from Moderate than Low BCS heifers (10.5 vs 3.8, SED=2.84) and from the F than the S diet (10.4 vs 3.9, SED=2.84; P=0.056).

Table 5.1. Total fatty acids and percentages (w/w) of saturated, mono- and polyunsaturated fatty acids (PUFA), families of n-6 and n-3 PUFA and ratio between n-6/n-3, in plasma, GCs, COCs and embryos induced by the main factors that is BCS, principle carbohydrate and addition of Megalac.

		Body condition score		Principle carbohydrate		Megalac (%)		SED
		Low	Moderate	Fibre	Starch	0	6	
Plasma	Total fatty acids (μg/ml)	1.08	1.16	1.29^{A}	0.95^{B}	0.63^{A}	1.61^{B}	0.05
	Saturated	53.96^{A}	49.14^{B}	50.06	53.05	54.17^{A}	48.94^{B}	1.54
	Monounsaturated	22.36	18.20	18.84	21.73	19.54	21.03	0.73
	PUFA	23.66^{A}	32.75^{B}	31.15^{A}	25.26^{B}	26.37^{a}	30.03^{b}	1.61
	n-6 PUFA	21.93^{A}	30.94^{B}	29.35^{A}	23.52^{B}	24.78^{a}	28.09^{b}	1.58
	n-3 PUFA	1.73	1.81	1.79	1.74	1.60	1.94	0.22
	Ratio of n-6/n-3	15.10	18.00	18.60^{a}	14.50^{b}	18.00	15.10	2.01
GCs	Saturated	49.27^{A}	63.80^{B}	55.62	57.45	56.04	57.02	1.47
	Monounsaturated	32.55	33.08	32.76	32.87	34.52^{A}	31.11^{B}	1.03
	PUFA	18.16^{A}	3.09^{B}	11.58	9.67	9.42	11.83	1.64
	n-6 PUFA	16.96^{A}	2.54^{B}	10.57	8.93	8.41	11.09	1.61
	n-3 PUFA	1.20^{A}	0.56^{B}	1.01	0.74	1.00	0.75	0.16
	Ratio of n-6/n-3	14.86^{A}	4.42^{B}	8.79	10.49	6.25^{A}	13.04^{B}	1.98
COCs	Total fatty acids (ng/COC)	78.40^{A}	69.20^{B}	71.90	75.60	70.50^{a}	77.10^{b}	1.78
	Saturated	84.44^{A}	90.10^{B}	86.67	87.88	87.10	87.44	1.13
	Monounsaturated	11.98	9.96	11.36	10.59	10.56	11.39	0.88
	PUFA	3.23^{A}	1.13^{B}	2.30	2.07	2.26	2.10	0.51
	n-6 PUFA	2.37^{A}	0.90^{B}	1.75	1.51	1.91	1.36	0.22
	n-3 PUFA	0.86^{a}	0.23^{b}	0.54	0.55	0.35	0.75	0.20
	Ratio of n-6/n-3	5.41	3.01	4.09	4.33	4.58	3.84	1.09
Embryos	Total fatty acids (ng/embryo)	108.00	107.40	114.20^{A}	101.20^{B}	100.50^{A}	114.90^{B}	2.55
	Saturated	77.55^{A}	80.88^{B}	77.02^{A}	81.41^{B}	77.67^{a}	80.77^{b}	1.08
	Monounsaturated	12.06	12.84	14.28^{A}	10.63^{B}	13.31	11.59	1.11
	PUFA	10.38^{A}	6.28^{B}	8.70	7.96	9.03	7.64	0.64
	n-6 PUFA	4.15^{A}	2.52^{B}	3.76^{a}	2.92^{b}	3.54	3.14	0.35
	n-3 PUFA	6.23^{A}	3.76^{B}	4.95	5.05	5.49	4.50	0.70
	Ratio of n-6/n-3	0.73	0.69	0.84	0.58	0.72	0.70	0.13

Means with different superscripts are significantly different: a – b ($P<0.05$); A – B ($P<0.01$).

Table 5.2. Composition of fatty acids in plasma induced by the main factors (BCS, principle carbohydrate and addition of Megalac) applied in Chapter 3. Content of specific fatty acids is expressed as percentage (w/w) of methyl esters of fatty acids.

Fatty acids	Structure	Body condition score		Principle carbohydrate		Megalac (%)		SED
		Low	Moderate	Fibre	Starch	0	6	
Lauric	C12:0	0.16	0.11	0.10	0.17	0.18^{a}	0.09^{b}	0.04
Myristic	C14:0	0.94	0.90	0.85	0.98	0.16^{A}	0.68^{B}	0.12
Myristoleic	C14:1n-5	0.40	0.40	0.50	0.40	0.50^{a}	0.30^{b}	0.07
Palmitic	C16:0	23.59^{a}	21.43^{b}	21.25^{a}	23.76^{b}	22.42	22.60	0.99
Palmitoleic	C16:1n-7	0.60	0.80	0.60^{A}	0.80^{B}	0.60^{a}	0.80^{b}	0.08
Stearic	C18:0	28.91^{A}	25.93^{B}	27.27	27.56	29.83^{A}	25.00^{B}	0.95
Oleic	C18:1n-9	19.00^{A}	15.60^{B}	16.20^{A}	18.30^{B}	16.60	17.90	0.72
Vaccenic	C18:1n-7	0.70	0.70	0.70	0.80	0.70	0.70	0.11
Linoleic	C18:2n-6	18.67^{A}	25.36^{B}	24.70^{A}	19.34^{B}	20.39^{a}	23.64^{b}	1.58
γ-Linolenic	C18:3n-6	0.46	0.53	0.62^{A}	0.38^{B}	0.44	0.55	0.08
α-Linolenic	C18:3n-3	0.53^{a}	0.74^{b}	0.70	0.57	0.45^{A}	0.82^{B}	0.08
Arachidic	C20:0	0.15^{A}	0.56^{B}	0.35	0.36	0.33	0.38	0.07
Gadoleic	C20:1n-9	0.10	0.10	0.10	0.10	0.10	0.10	0.05
Eicosadienoic	C20:2n-6	0.28	0.26	0.34	0.20	0.20	0.34	0.18
Dihommo-γ-linolenic	C20:3n-6	0.66^{A}	2.02^{B}	1.31	1.37	1.25	1.42	0.12
Arachidonic	C20:4n-6	0.82^{A}	2.10^{B}	1.52	1.41	1.57	1.35	0.23
Eicosatrienoic	C20:3n-3	0.06^{A}	0.18^{B}	0.10	0.14	0.10	0.15	0.04
Eicosapentaenoic	C20:5n-3	1.00	0.69	0.83	0.87	0.88	0.82	0.19
Behenic	C22:0	0.23	0.22	0.24	0.22	0.25	0.20	0.04
Erucic	C22:1n-9	1.50^{A}	0.60^{B}	0.80^{a}	1.30^{b}	1.00	1.20	0.25
Docosadienoic	C22:2n-6	1.03^{a}	0.67^{b}	0.87	0.84	0.93	0.78	0.14
Docosahexanoic	C22:6n-3	0.13	0.19	0.17	0.15	0.16	0.15	0.04

Means with different superscripts are significantly different: a – b ($P<0.05$); A – B ($P<0.01$).

Table 5.3. Composition of fatty acids in GCs collected by OPU on Day 5 of oestrous cycle, from animals used in Chapter 3. Content of specific fatty acids is expressed as percentages (w/w) of methyl esters of total fatty acids.

Fatty acids	Structure	Body condition score		Principle carbohydrate		Megalac (%)		SED
		Low	Moderate	Fibre	Starch	0	6	
Lauric	C12:0	0.37	0.22	0.25	0.34	0.35	0.24	0.10
Myristic	C14:0	2.06	2.19	1.97	2.28	2.27	1.98	0.32
Myristoleic	C14:1n-5	0.40	0.60	0.50	0.50	0.40	0.60	0.29
Palmitic	C16:0	26.72^{A}	42.61^{B}	33.73	35.60	34.83	34.50	1.29
Palmitoleic	C16:1n-7	1.30	0.90	1.10	1.10	1.10	1.10	0.25
Stearic	C18:0	16.29^{a}	18.75^{b}	17.66	17.38	16.69	18.35	0.84
Oleic	C18:1n-9	26.65	29.14	27.05	28.75	30.24^{A}	25.56^{B}	1.25
Vaccenic	C18:1n-7	0.46^{a}	2.33^{b}	1.64	1.15	0.91	1.88	0.81
Linoleic	C18:2n-6	5.61^{A}	1.77^{B}	3.96	3.42	3.37	4.01	0.70
γ-Linolenic	C18:3n-6	0.06	0.05	0.05	0.06	0.06	0.06	0.02
α-Linolenic	C18:3n-3	0.10	0.08	0.09	0.08	0.11	0.07	0.03
Arachidic	C20:0	1.26^{A}	0.02^{B}	0.60	0.68	0.57	0.71	0.08
Gadoleic	C20:1n-9	0.36^{A}	0.07^{B}	0.25^{a}	0.18^{b}	0.16^{A}	0.27^{B}	0.03
Eicosadienoic	C20:2n-6	0.43^{A}	0.01^{B}	0.26	0.18	0.12^{a}	0.32^{b}	0.08
Dihommo-γ-linolenic	C20:3n-6	0.78^{A}	0.17^{B}	0.48	0.47	0.43	0.52	0.10
Arachidonic	C20:4n-6	7.73^{A}	0.34^{B}	4.34	3.73	3.07	4.99	0.91
Eicosatrienoic	C20:3n-3	0.46^{A}	0.09^{B}	0.28	0.27	0.35	0.20	0.11
Eicosapentaenoic	C20:5n-3	0.30	0.34	0.42	0.21	0.41	0.23	0.15
Behenic	C22:0	2.57^{A}	0.01^{B}	1.41	1.17	1.34	1.24	0.25
Erucic	C22:1n-9	3.40^{A}	0.06^{B}	2.27^{a}	1.20^{b}	1.75	1.72	0.45
Docosadienoic	C22:2n-6	2.35^{A}	0.20^{B}	1.47	1.08	1.36	1.20	0.37
Docosahexanoic	C22:6n-3	0.34^{A}	0.05^{B}	0.21	0.18	0.14	0.26	0.06

Means with different superscripts are significantly different: a – b ($P<0.05$); A – B ($P<0.01$).

Table 5.4. Fatty acid composition of COCs retrieved on Day 5 of the oestrous cycle by OPU. Oocytes originated from animals used in Chapter 3. Content of each fatty acid is expressed as percentage (w/w) of methyl esters of total fatty acids.

Fatty acids	Structure	Body condition score		Principle carbohydrate		Megalac (%)		SED
		Low	Moderate	Fibre	Starch	0	6	
Lauric	C12:0	1.28	1.42	1.16	1.54	1.30	1.40	0.36
Myristic	C14:0	3.42^{A}	5.40^{B}	4.44	4.38	4.74^{a}	4.07^{b}	0.21
Myristoleic	C14:1n-5	2.40	1.90	2.50	1.80	1.70^{a}	2.60^{b}	0.32
Palmitic	C16:0	39.28^{A}	42.97^{B}	41.49	40.76	40.83	41.42	0.62
Palmitoleic	C16:1n-7	0.60	0.60	0.50	0.70	0.60	0.60	0.11
Stearic	C18:0	37.65	35.21	35.99	36.87	36.25	36.61	1.00
Oleic	C18:1n-9	7.50	5.80	6.70	6.60	6.90	6.40	0.82
Vaccenic	C18:1n-7	0.10	ND	ND	ND	ND	ND	0.03
Linoleic	C18:2n-6	1.28	0.58	1.10^{a}	0.76^{b}	0.99	0.87	0.11
γ-Linolenic	C18:3n-6	0.35^{a}	0.07^{b}	0.20	0.22	0.25	0.17	0.08
α-Linolenic	C18:3n-3	0.56	0.13	0.32	0.38	0.13	0.57	0.18
Arachidic	C20:0	1.01	0.90	1.10	0.81	0.94	0.97	0.13
Gadoleic	C20:1n-9	0.20	0.50	0.40	0.30	0.40	0.20	0.13
Eicosadienoic	C20:2n-6	0.14	0.06	0.10	0.10	0.13	0.07	0.05
Dihommo-γ-linolenic	C20:3n-6	0.15	0.06	0.11	0.10	0.15	0.06	0.15
Arachidonic	C20:4n-6	0.46^{A}	0.03^{B}	0.15	0.34	0.33	0.16	0.11
Eicosatrienoic	C20:3n-3	0.02	ND	0.02	0.001	0.02	0.006	0.01
Eicosapentaenoic	C20:5n-3	0.22	0.10	0.18	0.14	0.20	0.12	0.06
Behenic	C22:0	1.81^{A}	4.20^{B}	2.49	3.52	3.04	2.97	0.38
Erucic	C22:1n-9	1.30	1.10	1.20	1.20	1.00	1.40	0.25
Docosadienoic	C22:2n-6	ND	0.09	0.09	ND	0.07	0.02	0.05
Docosahexanoic	C22:6n-3	0.06	ND	0.02	0.04	0.01	0.05	0.02

Means with different superscripts are significantly different: a – b ($P<0.05$); A – B ($P<0.01$).

Table 5.5. Fatty acid composition of Day 8 blastocysts cultured in presence of serum (Chapter 4) collected from heifers used in Chapter 3. Content of each fatty acid is expressed as percentage (w/w) of methyl esters of total fatty acids.

Fatty acids	Structure	Body condition score		Principle carbohydrate		Megalac (%)		SED
		Low	Moderate	Fibre	Starch	0	6	
Lauric	C12:0	1.75	1.83	2.03^{A}	1.55^{B}	1.82	1.76	0.14
Myristic	C14:0	4.57	4.63	5.30^{a}	3.90^{b}	4.53	4.67	0.55
Myristoleic	C14:1n-5	2.60^{a}	1.90^{b}	3.20^{A}	1.40^{B}	2.20	2.40	0.55
Palmitic	C16:0	44.00	45.00	42.00^{a}	47.00^{b}	43.00	46.00	1.50
Palmitoleic	C16:1n-7	2.80	3.00	3.40	2.40	3.80^{a}	2.00^{b}	0.71
Stearic	C18:0	26.00^{a}	27.00^{b}	25.00^{A}	28.00^{B}	26.00	27.00	0.60
Oleic	C18:1n-9	5.51	6.73	6.77	5.47	5.83	6.42	0.94
Vaccenic	C18:1n-7	ND	0.10	0.10	ND	0.10	ND	0.10
Linoleic	C18:2n-6	1.85	1.79	2.25	1.40	1.75	1.89	0.39
γ-Linolenic	C18:3n-6	0.83^{A}	0.28^{B}	0.61	0.50	0.74^{A}	0.37^{B}	0.10
α-Linolenic	C18:3n-3	5.55^{A}	3.21^{B}	4.32	4.44	4.83	3.93	0.76
Arachidic	C20:0	1.29	1.22	1.43	1.07	1.26	1.25	0.24
Gadoleic	C20:1n-9	0.82	0.79	0.51^{a}	1.10^{b}	1.02	0.59	0.20
Eicosadienoic	C20:2n-6	0.59	0.22	0.53	0.28	0.45	0.36	0.24
Dihommo-γ-linolenic	C20:3n-6	0.28	ND	ND	0.28	0.10	0.18	0.20
Arachidonic	C20:4n-6	0.54^{a}	0.19^{b}	0.29	0.44	0.43	0.30	0.13
Eicosatrienoic	C20:3n-3	ND	ND	ND	ND	ND	ND	-
Eicosapentaenoic	C20:5n-3	0.07	0.25	0.07	0.24	0.21	0.11	0.09
Behenic	C22:0	0.70	1.09	0.96	0.83	0.83	0.97	0.26
Erucic	C22:1n-9	0.30	0.32	0.35	0.27	0.36	0.26	0.16
Docosadienoic	C22:2n-6	0.06	0.04	0.08	0.02	0.06	0.04	0.05
Docosahexanoic	C22:6n-3	0.61	0.30	0.55	0.36	0.45	0.46	0.19

Means with different superscripts are significantly different: a – b ($P<0.05$); A – B ($P<0.01$).

5.3.5. Differences in fatty acid profiles between specimen tested

Cumulus-oocyte complexes and embryos contained a higher ($P<0.001$) proportion of saturated fatty acids compared to plasma and GCs (Figure 5.2.a), but the proportion of saturates in plasma did not differ from that of GCs. In contrast, unsaturated fatty acids were greater ($P<0.01$) in plasma and GCs compared with oocytes and embryos. The highest ($P<0.001$) percentage of monounsaturated fatty acids was detected in GCs compared to all other tissues tested (Figure 5.2.b), whereas PUFA levels were gradually decreased ($P<0.001$) between plasma and COCs (Figure 5.2.c). The proportion of PUFA in embryos was similar to that of GCs. Furthermore, the ratio of n-6/n-3 PUFA was highest in plasma and it proportionately decreased from 16.6 for plasma to 9.6, 4.2 and 0.7 for GCs, COCs and embryos, respectively (Figure 5.2.f). This was mainly due to the decreasing levels of PUFA from the n-6 family (Figure 5.2.d).

Palmitic (16:0) and stearic (18:0) acids were the most abundant fatty acids within all the biological material tested. However, their abundance varied slightly between the tissues analysed. The percentage of palmitic acid (16:0) increased between plasma, GCs and COCs, with the highest levels detected within embryos (22.5±0.6%, 34.7±2.2%, 41.1±0.6% and 44.0±0.9%, respectively). In contrast, the percentage of stearic acid (18:0) was the highest in oocytes, moderate in plasma and the lowest percentage was detected in GCs (36.3±0.7, 27.4±0.7, 26.3±0.5 and 17.5±0.6% for COCs, plasma, embryos and GCs, respectively). Furthermore, the abundance of linoleic acid (18:2n-6) was higher in plasma (22.0±1.1%) compared to other tissues tested, in which its abundance did not exceed 5% of TFA. The highest percentage of γ-linolenic acid (18:3n-6) was detected in plasma and embryos compared to oocytes and GCs (0.5±0.04 and 0.6±0.10%, 0.2±0.06 and 0.1±0.01 %, respectively). Whereas, the levels of arachidonic acid (20:4n-6) were the highest in GCs and plasma (4.3±1.1 and 1.5±0.2%) but within oocytes and embryos it comprised less than 1% of TFA.

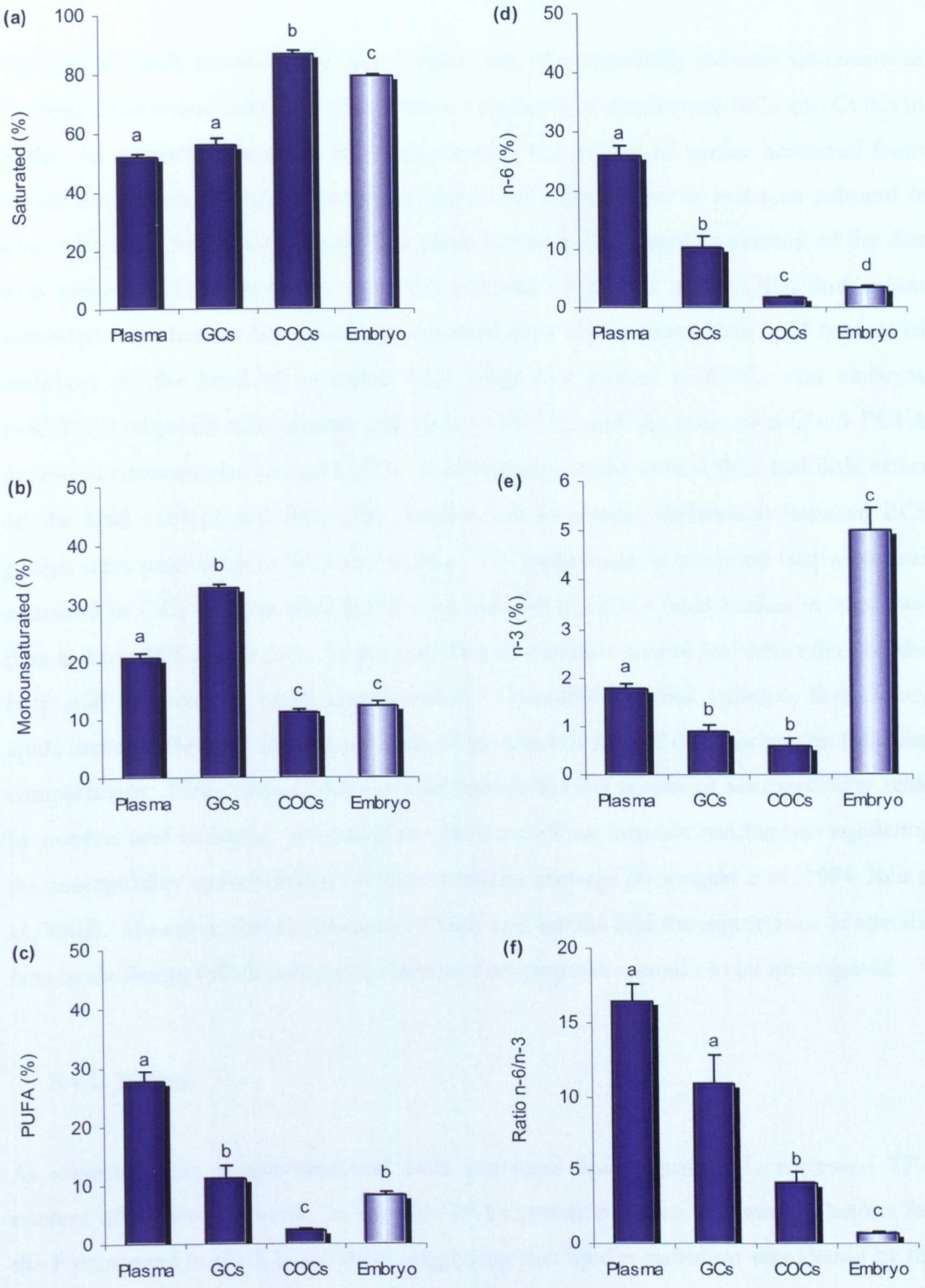

Figure 5.2. Fatty acid profiles of plasma, GCs, COCs and embryos for (a) saturated, (b) monounsaturated, (c) PUFA and families within PUFA (d) n-6 and (e) n-3 families; and the ratio of n-6 to n-3 families (f).

5.4. Discussion

The current study produces the first detailed data of nutritionally induced alterations in the lipid content and fatty acid composition of plasma, *in vivo* derived GCs and COCs in cattle. In addition, this study has demonstrated the effects of serum harvested from oocyte donors on the lipid content and fatty acid composition of embryos cultured *in vitro*. The main findings of the present studies were (i) that supplementation of the diet with protected lipid increased the TFA content of plasma and COCs, and serum harvested from heifers fed these supplemented diets also increased the lipid content of embryos; (ii) the level of saturated fatty acids was greater in COCs and embryos (~83.3%) compared with plasma and GCs (~53.9%), and the ratio of n-6/n-3 PUFA decreased between plasma and COCs. Furthermore, whilst animal BCS had little effect on the lipid content and fatty acid composition in plasma, differences between BCS groups were prominent in GCs and COCs. The percentage of saturated fatty acids was increased in GCs whereas total lipids were reduced in COCs from heifers in Moderate than in Low BCS (Table 5.1). In general, the carbohydrate source had little effect on the fatty acid composition of all tissues tested. Overall these data indicates that dietary lipids increase the lipid content not only of plasma but also of cells within the follicular compartment. Nevertheless, there is a preference for the uptake of saturated fatty acids by oocytes and embryos, indicating the presence of an intrinsic mechanism regulating the susceptibility to free radical or other oxidative damage (Nonogaki *et al.*, 1994; Reis *et al.*, 2003). However, the mechanisms of fatty acid uptake and the importance of specific fatty acids during follicle, oocyte and embryo development remain to be investigated.

5.4.1. Plasma

As expected, diet supplementation with protected lipid significantly increased TFA content of all tissues tested. In plasma, TFA content was also increased in heifers fed the F compared to the S based diets, suggesting that lipid metabolism was altered by the source of carbohydrate and that the F based diets increased the release of fatty acids into the circulation. Although, in the present study, we did not identify specific groups of lipids, the available evidence suggests that dietary fat leads to an increase in peripheral cholesterol (Lammoglia *et al.*, 1997) and high density lipoprotein (HDL) – cholesterol (Hawkins *et al.*, 1995; Thomas *et al.*, 1997). Cholesterol is the main precursor of steroid

synthesis and a component of cellular membranes (Mattos *et al.*, 2000), therefore it was not surprising that elevated concentrations of cholesterol have been related with increased progesterone secretion (Staples *et al.*, 1998). We can presume that dietary lipid supplementation in the present study also resulted in elevated peripheral concentrations of cholesterol and HDL which might have had an effect on follicular proliferation and steroidogenesis (Bao *et al.*, 1995, 1997). However, as reported in Chapter 3, only in Low BCS heifers fed the Fibre diet did the inclusion of Megalac (treatment F6) result in an increase in progesterone secretion. This finding indicates that diet and animal body composition may alter lipid metabolism and possibly cholesterol availability for steroid synthesis and/or reduce the clearance rate of progesterone from the circulation (Hawkins *et al.*, 1995).

Palmitic and oleic acids were the predominant fatty acids within the lipid supplement whereas stearic acid was the primary fatty acid in plasma (27.4%), followed by palmitic (22.5%), linoleic (22.0%) and oleic (17.3%) acids. From the studies of Lessard *et al.* (2003) it appears that the fatty acid composition of plasma is not only dependent on the composition of lipid supplement, but also on the diet itself. Interestingly, the percentage of the most abundant fatty acids in plasma was greater in Low than in Moderate BCS heifers and was reduced by the inclusion of protected lipid in the diet, indicating that lipid metabolism is dependent on animal body composition and fatty acid uptake and/or release is dependent on the presence or absence of fats in the diet. The fatty acid composition of foetal calf serum (Sata *et al.*, 1999; Kim *et al.*, 2001b) and also serum from Megalac-fed dairy cows (Lessard *et al.*, 2003) differed from the plasma reported here. Although earlier studies reported that linoleic acid was the most abundant fatty acid, there have been discrepancies between studies in terms of the remaining predominant fatty acids. Age difference and, most importantly, the metabolic activity of serum donors may have been responsible for the discrepancies between the previous and the present studies.

Linoleic acid and eicosapentaenoic acid are proven inhibitors of cyclooxygenase in endometrial tissue of dairy cows. As a result, endometrial secretion of prostaglandins can be suppressed, thus potentially preventing early embryonic death (Staples *et al.*, 1998). Hence, diets rich in PUFA have been proposed to have a beneficial effect on early embryo development and survival (Thatcher *et al.*, 2003). However, as reported in

Chapter 4, supplementation of embryo culture media with serum from Moderate BCS (with greater content of PUFA, mostly of n-6 series) reduced post-fertilisation development, indicating that while increased content of PUFA can have a positive effect on the later stages of pregnancy, they can be detrimental to oocyte and early embryo development.

5.4.2. Granulosa cells and COCs

Intracellular lipids not only provide energy storage but also play an important role in regulating the properties and functions of cell membranes i.e. cell-cell interactions, cell proliferation and transport (Stubbs and Smith, 1984). They can also influence sensitivity response of cells to freezing (e.g. Otoi *et al.*, 1997; Sata *et al.*, 1999; Kim *et al.*, 2001b). However, the importance of lipids in GCs and oocyte physiology is still not known. As mentioned earlier, the objective of this study was to investigate nutritionally induced alterations in fatty acid composition of GCs and COCs originating from the same donors. The content of saturated fatty acids in GCs was similar to that of plasma, but unlike plasma it was significantly greater ($P<0.01$) in Moderate than in Low BCS heifers, mainly due to increased palmitic acid (42.6 vs 26.7%). Furthermore, monounsaturates, mostly represented by oleic acid (27.7%), were greater and PUFA were lower in GCs than in plasma. Nutritional treatments had little effect on fatty acid composition of GCs, suggesting that the follicular compartment has a selective mechanism of fatty acid uptake. In accord with studies of Zeron *et al.* (2001) the content of arachidonic acid was significantly greater in GCs compared to oocytes (4.3 vs 0.3%) and, in the present study, was also increased by the inclusion of protected lipid, but only in Low BCS heifers. As recently reported, arachidonic acid and its metabolites play a role in ovulation and maturation of Atlantic Croaker oocytes (Patiño *et al.*, 2003) and are also involved in the ovulatory process in the pig (Downey *et al.*, 1998). It is tempting to speculate that in thinner animals by increasing the abundance of arachidonic acid within follicles dietary fat might enhance ovulation rates in cattle. However, final conclusions on the importance of arachidonic acid in bovine follicle and oocyte development are difficult to make, due to limited evidence in the literature and insufficient information from the current study.

As stated earlier, the presence of protected lipid in the diet increased total lipid content within COCs. In contrast to the studies of Zeron *et al.* (2001), however, the fatty acid composition of COCs was somewhat different from that of GCs, indicating that follicular cells possibly act as a filter for fatty acids and regulate their uptake by the oocyte. To further support this hypothesis, the fatty acid composition of plasma was different from GCs which appear to be in between that of plasma and COCs (Figure 5.2). Interestingly, although the lipid content of COCs harvested from Low BCS heifers was greater than that of heifers in Moderate BCS, the reason for this variation is unclear and there is no other evidence to explain the influence of animal body composition on oocyte lipid content. It was demonstrated in Chapter 3 that oocyte developmental competence was unaffected by animal BCS group. However, lipid supplementation and hence the increased lipid content within oocytes from Low BCS heifers, was associated with reduced oocyte developmental competence but not in Moderate BCS heifers. This suggests that body composition may have a role in determining the effects of lipid content on oocyte quality, but the mechanisms remain unclear.

The phospholipid and triglyceride fractions of TFA within immature cattle oocytes comprise 25% and 36% of TFA, respectively (McEvoy *et al.*, 2000). Within these fractions, saturated, monounsaturated and PUFA comprised 41-49%, 38-46% and 13-14% of TFA, respectively. Therefore we can presume that the major lipid groups were similarly distributed within oocytes tested in the present study. Phospholipids are the main components of the cellular membranes and the source of fatty acids necessary for the synthesis of prostaglandins (Mattos *et al.*, 2000; Berg *et al.*, 2002), whereas triglycerides are a major source of energy for developing oocytes, since their content reduces following oocyte maturation *in vitro* (Ferguson and Leese, 1999). In agreement with McEvoy *et al.* (2000), Kim *et al.* (2001b) and Zeron *et al.* (2001), but in contrast to studies of Sata *et al.* (1999), the most abundant fatty acid in COCs in the present study was palmitic acid (comprising 41.2% of TFA) followed by stearic acid (36.3%), which together represented almost 89% of saturated fatty acids. Saturated fatty acids (87.2%) were more abundant than monounsaturates (11.1%) which, in accord with the previous studies (Matorras *et al.*, 1998; McEvoy *et al.*, 2000), were more abundant than PUFA (2.2%). The n-6 family (mostly linoleic acid (0.9%)) contributed mostly to the PUFA (1.6%), whereas the n-3 family contributed only 0.5% to PUFA. Surprisingly, the content of saturated fatty acids in COCs was significantly greater and at the same time

PUFA was lower, compared to that of GCs, indicating that oocytes may benefit by storing the preferential precursors which are at lower risk of free radical or other oxidative damage (Kim and Parthasarathy, 1998; Baker and Aitken, 2004). It has previously been suggested (Kim *et al.*, 2001b) that the fatty acid composition might be an important factor in determining oocyte developmental competence. The results of the previous studies (Chapters 3 and 4), however, indicated that fatty acid composition of COCs was generally unaffected by nutritional treatments and was not correlated with oocyte quality. This also suggests that more extreme dietary treatments could induce changes in oocyte fatty acid composition or one could hypothesise that oocyte fatty acid composition is regulated by follicular cells or even the oocyte itself, and that there is an optimal level of fatty acids for oocyte development which is difficult to alter.

5.4.3. Embryos

Supplementation of embryo culture media with serum has previously been associated with the increased accumulation of lipids (Abe *et al.*, 1999b, 2002; Ferguson and Leese, 1999; Abd El Razek *et al.*, 2000; Kim *et al.*, 2001b; Reis *et al.*, 2003; Leroy *et al.*, 2005) through either increased lipid uptake from the medium or due to disturbance of mitochondrial metabolism (Crosier *et al.*, 2001). Furthermore, inclusion of serum can also lead to alterations in embryo energy metabolism, gene expression and abnormalities in foetal birth weights, recognised as the large offspring syndrome (Thompson *et al.*, 1995; Walker *et al.*, 1996; Young *et al.*, 1998; Sinclair *et al.*, 2000b). Moreover, the presence of serum in embryo culture medium can increase embryo sensitivity to cryopreservation (Massip *et al.*, 1995; Yamashita *et al.*, 1999), what may be directly related to the fatty acid composition of the embryo. As demonstrated in the present study, lipid accumulation by embryos is also dependent on the fatty acid composition of serum, indicating that dietary intake may affect the lipid content and composition of *in vivo* produced embryos. This may have serious implications in the later stages of embryo development.

It was demonstrated in the previous Chapter that embryo quality can be influenced by serum composition. The percentage of saturated fatty acids in embryos (79.2%), mostly composed of palmitic (44.4%) and stearic (26.3%) acids, was greater than in plasma (79.2 vs 51.8%), but was lower than in COCs (79.2 vs 87.1%). Also, Sata *et al.* (1999)

reported that palmitic (44.0%) and stearic (26.0%) acids were the most abundant fatty acids within bovine blastocysts cultured in the presence of serum. Whilst monounsaturates were greater in embryos cultured with serum from heifers fed the Fibre than the Starch based diet, they were unaffected by the lipid supplement, whereas PUFA were reduced by the presence of Megalac and unaffected by carbohydrate source. This suggests that different groups of lipids can be differentially affected by the dietary composition. The fact that embryos cultured in the presence of serum from heifers in Low than in Moderate BCS were more competent to develop to the blastocyst stage *in vitro*, may be associated with reduced saturates and increased PUFA in embryos. Dietary induced increases in PUFA *in vivo* have previously been related to reduced prostaglandin synthesis and hence improved embryo survival (e.g. Thatcher *et al.*, 1994; Abayasekara and Wathes, 1999; Mattos *et al.*, 2000). However, there is no evidence to suggest that increased PUFA in plasma are associated with an increase in PUFA within *in vivo* derived embryos, therefore not supporting this conclusion. In fact, in the present study, it appears that, whilst the percentage of PUFA in plasma was increased, PUFA percentage in embryos decreased. However, it should be noted that *in vitro* embryo culture conditions do not create the exact *in vivo* oviductal environment, where possibly fatty acids derived from plasma might be altered/metabolised by oviductal cells prior to their release into the oviductal fluid. In general, the percentages of specific fatty acids were increased by the F based diets and, surprisingly, by the absence of the protected lipid supplement, suggesting that fatty acid uptake by embryos was increased in the presence of serum containing less fatty acids. As mentioned earlier, serum originating from different nutritional backgrounds had an influence on embryo quality, however, it cannot be directly related to the fatty acid composition of embryos.

In contrast to all other tissues tested, the content of n-3 PUFA within embryos (5.0%), comprising mainly of α-linolenic acid (4.4%), was greater than the n-6 family (3.3%). Therefore the n-6/n-3 ratio was significantly lower in embryos than in plasma, GCs and COCs (Figure 5.2.f). It is well documented in humans that increased consumption of n-3 PUFA is associated with an increase in health (e.g. Connor, 2000; Morimoto *et al.*, 2005). Furthermore, it has recently been suggested that the n-3 PUFA play an important role in determining gestational duration (e.g. Baguma-Nibasheka *et al.*, 1999; Allen and Harris, 2001) and are also required for normal foetal retina and brain development (e.g. Hoffman *et al.*, 1993; Connor, 2000). However, there is no

information in the literature on the importance of the n-3 family PUFA during early embryo development. We can only hypothesise that even during the early stages of embryo development n-3 PUFA are accumulated and required for normal embryo growth.

5.5. Conclusions

Dietary supplementation of fatty acids significantly increased the lipid content of plasma, GCs, COCs and embryos; the latter cultured in the presence of serum from animals fed such diets. Nutritional treatments had little effect on the abundance of specific fatty acids in ovarian cells and, in general, were not correlated with oocyte and embryo quality. There were, however, differences between animal BCS groups, but the underlying mechanisms and reasons for these discrepancies are unknown. The fatty acid composition of plasma, however, differed from that of GCs and COCs indicting selective uptake of fatty acids with preference for saturates. Further studies are required to establish the importance and function of specific fatty acids in the oocyte and early embryo.

CHAPTER 6

General discussion and conclusions

6.1. General discussion

The relationship between nutrition and reproduction is well established, the mechanisms, however, remain poorly understood (e.g. Lucy, 2001). Over the last 20 years the dairy industries of the UK and other developed countries have been faced with a problem of declining fertility. One of approaches to counteract this decline is to regulate dietary composition in order to improve oocyte quality, which is the key to successful fertilisation and subsequent embryo development.

For an annual calving interval in dairy cows pregnancy must be re-established within a matter of weeks from calving, a period that coincides with NEBAL. This is characterised by an altered endocrine milieu, including increased peripheral concentrations of GH and NEFA and reduced peripheral concentrations of insulin and IGF-I (e.g. McGuire *et al.*, 1995; Rukkwamsuk *et al.*, 1999; Jorritsma *et al.*, 2003; Lucy, 2003), which are negatively correlated with fertility. Nutrition can act at different levels within the hypothalamus-pituitary-ovarian axis to control ovarian function (O'Callaghan and Boland, 1999; Armstrong *et al.*, 2003), but there is a dearth of information on the direct effects of nutrition on the follicle-enclosed oocyte and the implications that this has for subsequent embryo development. In ruminants, gonadotrophin secretion is not affected by nutrition to the same extent as in monogastric animals (O'Callaghan and Boland, 1999), suggesting that other factors are involved in the regulation of ovarian function and fertility (Gong, 2002). Recent studies, therefore, have focused on the local actions of metabolic hormones and growth factors, such as insulin, IGFs and leptin, the secretion of which are altered during NEBAL.

The majority of studies to date, however, have mainly considered the effects of plane of nutrition in moderately fat non-lactating animals (e.g. Nolan *et al.*, 1998; Armstrong *et al.*, 2001), in which high levels of feeding are associated with impaired fertility and oocyte viability (McEvoy *et al.*, 1995; Yaakub *et al.*, 1999a). Furthermore, the mechanisms that lead to impaired reproductive performance in nutritionally compromised post-partum cattle have also been investigated intensively (e.g. Mackey *et al.*, 2000; Butler, 2000; Lucy, 2003). However, as demonstrated in Chapter 2, the effects of plane of nutrition are dependent on animal BCS. Whilst high levels of feeding (2M) were detrimental to oocyte quality in moderately fat animals (in agreement with previous reports) they were

beneficial to oocyte quality in the thinner animals. More importantly, as data showed (Figure 2.7.b) these effects accumulated over time period, indicating that oocyte quality/development is gradually altered by dietary intake. Furthermore, in 8/12 animals within the Moderate BCS group, the high plane of nutrition led to hyperinsulinaemia, which was associated with impaired oocyte quality and subsequent embryo development. Chapter 3 further emphasised the importance of animal BCS in the nutritional regulation of oocyte competence, in which the effects of altering dietary composition (both carbohydrate type and the level of fatty acids) were also dependent on animal BCS. High starch diets in ruminants alter the proportion of volatile fatty acids resulting in an increase in propionate production and glucose synthesis in the liver (Lees *et al.*, 1990). Consequently, high glucose concentrations lead to increased post-prandial insulin concentrations. As reported previously, elevated plasma insulin concentrations can reduce the interval from calving to first ovulation (Gong *et al.*, 2002), in part by regulating cellular function within the ovarian follicle (Gutierrez *et al.*, 1997a; Armstrong *et al.*, 2002a).

The inclusion of protected lipid within the ruminant diet has also been associated with improved fertility and whole animal body energy metabolism (Staples *et al.*, 1998; Mattos *et al.*, 2000). Dietary lipids can increase the number of follicles and can have a beneficial effect on corpus luteum function (e.g. Williams, 1989; Lammoglia *et al.*, 1997; Petit *et al.*, 2002; Robinson *et al.*, 2002). In the present study, however, both starch based diets and the inclusion of rumen protected palm oil fatty acids (Megalac) had a negative effect on oocyte quality in Low BCS heifers. In contrast, oocyte quality in Moderate BCS heifers was unaffected by the dietary composition. These effects, however, were not observed during the final stages of oocyte maturation and during post-fertilisation development *in vitro*. In fact, there was a suggestion that serum from Low BCS heifers offered protected fatty acids had a beneficial effect on oocyte maturation *in vitro*, although the differences were not statistically significant.

Embryo quality, on the other hand, was impaired by the inclusion of sera from heifers fed Starch based diets and those offered the protected fatty acids supplement fed together with the Fibre based diets (Chapter 4; Figure 4.2). In addition, blastocyst yields were significantly greater, when zygotes were cultured in the presence of serum from Low compared to Moderate BCS heifers, suggesting that early embryo development and

embryo survival are dependent on animal BCS. Taken together, these findings demonstrate that oocyte developmental competence is mostly influenced by nutrition during the antral stages of follicular development, but these effects may differ during oocyte maturation and early embryo development. Nevertheless, dietary-induced effects on the oocyte and embryo development are dependent on animal BCS. The underlying mechanisms and specific factors responsible for discrepancies between BCS groups, however, remain unknown.

To help characterise the influence of nutrition on ovarian function and oocyte quality, changes in steroid hormones, such as progesterone and oestradiol, and metabolic hormones including insulin, IGF-I and leptin, were monitored. It was previously demonstrated that secretion of these hormones can be influenced by dietary intake. Furthermore, they can have a direct effect on the intra-follicular environment and hence oocyte quality (e.g. Gutierrez *et al.*, 1997a; Armstrong *et al.*, 2002a; Lucy, 2003). In the first study (Chapter 3), as predicted, the high level of feeding (2M) for heifers of Moderate BCS significantly increased plasma insulin concentrations, but at the same time, plasma leptin concentrations were also increased, demonstrating that insulin and leptin secretion are correlated rendering it difficult to separate their effects on ovarian function *in vivo*. In agreement with previous reports, plasma leptin concentrations in Chapters 2 and 3 were positively correlated with body fatness, level of feeding (Ehrhardt *et al.*, 2000; Delavaud *et al.*, 2000, 2002; Thomas *et al.*, 2001; León *et al.*, 2004) and plasma insulin (Kamoda *et al.*, 1998; Saad *et al.*, 1998; Fernandez-Real *et al.*, 2000; Sonnenberg *et al.*, 2001; Block *et al.*, 2003). From the available evidence it is tempting to speculate that moderate concentrations of both insulin and leptin, observed in Low BCS heifers fed 2M diets, may improve oocyte quality, whereas excess concentrations of these hormones, induced by 2M diets fed to moderately fat heifers, have a deleterious effect on oocyte developmental competence. However, in the latter study the starch-induced increase in insulin, which was similar to that of Low BCS fed 2M in Chapter 2, was associated with reduced post-fertilisation development *in vitro*, making it difficult to draw final conclusions. Therefore, it remains unclear as to what factors act as the main determinants of oocyte quality. There is possibly a fine balance between all of these endocrine factors and possibly other factors not monitored in the present studies acting to regulate oocyte quality. There is little information on the interaction between insulin and leptin, and their combined effects on oocyte and embryo development. Therefore,

future studies should establish the exact relationship between these two metabolic hormones and their impact on ovarian function.

In terms of follicular development, in Chapter 2 the high (2M) level of feeding increased the number of medium- (4 to 8 mm) and large-sized (> 8 mm) follicles (Table 2.4), and this may be correlated with increased concentrations of insulin as suggested by others (Gutierrez *et al.*, 1997a; Yu *et al.*, 1999; Armstrong *et al.*, 2002a) and leptin. According to Gong *et al.* (2002), however, heifers fed at 2M compared to M, had significantly more small and large follicles following FSH treatment. The duration of exposure to the level of feeding, however, varied between studies and may be responsible for these discrepancies. It was demonstrated in Chapter 3 that the total number of follicles increased following stimulation with FSH and GnRH (Figure 3.5.a). In general, the Fibre based diets increased the number of medium-sized follicles, but only in Low BCS heifers, whereas the inclusion of protected fatty acids did not affect follicle number and diameter. These observations are in contrast to the studies of Lammoglia *et al.* (1997) and Thomas *et al.* (1997), where cows fed diets containing lipid supplement had increased follicular population. In the study described in Chapter 3, an increase in the number of medium-sized follicles could not be related to increased plasma concentrations of insulin and/or leptin, indicating that other factors may be involved in the regulation of follicular development. Overall, it seems likely that the dietary treatments employed in the present series of studies had a more pronounced effect on the oocyte than the follicle. This may have arisen because nutrition either directly affects oocyte quality and/or alters the intra-follicular environment. Throughout these studies, however, animal BCS appeared to play a significant role in the regulation of follicular, oocyte and embryo development.

Our knowledge of fatty acid uptake and metabolism, and its importance during oocyte and embryo development is limited. There are only a few reports on the lipid composition of ovine, porcine and bovine oocytes (Ferguson and Leese, 1999; McEvoy *et al.*, 2000; Kim *et al.*, 2001b). Nevertheless, fatty acids have been suggested to play an important role in determining oocyte developmental competence (Kim *et al.*, 2001b). Chapter 5 presented data on nutritionally induced alterations to the lipid content and the fatty acid composition of plasma, GCs and COCs, all originating from the same donors, and also embryos cultured in the presence of serum from these donors. The inclusion

of protected fatty acids increased the lipid content not only of plasma, but also of oocytes and embryos (Table 5.1). Despite the fact that follicular growth was unaffected by dietary fatty acids oocyte quality was compromised, indicating that excess fatty acids may alter the intra-follicular environment. Or possibly the increased lipid content of the oocyte itself can have a negative effect on its developmental competence. However, the fact that similar increases in the lipid content of oocytes from Moderate BCS heifers was not associated with reduced developmental competence makes a final conclusion difficult to make. Nevertheless, as mentioned earlier, the same dietary fatty acids may have a different effect during the final stages of oocyte maturation and during early embryo development. In fact, dietary fatty acids when fed with the Starch based diets had a positive effect on embryo quality and could possibly increase its chances of survival. It has been demonstrated previously that PUFA are essential for the synthesis of steroid hormones (e.g. Mattos *et al.*, 2000; Robinson *et al.*, 2002) and have an inhibitory effect on prostaglandin synthesis, therefore creating a more favourable environment for embryo development and the successful establishment of pregnancy (Thatcher *et al.*, 1994; Abayasekara and Wathes, 1999; Mattos *et al.*, 2000). Although the inclusion of protected fatty acids increased the lipid content of all tissues tested, with the exception of plasma, fatty acid composition was not greatly affected by the dietary treatments. Differences in the fatty acid composition of plasma were not observed within GCs and COCs. In fact, the fatty acid composition of GCs differed from that of plasma, and COCs were different again, indicating the selective uptake of fatty acids by GCs and COCs, where the preference is for saturated fatty acids. This increase in saturation suggests that it may be an intrinsic mechanism protecting the follicle and oocyte from free radical and other oxidative damage (Kim and Parthasarathy, 1998; Baker and Aitken, 2004). The fatty acid composition of embryos was different yet again from that of oocytes, and was dependent on the source of serum, possibly because the serum supplement was unaltered by the oviductal cells, as it might have been *in vivo*. However, the fatty acid composition of COCs and embryos was not related to their quality. This study demonstrated that the lipid content of oocytes can be altered by dietary composition, but further studies are required to elucidate the function and importance of specific fatty acids on oocyte and embryo development.

6.2. General conclusions

In summary, this thesis has demonstrated that:

(i) Animal body composition is an important factor in the nutritional regulation of oocyte and early embryo development. However, it remains unclear as to what aspects of BCS are responsible for these effects.

(ii) High levels of feeding are beneficial to oocyte developmental competence in thin heifers, but have a deleterious effect on oocyte quality in moderately fat heifers. Moreover, these effects accumulate over time period.

(iii) Fibre based diets and diets without protected lipid supplements enhance oocyte quality in thin heifers, but in moderately fat heifers diet composition has little or no effect on oocyte quality.

(iv) The effects of diet composition on the final stages of oocyte maturation *in vitro* differ from those induced during pre-ovulation development.

(v) Serum from thin heifers has a beneficial effect on early embryo development, and whilst diet composition has little effect on the blastocyst formation, it significantly alters embryo quality and hence may affect survival *in vivo*.

(vi) The total lipid content of plasma, GCs, COCs and embryos is increased by the inclusion of rumen protected palm oil fatty acids, but fatty acid composition is not affected by diet composition to the same extent. There appears to be a selective mechanism of fatty acid uptake by the follicle and/or oocyte with preference for saturated fatty acids. This may serve to protect the oocyte from oxidative damage.

(vii) In general, moderately fat animals are less responsive to alterations to diet composition. However, thin heifers are more representative of lactating dairy cows. Oocyte quality in such animals is sensitive to alterations in dietary energy intake and diet composition.

To conclude, this thesis has highlighted the importance of animal body composition in the nutritional regulation of oocyte quality in cattle. Furthermore, oocyte developmental competence can be enhanced by dietary energy intake and diet composition, depending

on animal BCS. The specific factors and mechanisms responsible for these differences, however, remain to be determined. Further investigations are required in order to establish the interactive effects of insulin and leptin on the ovarian function, and to identify other elements which may be involved in the nutritional regulation of oocyte quality.

APPENDIX 1

In vitro embryo production

A.1.1. Ultrasound guided transvaginal aspiration of follicles – OPU

SOLUTIONS AND MEDIA REQUIRED:

Table A.1.1. The composition of aspiration and search media required during OPU procedure.

Reagent	Aspiration medium	Wash/Search medium
PBS	100 ml	100 ml
BSA (Fraction V or FAF)	0.1% w/v	0.2% w/v
Heparin (50 IU/ml)	0.2 ml	-
Pen/strep/neomycin (50 IU/ml penicillin, 50 µg/ml streptomycin and 100 µg/ml neomycin)	1.0 ml	1.0 ml

Prepared media was mixed, filtered and stored at 4°C for up to one week. Before use media was warmed to 38.5°C.

PROCEDURE:

Prior to the OPU procedure all heifers were sedated with 28 mg xylazine hydrochloride (Rampun, Bayer AG, Leverkusen) before receiving an epidural anaesthetic of 5 ml of lignocaine hydrochloride (30 mg/ml, Locovetic, Bimeda, UK). The rectum was cleaned from any remaining faeces and gasses to enable better manipulation and visualisation of the ovaries. The rectum and vagina were sterilised using cotton wool soaked with alcohol. The ovaries were visualised using 5MHz transvaginal curvilinear ultrasound transducer (Aloka SSD-500V; BCF, Livingstone, UK). All accessible follicles were aspirated with a 55 cm long, 20 gauge single lumen needle attached to the vacuum pump (Craft Duo-vac, Rocket, Watford, UK) with set pressure of 70 mmHg applied throughout the procedure. Follicular aspirants were collected into 50-ml conical tubes containing 5 ml of warm (38°C) Aspiration medium (Table A.1.1), kept on a heating block throughout the procedure. Once aspiration was completed each heifer received 15 ml penicillin, i.m. (150 mg/ml procaine penicillin, 112.5 mg/ml benzathine penicillin; Duphapen LA; Solvay Duphar BV, Holland).

Within 30 minutes from collection, tubes containing follicular fluid were transported in a polystyrene box to the laboratory, where the aspirate was filtered through a sterile 70 μm cell strainer (BD Falcon, USA). The sediment on the filter was rinsed out into a Petri dish with fresh Wash/Search medium (Table A.1.1) using 20 gauge needle attached to a sterile 10 ml syringe, and then searched for COCs.

A.1.2. Collection of COCs from the abattoir derived ovaries

SOLUTION REQUIRED:

PBS/Kanamycin – 1 tablet of PBS was dissolved in 100 ml of double distilled water and 50 mg/ml of Kanamycin was added.

pH=7.4

Glass bottles containing prepared PBS were autoclaved for 1.5 h at 123°C. Once cooled, Kanamycin was added. Bottles with sterile PBS/Kanamycin solution were stored at room temperature until needed.

PROCEDURE:

Ovaries collected from a nearby abattoir, within 2 h from collection were transported in a flask containing warm (37°C) PBS/Kanamycin to the laboratory. On arrival ovaries were rinsed with 70% ethanol solution and then with fresh PBS/Kanamycin. Clean ovaries were transferred into a beaker containing fresh, warm PBS/Kanamycin (37°C) and kept on a heating block while aspiration was carried out.

Only small (< 4 mm in diameter) and medium sized (4 – 8 mm) follicles were aspirated using 18 gauge needle attached to a 5 ml syringe. Follicular fluid was transferred into a universal tube containing 1 ml of Search medium (Table A.1.2). Once aspiration was completed, collected follicular fluid was transferred into a 45 mm Petri dish and then searched for COCs.

A.1.3. *In vitro* oocyte maturation – IVM

SOLUTIONS AND MEDIA REQUIRED:

Table A.1.2. The composition of media required during IVM.

Reagent	Search medium	Maturation medium
TCM199 with Hepes	20 ml	-
TCM199	-	10 ml
BSA (Fraction V or FAF)	0.2 % w/v	0.4% w/v
or FCS/treatment sera	5% v/v	10% v/v
FSH (from porcine pituitary)	-	10 μg/ml
LH (from horse pituitary)	-	10 μg/ml
Pen/strep (50 IU/ml penicillin and 50 μg/ml streptomycin)	100 μl	50 μl
pH	7.3 – 7.4	7.3 – 7.4
mOsmo	290 – 300	290 – 300

Search medium supplemented with BSA was filtered and stored in a fridge for up to 1 week. When using Serum supplement, the media was prepared on a day of use. The search medium was warmed at 37°C for at least 1 h before use. The Maturation medium was prepared on a day of use, filtered, warmed and equilibrated in 5% CO_2 in air, for at least 1 h before use.

FSH and LH stocks:

FSH from porcine pituitary (biological specificity attributed to the β-chain):

Supplied 50 units (equal to 23.1 mg) per vial were dissolved in 25 ml of filtered TCM199 to give 2 units/ml stock solution. One unit was the activity of 1 mg of Armour FSH Standard G-94 by the method of Steelman, S.L., and Pohley, F.M., *Endocrinology*, **53**: 604 (1953). Stock solution was aliquoted into 110 μl lots and stored at –20°C.

LH from horse pituitary (biological specificity attributed to the β-chain):

Supplied 15 units (equal to 8.5 mg) per vial (in terms of NIH standard LH-S1 using the rat Leydig cell assay) were dissolved in 7.5 ml of filtered TCM199 to give 2 units/ml stock solution. Stock was aliquoted into 110 μl lots and stored at –20°C.

PROCEDURE:

Harvested COCs were graded according to the criteria of Goodhand *et al.* (1999) based on the number of compact cumulus cell layers and appearance of the cytoplasm.

Grade 1 – >4 layers of compact, light or transparent cumulus with a clear, even cytoplasm

Grade 2 – compact, <4 layers of cumulus and/or homogenous cytoplasm but with a coarser appearance

Grade 3 – <4 layers or expanded cumulus, cytoplasm of uneven appearance with dark areas, cumulus and cytoplasm generally darker than previous two categories

Grade 4 – no cumulus

Only grades 1 and 2 COCs (Figure A.1.1.a) were selected for maturation. Selected oocytes were washed twice in Search and then twice in Maturation medium. Less than 20 oocytes per batch/treatment were matured in 50 µl drops with granulosa cell monolayers (procedure described in A.1.3.1), 20 – 40 oocytes were matured in 4 well plates with 500 µl of Maturation medium per well, and more than 120 oocytes were matured in a small (35 mm) Petri dish containing 3 ml of Maturation medium. Oocytes were matured for 24 h at 38.8°C and 5% CO_2 in a humidified atmosphere.

The handling of oocytes from the beginning of aspiration until placing them into Maturation medium took less than 1 hour.

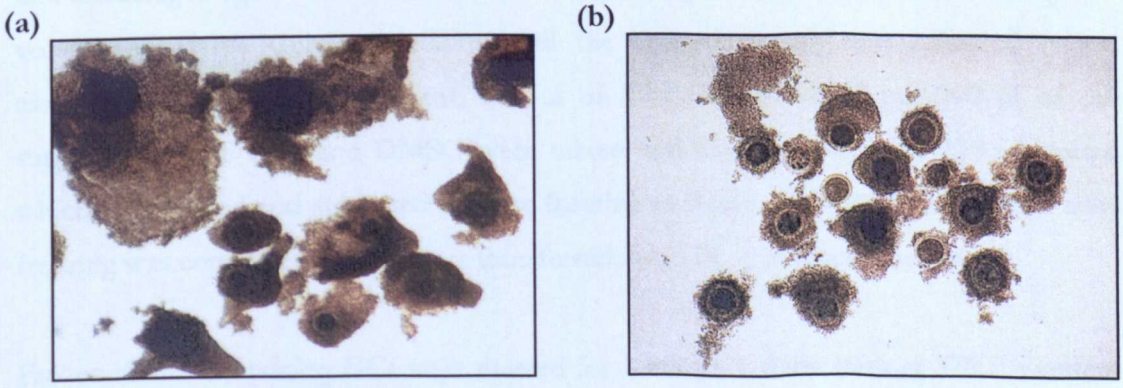

Figure A.1.1. (a) Good quality COCs – Grades 1 and 2; (b) Poor quality oocytes – Grades 3 and 4.

A.1.4. Granulosa cell monolayers

SOLUTIONS AND MEDIA REQUIRED:

PBS/Kanamycin – as previously.

Tissue Culture Medium

TCM199

5% v/v FCS or 0.2% w/v BSA (Fraction V or FAF)

Pen/strep (50 IU/ml penicillin and 50 μg/ml streptomycin)

pH=7.3; mOsmo=290 – 300

Prepared media was filtered, warmed and equilibrated in 5% CO_2 in air, for at least 1 h before use.

PROCEDURE:

Ovaries collected from a local abattoir, were transported to the laboratory in a flask containing warm (37°C) PBS/Kanamycin within 2 h from collection. Ovaries were washed on arrival and follicles aspirated as described previously. Follicular fluid was transferred into conical tubes and centrifuged for 5 min at 300 x g. After 5 min the supernatant was removed and the cell pellets resuspended in 2 ml of Tissue Culture Medium and centrifuged again for 5 min. To separate GCs from red blood cells and oocytes, tubes were gently tapped with a finger before removing the supernatant. The supernatant and top layer containing GCs were transferred into a clean centrifuge tube and centrifuged again. After this wash, the final cell pellet was resuspended in required volume of Tissue Culture Medium until the desired density was achieved (visual assessment). As a cryoprotectant, 100 μl of DMSO was added per 900 μl of cell suspension. The GCs and DMSO were mixed and loaded into sterile 250 μl straws, which were sealed and subjected to slow freezing in liquid nitrogen (LN_2). Once slow freezing was completed, straws were transferred into LN_2 containers for storage.

Frozen straws containing GCs were thawed for 1 min in a water bath at 37°C. Content of each straw was then released into warm centrifuge tube containing 1 ml of Tissue Culture Medium and washed three times by centrifugation for 5 min at 300 x g. The final cell pellet was resuspended in 900 μl of Tissue Culture Medium and 50 μl drops of

cell suspension were overlaid with mineral oil. Granulosa cell monolayers were incubated for 96 h at 38.8°C and 5% CO_2 in a humidified atmosphere. Half of a spent media was replaced with fresh media every 48 h (termed 'feeding'). Just before use for oocyte maturation, monolayers were fed with Maturation medium containing a double concentration of hormones (FSH and LH).

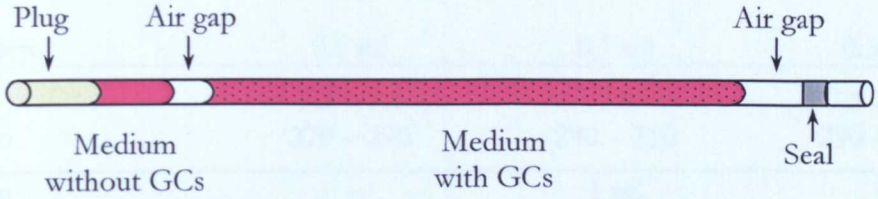

Figure A.1.2. Straw containing suspension of GCs in Tissue Culture Medium and DMSO, ready for freezing.

A.1.5. *In vitro* fertilisation – IVF

SOLUTIONS AND MEDIA REQUIRED:

Stock solutions:

Table A.1.3. The reagents and their quantities required to make up 500 ml of stock solutions of Oocyte Wash, Fertilisation and Capacitation media. All reagents were dissolved in double distilled water, then filtered and stored in a fridge for up to 2 months.

Reagents	Oocyte Wash	Fertilisation	Capacitation
NaCl	3330 mg	3330 mg	3225 mg
KCl	119 mg	119 mg	100 mg
$NaHCO_3$	84 mg	1045 mg	1050 mg
NaH_2PO_4	23.85 mg	23.85 mg	23.85 mg
$MgCl_2 \cdot 6H_2O$	50 mg	50 mg	50 mg
$CaCl_2 \cdot 2H_2O$	147 mg	147 mg	-
HEPES	1200 mg	1200 mg	600 mg
Phenol Red	5 mg	5 mg	-

Working solutions:

Table A.1.4. The list of reagents added to 100 ml of stock solutions, to make up working solutions of Oocyte Wash, Fertilisation and Capacitation media. Prepared media were filtered and stored in a fridge for up to 1 week.

Reagents	Oocyte Wash	Fertilisation	Capacitation
Sodium pyruvate	5.5 mg	5.5 mg	11 mg
Glucose	-	-	125 mg
Lactic acid	300 µl	300 µl	300 µl
BSA (Fraction V or FAF)	300 mg	600 mg (FAF)	600 mg
Pen/strep	0.5 ml	0.5 ml	0.5 ml
pH	7.3 – 7.4	7.8	7.3 – 7.4
mOsmo	270 – 290	290 – 310	290 – 310
Heparin	-	1 ml (3 µg/ml aliquot)	-
PHE (Penicillamine, Hypotaurine, Epinephrine solution) or	-	4 ml (stock aliquot)	-
Heparin	-	100 µl of stock	-
Hypotaurine	-	50 µl of stock	-
Epinephrine	-	50 µl of stock	-

Oocyte Wash medium was warmed for at least 1 h before use, and Fertilisation and Capacitation media were warmed and equilibrated in 5% CO_2 in air, for at least 1 h before use.

Stocks of heparin and PHE (Penicillamine, Hypotaurine, Epinephrine solution):

Heparin: 3 mg were dissolved in 10 ml of Fertilisation stock solution. Aliquoted into 500 µl lots and stored at –20°C for up to 2 months.

PHE stocks: Stock 1 - 0.9 g NaCl was dissolved in 100 ml of double distilled water

Stock 2 - 2.72 mg Hypotaurine was dissolved in 25 ml of Stock 1

Stock 3 - 7.5 mg Penicillamine was dissolved in 25 ml of Stock 1

Stock 4 - 50 mg Sodium metabisulphate and 0.37 ml of Lactic acid were dissolved in 50 ml of double distilled water and adjusted pH to 4.0 with HCl if needed.

1.75 mg Epinephrine was added to Stock 4; carried out in a dark room, using gas mask and gloves.

For PHE solution specified volumes of Stocks were mixed together:

30 ml of Stock 1
25 ml of Stock 2
25 ml of Stock 3
20 ml of Stock 4
} PHE

PHE solution was filtered and stored in 4 ml aliquots at –20°C for up to 2 months, protected from light.

Stocks of heparin, hypotaurine and epinephrine:

Heparin	10 mg/ml	(Added 100 mg to 10 ml of double distilled water)
Hypotaurine	1 mg/ml	(Added 20 mg to 20 ml of double distilled water)
Epinephrine	1 mg/ml	(Added 20 mg to 20 ml of double distilled water); protected from light.

Prepared stock solutions were filtered and stored in a fridge for up to 3 months.

PROCEDURE:

Oocyte preparation:

After 24 h maturation (from the end of aspiration), cumulus cells became expanded and sticky. Matured oocytes were transferred into a small Petri dish containing warm Oocyte Wash medium and were partially denuded from attached cumulus cells by gentle pipetting, leaving 2 – 3 layers of cumulus cells. These oocytes were washed again in Oocyte Wash medium and then twice in Fertilisation medium (Table A.1.4) before being placed in 46 μl drops (up to 20 oocytes per drop) or 460 μl of Fertilisation medium per well (30 – 40 oocytes per well).

Semen preparation:

Semen from two bulls was used for all inseminations: Beechpike Energy (SAC, Aberdeen laboratory) and Eugene (University of Nottingham laboratory) and prepared

by 'swim-up' procedure (Parrish *et al.*, 1986) described below. All straws were stored in LN_2 containers.

Straws containing semen were thawed for 30 sec in a water bath at 35°C, wiped with tissue soaked with 70% ethanol solution and finally dried with tissue. The content of each straw was then released into a round-bottom centrifuge tube ('swim-up tube') and rinsed out with 100 µl of Capacitation medium (Table A.1.4). A small drop of semen was placed onto a glass slide to check motility. Once motility was confirmed, 85 µl of semen was layered under 1 ml of Capacitation medium per tube. All three tubes were incubated for 30 min (swim-up) at 38°C and 5% CO_2 in a humidified atmosphere. After 30 min the top layer containing live/motile spermatozoa was transferred into a clean conical tube and centrifuged for 10 min at 300 x g. After 10 min the supernatant was removed and the pellet of spermatozoa was resuspended in 150 – 200 µl of remaining Capacitation medium. To establish the sperm concentration, 10 µl of sperm suspension was diluted in 90 µl of water. With the use of a haemocytometer the number of sperm in 5 squares was counted. One hundred divided by the mean number of sperm counted, gave the final volume of sperm to be added into each drop or well (x10) and giving final concentration of $1x10^6$ sperm/ml. Finally, the calculated volume of sperm suspension was added into each drop (or well) containing oocytes. Oocytes were co-incubated with sperm (Figure A.1.2) for 18 – 22 h at 38°C and 5% CO_2 in a humidified air.

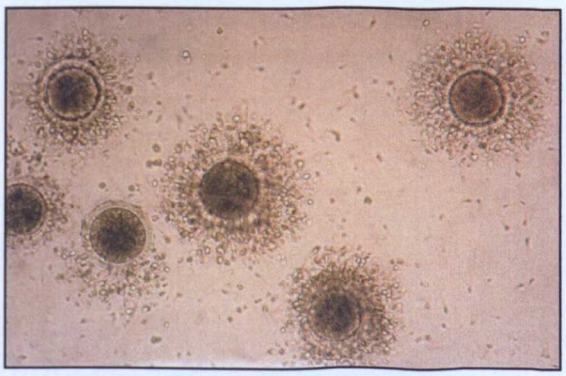

Figure A.1.2. Partially denuded COCs co-incubated with sperm in Fertilisation medium.

A.1.6. *In vitro* embryo culture – IVC

SOLUTIONS AND MEDIA REQUIRED:

Stocks for Synthetic Oviductal Fluid (SOF)

Table A.1.5. The list of reagents, their concentration and the storage time of stock solutions for SOF. Stocks were filtered and stored in a fridge for an indicated period of time.

Stock name	Reagent	Conc.	Quantity	Water	Storage time
Stock S3	NaCl		6.294 g	100 ml	3 months
	KCl		0.534 g		
	KH_2PO_4		0.162 g		
Stock D	$CaCl_2 \cdot 2H_2O$	171 mM	1.260 g	50 ml	3 months
Stock M	$MgCl_2 \cdot 6H_2O$	49 mM	500 mg	50 ml	3 months
Stock G	Glucose	60 mM	540 mg	50 ml	3 months
Stock C	Sodium pyruvate	33 mM	36 mg	10 ml	1 week
Stock GLN	Glutamine	10 mM	73 mg	50 ml	1 week
Stock L	Lactic acid	330 mM	1.41 ml	30 ml	2-4 weeks
Stock B	$NaHCO_3$	250 mM	1.0505 g	50 ml	Made fresh
	Phenol red		3 drops		
Stock H	Hepes	250 mM	2.98 g	50 ml	1 month

SOF Basic:

To 50 ml of double distilled water was added:

Stock S3	10.0 ml
Stock B	10.0 ml (or 210 mg of $NaHCO_3$ and 2 drops of phenol red)
Stock C	3.0 ml (or 10.8 mg of Sodium pyruvate)
Stock D	1.0 ml
Stock L	3.0 ml
Stock M	1.0 ml
Stock G	2.5 ml
Stock GLN	10.0 ml (or 14.6 mg of Glutamine)
Pen/strep	0.5 ml

These were mixed well and made up to 100 ml with double distilled water.

SOF + (with serum)

To 50 ml of SOF Basic was added 10% v/v of FCS or treatment sera

pH=7.4 and mOsmo=270 – 280.

This was prepared on the day of use, filtered, warmed and equilibrated in 5% CO_2 and 5% O_2 in air, for at least 1 hour before use.

SOF – (without serum)

To 50 ml of Basic SOF was added:

0.5 ml neaa's (non-essential amino acids)

1.0 ml eaa's (essential amino acids)

0.3% w/v BSA (FAF)

pH=7.4 and mOsmo=270 – 280

Prepared media was filtered and stored in a fridge for up to 1 week. On the day of use it was warmed and equilibrated in 5% CO_2 and 5% O_2 in air, for at least 1 h before use.

PROCEDURE:

Within 20 – 22 hours after fertilisation presumptive zygotes were transferred into a small Petri dish containing warmed and gassed SOF– or SOF+. Zygotes were denuded from attached cumulus cells and sperm by gentle pipetting, washed again in fresh SOF and finally transferred into 20 µl (10 zygotes per drop) or 30 µl (up to 20 zygotes per drop) drops of SOF overlaid with mineral oil. Zygotes were incubated at 38.8°C, 5% CO_2 and 5% O_2 in a humidified air until Day 8 of development. Cleavage rates were recorded 48 h post-fertilisation (Figure A.1.3). Uncleaved oocytes were removed from culture drops. Embryos were transferred into fresh drops of SOF every 48 h (termed 'feeding').

Blastocyst yields were recorded on Day 7 and Day 8 of development and individual blastocyst developmental stage were evaluated according to criteria of Lindner and Wright (1983).

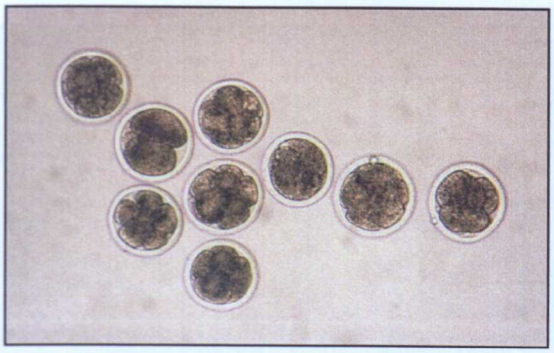

Figure A.1.3. Cleaved zygotes at 2-, 4- and 8-cell stage, assessed 48 h after fertilisation.

STAGES OF EMBRYO DEVELOPMENT:

Morulae – individual blastomeres difficult to discern from one another and the cellular mass of the embryo occupies most of the perivitelline space.

Compact morulae – individual blastomeres coalesced, forming a compact mass. The embryo occupies 60 to 70% of the perivitelline space.

Early blastocyst – an embryo that has formed a fluid-filled cavity called a blastocoele. There may be a possible visual differentiation between TE and ICM.

Blastocyst – pronounced differentiation of the outer TE layer and the darker, more compact ICM is evident. The blastocoele is highly prominent with the embryo occupying most of the perivitelline space.

Expanding blastocyst – the diameter of the embryo significantly increases with a concurrent thinning of the zona pellucida. At this stage some blastocysts may appear collapsed due to a complete or partial loss of the blastocoele, however, the zona pellucida rarely regains its original thickness.

Hatched blastocyst – the embryo undergoing the process of hatching or completely hatched from the zona pellucida. Hatched blastocyst may be spherical with a well defined blastocoele or collapsed (Figure A.1.4.a)

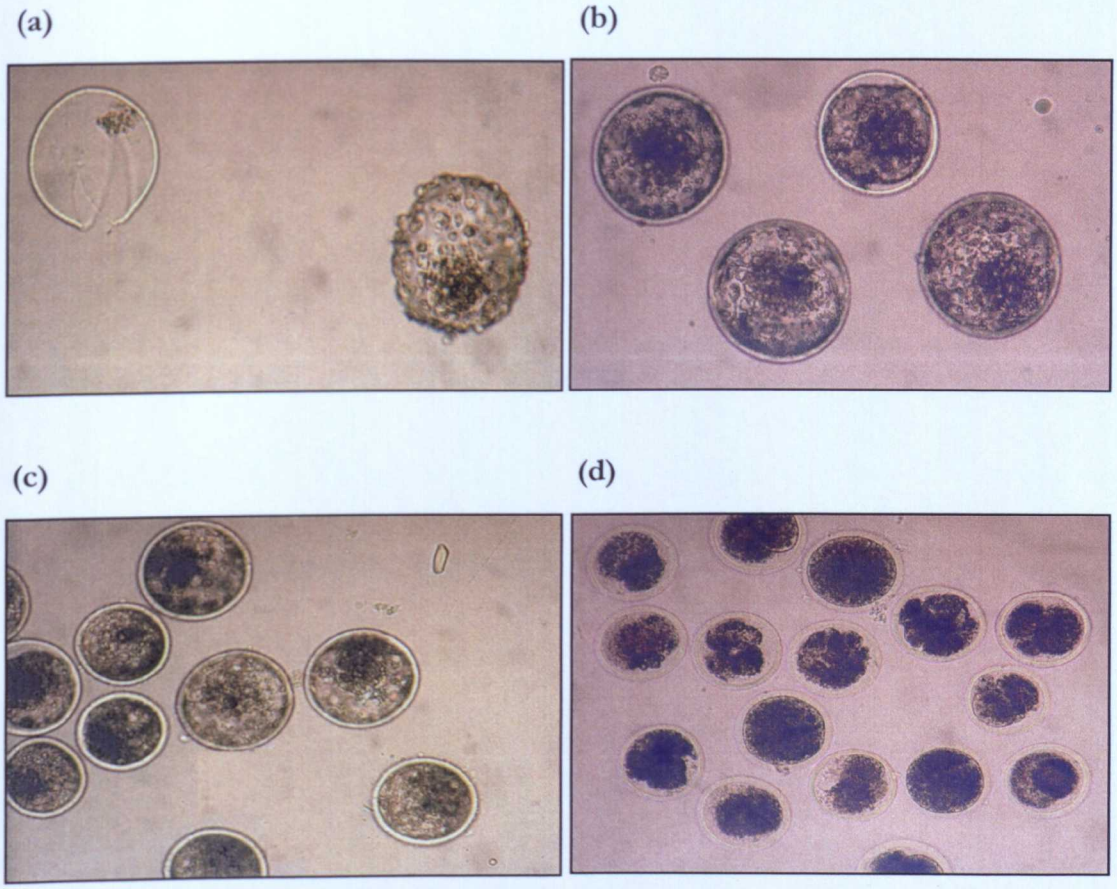

Figure A.1.4. (a) Hatched blastocyst and an empty zona pellucida; (b) Grades 1 and 2 blastocysts; (c) Expanding, Mid and Early blastocysts; (d) Degenerated zygotes, arrested at 2 to 8 cell stage of development.

APPENDIX 2

Blastocyst and granulosa cell assessments

A.2.1. Staining for total cell counts using Hoechst 33342

SOLUTIONS REQUIRED:

3:1 solution of Acetic acid:ethanol

2.3% Sodium citrate

1 mg/ml stock solution of Hoechst 33342 (protected from light exposure)

All solutions were stored in a fridge for up to 3 months.

On a day of staining, working solution (10 μg/ml) of Hoechst 33342 was prepared by adding 5 μl of Hoechst stock solution to 500 μl of 2.3% sodium citrate.

PROCEDURE:

On Day 8 of development individual blastocysts were evaluated and then fixed in acetic acid:ethanol solution (1 ml per well) for at least 1 h. Once fixed, embryos were stored in a cool room for up to 2 weeks.

Fixed blastocysts were individually transferred in a minimum volume onto a glass slide and left to dry for 1 min. Once dried, a 14 μl drop of Hoechst working solution was gently applied over the embryo and covered with a glass cover slip. Prepared slides were immediately visualised by fluorescence microscopy. Total cell numbers were promptly counted and recorded.

(a) (b)

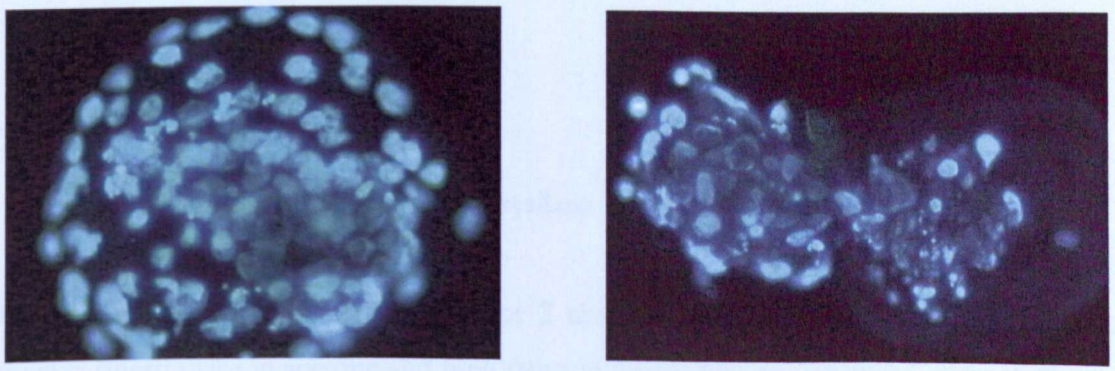

Figure A.2.1. (a) Expanding and (b) hatching Day 8 blastocysts stained with Hoechst 33342 for total cell counts and visualised under fluorescence microscope.

A.2.2. TUNEL assay – using ApopTag® staining kit (Intergen, USA)

SOLUTIONS AND REAGENTS REQUIRED:

PBS with 1 mg/ml PVP

10x PBS: Na_2HPO_4 55.0g

NaH_2PO_4 13.5g

NaCl 117.0g

pH 7.4, make up to one litre with distilled water.

PBS with 0.1% w/v PVP and 0.1% v/v Tween20 (PPT buffer)

8% w/v Paraformaldehyde in PBS

1% w/v Saponin in PPT

ApopTag red staining kit:

TdT enzyme: 70% reaction buffer

30% TdT (very viscous)

Solution was mixed by vortexing and stored on ice until use.

Stop/wash buffer: 1 µl stop wash buffer

34 µl distilled water

Both were mixed together and stored on ice until use.

Anti-digoxigenin conjugate: 53% blocking solution

47% anti-DIG conjugate

Both were mixed by vortexing and stored on ice until use, avoiding exposure to light.

To silanize glass slides:

2% solution of 3-aminopropyltriethoxysilane in acetone (TESPA)

Using Coplin jars slides were soaked for 2 min in TESPA. After 2 min slides were quickly rinsed twice in acetone and then twice in distilled water. Silanized slides were air dried for a few minutes and then dried overnight in the oven at 37°C.

To siliconize glassware and pipette tips:

10% solution of Sigmacote in ethanol

The mixture was applied onto glassware, spread evenly and left for 2 min. After 2 min excess was removed and glassware and pipette tips left to air dry for a few minutes.

PROCEDURE:

- ⇨ Day 8 blastocysts were assessed and washed three times in warm PBS/PVP to remove culture medium.
- ⇨ Washed blastocysts were fixed in 4% paraformaldehyde for at least 1 h at room temperature.
- ⇨ After 1 h embryos were washed in PBS/PVP 5 times for 2 minutes, and embryos were stored at 4°C until staining was conducted.
- ⇨ Fixed and washed embryos were permeabilised by incubating in 1% saponin for at least 1 h at room temperature.
- ⇨ After 1 h, embryos were washed 5 times in individual 20 µl drops of PPT.
- ⇨ Each embryo was incubated in 20 µl drop of equilibration buffer (neat, as supplied) for 10 minutes at room temperature.
- ⇨ Embryos were transferred after 10 min into 20 µl drops of working strength TdT enzyme and incubated in a humidified chamber at 37°C for 1 h.
- ⇨ Once incubation was completed, embryos were transferred into 20 µl drops of working strength stop/wash buffer and agitated briskly for 15 sec and then transferred into a second, fresh drop of stop/wash buffer and incubated at room temperature for 10 min.
- ⇨ At the end of the incubation period embryos were washed 3 times in PPT.
- ⇨ Once washed, embryos were transferred into 20 µl drops of working strength anti-digoxigenin conjugate and incubated in a darkened humidified chamber for 1 h at room temperature.
- ⇨ After 1 h embryos were washed 4 times in PPT protecting from light and then transferred into 20 µl drops of Hoechst 33342 solution (10 µg/ml in 2.3% sodium citrate) for 15 minutes at room temperature, protecting from light.
- ⇨ Once the staining procedure was completed, individual embryos were placed onto a silanized glass slides and allowed to dry for 1 to 3 minutes (so that they did not

become entirely dried out). A drop (about 30 µl) of citifluor mounting medium (proportions: 100 µl to 900 µl) was then added and allowed to stand for a further 3 to 5 minutes before a glass cover-slip was carefully applied.

⇨ The slides were finally left to dry overnight at room temperature, protecting from light until observation. Prepared slides were visualised by fluorescence microscopy. Total cell counts and TUNEL positive cells were counted and recorded.

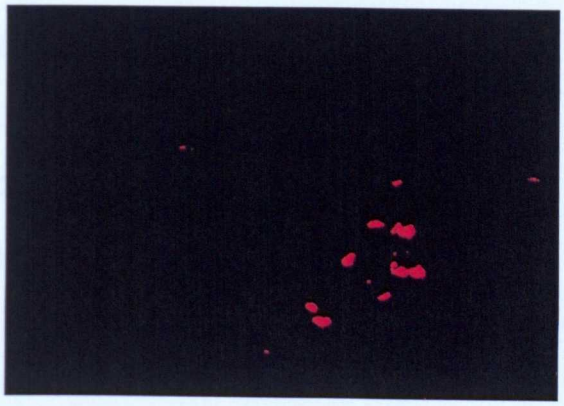

Figure A.2.2. Expanding Day 8 blastocyst stained for DNA fragmentation using ApopTag® Apoptosis detection kit.

A.2.3. Differential staining and TUNEL assay using In Situ Cell Death Detection Kit – Fluorescein (Roche, UK)

SOLUTIONS AND REAGENTS REQUIRED:

Stock solutions:

1 mg/ml of Propidium iodide (PI) was dissolved in distilled water, aliquoted and stored at –20°C protecting from light.

1 mg/ml Hoechst 33342 was dissolved in distilled water, aliquoted and stored at –20°C, protected from light.

8% w/v Paraformaldehyde (PFA) was dissolved in distilled water, aliquoted and stored at –20°C.

PBS with 2 mg/ml of PVA (PBS tablet was dissolved in 200 ml of distilled water and adjusted pH to 7.4 if needed)

2 x PBS with 4 mg/ml of PVA.

Working solutions:

0.2% v/v Triton-X100 in PBS/PVA

0.1% v/v Triton-X100 in 0.1% of Sodium citrate solution

4% w/v PFA (8% w/v PFA in 2 x PBS/PVA)

30 μg/ml PI in PBS/PVA

10 μg/ml Hoechst 33342 in 4% PFA

TUNEL mixture – 10 μl of enzyme + 90 μl of label, mixed well

PROCEDURE:

- ⇨ Embryos were washed twice in warm PBS/PVA to remove culture media.
- ⇨ Embryos were permeabilised in 0.2% Triton-X100 in PBS/PVA for 30 sec.
- ⇨ Embryos were washed twice in warm PBS/PVA.
- ⇨ Embryos were incubated with PI (30 μg/ml) for 5 – 10 min at 37°C protecting from light.
- ⇨ Washed twice in warm PBS/PVA.
- ⇨ Embryos were fixed in 4% PFA + Hoechst 33342 (10 μg/ml) for 30 min at room temperature protecting from light.
- ⇨ They were washed twice in PBS/PVA.
- ⇨ A second permeabilisation in 0.1% Triton-X100 with 0.1% sodium citrate for 5 min then took place.
- ⇨ Embryos were washed twice in PBS/PVA.
- ⇨ TUNEL. Embryos were incubated in 20μl drops of TUNEL mixture at 37°C in a humidified chamber for 30-60 min in the dark.
- ⇨ They were washed three times in PBS/PVA.
- ⇨ Embryos were finally mounted onto glass slides and allowed to dry for 1 to 3 minutes, but not allowed to dry out. A drop of Vectashield mounting medium was then added and a cover-slip carefully applied.

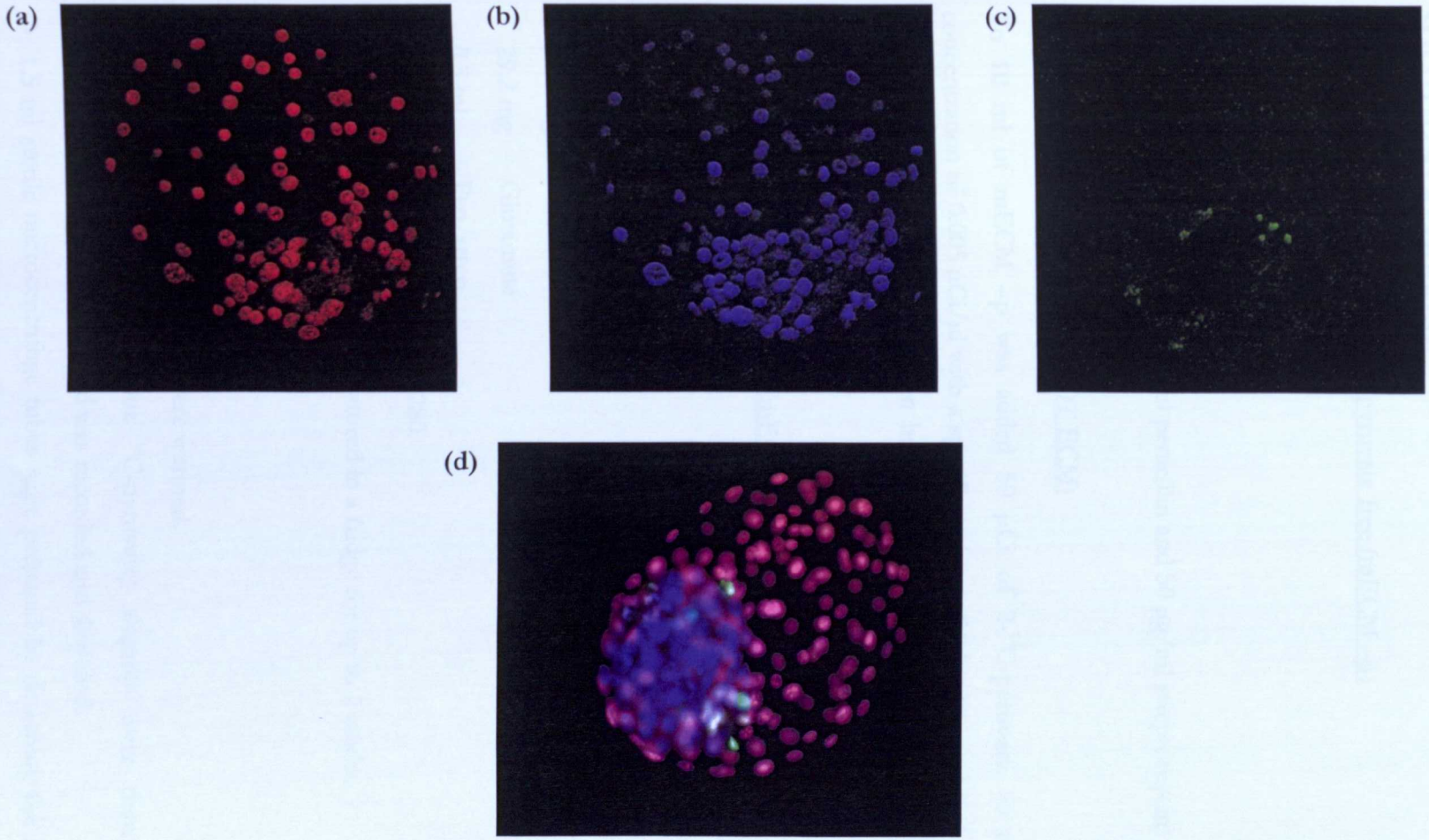

Figure A.2.3. Differential staining of expanding Day 8 blastocysts. Confocal images: (a) Red stain – PI, TE; (b) Blue stain – Hoechst 33342, ICM and (c) Green stain – fluorescein, apoptotic cells. (d) Combined image of differential staining of Day 8 blastocyst, visualised by a fluorescence microscopy.

A.2.4. ^{14}C-Pyruvate metabolism assay

$CH_3 - CO - COOH$

SOLUTIONS REQUIRED:

Modified embryo culture medium, pyruvate free (mECM –p)

To 100 ml of PBS was added:

0.4% w/v	BSA (Fraction V)
18.04 mg	Glucose
0.5 ml	Pen/strep (50 IU/ml penicillin and 50 μg/ml streptomycin)

HOT embryo culture medium (HOT ECM)

To 10 ml of mECM –p was added 50 μCi of 2-^{14}C-pyruvate, to make a final concentration of 0.005 μCi/μl with a specific activity of 5.5 mCi/mmol. Aliquots (50 μl lots) were stored at –20°C. When handling, filtered tips and protective gloves were worn.

Modified embryo culture medium (mECM +p)

To 100 ml of PBS was added:

0.4% w/v	BSA (Fraction V)
18.04 mg	Glucose
10.8 mg	Sodium pyruvate
29.2 mg	Glutamine
0.5 ml	Pen/strep

pH=7.2 – 7.4 and mOsmo=270 – 280.

Prepared solution was filtered and stored in a fridge for up to 2 weeks.

PROCEDURE:

- ⇨ mECM +p and a 4-well dish were warmed.
- ⇨ HOT ECM (mECM –p plus ^{14}C-pyruvate) aliquots were thawed at room temperature. The quantity used was recorded and disposed.
- ⇨ 1.5 ml sterile microcentrifuge tubes were prepared by detaching the lids from the tubes using scissors. Tubes were numbered, placed in racks and 1 ml of 0.1M

NaOH was added to each tube. All tubes were placed in a heated block to warm (37°C).

- ⇨ Embryos were washed three times in mECM +p.
- ⇨ Embryos from the last wash were transferred in a 3 µl drop and placed on the lid (inner surface) of the eppendorf tube, and 3 µl of the HOT ECM was added and mixed well. The final specific activity was 16 mCi/mmol.
- ⇨ The lids were placed on the 1 ml of 0.1M NaOH tubes (Figure A.2.4). When the incubator was running with CO_2, the lids were closed outside the incubator to minimise presence of CO_2 in the tube during the assay.
- ⇨ The sequence was: sham, sham, embryo 1, embryo 2, ……., last embryo, sham,sham. Sham tubes were placed every 20 embryos (or less) when large number were assayed.
- ⇨ Sham: no embryo in 3 µl mECM +p and 3 µl HOT ECM.
- ⇨ The timer was set and individual lid closure times recorded.
- ⇨ The tubes were incubated for exactly 3 h at 38.5°C.
- ⇨ Scintillation tubes were prepared by adding 4 ml of scintillation fluid (Emulsifier Safe) to each vial (2 vials per each eppendorf tube used).
- ⇨ 4-well dish with PBS or SOF were also prepared and kept warm.
- ⇨ After 3 h, the lids were removed and embryos transferred in a minimum volume (<1 µl) to 4-well dish. They were washed three times and embryo morphology was recorded.
- ⇨ The lids of scintillation vials were numbered and placed securely on the vials. Each vial was inverted prior to placing in racks. Each vial was dipped in water without submerging the lid and wiped gently.
- ⇨ Each vial was counted for 5 minutes on a beta counter.

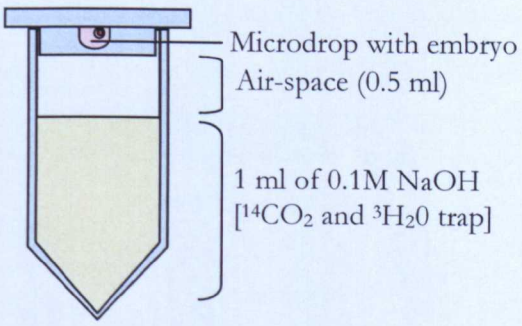

Figure A.2.4. Cross-section of 1.5 ml microcentrifuge tube and a hanging drop containing embryo.

A.2.5. Assessment of GCs proliferation and viability

A.2.5.1. Proliferation – Trypan blue exclusion

SOLUTIONS REQUIRED:

2.5% w/v Trypsin solution

0.4% w/v Trypan blue solution

Cell Culture medium as described in Chapter 2, section 2.2.6.

PROCEDURE:

At the end of incubation period (96 h) 400 µl of spent media was removed from each well and 250 µl of 2.5% trypsin solution was added per well in order to detach cells from the bottom. The cells were incubated with trypsin for 30 min at 38.8°C and 5% CO_2 in humidified air. After 30 min the cells (4 wells per treatment) were transferred into a microcentrifuge tube and centrifuged for 4 min and 800 x g. The supernatant was decanted and the cell pellet resuspended in 1 ml of warm Cell Culture medium. A sample was taken for trypan blue exclusion.

100 µl of trypan blue and 100 µl of cell suspension (x 2 dilution factor) were mixed and 2 x 10 µl of a mixture were used to load the chambers of haemocytometer. The number of cells was counted in 25 quadrates then multiplied by dilution factor and the area of the chamber (10^4 mm) to calculate final concentrations of cells in 1 ml, knowing that initial seeding density was 4 x 10^5 live cells/ml.

A.2.5.2. Viability – MTT assay

Cell viability is indicated by its metabolic activity, which can be measured in population of cells per well by incubating the cells with a tetrazolium salt (MTT) that is cleaved into a coloured formazan product by metabolic activity (MTT assay (Mosmann, 1983)).

SOLUTIONS REQUIRED:

5 mg/ml MTT solution in PBS

0.1N HCl in isopropanol

PROCEDURE:

At the end of the incubation period (96 h) 400 µl of spent media per well was replaced with 100 µl of MTT solution and incubated for 4 h at 38.8% and 5% CO_2 in a humidified air. After 4 h incubation, 400 µl of isopropanol was added per well in order to stop the reaction. The plate was allowed to rest for 20 min and the content of each well mixed and transferred in duplicate (2 x 200 µl) into 96-well plate for the spectrophotometric analysis. The absorbance of each plate was determined on a microplate reader. The coloured product (formazan) of MTT reduction was measured in two single wavelengths: 630 and 550. The final results of viability test were calculated by subtracting values of 630 from 550 wavelength values.

APPENDIX 3

Radioimmunoassays (RIAs)

A.3.1. Progesterone

SOLUTIONS REQUIRED:

Phosphate Citrate Buffer with Gelatin (PCBG)

17.85 g	Di-sodium hydrogen phosphate (anhydrous), Na_2HPO_4
7.75 g	Citric acid
1.0 g	Gelatin
1.0 g	Sodium azide, NaN_3

The above was added to 800 ml of double distilled water, once the solids dissolved, and adjusted the pH to 6.0 when necessary. It was made up to a final volume of 1 litre and stored at 4°C.

ANS Tracer Buffer

8-Anilino-1 Napthalene Sulphonic Acid (ANS) was added to PCBG (a ratio of 1 mg ANS to 1 ml PCBG) and stirred until dissolved. The required amount of ANS Tracer Buffer was prepared for the assays (100 µl per tube).

Separation Solution

9.0 g	Sodium chloride, NaCl
40.0 g	Polyethylene glycol, PEG-8000
2.0 ml	Triton X-100

NaCl, PEG and Triton X were added to 800 ml of double distilled water and stirred until dissolved/mixed. This was made up to a final volume of 1 litre.

STANDARDS:

Stock Solution:

50 mg of progesterone was dissolved in 50 ml of ethanol giving a concentration of 1 mg/1 ml solution of progesterone which was stored at 4°C.

Sub-Stock Solutions:

(a) (20 µg/ml solution). 100 µl of stock solution was diluted to 5 ml with PCBG, aliquoted into 150 µl lots and stored at –20°C.

(b) (200 ng/ml solution). 100 µl of (a) was added to 10 ml with PCBG, aliquoted into 1250 µl lots and stored at –20°C.

Working Solutions:

(c) (20 ng/ml solution). On the day of use, 1000 µl of (b) was diluted to 10 ml with PCBG. Assay standards were prepared by double diluting from the (c) standard as outlined in the table below.

	Reagents for 4 Std Curves		(100 µl sample x10 multiplication factor)	
Std	**Working Solution**	**PCBG**	**Dose ng/ml**	**Defined Dose ng/tube**
1	2000 µl (c)	- - - -	20	2.0
2	1000 µl (1)	1000 µl	10	1.0
3	1000 µl (2)	1000 µl	5	0.5
4	1000 µl (3)	1000 µl	2.5	0.25
5	1000 µl (4)	1000 µl	1.25	0.125
6	1000 µl (5)	1000 µl	0.625	0.0625
7	1000 µl (6)	1000 µl	0.312	0.0312
8	1000 µl (7)	1000 µl	0.156	0.0156

QUALITY CONTROLS (QC):

Those were prepared in house and stored at –20°C in aliquots of low, medium and high quality controls. They were treated as for samples in the assay.

CHARCOAL STRIPPED PLASMA (CSP):

Plasma was prepared in house to remove progesterone and aliquots were stored at –20°C.

1ST ANTIBODY SOLUTION – Rabbit anti-progesterone

Stock Solution:

Supplied in 1 ml vials and stored at 4°C.

Sub-Stock Solution:

1 ml of stock solution was diluted with 99 ml PCBG to give a 1/100 sub-stock solution which was aliquoted into 200 µl lots and stored at –20°C.

Working Solution:

A working dilution of 1:30,000 was used. 33 µl of the 1:100 sub-stock solution was added to 10 ml of PCBG and stored for up to 1 week at 4°C.

TRACER:

Stock Solution:

Supplied in 100 µl or 250 µl vials. PCBG was added to give a total stock volume of 200 µl or 500 µl and stored at 4°C.

Working Solution:

^{125}I-progesterone stock solution was diluted in a glass beaker with ANS Tracer Buffer to give a working solution of 15,000 cpm/100 µl in a glass tube. The number of counts in 100 µl of working solution was tested in a glass tube, before addition to the assay.

2ND ANTIBODY SOLUTION:

Donkey anti-rabbit IgG serum and normal rabbit serum were used at dilutions of 1:120 and 1:600 respectively. 167 µl of antibody and 33 µl of normal serum were added to 20 ml of PCBG (8.35 µl:1.65 µl:1 ml).

ASSAY METHOD:

Day 1

- ⇨ The following were remove from the fridge/freezer and allowed to come to room temperature: samples, QC, standard (b), CSP and PCBG.
- ⇨ Assay glass tubes were prepared in duplicate for the ^{125}I-total count (^{125}I-TC), non-specific binding (NSB), zero standard (Bo), standards 1-8, QC at the beginning and end of the assay and the samples.
- ⇨ Eight polystyrene tubes or cuvets were labelled and the standards double diluted from standard (c) as shown in the table.
- ⇨ 100 µl of each standard was pipetted in duplicate into the glass tubes.
- ⇨ 100 µl of PCBG was pipetted into the NSB and Bo tubes.
- ⇨ 100 µl of charcoal stripped plasma was added to the NSB, Bo and standard 1-8 tubes.
- ⇨ Any fibrinogen clots were removed and the samples and quality controls mixed.
- ⇨ 100 µl of each quality control or sample were pipetted in duplicate into the glass tubes.
- ⇨ 100 µl of PCBG was added to the QC and sample tubes. This step was omitted when using a diluter.

- ⇨ 100 µl of antiserum was added to all tubes except the ^{125}I-TC and NSB tubes.
- ⇨ 100 µl of PCBG was added to the NSB tubes.
- ⇨ 100 µl of ^{125}I-tracer was added to all tubes and the ^{125}I-TC tubes were capped.
- ⇨ The tubes were covered with cling film, vortexed for 10 sec and incubated overnight at 4°C.

Day 2

- ⇨ 1 ml of the Separating Solution was added to all tubes except the ^{125}I-TC.
- ⇨ These were incubated at room temperature for 10 minutes.
- ⇨ The tubes were centrifuged at 3,000 rpm (Jouan) or 3,300 rpm (Sorvall) at 4°C for 30 minutes.
- ⇨ The supernatant was decanted by tipping the tubes upside down. The tubes were kept upside down and allowed to dry (approximately 30 minutes).
- ⇨ The precipitate in the tubes was counted for 1 minute in the gamma counter.

REAGENT SOURCES:

1. Progesterone, Ref No. P0130, PEG-8000, Ref No. P2139 and ANS, Ref No. A1028, were obtained from Sigma Chemical Company Ltd, Poole, Dorset, BH17 7NH.
2. The tracer, Progesterone-1 1α-Glucuronide-[^{125}I] iodotyramine, Ref No. IM140, was obtained from Amersham International PLC, Little Chalfont, Buckinghamshire, HP7 9NA.
3. The rabbit anti-progesterone (Batch No.7044X), donkey anti-rabbit IgG (Cat. No. S022-220) and the normal rabbit serum (Cat. No. S030-220) were obtained as a gift from the Scottish Antibody Production Unit, Law Hospital, Carluke, Lanarkshire, Scotland, ML8 5ES.
4. The Triton X-100 was obtained from ICN, Cat. No. 807423.
5. The quality controls were prepared from bulk blood obtained from the abattoir, farm or SAPU.

EQUIPMENT:

COBRA II Auto Gamma Counter, Canberra Packard, Pangbourne, Berks, RG8 7DT.

Jouan KR422 Centrifuge and Sorvall RC3C Plus Centrifuge

A.3.2. Oestradiol (Iodinated oestradiol)

SOLUTIONS REQUIRED:

0.1M Phosphate buffer – stock solution

12.16 g Sodium dihydrogen orthophosphate dehydrate, $NaH_2PO_4 \cdot 2H_2O$

17.32 g Di-sodium hydrogen phosphate (anhydrous), Na_2HPO_4

2.0 g Sodium azide, NaN_3

All reagents were dissolved in double distilled water and made up to a final volume of 2.0 litres and stored at 4°C for up to 6 months.

Phosphate buffered saline – gel (PBS-Gel)

16.0 g Sodium chloride, NaCl

6.0 g Ethylenediaminetetra-acetic acid disodium salt (EDTA)

2.0 g Gelatin

All reagents were dissolved in 1,600 ml of warm 0.1M Phosphate buffer stock, adjusted the pH to 7.6 and made up to a final volume of 2.0 litres with 0.1M Phosphate buffer stock. Solution was stored at 4°C for up to one week.

QUALITY CONTROLS:

These were prepared in house and stored at –20°C in aliquots of low, medium and high quality controls. They were treated as for samples in the assay.

STANDARDS:

Stock solution:

5,000 pg/ml

Kit standard No.6, 500 µl supplied and stored at 4°C.

Sub-stock solution:

100 pg/ml

30 µl of stock was diluted with 1,500 µl of PBS-Gel.

	Reagents for 1 Std Curves		(x2 multiplier for 500 µl extracted sample)	
Std	Working Solution	PBS-Gel	Dose pg/ml	Defined Dose pg/tube
1	1500 µl (c)	- - - -	50	25
2	750 µl (1)	750 µl	25	12.5
3	750 µl (2)	750 µl	12.5	6.25
4	750 µl (3)	750 µl	6.25	3.125
5	750 µl (4)	750 µl	3.125	1.563
6	750 µl (5)	750 µl	1.563	0.781
7	750 µl (6)	750 µl	0.781	0.391
8	750 µl (7)	750 µl	0.391	0.195
9	750 µl (8)	750 µl	0.195	0.098
10	750 µl (9)	750 µl	0.098	0.049

1ST ANTIBODY SOLUTION – Rabbit anti-oestradiol

Stock solution:

12 ml was supplied per vial and stored at 4°C.

Working solution:

The dilution used was 1:6 which gave 30 – 35% of total binding.

1 ml of stock solution was added to 5 ml of PBS-Gel. On the day of use, the required amount of working antiserum solution was prepared and stored for up to 2 days at 4°C.

TRACER:

The ^{125}I-oestradiol tracer was supplied in 12.5 ml of phosphate citrate BSA buffer and contained up to a maximum of 170 kBq radioactivity. Prior to use in the assay, each batch of tracer had ^{125}I-oestradiol extracted from the manufacturer's buffer and then reconstituted in PBS-Gel assay buffer.

EXTRACTION OF ^{125}I-OESTRADIOL TRACER:

The ^{125}I-oestradiol was extracted on a 20 ml/5 g C18 Sep-pak cartridge attached to a vacuum manifold.

- ⇨ The Sep-pak cartridge was pre-wet with 20 ml methanol and pulled through under vacuum and then washed with 40 ml of double distilled water.
- ⇨ 250 ml beaker was placed into a manifold under the Sep-pak.
- ⇨ One bottle (12.5 ml) of tracer was added and pulled through under vacuum.
- ⇨ Tracer was washed out with 40 ml of double distilled water.

- ⇨ 4 ml of acetone was added and eluted to the waste collection beaker.
- ⇨ The waste collection beaker was then replaced with a rack containing six 12x75 mm glass tubes and eluted with six separate 2 ml aliquots of acetone, remembering to move the Sep-pak to the next tube position between each addition.
- ⇨ The rack containing the collection tubes was removed and the waste collection beaker was replaced.
- ⇨ When required, the Sep-pak was washed with 20 ml of acetone and reused immediately, starting at step 1.
- ⇨ The total volume of the liquid waste collected was measured and two 100 µl aliquots were counted in polystyrene tubes, prior to disposal.

500 µl of PBS-Gel buffer was added to the ^{125}I-oestradiol acetone extracts in tubes 2 to 6 and 100 µl to tube 1 (tube 1 had carry over water present) and all six were evaporated on a heating block at 35°C under a gentle stream of compressed air, until only the aqueous portion remained. A further 500 µl of PBS-Gel buffer was added to each tube, vortexed and pooled together. Tubes 1 and 2 were rinsed with an aliquot of the pooled tracer, as they contained the greatest activity. The volume of the pooled extracted tracer was measured and a 50 µl aliquot counted in a polystyrene tube. The tracer was stored at 4°C until the manufacturer's expiry date.

The tracer solution was diluted on a day of assay with PBS-Gel buffer to give a working solution containing 10,000 cpm per 50 µl. The number of counts was tested by counting 50 µl in a glass tube before addition to the assay. Working solution was stored at 4°C.

^{3}H-OESTRADIOL TRACER:

Stock solution:

5ml of ethanol was added to 9.25 MBq (250 µCi) vial of ^{3}H-oestradiol tracer and stored at –20°C.

Sub-stock solution:

The ^{3}H-oestradiol stock solution was diluted with PBS-Gel (in a glass tube) to give a working solution of 10,000 cpm per 50 µl. Before addition to the assay, the number of counts was tested in 50 µl of working solution as follows: 950 µl of PBS-Gel was

pipetted into a glass tube then added 50 µl of working tracer solution. The content was vortexed and tipped into a scintillation vial; 2,500 µl of scintillant was added, mixed and counted.

SEPARATION SOLUTION:

Goat anti-rabbit, supplied ready to use, was stored at 4°C. During dispensing, the vial was swirled occasionally to ensure homogeneity.

ASSAY METHOD:

Extraction:

- 500 µl of each quality control or sample were pipetted in to the duplicate glass extraction tubes followed by 500 µl of PBS-Gel buffer.
- 1,000 µl of PBS-Gel were pipetted into the extraction blank tubes.
- 950 µl of PBS-Gel and 50 µl of working ^{3}H tracer solution were pipetted into the extraction recovery tubes.
- 200 µl of PBS-Gel and 50 µl of working ^{3}H tracer solution were pipetted into the ^{3}H-TC glass assay tubes.
- Working in the fume hood, 2.5 ml of Fisher AR grade diethyl ether was added to each glass extraction tube.
- All tubes were covered with cling film and vortexed for 3 min.
- The tubes were allowed to stand in a fume hood for 20 min to enable the ether and aqueous layers to separate. When the layers failed to separate properly the tubes were capped and centrifuged at 1,000 rpm at 4°C.
- The tubes were then immersed in the freezing bath until the lower aqueous layer was completely frozen (10 – 20 min).
- The upper ether layer was decanted into the corresponding glass assay tube.
- The tubes were transferred into the heating blocks (set at 40°C) and the ether was evaporated at 40°C under a stream of air (20 min).
- Dry extract of each tube was reconstituted by adding 250 µl of PBS-Gel and vortexed for 10 sec.

Assay method:

- During the sample freezing/drying process, the standards were prepared by double dilution.

- ⇨ 250 µl of each standard was pipetted in duplicate into the corresponding glass assay tubes.
- ⇨ 250 µl of PBS-Gel was pipetted into the NSB and Bo tubes.
- ⇨ 50 µl of working antiserum solution was added to all tubes except the ^{125}I-TC, NSB, RECOVERY and ^{3}H-TC tubes and incubated for 15 min prior to tracer addition.
- ⇨ 50 µl of PBS-Gel was added to the NSB tubes.
- ⇨ 50 µl of ^{125}I-tracer was then added to all tubes except the RECOVERY and ^{3}H-TC tubes. The ^{125}I-TC tubes were capped.
- ⇨ The tubes were covered with cling film, vortexed for 10 sec and incubated for 2 h at room temperature.

- ⇨ After 2 h, 100 µl of the separation solution was added to all tubes except the ^{125}I-TC, RECOVERY and ^{3}H-TC tubes. The tubes were vortexed for 10 sec and incubated at room temperature for 20 min.
- ⇨ 2 ml of PBS-Gel was added to all tubes except the ^{125}I-TC, RECOVERY and ^{3}H-TC tubes. The tubes were then placed onto the magnetic rack, ensuring that they were touching the base plate and allowed to stand for 20 min at room temperature.
- ⇨ Whilst holding onto the magnetic portion of the rack, the supernatant was poured off and the tubes were blotted onto paper.
- ⇨ The ^{125}I-TC tubes were combined and counted for 1 min in the gamma counter.

- ⇨ 750 µl of PBS-Gel was added to the RECOVERY and ^{3}H-TC tubes and vortexed for 10 sec.
- ⇨ The contents of the RECOVERY and ^{3}H-TC tubes were decanted into scintillation vials.
- ⇨ 2.5 ml of scintillant was added to each vial, which were then capped and their content mixed thoroughly.
- ⇨ Each vial was counted for 5 min in the liquid scintillation counter.
- ⇨ Finally, the percentage of extraction recovery was determined by dividing the mean recovery dpm by the mean ^{3}H-TC dpm and multiplying by 100.

REAGENT SOURCES:

1. The oestradiol Maia Kit was manufactured by BioChem ImmunoSystems, Italy and distributed by BIO-STAT diagnostic Systems, Stockport, Chester, Uk. The kit has been adapted for ovine, bovine and porcine use, with the 120 Tube Kit (Code No. 370001) able to assay approximately 400 tubes and 1200 tubes when two extra bottles of Maia tracer (Code No. 370037) were purchased.
2. The Sep-pak 20 ml/5 g C18 cartridges (Cat. No. WAT036925) were obtained from Waters Ltd.
3. The tracer (2,4,6,7-^{3}H) oestradiol (Ref. No. TRK 322) was obtained from Amersham International PLC, Little Chalfont, UK.
4. Ultim Gold Scintillant was obtained from Canberra Packard, Pangbourne, Berks, UK.
5. Diethyl ether (certified AR grade) was obtained from Fisher Scientific UK Ltd.
6. The quality controls were prepared in house from plasma obtained from the abattoir or farm.

EQUIPMENT:

Cobra II Auto Gamma Counter, Canberra Packard, Pangbourne, UK.

Liquid Scintillation Analyser, 2000CA TRI-CARB, Canberra Packard, Pangbourne, UK.

Centrifuges – same as previously.

Magnetic Separator Racks, BIO-STAT Diagnostic Systems, Stockport, Chester, UK.

A.3.3. Insulin

SOLUTIONS REQUIED:

0.1M Phosphate buffer – stock solution

12.16 g Sodium dihydrogen orthophosphate, dehydrate, $NaH_2PO_4 \cdot 2H_2O$

17.32 g Di-sodium hydrogen phosphate, anhydrous, Na_2HPO_4

2.00 g Sodium azide, NaN_3

Those were added to 800 ml of double distilled water whilst stirring on a magnetic stirrer and once the solids had dissolved the solution was diluted to 2.0 litres and stored at 4°C for up to 6 months.

Phosphate BSA buffer – working solution

To 1,000 ml of 0.1M phosphate buffer was added:

18.0 g Sodium chloride, NaCl

10.0 g BSA (Fraction V)

1.0 g Sodium azide, NaN_3

This was diluted to 1,800 ml with double distilled water, the pH was adjusted to 7.4 with sodium hydroxide (40% w/v) and made up to a final volume of 2 litres. It was stored at 4°C for up to 2 weeks.

Separation solution

9.0 g Sodium chloride, NaCl

40.0 g Polyethylene glycol, PEG-8000

Sodium chloride and PEG were added to 800 ml of double distilled water, stirred until dissolved and made up to a final volume of 1 litre.

STANDARDS:

Stock solution:

1 mg/ml (27.8 IU/1 mg) solution of porcine insulin

1 mg of porcine insulin was dissolved in 1 ml of 0.01M hydrochloric acid.

Sub-stock solution:

(a) 100 µg/ml (2.78 IU/ml). 100 µl of the Stock solution was added to 900 µl of 0.05M phosphate buffer, aliquoted into 100 µl lots and stored at –20°C.

(b) 0.6 µg/ml (16.68 mIU/ml). 60 µl of Sub-stock (a) was added to 9,940 µl of 0.05M phosphate buffer, aliquoted into 150 µl lots and stored at –20°C.

Working standard solutions:

These were prepared on the day of use, as outlined in the table below.

	Reagents for 4 Std Curves			(100 µl sample x10 multiplication factor)	
Std	**Working Solution**	**0.05M Phos. BSA buffer**	**Defined dose µIU/tube**	**Dose µIU/ml**	**Dose ng/ml**
1	100 µl Sub-stock (b)	9,900 µl	16.68	166.8	6.0
2	1000 µl (1)	1000 µl	8.34	83.4	3.0
3	1000 µl (2)	1000 µl	4.17	41.7	1.5
4	1000 µl (3)	1000 µl	2.085	20.85	0.75
5	1000 µl (4)	1000 µl	1.0425	10.425	0.375
6	1000 µl (5)	1000 µl	0.5212	5.2125	0.1875
7	1000 µl (6)	1000 µl	0.2606	2.60625	0.0938
8	1000 µl (7)	1000 µl	0.1303	1.303125	0.0469

QUALITY CONTROLS:

These were stored at –20°C in 300 µl aliquots of low, medium and high quality controls and treated as for samples in the assay.

1ST ANTIBODY SOLUTION:

Stock solution:

1/100 dilution of guinea-pig anti-porcine insulin, supplied lyophilised.

Reconstituted with 1 ml of double distilled water and allowed to stand for 30 minutes.

Sub-stock solution:

1 ml of stock solution was diluted with 9 ml of 0.05M phosphate BSA buffer to give a 1/1,000 dilution. It was aliquoted into 1 ml lots and stored at –20°C.

Working solution:

345 µl of the 1/1,000 sub-stock solution was diluted in 10 ml of 0.05M phosphate buffer to give 1/30,000 dilution.

TRACER:

The ^{125}I-porcine insulin stock solution was diluted with 0.05M phosphate buffer to give a working solution of 10,000 cpm/100 µl. The number of counts were tested in 100 µl of working solution before addition to the assay.

2ND ANTIBODY SOLUTION:

A working solution of 1/80 sheep anti-guinea-pig 2nd antibody and 1/400 normal guinea-pig serum was prepared. 125 µl of sheep anti-guinea-pig and 25 µl of normal guinea-pig serum were added to 10 ml of 0.05M phosphate buffer.

ASSAY METHOD:

Day 1:

- ⇨ Polystyrene tubes were prepared in duplicate for the total counts (TC), NSB, Bo, standards, low, medium and high quality controls and samples.
- ⇨ Polystyrene tubes were labelled and the standards prepared.
- ⇨ 100 µl of standard, quality control or sample were pipetted in duplicate into tubes and 100 µl of 0.05M phosphate buffer was pipetted in duplicate into the Bo and NSB tubes.
- ⇨ 200 µl of 0.05M phosphate buffer was added into all tubes except the TC tubes.
- ⇨ 100 µl of the 1st antibody solution was added to all tubes except the NSB and TC tubes and 100 µl of 0.05M phosphate buffer was added to the NSB tubes.
- ⇨ All tubes were vortexed and incubated at room temperature overnight.

Day 2:

- ⇨ 100 µl of tracer was added to all tubes and the TC tubes were capped.
- ⇨ All tubes were vortexed and incubated at room temperature overnight.

Day 3:

⇨ 200 µl of 2nd antibody solution was added to all tubes except the TC tubes and incubated at 4°C overnight.

Day 4:

⇨ 1 ml of separation solution was added to all tubes except the TC tubes and incubated at room temperature for 10 minutes.

⇨ The tubes were then centrifuged at 3,000 rpm (Jouan) or 3,300 rpm (Sorvall), at 4°C for 30 minutes.

⇨ The supernatant was decanted by tipping the tubes upside down. The tubes were kept upside down and allowed to dry.

⇨ The precipitate in the tubes was counted for 1 min in the gamma counter.

REAGENT SOURCES:

1. Porcine insulin (I5523, 27.8 IU/mg) was obtained from Sigma Chemical Co Ltd, Poole, Dorset.
2. Guinea-pig anti-porcine insulin, normal guinea-pig serum and sheep anti-guinea-pig IgG were obtained as a gift from the Scottish Antibody Production Unit, Law Hospital, Carluke, Lanarkshire, Scotland.
3. The ^{125}I-porcine insulin solution was prepared using Chloramine T method (Greenwood *et al.*, 1963).
4. The quality controls were prepared from bulk blood obtained from the abattoir, farm or SAPU.

A.3.4. Insulin-like growth factor I (IGF-I) (Porcine/Bovine)

SOLUTIONS REQUIRED:

EDTA phosphate BSA buffer

0.03M Phosphate buffer, pH=7.5, containing 0.1% w/v BSA

The following was added to 800 ml of double distilled water while stirring:

4.68 g	Sodium hydrogen orthophosphate, dehydrate, $NaH_2PO_4 \cdot 2H_2O$
3.72 g	Ethylenediaminetetra-acetic acid, disodium salt (EDTA)
1.0 g	BSA (Fraction V)
0.2 g	Sodium azide, NaN_3
0.2 g	Protamine sulphate, grade X from salmon (Sigma, P4020)
500 µl	Tween 20 (Polyoxyethylene sorbitan monolaurate)

Once dissolved the pH was adjusted to 7.5 with sodium hydroxide (40% w/v), made up to a final volume of 1 litre and stored at 4°C for up to 2 weeks.

0.4M Tris-Base

4.85 g of Tris(hydroxymethyl)aminomethane was dissolved in double distilled water and made up to final volume of 100 ml.

Mobile phase buffer

0.2M Acetic acid containing 0.05M trimethylamine and 0.05% v/v Tween 20

Stock solution:

To 1,900 ml of water were added 22.9 ml glacial acetic acid, 13.25 ml 45% v/v trimethylamine and 7 ml hydrochloric acid. These were mixed well, made up to a final volume of 2 litres and stored in a glass container at room temperature. The stock solution was prepared fresh every few days.

Working solution:

Sufficient stock buffer for a single day use was filtered and degassed, using a 0.22 µm Millipore membrane filter and the pH was adjusted to 2.5 using hydrochloric acid. 50 µl of Tween 20 was added to 100 ml of buffer and mixed gently without causing undue aeration. A 10 ml sample was retained for use in quantitative IGF-I/IGF-II assay of samples.

Neutralised mobile phosphate buffer (NMP)

Mobile phase buffer and 0.4M Tris-Base were combined in a ratio of 250 µl:150 µl.

Separation solution

9.0 g Sodium chloride, NaCl

40.0 g Polyethylene glycol, PEG-8000

NaCl and PEG were added to 800 ml of double distilled water, stirred until dissolved and made up to final volume of 1 litre. The separation solution was stored at 4°C and was added cold (4°C) to the assay tubes in order to be effective as a precipitating agent.

0.01M hydrochloric acid

430 µl of hydrochloric acid was made up to 500 ml with double distilled water.

STANDARDS:

Stock solution:

1 mg/ml solution of recombinant human IGF-I (receptor grade)

The 100 µg vial of IGF-I was reconstituted with 100 µl of 0.01M hydrochloric acid, aliquoted into microcentrifuge tubes of 5 µl and 10 µl lots for iodination and standards, which were stored at –20°C.

Sub-stock solution:

1 µg/1 ml of stock solution

10 µl of the Stock solution was added to 9,990 µl of assay buffer in a polypropylene tube, aliquoted into 400 µl lots and stored at –20°C.

Working standard solutions:

These were prepared in polypropylene tubes on the day of use, as specified in the table below.

	Reagents for 1 Standard curve		Reagents for 3 Standard curves		120 µl extracted/250 µl of 4 ml assayed x 267 multiplication factor	
Std	Working solution	NMP buffer	Working solution	NMP buffer	Defined dose ng/tube	Dose ng/tube
1	320 µl sub-stock	1680 µl	640 µl sub-stock	3360 µl	32	160
2	1000 µl (1)	1000 µl	2000 µl (1)	2000 µl	16	80
3	1000 µl (2)	1000 µl	2000 µl (2)	2000 µl	8	40
4	1000 µl (3)	1000 µl	2000 µl (3)	2000 µl	4	20
5	1000 µl (4)	1000 µl	2000 µl (4)	2000 µl	2	10
6	1000 µl (5)	1000 µl	2000 µl (5)	2000 µl	1	5
7	1000 µl (6)	1000 µl	2000 µl (6)	2000 µl	0.5	2.5
8	1000 µl (7)	1000 µl	2000 µl (7)	2000 µl	0.25	1.25
9	1000 µl (8)	1000 µl	2000 µl (8)	2000 µl	0.125	0.625
10	1000 µl (9)	1000 µl	2000 µl (9)	2000 µl	0.0625	0.3125
11	1000 µl (10)	1000 µl	2000 µl (10)	2000 µl	0.03125	0.15625
12	1000 µl (11)	1000 µl	2000 µl (11)	2000 µl	0.01562	0.07812

^{125}I IGF-I TRACER RECOVERY

A 1 ml solution of ^{125}I-human IGF-I sample medium (plasma, serum etc.) was prepared in a polypropylene vessel to give 45,000 cpm/120 µl and was stored at 4°C. This solution was treated as a sample and one recovery was extracted from every batch of samples processed. At the time of extraction, 120 µl aliquots were pipetted in duplicate into a polypropylene tubes, which were capped and stored at 4°C for recovery determination.

EXTRACTION BLANK

Double distilled water was extracted in place of the sample. One blank, for every batch of samples processed, was extracted.

QUALITY CONTROLS:

These were stored at –20°C in 400 µl aliquots of low, medium and high quality controls and extracted as for samples prior to assay.

1ST ANTIBODY SOLUTION:

Stock solution:

1/50 dilution

Rabbit anti-human IGF-I, supplied lyophilised and containing the equivalent of 5 µl serum was reconstituted with 250 µl of assay buffer, ensuring that the lyophilised pellet was completely dissolved. Once reconstituted, the antibody was stored in the freezer.

Working solution:

1/3,500 dilution

Using a polypropylene or glass vessel, 71 µl of the stock solution was diluted to 5 ml with assay buffer and stored at 4°C for up to 5 days.

TRACER:

Using a polypropylene vessel, the ^{125}I-human IGF-I stock solution was diluted with assay buffer to give a working solution of 15,000 cpm/50 µl. The number of counts was tested in 50 µl of working solution before addition to the assay.

2ND ANTIBODY SOLUTION:

A working solution of 1/80 donkey anti-rabbit 2nd antibody and 1/800 normal rabbit serum was prepared by adding 125 µl of donkey anti-rabbit and 12.5 µl of normal rabbit serum to 10 ml of assay buffer.

EXTRACTION PROCEDURE:

Samples were extracted as detailed in the protocol 'Separation of IGFs from IGFBPs by HPLC'. Up to 20 samples were extracted per batch along with one blank, low and high quality controls and one tracer recovery, using a 24 place microcentrifuge. Using the Foxy Jr. fraction collector up to four batches (80 actual samples) were extracted and separated on the HPLC in a normal working day (one batch during the day and three overnight). The IGF fractions were stored capped at 4°C and assayed within five days of extraction. The IGF fraction was collected in 4 ml between 11 and 15 minutes.

ASSAY METHOD:

Day 1:

- ⇨ Polypropylene tubes were prepared in triplicate for non-specific binding (NSB), zero standard (Bo), standards and in duplicate for total counts (TC) and for low, medium and high quality controls and the samples.
- ⇨ Standards were prepared in polypropylene tubes.
- ⇨ 200 µl of standard was pipetted in duplicate into tubes.
- ⇨ 200 µl of NMP buffer was added into each standard tube and 400 µl of NMP buffer was pipetted in duplicate into the Bo and NSB tubes.
- ⇨ 250 µl of each sample or quality control was pipetted in duplicate into tubes.
- ⇨ 150 µl of 0.4M Tris-Base was added to neutralise each sample and quality control tube.
- ⇨ 50 µl of the 1st antibody solution was added to all tubes except the NSB and TC tubes and 50 µl of assay buffer was added to the NSB tubes.
- ⇨ All tubes were vortexed and incubated overnight (16 – 22 h) at room temperature.

Day 2:

- ⇨ 50 µl of tracer was added to all tubes and the TC tubes were capped.
- ⇨ All tubes were vortexed and incubated overnight (16 – 22 h) at room temperature.

Day 3:

- ⇨ 100 µl of 2nd antibody solution was added to all the tubes except the TC tubes.
- ⇨ All tubes were vortexed and incubated for 3 h at room temperature.
- ⇨ 1 ml of 4°C separation solution was added to all tubes except the TC tubes.
- ⇨ All tubes were incubated at room temperature for 10 min.
- ⇨ These tubes were then centrifuged at 3,000 rpm (Jouan) or 3,300 rpm (Sorvall) at 4°C for 30 min.
- ⇨ The supernatant was decanted and the pellet in the tubes counted for 1 minute on the gamma counter.

REAGENT SOURCES:

1. IGF-I receptor grade (CU100, 100 mg) and Rabbit anti-human IGF-I (5PAA1) were obtained from GroPep limited, Adelaide, Australia.

2. The ^{125}I human IGF-I solution was prepared using Chloramine T.
3. The donkey anti-rabbit and normal rabbit serums were obtained from Diagnostics Scotland, Ellen's Glen Road, Edinburgh.

A.3.5. Leptin (Emu Ovine/Bovine)

SOLUTIONS REQUIRED:

Working dilution of emu reagents:

Label	10,000 cpm/50 µl
1st Antibody	1:5000
Normal emu serum (NES)	1:500
2nd Antibody	1:12

All of the above were diluted in assay buffer.

The primary anti-serum, normal emu serum (NES) and sheep anti-emu immunoglobulin serum (SAES) used in the assay were provided by Dr D. Blache (Faculty of Agriculture, The University of Western Australia, Nedlands 6907, Australia). Ovine leptin (supplied by Dr. D. Keisler (University of Missouri, Columbia 65211, USA) was iodinated in house with ^{125}I and reconstituted in assay buffer as appropriate to give approximately 10,000 cpm per 50 µl.

Leptin Assay Buffer

To prepare 1 litre:

100 ml	0.5M PO_4	(0.05M)
9.0 g	NaCl	(0.154M)
9.306 g	EDTA	(0.025M)

The above were added to approximately 800 ml of deionized water and the pH was adjusted to 7.4.

Then the following were added:

250 mg	NaN_3	(0.025%)
500 µl	TritonX-100	(0.05%)
5.0 g	BSA	(0.5%)

Once dissolved, the solution was made up to a final volume of 1 litre and the pH was checked and adjusted to 7.4 when required.

ASSAY METHOD:

Day 1.

- ⇨ Polypropylene tubes were labelled.
- ⇨ The standards were aliquot in triplicate and 100 µl of unknown samples in duplicate.
- ⇨ 50 µl of 1st antibody o-leptin (1:5,000) was added to all tubes except the TC and the NSB tubes.
- ⇨ 50 µl NES (1:500) was added to all tubes except the TC tubes.
- ⇨ All tubes were incubated overnight at 4°C.

Day 2.

- ⇨ 50 µl of label was add (10,000 cpm) to all tubes and incubated at 4°C for 48 h.

Day 4.

- ⇨ The 2nd antibody (SAES) was diluted to 1:12 and added to all tubes except the TC tubes.
- ⇨ All tubes were incubated at 4°C for 48 h.

Day 6.

- ⇨ 1 ml of chilled 3% (w/v) PEG in 0.01M PO_4 buffer was added to all tubes except the TC tubes.
- ⇨ These were then centrifuged at 3,000 rpm for 30 min.
- ⇨ The supernatant (Aspirate) was decanted and the pellet counted in the gamma counter.

APPENDIX 4

Lipid extraction and analysis

A.4.1. Separation of lipid classes in oocytes, blastocysts and media.

EQUIPMENT:

Glassware

Before use all glassware was soaked in decon, nitric acid washed and rinsed thoroughly in distilled water. Glassware was kept separately and only unmarked and new glassware was used.

Tube preparation

An adequate number of tubes were prepared prior to collection day. Twenty five ml of 2:1 chloroform:methanol solution was added into each tube using a measuring cylinder. Known amount of C15:0 was added into each tube using graduated glass pipette. Such prepared tubes were stored stoppered in a darkened fridge.

SOLUTIONS:

2:1 Chloroform:methanol

500 ml of chloroform, mixed with 250 ml methanol

2:1:0.1 methanol:toluene:sulphuric acid

10 ml of sulphuric acid was added carefully to 200 ml of dry methanol and 100 ml of toluene was then added. This solution was prepared fresh each time.

Internal standards:

Pentadecanoic acid (C15:0): Stock (10 μg/ml) – 2 mg of C15:0 was weighed out and dissolved with methanol and made up to 100 ml, in a volumetric flask with methanol. Sub-stock (100 ng/ml) solution was prepared by diluting 500 μl of Stock (using a glass pipette) to 50 ml with methanol.

Supelco 37 (10 mg/ml): glass vial was snap opened and content transferred to a 10 ml volumetric flask, vial was washed with a few ml of hexane and made up to 10 ml. The solution was aliquoted into glass vials and stored at –80°C. Before injection the aliquot was allowed to come to room temperature. Depending on condition and injection volumes Supelco 37 needed further diluting.

PROCEDURE:

⇨ A minimum of 5 – 10 oocytes or 5 – 10 blastocysts were added per tube (containing 2:1 chloroform:methanol) using a glass pipette. The oocytes or blastocysts were picked up in a minimal volume (<5 µl) of PBS/BSA. A reagent blanks to check BSA and also other reagents, were carried out routinely. When samples were incubated for a period of time, also incubation blanks (e.g. for blastocyst culture) were carried out.

⇨ The tube content was mixed and stored overnight at 4°C.

⇨ Using a finnipipette 6 ml 0.88% KCl was added into each tube, which was then stoppered, mixed and allowed to separate for 1 h or overnight at room temperature.

⇨ After separation, the aqueous layer (top) was aspirated off and the chloroform layer was transferred into a 50 ml Quickfit flask. The chloroform was evaporated at 40°C on a rotary evaporator.

Methylation:

⇨ The reagents were prepared immediately before use.

⇨ 4 ml of 2:1:0.1 methanol:toluene:sulphuric acid solution was added to the round bottom flasks, mixed by swirling and refluxed gently for 30 min. After 30 min flasks were removed from heat and allowed to cool.

⇨ 10 ml of distilled water and 2 ml of hexane were added into each tube, which were then stopper and mixed thoroughly, allowing the two layers to separate.

⇨ Using a glass pipette, the hexane layer was aspirated, ensuring that no aqueous layer was drawn up.

⇨ Hexane layer was then transferred to Quickfit tube containing sodium sulphate for 30 min x 2 (a round bottom flask and evaporated once more).

⇨ 150 µl of hexane was added into each tube, mixed quickly and transferred to a chromacol vial with insert and analysed immediately.

Recoveries:

C17:0 phospholipid and C19:0 triglyceride also went through the system, but were not added to the samples as they were not pure enough.

Both routinely 88 – 104% recovery.

Analysis:

Splitless injection method was used for oocytes and blastocysts.

Each sample was injected in duplicate, with duplicate hexane blanks between each sample. A Supelco standard was also injected, approximately every 10 samples, to check retention times of individual fatty acids. Injection volumes varied between 2 µl – 0.2 µl, dependent on sample concentration. When analysing a new sample type, it was considered best to inject the sample at a range of injection volumes.

APPENDIX 5

Equipment and reagents

A.5.1. Equipment

	Supplier	Cat. No.
Auto analyser cups 2 ml	Fisher, UK	DIS-045-040G
Bijous	Fisher, UK	DIS-080-010R
Blunt Needles, 18G x 1½"	Grampian Hospital	FTR591
Cell strainer 70 µm	BD Biosciences, UK	352350
Centrifuge IEC Centra-4R	Dunstable, England	
Centrifuge tubes (50 ml, free standing)	Fisher, UK	CFT-900-031F
Filtered tips	Eppendorf UK, Ltd	
Filters 0.2 µm: Syringe attachable	Fisher, UK	FDP-625-030N
150 ml	Fisher, UK	FDM-105-010L
500 ml	Fisher, UK	TKV-700-016X
Gas Chromatograph	CP3800, Varian Analytical Instruments HP225, Hewlett Packard Ltd, Cheshire, UK	
Glass cover slips square 22 x 22 mm	Fisher, UK	MNJ-350-020H
Glass slides	Fisher, UK	MNJ-150-020A
Glass slides, Superfrost Plus	Fisher, UK	MNJ-700-010N
Haemocytometer	Weber, England, UK	
Image analysis and capture Software	ImageJ, version 1.19q or 1.28u, National WinTV, version 1.9A, Hauppage, NY, USA	
Incubators (low and high oxygen)	Heraeus Instruments, Institutes of Health, USA	
Inverted microscope	Labovert FS, Leitz Wetzlar, Germany	
Lipid classes - columns	Capillary column, 30 mm, 0.25 mm internal diameter, 0.25 m film thickness;Varian Bond Elut Aminopropyl Silica columns, 100 mg packed silica per 1 ml cartridges.	
Liquid scintillation analyser	Tri-carb 2300TR, Packard BioScience	
Micro-centrifuge tubes 0.5 ml	Fisher, UK	FB56005
1.5 ml	Fisher, UK	FB56023
Multidishes 24-well plates	NUNC	142475
Multidishes 4- well plates	NUNC	176740

Needles, 18G x 1½"	Sherwood Medical, Hampshire, UK	
Petri dishes 60 mm diameter	NUNC	150288
30 mm	NUNC	153066
Pipettes from 0.2 µl to 10 ml	Gilson, Anachem Ltd, UK	
Pipette tips: 5 ml	SLS, UK	PIP7904
1 ml	SLS, UK	PIP7880
10 µl	SLS, UK	PIP8802
Round-bottom flasks, 50 ml	Quickfit/Fisher, UK	QFH-092-L
Scintillation vials, 6 ml	Zinsser Analytic, Germany	
Syringes 10 ml (Plastipak)	Fisher, UK	SZR-150-041H
20 ml (Plastipak)	Fisher, UK	SZR-150-080V
5 ml (Plastipak)	Fisher, UK	SZR-150-031K
Test tubes, 50 ml	Quickfit/Fisher Version 5.5, Varian Analytical Instruments	
Universal containers, 30 ml	Fisher, UK	FB55151
Video Camera	JVC TK-C1381, Japan	

A.5.2. Reagents

	Supplier:	**Cat. No.:**
[2-^{14}C] pyruvic acid	American Radiolabelled Chemicals, St. Louis, MO, USA	
[^{3}H] glucose	Amersham, UK	
3-aminopropyltriethoxysilane	Sigma	A-3648
Acetic acid glacial	Fisher, UK	A/0360/PB17
Acetone	BDH, Pool, UK	100035R
Amino acids - essential	Sigma	B6766
Amino acids - non-essential	Sigma	M7145
Amphotericin	Sigma	A2942
Apoptag Red - detection kit	Intergen, NY, USA	S7165
BSA (Fatty Acid Free)	Sigma	A8806
BSA (Fraction V)	Sigma	A3311
$CaCl_2 \cdot 2H_2O$	Sigma	C7902
Chloroform, Aristar	BDH	152834E

Citifluor Solid Mountant Kit	Agar Scientific, Essex, UK	R1326
Dimethyl sulfoxide	Sigma	D5879
Dulbecco's PBS 1x with Ca and Mg	Sigma	D8537
Emulsifier Safe scintillation fluid	Packard BioScience, UK	6013389
Epinephrine	Sigma	E1635
Ethanol	BDH, Pool, UK	10107
Foetal Bovine Serum	Sigma	F9665
FSH (porcine pituitary)	Sigma	F2293
Glucose	Sigma	G6152
HCl solution	Sigma	H9892
Heparin (sodium salt)	Sigma	H3149
HEPES	Sigma	H4034
Hexane, HPLC grade	Rathburn Chemicals Ltd, Scotland, UK	
Histopaque 1077	Sigma	H8889
Hoechst 33342	Sigma	B2261
Hypotaurine	Sigma	H1384
In Situ Cell Death kit (Fluorescein)	Roche Applied Science, UK	1684795
Insulin	Sigma	I1882
Kanamycin	Sigma	K1876
KCl	Sigma	P5405
Lactic acid (sodium salt)	Sigma	L7900
L-glutamine	Sigma	G5763
LH (horse pituitary)	Sigma	L9773
Methanol, Aristar	BDH	451024P
$MgCl_2 \cdot 6H_2O$	Sigma	M2393
Mineral oil	Sigma	M8410
MTT	Sigma	M5655
NaCl	Sigma	S5886
NaH_2PO_4	Sigma	S5011
$NaHCO_3$	Sigma	S5761
NaOH solution	Sigma	S2770
Paraformaldehyde (PFA)	Sigma	44,124-4
PBS tablets	Oxoid	BR0014G
PBS tablets	Sigma	P4417

Pen/strep/neomycin (solution)	Sigma	P4083
Penicillin	Sigma	P4875
Penicillin/streptomycin (solution)	Sigma	P4333
Pentadecanoic acid (C15:0)	Sigma	P6125
Phenol Red 0.5% solution	Sigma	P0290
Phenol Red powder	Sigma	P5530
Polyvinyl alcohol (PVA)	Sigma	P8136
Polyvinylpyrrolidone (PVP)	Sigma	P0930
Propidium Iodide (PI)	Sigma	P4170
Pyruvic acid (sodium salt)	Sigma	P4562
Saponin	Sigma	S4521
Sigmacote	Sigma	SL2
Sodium citrate tribasic dihydrate	Sigma	S4641
Sodium selenite	Sigma	S5261
Streptomycin	Sigma	S1277
Sulphuric acid, GPR	BDH	303253D
Supelco 37 Standard Mix	Supelco, Poole, Dorset, UK	
TCM 199 with Hepes	Sigma	M7528
TCM 199	Sigma	M2154
Toluene, GPR	BDH	304526N
Transferrin	Sigma	T1283
TritonX-100	Sigma	X-100
Trypan blue solution	Sigma	T8154
Trypsin solution	Sigma	T4174
Tween20	Sigma	P1379
Vectashield Mounting medium	Vector Laboratories	H1000

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