THE EFFECT OF DIETARY OMEGA-3 AND -6 POLYUNSATURATED FATTY ACIDS ON OVINE OVARIAN FUNCTION AND THE PRE-IMPLANTATION EMBRYO

By

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DECLARATION

I hereby declare that this thesis is my own work and that it has not been submitted anywhere for any other degree or award. The work presented herein is my own work and where other sources of information have been used, they have been duly acknowledged.

Jaime Hughes

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ABSTRACT

There is considerable interest in the beneficial role of dietary polyunsaturated fatty acids (PUFA) on reproduction in ruminants. Detailed information regarding the mechanisms behind this beneficial effect is limited. The main objective of this thesis was to test the effects of dietary supplementation with omega-3 (n-3) or -6 (n-6) PUFA on gene expression, fatty acid (FA) composition and steroidogenesis in granulosa and theca cells and pre-implantation embryo development.

A previous study in our laboratory reported increased follicular-fluid progesterone concentrations in ewes fed an n-3 compared to an n-6 PUFA-enriched diet, but detected no differential effect of n-3 and n-6 PUFA enriched high-density lipoproteins (HDL) on granulosa cell steroidogenesis in vitro. Also, n-6 PUFA enriched HDL reduced early embryo development, but in the absence of a net uptake of FA. In view of these observations it was hypothesised that (i) effects of n-3 PUFA on ovarian steroidogenesis are mediated by theca rather than granulosa cells and (ii) during embryo culture lipids are acquired solely from the albumin fraction of serum, so that albumin delivered n- 3 and n-6 PUFA would exert a greater differential effect on embryo development than either LDL or HDL delivered PUFA.

Initial investigations into granulosa cell gene expression profiles using an ovine gonad-targeted cDNA macroarray were unsuccessful, highlighted by subsequent qRTPCR analysis. A thorough investigation confirmed that inconsistencies were due to poor array hybridisation. In vitro data confirmed that n-3 PUFA, via delivery by HDL, increase progesterone production solely in theca cells and that this is associated with an increase in STAR transcript expression. We also demonstrate that albumin is the only serum fraction that leads to a net uptake of FA during embryo culture. PUFA enriched serum and albumin accelerated the development of embryos and increased the yield of morphologically poorer quality blastocysts with increased transcript expression for the antioxidant enzyme SOD1. Important differential effects of n-3 and n-6 PUFA on ovarian steroidogenesis acting solely on theca cells are identified, but differentially effects of PUFA on embryo development are less apparent.

PUBLICATIONS

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LIST OF ABBREVIATIONS

aa	Amino acid
AA	Arachidonic acid
ACTB	Actin, beta (gene)
ALOX	Lipoxygenase
ANOVA	Analysis of variance
B-actin	Actin, beta
BMP	Bone morphogenetic factor
BSA	Bovine serum albumin
C	Centigrade
CAL	Calibrator
cDNA	Complementary DNA
CL	Corpus luteum
CLA	Conjugated linoleic acid
cm	Centimetre
CO_2	Carbon dioxide
CoA	Coenzyme A
COC	Cumulus oocyte complex
COX2	Cyclooxygenase 2
Cpm	Counts per minute
cRNA	complementary RNA
CV	Coefficient of variation
CYP11A1	Cytochrome P450, family 11, subfamily A, polypeptide 1 (cholesterol side chain cleavage)
CYP17A	Cytochrome P450, family 17, subfamily A (17-alpha-hydroxylase)
CYP19A	Cytochrome P450, family 19, subfamily A (aromatase)
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
DHA	Docosahexanoic acid
DHEA	Dehydroepiandrosterone
DM	Dry matter

DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
dTTP	Deoxythymidine triphosphate
E2	Oestradiol
EDTA	Ethylenediaminetetraacetic acid
EEF1a1	Eukaryotic translation elongation factor 1 alpha 1
EFA	Essential fatty acids
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
ELOVL	Elongation of very long chain fatty acids
EPA	Eicosapentanoic acid
ER	Endoplasmic reticulum
ESR1	Oestrogen receptor alpha
ESR2	Oestrogen receptor beta
FA	Fatty acid
FADS	Fatty acid desaturase
FAF	Fatty acid free
FAM	5’-6’-carboxyfluorescin
FAME	Fatty acid methyl ester
FAS	Fatty acids synthase
FATP	Fatty acid transport protein
FCS	Fetal calf serum
FF	Follicular fluid
FFA	Free fatty acids
FGF	Fibroblast growth factor
FSH	Follicle stimulating hormone
g	G force
g	Gram
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GC	Gas chromatography
GDF	Growth differentiating factor

gDNA	genomic DNA
GJ	Gap junction protein
GLM	Generalized linear model
GnRH	Gonadotrophin releasing hormone
GV	Germinal vesicle
GVBD	Germinal vesicle breakdown
h	Hour
H_2SO_4	Suphuric acid
HCL	Hydrochloric acid
HDL	High density lipoprotein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMG	Hydroxymethyl glutaryl
HPLC	High pressure liquid chromatography
HPRT	Hypoxanthine phosphoribosyltransferase
HRP	Horseradish peroxidase
HSD3B1	3β - hydroxysteroid dehydrogenase
HSD17B	17β - hydroxysteroid dehydrogenase
ICM	Inner cell mass
IDL	Intermediate density lipoprotiens
IETS	International Embryo Transfer Society
IFN	Interferon
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor binding protein
I-κB	Nuclear factor kappa B inhibitory subunit
IMS	Industrial methylated spirits
iNOS	Inducible nitric oxide synthase
INRA	French National Institute for Agricultural Research
IL	Interleukin
IU	International unit
IVF	In vitro fertilization
IVM	In vitro maturation
IVT	In vitro transcription
KBr	Potassium bromide
KCl	Potassium Chloride

kDa	Kilodalton
kg	Kilogram
LA	Linoleic acid
LDL	Low density lipoprotein
LDLR	Low density lipoprotein receptor
LH	Luteinising hormone
LNA	Linolenic acid
Log	Logarithm
LT	Leukotreine
M	Molar
ME	Metabolisable energy
mg	Milligram
ml	Millilitre
mM	Millimolar
mmol	Millimoles
MMP	Matrix metalloproteinase
mOsm	Milliosmolarity
MPF	Meiosis promoting factor
MPL	Membrane phopholipids
mRNA	Messenger ribonucleic acid
MUFA	Monounsaturated fatty acids
N_2	Nitrogen
n-3	Omega-3 polyunsaturated fatty acid
n-6	Omega-6 polyunsaturated fatty acid
n-9	Omega-9 polyunsaturated fatty acid
NaCl	Sodium chloride
NADH	Nicotine adenine dinucleotide, reduced
NCGD	Neutral cellulose gammanase digestibility
ND	Not detectable
NDF	Neutral detergent fibre
NEB	Negative energy balance
NFκB	Nuclear factor kappa B
ng	Nanogram
nm	Nanometer

NOS2	Nitric oxide synthetase-2
NS	Not significant
P4	Progesterone
P450scc	Cholesterol side chain cleavage enzyme (CYP11A1)
PBS	Phosphate buffered saline
PCOS	Polycystic ovarian syndrome
PCR	Polymerase chain reaction
pg	Picogram
PG	Prostaglandin
pH	Potential hydrogen
pmol	Picomoles
PO_4	Phosphate
PPAR	Peroxisome proliferator activated receptor
PPRE	Peroxisome proliferator response element
psi	Pounds per square inch
PTGS2	Prostaglandin-endoperoxidase synthase-2
PUFA	Polyunsaturated fatty acids
PVA	Polyvinyl alcohol
QC	Quality control
qRT-PCR	Quantitative real time polymerase chain reaction
RIA	Radioimmunoassay
RIN	RNA integrity number
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
RT	Reverse transcription
RXR	Retinoid X receptor
SA	Stearic acid
SCAP	SREBP-cleavage activating protein
SCARB1	Scavenger receptor, class B1
SCD	Stearoyl CoA desaturase
SDS	Sodium dodecyl sulphate
SED	Standard error of the difference
SEM	Standard error of the mean

SOD1	Superoxide dismutase 1
SOF	Synthetic oviductal fluid
SRE	Sterol regulatory element
SREBP	Sterol regulatory element binding protein
SSC	Saline sodium citrate
STAR	Steroidogenic acute regulatory protein
TAMRA	3'-6'-carboxy-N,N,N',N'-tetramethylrhodamine
TCM199	Tissue culture media 199
TFA	Total fatty acids
TIMP	Tissue inhibitors of metalloproteinases
TMB	Tetramethylbenzidine
TNF	Tumour necrosis factor
TX	Thromboxane
VA	Vaccenic acid
VEGF	Vascular endothelial growth factor
VLCFA	Very long chain fatty acids
VLDL	Very low density lipoprotein
vs	Versus
v/v	Volume to volume
Δ	Delta
µg	Micrograms
µl	Microlitre

CHAPTER 1

Literature Review

1.1 Introduction

Low reproduction rate in ruminants is a major restriction in the production of these species (Sinclair and Webb, 2005). Declining fertility in cattle, especially dairy cows, has become an international problem, despite variations in breed and management systems (Beam and Butler, 1999; Harris and Kolver, 2001; Lucy, 2003; Chagas et al., 2007). Reproductive performance has declined with genetic selection for high milk yield (Berry et al., 2003; Chagas et al., 2007). It is thought that this process selects for metabolic processes that are beneficial for milk production but detrimental to reproduction (Butler and Smith, 1989; Gutierrez et al., 2006). Additional to genetic variation, nutrition has been recognised as a major factor affecting fertility, with nutritional status varying greatly between herds (Beever, 2006). There have been a multitude of varied nutritional studies focussing on improving reproductive function in cattle. For example correlations have been observed between negative energy balance (NEB) and reduced reproductive performance (Butler and Smith, 1989), high dietary protein levels and decreased reproductive efficiency (Ferguson and Chalupa, 1989) and vitamin supplementation and improved reproductive function (Hemingway, 2003). One of the major constraints in dairy cow fertility is the problem of NEB brought on by high milk production and food intake too low to compensate for the energy expended by these high production animals (Pryce et al. 2001). NEB can affect many factors such as follicle growth, oocyte maturation and changes in follicular fluid composition including fatty acid composition (Leroy et al., 2005; Llewellyn et al., 2007; Leroy et al., 2008). An increase in dietary fat in high energy diets seems to have abated the severity of NEB (Andersen et al. 2008) and there is recent evidence which supports supplemental fat having a positive effect on cow fertility (Wathes et al., 2007). Protecting fats against rumen biohydrogenation have shown beneficial effects on energy metabolism and ovarian function (Mattos et al., 2000; Adamiak et al., 2006). In addition to increasing energy stores, fatty acids (FA) are known to affect reproduction by moderating prostaglandin production,

steroidogenesis, maintenance of cell membrane properties and cholesterol metabolism (Worgall et al., 1998; Abayasekara and Wathes, 1999; Jump and Clarke, 1999; Elmes et al., 2004; Vanholder et al., 2005). Therefore, the positive effects seen by additional fat in the diet of these animals could be due to specific FA acting via these complex mechanisms as opposed to increased energy availability. Many studies to date that have reported effects of polyunsaturated fatty acids (PUFA) on fertility have been inconsistent and have varied between species (Wathes et al. 2007). Due to the vast number of pathways PUFAs are able to affect, studies are only able to focus on a few of these aspects and the larger picture of the interactions of PUFA in the ovary remains unclear.

1.2 Reproductive physiology

1.2.1 The oestrous cycle

The oestrous cycle consists of a series of physiological events that begin and end with the onset of oestrus (heat). These reproductive events continue throughout the life of the female in a cyclical manner and are interrupted only by pregnancy, anoestrous (in seasonal breeders and during lactation), poor nutrition and some pathological conditions. The oestrous cycle can be divided into two parts; the follicular and luteal phases. The follicular phase refers to the point after luteolysis when progesterone levels begin to fall and oestradiol secretion increases due to follicular growth, ending with ovulation. The luteal phase begins as the ruptured, ovulated follicle transforms into a functioning corpus luteum (CL) (luteinisation) along with an increase in progesterone, ending with luteolysis.

1.2.2 Hormonal control of the oestrous cycle

The oestrous cycle is regulated by many inter-relationships between the hypothalamus-pituitary-ovarian axis, and the various hormonal and chemical signalling messages between them. The oestrous cycle is controlled mainly by hypothalamic gonadotrophin releasing hormone (GnRH) which stimulates the secretion of gonadotrophins from the anterior pituitary gland and initiates the follicular phase (Figure 1.1). GnRH is released into the portal blood in a pulsatile

fashion (Scaramuzzi et al., 1993) which then stimulates the synthesis and release of the gonadotrophins, luteinising hormone (LH) and follicle stimulating hormone (FSH), thereby promoting follicle development (McNeilly, 1991). Despite the initial stages of follicle development occurring independently of gonadotropic hormones, FSH is necessary for follicular emergence, whereby antral follicles become responsive to and then ultimately dependant on gonadotrophins (Roche, 1996). As follicles develop the production of oestradiol increases, synthesised by the increasing population of granulosa cells. At the same time, inhibin and activin are synthesised by granulosa cells which inhibit and stimulate the synthesis and secretion of FSH, respectively, maintaining correct levels. Inhibin and activin are two closely related glycoprotein complexes which appear to have multiple endocrine and paracrine functions in follicle development (Klein et al., 2000). LH receptors begin to be expressed by fully differentiated granulosa cells of large preovulatory follicles (Baird, 1991) and, along with maximal levels of oestradiol and low levels of

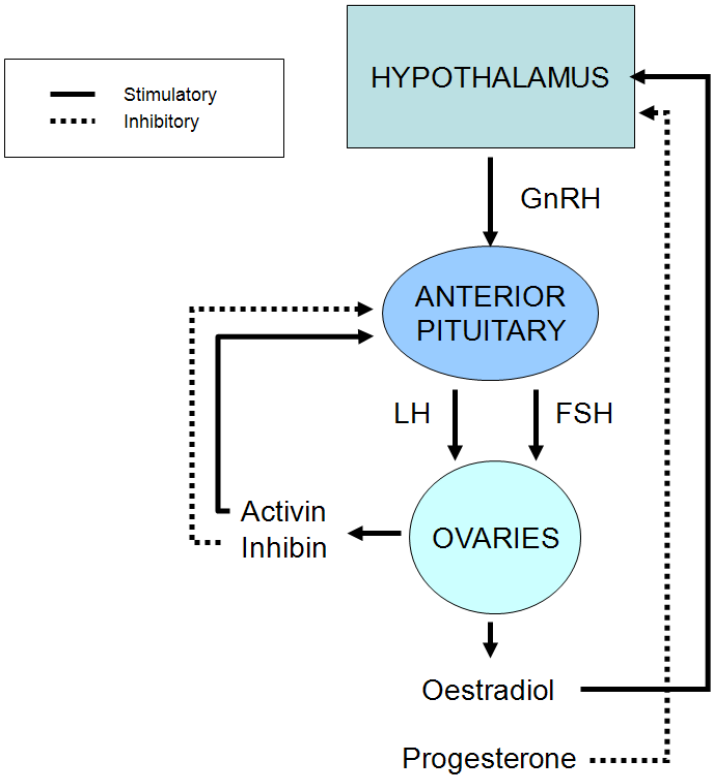

Figure 1.1. Endocrine feedback mechanisms and relationship between the hypothalamus, pituitary and ovaries. LH = luteinising hormone; FSH = follicle stimulating hormone; GnRH = gonadotrophin releasing hormone.

progesterone, a positive feedback effect of elevated oestradiol stimulates GnRH to induce the release of surge levels of LH from the pituitary. This surge in LH triggers a cascade of events which ends in ovulation and the formation of a CL.

1.2.3 Folliculogenesis

Folliculogenesis is an established pattern of follicle growth whereby follicles undergo stages of development termed recruitment, selection and dominance. Quiescent primordial follicles contain an oocyte that has no zona pellucida but is surrounded by a layer of flattened granulosa cells contained within a basal lamina (Scaramuzzi, 1993). The entire pool of primordial follicles develops in ruminants during foetal life (Fortune, 1994), and once committed to growth the follicles grow until they become atretic or they become dominant and ovulate. Once this pool of growing follicles has been established, a cohort is recruited by mechanisms that still remain unclear and become irreversibly committed to growth (recruitment). Studies indicate that these initial stages are not controlled by gonadotrophins (McNatty et al., 1999; Abel et al., 2000; Campbell et al., 2000), but follicular growth probably depends on the presence of oocyte/granulosa cell interactions and the secretion of a range of local factors (e.g., growth differentiating factor (GDF), bone morphogenetic proteins (BMP), activins, inhibins, fibroblast growth factor (FGF), and epidermal growth factor (EGF); (McNatty et al., 1999; Knight and Glister, 2001; Webb et al., 2003). As these committed follicles become primary follicles (Figure 1.2), several changes are detected; oocyte enlargement, development of flattened to cuboidal granulosa cells, formation of the zona pellucida, mitotic activity and RNA synthesis by the oocyte. Theca cells begin to differentiate from the surrounding stromal tissue as the granulosa cells become 2-3 layers thick (Scaramuzzi, 1993). The developing follicles become gondatropin-responsive once the granulosa and theca cells begin to express FSH and LH receptors, respectively (Erickson and Magoffin, 1983; McNatty et al., 1999). Evidence indicates that follicles act in a hierarchical pattern, dependant on their individual responses to FSH and their synthesis of androgens and related enzyme activity (McNatty, 1992; Scarramuzzi, 1993). Those that do not fit the profile, and have a lower expression of receptors and enzymes, become atretic (selection). As the follicles become gonadotrophin-dependant the antrum steadily grows from fluid-filled cavities, 40µm in diameter that aggregate together (Gougeon,

1996). These follicles have a greater requirement for FSH and theca cells begin to express LH receptors, which further stimulate the differentiation of theca layers, including increased capacity to secrete androgens (Fortune, 1994). LH is released in a pulsatile fashion from the pituitary, it binds to receptors on theca cells inducing testosterone synthesis, which in turn diffuses into the granulosa cells where it is converted to oestradiol (Filicori, 1999) (Figure 1.3). The aromatization of androgens in granulosa cells greatly increases oestradiol production which is crucial for further differentiation of granulosa cells. A follicle becomes dominant as the signals that continue its development are no longer strong enough to maintain the development of other, subordinate, large antral follicles. Zeleznik and Kubik (1986) showed that levels of FSH which are insufficient to recruit follicles are sufficient to maintain growth once it has been initiated. Also, the expression of granulosa cell LH receptors allows larger follicles to continue growing and become dominant over smaller follicles whose granulosa cells are lacking LH receptors (Campbell et al., 1999).

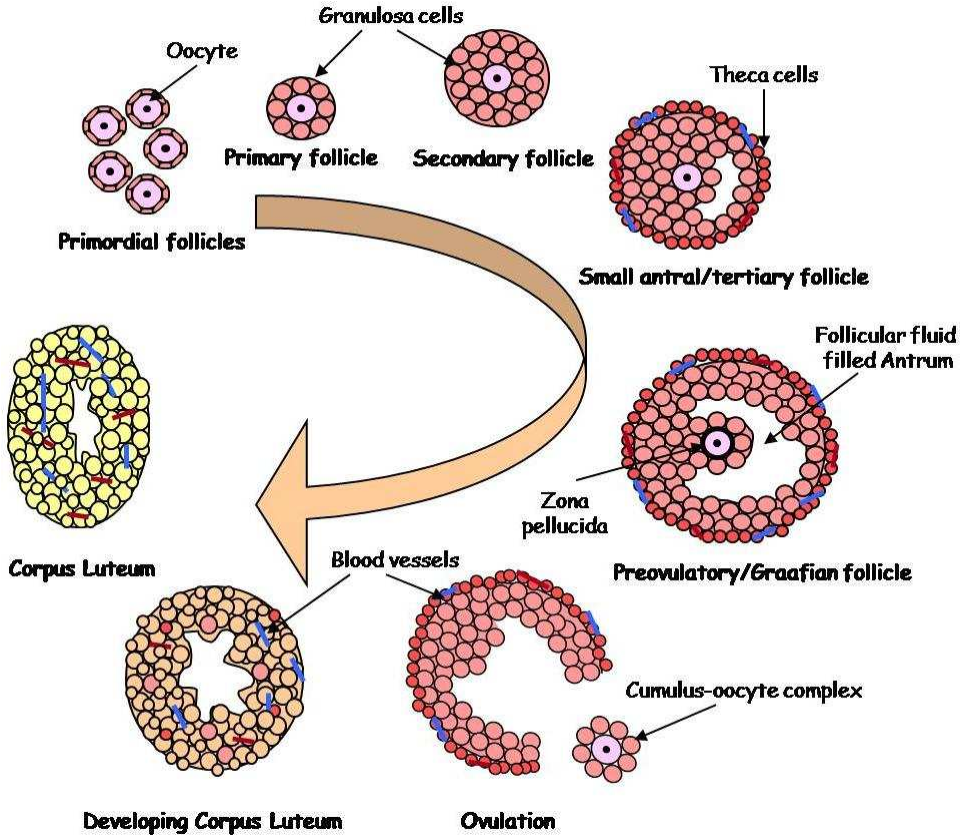

Figure 1.2 Schematic representation of the stages of follicle growth, ovulation and corpus luteum development.

Table 1.1. Comparison between cattle, sheep and human of reproductive cycles, follicular waves and ovulatory follicles. (Data adapted from Fortune, 1994; Adams, 1999; Hunter et al., 2004 and Johnson, 2007).

	Reproductive Cycles				**Follicular waves and ovulation**				
Species	Length of cycle (days)	Length of follicular phase (days)	Length of luteal phase (days)	Type of cycle	Number of waves	Length of folliculogenesis	Max follicle diameter (mm)		Ovulation rate
							non-ovulatory	ovulatory	
Cattle	20-21	2-3	18-19	Polyoestrous	2 or 3	4-6 months	10-15	12-20	1
Sheep	16-17	1-2	14-15	Seasonal polyoestrous	2-4	4-6 months	5-7	6-7	1-3
Human	24-32	10-14	12-15	Menstrual	2 or 3	11 months	10-20	23	1

1.2.4 Follicle waves

Ultrasound imaging has allowed the investigation of patterns of development of large antral follicles in domestic animals. These studies have shown that recruitment, selection and dominance of follicles occurs throughout the oestrous cycle (Lussier et al., 1987; Sirois and Fortune, 1988), but only the dominant follicle that is present at the time of luteal regression ovulates (Fortune, 2004). This is due to persistent high levels of progesterone synthesised by the corpus luteum which prevents the development of follicles past a certain stage (Figure 1.3). Typically two or three successive waves are observed during ruminant oestrous cycles, however in other species, such as primates, pigs and rats, the dominance of follicles is suppressed except during the follicular phase of the cycle (Fortune, 1994) (Table 1.1).

1.2.5 Ovulation and luteinisation

The LH surge is the physiological trigger that stimulates ovulation of preovulatory follicles, and the expulsion of the cumulus-oocyte-complex from the ruptured follicle (Richards et al., 2002). The transition of a follicle to one that can ovulate is a complex multi-step process involving a wide array of mechanisms (Richards, 1994) including; an increase in angiogenesis, cytokine signalling, synthesis of collagenase, inflammatory mediator interactions and extracellular matrix remodelling. A fine network of capillaries develops within the thecal layer of the preovulatory follicle (Yamada et al., 1994) which increases the supply of hormones and other controlling factors to the immediate area. Connective tissue lysis is dependent on proteolytic enzymes which are released and activated by FSH and LH. LH induces the production of prostaglandins by theca cells which, in turn, stimulate collagenase and proteoglycanase secretion by granulosa cells (Franchimont et al., 1988). Immediately following ovulation, luteinisation is a tissue remodelling process by which the follicle loses its defined concentric structure and granulosa and thecal cells differentiate, culminating in the irreversible transition into a corpus luteum (Richards et al., 1998). Granulosa cells begin differentiation into luteal cells within 7 hours of the LH surge and are reprogrammed to express luteal-specific pattern of genes (Richards, 1994).

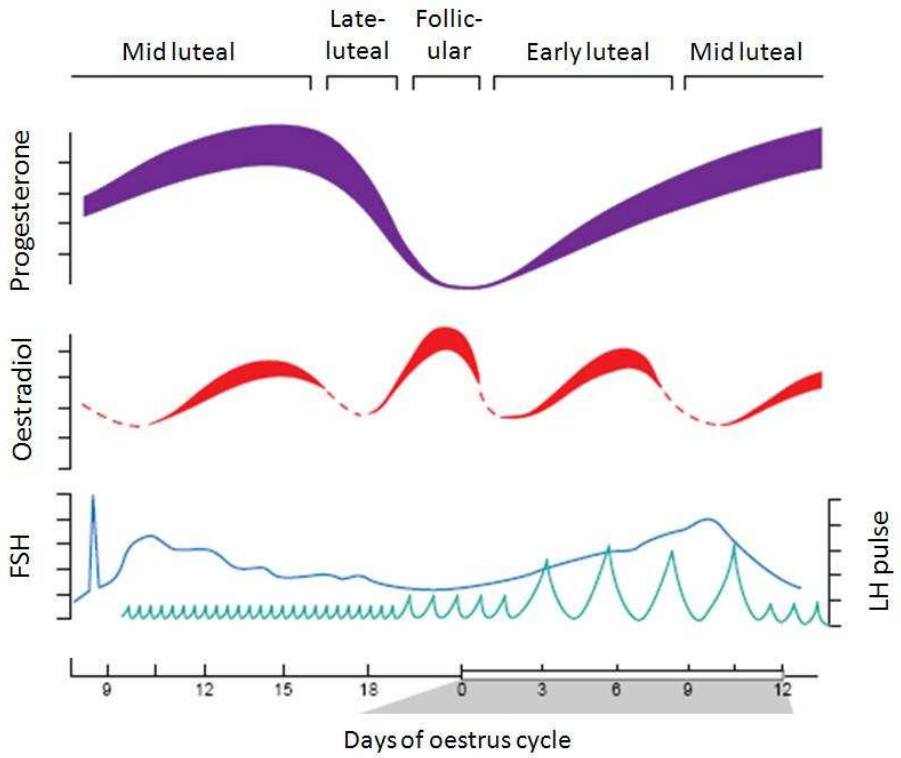

Figure 1.3 Phases of the bovine oestrous cycle and corresponding relative levels of progesterone, oestradiol, FSH and LH detected in serum. Adapted from Roche, 1996.

1.2.6 Oocyte maturation

Mammalian oocytes are formed during foetal life and are arrested at prophase of the first meiotic division until around the time of ovulation. Resumption of meiosis is brought about by the action of gonadotropic hormones which causes ovarian follicle cells to produce progesterone which acts directly on the oocyte to initiate oocyte maturation (Smith, 1989). This hormonal stimulation leads to germinal vesicle breakdown (GVBD) and chromosomal condensation, then progression through metaphase of the first meiosis, release of the first polar body then arrest at metaphase of the second meiosis (Wu et al., 1997). The induction of oocyte maturation is known to involve an initial action of agonists at the oocyte surface, which in turn leads to the activation of a cytoplasmic maturation or meiosis promoting factor (MPF) (Smith, 1989). MPF is a factor that is capable of inducing the M-phase of cell cycle without protein synthesis (Parrish et al., 1992). MPF induces chromosomal condensation, nuclear envelope breakdown and cytoplasmic reorganisation (Nurse, 1990; Parrish et al., 1992). GVBD is one of the events routinely used to score that

maturation is underway (Smith, 1989). GVBD is accompanied by expansion of the cumulus cell layer surrounding the oocyte, and in cattle occurs within hours of the ovulatory LH surge or after removal from the follicle (Sirard and First, 1988). Cumulus expansion is a more visible sign of oocyte maturation and is often been considered a reliable index to asses the quality of a mature oocyte and indirectly its developmental competence (Mattioli and Barboni, 2000). Cultured bovine oocytes will undergo spontaneous maturation 80% of the time, but gonadotropins are added to culture to ensure cytoplasmic maturation and cumulus expansion and to improve embryonic development (Sirard et al., 1988). Studies have shown that a greater proportion of in vivo matured oocytes reach the embryo stage compared to those produced in vitro (Leibfried-Rutledge et al., 1987), with the intrinsic quality of the oocyte being a key factor in this. It has been reported that oocytes derived from large follicles are more competent than those derived from small follicles (Pavlok et al., 1992, Lonergan et al., 1994; Blondin and Sirard, 1995). It seems that whilst still in the follicle the oocyte undergoes a series of significant changes that enable it to become developmentally competent, including a number of ultrastructural and molecular alterations (Rizos et al., 2002). As most oocytes collected for in vitro fertilization are from subordinate follicles, the majority of these will not develop to the blastocyst stage.

1.2.7 Fertilization and embryo development

At the point of ovulation, the oocyte is gathered by the fimbriated ositum of the oviduct and is transported down the oviduct by cilia. This transport can be severely affected if cumulus cells are lacking and/or if the oviductal cilia are malfunctioning (Johnson, 2007). When the spermatozoa encounter the oocyte in the first third of the oviduct (ampula) they undergo a series of reactions. They firstly bind to the zona pellucida by specific proteins overlying the acrosome. This binding then initiates the acrosome reaction (Breitbart et al., 2006), where the two membranes fuse and the acrosomal contents are dispersed into the oocyte (Senger, 2005). Following fertilisation, the zygote is transported towards the uterus during which time it undergoes a series of mitotic cleavage divisions. Each cleavage division doubles the number of cells (blastomeres) without increasing the total size of the zygote or cytoplasmic content, which is maintained by the fixed size of the zona pellucida. In

cattle and sheep, after 3-4 cleavage divisions, control of development is taken over by the expression of genes from the embryonic genome as maternally-derived transcripts become degraded (Hyttel et al., 2000). At around the 16 cell stage, the embryo undergoes compaction and becomes a morula, where the number of blastomeres can no longer be counted accurately. Gap junctions begin to form between the cells causing permeability changes. Fluid accumulates between the cells due to an increase in ions. A distinct cavity called the blastocoele is formed by which time the embryo is now called a blastocyst. Two distinct cell groups are formed; the inner cell mass (ICM) which gives rise to the body of the embryo, and the outer trophoblast which forms the extra embryonic membranes such as the amnion and placental tissues. Due to the increased pressure and growth, the zona pellucida splits and the blastocyst "hatches". The survival of the embryo is now dependant on luteal progesterone synthesis and the responsiveness of the uterus to progesterone (Senger, 2005).

1.3 Lipids

1.3.1 Structure and function

Lipids comprise a broad group of naturally-occurring molecules which includes fats, waxes, sterols, monoglycerides, triglycerides, phospholipids, glycolipids amongst others. They can be defined as 'fatty acids and their derivatives, and substances related biosynthetically or functionally to these compounds' (Christie, 2003). Lipids have many roles within the body including; constituents of cellular membranes, energy storage, regulation of gene transcription, metabolic pathways and cell signalling (Gurr et al., 2002, Hames and Hooper, 2004). There are four major groups of lipids found in the blood; phospholipids, triglycerides, free fatty acids and cholesterol (McDonald et al., 2002).

1.3.2 Phospholipids

Phospholipids consist of a hydrophobic tail of long fatty acid carbon chains, and a hydrophilic head containing a negatively charged phosphate group. The majority of phospholipids are synthesised in the liver, on the membrane of the endoplasmic

reticulum (ER) (Vance and Vance, 1996). The roles of phospholipids are primarily as constituents of biological membranes. Most phospholipids contain a diglyceride, a phosphate group, and a simple organic molecule such as choline. As part of cell membranes, phospholipids are a constant source of fatty acids for the synthesis of a variety of effector molecules such as the eicosanoids (Mattos et al., 2000).

1.3.3 Triglycerides

Triglycerides (or triacylglycerols) are a major fuel store and comprise of three fatty acid chains esterified to a glycerol backbone. The FA molecules can vary in length and saturation and can be removed from the glycerol backbone by the action of lipases. The free fatty acids (FFA) can then be utilised to produce energy by β-oxidation which is controlled mainly by the concentration of FFA in the blood which is in turn controlled by the hydrolysis rate of triglycerides in adipose tissue by hormone sensitive triacylglycerol lipase (Hames and Hooper, 2004).

1.3.4 Fatty acids

A fatty acid comprises of a hydrocarbon chain of varying length, a methyl group and a terminal carboxylic acid group. Most naturally occurring fatty acids have an even number of carbon atoms arranged in an unbranched chain. This chain can vary in length, but usually ranges between 14 to 24 carbon atoms, the most common FA containing 16 or 18 carbon atoms. FA differ in the number of carbon atoms and the number and position of unsaturated carbon-carbon double bonds. Saturated fatty acids contain no double bonds as all carbon atoms are saturated with hydrogen atoms. This gives the general formula of $CH_3(CH_2)_nCOOH$ where n is an even number. Monounsaturated fatty acids (MUFA) contain one carbon-carbon double bond and polyunsaturated fatty acids (PUFA) contain two or more. These double bonds have two configurations, cis and trans, however the vast majority of naturally occurring FA arise in the cis form. Trans FA occur as intermediates in the biohydrogenation of FA in the rumen and in some plant lipids (Christie, 2003). Some common naturally occurring fatty acids are shown in Table 1.2. PUFA are grouped depending on the position of the first carbon double bond from the methyl end or omega carbon atom, giving the nomenclature omega-3, -6 and -9 (or n-3, -6 or

-9). Linoleic acid (C18:2, n-6) and α-linolenic acid (C18:3, n-3) are classed as essential fatty acids (EFA) in mammals. These fatty acids cannot be synthesised by the body and must be included in the diet. This is due to a lack of the Δ^{12} and Δ^{15} desaturase enzymes and the inability to synthesise fatty acids with double bonds closer than carbon number 9 from the methyl terminus. EFA deficiency can lead to growth retardation, dermatosis, impaired reproduction and impairment of function in most organs throughout the body (Gurr et al., 2002).

1.3.5 Cholesterol

Cholesterol is a 27 carbon steroid molecule and has several important functions; a constituent of cell membranes, a precursor for the five major classes of steroid hormones (prostagens, oestrogens, androgens, glucocorticoids and mineralcorticoids), and a precursor of bile salts and vitamin D. It can be synthesised de novo, mainly in the liver or be obtained in the diet. Most cholesterol in the blood is in the form of cholesterol esters, formed by the addition of a fatty acid to the C3 hydroxyl group. This makes the cholesterol more hydrophobic enabling it to be stored and transported more easily (Lim and Roach, 2007). Cholesterol is transported around the body in lipoprotein particles and imported into cells utilising their corresponding receptors, although the preference for high- or low-density lipoproteins differs between species (Grummer and Carroll, 1988).

1.3.6 Lipoproteins

Lipoproteins are globular particles containing a hydrophobic core consisting of triacylglycerols and cholesterol esters, and a surrounding amphipathic coat of proteins (apolipoproteins), phospholipids and cholesterol. The apolipoproteins present in the structure help to solubilise the lipids in biological fluids. The ratio of lipids, proteins, size and specific proteins and lipids within the particles differ and can be classed into 5 types (see below). Differences in density and size allow lipoproteins to be isolated and characterized by sequential ultracentrifugation, electrophoresis or immunoelectrophoresis (Bauchart, 1993).

Table 1.2. Some naturally occurring and common fatty acids (Adapted from Gurr et al., 2002)

Molecular name	Common name	Systemic name	Occurrence
C16:0	Palmitic	Hexadecanoic	Most common saturated FA in animals and plants
C18:0	Stearic	Octadecanoic	Major component of animals and fungi
C18:1 n-9	Oleic	9-octadecadienoic	Most common MUFA in plants and animals
C18:2 n-6	Linoleic	9,12-octadecatrienoic	Major component of plant lipid. In mammals only derived from dietary plants and marine oils
C18:3 n-3	α-Linolenic	9,12,15-octadecatrienoic	Component of plant and seed oils
C20:4 n-6	Arachidonic	5,8,11,14-Eicosatetraenoic	Major component of animal phospholipid
C20:5 n-3	Eicosapentanoic	5,8,11,14,17-Eicosapentaenoic	Major component of marine animals and oils

1) High density lipoprotein (HDL). Highest in density (1.063-1.24g.ml) due to high protein-to-lipid ratio. HDL are the major lipoprotein in plasma (>80%) in ruminants. They are synthesized in the liver and remove excess cholesterol from cells and transport it to the liver (Bauchart, 1993).
2) Low density lipoprotein (LDL). End product of the degradation of (very low density lipoproteins) VLDL. Density 1.019-1.063g/ml. LDL represents <10% of total lipoproteins in plasma in ruminants but contains highest cholesterol ester content as a proportion of weight. They carry cholesterol from the liver to cells of the body. LDL contains only apoB-100 apolipoproteins which is recognised by LDL receptor allowing uptake of LDL into cells.
3) Intermediate density lipoproteins (IDL). Intermediate between VLDL and LDL and are generated during lipolysis (density 1.006-1.019g/ml). This is not generally detectable in blood.

4) Very low density lipoproteins (VLDL). The second largest and dense (<1.006g/ml) lipoprotein in ruminants. Synthesized in the liver, VLDL transports triglycerides, cholesterol and phospholipids to other tissues. The VLDL remnants remain in the blood first as IDL then as LDL where the cholesterol esters become esterified in the transformation.
5) Chylomicrons: Largest and least dense (<0.95g/ml) due to high lipid-to-protein ratio. They transport triglycerides from the intestines to skeletal muscle and adipose tissue and cholesterol to the liver.

Steroid-producing cells require cholesterol for the initial steps of the steroidogenic pathway and it seems that both LDL and HDL lipoproteins effectively support androgen production in these cells (Dyer and Curtiss, 1988; Magoffin and Erickson, 1988). The uptake of LDL-derived cholesterol involves specific binding to cell surface LDL receptors (LDLR) followed by internalization and lysosomal hydrolysis to release cholesterol esters (Brown and Goldstein, 1986). The HDL receptor SCARB1 (scavenger receptor, class B, type 1) preferentially uptakes cholesterol esters without the parallel uptake and degradation of the entire lipoprotein particle (Pittman et al., 1987). Both LDLR and SCARB1 are found to be localised in theca and luteinised granulosa cells (Li et al., 1998) and expression is reported to be increased in vitro with insulin (Li et al., 2001). Moreover, the addition of HDL and insulin to in vitro cultured theca-interstitial cells increases LH-stimulated androsterone production in a synergistic manner (Magoffin and Erickson, 1988), suggesting that lipoproteins are important physiological stimulators of ovarian androgen biosynthesis.

1.3.7 Albumin as a fatty acid carrier

The ability of serum albumin to bind fatty acids was discovered in the 1940s (Kendall, 1941). Since then extensive investigations have been carried out which have mapped the presence of seven albumin FA-binding domains which vary in affinity (Curry et al., 1998). Fatty acid binding affinity increases with increasing carbon chain length, however, FA binding diminishes when a chain length of 20 carbons is reached (Spector, 1975). Studies have shown that signalling events that have been mediated by unbound FFA have been abolished by the addition of FA-free

bovine serum albumin (BSA) to culture which reduced the unbound FFA without reducing total FFA concentration (Huber et al., 2006). Albumin is able to deliver FA to a wide range of cell and tissue types (van der Vusse, 2009), however its role in reproductive processes remains unknown.

1.4 Fatty acid metabolism and biosynthesis

Fatty acid biosynthesis consists of a series of reactions in which a fatty acid molecule is sequentially lengthened by the addition of two carbon units to give rise to 16C and 18C products. Once long-chain fatty acids have been synthesised various modifications can then take place, the most important being elongation and desaturation (Figure 1.4).

1.4.1 Elongation

In mammals, FA consisting up to 16 carbons in length (palmitate) are synthesised by fatty acid synthase (FAS). FAS functions as a homodimeric and multifunctional complex of around 250 kDa found in the cytoplasm (Jakobsson et al., 2006). FAS contains all the catalytic components required to direct a series of 37 sequential reactions leading to the formation of palmitic acid from acetyl and malonyl-CoA (Smith, 1994). However, most tissues contain longer-chain fatty acids in their membrane lipids. The formation of these very long-chain fatty acids (VLCFA) is catalysed by a group of enzymes termed elongases due to their ability to lengthen pre-formed fatty acids (Gurr et al., 2002). Elongases are present in the endoplasmic reticulum and their functions are principally the same as FAS, but each step is performed by individual proteins (Jakobsson et al., 2006). The enzymes which catalyse the elongation of very long chain fatty acids (ELOVL) are expressed throughout the body, with ELOVL1, 5 and 6 being expressed ubiquitously and ELOVL2, 3, 4 and 7 displaying tissue-specific level of expression, including the ovary (see below). ELOVL3, 6 and 7 have a preference for saturated and MUFA as substrates whereas ELOVL2, 4 and 5 are selective for PUFA (Guillou et al., 2010). Consequently the profile of ELOVL's present in cells will markedly impact on synthetic capacity and overall cellular function.

1.4.2 Desaturation

Desaturation is a reaction where a double bond is inserted between two carbon atoms of a fatty acid molecule. The enzymes responsible for these desaturation reactions are the Δ5, Δ6 and Δ9 desaturases, coded for by the FADS1, FADS2 and SCD genes, respectively. They are membrane-bound enzymes of the endoplasmic reticulum that require an electron transport chain for NADH which is catalyzed by the NADH cytochrome b5 reductase and cytochrome b5 (de Alaniz and Marra, 2003). SCD mediates a rate limiting reaction catalysing the conversion of saturated fats (preferably palmitic acid (16:0) and stearic acid (18:0)) to MUFA by inserting a cis double bond between the two carbon atoms at the Δ9 position.

Desaturases and elongases have been found to be expressed in cumulus cells in the rat (Moreau et al., 2006) and human (Feuerstein et al., 2007) and seem to play a role in follicular development and oocyte quality. Mouse studies have shown that mutant FADS2 -/- knockout mice have impaired gap junction networks between granulosa cells leading ultimately to sterility (Stoffel et al., 2008) with all tissues and fluids being devoid of PUFA. ELOVL5 -/- transgenic mice also display infertility in females (Moon et al., 2009), however the mechanism for this remains unknown. Although these studies suggest that ovarian function depends on the presence of both elongase and desaturase enzymes, there have been few studies characterising the profile of these factors and relating altered expression to ovarian function. The relative positions of the enzymes in the n-3 and n-6 metabolic pathways are illustrated in Figure 1.4.

1.4.3 Eicosanoids

The 20-carbon fatty acids arachidonic acid (AA) (20:4, n-6) and eicosapentanoic acid (EPA) (20:5, n-3) are precursors for eicosanoids (Figure 1.5), which include the prostaglandins, leukotrienes and thromboxanes. These factors are produced through the prostaglandin synthase and lipoxygenase pathways. Prostaglandin endoperoxidase synthase 2 (PTGS2, also known as cyclo-oxygenase-2 or COX-2) is not only responsible for the production of inflammatory eicosanoids from PUFAs, but the expression of PTGS2 can be directly altered by PUFAs in vivo

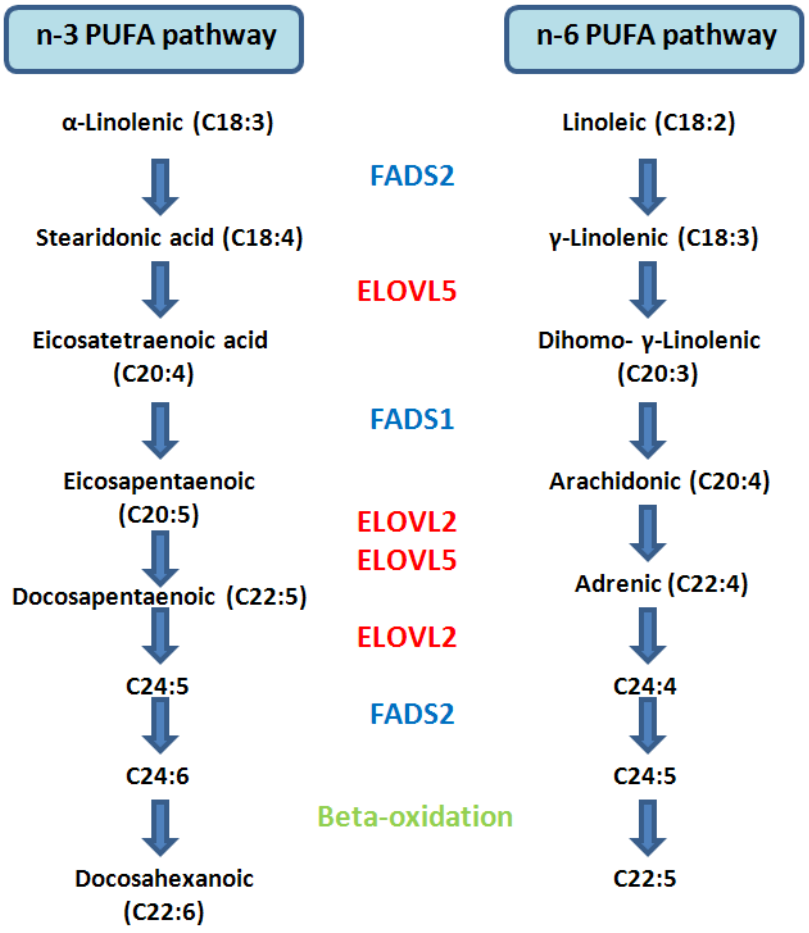

Figure 1.4 Summary of dietary PUFA modifications of the n-3 and n-6 families through desaturase (FADS) and elongase (ELOVL) reactions.

and in vitro. For example, it has been reported that n-3 PUFA down regulate, whereas n-6 PUFA up regulate the expression of the gene. AA (n-6) increased PTGS2 expression in cultured mouse bone marrow stromal cells (Shen et al., 2008) and EPA and docosahexanoic acid (DHA) (both n-3) caused a dose-dependent reduction in PTGS2 expression in cultured bovine chondrocytes (Curtis et al., 2000). Also, the fat-1 transgenic mouse which is able to convert n-6 to n-3 PUFA expresses approximately 25% reduction in PTGS2 transcript compared with the wild type mouse concomitantly with a 200% increase in tissue levels of n-3 PUFA (Boudrault et al., 2010). Eicosanoids derived from n-6 PUFAs are pro-inflammatory and those derived from n-3 PUFAs are anti-inflammatory and/or reduced pro-inflammatory. Due to AA being the major 20-carbon PUFA present in cells and their membranes, it tends to be the preferred substrate for eicosanoid synthesis. By changing the n-3:n-6

dietary ratio, the composition of the phospholipid membrane can change thus altering the proportion of available n-3 and n-6 PUFAs for eicosanoid production (Simopoulos, 2006).

$PGF_{2\alpha}$ is a key prostaglandin in reproduction. Produced by the uterus (also the ovary in the human), $PGF_{2\alpha}$ causes luteolysis and in domestic animals can be used pharmacologically to shorten the luteal phase if given during the mid or late stages. It has been reported that n-3 PUFAs can inhibit $PGF_{2\alpha}$ production in cultured bovine endometrial cells (Mattos et al., 2003), therefore n-3 PUFAs could inhibit luteolysis, thus impacting on subsequent follicular development. PGE_2 has been shown to be expressed in maturing oocytes (Neal et al., 1975) and developing follicles and seems to coordinate oocyte meiotic maturation and cumulus cell expansion (Takahashi et al., 2006).

The process of ovulation has been compared to a controlled inflammatory process (Espey, 1980), with marked changes seen in the ovarian vasculature and extracellular matrix after the LH surge; vasodilatation and increased vasculature, infiltration of immune cells, increased synthesis of inflammatory mediators, proteolytic activity leading to tissue damage and the subsequent healing. Some of these changes are thought to be attributable, at least partly, to mediation by eicosanoids. In a study by Matousek (2001), a leukotreine B_4 (LTB_4) receptor agonist inhibited ovulation through inhibition of matrix metalloproteinase (MMP)-2, which degrades collagen in basement membranes. A similar study using a lipoxygenase inhibitor also saw inhibition of ovulation which was reversed through administration of an exogenous LTB_4 (Mikuni et al., 1998). Prostaglandins are also essential for ovulation to take place. The preovulatory LH surge stimulates granulosa cells to express PTGS2 and phospholipase A2, the enzymes needed for prostaglandin synthesis. PGE_2 levels increase in follicles during the first several hours of ovulation and reach a peak at the time the follicles begin to rupture (Espey, 1994). Follicle rupture is prevented by PTGS2 inhibitors (Pall et al., 2001, Salhab et al., 2001), and can be restored by injection of PGE_2 (Duffy and Stouffer, 2002). Progesterone enhances the activity of the enzyme responsible for converting PGE_2 to $PGF_{2\alpha}$ (PGE2-9- ketoreductase). $PGF_{2\alpha}$ has been shown to increase the collagenolytic activity within follicles (Murdoch et al., 1986), as well as increasing the contraction of ovarian smooth

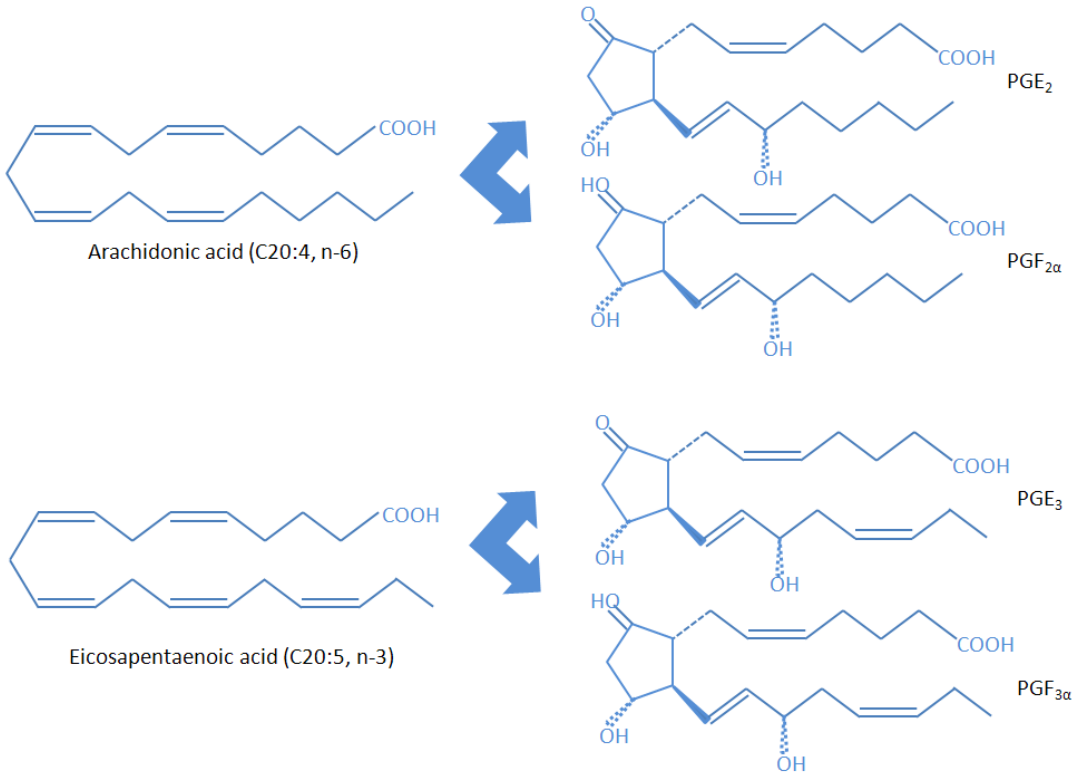

Figure 1.5 Structure of arachidonic acid (n-6) and eicosapentanoic acid (n-3) and the metabolites PGE_2, $PGF_{2\alpha}$, PGE_3 and $PGF_{3\alpha}$ synthesised through the PTGS2 pathway.

muscle (increasing follicular pressure) and stimulating the increase of lysosomal enzymes in the immediate area (Senger, 2003), showing it to be an integral factor of successful ovulation. Thromboxanes also increase in synthesis before ovulation takes place, but specific agonists to block TXB_2 do not prevent follicle rupture (Wilken et al., 1990), however detailed information about their role in the ovary is limited.

1.4.4 Steroidogenesis

In terms of reproduction, cholesterol is vital as it is a precursor for the synthesis of all steroid hormones (Figure 1.6). This process is initiated by the rate limiting transfer of cholesterol from the outer to the inner mitochondrial membrane by steroidogenic acute regulatory protein (STAR) (Stocco and Clark, 1996). Once in the mitochondria the synthesis of steroid hormones begins by the removal of a six carbon unit from the cholesterol side chain by cholesterol side chain cleavage enzyme (P450scc or CYP11A1) to form pregnenelone. Steroidogenesis in ovarian theca and granulosa

cells is regulated by the interaction of the gonadotrophins LH and FSH with their receptors. The STAR gene is expressed in theca cells under the influence of LH but not in granulosa cells until the preovulatory LH surge, where the expression increases and continues to be strongly expressed throughout luteinisation (Bao and Garverick, 1998). As granulosa cells do not synthesise the STAR protein, the androgens produced by the theca cells via LH stimulation diffuse into the FSH-exposed granulosa cells where they are aromatized into oestradiol. This mechanism of oestrogen production is termed the two-cell two-gonadotrophin model for steroidogenesis (Fortune and Quirk 1988) (Figure 1.7).

It is well documented that hormones such as LH induce AA release in steroidogenic cells and that this release is essential for steroid biosynthesis (Wang et al., 2000), as metabolic derivatives of AA regulate STAR gene expression (Wang et al., 2006). However little is known about the consequences on steroidogenesis of reducing the amount of AA availability through dietary modification. One group has investigated plasma progesterone and oestradiol concentrations in n-3 and n-6 PUFA fed dairy cows. Cows were fed either a high linolenic acid (LNA) (n-3) or high linoleic acid (LA) (n-6) diet and serum samples were taken throughout the oestrous cycle. They saw increased oestradiol concentrations in the n-3 group and an increase in progesterone concentration in the late luteal phase of the n-6 group (Robinson et al., 2002). However the underlying mechanisms or steroid regulation through dietary PUFA supplementation remain unclear. Another study investigated the role of HDL and LDL on steroidogenesis in cultured bovine theca cells (Bao et al., 1995). They found that HDL significantly increased progesterone synthesis at 96 hours. This study focussed on the effects of cholesterol and each lipoprotein treatment was equilibrated to deliver equal cholesterol levels. This shows that together with the type of FA present, the type of vehicle (i.e. HDL, LDL) in which cholesterol is delivered to these steroidogenic cells is important for optimal steroidogenesis.

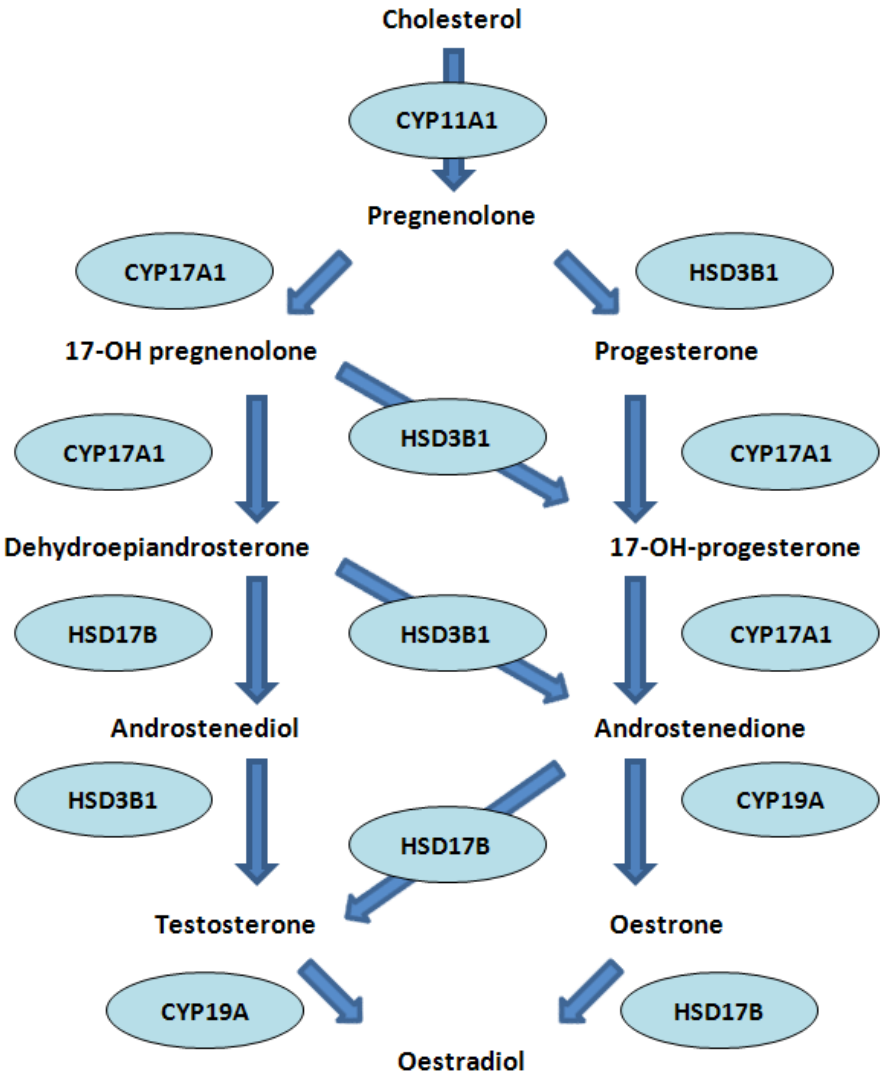

Figure 1.6 Steroidogenic pathways in the ovary. CYP11A1 = cholesterol side chain cleavage, CYP17A1 = 17α – hydroxylase, HSD3B1 = 3β - hydroxysteroid dehydrogenase, HSD17B = 17β - hydroxysteroid dehydrogenase, CYP19A = aromatase.

1.4.5 Metabolism of fatty acids in ruminants

Lipids in animal diets can affect productive efficiency through caloric and regulatory effects and any attempt to improve productive efficiency of ruminants by modifying dietary lipids must take into account ruminal metabolism of lipids (Jenkins, 1994). In non-ruminant species, the fatty acid composition of circulatory lipids are influenced

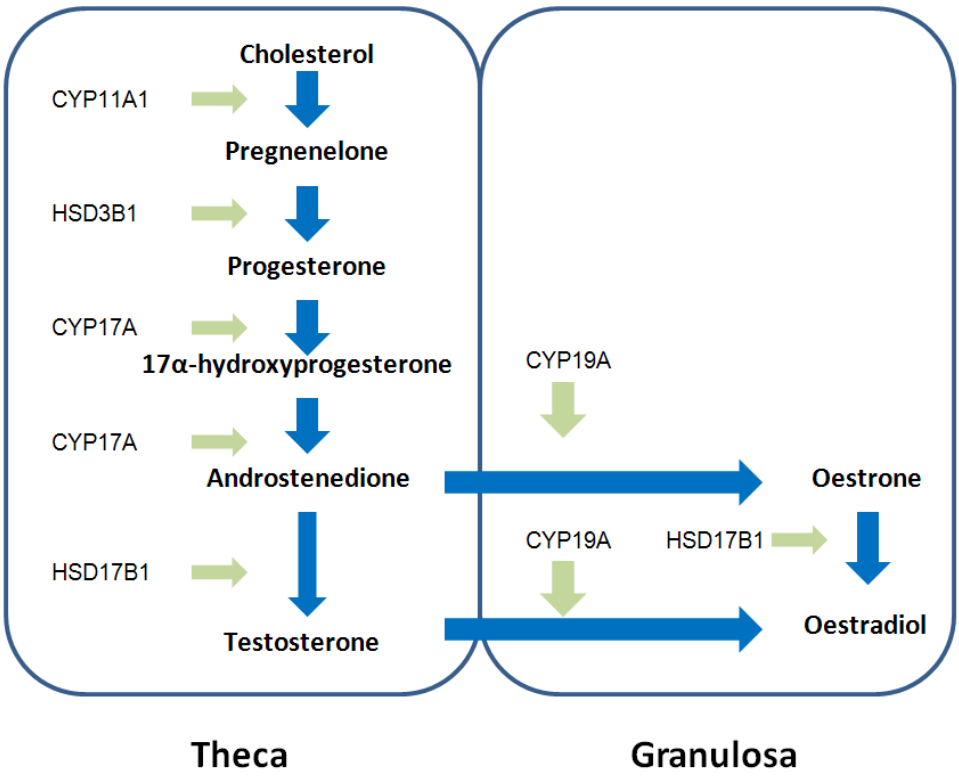

Figure 1.7 Schematic representation of the two-cell two-gonadotropin model for ovarian steroidogenesis (Adapted from Senthilkumaran et al., 2004).

by the fatty acid composition of the diet, but this is not true of ruminant animals. Unsaturated fatty acids, including LNA (n-3) and LA (n-6), are abundant in grass and certain other ruminant feedstuffs, yet are present at low concentrations in meat and milk. Ruminants have evolved a system of digestion that involves microbial fermentation of food prior to exposure to their own digestive enzymes. Bacteria number 10^9-10^{10} per ml of rumen contents, protozoa number 10^6 per ml and at least 12 species of anaerobic fungi have been identified (McDonald et al., 2002). As this microbial mass synthesises about 20% of nutrients absorbed by the host animal the composition of these microorganisms is important. Two major processes occur within the rumen which influences the composition and distribution of lipids and their subsequent metabolism within the intestine, these are hydrolysis and biohydrogenation.

1.4.6 Hydrolysis

Microbial lipases catalyse hydrolysis reactions whereby phospholipids, di- and triglycerides are broken down into glycerol and FFA molecules. Glycerol molecules are fermented rapidly, producing propionic acid as a major end product (Jenkins, 1992) and FA enter the small intestine as FFA. Hydrolysis is a prerequisite for biohydrogenation as it provides a free carboxyl group for isomerisation.

1.4.7 Biohydrogenation

After hydrolysis, unsaturated FFA are hydrogenated into more a saturated end point by ruminal microbes. The rates of hydrolysis and biohydrogenation are dependent on the type and amount of fat delivered to the rumen (Beam et al., 2000) and ruminal pH (Van Nevel and Demeyer, 1996). The initial step of biohydrogenation is an isomerisation reaction that converts cis-12 double bond to a trans-11 isomer followed by hydrogenation of the double bonds from an unsaturated to a saturated bond. The major substrates for biohydrogenation are linoleic and linolenic acids. They convert C18 unsaturated fatty acids to stearic acid (SA), via a number of intermediates. The main intermediates of linoleic acid biohydrogenation are cis-9, trans-11 conjugated linoleic acid (CLA) and vaccenic acid (VA). The main intermediates of a-linolenic acid biohydrogenation are cis-9, trans-11, cis-15 CLA, trans-11, cis-15 LA and VA (Nam and Garnsworthy, 2007). Despite the dramatic alteration of dietary fatty acids, ruminants do not appear to suffer from EFA deficiency. It seems the amount of unchanged EFA passing through the rumen (up to 4% of dietary intake) is sufficient for the needs of the animal (Gurr et al., 2002).

1.5 Metabolic hormones

1.5.1 Insulin

Insulin is a hormone synthesised by the β-cells of the pancreas and is central to regulating energy and glucose metabolism. Both in humans and in animal models, insulin receptors are widely distributed throughout all ovarian compartments, including granulosa, thecal, and stromal tissues (Poretsky et al., 1999). There is

evidence that insulin may support the growth and maturation of preovulatory follicles (Shimizu et al., 2008), with reported effects on steroid hormone production (Barbieri, 1994), steroidogenic enzyme synthesis (McGee et al., 1995; Nestler and Jakubowicz, 1996) and the regulation of LH receptors (Adashi et al., 1985). Negative energy balance and dietary restriction have been shown to reduce levels of insulin in the body (Sinclair et al., 2000). In vivo experiments in cattle have also shown an improvement in reproductive function with increased circulating levels of insulin (Armstrong et al, 2002; Gong, 2002). Any alterations in the synthesis and regulation of insulin have strong links with ovarian dysfunction observed in a number of human disorders such as diabetes mellitus, obesity and polycystic ovarian syndrome (PCOS) (Poretsky et al., 1999).

1.5.2 Insulin-like growth factors

The insulin like growth factor (IGF) family consist of two single chain polypeptide ligands (IGF-1 and -2), two cell surface receptor types (types I and II IGF-R), and at least six IGF-binding proteins (IGFBP), which appear to adapt the actions of the ligands. The presence of the components of the IGF family seems to be tissue and species specific but have been detected in the bovine and ovine ovary (Campbell et al., 1995; Spicer et al., 1993). Hastie and Haresign (2006) have identified, in great detail, mRNA expression for all components of the IGF system in the ovine ovary with respect to follicle size and health status, and a recent study has shown that FSH is a potent inducer of IGF-R expression in granulosa cells of bovine ovaries (Sudo et al., 2007). In all mammalian species IGF-1 stimulates granulosa cell proliferation and steroidogenesis (Monget et al., 2002) and the presence of certain IGFBP are characteristic of follicle growth and atresia (Monget et al., 1996).

1.5.3 Leptin

Leptin is the 16kDa protein product of the obese (ob) gene. It is highly conserved between species, is secreted predominantly by adipocytes and plays a key role in energy intake and expenditure by controlling appetite and metabolism. It has been demonstrated that PUFA intake influences adipose tissue expression of leptin, and of several lipogenic enzymes and transcription factors. In addition, leptin stimulates

triglyceride depletion in white adipose tissue without increasing free fatty acid release, thus favouring fatty acids as a fuel source (Hynes and Jones, 2001). In most domestic animals, reproductive function is dramatically influenced by nutrition. Leptin receptors have been found in the brain and hypothalamus, where it has a direct action on GnRH secretion, inhibiting or stimulating release in a dose-dependant manner (Spicer, 2001). Receptors for leptin have also been found in the ovary (Cioffi et al., 1996), and more specifically in ruminant granulosa (Spicer and Francisco, 1997) and theca cells (Spicer and Francisco, 1998). The role of leptin in reproduction was first highlighted in homozygous (ob/ob) genetically obese mice which lack endogenous leptin. These mice were sterile but reproductive function resumed when given exogenous leptin (Chehab et al., 1996). In the bovine ovary, leptin directly antagonizes insulin's stimulatory effect on granulosa cell steroidogenesis, and in the absence of insulin, had little effect on granulosa cells steroidogenesis (Spicer and Francisco, 1997; Spicer et al., 2000). Muñoz-Gutiérrez and colleagues (2005) indicated that the actions of leptin in ovarian follicles are mediated by the IGF system, and that reduced oestradiol production by leptin-treated granulosa cells is due to a decrease in bioavailablity of IGF-1. The leptin receptor has also been detected in oocytes and pre-implantation embryos, implying the role of leptin in oocyte development, maturation and cleavage of embryos (Brannian and Hansen, 2002; Herrid et al., 2006).

1.6 Polyunsaturated fatty acids and gene expression

It was previously thought that PUFAs regulated gene expression by altering the make-up of phospholipid membranes or through eicosanoid production. However the existence of nuclear receptors capable of binding fatty acids which therefore affected gene transcription was discovered by Gottlicher and colleagues in 1992. Since then, other transcription factors that utilise fatty acids to control gene expression have been discovered, and it seems that these factors are involved in a complex interplay to maintain tissue-specific biological functions throughout the body. Within the context of reproductive function very few studies have investigated the role of dietary PUFAs on gene expression (Childs et al., 2008; Coyne et al., 2008), but PUFAs are known to regulate a wide range of genes which are expressed within the ovary, oocyte and embryo. Indeed, the examination of gene expression is now

acknowledged as a valuable tool to assess the viability of cultured embryos (Childs et al., 2008), and could be used to a much greater extent to investigate the role of PUFA on the multitude of genes they can affect throughout reproductive tissues.

1.6.1 Peroxisome proliferator-activated receptors (PPAR)

PUFAs are one subset of a wide range of ligands and activators for PPARs, others include; hypolipidemic drugs (clofibrate, bezafibrate), thiazolinediones (ciglitazone, troglitazone), eicosanoids (PGJ_2, LTB_4), and steroids (DHEA) (Jump and Clarke, 1999; Moraes et al., 2006). PPARs were discovered in 1990 (Issemann and Green, 1990), and belong to a family of steroid nuclear receptors, which act as ligand activated transcription factors. When bound to its ligand, PPARs form heterodimers with retinoid X receptors (RXRs) which then in turn regulate transcription and gene expression. There are 3 isoforms in the PPAR family; PPARα PPARβ and PPARγ each being expressed at varying levels in different tissues, and each having specific physiological functions which are attributable to the specific genes they modulate (Desvergne and Wahli, 1999). PPARs contain two zinc-finger motifs in their DNA binding domain which allow them to bind to peroxisome proliferator response elements (PPREs) upstream from responsive genes (IJpenberg et al., 1997) (Figure 1.8).

PPARα is an important factor in regulating fatty acid metabolism and has been detected at high levels in liver, heart, kidney and brown adipose tissue (Escher and Wahli, 2000). PPARβ is ubiquitously expressed with higher amounts found in brain, adipose tissue and skin (Braissant et al., 1996). PPARγ is found in most tissues, with higher expression found in adipose tissue, skeletal muscle, liver, heart and colonic epithelium (Braissant et al., 1996). In the context of ovarian expression PPARα and β have been detected in ovarian stroma and theca cells, whereas PPARγ has been found to a much greater extent in granulosa cells of developing follicles (Komar et al. 2001; Froment et al. 2003). In addition, granulosa cell PPARγ expression increases with follicular development until the large antral stage and then rapidly declines after the luteinising hormone (LH) surge (Komar et al., 2001), but only in follicles that have responded to the LH surge (Komar and Curry, 2003). Some genes which are important for normal ovarian follicle development are directly regulated

Table 1.3. A selection of genes involved in ovarian function regulated by PUFA-sensitive transcription factors; peroxisome proliferator activated receptors (PPARs), sterol regulatory element binding protein (SREBPs) and nuclear factor kappa-B (NFκB).

Type of molecule/ Pathway	Gene	Transcription Factor Family	Reference
Inflammatory/	IL-1, 2, 6, 8, 12	NFκB, PPAR	McKay and Cidlowski 1999
Immune	TNF-α	NFκB, PPAR	Minge et al. 2006
	IFN-γ, -β	NFκB	McKay and Cidlowski 1999
	PTGS2	PPAR	Froment et al. 2006
	iNOS	NFκB	Wahle et al 2003
Lipid metabolism	FAS	SREBP	Xu et al 1999
	SCD	SREBP	Ntambi et al 2002
	Acyl CoA synthetase	PPAR	Schoonjans et al 1995
	Acyl CoA carboxylase	SREBP	Xu et al 1999
	FATP	PPAR	Frohnert et al 1999
	LDLR	SREBP	Sato 2009
	SREBP	PPAR	Xu et al 1999
	Elongases	SREBP	Moon et al 2001
	HMG CoA synthase	SREBP	Worgall et al 1998
	Leptin	SREBP	Gurr et al 2002
Steroidogenesis	CYP17A1	PPAR, SREBP	Froment et al 2006, Ozbay et al 2006
	CYP19	PPAR	Froment et al 2006
	HSD3B1	PPAR	Froment et al 2006
Others	Plasminogen activator	PPAR	Froment et al 2006
	VEGF	PPAR	Yamakawa et al 2000
	MMP3	PPAR	Froment et al 2006
	MMP9	PPAR	Yee et al 1997

by PPAR transcriptional activation (see Table 1.3). Consequently, restricting the ligands available to these nuclear receptors could potentially alter the expression of these genes. PPARγ is known to inhibit the expression of aromatase by disrupting the interaction of the transcription factor NFκB with the aromatase promoter (Fan et al., 2005). In one ovine in vitro study PPARγ was reported to significantly inhibit granulosa cell proliferation and to stimulate follicular differentiation (Froment et al.,

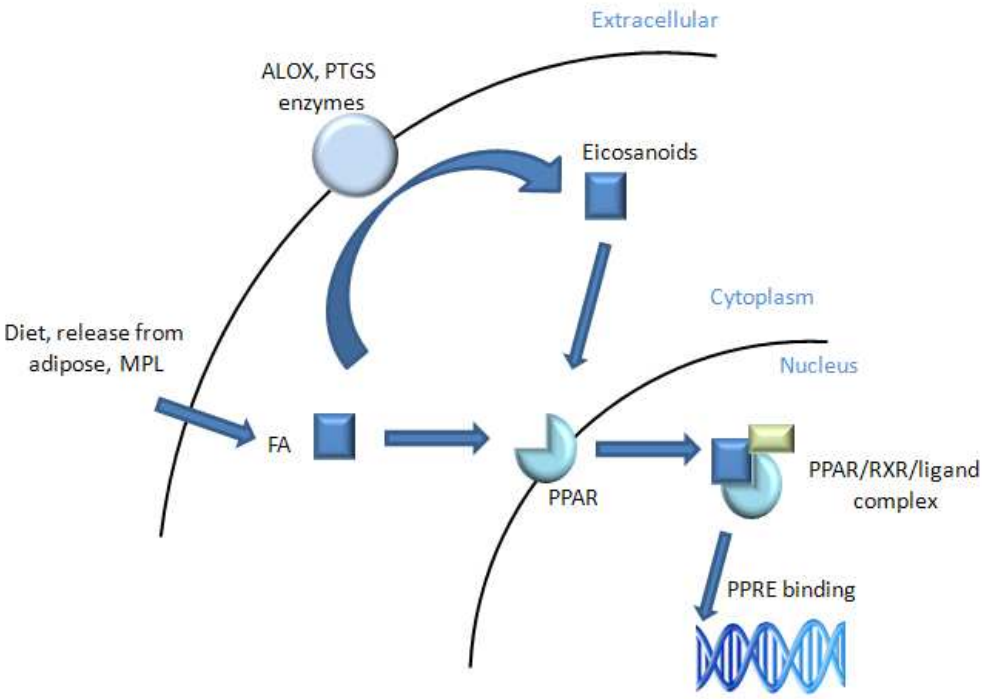

Figure 1.8 General overview of the transcriptional activation of PPAR. FA and their metabolites (e.g. eicosanoids) act as ligands for the PPARs. Once bound to a ligand, PPARs translocate to the nucleus where they form a heterodimer with RXR. This active complex binds to the DNA PPRE promoter region to activate gene transcription. MPL = membrane phospholipids; FA = fatty acids; ALOX = lipoxygenase; PTGS = prostaglandin synthase; RXR = retinoid X receptor; PPRE = peroxisome proliferator response element.

2003). In mice PPARγ and PPARα have been found to be expressed in ovarian macrophages at levels dramatically higher than macrophages present in non-reproductive tissues. Treatment with troglitazone (a potent activator of PPARγ) reduces the activation of nitric oxide synthase-2 (NOS2) (an important proinflammatory enzyme that regulates ovulation) in macrophages present only in the preovulatory follicle, indicating that PPARs are hormonally regulated. In addition, a transient suppression of PPARγ expression occurs which coincides with the structural reorganization of ovulation, requiring the production of inflammatory cytokines (Minge et al., 2006).

1.6.2 Sterol regulatory-element binding proteins (SREBP)

SREBPs are a group of transcription factors which bind to the sterol regulatory element (SRE) DNA binding domain. Three isoforms have been identified; SREBP1a and 1c, which are encoded by the same gene but differ at the N-terminus, and SREBP2 (Sampath and Ntambi, 2004). SREBPs are endoplasmic reticulum membrane-bound transcription factors which, when activated, undergo proteolytic cleavage. This allows them to move into the nucleus where they bind to SREs, activating transcription of genes involved in cholesterol, triglyceride and fatty acid synthesis (Brown and Goldstein, 1997) (Table 1.3) (Figure 1.9). It has been shown that a diet supplemented with PUFAs showed decreased levels of SREBP within the nucleus of up to 85% and lower levels of SRE-regulated genes (Xu et al., 1999). This mechanism still remains unclear but PUFAs seems to regulate SREBP expression by interfering with the post-transcriptional regulation of the gene and by blocking proteolytic modifications of the membrane bound protein (Hannah et al., 2001; Xu et al., 2001). Experiments have also shown that SREBPs activate transcription of low density lipoprotein (LDL) receptor (LDLR), high density lipoprotein (HDL) scavenger receptor (SCARB1) and 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) synthase genes (Hua et al., 1993; Yokoyama et al., 1993; Lopez and McLean, 1999), all of which are important in cholesterol metabolism and steroidogenesis in the ovary. Also, it seems that gonadotropic hormones enhance SREBP-dependent transcriptional activity of STAR, SCARB1, and LDLR promoters (Sekar and Veldhuis, 2001; Shea-Eaton et al., 2001).

1.6.3 Nuclear factor- kappaB (NFκB)

NFκB is made from two subunits (p50 and p65) which bind to an inhibitory subunit (I-κB) to form an inactive heterodimer in the cytoplasm of cells. The degredation of I-κB requires phosphorylation, allowing NFκB to separate from I-κB, translocate to the nucleus and regulate the expression of a variety of genes (Figure 1.10). NFκB is a

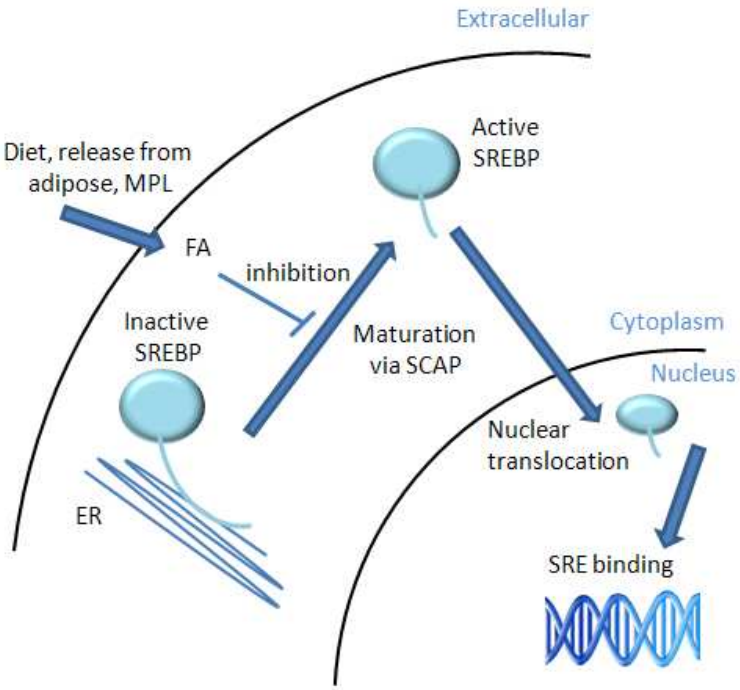

Figure 1.9 General overview of the transcriptional activation of SREBP and FA inhibition. SREBPs require activation by a cleavage step catalysed by SCAP. Once cleaved SREBP can translocate to the nucleus and bind to the DNA SRE promoter region to active gene transcription. FAs can inhibit the SCAP activation step. SREBP = sterol regulatory element binding protein; MPL = membrane phospholipids; FA = fatty acids; ER = endoplasmic reticulum; SRE = sterol regulatory element; SCAP = SREBP-cleavage activating protein.

major component of the stress cascade and is important in the regulation of inflammatory cytokines/chemokines, cyclooxygenases, nitric oxide synthases, growth factors and adhesion molecules (McKay and Cidlowski, 1999, Wahle et al., 2003) (Table1.3). PUFAs have been shown to regulate NFκB translocation both negatively and positively. Camandola et al (1996) have shown that AA (20:4,n-6) induces NFκB translocation possibly by indirect action through AA metabolites such as PGE_2 which increases the availability of the p65 subunit. However, this effect was not observed with EPA (20:5n-3). Several groups have shown that EPA prevents NFκB activation by decreasing phophorylation of I-κB in cultured cells (Lo et al., 1999; Zhao et al., 2004). This is supported by a study showing that transgenic mice that endogenously biosynthesise n-3 PUFA from n-6 PUFA are protected from colitis

through a decrease in NFκB activity (Hudert et al., 2006). NFκB is expressed by many cell types and is known to be endogenously expressed by granulosa cells (Fan et al., 2005) as well as the many immune cells that are present in the ovary (Brännström and Norman, 1993; Bukovsky, 2006;). NFκB plays a key role in ovulation as it regulates the expression of many inflammatory genes required for this process. However, the role of NFκB in ovarian function is complicated by interactions with steroid hormones and their receptors. It has been established that the progesterone and oestrogen receptor can alter the activity of NFκB, which could provide an explanation for the inflammatory modulatory role of steroids. For example, the increase in immunoresponsiveness prior to ovulation is linked with the increase in oestradiol, while after ovulation this responsiveness decreases along with rising progesterone levels (van der Burg and van der Saag, 1996). It seems that not only do progestins suppress NFκB activity, but NFκB can repress the activity of the progesterone receptor, therefore a balance is required for regulating successful ovulation (van der Berg and van der Saag, 1996). As previously mentioned, PPARγ can inhibit aromatase synthesis indirectly by suppression of NFκB, possibly through the physical interaction of PPARγ with the p65 subunit of NFκB. This same mechanism, it seems, has an important role in the inhibition of inflammatory cytokines leading up to ovulation. Once levels of PPARγ begin to drop in the periovulatory period, NFκB expression increases along with the regulation of inflammatory mediators required for successful ovulation (Fan et al., 2005).

In the ovary PPARs, SREBPs and NFκB play important roles in the regulation of gene expression. Through various mechanisms and pathways these transcription factors are vital in all aspects of ovarian function, from the production of cytokines, and tissue remodelling factors to enzymes involved in steroidogenesis and eicosanoid production. The n-3:n-6 ratio content in cells strongly influences numerous cellular processes by affecting gene expression through many pathways. PUFA interaction can lead to a change in cellular function depending on the transcription factor, gene and the type of PUFA in question. Due to the important role PUFAs have in the activation or suppression of these genes, they have been termed as being “master switches” in the regulation of these factors (Deckelbaum et al., 2006).

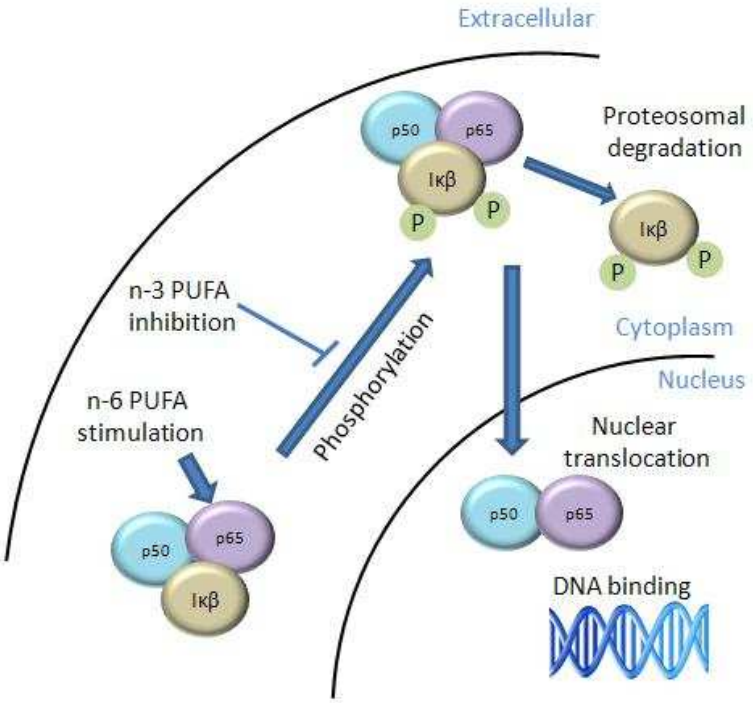

Figure 1.10 General overview of the transcriptional activation of NFκB and PUFA interaction. To activate NFκB, IκB needs to be phosphorylated. This allows the active NFκB to translocate to the nucleus which then activates gene transcription. n-6 PUFA and metabolites can increase availability of p65 subunit whereas n-3 PUFA can inhibit activation by inhibiting the phosphorylation of IκB. p50/p65 = NFκB subunits; IκB = NFκB inhibitory subunit; P = phosphorylation.

1.7 Development of working hypotheses

There have been a number of studies which have investigated the effects of FA on reproductive parameters in ruminants. However, few have identified mechanistic explanations for any changes observed in these studies. FAs are able to act directly on the follicle and those effects can be altered depending on the type of FAs available to the tissue. Few studies have looked at FA composition of ovarian cells and oocytes and to date results have been inconsistent. Omega-3 and -6 PUFAs are important biologically active FA involved in a range of reproductive processes. Altering the ratio of available PUFA can affect these processes and this thesis attempts to further investigate the mechanistic role of these FA in greater detail at

two different reproductive time points; steroidogenic cells of the pre-ovulatory ovarian follicle and the pre-implantation embryo.

An in vivo experiment was designed which consisted of ewes fed one of two experimental diets; high n-3 or high n-6 PUFA. The experimental diets included bioactive fish oils as part of the n-3 PUFA source. Fish oils are rich in long chain PUFA such as EPA and DHA and were included to maximise any differential effects between dietary treatments and to assess the ability of the ovarian cell types to uptake these long chain pre-formed PUFA. Previous work in our laboratory highlighted an important observation from this study; ewes offered the n-3 enriched diet had increased levels of progesterone in the follicular fluid (Wonnacott et al., 2010). This study failed to detect any alteration in steroid output in aspirated granulosa cells cultured with HDL from the sera of ewes fed n-3 or n-6 enriched diets. This study also sought to asses the mechanisms of FA uptake in the pre-implantation embryo by culturing fractionated n-3 or n-6 HDL with IVM and IVF zygotes. Despite alterations in some transcripts between treatments there was no net uptake of FA.

Very few studies have investigated the effect of PUFA on gene expression in the ovary. Those that have, focussed on genes involved with eicosanoid production and steroid hormone synthesis. In order to identify other genes which may be differentially altered by n-3 vs n-6 PUFA, Chapter 2 focuses on an ovine gonad-targeted cDNA macroarray. RNA from the granulosa cells of ewes fed either the n-3 or n-6 experimental diet would be used in an attempt to identify changes in gene expression between the experimental diets. Chapter 3 sought to assess the effects of culturing primary granulosa and theca cells with LDL and HDL fractionated from the sera of ewes fed n-3 or n-6 enriched diets from chapter 2. LDL and HDL are the two most abundant lipoproteins in plasma and follicular fluid and were incorporated into culture media at physiological concentrations. The current thesis extended previous work by (i) including LDL as a potential source of PUFA delivery and also by investigating effects in (ii) theca cells and (iii) a different sub-population of granulosa cells (mural as opposed to aspirated antral cells). These particular experiments were designed to test the hypothesis that PUFA, delivered by lipoproteins, are able to differentially alter steroid hormone synthesis in theca cells.

These experiments allowed a more detailed investigation into the uptake and metabolism of PUFA together with other FA and cholesterol, and the subsequent effects on gene expression, FA composition and steroid hormone production. Chapter 4 extended this investigation further by assessing the effects of culturing sheep zygotes in the presence of LDL and albumin fractionated from the sera of ewes fed n-3 or n-6 enriched diets from Chapter 2. This experiment tested the hypothesis that albumin is the main FA transporter delivering PUFA to developing pre-implantation embryos. These experiments facilitated a detailed investigation into the consequences for the pre-elongation embryo in terms of developmental potential, FA uptake and the expression of genes involved in FA uptake and metabolism.

In summary, this thesis aims to address the effect of dietary n-3 and n-6 PUFA on the ruminant ovary, particularly granulosa cells and theca cells, together with the pre-implantation embryo. The main objective of this project is to build upon previous work in our laboratory and to further investigate the role of n-3 and n-6 PUFA on reproductive parameters in the ovine ovary. Studying how these PUFAs are taken up into these reproductive tissues, how they differentially alter gene expression and lipid metabolism, as well as activating down-stream signalling pathways, will allow us to explore and dissect the possible mechanisms of their action.

CHAPTER 2

Validation of a cDNA macroarray for use in the detection of dietary PUFA induced alterations to gene expression.

2.1 Introduction

Omega-3 and -6 PUFAs are vital for a multitude of cellular mechanisms, all of which are involved in maintaining ovarian function. The PUFA regulation of gene expression has been widely reviewed (Jump et al., 1996; Wahle et al., 2003; Sampath and Ntambi, 2005; Schmitz and Ecker, 2008). PUFA-mediated gene expression can occur by two different mechanisms; firstly by the direct activation or inhibition of PUFA-sensitive transcription factors (e.g. PPARs, SREBPs and NFκB), or by the indirect action of altering the availability of specific metabolites (e.g. eicosanoids) which in turn regulate the expression of genes by a series of complex signalling pathways. In terms of the ovary, these mechanisms could all potentially play a role in maintaining correct follicle development, oocyte maturation, steroidogenesis and embryo development The situation is complicated by the sometimes contrasting effects of n-3 vs n-6 PUFA and again by the levels of PUFA and ratio of n-3:n-6 which is available to the animal and tissues in vivo.

The aim of this chapter was to investigate the effect of dietary n-3 vs n-6 PUFAs on gene expression in the ovine ovary, specifically granulosa cells. Firstly, an in vivo feeding study was conducted, with two dietary regimes created to offer an n-3 or n-6 enriched diet to non-lactating ewes. Ovaries and cells were harvested for use in gene expression analysis. As PUFAs are involved in many cellular functions, the list of molecular targets which potentially could be investigated was extensive. Consequently, an ovine gonad-specific cDNA macro-array was used to utilise minimal sample quantities for maximal expression analysis. The array used for this study was received from Dr Olivier Sandra, INRA, Paris, France, and allowed a more focussed and targeted approach to the experiment than might be afforded by commercially available micro-arrays. The macroarray in question contained 88 target genes, 4 housekeeping or positive control genes and 2 vector-only or negative

controls. The target genes in the array covered a wide range of functions including steroidogenic enzymes, cytokines and inflammatory enzymes, tissue remodelling proteins, signalling molecules, DNA repair and growth factors. The majority of these genes are involved in follicle development, oocyte maturation and ovulation. For example; steroidogenic genes such as aromatase (CYP19A1), steroidogenic acute regulatory protein (STAR), 3-beta-hydroxysteroid dehydrogenase (HSD3B1), oestrogen receptors (ESR1 and ESR2) and the progesterone receptor (PGR) are vital components of follicular function and altered expression in the genes can ultimately lead to reproductive dysfunction (Britt et al., 2001; Manna and Stocco, 2005; Szoke et al., 2009; Drummond and Fuller, 2010). Cytokines and inflammatory mediators (such as the interleukins (IL), interferons (IFN) and tumour necrosis factors (TNF)) are a vital part of reproductive function and have pivotal roles in ovulation and CL regression (Vinatier et al., 1995; Bukulmez and Arici, 2000). Tissue remodelling proteins such as the matrix metalloproteinases (MMP) and the tissue inhibitors of metalloproteinases (TIMP) carefully regulate the breakdown of the extracellular matrix in ovulatory follicles to ensure successful ovulation (Goldman and Shalev, 2004). Signalling molecules such as the gap junction (GJ) proteins are vital for maintaining correct cross-talk between the cell types for successful follicle and oocyte development (Gershon et al., 2008).

During the analysis of data from the in vivo feeding experiment described above, it became apparent that data generated from the array did not correlate with qRT-PCR data generated against specific genes also found on the array. Consequently an investigative experiment was designed to dissect out the nature of this inconsistency. This was achieved through the design of an in vitro study culturing ovine granulosa cells in conditions known to alter gene expression. This would allow for the production of large volumes of treated samples under defined culture conditions, which would allow a thorough investigation of all aspects of the methodology and allow us to ascertain where the problem lay.

2.2 Ovine in vivo study– Experiment 1

2.2.1 Materials and methods

2.2.1.1 Animals and experimental diets

Thirty six mature Blackface ewes were randomly assigned to one of two dietary treatment groups, 18 animals per group. The diets contained oils rich in either n-3 or n-6 PUFA. Dietary formulations are presented in Table 2.1. Live weight was recorded every two weeks throughout the experimental period and on the day of slaughter. Ewes were introduced to diets over a period of 12 days with the amount of diet increasing every 2 days until all animals received 1 kg/day (2 x 500g per day). Hay was offered at 100g/day with the second daily feed to provide a source of roughage overnight. Ewes were offered 200g of one of the diets in the first week, increasing to 400g and 800g in the second week, and reaching the final amount of 1kg in the third week. This amount of feed was maintained for a further 4 weeks. Blood samples were taken weekly throughout the study and on the day of slaughter. Weight and body condition score was measured fortnightly throughout the study.

2.2.1.2 Oestrous synchronization

Oestrous was synchronized with an intravaginal sponge impregnated with the synthetic progesterone analogue, Chronogest CR (30 mg fluorogestone acetate) (Intervet, Buckinghamshire, UK) inserted for 12 days with the sponges renewed on day 7. Ewes were given an intramuscular injection of the synthetic prostaglandin Estrumate®, 1ml per ewe at a concentration of 250µg/ml (Schering-Plough Animal Health Ltd, Middlesex, UK) at sponge insertion to induce luteolysis of any persistent corpora lutea. All ewes were treated with 8 equal doses of FSH (Ovagen, ICP Bio Ltd, Aukland, New Zealand), one every 12 hours, over 4 days starting on day 10 after the initial sponge insertion, to stimulate superovulation. Animals were slaughtered by captive bolt and exsanguination on average 26 hours after removal of second sponge and 17 hours after the last FSH injection.

Table 2.1. Ingredients and chemical analysis of experimental n-3 and n-6 diets.

	n-3	**n-6**
Ingredient (g/kg)		
Nutritionally improved straw	250	250
Oat feed	190	190
Barley	225	225
Molassed Beet Pulp	100	100
Hipro Soya	150	150
Linseed Oil	**10**	**-**
Sunflower Oil	**-**	**45**
Fish Oil	**35**	**-**
Molasses	20	20
Minerals and Vitamins†	20	20
Chemical analysis††		
Dry matter, g/kg	889	896
Crude protein, g/kg DM	134	129
NDF, g/kg DM	371	383
Acid hydrolysed, g/kg DM	64.1	57.3
Starch, g/kg DM	172	161
ME, MJ/kg DM	10.7	10.2
NCGD %DM	65.2	62.7

†Minerals and vitamins: Calcium 170g/kg, Phosphorous 60g/kg, Sodium 70g/kg, Magnesium 100g/kg, Cobalt 150mg/kg, Manganese 6g/kg, vitamin A 500,000 IU/kg, Vitamin D3 100,000 IU/kg, Vitamin E 1,200 IU/Kg. ††DM, dry matter; NDF, neutral detergent fibre; ME, metabolisable energy; NCGD, Neutral Cellulase Gammanase Digestibility.

2.2.1.3 Tissue collection and storage

Tissues were recovered on day 14 after the initial chronogest treatment before ovulation took place to ensure maximal tissue recovery. Each ovary was examined and information such as number and size of follicles recorded. Ovaries were collected and follicular fluid along with granulosa cells and oocytes were aspirated from medium to large follicles and separated in the lab. Oocytes were denuded of

cumulus cells, washed in PBS containing 0.1% PVA and then snap frozen in liquid nitrogen in a minimal volume (typically <2µl). Follicular fluid was snap frozen and stored at -80^{o}C. Granulosa cells were washed 3 times in PBS/0.1% PVA. Red blood cells were removed via lysis and snap frozen in minimal volume. Some ovarian tissues containing follicles of various sizes were fixed in Bouins solution for 6 hours and transferred to 70% ethanol for storage at room temperature. A sample of liver was taken as control tissue which was snap frozen in liquid nitrogen and stored at -80^{o}C. Blood samples were collected into heparinised vacuum tubes. Blood was then centrifuged at 575g for 15 minutes and plasma stored at -20°C. For serum samples, blood was left to stand at room temperature in plain vacuum tubes for a minimum of 3 hours. Samples were then centrifuged at 575g for 15 minutes. Serum was transferred into 30ml universals and stored at -20°C.

2.2.1.4 RNA extraction

From the initial 36 animals granulosa cells from 15 were used in a parallel study to measure the fatty acid composition by gas chromatography (Wonnacott et al., 2010), leaving 21 samples for RNA extraction. RNA was extracted from harvested granulosa cells using the RNeasy Mini Kit (Qiagen Ltd., West Sussex, UK). In brief, samples were lysed in 350µl lysis buffer (RLT, supplied with kit) containing β-mercaptoethanol (10µl βME per 1ml RLT buffer). The lysate was passed through a 19 gauge needle several times then added directly into a QIAshredder spin column (Qiagen Ltd., West Sussex, UK) for compete disruption of material. The lysate was then passed through an RNeasy spin column, where total RNA binds to column. After several wash steps with RW1 and RPE wash buffers, RNA was eluted from the column in 30µl RNase-free water. Eluted samples were treated with RQ1 RNase-free DNase (Promega, Southampton, UK). The mix was incubated at 37°C for 30 minutes, then 1µl of DNase stop solution was added to terminate the reaction. The mix was incubated at 65°C for 10 minutes to inactivate the enzyme. Samples were quantified by Nanodrop spectrophotometer (ND1000, Nanodrop, Wilmington, USA) and RNA was stored at -80°C.

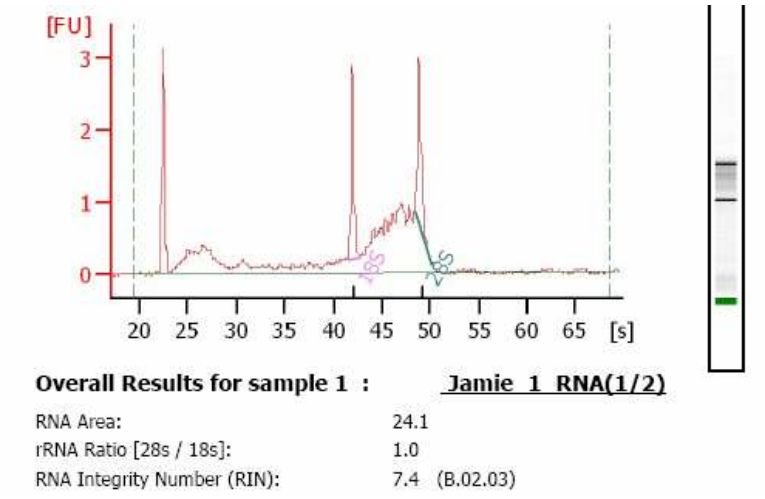

Figure 2.1. An example of an Agilent bioanalyser electrophoresis trace showing RNA quality (RIN).

2.2.1.5 RNA quality testing

For the purpose of macroarray analysis, RNA was required to be of a certain quality and concentration. Those samples which were deemed to have a high enough concentration (>100ng/µl) were analysed for quality and degradation using the Agilent 2100 Bioanalyser system (Agilent Technologies UK Ltd, Cheshire, UK). The quality of RNA is determined through the RNA Integrity Number (RIN), which is a measure of any degradation products present in the electrophoresis trace of the sample (Figure 2.1). For macroarray analysis a RIN of around 7 and above is required (O Sandra, INRA, Paris, personal communication). Of the 21 samples available, only 13 were suitable for array analysis due to limited RNA concentration and quality (n-3, n=8; n-6, n=5).

2.2.1.6 Sample preparation – cDNA synthesis and amplification

Extracted RNA was reverse transcribed into cDNA using the SMART PCR cDNA synthesis kit (Clontech Europe, Saint Germain-en-Laye, France) following manufacturers guidelines. 300ng total RNA in a maximum volume of 3µl was reverse transcribed to single stranded cDNA. RNA (made up to 3µl with deionised

PCR-grade water), 1µl 3' SMART CDS Primer IIA (12µM), 1µl SMART IIA oligonucleotide (12µM) were mixed and incubated at 70°C for 2 minutes. After denaturation, a reaction mix containing 2µl 5X first strand buffer, 1µl DTT (20mM), 1µl dNTP mix (10mM each) and 1µl of superscript II reverse transcriptase (200U/µl) (Invitrogen Ltd., Paisley, UK), was added to the cDNA samples and incubated at 42°C for 1 hour. 40µl Tris EDTA buffer was added to each of the samples and incubated at 72°C for 7 minutes to terminate the reaction. The RT reaction adds a short chain of deoxycytidines to the 3' end of the DNA sequence allowing an oligo containing an extended deoxyguanine sequence to bind to that end extending the template to include a new primer sequence. Amplification of the cDNA was carried out using the Advantage 2 PCR kit (Clontech Europe, Saint Germain-en-Laye, France) following the manufacturers guidelines. 4µl of cDNA mix was used for the PCR amplification step. The PCR reaction mix contained 10µl 10X Advantage 2 PCR buffer, 2µl 50X dNTP (10mM each), 2µl 5' PCR Primer IIA (12µM), 2µl 50X Advantage 2 Polymerase mix. This mix was added to 4ul cDNA and made up to 100µl with deionised PCR-grade water. The samples were then PCR amplified for 17 cycles (see section 2.2.1.7 for optimization explanation) each consisting of 15 seconds denaturation at 95°C, 30 seconds amplification at 65°C and 6 minutes elongation at 68°C. After PCR amplification was completed, 2µl of EDTA was added to terminate the reaction. The RT reaction CDS primer along with the SMART IIA oligo creates a priming site at both the 3' and 5' ends of the molecule. This primer sequence is targeted during the PCR stage allowing end to end cDNA amplification. PCR products were purified using Qiaquick spin column (Qiagen Ltd., West Sussex, UK). The PCR product was mixed with 500µl PB buffer, added to the Qiaquick spin column and centrifuged. Column was washed with 750µl PE buffer and the DNA sample was eluted in 30µl EB buffer.

2.2.1.7 Optimization of cDNA amplification

The key optimization step for the amplification of cDNA for array work is to amplify the cDNA to a point where the PCR reaction is still in the log phase. If the sample is left to amplify to the plateau stage then the amounts of various cDNAs in the reaction could become disproportionate, thereby potentially nullifying any expression differences. During this study, Clontech withdrew the enzyme optimised for the

SMART PCR cDNA synthesis kit. Consequently, an alternative enzyme was sourced from Invitrogen which required further optimisation work to determine compatibility with the Clontech kit. Three cDNA synthesis reactions were set up; A) Invitrogen enzyme with Clontech kit buffer, B) Invitrogen enzyme with Invitrogen buffer, and C) Clontech Kit-supplied enzyme with kit buffer (a small amount of this remained from when the original enzyme was commercially available. A PCR reaction of 100µl was set up for each reaction. Starting at 15 cycles, 15µl was removed every 3 cycles until 30 cycles. Samples were run out on a gel, (Figure 2.2). The combination of reagents seen in figure 2.2 (run A) seems to have the best overall PCR efficiency, due to more observable PCR product. From the PCR products on the gel an optimal cycle number of 17 calculated. This was measured by taking the number of cycles taken to not quite reach the plateau stage and subtracting 1 away. Considering that the cycle number 18 shows a high amount of amplification, but not quite as high as 21, it was decided to use 17 as the optimal cycle number.

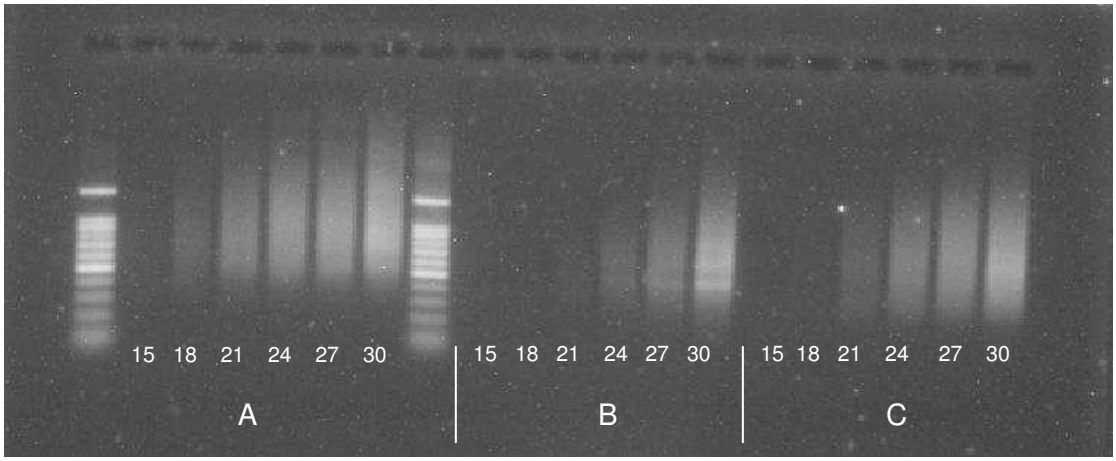

Figure 2.2 Agarose gel electrophoresis with optimization of PCR products. Samples from 15, 18, 21, 24 and 30 PCR cycles. A, B and C refer to different optimization conditions (refer to text).

2.2.1.8 Array manufacture

Ovine cDNA arrays were received from INRA (kindly supplied by Dr Olivier Sandra, French National Institute for Agricultural Research, Jouy-en-Josas, Paris, France). 140 genes were selected from the literature for their involvement in gonad development and function and pregnancy in mammals. RNA from tissues of interest (ovary and uterus) was extracted and reverse transcribed. PCR amplification of each gene was carried out and a product of expected size was obtained for 83% of genes (116/140). Each of these products was sequenced and compared with existing sequence databases and 80% (94/116) of these were found to be homologous with sheep gene sequences. Each PCR product was inserted into a pCR®2.1-TOPO vector (Invitrogen Ltd., Paisley, UK) using TOPO cloning (Invitrogen Ltd., Paisley, UK) and after purification were deposited on nylon membranes in a 96-well format (94 genes and 2 vector-only controls) by robot, with 4 spots for each gene at its location (Figure 2.3).

2.2.1.9 Array labelling

Membranes were firstly prehybridized in ExpressHyb hybridization buffer (Clontech Europe, Saint Germain-en-Laye, France) with added denatured single stranded salmon sperm DNA (Sigma-Aldrich Company Ltd, Dorset, UK) for blocking for 3 hours at 68^{o}C. A total of 500 ng of purified amplified double stranded cDNA for each sample was needed. cDNA was incubated with 2.42µl random primer 9 (New England Biolabs, Ipswich, UK) for 8 minutes at 100^{o}C, cooled on ice and incubated at 50^{o}C for 45 minutes with α-33P dATP (GE Healthcare Life Sciences, Buckinghamshire, UK), 4µl Klenow enzyme (New England Biolabs, Ipswich, UK) and unlabelled dGTP, dCTP and dTTP (10µM each) (MP Biomedicals, Cambridge, UK). The reaction was stopped by placing on ice and adding 2µl 0.5M EDTA. At this stage 1µl of reaction mix is added to 2ml scintillant for counting later to determine percentage incorporation. Samples were purified using Nucleospin Extraction Kit (Machary Nagel, Düren, Germany) following manufacturers guidelines. One µl of eluted sample was placed in 2ml scintillation fluid for testing. From these values a percentage incorporation value was determined. This was needed to check for poor incorporated samples, which were then repeated. The total

reaction volume was added to the pre-hybridized membranes and incubated at 68^{o}C for 18-20 hours. The membranes were washed twice for 30 minutes at 68^{o}C in 1 x SSC, 0.5% SDS, then washed once for 30 minutes at 68^{o}C in 0.1 x SSC, 0.5% SDS. After washing, membranes were kept damp by placing on 3mm chromatography paper, soaked in 0.1 x SSC, 0.5% SDS, wrapped in cling film. Membranes were exposed to a phosphor-imaging screen for 72 hours at room temperature and imaged using a Typhoon Trio+ scanner (GE Healthcare, Buckinghamshire, UK).

2.2.1.10 Reprobing

Due to pipetting errors and inaccuracies created by differences in sequence lengths (150-500bp) and therefore concentration differences, each gene spot on the array will contain variations in the total amount of cDNA spotted in each region. Due to these variations, the array must be reprobed with a vector-specific probe to quantify these differences. The data from the first experiment can then be normalized against the reprobed data to give a better representation of actual gene expression levels in each sample. The vector-specific oligo that was used to reprobe the array membranes is the T7 promoter sequence;

5' TAATACGACTCACTATAGGG 3'

The membranes were thawed and stripped of any residual radioisotope left over from the initial hybridization, by washing three times with stripping buffer (0.1X SSC, 0.2% SDS) for 1 hour each at 68°C. Membranes were then exposed in cassettes for 24 hours to check for presence of remaining ^{33}P. Membranes were prehybridized in ExpressHyb hybridization buffer (Clontech Europe, Saint Germain-en-Laye, France) with added denatured single stranded salmon sperm DNA (Sigma) for blocking for 3 hours at 42°C. The probe was labelled using γ^{33}P labelled dATP (Perkin Elmer, Massachusetts, USA) and a T4 polynucleotide kinase (New England Biolabs, Ipswich, UK) incubated at 37°C for 45 minutes. No other dNTPs are necessary as the reaction is an end-labelling reaction only. The reaction was stopped by placing on ice and purified using Mini Quick Spin Oligo Columns (Roche Diagnostics Ltd, West Sussex, UK), following manufacturer's guidelines to remove any unincorporated label from the mix. The amount of incorporated label was determined by scintillation

Vector	DAZL	HSD3B1	DMRT1	INHA	TGFb2	TNF	CSF-3	IL12B	SOCS1	PTGDS	TIMP2
Vector	MEL1A	CYP19A1	WT1	INHBa	TGFb3	BMPR1A	IL1A	IL13	SOCS2	PTGFR	MMP3
MSH4	ESR1	STAR	SRY	INHBb	GDF9	BMPR1B	IL1B	IL16	SOCS3	OSBP2	VIM
MSH5	ESR2	NR5A1	GATA4	INHBc	AMH	BMPR2	IL2	IL17	ALOX5AP	GJA1	PAPPA
SPO11	LHR	FOXL2	TCF21	BMP4	IGF1	IGF2R	IL7	Il18	ALOX12	GJC1	ACTB
DDX4	PGR	SOX2	CAV1	BMP7	IGF2	IGFBP1	IL8	IFNA	PTGS1	GJB1	EEF1a1
c-kit	POU5F1	SOX9	PHB	BMP15	PGF	IGFBP2	IL10	IFNB	HSL	GJB2	GAPDH
ZFX	PTPRG	SOX14	SDHA	TGFb1	STC1	CISH	IL12A	IFNG	PTGER3	TIMP1	HPRT1

Figure 2.3. Ovine gonad targeted cDNA macroarray layout. Endogenous/positive control genes highlighted in yellow. Vector-only/negative controls highlighted in blue. Other key gene groups include; steroidogenesis (pink); inflammatory and immune regulators (green); TGF-beta family members (orange). For detailed gene information refer to Appendix 1.

counter. 2µl of sample was placed in 2 ml scintillant and ran in duplicate and the counts per minute from the two samples were averaged and adjusted for volume. A count of $2x10^7$ was required for each membrane. The appropriate amount of labelled probe was denatured for 10 minutes at 100°C, cooled on ice and placed directly into the hybridization tubes. The membranes were incubated at 42°C for 24 hours in a rotating incubator. Membranes were washed twice with wash buffer (2XSSC, 0.1% SDS) at 42°C, rinsed with 2XSSC and exposed for 2 hours in an exposure cassette. Membranes were imaged using a Typhoon Trio+ Scanner (GE Healthcare, Buckinghamshire, UK).

2.2.1.11 Array image analysis

Phosphor-storage screens were scanned using a Typhoon Trio+ scanner and Typhoon Scanner Control V5.0 software. The images were analysed using Image Quant TL V2005 software with the Array Analysis option (all GE Healthcare, Buckinghamshire, UK) (Figure 2.4). The image analysis software bases the analysis on the total pixel intensity on a greyscale range over a given area. Each group of four spots representing a gene is assigned an area around it. The software measures the pixel intensity reading of the total area. Due to variations in background noise across the image, each spot area has a separate background value assigned to it, which is subtracted from the total area value.

The value given for any one gene from the original experiment was normalised against the reprobed data for the same gene to account for spot-to-spot differences within the array. This normalised value was then normalised again to a positive control on the array to account for array-to-array differences. The positive control gene chosen for this purpose was *beta*-actin (ACTB). All housekeeping genes (ACTB, EEF1a1, HPRT and GAPDH) were highly correlated with each other (Figure 2.5) and none were affected by treatment.

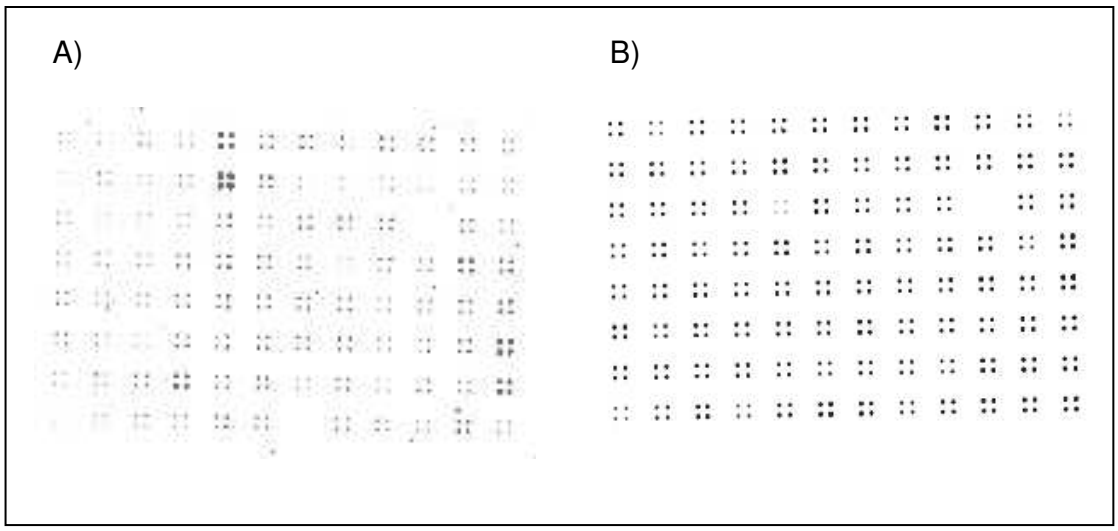

Figure 2.4 Example of scanned array images; labelled sample hybridisation (A) and reprobed image (B).

2.2.1.12 Quantitative real time-PCR

Complementary DNA was quantitatively PCR amplified with gene specific primers and fluorescently-tagged hydrolysis probes using the Roche Lightcycler® 480 amplification system. Probes used were either TaqMan (Eurofins MWG Operon, London, UK) or Universal Probe Library (UPL) (Roche Diagnostics Ltd., West Sussex, UK). Primer and probe sequences are shown in Table 2.2. UPL probes were used initially due to limitations in available reagents at that time. Primers were designed through either Primer Express (Applied Biosystems, Foster City, CA) or Roche Probe Finder software on the Roche Applied Science website. All Taqman probes were 5' labelled with 5'-6'-carboxyfluorescin (FAM) (reporter) and 3' labelled with 3'-6'-carboxy-N,N,N',N'-tetramthylrhodamine (TAMRA) (quencher). All Roche probes were 5' labelled with FAM and 3' labelled with an unreported quencher dye. Sequence homology for each target gene was matched to the ovine (Ovis Aries) sequence, but when this sequence was not available, the bovine (Bos Taurus) sequence was used. If possible, primers were designed so that they span two exons to prevent amplification of any genomic DNA contamination in the sample.

Table 2.2 Alphabetical list of primer and probe sequences used for qRT-PCR analysis. F, forward primer, R, reverse primer.

Name	Sequence 5'-3'	NCBI Accession	Primer Efficiency
ACTB†			
F	TGTGCGTGACATCAAGGAGAA	AF129289	1.92
R	CGCAGTGGCCATCTCCTG	Ovis aries	
Probe	**FAM**-CTGCTACGTGGCCCTGGACTTCGA-**TAMRA**		
IL18			
F	GAAGCTATTGAGCACAGGCATA	NM_001009263.1	1.78
R	TTCCACAAAGCTGATGCAAT	Ovis aries	
Probe	Roche Universal probe library probe #46 (ATGGCTGC)		
PPARγ†			
F	AGAGATCTCCAGCGACATCGA	AY137204	1.94
R	TCAGCGGGAAGGACTTTATGTAC	Ovis aries	
Probe	**FAM**-CAGAGTCTGCTGACCTCCGGGCC-**TAMRA**		
PTGS2			
F	GCACAAATCTGATGTTTGCAT	U68486	1.92
R	AGCTGGTCCTCGTTCAATATCTG	Ovis aries	
Probe	**FAM**-TGCCCAGCACTTCACCCATCAATTTTT-**TAMRA**		
STAR			
F	GCGACGTTTAAGCTGTGTGCT	AF290202	1.87
R	TGCTGCCGCAGCCC	Ovis aries	
Probe	**FAM**-AGCTCCTATAGACACGTGCGCAGCATG-**TAMRA**		

† Sequences for ACTB and PPARγ were obtained from Wynn et al. (2006).

Prior optimization was carried out for each primer and probe set to determine optimal primer and probe concentration. Optimised PCR products were run on a 1.5% agarose gel to confirm specificity and product size (Figure 2.6). A standard curve from combined ovine liver, theca and granulosa cell cDNA was used to ensure adequate expression levels, check assay linearity and determine primer efficiency values (Figure 2.7). A calibrator sample (sample from the midpoint of standard curve) and reagent blanks was run on each plate. The calibrator was used to adjust for any plate-to-plate variation in PCR efficiency. Relative expression of each gene was normalised against an endogenous control gene, beta actin (ACTB). The PCR reaction mix comprised the Roche Lightcycler 480 Probes Master mastermix (Roche Diagnostics Ltd., West Sussex, UK). A total reaction volume of 20µl was prepared using 10µl Probes Master 2X mix, 0.6µl (10µM) forward and reverse primers, 0.4µl (10µM) probe, 1.5µl cDNA and PCR H_2O to make up the volume. All samples were run in duplicate. The 96 well PCR plate (LightCycler® 480 Multiwell Plate, Roche) was sealed using sealing foil (LightCycler® 480 sealing foil, Roche) and centrifuged for 2 minutes at 1500 x g in a swing bucket centrifuge. The plate was loaded into a Lightcycler® 480 and run using the parameters in Table 2.3.

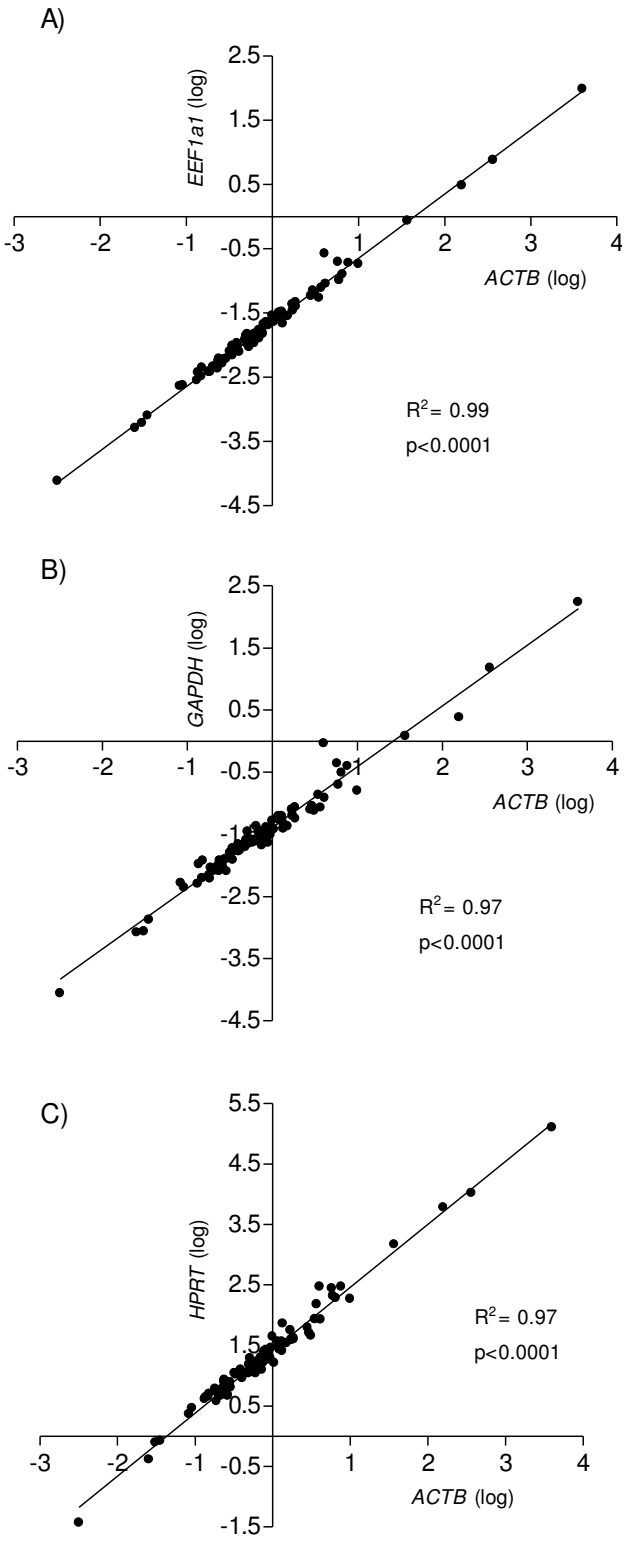

Figure 2.5 Array data expressed relative to ACTB correlated against data relative to the other control genes; EEF1a1 (A), GAPDH (B) and HPRT (C).

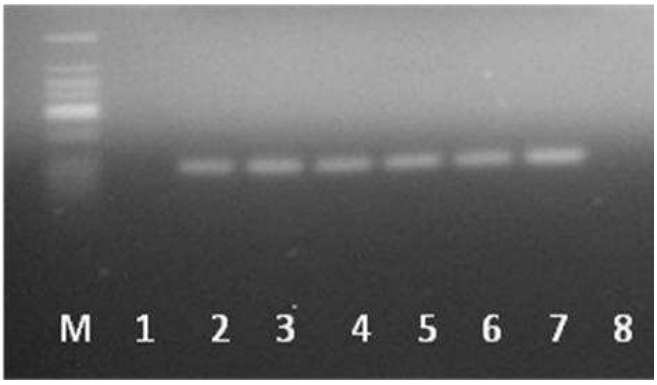

Figure 2.6 Agarose gel electrophoresis to confirm primer specificity and PCR product size. M = DNA marker, wells 1 and 8 PCR negatives, wells 2-7 single band of PCR product of PTGS2 at correct size (96bp).

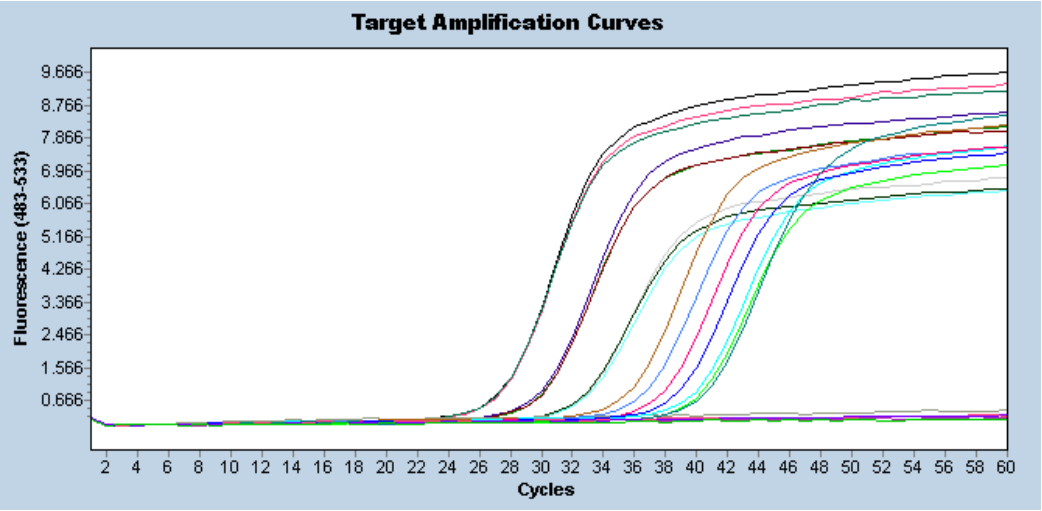

Figure 2.7 Snapshot of an example of a standard curve, in this case PTGS2 with a primer efficiency of 1.91 using Roche 480 Lightcycler® software. The flat lines extending beyond cycle 40 are negatives containing no signal.

Table 2.3. Programme and cycling parameters for qRT-PCR analysis using Roche Lightcycler® 480.

Detection format		**Block type**		**Reaction volume**
TaqMan probe		96		20µl
Programme name		**Cycles**		**Analysis mode**
Pre-incubation		1		None
Amplification		45		Quantification
Cooling		1		None
Temperature targets				
Target (°C)	**Acquisition mode**	**Hold (hh:mm:ss)**	**Ramp rate (°C/sec)**	**Acquisitions (per °C)**
Pre-incubation				
95	None	00:10:00	4.4	-
Amplification				
95	None	00:00:10	4.4	-
60	None	00:00:50	2.2	-
72	Single	00:00:01	4.4	-
Cooling				
40	None	00:00:10	1.5	-

2.2.1.13 Statistical analysis

Follicular fluid concentrations of steroids were analysed by ANOVA. Oestradiol and progesterone data were transformed (log_{10}) prior to analysis to correct for differences in means and variance between treatments. Data are presented as geometric means with log_{10} means presented in italics. Standard errors of differences (S.E.D) in this case are presented on the log scale. Transcript expression (relative to that of ACTB) for each of the genes on the array was analysed by ANOVA using Genstat release 11 (Genstat 2009). Subsequent qRTPCR analyses again were conducted by ANOVA. The term fitted to these models was Diet (n-3 vs. n-6). Data are presented as predicted means ± S.E.M.

2.2.2 Results

2.2.2.1 Phenotypic and hormonal effects of dietary treatments in stimulated ovaries

Follicle number and size were measured in all 36 experimental animals. There was no significant effect on follicle number or follicle size between the two experimental groups (Figure 2.8). Concentrations of progesterone detected in follicular fluid were greater ($P<0.001$) for ewes offered the n-3 PUFA diet than the n-6 PUFA diet (Table 2.4). Oestradiol concentrations were not altered between treatment groups.

2.2.2.2 Gene expression in granulosa cells by ovine cDNA array analysis

Macroarray data was analysed to detect differences in gene expression in granulosa cells between the two dietary treatment groups. Two out of the 88 genes present on the array (SOCS3 and CISH) were discounted due to problems when the array was constructed (O Sandra, personal communication). Two genes were significantly altered in granulosa cells from n-3 vs n-6 PUFA fed ewes; IL18 ($P=0.03$) and STAR ($P=0.05$), (Figure 2.9 (A) and (B)). In both cases transcript expression was greater in n-3 group than n-6 fed ewes; with IL18 having a 3 fold increase and STAR a 2.5 fold increase in expression.

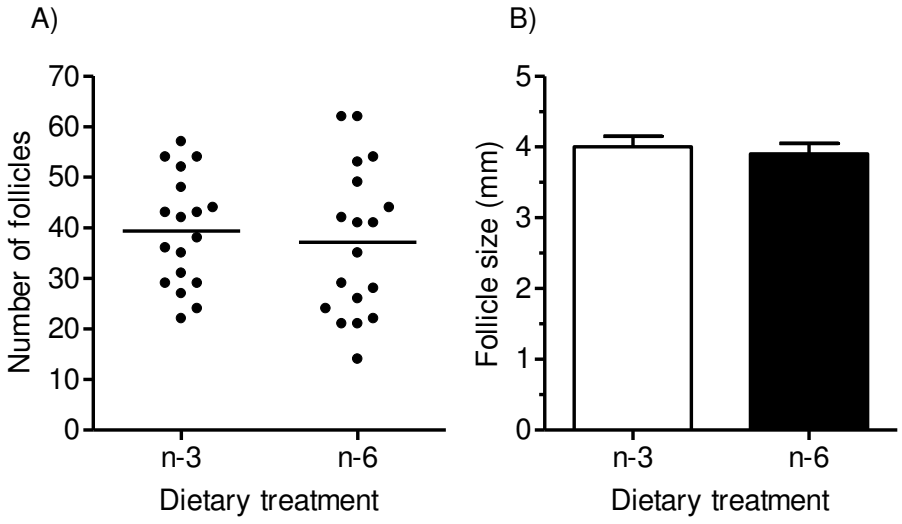

Figure 2.8. The effect of dietary treatment (n-3 vs n-6 PUFA) on follicle number (A) and mean follicle diameter (B) following oestrous synchronisation and ovarian stimulation with FSH.

Table 2.4 Concentrations of steroid hormones in follicular fluid of ewes offered diets enriched with n-3 or n-6 PUFA (Wonnacott et al., 2010).

Experimental diet	**n-3**	**n-6**	**S.E.D.**	**P**
Steroid hormone				
Oestradiol (E2) (ng/ml)	30.2	23.4		
(log_{10})	(1.48)	(1.37)	(0.186)	-
Progesterone (P4) (ng/ml)	52.5	22.4		
(log_{10})	(1.72)	(1.35)	(0.092)	<0.001
E2:P4	0.56	1.05		
(log_{10})	(-0.25)	(0.02)	(0.156)	-

E2, P4 and E2:P4 expressed as geometric means, with means and S.E.D. on a log_{10} scale.

2.2.2.3 Gene expression in granulosa cells by quantitative real time PCR

Quantitative RTPCR analysis failed to detect any significant differences in the expression of IL18 and STAR between the two dietary groups (Figure 2.9 (C) and (D)). This illustrated that the expression pattern for these genes detected by the array and qRTPCR did not correlate.

Due to their known relationship with PUFAs, two other gene candidates PPAR-γ and PTGS2 were analysed by qRTPCR (Figure 2.10). Due to limitations in available gene sequences when the array was constructed, these genes were not included. Despite a trend of increased expression in both genes in the granulosa cells of n-6 PUFA fed ewes, there was no significant effect of dietary treatment on transcript expression.

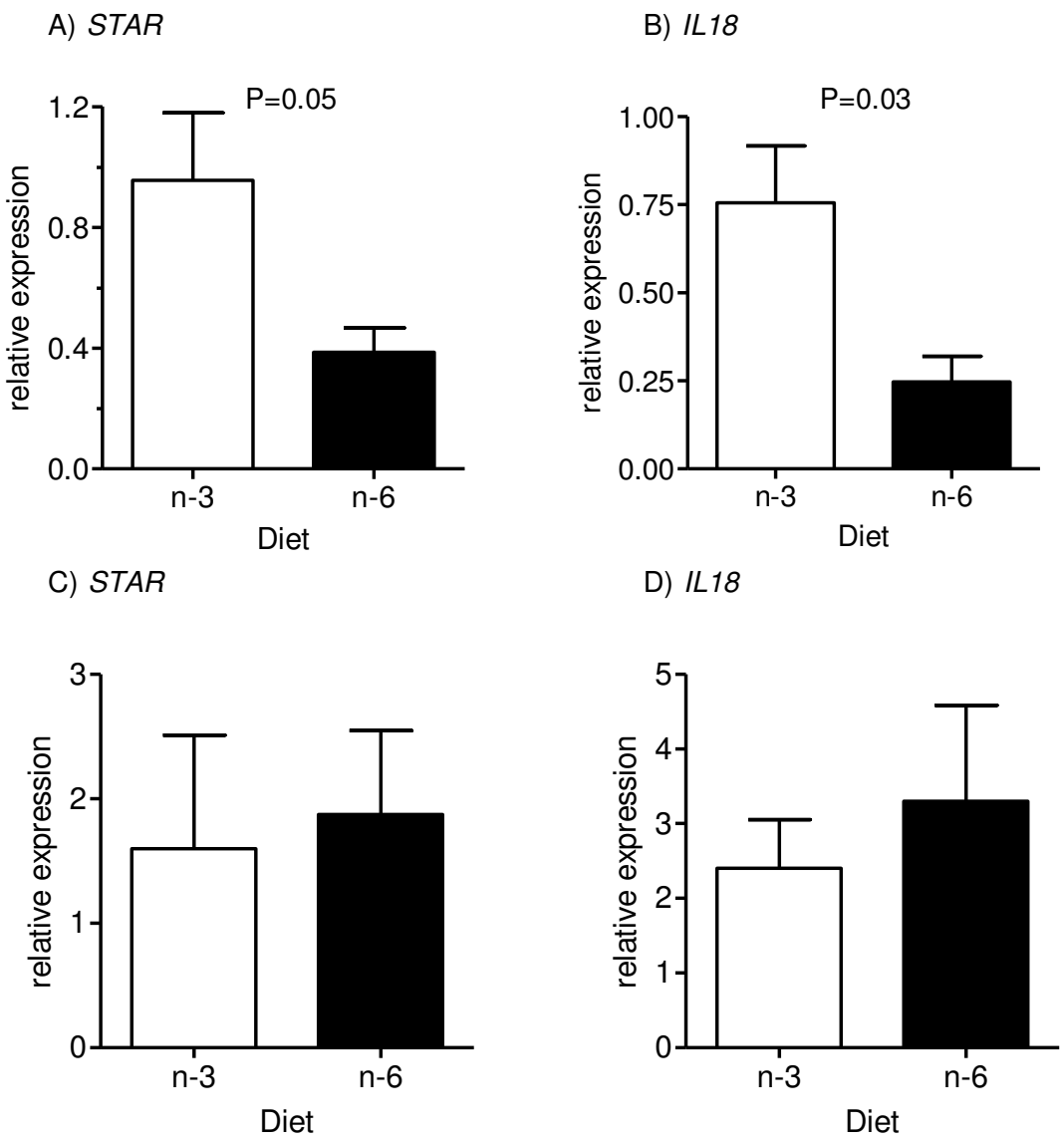

Figure 2.9 The effect of diet (n-3 vs. n-6 PUFA) on STAR and IL18 gene expression in ovine granulosa cells of ewes fed high n-3 or high n-6 diet. Gene expression was measured by cDNA macroarray (STAR: A, IL18: B) and qRTPCR analysis (STAR: C and IL18: D). Data expressed relative to ACTB + S.E.M. Note the lack of correlation between the 2 methods.

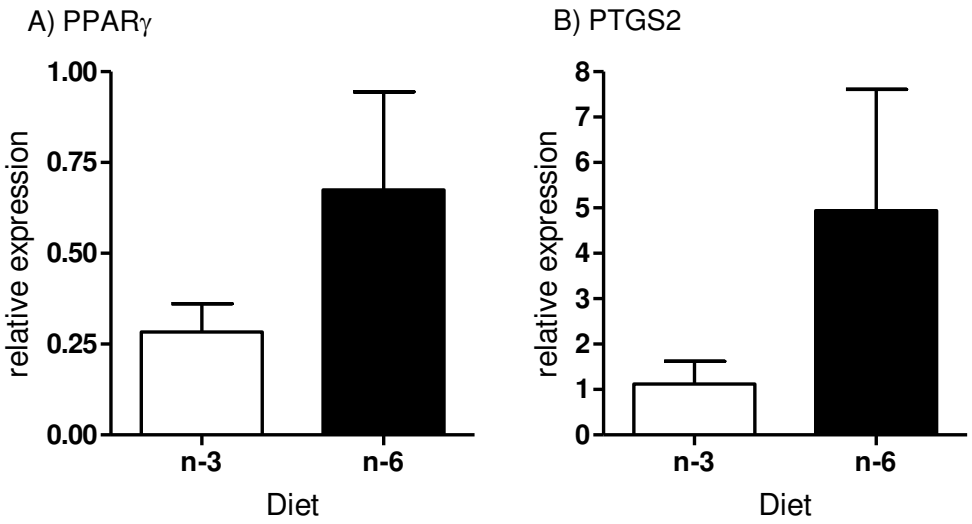

Figure 2.10 qRTPCR expression of PPAR-γ (A) and PTGS2 (B) (relative to ACTB) in granulosa cells from ovaries of ewes offered either a high n-3 or n-6 PUFA diet.

2.2.3 Discussion

From the observed changes in progesterone output in follicular fluid of the experimental ewes it is clear that a mechanism controlling steroidogenic output has been altered by dietary treatments. Unfortunately the current methodology has been unable to highlight any potential candidates for further study. This line of study will be readdressed in Chapter 3.

It was clear from these observations that the suitability of this particular cDNA macroarray for use in this study was in question. The differences observed between the array and qRTPCR data highlights a problem somewhere in the methodology. The second part of this chapter highlights these potential issues and thoroughly tests the array methodologies.

2.3 In vitro validation – Experiment 2

2.3.1 Introduction

Due to the inconsistencies between the array and qRTPCR data, and since the qRTPCR methodology is well established, it was likely that the problem lay with the array methodology. Consequently, further investigations focussed on the array

methodology including sample preparation. Possible reasons for a lack of correlation were hypothesised: (i) the amplification method of sample preparation may not be suitable. Many array techniques utilise a PCR-amplification method to increase the amount of cDNA for hybridizing. There are arguments as to whether this technique gives a true representation of the relative levels of transcripts in the original sample, and some commentators have proposed that, due to the differences between transcripts (such as length, tertiary structure, GC content etc), this PCR step could potentially amplify transcripts disproportionately (Baugh et al., 2001; Degrelle et al., 2008). (ii) The array is not sensitive enough to detect gene expression differences. Most commercial array technologies use a twofold difference in expression as the threshold for gene expression scoring (Clarke et al., 2001; Jenson et al., 2003). Since the array used for this study is not a commercially available platform, the question regarding the sensitivity of fold difference detection level needed to be addressed.

2.3.2 Materials and Methods

2.3.2.1 Experimental design

A cell culture experiment was designed to create a substantial amount of material in order to fully test the array methodologies. Granulosa cells obtained from abattoir derived ovaries were cultured in vitro for 144 hours in the presence of either 0, 10 or 20% FCS. Media were harvested at 48, 96 and 144 h, and cells at 144 h for cell counts and RNA extraction. The experiment was conducted in a 24-well plate on three separate occasions. Serum in culture is known to affect granulosa cell differentiation and steroid hormone output (Luck 1990), thus we predicted that genes involved in these mechanisms would be differentially altered between treatments. This produced enough experimental material to not only thoroughly test the array but also the PCR amplification steps used prior to array hybridization. The focus areas for testing were as follows;

1. Testing the ability of the PCR amplification method to amplify all transcripts in a linear manner. Due to the large volume of cDNA required for hybridization to arrays, PCR amplification is required when sample volumes are low. This could potentially amplify cDNA disproportionately. Amplified

samples were tested against non-amplified samples by qRTPCR (Figure 2.11). Any indication of disproportional amplification would explain the lack of correlation between the array and qRTPCR methods.

2. Testing the ability of the array to detect differences in gene expression in cultured cells without prior pre-amplification by PCR. The culture experiment assured enough sample without the need for PCR amplification. We were able to hybridise experimental cDNA direct to the array. The resulting data was then compared with qRTPCR analysis of the same samples (Figure 2.11).

Figure 2.11 outlines the 2 in vitro approaches described above and how this compares to the original array method using in vivo generated samples. Of the 88 genes present on the array a small selection were used for analysis in this study. Transcripts for qRTPCR analysis included the steroidogenic genes, CYP19A1, HSD3B1, and STAR. Others include, insulin growth factors 1 (IGF1) and 2 (IGF2) and tissue inhibitor of metalloproteinases (TIMP1). IGF1 and 2 have been reported to be involved in ovarian steroidogenesis by stimulating progesterone synthesis as well as enhancing the proliferation of granulosa cells in vitro (Sekar et al., 2000; Demeestere et al., 2004). Additional to its role in tissue remodelling at ovulation TIMP1 expression has been reported to be stimulated by progesterone production in granulosa (Boujrad et al., 1995) and theca cells (Shores and Hunter, 2000).

2.3.2.2 Granulosa cell culture

Ovine ovaries were obtained from an abattoir and washed briefly with 70% industrial methylated spirits (IMS) before washing in warm PBS (37^{o}C). Granulosa cells were harvested by aspiration with a syringe. Follicular contents were placed into a sterile petri dish and oocytes were removed. The remaining follicular fluid and cells were spun down and follicular fluid discarded. The cell pellet was resuspended briefly in water to lyse any blood cells present, then 10X PBS immediately added to regain isotonicity. Cells were then washed in PBS/PVA (0.1%) to help reduce the viscosity of the sample. Cells were resuspended in medium and a viable cell count was carried out by trypan-blue exclusion. From the cell counts, a cell suspension was created to provide a seeding density of $3.5x10^5$ cells per well in 500µl of a 24 well

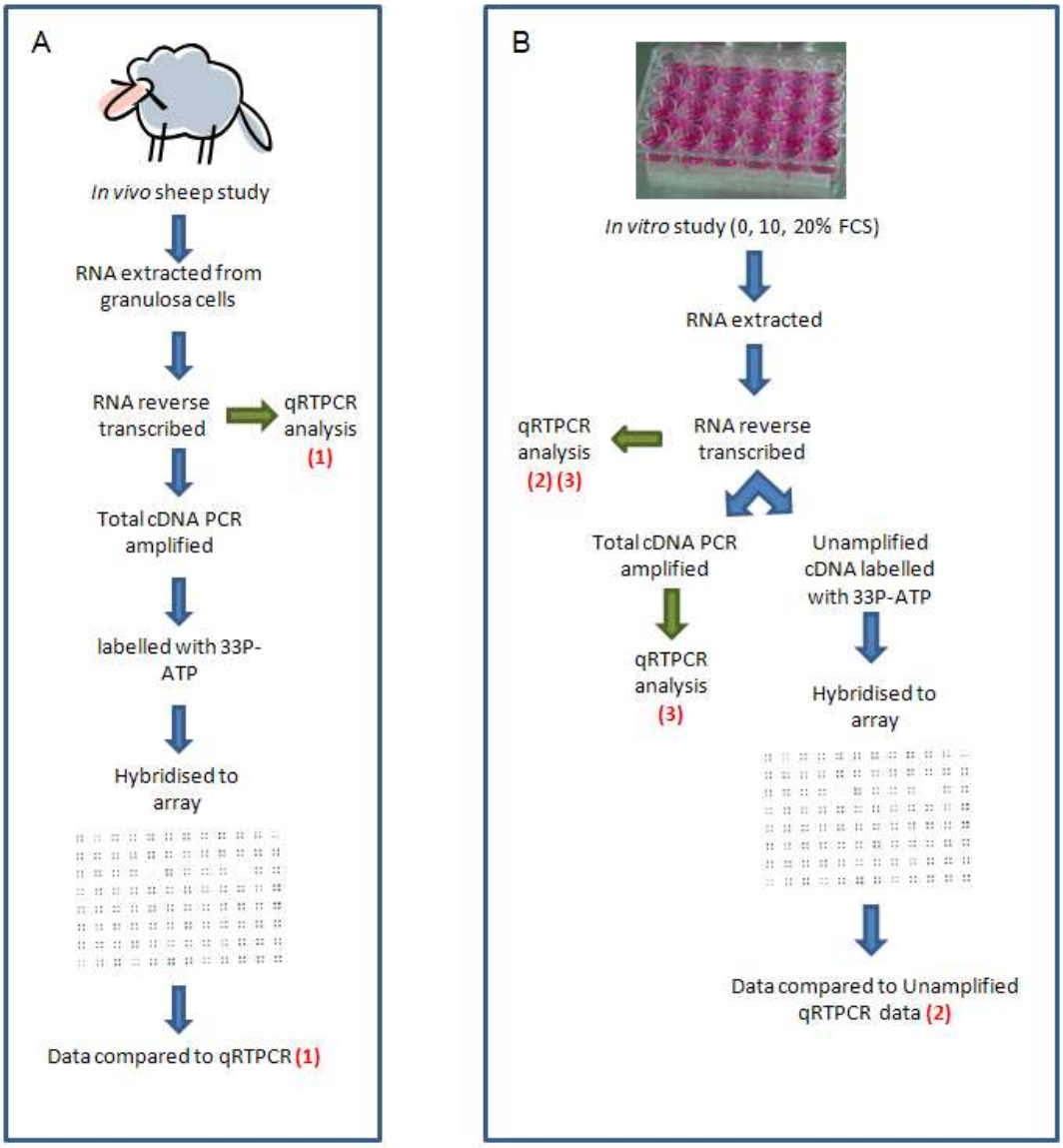

Figure 2.11 Flow diagram representing experimental procedure for the original in vivo sheep study (Box A) and subsequent investigative in vitro experiments (Box B). Equivalent numbers in red indicate where data comparisons were made.

culture plate. Cell culture medium consisted of; 90% v/v TCM199 (Sigma) and 10% v/v double distilled water. Medium was supplemented with 1mg/ml fatty acid free (FAF) BSA, 100ng/ml L-glutamine, 10ng/ml insulin, 0.5µg/ml transferrin, 0.5ng/ml sodium selenite, 50IU/ml penicillin, 50µg/ml streptomycin, 1ng/ml FSH and 100ng/ml testosterone. Media was supplemented with 0, 10 or 20% FCS. Cells were incubated at 38.8°C in a humidified atmosphere of 5% CO_2 for 144 h. Eighty percent of media was collected at 48 h and replaced with fresh media, all spent media was stored at -20°C, further media was collected at 96 and 144 h. Cells were harvested

with trypsin EDTA and cell counts performed at 144 h. All cells were snap-frozen in liquid nitrogen prior to storage at -80°C. A total of 3 experimental replicates were run with 2 wells per treatment per experiment.

2.3.2.3 Progesterone ELISA

Progesterone concentrations in spent media samples were determined by enzyme linked immunosorbent assay (ELISA). Progesterone ELISA kit was purchased from Ridgeway Science (Ridgeway Research Ltd, Gloucesershire). Media samples were diluted in PBS in the range of 1:5 to 1:100 (determined by time point and serum concentration of sample). Samples were run in duplicate along with standards (0, 0.5, 1, 2, 5, 10, and 20ng/µl) and QCs (2 and 4 ng/µl), both supplied with the kit. All ELISA plates, samples and reagents were brought to room temperature. Protective foil and buffer were removed from the plate. 10µl of either standard, QC or sample were added to the wells and 200µl progesterone-enzyme label was added to each well. The plate was then mixed gently on a plate vortex for 10 minutes and left at room temperature for 2 hours and 15 minutes. All wells were washed three times in wash buffer (200µl per well for 2 minutes) and the plates tapped dry on tissue paper after each wash. 200µl alkaline phosphatase substrate was added to each well (substrate was dissolved in 25ml substrate buffer 30 minutes before use and kept in the dark until needed). The plate was mixed gently on a plate vortex for 30 seconds and incubated at room temperature for 30 minutes. The plate was read using Thermo Labsystems Multiscan Ascent plate reader and Ascent Software Version 2.6 (Thermo-Fisher, Loughborough, UK) at an optical density of 570nm. Concentrations of samples were calculated from the standard curve with a minimum detection limit of 1ng/ml. Intra and inter-assay CV were 8.2% and 5.4% respectively.

2.3.2.4 Oestradiol radioimmunoassay

Spent media was diluted in assay buffer (0.05M PBS, 0.1% gelatine, 0.1% sodium azide). All samples (total counts, standards, non specific binding, total binding) were set up in triplicate. Standard concentrations were 1.95, 3.9, 7.8, 15.6, 31.3, 62.5, 125 and 250pg/100µl. Quality control concentrations were 5, 25 and 50 pg/100µl and run at the front and back of the assay and also at every 100 tubes. Oestradiol was

iodinated using chloramine T, which is an oxidising agent capable of converting iodide to a more reactive form. The reaction is terminated by adding sodium metabisulphate. The following was added in sequence: 10µl E2 TME buffer, 30µl 0.5M PO_4 buffer (pH 7.5), 10µl I^{125} (1mCi), 10µl chloramine T (10µg) in 0.05M PO_4 buffer (pH 7.5). After addition of chloramine T, solution was left for 2 minutes to react. 10µl sodium metabisulphate (10µg) in 0.05M PO_4 buffer (pH 7.5) and 200µl 0.05M PO_4 buffer (pH 7.5) were then added. The reaction was transferred to a 10cm Sephadex G 25 column equilibrated in 0.05M PO_4 buffer (pH 7.5). The reaction was then rinsed with 200µl 0.05M PO_4 buffer (pH 7.5) and applied to the column. Column was eluted with 0.05M PO_4 buffer (pH 7.5) and 1ml fractions were collected into numbered LP3 tubes containing 200µl of 0.5M PO_4 buffer (pH 7.5). The radioactivity of each fraction was counted and the fractions that showed the highest peak were collected and stored at 4ºC. Rabbit anti-oestradiol serum was used as primary antibody at 1:40,000 dilution from stock and was added to total binding, standards, QCs and sample tubes. Iodinated oestradiol label with a count of 10,000-12,000 was added to each tube after the addition of primary antibody, mixed on a vortex mixer and incubated at room temperature for 4 hours. Bound and unbound hormone were separated by the addition of a second antibody, donkey anti-rabbit serum at 1:80 dilution combined with normal rabbit serum at 1:400 dilution. This was added to the tubes and incubated overnight at 4ºC. After incubation, 250µl of 1% Tween 20 in assay buffer was added to same tubes as second antibody (i.e. all but total counts) as a detergent to reduce non-specific bonding and tubes were centrifuged at 3000rpm at 4ºC for 30 minutes. The supernatant was removed. The radioactivity of the remaining pellet was counted in an automatic gamma counter. The unlabelled antigen in the sample was quantified using the standard curve. The minimum detection limit was 4.4pg/100µl. Intra and inter-assay CV were 5.6% and 6.9% respectively.

2.3.2.5 RNA extractions and cDNA synthesis

RNA was extracted as described in section 2.2.1.4 using RNeasy mini kit (Qiagen, West Sussex, UK). The RNA to cDNA reverse transcription reaction was carried out using Superscript™ II (Invitrogen Ltd., Paisley, UK) following manufacturers instructions. For each cDNA reaction 5µl of RNA (concentration > 300ng/µl) was

added to 1µl Oligo(dT)$_{12-18}$ primers (500µg/ml), 1µl dNTP mix (10mM each) and nuclease free water made up to a final volume of 12µl. Samples were heated to 65°C for 5 minutes and quick chilled on ice. Following centrifugation, 4µl 5X first strand buffer and 2µl 0.1M DTT was added to each sample. The contents were mixed and heated to 42°C for 2 minutes. One µl Superscript™ II reverse transcriptase was added to each sample which were then incubated for a further 50 minutes at 42°C. The reaction was inactivated by heating to 70°C and stored at -20°C.

2.3.2.6 Array labelling and reprobing

Sample labelling and array hybridisation was carried out as detailed in section 2.2.1.9. To ensure adequate coverage of all transcripts 5µg of unamplified cDNA was used per membrane. Array reprobing and image analysis was carried out as detailed in sections 2.2.1.10 and 2.2.1.11. A total of 3 samples per treatment group were used (0, 10 and 20% serum), equalling 12 membranes in total.

2.3.2.7 Quantitative real-time PCR

Quantitative RTPCR was carried out as described in section 2.2.1.12. Details of primers and probes can be found in Tables 2.2 and 2.5. Cycling parameters can be found in Table 2.3. Transcripts were analysed relative to ACTB.

2.3.2.8 Statistical analysis

Progesterone and oestradiol concentrations in spent media from 24-well plates were analysed by repeated measures ANOVA using Genstat release 11 (Genstat 2009). To recap, there were three plates and three treatments (0, 10 and 20% serum) within plate. Media was pooled from a pair of wells within treatment at three time points (48, 96 or 144 h) within plate. This, therefore, led to there being 4 replicates within plate. Cell counts and transcript expression (relative to that of ACTB) in cells harvested at 144 h for each of the genes on the array was analysed by ANOVA. Analysis was based on cells pooled from the same pairs of wells within treatment (giving 4 replicates within plate). Data are presented as means ± S.E.M.

Table 2.5 Alphabetical list of primer and probe sequences used for qRTPCR analysis. F, forward primer, R, reverse primer.

Name	Sequence 5'-3'	NCBI Accession	Primer Efficiency
CYP19A			
F	TGGGTTGCCATTGCCTTC	AJ012152	1.99
R	GGACAGTAAGGAGCTGGAGTGAG	Ovis aries	
Probe	**FAM**-CCGTTGGAAAAGACAAGTCACCAGCAA-**TAMRA**		
HSD3B1			
F	CCAGGCTAGTGTGCCAGTCTTT	NM_001135932	1.97
R	CTCCCTGTAGGCGTTGGGCC	Ovis aries	
Probe	**FAM**-TCCACACCAGCACCATAGAGGTGGCT-**TAMRA**		
IGF1			
F	TCCGTGCCCAGCGC	M31736	1.99
R	TGCTTGTGTTCTTCAAATGTACTTCC	Ovis aries	
Probe	**FAM**-CACCGACATGCCCAAGGCTCAGA-**TAMRA**		
IGF2			
F	CCGCGGCTTCTACTTCAGC	X53554	1.99
R	ACTCTTCCACGATGCCAC	Ovis aries	
Probe	**FAM**-CCAGCCGCATAAACCGACGCA-**TAMRA**		
TIMP1			
F	CACCCACAGACGGCCTTCT	S67450	1.99
R	TGCGGTCCCCACGAAC	Ovis aries	
Probe	**FAM**-AACTCCGAAGTCGTCATCAGGGCCA-**TAMRA**		

2.3.3 Results

2.3.3.1 Phenotypic effects of serum on cell proliferation, oestradiol and progesterone levels in cultured granulosa cells

To determine if the period of cell culture and serum treatment induced luteinisation and associated changes in steroid hormone secretion, steroid hormone levels were measured at 48, 96 and 144 hours (Figure 2.12). Progesterone concentrations generally increased ($P<0.001$) whilst oestradiol concentrations decreased ($P<0.001$) with time during culture. Cells treated with 20% serum produced lower levels of progesterone ($P<0.001$) and oestradiol ($P=0.01$) over all time points. Cells were counted at 144 hours and revealed that cell proliferation increased ($P<0.001$) with serum concentration. Furthermore, when steroid levels were adjusted for cell number, the pattern of hormone production was maintained (Figure 2.13).

2.3.3.2 Testing the PCR amplification step

Quantitative RT-PCR analysis was carried out on cDNA from granulosa cells and on cDNA which was previously PCR amplified by the method detailed in section 2.2.1.6. This was carried out to determine if the transcript abundance profiles remained the same after PCR amplification. Correlations between data sets show a close relationship between both methods (Figure 2.14) with HSD3B1, TIMP1, IGF1 and STAR expression being highly correlated ($R^2 > 0.75$, $P<0.01$).

2.3.3.3 Testing the ability of the array to detect differences in transcript levels

Firstly an experiment was set up in which doubling concentrations of cDNA were labelled and hybridised to the array to check for linearity. Samples containing 500, 1000 or 2000ng cDNA from one sample were analysed as in section 2.2.1.11 (Figure 2.15. Data was not expressed relative to ACTB as absolute levels only were required. The array failed to detect doubling concentrations of cDNA and no linearity was observed.

A second experiment used cDNA from granulosa cells cultured with either 0, 10 or 20% FCS for 144 hours. Since differences in hormone synthesis and cell proliferation had been demonstrated (Fig 2.13), it was anticipated that would be reflected by differences in gene expression. The cDNA was hybridised directly to the array membrane after radioisotope labelling without any previous amplification step. The array failed to detect any significant differences in gene expression in any gene between treatment groups (Figure 2.16). Subsequent qRT-PCR analysis was carried out on the same cDNA samples and all transcripts showed a significant reduction ($P<0.001$) in expression between 0% and 10% and between 0% and 20% treatment groups (Figure 2.17). CYP19A was not detected with treatment of 10 and 20 % FCS, while HSD3B1 shows approximately a 30 fold reduction in expression with treatment of 10 and 20 % FCS. All other transcripts (IGF1, IGF2, TIMP1 and STAR) showed a reduction with inclusion of 10 and 20% FCS compared to untreated cells, albeit a much smaller decrease; STAR (1.5 fold), TIMP1 (2.1 fold), IGF1 (2.7 fold) and IGF2 (4 fold).

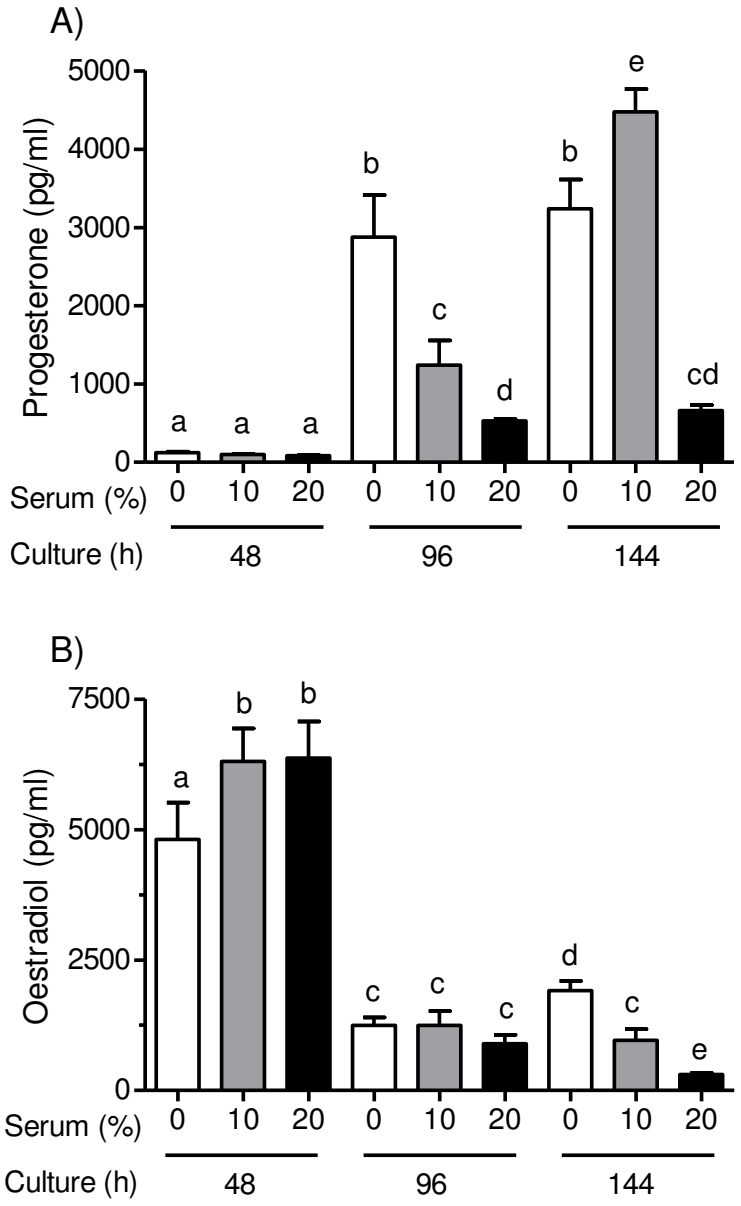

Figure 2.12 Effect of 0, 10 or 20% serum on progesterone (A) and oestradiol (B) production after 48, 96 and 144 hours of culture. Data are the average of 6 replicates +S.E.M. Different superscripts indicate $P<0.05$.

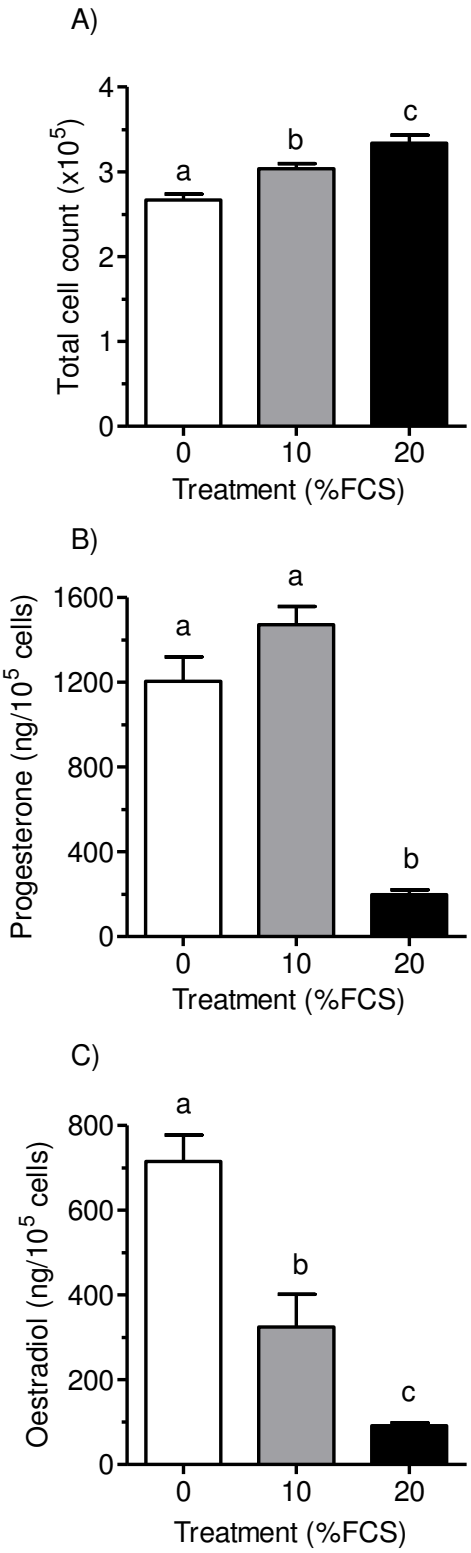

Figure 2.13 Cell count data at 144 hours after treatment with 3 serum concentrations (A). Progesterone (B) and Oestradiol (C) levels in culture media at 144 hours, adjusted for cell number. Data average of 6 replicates + S.E.M. Different superscripts indicate $P<0.01$.

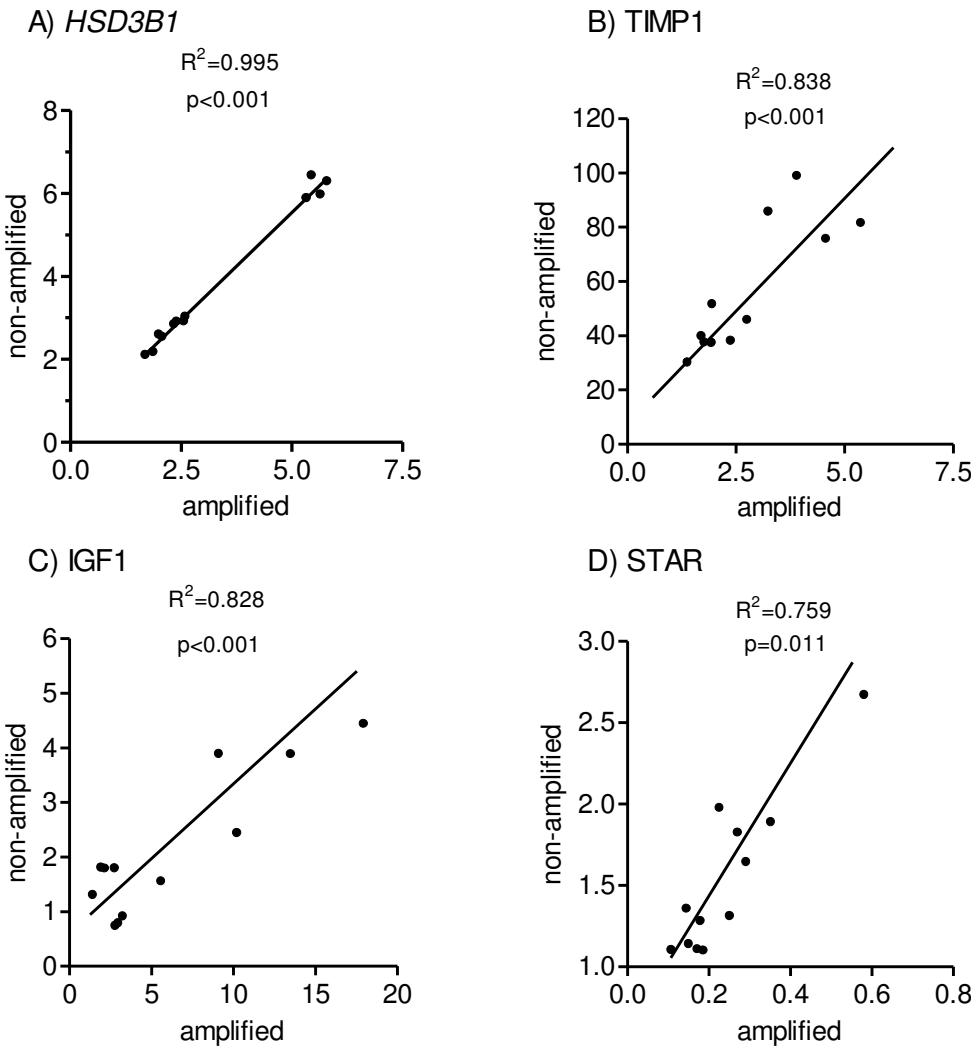

Figure 2.14 Correlations of qRTPCR data for pre-amplified vs non-amplified cDNA for HSD3B1 (A), TIMP1 (B), IGF1 (C) and STAR (D) expression relative to ACTB.

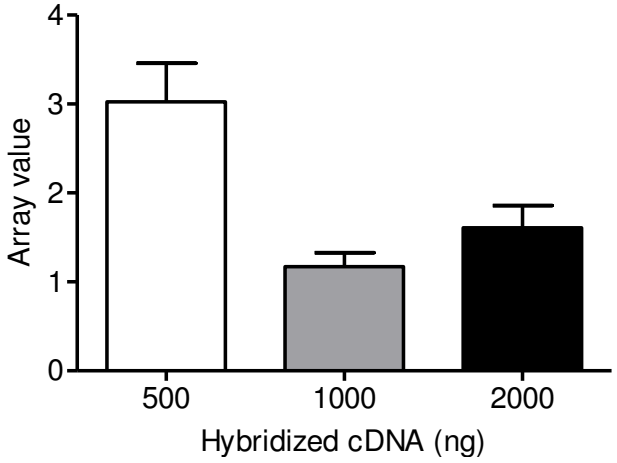

Figure 2.15 Array data showing absolute levels of detectable transcript after hybridisation with 500, 1000 or 2000 ng of labelled cDNA from a single cDNA sample. Data average of all genes + S.E.M.

2.4 Discussion

2.4.1 cDNA macroarray vs. qRT-PCR for detection of gene expression differences in ovine granulosa cells

From the samples analysed in the original in vivo sheep study, none of the genes selected for qRT-PCR analysis showed a correlation with data from the cDNA macroarray. Given the subtle changes in gene expression that may result from the dietary study described, cell culture experiments were set up to ensure a series of samples containing measurable changes in gene expression. Quantitative RT-PCR analysis showed that this was the case, with all chosen genes showing a reproducible expression profile across treatment groups. These expression differences were not detected by the array.

There are a number of possible reasons as to why the array and qRT-PCR results did not correlate. The first potential reason for the observed discrepancies was the method of cDNA amplification and whether it was disproportionately amplifying transcripts. It has been reported that cDNAs with differing lengths and compositions

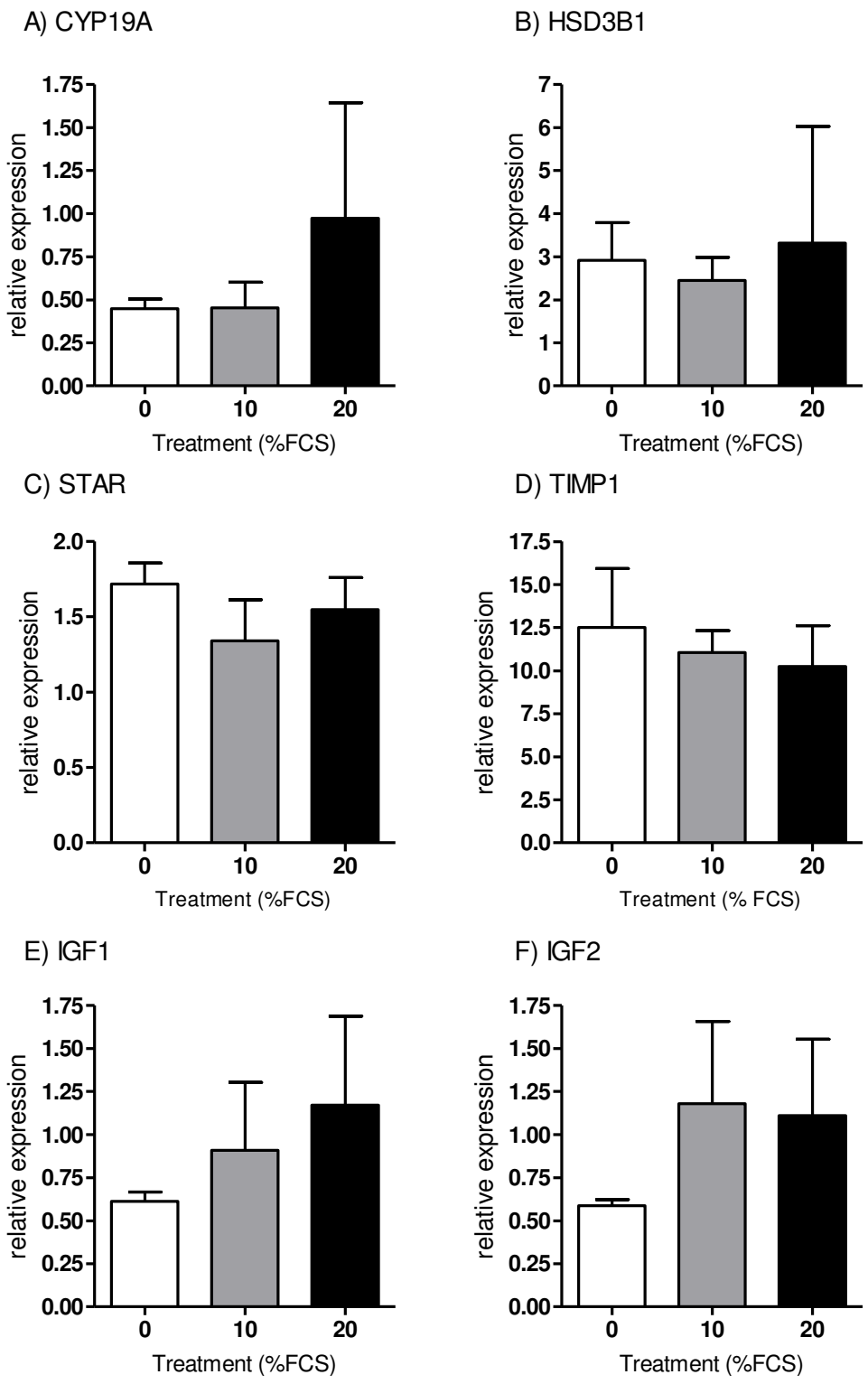

Figure 2.16 cDNA macroarray analysis of the effect of treatment on the expression of CYP19A, HSD3B1, STAR, TIMP1, IGF1 and IGF2 in granulosa cells cultured with either 0, 10 or 20% FCS supplement at 144 h. cDNA was not amplified prior to hybridisation. Data are the mean of 3 replicates + S.E.M.

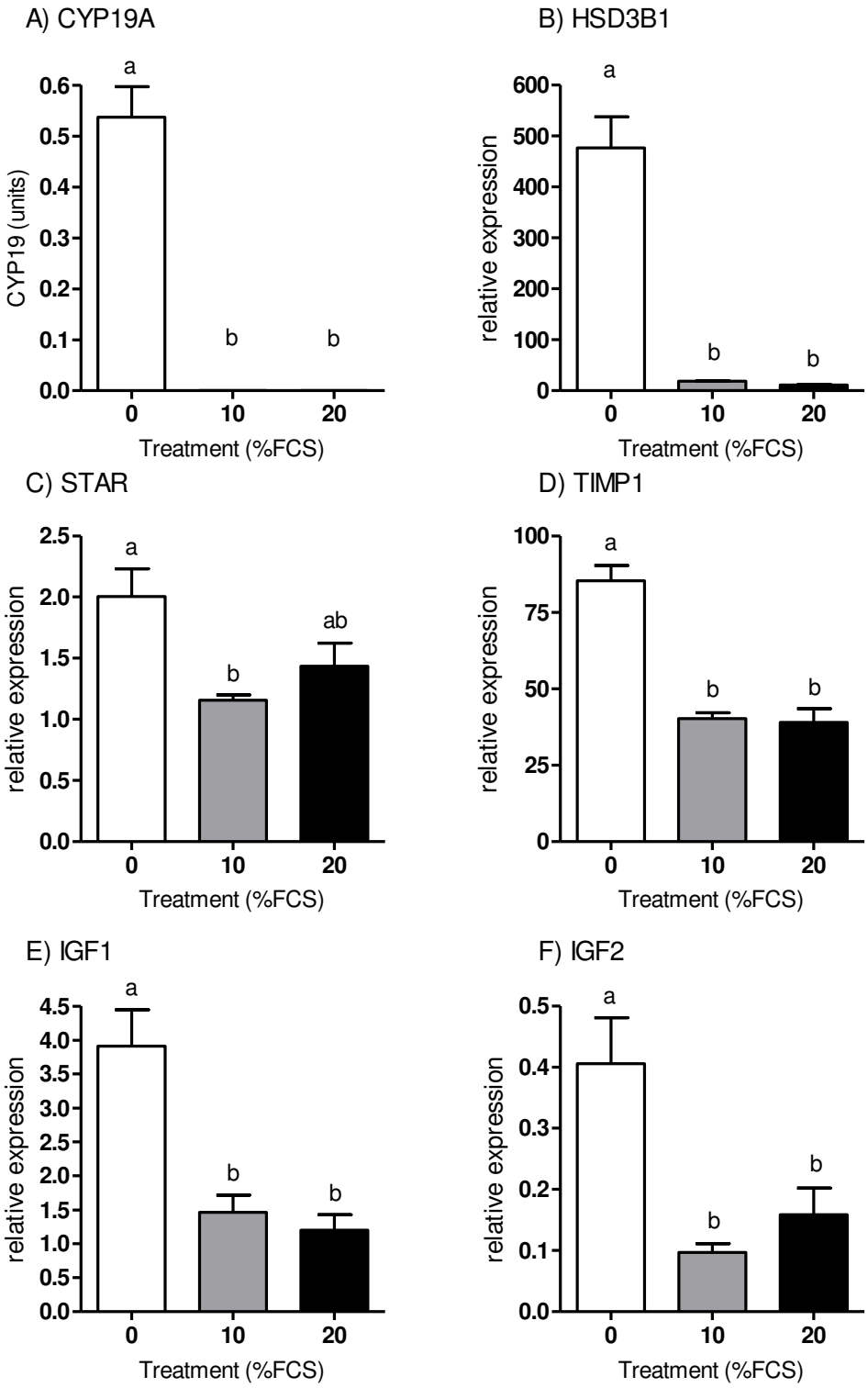

Figure 2.17 qRT-PCR analysis of the effect of treatment on the expression of CYP19A, HSD3B1, STAR, TIMP1, IGF1 and IGF2 in granulosa cells cultured with either 0, 10 or 20% FCS supplement at 144 h. Data are the mean of 3 replicates + S.E.M. Different superscripts indicate $P<0.001$.

could be amplified with differing efficiencies therefore skewing expression data (Lockhart et al., 1996; Baugh et al., 2001). Microarray expression profiling experiments usually require RNA in larger quantities than are available from small biological samples (Jenson et al., 2003). There are two main methods for increasing nucleic acid concentration, the first being a linear amplification method utilising in vitro transcription (IVT) which involves cDNA being synthesized from RNA to provide a template for amplification by T7 RNA polymerase. An IVT reaction is then performed during which complementary RNA (cRNA) transcript is generated. However, since the second strand is synthesised using random priming, IVT products are not always full length copies and 5' end sequences are often underrepresented (Wilhelm et al., 2006). The PCR-based non-linear amplification method used for this study overcomes these issues and a limited number of studies indicate that the method preserves abundance relationships (Iscove et al., 2002; Ji et al., 2004; Wilhelm et al., 2006). The method also works with markedly less RNA reducing the amount of starting material by up to a million-fold (Iscove et al., 2002).

The second potential reason is that the array may not be sensitive enough to detect subtle dietary-induced expression changes. From the series of culture experiments it was clear from the qRT-PCR data that the expression profiles of those genes chosen were clearly altered between groups. These changes were not subtle (e.g. a 30 fold difference was detected in HSD3B1 through qRT-PCR), yet were still not detectable by the array despite most array technologies using a twofold difference in expression as the threshold for gene expression scoring (Jenson et al., 2003; Clarke et al., 2001). These conclusions were backed up by the results of the experiment set up to detect doubling dilutions of cDNA, in which no linearity following the increasing levels of cDNA was detected by the array.

Given the lack of correlation between the array and qRT-PCR data, this study has dissected each of the possible steps including the amplification method. The qRT-PCR data presented in this chapter conclusively shows that the amplification step maintains gene expression profiles and therefore supports previous published studies. From the comparison of qRT-PCR analysis using amplified vs. non-amplified cDNA we have shown that this particular method of sample preparation maintains the

original expression profile of transcripts (R^2 values all > 0.75, p values all ≤0.01) indicating that the pre amplification method by PCR used in this study did not disproportionately alter transcript levels and can be used reliably to increase low sample levels for array hybridisation. This is consistent with a study which concludes that a non linear amplification method (specifically the Clontech SMART system as used in this project) was more comparable with unamplified samples than IVT (Wilhelm et al., 2006).

Despite the evidence pointing towards a robust sample preparation but a potentially poor array platform we must also consider the problems arising from low sample numbers. Only 13 (8 x n-3, 5 x n-6) out of a possible 21 samples were analysed by array in this study due to insufficient RNA yields and quality. Furthermore, in the validation experiments only 9 samples (3 per treatment group) were analysed. This number of replicates may be enough to detect expression differences by qRT-PCR but this type of array may need substantially more. Due to noise and variability, array technologies highlight a certain percentage of false positives and negatives which could mask true results (Hatfield et al., 2003). This false discovery rate can change depending on experimental variability and the type of statistical analysis used (Zhou et al., 2007), and potentially can fail to detect up to 70% of genes showing a 2 fold change in expression (Cole et al., 2003). However, the array may have been unsuccessful for several other reasons; degraded cDNA on the array causing poor hybridisation; lack of sequence specificity (bovine vs ovine); or underlying analysis software issues (poor sensitivity). Unfortunately these issues were out of the confines of this thesis to investigate.

2.4.2 Effect of serum on granulosa cells in culture

An added benefit to the optimisation studies described is that data was generated on the actual effect of serum in granulosa culture on the expression of steroidogenic related genes. The study demonstrated that progesterone levels generally increased over time whilst oestradiol levels fell, which is consistent with published data (Henderson and Moon, 1979; Henderson et al., 1987; Luck et al., 1990). Patterns of hormone production observed in this study are consistent with the luteinisation of granulosa cells in vitro over time as serum in culture is known to augment the

luteinisation of granulosa cells (Luck, 1989; Luck, 1990), which is reflected in the reduced levels of oestradiol. However, progesterone levels are lower than expected with 20% serum. Conversely, it has been noted that high levels of serum in granulosa culture could reduce hormone production (Luck 1989), although the mechanism remains unclear. This is consistent with the reduced level of progesterone that was detected at 144 hours with 20% FCS. Levels of oestradiol also drop to the lowest concentrations detected at 144 hours with 20% FCS. This at first seemed consistent with the luteinisation of the cells but could in fact be at such low levels due to this inhibitory mechanism brought on by high serum levels in the culture media. Unfortunately, due to cell number restraints we were unable to set up a time course study for a more in depth look at gene expression leading up to this point.

All transcripts show higher expression in cultures absent of serum. The reduction in these steroidogenic-related genes with inclusion of serum in culture media is consistent with the observed reduction of oestradiol levels. However, despite the reduction in STAR and HSD3B1 transcripts, progesterone levels were greater with 10% serum at 144 hours. This could indicate that either the level of transcript has not yet affected the translated protein levels and/or downstream pathways or that other factors which have not been measured are compensating in some way to maintain hormone levels.

2.5 Conclusions

After a thorough investigation of the macroarray associated methodologies, several issues regarding its sensitivity have been highlighted. The amplification system used for sample preparation has been shown to maintain true abundance relationships within samples, allowing us to disregard this as the cause for the discrepancies seen. The main cause for concern is that the array itself fails to detect differences in expression up to 30 fold (HSD3B1), even despite low replicate numbers, a sizable difference such as this should be picked up when most array platforms set a biologically significant fold difference of 2 as a baseline. Additional to this, with the lack of correlation between the macroarray data and qRT-PCR, one can conclude that this particular cDNA macroarray is unsuitable for quantitative gene expression analysis.

CHAPTER 3

The effect of LDL and HDL obtained from serum of ewes fed n-3 or n-6 PUFA enriched diets on granulosa and theca cells cultured in vitro

3.1 Introduction

We have previously reported a significant increase in progesterone levels in the follicular fluid of ewes fed a high n-3 compared to a high n-6 PUFA diet, section 2.2.1.1 (Wonnacott et al., 2010). Ovine granulosa cells aspirated from abattoir derived ovaries were cultured in the presence of HDL derived from n-3 or n-6 PUFA fed ewes in that study, but steroid production was unaltered by PUFA source. Lipoproteins are the main transporter of FA in ruminants, with HDL being the most abundant comprising over 80% of the total, and LDL accounting for less than 10% (Palmquist 1976; Bauchart 1993). Both HDL and LDL have been shown to be involved in ovarian steroid hormone production in humans and non-primate animals (Carr et al., 1982; Veldhuis et al., 1984; O'Shaughnessy et al., 1990; Bao et al., 1995; Ragoobir et al., 2002). HDL is present in ruminant follicular fluid at high levels prior to ovulation (O'Shaughnessy et al., 1990), with the concentration of HDL being up to 20-fold greater than LDL (Wonnacott et al., 2010). However, despite low levels, one of the major functions of LDL is cholesterol tissue distribution and delivery; it is therefore important for steroidogenic tissues and has been implicated as such in ruminants (Bauchart, 1993).

The in vivo ovine study described in Chapter 2 was extended to consider the effect of n-3 and n-6 PUFA enriched LDL and HDL fractions on cultured granulosa and theca cells in the current chapter. As HDL did not seem to affect steroidogenesis in aspirated granulosa cells (Wonnacott et al., 2010), a different sub-population was used; mural granulosa cells, collected via scraping the follicle wall. Theca cells are a highly vascularised sub-layer of the ovarian follicle and are key regulators of ovarian steroidogenesis. Therefore it was important to culture these cells with the same lipoprotein and PUFA treatments as granulosa cells, as the in vivo effects reported by Wonnacott et al. (2010) may have been due to this population of cells rather than granulosa. Experiments in this chapter were designed to assess the effects of

culturing ovine mural granulosa and theca cells in the presence of physiological levels of LDL and HDL derived from sera of ewes offered enriched n-3 and n-6 PUFA diets described in Chapter 2.

The expression of genes involved in steroidogenesis and lipid metabolism was analysed by quantitative real-time PCR. Transcripts included; cholesterol side chain cleavage (CYP11A1), 17-alpha-hydroxylase (CYP17A), steroidogenic acute regulatory protein (STAR), 3 beta-hydroxysteroid dehydrogenase (HSD3B1), scavenger receptor class B member 1 (SCARB1), low density lipoprotein receptor (LDLR), stearoyl-CoA desaturase (delta-9-desaturase) (SCD), elongase 2 (ELOVL2), elongase 5 (ELOVL5), delta-5-desaturase (FADS1) and delta-6-desaturase (FADS2). The molecular analysis was carried out to determine (i) the mechanism by which PUFA alter steroid output in the follicle and (ii) how theca and granulosa cells differ in the uptake and metabolism of FA by expression of key metabolic genes. It was important to look at expression levels for the steroidogenic enzymes CYP11A1, CYP17A and HSD3B1 and also STAR (regulator of cholesterol transfer into mitochondria) to determine the mechanism by which progesterone is increased in ovarian steroidogenic cells. SCARB1 (the HDL scavenger receptor) sequesters cholesterol by selectively removing cholesterol esters from the HDL molecule. LDLR mediates cholesterol uptake via internalisation of the entire LDL particle and its receptor. The desaturase (SCD, FADS1 and FADS2) and elongase (ELOVL2 and ELOVL5) enzymes are important in the synthesis of long chain fatty acids and it was important to identify the expression of these transcripts in granulosa and theca cells. FA analysis was carried out (i) to determine if both cell types are able to sequester and utilise FA from the culture media and (ii) to ascertain which lipoprotein (i.e. LDL or HDL) is the most efficient at delivering FA to follicular cells.

3.2 Materials and methods

All reagents were obtained from Sigma-Aldrich unless otherwise stated. Granulosa- and theca-cell cultures were carried out using four treatments. Standard culture media (TCM199 for granulosa cells and DMEM/F12 Hams for theca cells) were supplemented with either LDL or HDL fractions obtained from serum of ewes fed n-3 or n-6 PUFA enriched diets (sections 2.2.1.1 and 2.2.1.3). The culture experiments,

therefore, were a 2x2 factorial arrangement (LDL vs HDL and n-3 vs n-6 PUFA) and were conducted in 24-well plates (Nunc, Thermo fisher Scientific, Loughborough, UK). Cells within plate and within treatment combination were harvested at 48, 96 and 144 h, giving 2 replicates per treatment combination and per time point per plate. Two plates were cultured on any one occasion and there were 3 occasions, given a total of 6 plates per cell type (experiment).

3.2.1 Fatty acid analysis

3.2.1.1 Fractionation of lipoproteins from serum

Serum from ewes offered n-3 and n-6 PUFA diets (study detailed in sections 2.2.1.1 and 2.2.1.3) were pooled according to dietary group. Lipoproteins were fractionated according to density by sequential ultracentrifugation. Serum was inverted to maximise homogenicity. Potassium bromide (KBr) was added to achieve the required density according to the Radding-Steinberg Formula:

$$X = [(V(d_f - d_i)] / [1- (0.312 \times d_f)]$$

Where X= grams of KBr; V=volume of serum (ml); d_i=initial density; d_f=final density; 0.312 = partial specific volume of KBr.

Serum samples were added to Beckman quick seal ultracentrifuge tubes (Beckman Coulter (UK) Ltd., Buckinghamshire, UK) using a 10ml syringe and needle. To obtain the VLDL fraction, samples were topped up with 1.006g/ml KBr solution (Max 12ml per tube in total). Tubes were sealed with Beckman Tube sealer and samples spun at 39,000 RPM for 16 hours under vacuum at 12°C using a 70.1Ti rotor in a Beckman XL-70 Preparative ultracentrifuge with no brake on deceleration. After centrifugation, the top fraction (VLDL) was removed by cutting the top of the tube in a Kontron bench top tube slicer. The top fraction was removed and stored at -20°C. The remaining sample was resuspended from the pellet at bottom of tube and density adjusted to 1.063g/ml by adding solid KBr using above equation. Samples were added to ultracentrifuge tubes and topped up with 1.060g/ml KBr solution and sealed. Tubes were spun under the same conditions as before but for 20 hours. The

top fraction (LDL) was removed as before and stored at -20°C. The density of the remaining sample was adjusted to 1.21g/ml by adding solid KBr, added to new centrifuge tubes and topped up with 1.24g/ml KBr. The tubes were centrifuged under the same conditions as before but for 40 hours. The top fraction was removed (HDL) and stored at -20°C. The remaining sample was a lipoprotein deficient fraction containing mainly albumin and this was also stored at -20°C.

3.2.1.2 Purification of lipoprotein fractions via dialysis

In order to remove excess KBr from fractionated lipoproteins, samples required dialysis. Dialysis tubing was placed in boiling water for 10 minutes to soften. Approximately 10 inches of 25mm tubing was used for 15 mls of sample. One end of tube was clipped and the tube was rinsed out with 0.15M Sodium chloride (NaCl). Sample was then added to the empty tube, bubbles were removed and the tube closed with a second clip. All dialysis tubes and samples were added to 5 litres 0.15M NaCl, 1mM EDTA, pH 7.4 and placed at 4°C with stirring. After 1 hour, the NaCl solution was replaced, and this was repeated an hour later followed by an overnight incubation. After this dialysis period, samples were removed from the solution and transferred into labelled 15ml falcon tubes for storage at -20°C.

3.2.1.3 Analysis of serum and serum fractions by gas chromatography

One ml serum or serum fraction was pipetted directly into a labelled tube together with 3.75mls of cold, freshly made chloroform:methanol (1:2 v/v) and 200µl 200µg/ml pentadecanoic acid (C15:0; internal standard). Samples were then vortexed for 1 minute. A further 1.25mls chloroform and 1.25 mls HPLC grade water was then added and following a second vortex tubes were centrifuged at 3400 x g at 15°C for 15 minutes. The top water layer was then removed and discarded and the lower organic chloroform phase was carefully collected into a clean labelled tube, taking care not to disturb the mid protein layer. The chloroform was then evaporated to dryness under a steady stream of nitrogen at 40°C. Dried samples were then methylated by initially adding 3ml of methanolic HCL. Samples were then purged with N_2 gas to remove oxygen after which the tubes were sealed tightly and heated at 70°C for 2 hrs in a heated block. Tubes were then removed and left to equilibrate to

room temperature. To stop the methylation reaction 2ml HPLC water was added and vortexed to mix. 500µl HPLC hexane was added, vortexed and centrifuged 3400 x g at 15°C for 15 minutes. Using a glass Pasteur pipette the upper hexane layer was removed and transferred to a GC vial for immediate analysis or storage at -20°C.

3.2.1.4 Gas chromatograph instrumentation and run parameters

The instrument used was an Agilent 6890 series GC system with a 7683 series injector (Agilent Technologies, Cheadle, UK). The column used was a Varian CP-Sil 88 fused silica capillary column (100m x 0.25mm ID, 0.2µm film) (Varian Scientific, Oxford, UK). Fatty acid methyl esters (FAME) suspended in hexane were injected at a volume of 2µl. Two hexane blanks were injected between each sample to prevent carry-over contamination. Identification of individual FAME peaks was based on retention time with reference to a known standard (37 component mix, Supelco, Poole, UK) using Agilent HPCORE Chemstation software (version 1.1). Twenty-six FA from C16:0 upwards were identified. Calibration was achieved by comparison of peak areas for individual FA with peak area for a known concentration of internal standard (C15:0). Run parameters for gas chromatograph are detailed in Table 3.1.

3.2.2 Cell culture

3.2.2.1 Lipoprotein treatments in culture media

LDL and HDL lipoprotein fractions (A.2.3 and A.2.4) were added to culture based on their TFA content. The levels added were formulated to deliver physiological amounts of FA to the theca and granulosa cells. Fatty acid levels equivalent to 5% serum were added to culture media. Inclusion rates of up to 10% serum in culture media are standard in our laboratory and elsewhere (May and Schomberg, 1984; Spicer et al., 1993; Roberts and Echternkamp, 1994; Kayani et al., 2009). Serum TFA concentration (1442µg/ml) was determined before fractionation (A.2.1). Consequently 5% equivalent was calculated to deliver 72.1µg/ml of TFA. Therefore, on average, 57µl of either LDL fraction or 22µl of either HDL fraction was added to each ml of culture media.

Table 3.1 Gas chromatograph column, injection and oven parameters for FA analysis.

Inlet	**Mode:Splitless**	**Gas: H_2**		
Heater temperature	255°C			
Pressure	27.8 psi			
Total flow	67.7 ml/min			
Purge flow to split vent	60ml/min for 0.75 min			
Gas saver	20ml/min for 5 min			
Column				
Mode	Constant flow			
Pressure	28.7 psi			
Flow	2.0 ml/min			
Average velocity	36 cm/sec			
Oven				
Oven ramp	°C/min	Next °C	Hold min	Run time
Initial		59	0.75	0.75
Ramp 1	8	110	0	7.1
Ramp 2	6	170	10	27.1
Ramp 3	3	240	6	56.5
Detector				
Detector temperature	300°C			
H_2 flow	30 ml/min			
Air flow	400 ml/min			
N_2 makeup flow	30			
Lit offset	2			

3.2.2.2 Granulosa and theca cell culture

Sheep ovaries were collected from an abattoir and transported in a flask containing warm PBS to the laboratory within 2 hours. Ovaries were rinsed with 70% ethanol and then with fresh, sterile warm PBS and kept at 37°C in a heated box until needed. Large (>5mm) amber-coloured clear follicles were dissected from the ovaries and kept in warm PBS until the number required (i.e. 30-40) was collected. Follicles were placed in a petri dish containing warm PBS/0.1% PVA. Follicles were cut open

and granulosa cells scraped from inner follicle wall using a sterile inoculation loop. Both mural and antral granulosa cells were collected and searches were performed to remove oocytes. Cells were then spun at 800 x g for 5 minutes. Theca cells were removed by pinching the inner wall with curved forceps and peeling away from the outer shell. Theca sheets were kept in warm media containing 50 IU/ml penicillin and 50µg/ml streptomycin. Once all theca cells had been collected, they were spun at 800 x g for 5 minutes. Theca sheets were then added to a dissociation solution containing 18.5ml DMEM/Hams F12 media with 50IU/ml penicillin, 50µg/ml streptomycin, 5 mg/ml collagenase I, 1mg/ml hyaluronidase and 25µg/ml (188Units/ml) DNase1. Cells were incubated at 37°C for 30 min in shaking water bath. 5% FCS was added to terminate the reaction, and the cell suspension was passed through a 70µm filter to remove remaining clumps. Cells were spun again at 800 x g for 5 minutes and washed twice in warm PBS/0.1% PVA. The supernatant for both cell types were discarded and 9 mls of double-distilled water was added to the cells and mixed in order to lyse red blood cells. 1ml 10x Dulbecco's PBS was added to the tube and mixed again to restore isotonicity. Cells were centrifuged at 800 x g for 3 minutes and washed twice with warm PBS/0.1% PVA, then resuspended in 1ml warm basal culture media. 10µl suspension was removed for cell counts (see section 3.2.2.3) and cells plated down in a 24 well plate at an initial seeding density of $3.5x10^5$ (granulosa) and $2x10^5$ (theca) live cells per well in 500µl culture media in 24-well plates. Plates used for theca cultures were pre-coated with fibronectin (500µl of 6µg/ml fibronectin per well at 39°C for 4 h, removed then air dried for 2 h). Basal media for granulosa cultures consisted of 90% v/v TCM199 and 10% v/v double distilled water supplemented with 1mg/ml BSA, 100ng/ml L-glutamine, 10ng/ml insulin, 0.5µg/ml transferrin, 0.5ng/ml sodium selenite, 50IU/ml penicillin, 50µg/ml streptomycin, 1ng/ml FSH and 100ng/ml testosterone. Theca culture media consisted of DMEM/Hams F12 supplemented with 1mg/ml BSA, 100ng/ml L-glutamine, 10ng/ml insulin, 0.5µg/ml transferrin, 0.5ng/ml sodium selenite, 50IU/ml penicillin, 50µg/ml streptomycin, 1ng/ml LH. Both media were kept at pH 7.4 and 270-280 mOsm. Basal media was supplemented with either n-3 LDL, n-6 LDL, n-3 HDL or n-6 HDL. Lipoproteins were added at 5% serum fatty acid equivalent (LDL 57µl, HDL 22µl per 1ml media) (section 3.2.2.1). Cells were incubated at 38.8°C in a humidified atmosphere of 5% CO_2 for 144 h. Eighty percent of media was collected at 48-h intervals, and replaced with fresh media. Two wells

per treatment were harvested with trypsin EDTA and cell counts performed (section 3.2.2.3). Further cells and media were collected at 96 hours and 144 hours. All cells were snap-frozen in liquid nitrogen prior to storage at -80°C.

3.2.2.3 Cell counts via trypan blue exclusion and trypsin EDTA removal of cultured cells

Cells were resuspended in a known volume of culture media containing FAF-BSA (1%), 50IU/ml penicillin and 50µg/ml streptomycin. A 10µl aliquot was taken and added to 40µl of PBS and 50µl trypan blue solution and mixed thoroughly. The mixture was pipetted into each chamber of a haemocytometer and the number of viable (white) and non-viable (stained blue) cells were counted under x10 magnification of a microscope. Live cells per ml of suspension were then estimated.
Prior to addition of trypsin EDTA, all spent media was removed from 24-well plate wells. 200µl trypsin EDTA was added to each well and incubated at 38°C for 3 minutes. The well bottom was rinsed with a pipette using trypsin EDTA already present, until all cells had detached from the well, and then transferred to a labelled eppendorf. 100µl media containing 5% FCS was added to each well and used to rinse the bottom to ensure maximal cell recovery. This was then added to the rest of the cells in labelled tubes. Cells were washed and resuspended in warm PBS/0.1% PVA and a 10µl aliquot was taken and added to 15µl of PBS and 25µl trypan blue solution and used to perform cell counts as above. All cells were snap frozen in liquid nitrogen and stored at -80°C for later analysis.

3.2.3 Steroid hormone analysis

3.2.3.1 Oestradiol ELISA

Oestradiol concentrations in spent media were measured by ELISA. Media samples were diluted up to 1:10 in PBS. All samples were run in duplicate along with standards (3.9, 7.8, 15.6, 31.25, 62.5, 125, 250, 500 and 1000pg/100µl) and QCs (7.8 and 31.25 pg/100µl). 96-well plates (Immuno-Maxisorp, Nuncbrand Fisher Scientific) were coated with 17-beta Estradiol 3 antibody (Abcam) at a dilution of 1:64000 in coating buffer (50mM Sodium bicarbonate, 50mM Sodium carbonate, pH

9.6). Plates were sealed and incubated overnight at 4°C. Plates were washed 4 times in wash buffer (PBS, 0.05% Tween20, pH7.4). Plates were then tapped dry and 100µl blocking solution (PBS, 3%BSA) added to each well and incubated at room temperature for 2 h. Plates were then washed again 4 times and tapped dry. 100µl of either sample, standard or QC was added to each well together with 100µl HRP conjugate and incubated for 2 h at room temperature. Plates were washed 4 times and tapped dry. 50µl Tetramethylbenzidine (TMB) was added to each well and incubated for 12 min at room temperature. 50µl stop solution (1N H_2SO_4) was added to each well and plates read at 450nm. Intra and inter-assay CV were 9.8% and 4.2% respectively.

3.2.3.2 Progesterone ELISA

Progesterone ELISA was carried out on spent media as previously described in section 2.3.2.3. Samples were diluted up to 1:400 for theca cell media and up to 1:100 for granulosa cell media in PBS. Intra and inter-assay CV were 7.9% and 8.4% respectively.

3.2.4 Fatty acid analysis of granulosa and theca cells via gas chromatography

Frozen cells were thawed over ice and transferred to a labelled tube together with 3.75ml of cold, freshly made chloroform:methanol (2:1 v/v) and 100µl 200µg/ml pentadecanoic acid, and vortexed to mix. Samples were wrapped in foil and placed in a fridge overnight. 1.25ml of 0.88% KCl solution was added to each sample to aid quantitative transfer of residual material. Tubes were centrifuged at 800 x g at 8°C for 15 min. The top water layer was removed and discarded. The lower organic chloroform phase was collected into a clean labelled tube, careful not to disturb the mid protein layer. 1.25 ml cold chloroform was added to each tube containing protein and cell debris and mixed and centrifuged again to maximise extraction. The remaining chloroform was collected and added to the previously collected sample. Chloroform was evaporated to dryness at 40°C, under a steady stream of nitrogen gas. For methylation, 3ml of methanolic HCL (5% v/v) was added to the dried sample. Samples were purged with N_2 gas to remove oxygen and tubes sealed tightly then heated at 70°C for 2 h in a heated block. Tubes were removed and left to

equilibrate to room temperature. To stop the methylation reaction 2ml HPLC water was added and vortexed to mix. 200µl HPLC hexane was added, vortexed and centrifuged at 3400 x g at 15°C for 15 min. The upper hexane layer was removed using a glass pasteur pipette and transferred to a clean GC vial for immediate analysis or storage at -20°C. GC oven parameters are detailed in section 3.2.1.4 and Table 3.1.

3.2.5 RNA extraction and RT reaction

RNA was extracted from cells and reverse transcribed into cDNA as previously described in sections 2.2.1.4 and 2.3.2.5.

3.2.6 Quantitative RT-PCR

Transcripts were analysed by qRT-PCR as previously described in section 2.2.1.12. SCARB1, ELOVL2 and FADS1 used forward and reverse primer concentrations of 1.2µl (10pmol/µl); all other primers used 0.6µl (10pmol/µl) of each primer. Cycling parameters and primer and probe sequences can be found in Tables 2.3 and 3.2 (HSD3B1 details can be found in table 2.5). Transcripts were analysed relative to β-actin (ACTB) (Table 2.2).

3.2.7 Statistical analysis

To recap, granulosa and theca cell experiments were conducted separately, each as a 2 x 2 x 3 factorial (i.e. LDL vs HDL; n-3 vs n-6 PUFA; and three sample times (48, 96 and 144 h)) arrangement in 6 x 24-well plates. Cells within plate and within treatment combination were harvested from separate wells at 48, 96 and 144 h for FA and transcript analyses, and spent culture media analysed for steroid hormones.

All statistical analyses were conducted using Genstat release 11.1 (Genstat, 2009). FA and steroid concentrations in media and cells were analysed by ANOVA as a three-way factorial randomised block design. Progesterone data were transformed (log_{10}) prior to analysis to correct for differences in means and variance across the

Table 3.2 Alphabetical list of primers and probes used for qRT-PCR. (F = forward primer, R = reverse primer).

Name	Sequence 5'-3'	NCBI Accession	Primer Efficiency
ACTB			
F	TGTGCGTGACATCAAGGAGAA	AF129289	1.92
R	CGCAGTGGCCATCTCCTG	Ovis aries	
Probe	**FAM**-CTGCTACGTGGCCCTGGACTTCGA-**TAMRA**		
CYP11A1			
F	TCGGCAATTTGGAGTCAGTTT	D50057	1.96
R	GGTAGGATCCCTCGAACTTAAAGAG	Ovis aries	
Probe	**FAM**-ATCATTCACCCTGAAGGCGTGGCC-**TAMRA**		
CYP17A			
FP	TCATCTCGCCATCGTTAAGCT	AF251388	1.96
RP	CGGGCTAGCATCTCACCTACA	Ovis aries	
Probe	**FAM**-TTGCCCTTTGGAGCCGGACCC-**TAMRA**		
ELOVL2			
F	TTCCTTGTGGGCAAAGTTTCTT	BC114786	1.99
R	GCCCGTAGTAGGAGTACATGAGGAT	Bos Taurus	
Probe	**FAM**-ACGTCTACCACCACGCCTCCATGTTT-**TAMRA**		
ELOVL5			
F	TCACGGTCCTGCATGTGTATC	BC105391	1.83
R	GACCCAGTTCATCACGAACCA	Bos Taurus	
Probe	**FAM**-CCACGCCAGCATGCTCAACATCTG-**TAMRA**		
FADS1			
F	AGCTGCCCCTGAGGATATTTC	EU272818	1.91
R	CGACCACTCGAAGATGTCGTT	Ovis aries	
Probe	**FAM**-CTGCTGCCAACTCCATGGCTTTCC-**TAMRA**		
FADS2			
F	TGGCCTGCATGCTCATCA	BC133520	1.98
R	TGGAAGATGTTGACGTGGAGAT	Bos Taurus	
Probe	**FAM**-AGGTCCCTGCTGGCCCACCC-**TAMRA**		
LDLR			
F	GAGCGTGGGTGCCCTATACA	K01830	1.87
R	TTCCGAAGGCCAGGAGG	Bos Taurus	
Probe	**FAM**-CGTCCTCCCCATCGCACTGCTC-**TAMRA**		
SCARB1			
F	TCCAAGGCCAGAAGCCAC	AF019384	1.77
R	GCTCTTGTGCCTGAACTCCC	Bos Taurus	
Probe	**FAM**-TGCAGGAGCACGGGCCTTATGTGTA-**TAMRA**		
SCD			
F	CGAACCTACAAAGCTCGGCT	NM_001009254	1.97
R	TGGAACGCCATGGTGTTG	Ovis aries	
Probe	**FAM**-CCCCTACGGGTCTTCCTGATCATCG-**TAMRA**		
STAR			
F	GCGACGTTTAAGCTGTGTGCT	AF290202	1.87
R	TGCTGCCGCAGCCC	Ovis aries	
Probe	**FAM**-AGCTCCTATAGACACGTGCGCAGCATG-**TAMRA**		

three time points. Data are presented as geometric means with log_{10} means and SED presented in italics. Where a FA was not detectable in a single treatment (i.e. a FA percentage < 0.03g/100g TFA), then the FA percentage in the other treatment was analysed by using a two-tailed t-test to determine if it was significantly greater than the detection limit. Data are presented as predicted means ± SEM.

3.3 Results

3.3.1 Treatment effects on cell proliferation and steroid hormone synthesis

3.3.1.1 Granulosa cells

There was no effect of treatment on granulosa cell proliferation (Table 3.3a). Cell number peaked (P<0.001) at 96 h and decreased between 96 and 144 h. Cell number-adjusted oestradiol concentrations decreased (P<0.001) over time and were lowest at 144 h. In contrast, cell number-adjusted progesterone concentrations increased (P<0.001) over time and were maximal at 144 h. There was no effect of treatment on steroid hormone production in these cells.

3.3.1.2 Theca cells

Theca cell number increased (P<0.001) with time during culture (Table 3.3b), and interacted (P=0.012) with lipoprotein fraction to determine the rate of increase, which was greater for LDL than HDL supplemented cells (LDL supplemented cells witnessed a two fold increase between 48 and 144 h, whereas HDL supplemented cells witness a 1.6 fold increase over the same time period). Cell number adjusted progesterone concentrations increased (P<0.001) over time, and were influenced by dietary source (P<0.001), with n-3 PUFA producing greater levels of progesterone than n-6. When all time points considered there was no effect of lipoprotein on progesterone output, however at 144 hours there was a lipoprotein x dietary source interaction with an increase (P=0.037) in progesterone production with n-3 HDL compared to all other treatments (Figure 3.1). In contrast, cell number-adjusted oestradiol concentrations remained at a constant low level across all treatments and timepoints.

Tables 3.3 Effect on (a) granulosa and (b) theca cell proliferation, oestradiol (E_2) and progesterone (P_4) production during 144 h culture. Media supplemented with LDL or HDL from serum of ewes offered diets enriched with either n-3 or n-6 PUFA.

(a) Granulosa

Lipoprotein (L)	**LDL**		**HDL**			**Time of culture (hours) (T)**				**Significance**				
Dietary Source (D)	**n-3**	**n-6**	**n-3**	**n-6**	**SED**	**48**	**96**	**144**	**SED**	**L**	**D**	**T**	**LxD**	**LxT**
n	**36**	**36**	**36**	**36**		**48**	**48**	**48**						
Cell number (x10^5)	2.93	3.00	3.18	3.02	0.152	2.81^x	3.41^y	2.86^x	0.132	-	-	<0.001	-	-
E2 (pg/10^5 cells)	1289	1276	1259	1186	77.1	2337^x	847^y	566^z	66.8	-	-	<0.001	-	-
P4 (pg/10^5 cells)	345^a	281^{ab}	256^b	232^b		49^x	442^y	959^z						
(log_{10})	(2.538)	(2.450)	(2.408)	(2.366)	(0.0577)	(1.694)	(2.645)	(2.982)	(0.0499)	0.012	-	< 0.001	-	-

(b) Theca

Lipoprotein (L)	**LDL**		**HDL**			**Time of culture (hours) (T)**				**Significance**				
Dietary Source (D)	**n-3**	**n-6**	**n-3**	**n-6**	**SED**	**48**	**96**	**144**	**SED**	**L**	**D**	**T**	**LxD**	**LxT**
n	**36**	**36**	**36**	**36**		**48**	**48**	**48**						
Cell number (x10^5)	2.47^a	2.39^a	2.29^a	2.31^a	0.10	1.70^x	2.36^y	3.03^z	0.091	-	-	<0.001	-	0.012
E2 (pg/10^5 cells)	69	70	69	66	7.5	74	62	69	6.5	-	-	-	-	-
P4 (pg/10^5 cells)	149^a	100^a	218^b	105^a		14^x	231^y	783^z						
(log_{10})	(2.172)	(2.000)	(2.339)	(2.020)	(0.0842)	(1.139)	(2.364)	(2.894)	(0.0729)	-	< 0.001	< 0.001	-	-

Means within a row with different superscripts (i.e. a, b or c; x, y or z) are significantly different (P<0.05). SED, Standard error of difference.

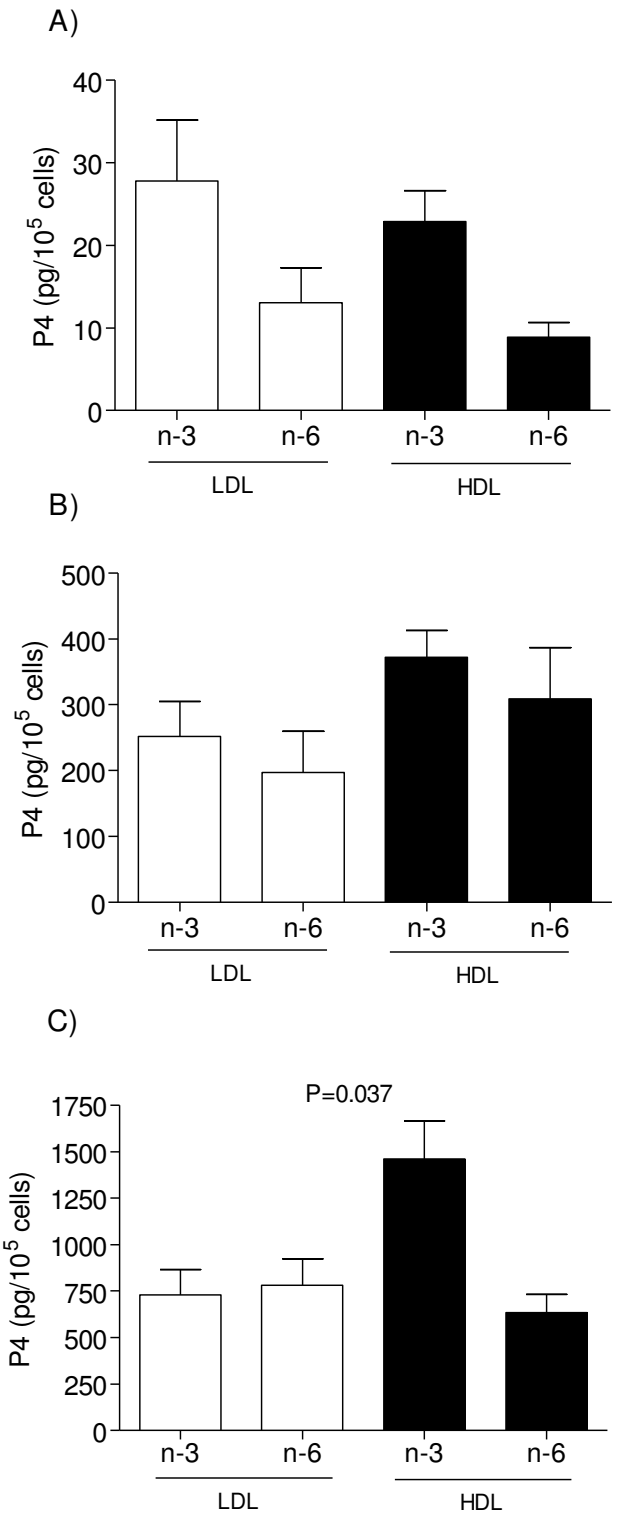

Figure 3.1 Progesterone production at (A) 48 hours, (B) 96 hours and (C) 144 hours by theca cells cultured in vitro supplemented with LDL or HDL from sera of ewes offered diets enriched with either n-3 or n-6 PUFA (n=12).

3.3.2 Fatty acid composition of in vitro cultured cells

3.3.2.1 Granulosa cells

Total FA content per 100,000 granulosa cells at 144 h of culture did not differ between treatment groups (Table 3.4). Proportions of saturated and total unsaturated FA also did not differ between culture groups. The proportional increase (P=0.04) in MUFA for LDL relative to HDL, was largely attributable to the n-6 LDL culture group. The proportion of PUFA was higher (P=0.004) in cells cultured with HDL irrespective of PUFA source. The proportion of n-6 PUFA increased (P=0.014) in cells cultured with n-6 LDL or HDL and the proportion n-3 PUFA increased (P=0.04) in the presence of either n-3 LDL or HDL. The ratio of n-6 to n-3 FA was higher (P=0.02) for cells cultured in the presence of n-6 compared to n-3 LDL or HDL.

In the present study, the most abundant FA in granulosa cells was oleic acid (C18:1n-9c), comprising on average 30.9 ± 0.35 g/100g of TFA (Table 3.5). Its content was greater (P<0.05) in LDL than HDL, and n-6 than n-3 cultured cells. In fact, its content was greatest for n-6 LDL supplemented group. The second most abundant FA was palmitic acid (C16:0) comprising 23.18 ± 0.18 g/100g TFA, and stearic acid (C18:0) was the third (11.5 ± 0.66 g/110g TFA). Of the seven n-6 PUFA identified, three were increased in cells cultured with n-6, regardless of lipoprotein delivery, Linoleic (C18:2n-6) (P<0.001), γ-linolenic (C18:3n-6) (P=0.02), and eicosadienoic (C20:2n-6) (P=0.02). The remaining four n-6 FA were numerically greater but not statistically significant. Similarly, of the five n-3 PUFA identified, four were increased in cells cultured with n-3; α-linolenic acid (C18:3n-3) (P=0.002), eicosapentanoic acid (C20:5n-3) (P<0.001), docosapentaenoic acid (C22:5n-3) (P=0.003) and docosahexaenoic acid (C22:6n-3) (P=0.02).

3.3.2.2 Theca cells

Total FA content per 100,000 granulosa cells at 144 h of culture did not differ between treatment groups (Table 3.4). Proportions of saturated, unsaturated FA and PUFA did not differ between culture groups. The proportional increase (P<0.001) in

MUFA for n-6 relative to n-3 was largely due to the n-6 LDL culture group. This increase led to a significant (P=0.004) interaction of lipoprotein vs dietary source (n-3 or n-6). The proportion of n-6 PUFA increased (P<0.001) in cells cultured with n-6 and n-3 increased (P<0.001) in n-3 treated cells irrespective of lipoprotein delivery. The ratio of n-6 to n-3 FA was higher (P<0.001) for cells cultured in the presence of n-6 compared to n-3.

As for granulosa cells, the most abundant FA was oleic acid (C18:1n-9c), comprising on average 31 ± 0.35 g/100g of TFA (Table 3.6). Its content was greatest (P<0.001) in cells cultured with n-6 PUFA. The second most abundant FA was palmitic acid (C16:0) comprising 21.2 ± 0.52 g/100g TFA, and stearic acid (C18:0) was the third (12.35 ± 0.26 g/110g TFA). Of the seven n-6 FA identified, four were increased in cells cultured with n-6; Linoleic (C18:2n-6) (P<0.001), eicosadienoic (C20:2n-6) (P=0.002), dihomo-γ-linolenic acid (C20:3n-6) (P=0.009) and arachidonic acid (P<0.001). Similarly, all five identified n-3 FA were increased in cells cultured with n-3 (all P<0.001). Lipoprotein did not have an effect on FA uptake in these cells.

3.3.3 Transcript expression of in vitro cultured cells

3.3.3.1 Granulosa cells

Transcript expression for SCARB1 and LDLR was unaffected by time or treatment (Table 3.7). Expression of SCD increased (P<0.001) by 7.5 fold between 48 and 144 h of culture, whereas FADS1 increased (P<0.001) at 144 h. Transcript expression for FADS2 increased (P<0.001) by 96 h and declined by 144 h. Levels of SCD, FADS1 and FADS2 expression were not altered by treatment and ELOVL5 expression was not affected by time or inclusion of culture treatment. By 96 h of culture, transcript levels of HSD3B1 had decreased (P<0.001) and increased again by 144 h, but was not affected by culture treatment. Inclusion of LDL during culture led to an increase (P=0.018) in transcript expression of STAR compared to HDL, dietary source (n-3 vs n-6) had no effect on transcript expression. STAR transcript levels also increased (P<0.001) over time. CYP11A1 expression was not affected by time or inclusion of culture treatment. Transcripts for ELOVL2 and CYP17A were not detected in these cells.

Table 3.4 Fatty acid composition (g/100g) of granulosa and theca cells cultured for 144 hours in media supplemented with LDL or HDL from serum of ewes offered diets enriched with either n-3 or n-6 PUFA (n=4).

Lipoprotein (L)	**LDL**		**HDL**		**SED**	**Significance**		
Dietary source (D)	**n-3**	**n-6**	**n-3**	**n-6**		**L**	**D**	**LxD**
Granulosa cells								
TFA (µg/10^5 cells)	5.55	6.5	6.11	6.04	0.7	-	-	-
Saturated	40.2	38.0	39.1	38.8	0.71	-	-	-
Unsaturated	54	56.2	55.2	55.6	0.76	-	-	-
MUFA	36.1^{a}	38.0^{b}	35.6^{a}	35.9^{a}	0.55	0.04	-	-
PUFA	17.9^{a}	18.2^{a}	19.6^{b}	19.6^{b}	0.41	0.004	-	-
n-6 series	10.4^{a}	12.3^{b}	10.8^{a}	13.4^{b}	0.73	-	0.014	-
n-3 series	7.51^{a}	5.95^{b}	8.82^{a}	6.22^{b}	0.86	-	0.04	-
Ratio n-6:n-3	1.44^{a}	2.24^{b}	1.25^{a}	2.23^{b}	0.3	-	0.02	-
Theca cells								
TFA (µg/10^5 cells)	3.62^{a}	4.76^{b}	4.33^{ab}	4.14^{ab}	0.42	-	-	0.04
Saturated	38.6	35.3	35.9	36.9	1.12	-	-	-
Unsaturated	55.6	58.8	58.3	57.3	1.06	-	-	-
MUFA	32.6^{a}	37.2^{b}	34.7^{c}	35.5^{c}	0.77	-	<0.001	0.004
PUFA	22.9	21.7	23.6	21.8	0.89	-	-	-
n-6 series	11.7^{a}	15.8^{b}	12.2^{a}	16.1^{b}	0.44	-	<0.001	-
n-3 series	11.3^{a}	5.8^{b}	11.4^{a}	5.7^{b}	0.5	-	<0.001	-
Ratio n-6:n-3	1.03^{a}	2.74^{b}	1.07^{a}	2.84^{b}	0.08	-	<0.001	-

Means within a row with different superscripts are significantly different (P<0.05). SED, standard error of the difference; TFA, total fatty acids.

Table 3.5 Fatty acid composition of ovine granulosa cells cultured for 144 hours in vitro in media supplemented with low or high density lipoprotein (LDL, HDL) from serum harvested from ewes offered diets enriched in n-3 or n-6 PUFA (n=4).

Lipoprotein (L)	**LDL**		**HDL**		**SED**	**Significance**		
Dietary source (D)	**n-3**	**n-6**	**n-3**	**n-6**		**L**	**D**	**LxD**
Fatty acids	**g/100g TFA**							
C16:0	23.4	22.4	23.5	23.4	0.31	-	-	-
C17:0	0.54	0.49	0.56	0.52	0.03	-	-	-
C18:0	11.4	11.8	11.4	11.4	0.17	-	-	-
C20:0	0.27	0.25	0.26	0.24	0.01	-	-	-
C21:0	0.42^{a}	0.41^{a}	0.28^{b}	0.33^{ab}	0.05	0.05	-	-
C22:0	0.12	0.12	0.13	0.1	0.01	-	-	-
C23:0	3.94	2.57	2.91	2.55	0.98	-	-	-
C24:0	0.1	0.06	0.12	0.26	0.07	-	-	-
C16:1	2.49	2.11	2.4	2.31	0.11	-	-	-
C17:1	0.47	0.62	0.55	0.77	0.15	-	-	-
C18:1n-9t	0.6	0.51	0.61	0.56	0.36	-	-	-
C18:1n-9c	30.5^{a}	32.8^{b}	30.1^{a}	30.4^{a}	0.55	0.03	0.04	-
C20:1	1.27^{a}	1.35^{b}	1.18^{c}	1.20^{ac}	0.03	0.005	-	-
C22:1n-9	0.73^{a}	0.63^{b}	0.76^{a}	0.63^{b}	0.04	-	0.02	-
C18:2n-6t	0.51	0.21	0.22	0.28	0.08	-	-	-
C18:2n-6c	4.95^{a}	5.72^{b}	5.26^{a}	5.70^{b}	0.1	-	<0.001	-
C18:3n-6	0.37^{a}	0.45^{b}	0.33^{a}	0.41^{ab}	0.03	-	0.02	-
C20:2n-6	0.40^{a}	0.46^{b}	0.42^{a}	0.47^{b}	0.02	-	0.02	-
C20:3n-6	0.51	0.87	0.62	0.69	0.2	-	-	-
C20:4n-6	3.52	4.38	3.75	5.67	0.63	-	-	-
C22:2n-6	0.19	0.19	0.21	0.2	0.02	-	-	-
C18:3n-3	0.52^{a}	0.37^{b}	0.54^{a}	0.38^{b}	0.04	-	0.002	-
C20:3n-3	0.13	1.05	0.12	0.07	0.49	-	-	-
C20:5n-3	1.63^{a}	0.58^{b}	2.11^{a}	1.02^{b}	0.21	-	<0.001	-
C22:5n-3	3.31^{a}	2.35^{b}	3.77^{a}	2.81^{b}	0.24	-	0.003	-
C22:6n-3	1.91^{a}	1.60^{b}	2.27^{c}	1.95^{ab}	0.11	0.01	0.02	-
Unidentified	5.82	5.77	5.62	5.62	0.22	-	-	-

Means within a row with different superscripts are significantly different (P<0.05). SED, standard error of the difference; TFA, total fatty acids.

Table 3.6 Fatty acid composition of ovine theca cells cultured for 144 hours in media supplemented with low or high density lipoprotein (LDL, HDL) from serum harvested from ewes offered diets enriched in n-3 or n-6 PUFA (n=4).

Lipoprotein (L)	**LDL**		**HDL**		**SED**	**Significance**		
Dietary source (D)	**n-3**	**n-6**	**n-3**	**n-6**		**L**	**D**	**LxD**
Fatty acids	**g/100g TFA**							
C16:0	23.3[a]	21.1[b]	19.6[b]	20.8[b]	0.89	-	0.037	-
C17:0	0.66	0.68	0.74	0.66	0.05	-	-	-
C18:0	12.3	12.5	11.7	12.9	0.39	-	-	-
C20:0	0.23	0.24	0.22	0.25	0.01	-	-	-
C21:0	0.18	0.16	0.12	0.2	0.03	-	-	-
C22:0	0.23	0.23	0.22	0.24	0.01	-	-	-
C23:0	1.41	1.44	1.54	1.31	0.14	-	-	-
C24:0	0.22[a]	0.47[b]	0.26[a]	0.51[b]	0.06	-	<0.001	-
C16:1	0.89	0.74	1.11	0.88	0.09	-	-	-
C17:1	0.42	0.57	0.36	0.42	0.08	-	-	-
C18:1n-9t	0.21[a]	0.37[b]	0.25[a]	0.31[b]	0.04	-	0.01	-
C18:1n-9c	29.2[a]	32.7[b]	30.8[b]	31.3[b]	0.51	-	<0.001	0.012
C20:1	1.10[a]	1.54[b]	1.07[a]	1.34[b]	0.08	-	<0.001	-
C22:1n-9	0.83	1.3	1.13	1.19	0.13	-	-	-
C18:2n-6t	0.19	0.19	0.17	0.2	0.02	-	-	-
C18:2n-6c	6.92[a]	8.58[b]	7.23[a]	8.22[b]	0.25	-	<0.001	-
C18:3n-6	0.25[a]	0.15[b]	0.27[a]	0.11[b]	0.03	-	<0.001	-
C20:2n-6	0.62[a]	0.80[b]	0.52[a]	0.84[b]	0.07	-	0.002	-
C20:3n-6	0.90[a]	1.04[b]	1.00[b]	1.10[b]	0.04	-	0.009	-
C20:4n-6	2.55[a]	4.79[b]	2.78[a]	5.35[b]	0.28	-	<0.001	-
C22:2n-6	0.22	0.31	0.23	0.29	0.04	-	-	-
C18:3n-3	0.76[a]	0.50[b]	0.75[a]	0.62[b]	0.04	-	<0.001	-
C20:3n-3	0.24[a]	0.08[b]	0.22[a]	0.07[b]	0.01	-	<0.001	-
C20:5n-3	2.67[a]	0.65[b]	2.62[a]	0.40[b]	0.12	-	<0.001	-
C22:5n-3	5.18[a]	2.92[b]	5.26[a]	2.91[b]	0.26	-	<0.001	-
C22:6n-3	2.47[a]	1.66[b]	2.59[a]	1.72[b]	0.15	-	<0.001	-
Unidentified	5.78	5.86	5.74	5.85	0.19	-	-	-

Means within a row with different superscripts are significantly different (P<0.05). SED, standard error of the difference; TFA, total fatty acids.

3.3.3.2 Theca cells

Transcript expression for SCARB1 increased (P<0.001) over time (Table 3.8). There was a lipoprotein x diet interaction (P=0.008) with highest transcript levels observed with inclusion of n-3 LDL. LDLR was unaffected by culture treatment or time. Transcript levels increase for SCD (P>0.001), FADS1 (P<0.001) and ELOVL5 (P<0.001) over time, but none are affected by culture treatment. Expression of FADS2 was not affected by treatment or time. Transcripts expression for CYP11A1 increased (P<0.001) over time, whereas expression for CYP17A decreased (P<0.001) with time during culture so that, by 144 h, levels were around 8% of those at 48 h. HSD3B1 transcripts levels increased (P<0.001) by 8-fold between 48 and 144 h. Transcripts for CYP11A1, CYP17A and HSD3B1 were not affected by culture treatment. Transcripts levels for STAR increased (P<0.001) over time and inclusion of n-3 PUFA led to an increase (P=0.006) of expression compared to n-6 PUFA, lipoprotein source did not have an effect. Transcript for ELOVL2 was not detected in these cells.

3.4 Discussion

Experiments in this chapter demonstrated that both granulosa and theca cells cultured in the presence of LDL and HDL were able to successfully proliferate and undergo steroidogenesis. This study is the first to demonstrate the effects of LDL and HDL from either an n-3 or n-6 dietary source on fatty acid composition of theca cells cultured in vitro. It is also the first to demonstrate the effects of these culture conditions on transcripts expression of ELOVL5, FADS1 and FADS2 in both granulosa and theca cells and SCD in theca cells in vitro. The major finding of this study is that HDL from an n-3 PUFA background, but not an n-6 PUFA background, increases progesterone production at 144 hours in cultured theca, but not granulosa cells. This observation supports the increase in progesterone in follicular fluid observed in ewes offered an n-3 PUFA enriched diet, and our previous failure to detect differences in progesterone in cultured granulosa cells (Wonnacott et al., 2010). Data presented in this chapter indicates that this increase is mediated by an HDL-specific signalling mechanism and this is associated with an increase in STAR expression in response to n-3 PUFA.

Table 3.7 Granulosa cell transcript abundance (relative to ACTB) after culture in media supplemented with either HDL or LDL fractionated from sera of ewes offered n-3 or n-6 PUFA enriched diets (n=3).

Lipoprotein (L)	**LDL**		**HDL**			**Time of culture (hours) (T)**				**Significance**			
Dietary Source (D)	**n-3**	**n-6**	**n-3**	**n-6**	**SED**	**48**	**96**	**144**	**SED**	**L**	**D**	**T**	**LxD**
n	**9**	**9**	**9**	**9**		**12**	**12**	**12**					
SCARB1	0.545	0.537	0.516	0.604	0.1230	0.508	0.518	0.627	0.1066	-	-	-	-
LDLR	0.0175	0.0177	0.0179	0.0218	0.2792	0.0158	0.0217	0.0187	0.0024	-	-	-	-
SCD	0.0753	0.0704	0.0734	0.0925	0.0087	0.0197^{x}	0.0666^{y}	0.1475^{z}	0.0076	-	-	<0.001	-
FADS1	0.0036	0.003	0.0034	0.0038	0.0008	0.0024^{x}	0.0024^{x}	0.0056^{y}	0.0007	-	-	<0.001	-
FADS2	0.0007	0.0008	0.0008	0.0007	0.0001	0.0005^{x}	0.0010^{y}	0.0007^{z}	0.0001	-	-	<0.001	-
ELOVL5	0.166	0.14	0.149	0.166	0.0283	0.153	0.148	0.165	0.0245	-	-	-	-
CYP11A1	0.1127	0.1065	0.0997	0.1001	0.0222	0.108	0.1003	0.1059	0.0193	-	-	-	-
HSD3B1	0.0731	0.0677	0.0667	0.0782	0.0090	0.0796^{x}	0.0405^{y}	0.0941^{x}	0.0080	-	-	<0.001	-
STAR	0.0049^{a}	0.0044^{a}	0.0033^{b}	0.0033^{b}	0.0007	0.0015^{x}	0.0033^{y}	0.0069^{z}	0.0006	0.018	-	<0.001	-

Means within a row with different superscripts are significantly different (P<0.05), SED, standard error of differences.

Table 3.8 Theca cell transcript abundance (relative to ACTB) after culture in media supplemented with either HDL or LDL fractionated from sera of ewes offered n-3 or n-6 PUFA enriched diets (n=3).

Lipoprotein (L)	**LDL**		**HDL**			**Time of culture (hours) (T)**				**Significance**			
Dietary Source (D)	**n-3**	**n-6**	**n-3**	**n-6**	**SED**	**48**	**96**	**144**	**SED**	**L**	**D**	**T**	**LxD**
n	**9**	**9**	**9**	**9**		**12**	**12**	**12**					
SCARB1	0.2334^{a}	0.2017^{b}	0.1917^{b}	0.2208^{a}	0.0149	0.1226^{x}	0.2347^{y}	0.2785^{z}	0.0129	-	-	<0.001	0.008
LDLR	0.0059	0.0062	0.0063	0.0070	0.0011	0.0063	0.0056	0.0073	0.0008	-	-	-	-
SCD	0.0142	0.0177	0.0115	0.0132	0.0017	0.0085^{x}	0.0102^{x}	0.0191^{y}	0.0014	-	-	<0.001	-
FADS1	0.0029	0.0028	0.0029	0.0033	0.0003	0.0017^{x}	0.0031^{y}	0.0041^{z}	0.0003	-	-	<0.001	-
FADS2	0.0012	0.0013	0.0013	0.0013	0.0001	0.0013	0.0013	0.0013	0.0001	-	-	-	-
ELOVL5	0.1069^{a}	0.0776^{b}	0.0836^{b}	0.0898^{b}	0.0098	0.0741^{x}	0.0790^{x}	0.1153^{y}	0.0085	-	-	<0.001	0.018
CYP11A1	0.0633	0.0671	0.0642	0.0694	0.0062	0.0423^{x}	0.0612^{y}	0.0945^{z}	0.0053	-	-	<0.001	-
CYP17A	0.0052	0.0049	0.0039	0.0045	0.0012	0.0118^{x}	0.0012^{y}	0.0009^{y}	0.0010	-	-	<0.001	-
HSD3B1	0.0539	0.0612	0.0562	0.0655	0.0129	0.0127^{x}	0.0599^{y}	0.105^{z}	0.0112	-	-	<0.001	-
STAR	0.018^{a}	0.010^{b}	0.018^{a}	0.012^{b}	0.0031	0.004^{x}	0.012^{y}	0.027^{z}	0.0027	-	0.006	<0.001	-

Means within a row with different superscripts are significantly different (P<0.05), SED, standard error of differences.

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3.4.1 Cell proliferation and steroidogenesis

Several studies have reported that both LDL and HDL can provide cholesterol for steroid hormone synthesis and aid proliferation of theca and granulosa cells in culture (O'Shaughnessy et al., 1989; Bao et al., 1995; Bao et al., 1997; Ragoobir et al., 2002; Cherian-Shaw et al., 2009). However, studies so far have reported effects in granulosa and granulosa-lutein cells, and thus far effects in theca cells have been reported only by Bao et al. (1995). In agreement with Bao et al. (1995), who have shown HDL promoting progesterone production in granulosa cells having undergone partial luteinisation, the current study found progesterone levels increase and oestradiol decease over time in granulosa cells, which is indicative of luteinisation. However no difference between LDL and HDL were noted. Theca cells in this study produced oestradiol at very low levels throughout and progesterone levels increased over time. This is also in agreement with Bao et al. (1995), who also noted that HDL, but not LDL increased progesterone production in theca cells. In the current study HDL also increased progesterone production between 96 and 144 h compared to LDL, but only HDL from the n-3 source, and not the n-6, led to this increase. This indicates that HDL stimulates progesterone production by a mechanism not solely reliant on cholesterol delivery. To illustrate this, Chandras et al. (2004) found that lipid-free apolipoprotien-A1 (the major protein component of HDL) increased progesterone production in human granulosa-lutein cells following 3 days of culture.

3.4.2 Transcript expression

Transcript levels for HSD3B1 and STAR increased in granulosa cells over time, this is consistent with the increase in progesterone production over the same time frame. This finding is consistent with a study by Zheng et al. (2009) who noted a similar pattern in bovine granulosa cells over the same time scale. SCD transcript levels also increased over time, but were not altered by treatment. A study by Marra and de Alaniz (1989) reported a significant increase in SCD expression in the liver of rats given an injection of testosterone. Testosterone is included as standard in granulosa culture as a precursor for oestradiol synthesis, and is replenished every 48 hours with media changes. Insulin is also included in our cultures as standard and has been reported to increase SCD expression in adipose tissue of sheep (Daniel et al., 2004).

Transcript levels of FADS1 increased at 144 h, whereas FADS2 increased at 96 and dropped again by 144 h. These enzymes also have been shown to be upregulated by insulin (Brenner, 2003) and testosterone in rat sertoli cells (Hutado de Catalfo and de Gomez Dumm, 2005).

In theca cells, transcript levels for CYP11A, HSD3B1 and STAR increased over time. This again is consistent with the increase in progesterone production over time. LH and insulin have been reported to increase STAR expression in cultured porcine theca cells (Zhang et al., 2000). CYP11A1 and HSD3B1 have also been shown to be increased with addition of LH in culture (Keeney and Mason 1992; Clemens et al., 1994). Again, insulin and LH are standard additives for theca culture in our laboratory. Levels of CYP17A decreased over time to lowest levels at 144 h, suggesting that by this stage of culture theca cells have begun to luteinise (Demeter-Arlotto et al., 1993). Transcript levels for ELOVL5, SCD and FADS1 increased with time during culture. As mentioned previously, desaturases have been shown to be mediated by insulin (Brenner, 2003), but it seems that very few studies have investigated the regulation of elongase expression. It seems that the PUFA-sensitive transcription factors SREBP and PPAR play a role in the regulation of these genes (Jakobsson et al., 2006) but the time dependant increase seen in the current study is not yet fully understood.

An interesting effect on STAR transcript is that n-3 PUFA-enriched lipoproteins increased expression compared to n-6 PUFA-enriched lipoproteins. This would explain the increased progesterone production seen with inclusion of n-3 HDL, but would suggest that further mechanisms are involved which are active with delivery by HDL but not LDL. Published data have reported that arachidonic acid (AA, C20:4n-6) is essential for STAR expression (Wang et al., 2000), via a variety of complex mechanisms, none of which have been fully elucidated. All treatments in this study contained AA, and subsequent FA analysis revealed that theca cells contained approximately 2.6g/100g TFA in n-3 PUFA treatments and 5g/100g in n-6 PUFA treatments. The increase in STAR expression seen in both n-3 cultures relative to n-6 treatment could be explained, at least in part, by a reduction in levels of n-6 PUFA metabolites that are known to inhibit STAR expression (Wang et al., 2005; Wang et al., 2006). Other mechanisms could include the inhibition of transcription

factors by PUFA. The STAR gene is known to contain promoter regions for many transcription factors, including PPAR-γ (Kowalewski et al., 2009) and SREBP (Shea-Eaton et al., 2001), both of which can be positively and negatively regulated by PUFA (Jump et al., 1996; Duplus et al., 2000; Grimaldi, 2001; Sampath and Ntambi, 2004; Schmitz and Ecker, 2008). This PUFA-regulation of gene expression could also explain the differences observed in SCARB1 and ELOVL5 transcripts between treatments; both transcripts were expressed at their highest levels in the presence of n-3 PUFA from an LDL source (Table 3.8) and are known to be regulated by the SREBP family transcription factors (Lopez and McLean 1999; Moon et al., 2001).

3.4.3 Fatty acid composition

The proportions of n-3 and n-6 PUFA detected in granulosa and theca cells reflect the composition of the treatments added to culture media (A.2.3 and A.2.4). This demonstrates that both cell types are capable of taking up FA via LDL and HDL from the culture media. However, uptake of FA from lipoproteins by these cell types in vivo is not fully understood. It seems that the basement membrane could potentially restrict access by granulosa cells to the full complement of lipoproteins found in peripheral circulation, whereas thecal and luteal cells are exposed to all lipoprotein classes (Grummer and Carroll 1988). Only a few studies (Reis et al., 2002; Adamiak et al., 2006; Zachut et al., 2010; Wonnacott et al., 2010) have determined the FA composition profile of granulosa cells and none have reported this information for theca cells. Levels of MUFA and PUFA in granulosa cells are similar to that observed by Adamiak et al. (2006) and levels of saturates and unsaturates are similar to that reported by Wonnacott et al. (2010) and Reis et al. (2002). Proportions of PUFA, on average, are higher in theca cells than granulosa (Table 3.4) with 10 out of the 12 identified PUFA being significantly altered by dietary source (i.e. n-3 vs n-6), as opposed to 7 out of 12 in granulosa. This suggests a more efficient uptake mechanism is in place in theca compared to granulosa cells. SCD transcript levels increased by 7-fold from 48 to 144 h, which may indicate why oleic acid (C18:1n-9) constitutes such a large proportion of TFA (30.9 ± 0.35 g/100g) in granulosa cells at 144 h of culture. This observation is consistent with levels of oleic acid observed in a previous study in our laboratory (Wonnacott et al., 2010). Both theca and granulosa cells contain approximately 35g/100g MUFA, whereas the serum fractions added to

culture media contain approximately 20g/100g MUFA (A.2.1), suggesting a selective uptake or de novo synthesis of MUFA in these cells, which also may contribute towards the observed high levels of oleic acid in both cell types. A study by Mu et al. (2001) demonstrated that human granulosa cells cultured with palmitic (C16:0) and stearic (C18:0) acid underwent apoptosis in a dose-dependent manner, whereas other FA such as oleic (C18:1n-9), arachidonic (C20:4n-6) and linoleic acid (C18:2n-6) did not have the same effect. This may provide another reason for the high levels of SCD transcript and oleic acid observed in the current study, as SCD utilises stearic acid as preferred substrate for production of oleic acid, minimizing levels of potentially harmful FA.

3.5 Conclusions

In this study, HDL and LDL were added to culture at physiological levels, according to TFA concentration. LDL contains approximately four-fold higher levels of cholesterol than HDL. Therefore, the fact that HDL, on average, evoked a greater steroid response than LDL suggests that the efficiency of cholesterol delivery between these two particles differs, or that HDL-specific intra-cellular signalling is key for steroidogenesis. However, due to the greatest steroid response being observed with n-3 HDL, maximal steroid output seems to rely on other mechanisms which are stimulated by n-3 PUFA. Such mechanisms include; gene regulation through PUFA-sensitive transcription factors and/or alteration of PUFA-mediated signalling pathways. To the best of our knowledge this is the first time that n-3 PUFA-enriched HDL in combination with HDL-specific signalling has been demonstrated to increase progesterone production via mediation of STAR regulation. Also, transcript expression for ELOVL5, FADS1 and FADS2 has been reported, to the best of our knowledge, for the first time in mammalian granulosa and theca cells indicating that these cell types are actively metabolising FA.

CHAPTER 4

The effect of serum and serum derived LDL and albumin from ewes fed n-3 or n-6 PUFA enriched diets on the development, fatty acid composition and gene expression of ovine blastocysts

4.1 Introduction

The growth and maturation of the follicular-enclosed oocyte relies on a complex exchange of metabolites and molecules between the germ cell and surrounding somatic cells of the ovary (Cecconi et al., 2004). Adamiak et al. (2006) observed that oocytes assimilate and metabolise FA from the follicular environment which may affect the quality of the oocyte. The intrinsic quality of the oocyte is the key factor determining the proportion of zygotes developing to the blastocyst stage (Lonergan et al., 2003). A previous study in our laboratory cultured in vitro derived ovine embryos with HDL from an either n-3 or n-6 PUFA source (Wonnacott et al., 2010). There was no net uptake of FA by embryos in that study although PUFA supplementation altered the expression of several transcripts indicating that embryos were responsive to the culture conditions.

The current chapter considered the effects of physiological levels of n-3 and n-6 PUFA enriched serum, LDL and albumin fractions on in vitro cultured embryos. LDL is the second most abundant lipoprotein in the follicular fluid of ruminants (Argov et al., 2004; Wonnacott et al., 2010). Albumin is known to be a fatty acid transporter and is the predominant protein found in follicular fluid in humans and cattle where the concentration increases with increasing follicular size (Mortarino et al., 1999; Wen et al., 2009). Albumin has also been identified as one of the major lipid carriers in oviductal fluid and accounts for up to 80% of all oviductal proteins (Menezo and Guerin, 1997). Previous work has indicated that embryos cultured with individual FAs complexed with BSA have been able to assimilate FA (Reis et al., 2003). The current study tested the hypothesis that serum-derived lipids are delivered to developing embryos only by the albumin fraction. Therefore, albumin delivered n-3 and n-6 PUFA were expected to exert a greater differential effect on pre-

implantation embryo development than either LDL or HDL (the latter fraction was tested by Wonnacott et al., 2010).

FA analysis by gas chromatography was used to asses the composition of the embryos and therefore the ability of LDL and albumin fractionated from the sera of n-3 and n-6 PUFA fed ewes to successfully deliver FA to the embryo. Transcripts for the following genes involved in lipid uptake and metabolism were selected for analysis in blastocysts by qRT-PCR; SCARB1, LDLR, SCD, ELOVL2, ELOVL5, FADS1, FADS2 as previously described in section 3.1 and superoxide dismutase (SOD1). The desaturase enzymes (SCD, FADS1 and FADS2) and elongase (ELOVL2 and ELOVL5) enzymes are important in the synthesis of long chain fatty acids and the expression of these transcripts (except SCD) have never previously been reported in the pre-implantation embryo. SOD1 expression is a marker for cellular stress from reactive oxygen species which have the ability to induce damaging lipid peroxidation. The molecular analysis was carried out to determine (i) how LDL and albumin might differ in initiating intracellular signalling thus affecting the expression of the chosen transcripts and (ii) how n-3 and n-6 PUFA differ in their ability to regulate the expression of these genes.

4.2 Materials and methods

All reagents were obtained from Sigma-Aldrich unless otherwise stated. Embryo cultures were divided into two experiments; 1) FAF-BSA vs n-3 or n-6 serum, and 2) FAF-BSA vs n-3 or n-6 LDL or albumin. The second experiment was sub divided into 2 parts; samples to be used for FA analysis and those for qRT-PCR experiments. Samples to be used for FA compositional analysis were cultured with all 5 treatments running in parallel (i.e. FAF-BSA, n-3 LDL, n-6 LDL, n-3 albumin, n-6 albumin). Samples destined for qRT-PCR were run separately (i.e. (a) FAF-BSA with n-3 or n-6 LDL and (b) FAF-BSA with n-3 or n-6 albumin). Experiments were carried out using standard culture media (SOF, A.3.7) with supplements (i.e. serum, LDL or albumin). Omega-3 and -6 sera were obtained from ewes fed diets enriched with n-3 or n-6 PUFA (sections 2.2.1.1 and 2.2.1.3). LDL and albumin fractions were obtained from sera of n-3 or n-6 fed ewes via ultracentrifugation (sections 3.2.1.1 and 3.2.1.2).

4.2.1 Media supplements

For the first experiment, serum from n-3 or n-6 FA fed ewes (A.2.2) was added to SOF media at 5% v/v. LDL and albumin fractions were separated from serum from ewes fed either a high n-3 or high n-6 diet (A.2.3 and A.2.5). The LDL and albumin fraction treatments were added to culture based on their TFA content. The levels added were formulated to deliver physiological amounts of FA to the blastocysts. Fatty acid levels equivalent to 1% v/v serum were added to culture media. This was in keeping with previous work in our laboratory (Wonnacott et al., 2010). Levels of up to 10% serum in culture media are standard in our laboratory and elsewhere (Rizos et al., 2003; Carolan et al., 1995; Lonergan et al., 2004). TFA content of whole serum was calculated before fractionation (1442µg/ml) (Table A.2.1), consequently 1% equivalent was calculated to deliver 14.4µg/ml of TFA. Therefore, on average, 11.4µl of either LDL fraction or 160µl of either Albumin fraction was added to each ml of culture media.

4.2.2 Embryo culture

Ovine ovaries were collected from a local abattoir and transported in warm (37°C) sterile PBS to the laboratory. On arrival ovaries were rinsed with 70% ethanol and warm sterile PBS. Ovaries were kept at 37°C in PBS in a hot box during aspiration. Follicles were aspirated and cumulus-oocyte complexes (COCs) of grades 1 and 2 (IETS, 1998) were washed 3 times in pre-warmed search media (A.3.1) and washed once in pre-warmed and gas-equilibrated maturation media (A.3.2). Up to 70 COC were then transferred to a single well of a 4-well culture plate containing maturation medium and placed in a humidified incubator at 38.8°C and 5% CO_2 for 24 h. Matured oocytes were gently pipetted in the wells to remove expanded cumulus cells. Oocytes were washed twice in warmed PBS/0.1%PVA and once in fertilisation medium (A.3.5) before being placed in groups of up to 70 in wells containing 460µl equilibrated fertilization medium. A frozen semen pellet from a single ram was thawed by immersing a glass tube in water at 35°C for 30-60 seconds. 125µl of thawed semen was layered under 3ml of sperm wash media in conical tubes for swim-up procedure (A.3.4). Tubes were placed in a humidified atmosphere at 39°C and 5% CO_2 for 1 h. The top layer of the solution containing motile spermatozoa

was transferred to a second tube and centrifuged at 300xg for 10 min. Supernatants were removed leaving approximately 200µl, and pellets were resuspended. 10µl of this solution was added to 90µl water and sperm concentration was established using a haemocytometer. Spermatozoa were added to fertilization media to establish a final concentration of $1x10^6$ motile spermatozoa/ml (A.3.5). The oocytes and spermatozoa were incubated in a humidified atmosphere at 38.8°C and 5% CO_2 for 24 h. Presumptive zygotes were transferred to a 35mm petri dish containing HEPES SOF medium (A.3.6) and denuded of any remaining cumulus cells and attached sperm by gentle pipetting. Denuded zygotes were washed once in HEPES SOF and then transferred to fresh media and incubated at 38.8°C and 5% CO_2 for 24 hours. Cleaved embryos were then transferred to treatment media (either SOFaaBSA, SOFaaSerum n-3, SOFaaSerum n-6 SOFaaLDL n-3, SOFaaLDL n-6, SOFaaAlbumin n-3 or SOFaaAlbumin n-6) for 72 hours. Morphological assessments of embryo development and quality were carried out on days 5, 6 and 7 after IVF according to the International Embryo Transfer Society (IETS) classification system (IETS, 1998). On day 7 blastocysts were washed 6 times in PBS/0.1%PVA, quality was recorded and these embryos were then frozen in either liquid nitrogen or on dry ice for GC analysis or transcript analysis, respectively.

4.2.3 RNA extraction

RNA was extracted from ovine blastocysts using Dynabeads® mRNA DIRECT kit (Invitrogen, Paisley, UK). This method uses oligo (dT) primers, covalently bound to the surface of magnetic beads to hybridise the poly-A tail of mRNA for specific isolation. Blastocysts were lysed in 150µl of kit-supplied lysis buffer for 15 min at room temperature. Dynabeads were washed and suspended in lysis buffer and 40µl of bead solution added to each sample and incubated for 10 minutes at room temperature with rotation. The supernatant was removed by placing the tubes on a magnet (Dynal-MPC-S), after several seconds the beads form a pellet on the side of the tube allowing removal of supernatant by pipetting. The beads were washed 3 times (100µl each) with buffer A and 2 times with buffer B. The beads were then resuspended in 10µl of RNase-free water and incubated for 2 min at 65°C. Samples were transferred to ice immediately to prevent RNA re-annealing to beads. All

supernatants (10µl of RNase-free water containing mRNA) were removed and transferred to clean tube. RNA was stored at -80°C.

4.2.4 Reverse transcription

Reverse transcription of RNA to cDNA was carried out using the QuantiTect® RT kit (Qiagen Ltd., West Sussex, UK). This kit provides high yields of cDNA from low levels of RNA with removal of genomic DNA. RNA was thawed on ice and 10µl RNA (eluted from Dynabeads) was added to the following; 2µl gDNA Wipeout Buffer (x7) and 2µl RNase-free water. The mix was incubated for 2 min at 42°C and placed on ice immediately. For the –RT reaction, 1µl of the mix was removed and kept on ice. To the remaining 13µl the following was added; 1µl Quantiscript reverse transcriptase, 4µl Quantiscript RT buffer (x5), 1µl RT primer mix (contains both oligo(dT) and random primers), 1µl diluted gDNA wipeout buffer (2µl gDNA wipeout buffer and 12µl RNase free water), totalling 20µl reaction volume. Samples were incubated at 42°C for 30 min and then at 95°C for 3 min to inactivate the enzyme. Samples were stored at -20°C. For the –RT reaction, 7.4µl of RNase-free water was added to the 1µl saved from earlier and incubated at 95° for 3 min. The cDNA was diluted with water to 0.131 blastocysts per µl and stored at -20°C.

4.2.5 Quantitative RT-PCR

Transcripts were analysed by qRT-PCR as previously described (section 2.2.1.12). Cycling parameters and primer and probe sequences can be found in Tables 2.3, 3.2 and 4.1. SCARB1, ELOVL2 and FADS1 used forward and reverse primer concentrations of 1.2µl (10pmol/µl); all other primers used 0.6µl (10pmol/µl) of each primer. A total of 2µl cDNA was used per PCR reaction (0.26 blastocysts/reaction) and all samples were run in duplicate. All transcripts were measured relative to the reference gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Table 4.1 Primer and probe sequences for GAPDH and SOD1.

Name	Sequence 5'-3'	NCBI Accession	Primer Efficiency
GAPDH			
F	TCCGTTGTGGATCTGACCTG	AF030943	1.82
R	TGCTTCACCACCTTCTTGATCTC	Ovis aries	
Probe	**FAM**-CGCCTGGAGAAACCTGCCAAGTATGA-**TAMRA**		
SOD1			
F	GCCGTCTGCGTGCTGAA	XM 584414	1.99
R	ACTGTATTTCCCTTTGCCTCGA	Bos Taurus	
Probe	**FAM**-CCCGGTGCAAGGCACCATCC-**TAMRA**		

4.2.6 Analysis of embryos by gas chromatography

Procedures for the preparation of embryos for analysis by gas chromatography were described previously for theca and granulosa cells (Section 3.2.4), except that 50µl 200µg/ml pentadecanoic acid was added to each sample as an internal standard. Refer to section 3.2.1.4 and Table 3.1 for run and GC oven parameters.

4.2.7 Statistical analysis

All statistical analysis was conducted by Genstat release 11 (Genstat, 2009). The proportion of zygotes that developed to blastocysts was analysed by analysis of deviance using Generalized Linear Models (GLM) assuming binomial errors with logit link functions. The effects of culture treatment and morphological assessments of embryo stage and grade were assessed by ordinal regression analysis assuming multinomial errors with logit link functions. Transcript and FA data for Day 7 blastocysts were analysed by ANOVA.

To recap, two experiments were conducted to determine the FA composition of blastocysts (a serum experiment and an experiment which added LDL and albumin). The serum experiment consisted of three treatments FAF-BSA vs n-3 PUFA serum vs n-6 PUFA serum, which was replicated 4 times. The LDL and albumin

experiment consisted five treatment groups (FAF-BSA vs n-3 LDL vs n-6 LDL vs n-3 albumin vs n-6 albumin), which was replicated three times.

4.3 Results

4.3.1 In vitro embryo development

4.3.1.1 Experiment 1 (FAF-BSA vs n-3 or n-6 serum)

The proportion of cleaved zygotes that developed to the blastocyst stage by Day 6 of culture was greater ($P<0.001$) for the two serum treatments than for FAF-BSA (Figure 4.1 A). By day 7 the proportion of blastocysts in the FAF-BSA treatment group had increased but was still lower than in both serum groups ($P=0.048$). There was no effect of serum or PUFA source on the proportion of blastocysts classified (IETS, 1998) by stage of development (Figure 4.2A). The inclusion of PUFA enriched serum increased ($P<0.001$) the proportion of lower grade (IETS, 1998) Day 7 blastocysts (Figure 4.3 A), PUFA source had no effect on morphological grade.

4.3.1.2 Experiment 2a (FAF-BSA vs n-3 or n-6 LDL)

The proportion of cleaved zygotes that developed to the blastocyst stage by day 6 and 7 of culture in both LDL treatment groups was equal to those observed in the FAF-BSA control group (Fig 4.1B). There was no effect of LDL or of PUFA source on the proportion of blastocysts classified by stage of development (Figure 4.2B). The inclusion of PUFA enriched LDL also did not affect the proportion of Day 7 blastocysts classified by grade (Figure 4.3 C).

4.3.1.3 Experiment 2b (FAF-BSA vs n-3 or n-6 albumin)

The proportion of cleaved zygotes that developed to the blastocyst stage by Day 6 of culture was greater ($P<0.001$) for both albumin treatments compared to FAF-BSA control (Fig 4.1 C). By Day 7 the proportion of blastocysts in FAF-BSA group had increased but numbers were still greater for both albumin groups ($P<0.001$). PUFA source (i.e. n-3 vs n-6) did not affect development. There was no effect of albumin or PUFA source on the proportion of blastocysts classified by stage of development

(Figure 4.2C). The inclusion of PUFA enriched albumin increased (P<0.001) the proportion of lower grade Day 7 blastocysts (Figure 4.3 C). PUFA source once again had no effect.

4.3.2 Fatty acid composition of in vitro produced blastocysts

4.3.2.1 Experiment 1 (FAF-BSA vs n-3 or n-6 serum)

TFA content (ng/blastocyst) did not differ between treatments (Table 4.2). No significant difference was found between treatments for saturated, unsaturated MUFA and PUFA. There was a reduction (P=0.004) in n-6 PUFA with inclusion of serum compared to FAF-BSA. Compared to FAF-BSA control, inclusion of serum increased (P<0.001) n-3 PUFA and was greatest (P=0.004) with inclusion of sera from the n-3 PUFA source. The most abundant FA in these blastocysts was stearic acid (C18:0) comprising, on average, 30.5 ± 1.26 g/100 g TFA (Table 4.3). Oleic acid (C18:1,n-9) was the second most abundant FA at 29.1 ± 1.53 g / 100 g TFA and palmitic acid (C16:0) the third at 22.8 ± 0.76 g/100g TFA. Oleic acid was lowest (P=0.04) with inclusion of n-6 serum compared to FAF-BSA and n-3 serum treatments. Four out of the five identified n-3 PUFA were increased with inclusion of serum compared to FAF-BSA; eicosatrienoic acid (C20:3,n-3) (P=0.03), eicosapentanoic acid (C20:5,n-3) (P=0.004), docosapentaenoic acid (C22:5,n-3) (P=0.004) and docosahexaenoic acid (C22:6,n-3) (P<0.001). Of these four, three had significantly increased levels following inclusion of n-3 serum compared to n-6 serum; i.e. eicosapentanoic acid (C20:5,n-3) (P=0.004), docosapentaenoic acid (C22:5,n-3) (P=0.04) and docosahexaenoic acid (C22:6,n-3) (P<0.001).

4.3.2.2 Experiment 2 (FAF-BSA vs n-3 or n-6 LDL or albumin)

TFA (ng/blastocyst) was greater (P=0.024) with the inclusion of albumin compared to FAF-BSA and LDL treatments (Table 4.3). No significant difference was found between treatments for saturated, unsaturated, MUFA and PUFA. There was a reduction (P=0.014) in n-6 PUFA and an increase (P=0.001) in n-3 PUFA with inclusion of albumin, regardless of PUFA dietary source. The most abundant fatty

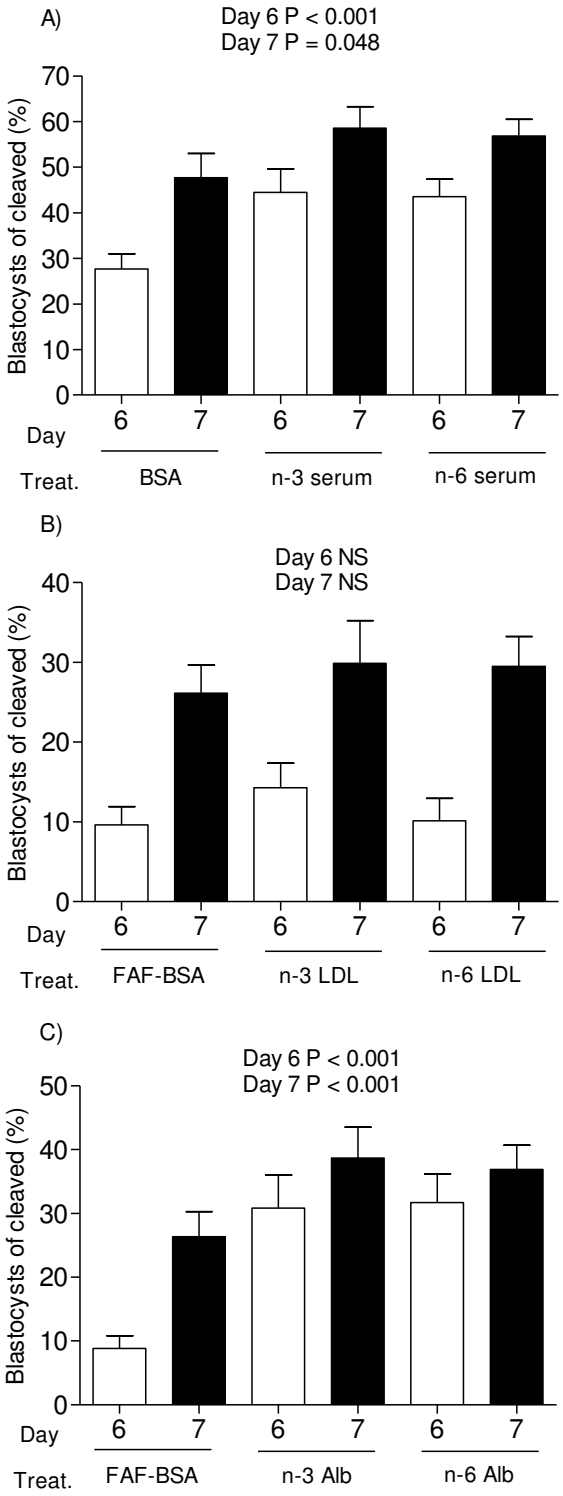

Figure 4.1 Mean (±SEM) blastocysts of cleaved following 6 or 7 days of culture in SOF media supplemented with A) FAF-BSA, n-3 or n-6 PUFA enriched serum, B) FAF-BSA, n-3 or n-6 PUFA enriched LDL and C) FAF-BSA, n-3 or n-6 PUFA enriched Albumin, all fractionated from sera of ewes offered n-3 or n-6 PUFA enriched diets.

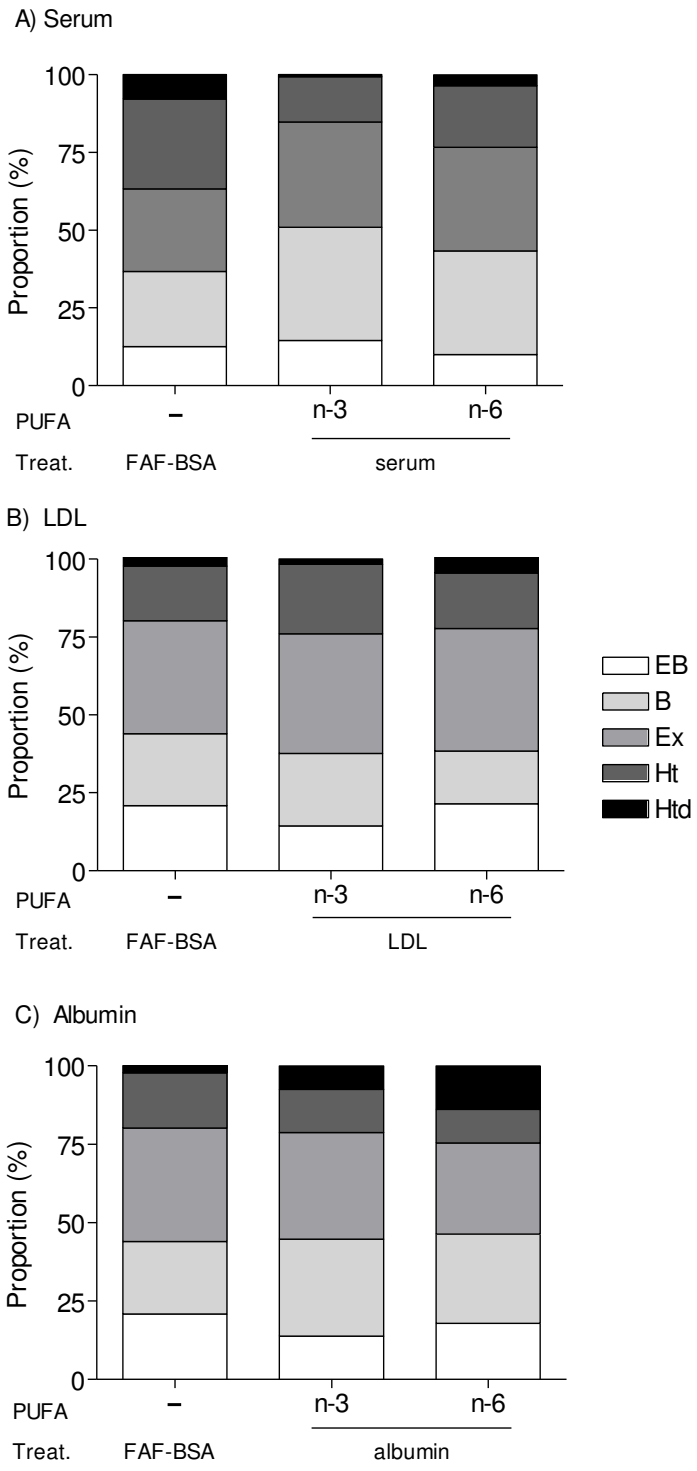

Figure 4.2. Effect of culture treatment on stage of development of Day 7 sheep blastocysts. (A) FAF-BSA, n-3 or n-6 PUFA enriched serum, (B) FAF-BSA, n-3 or n-6 PUFA enriched LDL and (C) FAF-BSA, n-3 or n-6 PUFA enriched albumin fractionated from sera of ewes offered n-3 or n-6 PUFA enriched diets. EB = early blastocyst; B = blastocyst; Ex = expanding blastocyst; Ht = hatching blastocyst; Htd = Hatched blastocyst.

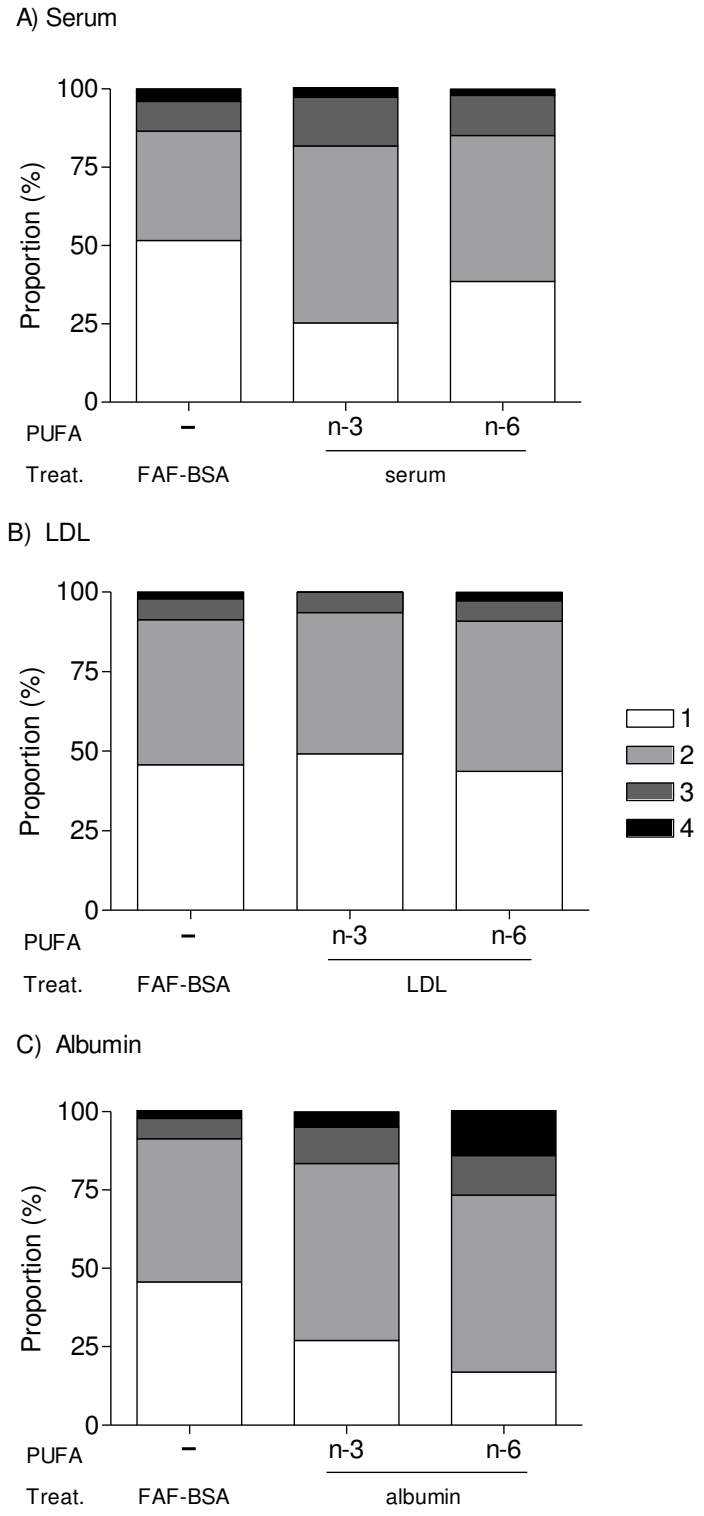

Figure 4.3. Effect of culture treatment on morphological grade of Day 7 sheep blastocysts. (A) FAF-BSA, n-3 or n-6 PUFA enriched serum, (B) FAF-BSA, n-3 or n-6 PUFA enriched LDL and (C) FAF-BSA, n-3 or n-6 PUFA enriched albumin fractionated from sera of ewes offered n-3 or n-6 PUFA enriched diets. Grade 1 = Excellent, grade 4 = Degenerating (IETS 1998).

acid in these blastocysts was oleic acid (C18:1n-9), comprising on average, 45.9 ± 0.51 g/100g TFA (Table 4.4). Stearic acid (C18:0) was the second most abundant FA at 14.9 ± 0.27 g/100g TFA and palmitic acid (C16:0) the third at 13.6 ± 0.23 g/100g TFA. None of these three FA differed between treatments. Two out of the twenty-six FA reported were not detected in these blastocysts from any treatment group. Four out of the five identified n-3 PUFA were significantly increased with inclusion of albumin compared with FAF-BSA and LDL; i.e. eicosatrienoic acid (C20:3,n-3) (P=0.02), eicosapentanoic acid (C20:5,n-3) (P=0.003), docosapentaenoic acid (C22:5,n-3) (P=0.003) and docosahexaenoic acid (C22:6,n-3) (P<0.001).

4.3.3 Transcript expression of in vitro produced blastocysts

4.3.3.1 FAF-BSA vs n-3 or n-6 serum

The addition of serum in culture reduced (P<0.001) LDLR transcript levels by 7-fold, dietary source (i.e. n-3 vs n-6) had no effect (Figure 4.4). SCARB1 expression was also not affected by treatment. Levels of SOD1 were increased (P=0.004) with the addition of serum in culture. Transcript expression for SCD decreased (P<0.001) with inclusion of serum, but again dietary source had no effect (Figure 4.5). Expression of FADS2 increased (P=0.012) with inclusion of n-6 PUFA serum compared to n-3PUFA serum and FAF-BSA control. Serum had no effect on expression of ELOVL5. Transcripts for FADS1 and ELOVL2 were not detected.

4.3.3.2 FAF-BSA vs n-3 or n-6 LDL

Inclusion of n-3 LDL in embryo culture media reduced LDLR (P=0.003) and SCARB1 (P=0.029) expression compared with FAF-BSA control (Fig 4.4). Dietary source of LDL also affected SCARB1 with addition of n-3 PUFA causing a reduction (P=0.008) in expression. SOD expression was not affected by treatment. SCD expression was reduced (P=0.014) with inclusion of n-3 compared with n-6 PUFA

Table 4.2 Fatty acid composition (g/100g TFA) of ovine blastocysts cultured in vitro in the presence of media supplemented with FAF-BSA or serum of ewes fed a high n-3 or n-6 diet (n=4).

Treatment (T)	**BSA**	**Serum**		**SED**	**Significance**	
Dietary Source (D)		**n-3**	**n-6**		**T**	**TxD**
TFA (ng/blastocyst)	150.6	157.8	166.6	5.17	-	-
Saturated	55.5	56.2	58.0	0.88	-	-
Unsaturated	40.7	40.2	38.3	0.82	-	-
MUFA	32.6	31.8	30.8	0.52	-	-
PUFA	8.10	8.32	7.49	0.35	-	-
n-6 series	7.17^{a}	6.11^{b}	6.10^{b}	0.25	0.004	-
n-3 series	0.93^{a}	2.21^{b}	1.48^{c}	0.16	<0.001	0.004
Ratio n-6:n-3	7.67^{a}	2.71^{b}	4.19^{c}	0.53	<0.001	0.05

Means within a row with different superscripts are significantly different (P<0.05): SED, standard error of difference; TFA, total fatty acids.

LDL and FAF-BSA control (Figure 4.5). FADS2 expression was not affected by treatment. Transcript expression for ELOVL5 was reduced (P=0.003) with inclusion of LDL from both dietary sources compared with FAF-BSA control. Transcripts for FADS1 and ELOVL2 were not detected.

4.3.3.3 FAF-BSA vs n-3 or n-6 albumin

Inclusion of n-3 PUFA enriched albumin from an n-3 source reduced transcript expression for LDLR (P=0.025) and SCARB1 (P=0.032) compared with n-6 PUFA albumin and FAF-BSA control (Fig 4.4). SOD1 expression was increased (P=0.05) with inclusion of albumin in culture compared to FAF-BSA; PUFA source had no effect. All other transcripts were not significantly altered. FADS1 and ELOVL2 transcripts were not detectable.

Table 4.3 Fatty acid composition (g/100g TFA) of ovine blastocysts cultured in vitro in the presence of media supplemented with FAF-BSA, LDL or albumin enriched with n-3 or n-6 PUFA (n=3).

Treatment (T)	BSA	Lipoprotein Fraction				SED	Significance		
Fraction (F)		LDL		Albumin					
Dietary Source (D)		n-3	n-6	n-3	n-6		T	TxF	TxD
TFA (ng/blastocyst)	119.2^{a}	115.3^{a}	121.1^{a}	126.6^{b}	131.1^{b}	5.43	-	0.024	-
Saturated	33.0	34.2	33.2	31.0	33.3	1.35	-	-	-
Unsaturated	63.2	61.9	63.1	65.4	62.7	1.06	-	-	-
MUFA	48.9	47.5	47.9	50.2	48.9	1.32	-	-	-
PUFA	14.2	14.4	15.2	15.2	13.9	0.83	-	-	-
n-6 series	12.9^{ab}	13.3^{a}	14.4^{a}	11.6^{b}	11.6^{b}	1.03	-	0.014	-
n-3 series	1.36^{a}	1.10^{a}	0.74^{a}	3.54^{b}	2.29^{b}	0.57	-	0.001	-
Ratio n-6:n-3	9.75^{a}	12.2^{b}	19.7^{c}	3.31^{d}	6.75^{d}	2.32	-	<0.001	0.01

Means within a row with different superscripts are significantly different (P<0.05): SED, standard error of difference; TFA, total fatty acids.

4.4 Discussion

The current study has demonstrated that pre-implantation embryos can assimilate FA from the surrounding environment via albumin binding and uptake. This is the first study to demonstrate the effects of LDL and albumin fractionated from the sera of experimental animals on fatty acid composition and transcript expression in blastocysts. The important finding from this study is that whilst sheep embryos can respond to LDL and HDL (HDL investigated previously in our laboratory, Wonnacott et al., 2010) in terms of altered transcript expression, only serum and albumin alters their FA composition. Also, this is the first study to report the presence of ELOVL5 and FADS2 transcripts in mammalian embryos.

Table 4.4. Fatty acid composition (g/100g TFA) of ovine blastocysts cultured in vitro in the presence of media supplemented with FAF-BSA or serum of ewes fed a high n-3 or n-6 PUFA diet (n=4).

Treatment (T)	**BSA**	**Serum**		**SED**	**Significance**	
Dietary Source (D)		**n-3**	**n-6**		**T**	**TxD**
C16:0	22.4	22.7	23.3	0.29	-	-
C17:0	ND	ND	ND	-	-	-
C18:0	30.0	30.4	31.1	0.29	-	-
C20:0	1.14	1.16	1.16	0.02	-	-
C21:0	0.13	0.22	0.18	0.06	-	-
C22:0	ND	ND	ND	-	-	-
C23:0	1.40	1.05	1.8	0.45	-	-
C24:0	0.39	0.59	0.57	0.12	-	-
C16:1	0.78	0.79	0.76	0.03	-	-
C17:1	ND	ND	ND	-	-	-
C18:1n-9t	1.59	1.55	1.46	0.12	-	-
C18:1n-9c	29.8^{a}	29.1^{a}	28.3^{b}	0.47	0.048	-
C20:1n-9	0.36	0.41	0.37	0.03	-	-
C22:1n-9	ND	ND	ND	-	-	-
C18:2n-6t	ND	ND	ND	-	-	-
C18:2n-6c	3.79^{a}	4.18^{b}	4.13^{b}	0.09	0.01	-
C18:3n-6	ND	ND	ND	-	-	-
C20:2n-6	0.03^{a}	0.04^{a}	0.18^{b}	0.05	-	0.046
C20:3n-6	0.35	ND	ND	-	-	-
C20:4n-6	2.95^{a}	1.89^{b}	1.67^{c}	0.19	<0.001	-
C22:2n-6	0.05	ND	0.03	-	-	-
C18:3n-3	0.33	0.41	0.36	0.02	-	-
C20:3n-3	0.24^{a}	0.34^{b}	0.32^{b}	0.03	0.034	-
C20:5n-3	0.11^{a}	0.35^{b}	0.18^{c}	0.04	0.004	0.004
C22:5n-3	0.05^{a}	0.57^{b}	0.30^{c}	0.10	0.004	0.04
C22:6n-3	0.21^{a}	0.55^{b}	0.32^{c}	0.03	<0.001	<0.001
Unidentified	3.83	3.67	3.63	0.23	-	-

Means within a row with different superscripts are significantly different (P<0.05): NS, not significant: SED, standard error of difference; ND, below detectable limit (<0.03):

Table 4.5 Fatty acid composition (g/100g TFA) of ovine blastocysts cultured in vitro in the presence of media supplemented with FAF-BSA, LDL or albumin enriched with n-3 or n-6 PUFA (n=3).

Treatment (T)	**BSA**	**Serum Fraction**							
Fraction (F)		**LDL**		**Albumin**		**SED**	**Significance**		
Dietary Source (D)		**n-3**	**n-6**	**n-3**	**n-6**		**T**	**TxF**	**TxD**
C16:0	13.5	14.3	13.2	12.8	13.9	0.54	-	-	-
C17:0	0.09	0.09	ND	0.14	ND	-	-	-	-
C18:0	15.0	15.2	15.0	13.9	15.3	0.77	-	-	-
C20:0	0.34	0.37	0.36	0.22	0.37	0.07	-	-	-
C21:0	0.07	ND	ND	ND	ND	-	-	-	-
C22:0	ND	ND	ND	ND	ND	-	-	-	-
C23:0	3.61^{a}	4.13^{b}	4.58^{b}	3.84^{a}	3.81^{a}	0.21	0.02	0.008	-
C24:0	0.33	0.07	ND	0.06	ND	0.06	<0.001	-	-
C16:1	1.4	1.25	0.75	1.19	1.09	0.29	-	-	-
C17:1	ND	0.2	ND	0.15	ND	-	-	-	-
C18:1n-9t	1.47	0.31	0.72	0.5	1.35	0.56	-	-	-
C18:1n-9c	45.2	45.1	45.7	47.9	45.9	1.44	-	-	-
C20:1n-9	0.59	0.58	0.59	0.51	0.53	0.04	-	-	-
C22:1n-9	0.16	ND	0.15	ND	ND	-	-	-	-
C18:2n-6t	0.22	0.12	ND	0.08	ND	-	-	-	-
C18:2n-6c	6.69	6.79	7.35	7.09	7.03	0.39	-	-	-
C18:3n-6	ND	ND	ND	ND	ND	-	-	-	-
C20:2n-6	ND	ND	0.11	ND	ND	-	-	-	-
C20:3n-6	ND	0.82	1.02	0.06	0.44	-	-	-	-
C20:4n-6	5.53^{a}	5.46^{a}	5.68^{a}	4.22^{b}	3.92^{b}	0.74	-	0.02	-
C22:2n-6	0.45	0.14	0.27	0.16	0.17	0.17	-	-	-
C18:3n-3	0.38	0.35	0.38	0.36	0.34	0.04	-	-	-
C20:3n-3	0.26^{a}	0.34^{a}	0.2^{a}	0.57^{b}	0.51^{b}	0.14	-	0.02	-
C20:5n-3	ND	ND	ND	0.73	0.23	-	-	0.003	-
C22:5n-3	0.43^{a}	0.11^{b}	0.17^{b}	0.89^{c}	0.53^{a}	0.19	-	0.003	-
C22:6n-3	0.29	0.3	ND	0.99	0.69	-	-	<0.001	0.05
Unidentified	3.85	3.88	3.72	3.66	3.94	0.37	-	-	-

Means within a row with different superscripts are significantly different (P<0.05): NS, not significant: SED, standard error of difference; ND, below detectable limit (<0.03).

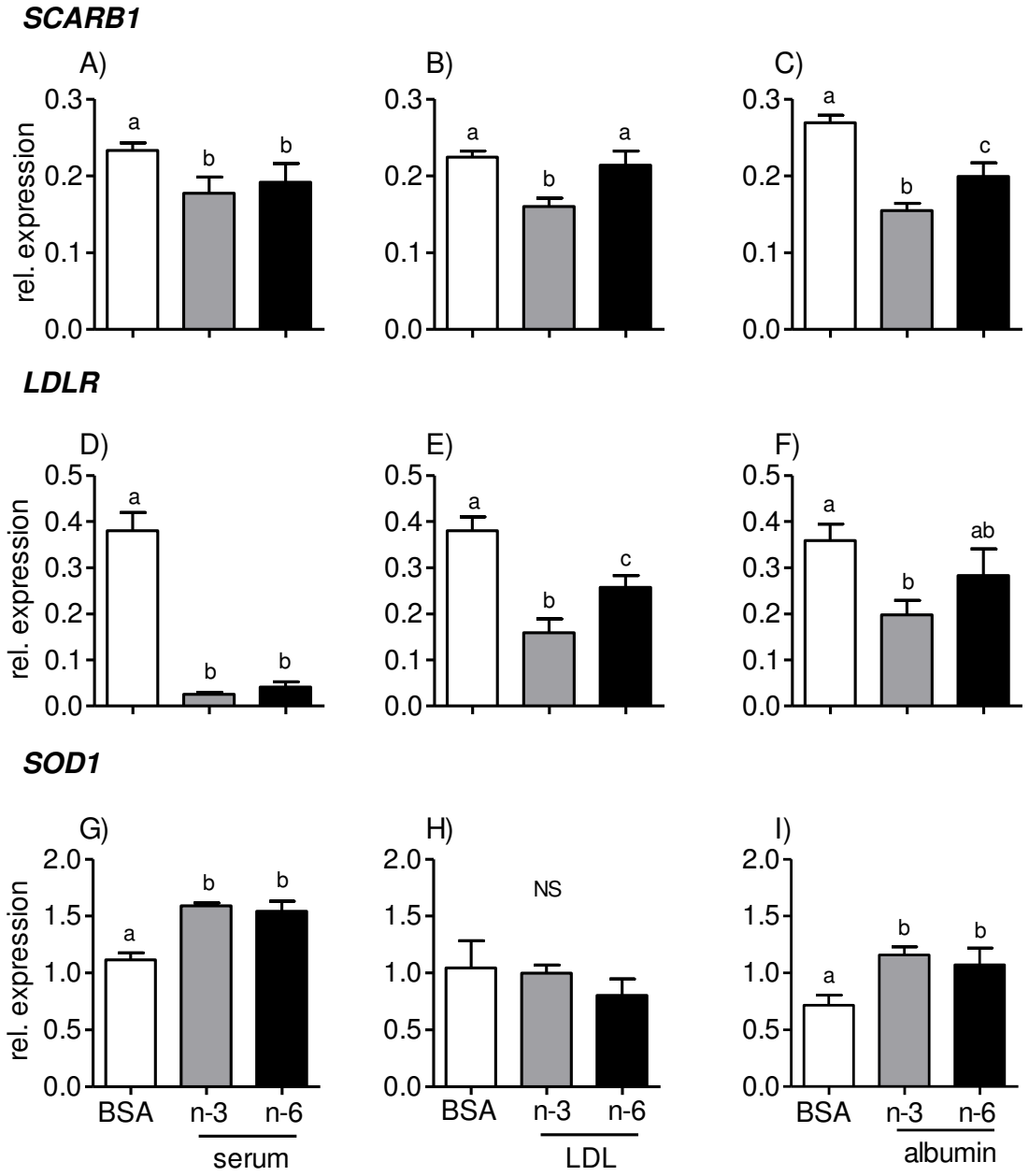

Figure 4.4 Transcript expression (relative to GAPDH) in Day 7 blastocysts cultured in synthetic oviductal fluid media supplemented with either fatty acid-free BSA (BSA, white) or one of three supplements (serum, left column; LDL, middle column; albumin, right column) from an n-3 (grey) or n-6 (black) PUFA source. Different superscripts indicate significance of $P<0.05$. NS = not significant (n=4).

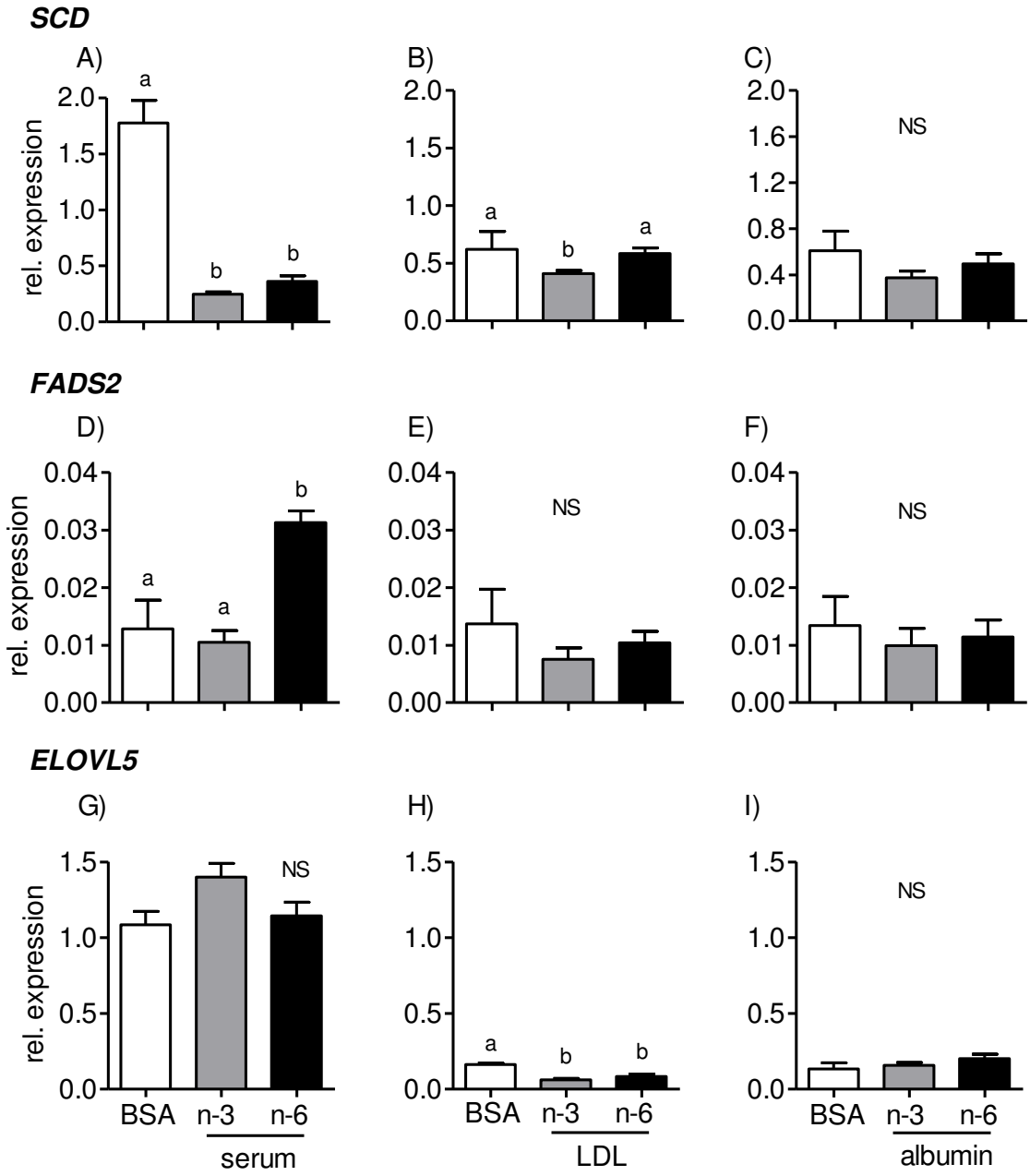

Figure 4.5 Transcript expression (relative to GAPDH) in Day 7 blastocysts cultured in synthetic oviductal fluid media supplemented with either fatty acid-free BSA (BSA, white) or one of three supplements (serum, left column; LDL, middle column; albumin, right column) from an n-3 (grey) or n-6 (black) PUFA source. Different superscripts indicate significance of $P<0.05$. NS = not significant (n=4).

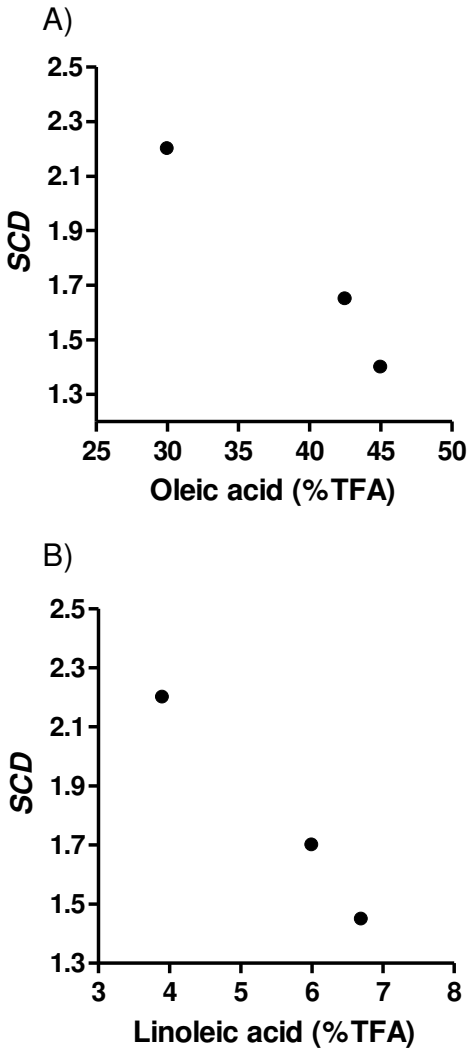

Figure 4.6 Relationship between oleic (A) and linoleic (B) acid and SCD transcript expression in Day 7 sheep blastocysts. Data points are means for embryos cultured in FAF-BSA for each of the three culture experiments (i.e. serum, LDL and albumin).

4.4.1 In vitro embryo development

A greater proportion of embryos cultured in the presence of either serum or albumin developed to the blastocyst stage by day 6 compared with LDL treated embryos. This suggests that the albumin portion of serum is the driving force behind this accelerated development. However, serum is an undefined supplement and contains many other components including growth factors, hormones and a range of proteins, vitamins and inorganic minerals (Freshney, 2005). The albumin fraction also is not

defined and may contain other serum components bound to albumin or unbound that perhaps could aide blastocyst development. The accelerated development brought about by the addition of serum to embryo culture media is in keeping with the observations of others (Thompson et al., 1995; Van Langendonckt et al., 1997; Lonergan et al., 1999). This accelerated development, however, was associated with poorer morphological grades. This is also in keeping with the observations of others who noted reduced embryo quality with inclusion of serum in culture (Thompson et al., 1995; Reis et al., 2005).

4.4.2 Fatty acid composition

TFA content (ng/blastocyst) was greatest in embryos cultured with the serum (158ng) (A.2.2) and the albumin (128ng) (A.2.5) fractions compared to LDL fraction (118ng) (A.2.3) or FAF-BSA control (119ng). The FA profile of embryos cultured with either serum or albumin was altered by culture treatment, showing differences in the n-3 and n-6 PUFA content. This was not observed in LDL-cultured embryos. Taken together, this indicates that there was no significant net uptake of FA by inclusion of LDL and that serum and albumin in culture facilitate the uptake of FA from the culture environment. A number of studies have reported lipid accumulation during in vitro embryo production supplemented with serum in ruminants (Thompson et al., 1995; Ferguson and Leese, 1999; Rizos et al., 2002; Reis et al., 2003). The increase in TFA content in the current study was associated with a visible increase in lipid droplet accumulation within the embryo (data not presented), again this is consistent with others (Thompson et al., 1995; Reis et al., 2005). The TFA content measured in these experiments is greater than that reported by Wonnacott et al. (2010) (60 to 80 ng/blastocyst) and Reis et al. (2002) (69 to 124 ng/blastocyst). Differences in FA composition in the FAF-BSA treated embryos over the various experiments (119 to 150ng/blastocyst) and the differences in total amounts of saturates and unsaturates detected in the same samples have highlighted fundamental differences in the composition of the oocytes from which these embryos were derived. The difference in FA composition in the control group is unlikely to come from the culture media as it contained only trace amounts of FA (Table A.2.6).

The FA composition of embryos is different to that observed in granulosa and theca cells (Chapter 3, Tables 3.4, 3.5 and 3.6). Levels of PUFA in blastocysts (average 11g/100g TFA) are approximately half that observed in somatic cells of the ovarian follicle (average 20g/100g TFA), however levels of n-6 PUFA are comparable (10 vs 12 g/100g TFA, respectively). Haggarty and colleagues (2006) observed that in discarded human pre-implantation embryos, those that developed beyond the 4-cell stage have a higher relative percentage of PUFA, specifically linoleic acid (C18:2,n-6). Levels of linoleic acid measured in blastocysts in this study were approximately 6g/100g TFA. This is comparable to that detected in sheep oocytes (6.9g/100g TFA) (McEvoy et al., 2000), and bovine cumulus oocyte complexes (5.7g/100g TFA) (Zachut et al., 2010). These data suggests an important role for n-6 PUFA, especially linoleic acid in oocyte maturation and embryo development; however recent data indicates that FA uptake by the maturing oocyte is limiting and additional dietary n-3 and n-6 PUFA have only a modest effect on embryo development (Fouladi-Nashta et al., 2009). In contrast, recent work has reported that inclusion of linoleic acid (n-6) and linolenic acid (n-3) in media for IVM and IVF of oocytes have adverse and beneficial effects on maturation and early embryo development respectively (Marei et al., 2009; Marei et al., 2010).

An interesting observation with the current study is the amount of n-3 PUFA detected in embryos cultured with albumin. The amount of individual n-6 PUFAs were not differentially altered with inclusion of n-6 albumin compared to n-3 albumin. However, four out of the five n-3 PUFAs were significantly altered. These data indicate that albumin may preferentially bind and transport n-3 PUFA. The affinity for albumin to bind FA increases as chain length increases (Spector, 1975), but to date no information is available regarding albumin binding affinities for n-3 vs n-6 PUFAs. However, along with chain length, the number and position of double bonds in the carbon chain may affect the binding efficiencies of FA to albumin (Petitpas et al., 2001) which could begin to explain the differences observed in the present study.

Lipid accumulation in oocytes and embryonic cells is an integral part of energy storage. These inclusions are metabolically active and the lipid is in a constant state of hydrolysis and resynthesis (Reis et al., 2005). It is probable that a selective uptake

mechanism has developed to ensure that minimal levels of potentially detrimental PUFA are maintained within the oocyte and developing embryo in vivo. However, it seems that this selective mechanism can be bypassed in vitro as demonstrated by the current study and others (Thompson et al., 1995, Kim et al., 2001; Reis et al., 2005). Excessive lipid accumulation in embryos cultured in vitro has been associated with reduced cryotolerance and elevated levels of stress responsive genes (Abe et al., 2002; Rizos et al., 2003).

Additional PUFA supplementation during oocyte maturation in vitro can improve early embryo development (Marei et al., 2010). However, this is not as clear-cut as it seems. Bovine oocyte exposure to α-linolenic acid can improve embryo development in vitro (Marei et al., 2010) whereas addition of linoleic or palmitic acid to mouse embryos inhibits development (Nonogaki et al., 1994). The FA composition of follicular fluid is a complex mixture of saturates, MUFA and PUFA of varying chain lengths and series (n-3, n-6, n-9 etc.), free or bound to transport molecules (Renaville et al., 2010; Sinclair et al., 2008; Jones and King 2009; Wonnacott et al., 2010; Zachut et al. 2010). In vivo exposure of oocytes to lipid never occurs in isolation of a single type of FA and optimal embryo development is likely to be associated with ratios between certain FAs and not the presence or absence of any single FA (Waterman and Wall, 1988).

4.4.3 Transcript expression

Wonnacott et al., (2010) demonstrated that despite no net uptake of FA from HDL, transcripts for LDLR and SCD were reduced, presumably acting via SCARB1, with little effect observed between n-3 vs n-6 PUFA. The same observations were seen in the present study with inclusion of LDL in the culture media, where LDLR and SCARB1 transcripts were reduced with inclusion of LDL compared with FAF-BSA control. The HDL signalling cascade is not yet fully elucidated (Fidge et al., 1999) and it could be that LDL signalling is more complicated than first thought. The LDLR is regulated by the SREBP family of transcription factors and requires several post translational modifications before it becomes active (Kong et al., 2006) and despite the detection of LDLR transcript, actual levels of the active protein may not correlate.

Expression levels for SOD1 were increased with inclusion of both serum and albumin, but not LDL, in culture media. Elevated levels of SOD1 are indicative of increased superoxide toxicity in embryos (Lequarre et al., 2001), and in the present study, heightened levels are associated with increased lipid accumulation and poorer morphological grades in embryos. PUFA source did not affect SOD1 transcript levels. FADS2 was not altered by treatment, however a substantial increase in expression was observed with addition of n-6 PUFA serum. This increase is inscrutable and would require further investigation to elucidate the mechanism involved.

SCD transcript has been shown to be regulated by PUFA (Landschulz et al., 1994; Keating et al., 2006), with different FA affecting this regulation with varying degrees of potency. A study by Landschulz et al. (1994) demonstrated that culturing hepatocytes with a range of individual FA at the same concentration varied the rate of suppression of SCD, with arachidonic (C20:4,n-6) and eicosapentanoic acid (C20:5,n-3) having the greatest effect. In fact, the potency of inhibition increased with the number of double bonds. In the current study, comparisons of SCD transcripts levels between experiments highlighted differences in expression. These differences had a negative correlation with levels of oleic and linoleic acid (i.e. high SCD, low FA) (Figure 4.6). Oleic acid was the most abundant FA within the embryo in all experiments with a range of 30-45g/100g TFA and concentrations were between 9 to 14 fold higher than arachidonic acid and 90 to 150 fold higher than eicosapentanoic acid. Keating et al. (2006) suggest that oleic acid, as the main product of SCD activity, could self regulate via a negative feedback mechanism, thereby maintaining the stearic acid to oleic acid ratio (also known as the desaturase index), which is a measurement of membrane fluidity, a key factor in maintaining normal cell function (Ntambi, 1995). This negative regulation of SCD by FA is most likely due to inhibition of SREBP transcription factors (Zulkifli et al., 2010) which are well known to be regulated post-transcriptionally by FA, by reducing the amount of active SREBP available for nuclear translocation (Jump and Clarke, 1999; Xu et al., 1999; Sampath and Ntambi, 2005).

Large differences in transcript expression between studies in the FAF-BSA control have highlighted fundamental differences in oocytes obtained from abattoir derived ovaries. Levels of ELOVL5 and SCD transcripts are much lower in the LDL and albumin experiments compared to the serum experiment. These experiments were conducted at different times of the year (serum experiment conducted between November and December 2009; LDL and albumin conducted in parallel between January and March 2010). This taken together with the FA composition of FAF-BSA control embryos of the same experiments suggests not only a seasonal effect but the composition of abattoir derived oocytes for IVF can vary greatly depending on diet, age and genotype of the animal. Experimental animals with controlled diets would reduce this effect, however in order to collect enough oocytes for IVF would make that option too expensive and beyond the confines of the current study.

4.5 Conclusions

The current study has provided evidence that albumin is the main FA transport mechanism for uptake into developing pre-implantation embryos. However FA uptake was associated with an observed increase in poor morphology and stress response. Nonogaki et al. (1994) suggested that lipid radicals can be generated easily in early stage embryos with blastomeres being the most sensitive to damage by lipid peroxidation. The current study was designed so that FAs were delivered to embryos at physiological concentrations, in fact less than that typically found in serum. However the amount of FA available to cells solely by albumin in vivo is substantially less. Therefore the detrimental effects observed may be due simply to over abundance of available FA. Transcript expression for ELOVL5 and FADS2 has, to the best of our knowledge, been described in mammalian embryos for the first time, indicating that FA metabolism is active in early blastocysts.

CHAPTER 5

General discussion and conclusions

5.1 General discussion

Declining fertility in cattle, especially dairy cows, has become an international problem due to the genetic selection for high milk yield. Studies have shown that supplementing ruminant diets with FA can have a range of beneficial effects on reproductive function. For example, positive effects have been observed with follicular development (Lammoglia et al., 1997) improved corpus luteum function (Petit et al., 2002), improved fertilization (Cerri et al., 2009) and enhancement of embryo quality and maintenance of pregnancy (Santos et al., 2008). PUFA are able to influence reproductive processes by a variety of mechanisms. They are precursors for inflammatory eicosanoids and can modulate the expression of a wide range of genes including those involved in steroid hormone synthesis. Alterations in the levels of dietary n-3 and n-6 PUFA could greatly affect these processes at the level of the ovary. FA compositional profiles have previously been investigated in granulosa cells, oocytes and embryos (Reis et al., 2002; Adamiak et al., 2006) and some studies have demonstrated that alterations in dietary FA can change the composition of these reproductive tissues (Wonnacott et al., 2010; Zachut et al., 2010). Studies indicate that the beneficial effects brought on by supplemental dietary FA may occur at the level of the ovary but there is a lack of information regarding the mechanisms by which dietary FA affect these tissues. There is also a paucity of information regarding the mechanisms by which n-3 and n-6 PUFAs are transported to and taken up by the ruminant ovary, and how the ovarian cells and developing pre-implantation embryo utilise these lipids.

Previous work in our laboratory have shown that follicular fluid progesterone is increased in n-3 compared to n-6 PUFA fed ewes (Wonnacott et al., 2010). This study showed that diets supplemented with contrasting sources of PUFA could alter the FA composition of plasma, follicular fluid, granulosa cells and oocytes of these animals. Surprisingly, when the serum from ewes fed on either the n-3 or n-6 enriched diet was HDL fractionated and used for the in vitro culture of granulosa

cells, no differences on progesterone secretion was observed. These data suggested that either granulosa cells were not responding to the dietary differences or that HDL was not the lipoprotein that delivered FA to these cells.

The experiments in this thesis used a combination of in vivo and in vitro studies to investigate the effects of dietary n-3 and n-6 PUFA on various ovine reproductive parameters. Granulosa cells and serum were collected from ewes fed n-3 and n-6 rich diets. Gene expression was investigated by array and qRTPCR in granulosa cells and serum was fractionated and analysed for FA composition by GC. PUFA enriched fractions were used to supplement granulosa, thecal and embryo cultures and molecular techniques used to elucidate the effects. The FA compositional profiles of granulosa cells, theca cells and pre-implantation embryos were also investigated. Specifically, the effects of n-3 and n-6 PUFA enriched HDL and LDL on granulosa and theca cell gene expression and steroidogenesis were explored. Sheep zygotes were cultured with LDL and albumin enriched with n-3 or n-6 PUFA to examine the mechanism of FA uptake by the pre-implantation embryo and subsequent development and gene expression.

This thesis sought to identify the mechanisms by which dietary n-3 and n-6 PUFA bind to HDL or LDL and alter steroid hormone synthesis in ovarian steroidogenic cells. Data presented in this thesis indicate that both HDL and LDL are able to deliver FA to both granulosa and theca cells in vitro. However, the FA composition of theca cells more closely resembled that of the culture treatments indicating a more effective uptake mechanism is in place in these cells. This may be explained by the fact that, in vivo, theca cells are exposed to the full range of lipoprotein classes found in the circulation as opposed to granulosa cells which have limited access due to the basement membrane (Grummer and Carroll, 1988). Also, this study is the first to report the FA compositional profile of theca cells.

A major finding from the in vitro studies is that n-3 PUFA increase STAR expression compared with n-6 PUFA in theca cells. This, together with an HDL-specific mechanism, may account for the increased progesterone production by n-3 HDL treated cells. These data suggest that a similar mechanism may account for the increased follicular fluid progesterone levels in ewes fed the n-3 PUFA enriched diet

(Chapter 2). Both the n-3 and n-6 PUFA culture media contained the full range of FA (including all n-3 and n-6 PUFA) and it seems that it is the n-6:n-3 ratio that drives the alteration seen in STAR expression. Without the inclusion of a 'control' group (i.e. no FA), however, the question remains as to whether n-3 PUFA increases or n-6 PUFA reduces STAR expression and subsequently progesterone production. In order to elucidate the mechanisms behind these alterations further analyses would be needed. Several directions would be beneficial; i) measuring arachidonic acid (AA) metabolites (e.g. prostaglandins) in in vitro media, and to extend this further by investigating concentrations in the follicular fluid from the in vivo study. Several studies have reported that AA metabolites have a critical role in steroid biosynthesis, acting both positively and negatively (Fiedler et al., 1999; Wang and Stocco, 1999; Zosmer et al., 2002; Wang et al., 2006), thus it would be interesting to observe how the n-6:n-3 PUFA ratio altered these metabolites. ii) Measuring protein levels of PUFA-sensitive transcription factors in in vitro cultured theca cells. The PPARs and SREBPs are the most likely candidates for investigation, as they are both regulated post-transcriptionally by PUFA with n-3 and n-6 PUFA having differential effects (Jump et al., 1996; Duplus et al., 2000; Grimaldi, 2001; Sampath and Ntambi, 2004; Schmitz and Ecker, 2008).

Data presented in this thesis (Chapter 4) indicate that embryos acquire FA from the surrounding environment via albumin. Wonnacott et al., (2010) found that pre-implantation sheep embryos responded to HDL enriched with n-3 or n-6 PUFA in terms of alterations in the expression of transcripts for LDLR and SCD but that the FA composition was not altered. This is in keeping with the current study where it was observed that embryos cultured with LDL enriched with n-3 or n-6 PUFA altered the expression of LDLR and SCD transcripts but FA profiles were not changed. When cultured with serum or albumin, fractionated from serum, both from ewes fed n-3 or n-6 PUFA, the FA composition of embryos was significantly altered together with an increase in TFA. The uptake of FA by serum and albumin also coincided with accelerated development, poorer quality embryos and an increase in oxidative stress (i.e. increase in SOD1 transcript expression). An interesting and unique observation from this study was that albumin tended to preferentially bind and transport n-3 PUFA to the pre-implantation embryo regardless of the dietary background source of the albumin. This may be due to varying binding efficiencies

of different FA (Spector, 1975; Petitpas et al., 2001), but to date no information is available regarding the binding of albumin to n-3 vs. n-6 PUFA. It is noteworthy that the albumin fraction in this study contained the lowest n-6:n-3 ratio out of all fractions measured (Table A.2.1). This may suggest that there is an evolutionary explanation as to why developing embryos utilise albumin to transport FA and not LDL or HDL, perhaps to limit the exposure to potentially harmful n-6 PUFA such as LA (Marei et al., 2010).

In order to further investigate the mechanisms behind gene expression alterations brought on by the binding of lipoproteins to their receptors, a detailed investigation into these complex signalling pathways would be beneficial. It is clear that HDL stimulates progesterone production, not necessarily by cholesterol delivery alone. One study suggests that apolipoprotien-A1, the major protein component of the HDL molecule is the inducer of steroidogenesis, without the need for parallel cholesterol uptake (Chandras et al., 2004) and that embryos respond to HDL (Wonnacott et al., 2010) and LDL in terms of altered transcript expression but without FA uptake. The importance of these mechanisms in ovarian function and embryo development requires further investigation.

Further investigations into the roles of individual PUFA on ovarian steroidogenic cells and developing pre-implantation embryos would also help to elucidate the mechanisms behind the observations in this thesis. Several groups have investigated individual PUFAs on reproductive parameters (Mattos et al., 2003; Sheldrick et al., 2007; Marei et al., 2009; Meier et al., 2009; Marei et al., 2010), but caution must be taken whilst interpreting these data as these FA do not occur in isolation in vivo, but as a complex mixture of FA with often opposing actions. In the studies described in this thesis, the use of fractionated serum lipoproteins more closely resembles the in vivo scenario. Moreover future work may utilise this knowledge of the FA content of in vivo derived serum by testing the effects of different combinations of PUFAs on granulosa cells and embryos in vitro.

One observation from these studies, which must be taken into account if further work is to be carried out, is seasonal differences in quality of abattoir derived material. Variations in the expression of transcripts for ELOVL5 and SCD and FA composition

in FAF-BSA treated blastocysts highlighted fundamental differences in the oocytes obtained from the abattoir over a period of 4 months (November 2009 to March 2010). Without any knowledge of age, genotype and dietary background of donor animals, the fundamental quality of the tissues can vary greatly, potentially affecting the outcome of experiments such as the ones described in this thesis. Differences in the FA composition of blastocysts in the current thesis and those of others (Reis et al., 2002; Wonnacott et al., 2010) could potentially be due to this seasonal effect and caution must be taken when interpreting these data.

This thesis also describes a series of experiments designed to optimise a custom-designed ovine-gonad specific cDNA macro-array gene for the purposes of identifying PUFA sensitive genes in granulosa cells. Although this approach was applied to both in vivo and in vitro derived granulosa cells, the non-commercial array was found to be unsuitable for quantitative gene expression analyses. Nevertheless, the use of an alternative array platform would be highly appropriate for the identification of novel PUFA sensitive ovarian genes.

In terms of the implications for ruminant reproduction published data suggests that supplementing diets with increased n-3 PUFA content could be beneficial. Several studies have investigated this hypothesis and have reported improvements in higher grade oocytes (Zeron et al., 2002), higher follicular phase oestradiol concentrations (Robinson et al., 2002), delayed onset of luteolysis (Mattos et al., 2004) and improved oocyte maturation and early embryo development (Marei et al., 2010). As cattle fertility is declining worldwide, a relatively cheap and simple option of dietary supplementation would be an attractive remedy. Several large-scale feeding studies have been completed to date (Armstrong et al., 1990; Carroll et al., 1994; Burke et al., 1997) but these studies have had limitations (low sample sizes, inconsistent results and undefined energy levels in feed) and therefore no distinct conclusions can be drawn.

Although caution must be taken when comparing ruminants to humans, this thesis may serve to encourage the consumption of a lower n-6:n-3 PUFA diet. The modern western diet comprises an n-6 to n-3 ratio ranging from 15-20 as opposed to a value of approximately 1 which several sources suggest is what humans became

accustomed to during the course of evolution (Eaton et al., 1998; Simopoulos, 2008). Studies have shown that reducing this ratio can have beneficial effects on chronic diseases such as rheumatoid arthritis and asthma, and a reduction in the risk of developing breast cancer (Broughton et al., 1997; James and Cleland, 1997; Maillard et al., 2002). In terms of human reproduction, Several papers suggest a beneficial role of n-3 PUFA on human reproduction, for example; increased birth weight (Muthayya et al., 2009) longer gestational age (Olsen et al., 1991) and improved spermatozoa quality (Safarinejad et al., 2010). However, more in depth studies are required to investigate the mechanistic role of PUFAs in the human reproductive system.

5.2 General conclusions

This thesis has focused on the effects of offering different sources of PUFA, namely the n-3 and n-6 series on FA composition and subsequent effects on gene expression in ovine granulosa and theca cells and the pre-implantation embryo. Additional to this, the effects of HDL and LDL fractions harvested from sera of ewes offered n-3 or n-6 PUFA on steroidogenesis were assessed in in vitro cultured granulosa and theca cells. The role of LDL and albumin fractions was investigated in the development, uptake and gene expression of pre-implantation embryos.

This study has demonstrated that:

i. Supplementation of culture media with LDL and HDL fractions obtained from ewes offered diets high in either n-3 or n-6 PUFA can support ovine granulosa and theca cell growth and function.
ii. The altered follicular-fluid progesterone levels observed in ewes fed n-3 PUFA diets can be reproduced in vitro and is mediated by actions on the theca layer of the follicle.
iii. Relative to n-6 PUFA, n-3 PUFA increases transcript expression for STAR in theca cells.
iv. HDL, but not LDL, stimulates progesterone output in theca but not granulosa cells.
v. Ovine granulosa and theca cells express transcripts for ELOVL5, FADS1 and FADS2 and SCD in theca cells indicating active FA metabolism.

vi. Ovine blastocysts utilise albumin but not LDL as a transport molecule for FA uptake, but this uptake is associated with poorer morphological grades and a heightened stress response.

vii. Ovine blastocysts are responsive to the presence of serum, albumin and LDL in culture in terms of altered transcript expression.

viii. Ovine blastocysts express transcripts for ELOVL5 and FADS2 indicating active FA metabolism in early mammalian embryos.

To conclude, this thesis has confirmed previous reports of HDL stimulated progesterone production in ovarian steroidogenic cells. In addition, it has been demonstrated that HDL from an n-3 PUFA enriched background can further improve progesterone production and that this is associated with an upregulation of the STAR transcript in cultured ovine theca cells. Low density lipoprotein was ineffective in delivering FA to cultured pre-implantation embryos, however, the albumin fraction of serum not only allowed uptake of FA to the embryos but preferentially bound n-3 over n-6 PUFA. This FA uptake by embryos via albumin however, was associated with poorer morphological grades and a heightened stress response manifest by increased expression of SOD1 transcript.

This thesis has highlighted the importance of n-3 PUFA in ruminant reproductive function in terms of improved steroidogenesis in theca cells both in vitro and in vivo and the preferential uptake by embryos via albumin. This taken together with published data reporting improved reproductive parameters in ruminants by n-3 PUFA supplementation may serve to encourage more research in this field to investigate the development of a relatively cheap method to begin to improve the worldwide decline in dairy cattle fertility.

APPENDIX 1

Table A.1.1 Ovine cDNA macroarray gene information

Abbreviation	Gene Name
ACTB	Actin, beta
ALOX12	Arachidonate 12-lipoxygenase
ALOX5AP	Arachidonate 5-lipoxygenase-activating protein
AMH	Anti mullerian hormone
BMP15	Bone morphogenetic protein 15
BMP4	Bone morphogenetic protein 4
BMP7	Bone morphogenetic protein 7
BMPR1A	Bone morphogenetic protein receptor IA
BMPR1B	Bone morphogenetic protein receptor IB
BMPR2	Bone morphogenetic protein receptor II
CAV1	Caveolin 1
CISH	Cytokine inducible SH2-containing protein
c-kit	c-kit receptor
CSF-3	Colony stimulating factor 3 (granulocyte)
CYP19A1	Cytochrome P450, family 19; aromatase
DAZL	Deleted in azoospermia-like
DDX4	DEAD box polypeptide 4
DMRT1	Doublesex and mab-3 related transcription factor 1
EEF1a1	Eukaryotic translation elongation factor 1 alpha 1
ESR1	Oestrogen receptor 1
ESR2	Oestrogen receptor 2 (ER beta)
FOXL2	Forkhead transcription factor
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GATA4	Gata-binding protien 4
GDF9	Growth differentiation factor 9
GJA1	Gap junction protien, alpha 1
GJB1	Gap junction protien, beta 1
GJB2	Gap junction protien, beta 2
GJC1	Gap junction protien, gamma 1
HPRT1	Hypoxanthine phosphoribosyltransferase 1

HSD3B1	3-beta-Hydroxysteroid dehydrogenase
HSL	Hormone sensitive lipase
IFNA	Interferon alpha
IFNB	Interferon beta
IFNG	Interferon gamma
IGF1	Insulin-like growth factor 1
IGF2	Insulin-like growth factor 2
IGF2R	Insulin-like growth factor 2 receptor
IGFBP1	Insulin-like growth factor binding protein 1
IGFBP2	Insulin-like growth factor binding protein 2
IL1A	Interleukin 1 alpha
IL1B	Interleukin 1 beta
IL10	Interleukin 10
IL12A	Interleukin 12 alpha
IL12B	Interleukin 12 beta
IL13	Interleukin 13
IL16	Interleukin 16
IL17	Interleukin 17
IL18	Interleukin 18
IL2	Interleukin 2
IL7	Interleukin 7
IL8	Interleukin 8
INHA	Inhibin alpha
INHBa	Inhibin beta a
INHBb	Inhibin beta b
INHBc	Inhibin beta c
LHR	Luteinizing hormone receptor
MEL1A	Melatonin receptor
MMP3	Matrix metalloproteinase 3
MSH4	MutS homolog 4
MSH5	MutS homolog 5
NR5A1	Nuclear receptor subfamily 5, group A, member 1
OSBP2	Oxysterol binding protein 2
PAPPA	Pregnancy-associated plasma protein A
PGF	Placental growth factor
PGR	Progesterone receptor
PHB	Prohibin

POU5F1	POU class 5 homeobox 1
PTGDS	Prostaglandin D2 synthase
PTGER3	Prostaglandin E receptor 3 (subtype EP3)
PTGFR	Prostaglandin F receptor
PTGS1	Prostaglandin synthase 1
PTPRG	Protein tyrosine phosphatase, receptor type, G
SDHA	Succinate dehydrogenase complex, subunit A
SOCS1	Suppressor of cytokine signalling 1
SOCS2	Suppressor of cytokine signalling 2
SOCS3	Suppressor of cytokine signalling 3
SOX14	SRY (sex determining region Y)-box 14
SOX2	SRY (sex determining region Y)-box 2
SOX9	SRY (sex determining region Y)-box 9
SPO11	Meiotic protein covalently bound to DSB homolog
SRY	Sex determining region Y
STAR	Steroidogenic acute regulatory protein
STC1	Stanniocalcin 1
TCF21	Transcription factor 21
TGFb1	Transforming growth factor beta 1
TGFb2	Transforming growth factor beta 2
TGFb3	Transforming growth factor beta 3
TIMP1	Tissue inhibitor of metalloproteinase 1
TIMP2	Tissue inhibitor of metalloproteinase 2
TNF	Tumor necrosis factor (alpha or member 2)
VIM	Vimentin
WT1	Wilms tumor 1
ZFX	Zinc finger protein, x-linked

APPENDIX 2

Fatty acid composition of serum, serum fractions and culture media.

Table A.2.1. Fatty acid (FA) composition of (a) Serum, (b) LDL, (c) HDL and (d) Albumin (all fractionated from pooled sera) from ewes offered diets differing in n-3 and n-6 PUFA (n=2/treatment).

Diet	n-3	n-6	S.E.D.	P
	g/100g TFA			
(a) Serum				
TFA (µg/ml)	**1439**	**1445**		
Saturated	36.51	36.50	0.189	-
Unsaturated	59.08	59.13	0.718	-
MUFA	17.29	18.72	0.618	-
PUFA	41.79	40.41	0.544	-
n-6 series	23.51	36.62	0.432	<0.001
n-3 series	18.28	3.79	0.133	<0.001
Ratio n-6:n-3	1.29	9.66	0.234	<0.001
(b) LDL				
TFA (µg/ml)	**1519**	**1012**		
Saturated	36.8	41.5	0.192	0.002
Unsaturated	58.8	53.9	0.624	0.015
MUFA	19.5	20.7	0.598	-
PUFA	39.3	33.1	0.149	<0.001
n-6 series	24.7	30.5	0.124	<0.001
n-3 series	14.6	2.63	0.045	<0.001
Ratio n-6:n-3	1.68	11.6	0.020	<0.001
(c) HDL				
TFA (µg/ml)	**3321**	**3171**		
Saturated	33.9	30.2	0.612	0.026
Unsaturated	61.6	64.9	0.521	0.024
MUFA	18.2	20.1	0.796	-
PUFA	43.5	44.8	0.276	0.039
n-6 series	27.7	41.5	0.267	<0.001
n-3 series	15.7	3.30	0.085	<0.001
Ratio n-6:n-3	1.76	12.5	0.034	<0.001
(d) Albumin				
TFA (µg/ml)	**88.6**	**89.6**		
Saturated	38.9	44.0	0.261	0.003
Unsaturated	57.1	51.4	0.258	0.002
MUFA	20.8	20.8	0.576	-
PUFA	36.3	30.6	0.754	0.017
n-6 series	16.7	25.7	0.298	<0.001
n-3 series	19.6	4.91	0.460	<0.001
Ratio n-6:n-3	0.85	5.24	0.073	<0.001

Table A.2.2 Fatty acid composition of ovine pooled serum of ewes offered diets enriched with either n-3 or n-6 PUFA (n=2/treatment)

Dietary Treatment	**n-3**	**n-6**	**S.E.D.**	**P**
Fatty acids	**g/100g TFA**			
C16:0	20.29	14.27	0.193	<0.001
C17:0	1.47	0.90	0.076	0.017
C18:0	13.45	20.48	0.167	<0.001
C20:0	0.22	0.15	0.044	-
C21:0	0.17	0.05	0.030	-
C22:0	0.25	0.21	0.007	0.031
C23:0	0.34	0.27	0.012	0.035
C24:0	0.33	0.17	0.005	<0.001
C16:1	3.99	1.98	0.112	0.003
C17:1	0.42	0.43	0.080	-
C18:1n-9t	0.66	0.56	0.026	-
C18:1n-9c	10.73	15.55	0.802	0.027
C20:1	1.33	0.14	0.020	<0.001
C22:1n-9	0.16	0.06	0.007	0.005
C18:2n-6t	0.21	0.19	0.091	-
C18:2n-6c	18.76	32.11	0.347	<0.001
C18:3n-6	0.20	0.72	0.019	0.002
C20:2n-6	0.86	0.17	0.061	0.008
C20:3n-6	0.83	0.42	0.002	<0.001
C20:4n-6	2.54	2.80	0.072	-
C22:2n-6	0.10	0.21	0.147	-
C18:3n-3	2.14	0.57	0.008	<0.001
C20:3n-3	0.37	0.04	0.037	0.013
C20:5n-3	11.11	1.43	0.096	<0.001
C22:6n-3	4.67	1.76	0.041	<0.001
Unidentified	4.41	4.37	0.084	-

Table A.2.3 Fatty acid composition of LDL fractionated from pooled sera of ewes offered diets enriched with either n-3 or n-6 PUFA (n=2/treatment)

Dietary Treatment	n-3	n-6	S.E.D.	P
Fatty acids	**g/100g TFA**			
C16:0	22.7	15.5	0.124	<0.001
C17:0	1.26	0.91	0.019	<0.001
C18:0	11.2	23.4	0.264	<0.001
C20:0	0.3	0.25	0.005	0.005
C21:0	0.22	0.18	0.008	0.014
C22:0	0.29	0.3	0.104	-
C23:0	0.63	0.62	0.018	-
C24:0	0.27	0.19	0.004	0.005
C16:1	3.79	2.38	0.540	-
C17:1	0.74	0.59	0.130	-
C18:1n-9t	0.48	0.72	0.060	0.035
C18:1n-9c	13.4	16.8	0.100	<0.001
C20:1	0.95	0.14	0.010	<0.001
C22:1n-9	0.16	0.05	0.010	0.009
C18:2n-6t	0.12	0.3	0.033	0.03
C18:2n-6c	20.1	26.3	0.141	<0.001
C18:3n-6	0.27	1.83	0.006	<0.001
C20:2n-6	0.95	0.14	0.015	<0.001
C20:3n-6	0.89	0.24	0.019	<0.001
C20:4n-6	2.23	1.61	0.007	<0.001
C22:2n-6	0.14	0.06	0.002	<0.001
C18:3n-3	2.24	0.54	0.008	<0.001
C20:3n-3	0.34	0.05	0.002	<0.001
C20:5n-3	8.86	0.96	0.031	<0.001
C22:6n-3	3.18	1.06	0.004	<0.001
Unidentified	3.86	3.72	0.091	-

Table A.2.4 Fatty acid composition of HDL fractionated from pooled sera of ewes offered diets enriched with either n-3 or n-6 PUFA (n=2/treatment)

Dietary Treatment	**n-3**	**n-6**	**S.E.D.**	**P**
Fatty acids	**g/100g TFA**			
C16:0	19.8	12.7	0.311	0.002
C17:0	1.06	0.63	0.072	0.004
C18:0	11.9	15.3	0.246	0.003
C20:0	0.05	0	0.101	-
C21:0	0	0.27	0.120	-
C22:0	0.26	0.26	0.004	-
C23:0	0.52	0.53	0.006	-
C24:0	0.27	0.21	0.008	-
C16:1	3.68	1.11	0.051	<0.001
C17:1	0	0.50	0.141	-
C18:1n-9t	0.43	0.31	0.050	-
C18:1n-9c	13.0	18.2	0.750	0.020
C20:1	0.92	0	0.022	<0.001
C22:1n-9	0.14	0	0.135	-
C18:2n-6t	0	0	-	-
C18:2n-6c	20.5	37.4	0.206	<0.001
C18:3n-6	0	0.44	0.021	0.002
C20:2n-6	0.78	0.40	0.013	0.001
C20:3n-6	2.46	2.98	0.035	0.004
C20:4n-6	0.72	0	0.025	0.001
C22:2n-6	3.25	0.28	0.040	<0.001
C18:3n-3	1.95	0.52	0.015	<0.001
C20:3n-3	0.19	0	0.003	<0.001
C20:5n-3	9.98	1.28	0.069	<0.001
C22:6n-3	3.61	1.52	0.021	<0.001
Unidentified	4.51	4.91	0.245	-

Table A.2.5 Fatty acid composition of Albumin fractionated from pooled sera of ewes offered diets enriched with either n-3 or n-6 PUFA (n=2/treatment)

Dietary Treatment	**n-3**	**n-6**	**S.E.D.**	**P**
Fatty acids	**g/100g TFA**			
C16:0	19.2	17.3	0.101	0.006
C17:0	1.30	0.91	0.124	0.015
C18:0	16.2	23.8	0.231	<0.001
C20:0	0.19	0.15	0.004	0.029
C21:0	0.36	0.26	0.061	-
C22:0	0.27	0.25	0.022	-
C23:0	1.20	1.12	0.114	-
C24:0	0.21	0.16	0.052	-
C16:1	2.21	1.12	0.23	0.042
C17:1	0.34	0.39	0.011	0.008
C18:1n-9t	0.68	0.42	0.105	-
C18:1n-9c	16.1	18.5	0.461	0.035
C20:1	1.23	0.26	0.034	<0.001
C22:1n-9	0.35	0.11	0.067	-
C18:2n-6t	0.12	0.09	0.005	0.027
C18:2n-6c	12.8	22.2	0.177	<0.001
C18:3n-6	0.15	0.12	0.064	-
C20:2n-6	0.61	0.11	0.036	0.005
C20:3n-6	0.66	0.34	0.09	-
C20:4n-6	2.13	2.53	0.034	0.007
C22:2n-6	0.12	0.31	0.029	0.023
C18:3n-3	2.13	0.85	0.086	0.004
C20:3n-3	0.29	0.15	0.021	0.019
C20:5n-3	10.6	1.72	0.16	<0.001
C22:6n-3	6.53	2.19	0.199	0.002
Unidentified	3.89	4.60	0.265	-

Table A.2.6 Fatty acid composition of culture media, basal synthetic oviductal fluid medium (SOF), SOF plus 0.1% w/v FA-free (FAF) BSA, basal DMEM and DMEM plus n-6 HDL incorporated at a FA level equivalent to that of 5% v/v serum. Values are means ± SEM from two technical replicates.

Media	**SOFM**	**SOFM + FAF-BSA**	**DMEM**	**DMEM + 5%**
		g/100g TFA		
Saturated	52.3±0.40	49.4±1.75	47.5±1.04	32.6±0.10
Unsaturated	43.6±0.10	45.4±0.88	49.7±0.42	63.1±0.03
MUFA	25.8±0.62	27.0±0.12	29.2±0.02	22.4±0.46
PUFA	17.8±0.05	18.4±0.30	20.6±0.27	40.7±0.03
n-6	15.0±0.28	14.8±0.07	11.8±0.34	35.8±0.92
n-3	2.8±0.12	3.6±0.29	8.8±0.18	4.9±0.35
ratio	5.4±0.13	4.2±0.32	1.4±0.01	7.23±0.34
C16:0	16.9±1.10	20.8±0.20	16.1±0.80	14.7±0.12
C18:0	20.6±0.14	11.4±0.14	22.8±0.41	17.3±0.19
C18:1n9c	20.9±1.05	21.0±0.07	22.0±0.81	19.6±0.48
C18:2n6c	12.1±0.11	9.7±0.06	7.1±0.04	32.2±0.01
C20:4n6	2.9±0.51	2.2±0.19	0.9±0.03	2.5±0.20
C18:3n3	2.2±0.25	0.8±0.01	0.2±0.00	1.1±0.03
C20:5n3	ND	ND	ND	1.1±0.03
C22:6n3	ND	ND	ND	1.6±0.05
TFA (µg/ml)	**3.57**	**3.79**	**4.20**	**73.00**

APPENDIX 3

Media for in vitro embryo culture

All reagents are from Sigma-Aldrich unless otherwise stated. All water used for media is tissue culture grade (TCG). All media is sterile filtered after pH and osmolarity checks and stored at 4°C. Note that Stock D can precipitate out of solution therefore should be added last and added slowly. Where stocks are used, refer to table A.3.1 for details.

A.3.1 Oocyte collection media

TCM199 with Hepes	19ml
Glutamine	2.0mg
Heparin	0.2mg
Pen/strep soln	100µl
FCS	1ml

pH = 7.2-7.3; mOsmo = 283 +/-10

Store for 1 week

A.3.2 Maturation media

TCM199	9 ml
Glutamine	1 mg
Pyruvate	1 mg
LH	100µl (10µg/ml)
FSH	1ml (10µg/ml)
Pen/strep	50µl

pH = 7.9; mOsmo = 283 +/- 10

Equilibrate at 5% CO2 for at least 1 hour before use.
Store for 1 week

Table A.3.1 List of stock components for embryo culture media. All media made up in tissue culture grade water. All stocks sterile filtered and stored at 4°C.

Stock	Component	Concentration	Amount	Volume	Storage
C	Na Pyruvate	33mM	36 mg	10ml	1 week
GLN	Glutamine	10mM	73 mg	50ml	1 week
B	$NaHCO_3$	250mM	1.05 g	50ml	1 week
	Phenol red		3 drops		
H	Hepes	250mM	5.957 g	100ml	2-4 weeks
L	Na Lactate	330mM	1.41 ml	30ml	2-4 weeks
S3	NaCl	1.1M	6.294 g	100ml	3 months
	KCl	70mM	0.534 g		
	KH_2PO_4	12mM	0.162 g		
D	$CaCl_2.2H_2O$	171mM	1.26 g	50ml	3 months
M	$MgCl_2.6H_2O$	49mM	500 mg	50ml	3 months
G	Glucose	60mM	540 mg	50ml	3 months

A.3.3 Sperm wash media

Stock S3	10ml
Stock H	8.4ml
Stock B	1.6ml
Stock C	1ml
Stock GLN	10ml
Stock M	1ml
Stock L	1ml
Stock G	4ml
Stock D	1ml
BSA (V/FAF)	300mg
Pen/strep	0.5ml
Water	61.5ml

Made up to 100ml with TCG grade water

pH = 7.2-7.4; mOsmo = 283+/- 10

Store for 1 week

A.3.4. Percoll medium for sperm separation

To make up 90% Percoll:

Percoll	53ml
Stock S3	5ml
Stock M	500µl
Stock D	500µl
$NaHCO_3$	96mg (22mM)
Hepes (free acid)	126mg (10.5mM)
Na Hepes (salt)	137mg (0.5mM)

pH = 7.2-7.4; mOsmo = 280-300

Store for 2 months

A.3.5. Fertilisation media

Stock S3	5ml
Stock B	5ml
Stock C	1.5ml
Stock L	1.5ml
Stock M	0.5ml
Stock GLN	5ml
Stock D	1ml
Pen/strep	0.25ml
Water	28.05ml

pH = 7.9; mOsmo = 283 +/- 10

Store for 2 weeks

For IVF – add 2% sheep serum to Fertilisation medium and equilibrate before use

A.3.6. Hepes SOF (for washing and transporting embryos)

Stock S3	10ml
Stock B	2ml
Stock H	8ml
Stock C	1ml
Stock D	1ml
Stock M	1ml
Stock L	1ml

Stock G	2.5ml
Stock GLN	10ml
Water	87.5ml

Mixed and added 300mg FAF-BSA

pH = 7.4; mOmol = 270+/-5

Store for 1-2 weeks

A.3.7. SOFaaBSA (standard embryo culture medium)

Stock S3	10ml
Stock B	10ml
Stock C	3ml
Stock D	1ml
Stock L	3ml
Stock M	1ml
Stock G	2.5ml
Stock GLN	10ml
Pen/strep	0.5ml
TCG water	50ml
Non-essential amino acids	0.5ml
Essential amino acids	1ml
FAF-BSA*	500mg

pH = 7.4; mOsmo = 270-280

Store for 1 week

* Note that SOFaa media containing treatments (i.e LDL, Albumin) included no FAF-BSA. Water volume was adjusted to allow for extra volume of LDL and Albumin fractions.

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