Pre-implantation maternal uterine effects on embryo growth and development: An investigation using models of maternal constraint in sheep

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Abstract

Prenatal development and growth are critical to survival of the fetus and neonate. Recent evidence suggests that a critical period for determining growth is the pre-implantation period of pregnancy during which differentiation, organogenesis and development of the embryo occur and the embryo is considerably vulnerable to uterine environmental factors. The objectives of the present study were to examine the effects of restrictive uterine environments on embryo development using two sheep models of maternal constraint: litter size and dam size, and to identify embryonic and maternally-driven mechanisms that regulate development of the peri-implantation sheep embryo.

Morphometric analysis (embryo length, width and heart bulge width) of the embryos in peri-implantation single and twin embryos was inconclusive; as was the transcriptomics analysis of whole embryos using RNA-seq to examine differential gene expression that may be responsible for differential regulation of growth.

In a dam size model, large-breed Suffolk embryos gestated in small-breed Cheviot ewes (constrained environment) were smaller than Suffolk embryos gestated in Suffolk ewes (control) at day 19 of pregnancy, confirming previous findings that maternal constraint is evident in early pregnancy when limitations of space are not of consequence. Progesterone administered in the post-ovulatory period, day 0 to 6, alleviates this apparent constraint such that Suffolk embryos gestated in Cheviot ewes that received progesterone are larger than those gestated in Cheviot ewes that did not. Further, differential gene expression analysis of maternal uterine tissues showed that at day 6 and day 19 endometrial genes that

encode for histotroph secretion and uterine receptivity are altered by post-ovulatory progesterone administration. Timing of administration of progesterone is critical not only to embryo growth but also to embryo survival. There were lower pregnancy rates in the ewes that received progesterone from day 0 than those that received progesterone from day 2.

The results of this thesis indicate that progesterone exerts its effects by regulation of genes that encode for uterine structural and secretory activity to advance the uterus. This likely forces the asynchronous embryo to accelerate its growth in order to adapt to its environment. These findings contribute to the knowledge of the regulatory mechanisms controlling early embryo growth and present a platform within the livestock industry and human reproductive technology practice to manipulate embryo growth to improve survival of offspring.

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List of Abbreviations

ACTB = Beta actin

AI = Artificial insemination

BNC = Binucleate cells

Bp = Base pair

C = Cheviot

cDNA = Complementary Deoxyribonucleic acid

CIDR = Controlled intravaginal progesterone drug releasing device

CL/s = corpus luteum/corpora lutea

Con1E1CL = Control singleton bearing ewe (single CL), no embryo transfer

Con 2E2CL = Control twin bearing ewe (two CLs), no embryo transfer

COX2 = Cyclooxygenase 2

CP4 = Cheviot ewe that receive progesterone from day 0 to day 6 of pregnancy

CnP4 = Cheviot ewe that did not receive progesterone from day 0 to day 6 of pregnancy

Ct = Quantification cycle

CTSL = Cathepsin L

CV% = coefficient of variance

DEG = differentially expressed gene

DGAT2 = diacylglycerol-O-acyltransferase

DKK4 = Dickkopf WNT signalling pathway inhibitor 4

EL = embryo length: distance from the medial aspect of the head to the tip of the embryonic tail, following the outer curvature of the embryo

ET = Embryo transfer

EGF = Epidermal growth factor

ER/ESR1 = Estrogen receptors

EW = Embryo width: distance between the two widest points of the embryo with the line passing just below the heart bulge

FDR = False discovery rate

FGF1 = Fibroblast growth factor 1

FGF2 = Fibroblast growth factor 2

FGF7 = Fibroblast growth factor 7

FGF10 = Fibroblast growth factor 10

GAPDH = Glyceralydehyde-3-phosphate dehydrogenase

GE = Glandular epithelium

GH = placental growth hormone

HB = Heart bulge width: distance between the two widest points of the heart bulge with the line passing through the midsection of the heart bulge

HGF = Hepatocyte growth factor

HPRT = Hypoxanthine phosphoribosyltransferase 1

IGFs = Insulin like growth factors

IGF1 = Insulin like growth factor 1

IGF2 = Insulin like growth factor 2

IGF1R = Insulin like growth factor 1 receptor

INFτ = Interferon tau

INFAR = Type 1 interferon receptors

IRF2 = Interferon regulatory factor 2

ISG17 = Interferon stimulated gene 17

IUGR = Intrauterine growth restriction

IV = Intravenous

LAPTM5 = Lysosomal-associated protein transmembrane 5

LE = Endometrial luminal epithelium

LGALS3 = Lectin galactoside-binding, soluble 3

LGALS15 = Endometrial galectin 15/ Lectin galactoside-binding soluble 15

LOC101103603 = Pregnancy associated glycoprotein-4 like

LOC101117738 = Pregnancy associated glycoprotein-1 like

LRRC32 = Leucine rich repeat containing 32

MET = C-met proto-oncogene

mRNA = Messenger RNA

MSTN = Myostatin

MUC1 = Mucin glycoprotein 1

NFW = Nuclease free water

OXTR = Oxytocin receptor

PBS = Phosphate buffered saline

PCR = Polymerase chain reaction

$PGF_{2\alpha}$ = Prostaglandin $F_{2\alpha}$

PL = Placental lactogen

PGR = Progesterone receptors

PTGS2 = Prostaglandin endoperoxide synthase 2

P4 = Progesterone

qPCR = Quantitative real time PCR

RIN = RNA Integrity number

RNA = Ribonucleic acid

RPL19 = Ribosomal protein L 19

RSAD2 = Radical S-adenosyl methionine domain containing 2

RT = Reverse transcriptase

RT-qPCR = Reverse transcriptase quantitative PCR

S = Suffolk

SinCP4 = Suffolk embryo that was gestated in a Cheviot ewe that receive progesterone from day 0 to day 6 of pregnancy

SinCP4$^{0-3}$ = Suffolk embryo that was gestated in a Cheviot ewe that receive progesterone from day 0 to day 3 of pregnancy

SinCP4$^{0-6}$ = Suffolk embryo that was gestated in a Cheviot ewe that receive progesterone from day 0 to day 6 of pregnancy

SinCP4$^{2-4}$ = Suffolk embryo that was gestated in a Cheviot ewe that receive progesterone from day 2 to day 4 of pregnancy

SinCP4$^{3-6}$ = Suffolk embryo that was gestated in a Cheviot ewe that receive progesterone from day 3 to day 6 of pregnancy

SinCnP4 = Suffolk embryo that was gestated in a Cheviot ewe that did not receive progesterone from day 0 to day 6 of pregnancy

SinSP4 = Suffolk embryo that was gestated in a Suffolk ewe that received progesterone from day 0 to day 6 of pregnancy

SinSnP4 = Suffolk embryo that was gestated in a Suffolk ewe that did not receive progesterone from day 0 to day 6 of pregnancy

SERPIN = Uterine serine proteinase inhibitor/ Uterine milk proteins

sGE = Superficial glandular epithelium

SPP1 = Secreted phosphoprotein 1/osteopontin

SP4 = Suffolk ewe that received progesterone from day 0 to day 6 of pregnancy

SnP4 = Suffolk ewe that did not receive progesterone from day 0 to day 6 of pregnancy

TGF = Transforming growth factor

TKDP = Trophoblast Kunitz domain protein-1

TP1 = Trophoblast protein 1

UGKO = Uterine gland knock out

UTMP = Uterine Milk Proteins

YWHAZ = Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta.

1E1CL = Singleton embryos (harvested from a ewe with a single CL) transferred into a ewe that was also identified as having a single CL, and a single embryo that was removed

1E2CL = Singleton embryos (harvested from a ewe with a single CL) transferred into a ewe that was identified as having two CLs, and twin embryos that were removed

2E1CL = Twin embryos (harvested from a ewe with two CLs) transferred into a ewe that was identified as having a single CL, and single embryo that was removed

2E2CL = Twin embryos (harvested from a ewe with two CLs) transferred into a ewe that was also identified as having two CLs, and twin embryos that were removed

1 Introduction

Reproductive performance, productivity and good health are important to the livestock industry and greatly influence long-term economic success. Prenatal development and growth is critical to fetal and neonatal survival, and poor development during this period is associated with low lamb birth weights and subsequent poor survival and growth (Huffman *et al.*, 1985; Jaquiery *et al.*, 2011). Recent evidence suggests that factors that regulate fetal growth and development patterns may have their most significant influence during the peri-conceptional period rather than later in pregnancy as was previously proposed (Hancock *et al.*, 2012; Sharma *et al.*, 2013).

Gluckman and Hanson's (2004) review described the interaction between the fetal genome and the environment in which the fetus is developing. They reported that the fetal environment, which is primarily determined by the maternal physiology and placental function, is as dominant as genetic influences in determining fetal growth (Gluckman and Hanson, 2004). This paradigm of maternal influence on fetal development known as "developmental origins of disease" reported by Barker (1995; 1998), and Barker and Clarke (1997) is supported by human epidemiological studies, which described increased risk to later-life cardiovascular and metabolic disease as a result of *in utero* developmental programming due to maternal dietary and physiological factors. Similarly, studies of production animals have also shown maternal nutrition, during critical periods of gestation, has long-term consequences to postnatal lifetime productivity and health (Wu *et al.*, 2004; Bell, 2006; Symonds *et al.*, 2006; Kenyon and Blair, 2014). This realisation that altering the uterine environment results in "developmental programming", and that this is a contributor to adult onset of cardiovascular disease and other metabolic syndromes, as well as lifetime

productivity and health of livestock, supports the need for further embryonic and fetal growth research.

"Maternal constraint" describes the non-genetic, non-pathological factors determined by the dam which may act to control development *in utero* (Gluckman and Hanson, 2004). It is suggested that these factors exert their action by limiting nutrient support to the developing conceptus. These factors include maternal size, nutrition, age, parity and litter size (Gluckman and Hanson, 2004; Gardner *et al.*, 2007). To date, there is a paucity of information on the mechanisms by which these factors may exert their effects on embryonic and early fetal development, although the consequences on neonatal survival and production have been demonstrated in humans (Smith *et al.*, 1998). Additionally, studies in animals have reported the effects of maternal constraint on fetal development, birth weight and post-natal growth: maternal size (Walton and Hammond, 1938; Dickinson *et al.*, 1962; Cowley *et al.*, 1989; Giussani *et al.*, 2003; Allen *et al.*, 2004; Sharma *et al.*, 2012a; 2013), litter size (Gootwine *et al.*, 2007; Hancock *et al.*, 2012), and age (Loureiro, 2014). Therefore, not only is it important to elucidate the mechanisms by which maternal constraint acts to restrict or reduce embryonic development, it is essential to examine situations in which embryos can overcome control exerted by the uterine environment.

The majority of studies that examine the mechanisms of fetal and neonatal development and the effects of various forms of maternal constraint are human-focused. However, it would be beneficial to examine the application of these principles to sheep production, since the understanding of fetal development could lead to improved pregnancy outcomes and production. In addition, any mechanisms that are elucidated may also be of benefit to advancing improvements in human reproduction.

The present study was undertaken to investigate the effects of genetically and physiologically restricted uterine environments on embryonic development and to identify maternal signals that drive differences in growth and development. The main objectives were to examine the mechanisms of early embryo growth using sheep models of maternal constraint (maternal size and litter size):

I. to compare embryonic gene expression levels using transcriptomics in embryos conceived as singles or twins.
II. to identify pathways associated with differences in growth and development
III. to investigate if administration of progesterone could overcome maternal constraint.
IV. to elucidate the molecular mechanisms through which progesterone may exert its effect on embryo growth by examination of maternal uterine tissue.

The outcome of this research will allow for further development of models of maternal constraint, provide a foundation for future research in the mechanisms and critical periods of gestation at which embryonic and fetal development are most vulnerable. In addition it has the potential to provide opportunities for manipulations that can be used by farmers to influence embryo growth and survival thereby improving livestock productivity and success.

2 Embryonic development and maternal-embryonic interactions during early pregnancy: A review of literature

2.1 Preamble

It has long been accepted that the critical period of fetal growth is during the last 6 weeks of pregnancy (Alexander, 1974). During this period lambs gain approximately 60% of their birth weight (Alexander, 1974) (average birth weight singleton, 4.9kg; twin, 3.7kg (West *et al.*, 2009)). Any alteration in fetal weight was therefore thought to be due to nutritional deprivation via placental restriction of nutrition, competition between littermates, or as a result of limitations of space. However, it has recently been established that fetal growth and development is influenced much earlier in pregnancy, possibly as early as the peri-conception/pre-implantation period (Watkins *et al.*, 2008; Hancock *et al.*, 2012). It is likely this period can significantly affect birth weight and consequently survival and productive performance postnatally and into adulthood (Todd *et al.*, 2009; Hancock *et al.*, 2012; Sharma *et al.*, 2012a). In human studies, examination of first trimester fetal size and its relationship to birth size, suggest that size at birth may, in part, be determined by variation in growth programmed during early gestation (Smith *et al.*, 1998; Bukowski *et al.*, 2007; Salomon *et al.*, 2011). The process whereby "a stimulus or insult at a sensitive or critical period of development has long-term effects is termed *programming*" (Godfrey and Barker, 2000, p. 1344s). The early embryonic period is critical as this aligns with an interval of rapid cell division, organogenesis, implantation and maternal recognition of pregnancy (Dziuk, 1992; Bazer *et al.*, 1997; van Mourik *et al.*, 2009). Therefore, it is conceivable that an environmental insult experienced in early pregnancy could result in a permanent alteration to embryo development, fetal growth, birth size and subsequent lifetime production and health.

The intrauterine environment plays a pivotal role in embryo-maternal interactions that drive early stage growth and development. In fact “maternal constraint” during early gestation is thought to exert a profound effect on the development and growth trajectory of embryos and as a consequence size at term, more so than even genetic factors (Brooks *et al.*, 1995; Gluckman and Hanson, 2004). Factors that contribute to maternal constraint include maternal size, nutrition, age, parity and as well as litter size which all have the underlying effect of altering the uterine environment and the supply of nutrients to the embryo or fetus during the critical time of development and growth (Gluckman and Hanson, 2004; Gardner *et al.*, 2007). However, little is known about the mechanisms by which these maternal constraint factors act to alter the prenatal environment to cause changes in embryo/fetal growth and development.

Although the effects of maternal constraint are now widely accepted, to date only a limited number of studies in this area have been undertaken in livestock species. Since optimal health and growth greatly influences survivability and productive performance of animals, understanding these early gestational effects offers significant potential to influence on farm economic success in the livestock industries. Additionally, further elucidation of the mechanisms involved in embryonic development and the effects of maternal constraint would go a long way to improving our understanding of pregnancy and influence successful outcomes.

The objective of this PhD study is to decipher the mechanisms involved in early embryonic growth. Therefore, this review will firstly describe the developmental process of embryogenesis. The scope of this review will be limited to the first two periods of prenatal development in the sheep, that of the ovum and embryo and will not extend beyond the

events occurring at day 34 of gestation. Embryo-dam interactions will then be discussed with a focus on maternal factors that may influence embryo development. A brief description of the regulation of embryo development via uterine structural changes, gene expression and cell signalling will follow, particularly with a focus on the role of progesterone in regulating embryo-maternal interactions. Finally the hypotheses and objectives of this thesis will be outlined.

2.2 Embryonic development in sheep: day 0 to 34

Embryonic and fetal growth have long been recognised as important factors determining birth weight and survival. Growth in late gestation was thought to play the major role in determining birth weight. However, recent evidence suggests that fetal growth trajectory may be set during the early pre-implantation period of embryonic growth. Little is known about the mechanisms and maternal-embryo interactions that occur during this period and how they programme growth and development of the embryo.

Bryden *et al*., (1972) suggested that development can be divided into three developmental periods based on the relative degree of development attained through each of the periods: the period of the ovum, the period of the embryo and the fetal period. Green and Winters, (1945) describe the period of the ovum as the period of time from ovulation until initiation of blastocyst attachment to the endometrium. The embryonic period follows for approximately three weeks during which genesis of the main organs and organ systems takes place. The third period is dedicated primarily to growth and development and secondarily to further differentiation of organs (Green and Winters, 1945), which continues

until parturition. The text below describes the events involved in the development of the sheep embryo from day zero (fertilisation) to the end of the embryonic period at day 34.

2.2.1 Period of the ovum: day 0 to 10

Cleavage

Cleavage involves a series of specialised mitotic divisions which increase the number of daughter cells or blastomeres (Boyd and Hamilton, 1952). In sheep, following fertilisation, the ova are considered to undergo almost equal or holoblastic cleavage typical of miolecithal eggs, containing a small amount of yolk that is uniformly dispersed throughout the egg (Figure 2.1) (Boyd and Hamilton, 1952), with the initial cell division occurring at approximately two days post-fertilisation (Clark, 1934). However, although early cleavage divisions have been demonstrated to occur synchronously, divisions at the eight to 16 cell stages have been demonstrated to result in intermediate, odd numbers of blastomeres and differences in size (Green and Winters, 1945; Boyd and Hamilton, 1952). This is typically observed at the morula stage and continues for subsequent cleavage divisions (Boyd and Hamilton, 1952). The morula consists of a compact sphere of cells consisting of an inner core surrounded by a superficial layer of cells (Figure 2.1). The superficial layer eventually differentiates into the epithelial layer known as the trophoblast or trophectoderm (McGeady *et al.*, 2006). The trophoblast ultimately forms the outer surface of the extra-embryonic membrane. At the same time as these multiple divisions occur, a fluid-filled cavity, the blastocoele, develops as fluid accumulates between the peripheral trophoblastic cells and the inner cell mass, producing what is known as the blastocyst stage which is observed at approximately day five to eight post-ovulation (Clark, 1934; Boyd and Hamilton, 1952).

Compaction

Descent of the fertilised ovum into the uterus takes place at approximately day three to four post-ovulation, during which the ovum is typically in the 16- to 32-cell morula stage (Clark, 1934; Spencer *et al.*, 2004a). Blastocoele formation is apparent at approximately day six. The process by which the blastomeres are organised within the zygote is termed compaction (McGeady *et al.*, 2006). Compaction occurs at the 16-cell stage in sheep; the blastomeres become more compressed against each other changing their shape, increasing cell-to-cell contact and forming tight junctions within the trophoblast layer (Ducibella, 1977; McGeady *et al.*, 2006). At this point and continuing to day 10, the zona pellucida thins and is lost; the blastocyst hatches from the zona pellucida allowing further growth and expansion of the embryo (Figure 2.1) (Rowson and Moor, 1966; Bindon, 1971b). In addition, hatching is necessary as the zona pellucida is thought to prevent the trophoblast from contacting and attaching to the maternal endometrium (Spencer *et al.*, 2004a). Simultaneously, further development of the trophoblast and inner cell mass occurs producing the spherical form characteristic of blastocysts, such that at the end of cleavage and compaction the compact morula consists of a superficial layer of cells and an inner core or the inner cell mass, which flattens forming a disk of cells, the embryonic disk, from which the embryo develops. The superficial layer will ultimately give rise to the epithelial or trophoblastic layer (Johnson, 1981; Fehilly and Willadsen, 1986). In addition, the cleavage and compaction stages terminate with degeneration of the layer of trophectoderm that lies immediately over the embryonic disk (Rauber's layer), exposing the outer layer of the embryonic disk to the maternal environment (Guillomot *et al.*, 2004).

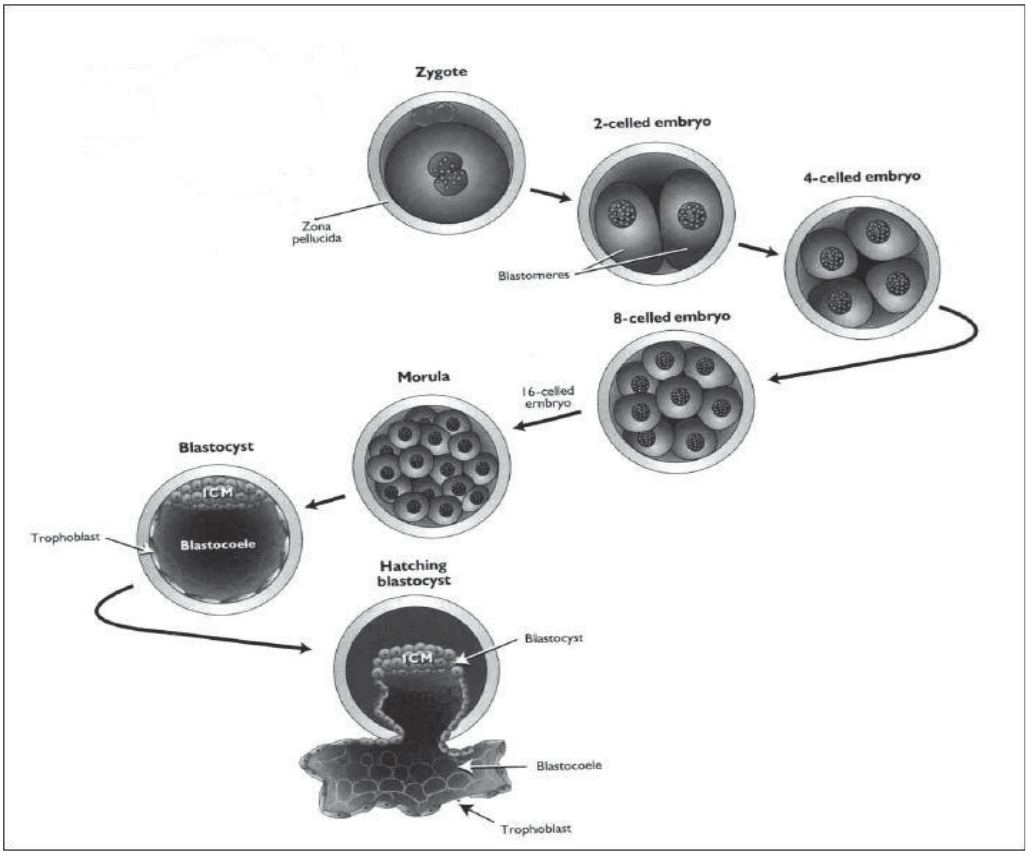

Figure 2.1 Development of the pre-implanted embryo from zygote (2-cell stage) to hatching: cleavage, compaction to morula stage and formation of blastocoele cavity. (Adapted from Anonymous)

2.2.2 Embryonic period: day 11 to 34

During the embryonic period of the sheep embryo grows from the simple form of a flattened embryonic disk to that of a structure that is considerably differentiated and characterised by the development of many internal and external organs (Wales and Cuneo, 1989). This is also the period during which attachment to the maternal endometrium takes place.

Gastrulation

At the end of the cleavage and compaction stages of ovum development, gastrulation occurs, whereby the single-layered blastula develops into the tri-layer structure that begins attachment to the endometrium of the uterus. Concomitantly, the trophectoderm begins a stage of rapid elongation (Stroband and Van der Lende, 1990; Blomberg *et al.*, 2008). This leads to initiation of the implantation stages, apposition and adhesion, that are characteristic of the day 11 to 18 period (Boyd and Hamilton, 1952). The three primary germ layers formed during gastrulation are the ectoderm, mesoderm and endoderm (Figure 2.2). The formation of these layers occurs through a process of cell migration (McGeady *et al.*, 2006). The inner cell layer (endoderm) migrates from the embryonic disk forming a continuous membrane enclosing the blastocoele (Bryden *et al.*, 1972), and thus forming the bilaminar yolk sac (archenteron or primitive gut). The second stage of gastrulation involves formation of the primitive streak. The formation of the primitive streak occurs through a process of thickening and further invagination of the cells that form the outermost layer of the embryonic disk (Boyd and Hamilton, 1952; McGeady *et al.*, 2006) into two parallel ridges on either side of a depression that run along the diameter of the disc (Latshaw, 1987). Subsequent to this, the intra-embryonic mesoderm arises in the region of the primitive streak via further invagination of the ectoderm, and results in formation of the notochord (Boyd and Hamilton, 1952). Extra-embryonic mesoderm simultaneously expands and forms an inner lining of the trophectoderm, and thus constitutes part of the placental chorion (Betteridge and Fléchon, 1988). This entire process of gastrulation occurs before implantation (Guillomot, 1995) and it is suggested that this process allows the organisation of organ primordia into position within the embryo (Boyd and Hamilton, 1952).

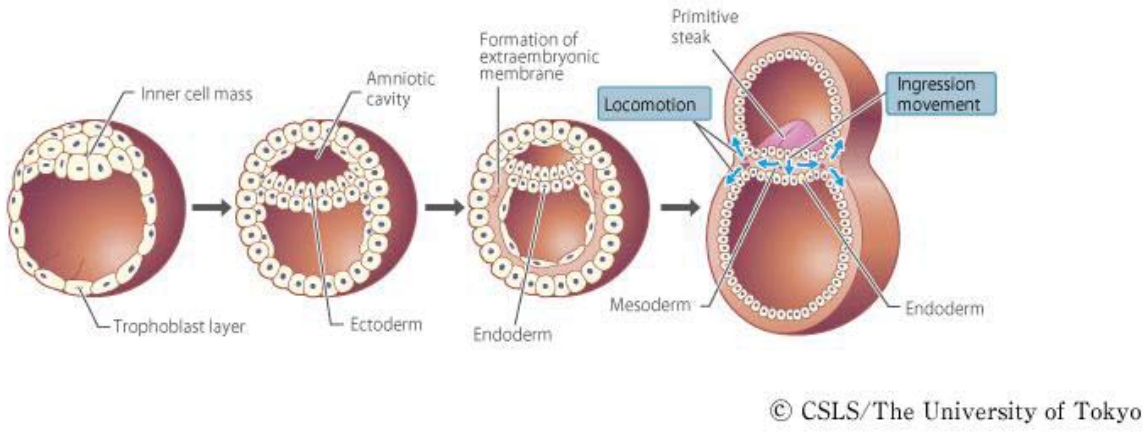

Figure 2.2 Illustration of mammalian gastrulation. Cells separate from the central part of the ectoderm and move into the interior of the embryo, becoming endoderm and mesoderm. (Adapted from College of Arts and Science, The University of Tokyo, 2011).

Elongation and attachment/implantation

Following loss of the zona pellucida, the embryo enters a rapid growth (cellular hyperplasia) phase which involves marked elongation of the trophoblast (chorionic vesicle) between day 11 and 13 (Boyd and Hamilton, 1952; Bindon, 1971b). It has been reported that at day 12 the ovine tubular conceptus is typically 10-22mm in length, and by day 14 becomes filamentous, reaching up to 100mm long (Wintenberger-Torrès and Fléchon, 1974). By day 15 the developing embryo has grown sufficiently within the uterine horn to come into close contact and form loose attachments to the uterine epithelium (Boshier, 1969; Guillomot, 1995). Guillomot *et al.*, (1981) described the attachment at day 14 as "punctate areas of contact" between the trophoblast and uterine epithelium that are loose and easily disrupted by flushing. Early reports by Bryden *et al.*, (1972) suggested that attachment takes place on day 10 of pregnancy. However, other investigators reported that attachment does not begin until day 16-18 following the expansion of the chorionic vesicle

to fill the entire uterine horn (Boyd and Hamilton, 1952; Chang and Rowson, 1965; Boshier, 1969). Further to this, Gaviria and Hernandez, (1994) demonstrated that trophoblastic attachment to the uterine epithelium is less than 50% prior to day 20, expanding to greater than 85% at day 24, when:

$$\text{percentage area of trophoblast attachment} = \frac{\text{area of trophoblast attachment}}{\text{area of internal uterine contour}} \times 100.$$

During the initial period of elongation, (day 10-14) there is limited or no increase in size of the embryonic disk (Rowson and Moor, 1966). Initially, the trophectodermal cells that are in apposition to the uterine epithelium are columnar in shape and mononucleate (Wooding, 1984). However, the trophoblastic cells undergo structural and functional modification, becoming flat and spindle-shaped (Guillomot *et al.*, 1981). These changes facilitate the attachment process and initially appear to be confined to the trophoblast immediately surrounding the embryo such that apposition starts in the area surrounding the embryo and extends from there to the ends of the conceptus (Guillomot, 1995). However, the rest of the trophoblastic cells retain their columnar shape. In addition, immobilisation and apposition is reported to be facilitated by the presence of villus projections (papillae) which develop and project into the opening of the uterine glands (Guillomot *et al.*, 1981; Wooding and Staples, 1981). These papillae are observed covering the embryonic region of the conceptus as early as day 13 and extend deep into the uterine glands by day 16 (Wooding and Staples, 1981).

Attachment between the embryo and maternal uterine epithelium is consolidated by specialised trophoblastic cells known as giant binucleate cells (BNC) (Amoroso, 1952; Boshier, 1969; Wooding, 1982). Mononucleate trophoblastic cells begin differentiation into BNC at about day 16 (Wooding, 1984; Guillomot, 1995). By day 20, these BNC proceed to migrate into and then fuse with the uterine epithelium to result in the formation of the

syncytium (Boshier, 1969; Wooding and Staples, 1981; Wooding, 1982). The implantation process in sheep culminates with the formation of the syncytium which is the most invasive step (Guillomot, 1995). BNC are thought to play a major role in the placental function of the sheep and produce various steroid hormones and proteins, such as placental lactogen (Watkins and Reddy, 1980; Wooding, 1981; Wooding *et al.*, 1992) and progesterone (Wooding, 1992; Wooding *et al.*, 1996) that regulate maternal uterine physiology (Hoffman and Wooding, 1993) and are therefore, likely to be directly or indirectly involved in regulating embryonic growth.

In order for attachment to be completed, structural modifications to the uterine endometrial tissue must also occur (Boshier, 1969; 1970; King *et al.*, 1982). This includes remodelling of the epithelium into crypts and caruncular depressions or endometrial grooves, increased vascularisation and cytoplasmic changes (Boshier, 1969; Reynolds and Redmer, 1992). Adhesion and attachment is further reinforced by growth of the uterine microvilli into the folds of trophoblastic cell plasma membrane (Guillomot, 1995) and denudation of the uterine epithelium in areas in contact with the trophoblast (Amoroso, 1952; Boshier, 1969; King *et al.*, 1982; Wooding, 1982). These modifications are important to ensure the formation of syncytium which is characteristic of the sheep placentation. The process of implantation involves a sequence of events beginning with apposition, through adhesion, and is only completed following attachment when the trophoblast is firmly adhered to the endometrial luminal epithelium at approximately day 22 (Spencer *et al.*, 2004a).

Embryo organ differentiation and body form changes

The earliest differentiation of the embryo is characterised by the formation of the ectodermal plate, somites, neural folds and the primitive streak (Green and Winters, 1945). Bryden *et al.* (1972) reported observation of these structures by day 14. The embryonic shield is observed to be slightly raised above the trophoblast (Bryden *et al.*, 1972; Guillomot *et al.*, 1981; Wales and Cuneo, 1989). Cranial migration of mesodermal cells takes place from Hansen's (primitive) node, which are a knot of cells at the cranial end of the primitive streak. These mesodermal cells move forward into and intersperse with the endodermal cells of the future head area (Latshaw, 1987). This forms a plate of cells known as the notochordal plate and this further differentiates into the notochord (a rod-shaped cell aggregation that lies along the entire length of the embryo) via dorsal folding (Latshaw, 1987). This notochord lies just ventral to a layer of neuro-ectodermal cells that proliferate and differentiate into the neural plate (McGeady *et al.*, 2006). Further, this neural plate differentiates into the neural grove, becoming the neural tube upon detachment from the overlying ectodermal cells. Concurrently, the primitive streak regresses, with caudal movement of Hansen's node. Continuing elongation, followed by rapid growth of the head region, produces a head fold at day 15, which then further expands to include neural ridges (future brain) and closure of the neural tube by day 16 (Bryden *et al.*, 1972).

This period of embryonic development (day 15 to 18) is characterised by caudal and cranial folding forming head and tail regions, as well as lateral folding, all involving differentiation of mesodermal cells. Of note at this stage is the anteroposterior elongation of the embryonic shield; while simultaneously the lateral mesoderm splits into splanchnic and somatic layers with formation of the coelom (the space between these layers) by day 14 (Bryden *et al.*, 1972). The splanchnic mesoderm goes on to become splanchnopleure, a

membrane formed with endoderm, and similarly somatopleure which comprises of somatic mesoderm and ectoderm (Bryden *et al.*, 1972).

On either side of the neural plate, somitomeres are formed via a spiralling aggregation of the paraxial mesoderm (McGeady *et al.*, 2006). This development begins in the cephalic region and is associated with regression of the primitive streak. Subsequently, caudal to the seventh somitomere these are further organised into discrete blocks known as somites (McGeady *et al.*, 2006). This occurs simultaneously with post-gastrulation trophoblastic elongation, such that at 16 to 17 days, when the chorionic sac occupies both uterine horns, the concurrent observation of increasing number of somites, ranging from eight to 25, was seen in the developing embryos (Chang and Rowson, 1965); similar observations were also reported by Bryden et al., 1972. Somites are the precursors to, and eventually form, skeletal, muscular and dermal tissue through a complex process of segmentation and differentiation (Latshaw, 1987). Likewise intermediate mesoderm, which connects the lateral mesoderm to the somites, is the precursor to kidneys and some of the genital organs. The embryonic somatopleure becomes the body wall and splanchnopleure becomes the gastrointestinal and respiratory tracts (Latshaw, 1987; McGeady *et al.*, 2006).

By day 17, the trilaminar embryonic disk has been converted into a three-layered tube, via the formation of head, tail and lateral folds, thus establishing the basic body plan of the embryo. Through migration of cells, using the notochord as the axis of reference for cranial-caudal orientation, each layer is transformed into the various cells, tissues and organs of the body (McGeady *et al.*, 2006). Following the formation of these structures the formation of new organs and organ systems ensues (Green and Winters, 1945). Indeed, Wales and Cuneo (1989) reported that 18 somites and the heart bulge were present by day 17. Further

development occurs such that the heart bulge is more prominent, as are pharyngeal (brachial) pouches, and the retinal vesicle and internal ear becoming visible by day 19 (Wales and Cuneo, 1989).

From approximately day 21 onwards many of the structures of the body cavity have formed, and developmental activity changes with rapid growth and further differentiation of those structures (Green and Winters, 1945; Robinson, 1951). During this period, formation of new, major organs is limited. This advanced internal organisation of embryonic body structures is further reflected in the external, dorso-ventral curvature "C" shape form of embryos that is evident by day 19 (Wales and Cuneo, 1989). Increasing torsion of the caudal one-third of the body is reported by Bryden et al. (1972) from day 18 to 21; as is the development and differentiation of nasal, lens and otic placodes and other external structures including limbs from limb buds (day 21 to 24) (Robinson, 1951). Body form changes from "C" shaped, to that almost resembling the fetus take place between day 25 and 29. Gonad and metanephros formation also begins at this time (Green and Winters, 1945; Robinson, 1951).

Table 2.1 lists the previously described developmental events in chronological order, including the appearance of various structures of which the formation is too detailed and therefore beyond the scope of this review.

2.2.3 Extra-embryonic (fetal) membranes

The fetal membranes comprise the amnion, yolk sac, chorion and allantois. These membranes are of embryonic origin and lie between the embryo and the dam. Whilst these membranes are of structural importance they do not form part of the embryo itself. Figure

2.3 demonstrates the arrangement of these extraembryonic membranes in the sheep, and the order of development is listed in Table 2.1.

The trophoblast is the first embryonic membrane to form through rapid expansion (days 11 to 13) (Boyd and Hamilton, 1952). During this elongation of the blastocysts, formation of the primitive yolk sac via migration of the extra-embryonic endoderm to line the trophoblast occurs. Then mesodermal migration, originating from the primitive streak, interposes between the endoderm and trophectoderm forming somatopleure and splanchnopleure (Spencer *et al.*, 2004a). Following this, formation of the chorion, which becomes directly involved with attachment to the maternal uterine tissue, requires folding of the trophoblast around the embryonic disc, at about the time that the primitive streak appears (day 13) (Bryden *et al.*, 1972; McGeady *et al.*, 2006). At the same time that growth of the embryo results in the head and tail pushing their way into the trophoblast, these folds continue to extend until they meet and fuse at a position dorsal to the embryo (day 15) (Bryden *et al.*, 1972; Latshaw, 1987). This folding results in the formation of the amnio-chorion (Figure 2.4) (Bryden *et al.*, 1972). The somatopleure separates into inner and outer layers which surround the embryo. The inner amnion layer remains attached to the embryo at the umbilicus and the outer chorionic layer completely surrounds the embryo, amnion and yolk sac (Latshaw, 1987).

Formation of the trilaminar yolk sac via the migration of the mesoderm to line the primitive yolk sac (endoderm lined blastocoele) is initiated during gastrulation (Boyd and Hamilton, 1952). By day 15, the yolk sac (Figure 2.4) can be seen as a separate membrane (Bryden *et al.*, 1972; Wales and Cuneo, 1989). This yolk sac is initially large, but does not persist throughout gestation, at day 17 it is completely separated from the chorion except

for a small “ab-embryonic” attachment (Bryden *et al.*, 1972). It is further reduced to a solid rod of cells which is partially enclosed within the umbilicus by the allantois at day 25 (Bryden *et al.*, 1972).

From day 17 the emerging “crescent-shaped” allantois (Figure 2.3) is visible at the caudal end of the embryo (Green and Winters, 1945; Boyd and Hamilton, 1952; Bryden *et al.*, 1972; Wales and Cuneo, 1989). This fluid-filled membrane first forms from an outgrowth of the splanchnopleure of the hindgut from day 15 (Boyd and Hamilton, 1952; McGeady *et al.*, 2006) and becomes vascularised by day 17 (Bryden *et al.*, 1972). The allantois forms a large, fluid-filled sac which fuses with the amnion and chorion and fills most of the extra-embryonic coelom (Bryden *et al.*, 1972). Umbilical vessels are derived from the splanchnic mesoderm of the allantois (Latshaw, 1987).

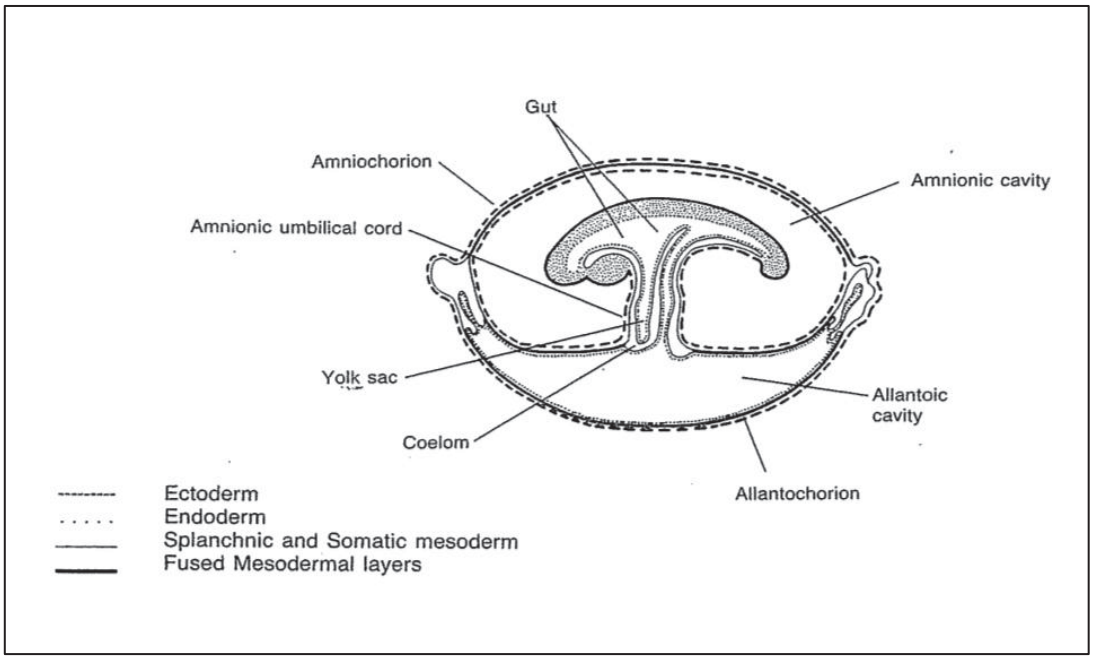

Figure 2.3 Arrangement of mid-gestation extraembryonic membranes of the sheep (Latshaw, 1987).

The formation of these extra-embryonic membranes occurs before implantation, and these structures provide protective, nutritive and excretory functions for the developing embryo, eventually forming a large portion of the placenta (Latshaw, 1987). From day 21-25, after most of the organs have been formed, growth appears to be the priority. During this time there is large expansion of the allantois and the yolk sac disappears (Robinson, 1951).

It is difficult to put a definitive age at which the conceptus ends its embryonic period and begins the fetal period. However, day 34 has been justified by Green and Winters (1945) as an appropriate time for the transition between the two periods. These authors demonstrated that prior to day 34, organogenesis and body form changes were the prominent activity, whereas fetal growth post day 34 is characterised by changes in size and density of organ systems rather than differentiation and formation of new organs and tissues typical of the embryonic period.

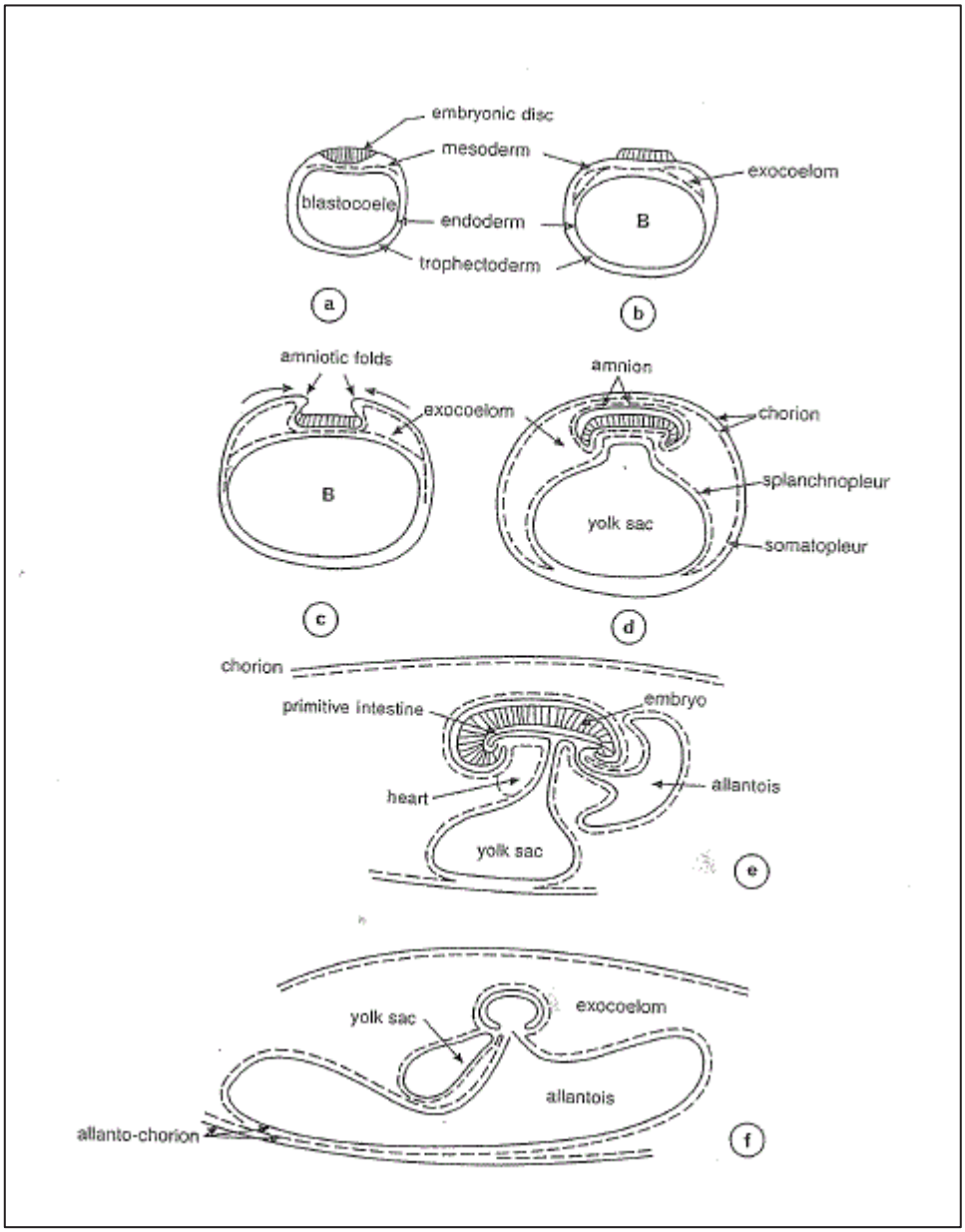

Figure 2.4 Formation of the extra-embryonic membranes (pig blastocyst model): a-d transverse sections showing the differentiation of the mesoderm from the embryonic disc, delamination of the mesoderm, and folding of the trophectoderm and mesoderm to form amnion and yolk sac. e-f longitudinal sections of allantois lined by endoderm and mesoderm, with further expansion to fuse with chorion. (Adapted from Guillomot *et al.*, 1993)

Table 2.1 Development of the sheep embryo and extraembryonic membranes. Adapted from Bryden *et al.*, 1972; Evans and Sack, 1973.

DAY	EMBRYOLOGICAL OBSERVATIONS	EXTRA-EMBRYONIC MEMBRANE OBSERVATIONS/EVENTS
2	Initial cell division	
4	Differentiation of trophoblast	
6	Blastocyst formed	
9	Zona pellucida lost	
10	Days 10-13, round flat embryonic disc present	Yolk sac formed.
12	Elongation of trophoblast begins	
14	End of very rapid elongation of trophoblast. Embryonic shield rises above trophoblast, becomes oval, formation of primitive streak and neural plate	Amniogenesis occurs.
15	Appearance of neural folds, formation of up to 7 somites	Amnion complete.
16	Head folding begins, closure of the neural groove at the level of the 6th somite	Allantois first appears grossly. Differentiation of binucleate cells in the trophoblast.
17	About 20 somites. Continued head folding, tail folding has begun.	Invasion of the uterine mucosa by chorion in the region of chorionic burrs. Allantois crescent-shaped. Separation of yolk sac complete.
18	About 25 somites. Torsion of caudal one-third of body, heart bulge seen, otic placode visible, appearance of first two brachial grooves.	Allantois becoming free from body wall.
19	Appearance of third brachial groove, partial differentiation of first brachial arch into maxillary and mandibular processes. Greater torsion of caudal one-third of body. Heart and liver bulges prominent. Optic vessel, otic placode present.	Allantois enlarging rapidly; appears as a semilunar sac, attached to hind gut by narrow stalk.
20	Posterior neuropore closes. Thick folds represent first sign of fore- and hind limb buds. Separation of maxillary and mandibular processes complete.	Migration of binucleate cells and formation of synctium with maternal endometrial tissue.
21	Embryo assumes "C" shape, with loss of body torsion. Formation of cervical sinus begins.	
22	Second brachial arch covers about one-third of cervical sinus, forelimb bud divided by constriction into proximal cylindrical part, and distal flattened part. Olfactory placodes present.	Trophoblast firmly attached to endometrial luminal epithelium.
23	Differentiation of hind limb buds into proximal and distal segments. Second brachial arch covers about half cervical sinus. Beginning of formation of olfactory pits.	
24	Forelimb buds have proximal, middle and distal divisions, with primitive elbow flexure and primitive hand. Mandibular arches fused. Olfactory pits deep.	
25	Aural hillocks indicate early development of auricle. Nasolacrimal groove deep.	Yolk sac reduced to solid rod of cells.
26	Back begins to straighten. Nasolacrimal groove closed ventrally. Folds representing eyelids present. Cervical sinus closed.	
27	Fusion of nasal and maxillary processes. Loss of segmentation mid-dorsally. Third and fourth digits become prominent in forelimb.	
29	Complete loss of external segmentation. Third and fourth digits of hind limb prominent. Relative narrowing of facial region and elongation of neck.	
30	Tongue projects from mouth. Nasolacrimal groove closed completely.	
31	Development of chorionic villi.	
31-34	Further growth and development of already formed organs.	

2.3 Embryo- maternal interactions: Factors involved in maternal constraint

Prior to implantation, early conceptus growth, that is growth and differentiation of the embryo through the phase of embryogenesis, is significantly different to the growth occurring in the later phases of fetal development once organogenesis is completed (Gluckman, 1997). Traditionally, it is thought that embryogenesis is largely independent of the environment, and driven by the fetal genome, while later fetal growth is influenced considerably by external influences and particularly the intrauterine environment (Johnson, 1979; Gluckman and Liggins, 1984; Gluckman, 1997).

The early embryonic period is a critical period in the developmental process, particularly as it is the time during which conceptus-maternal interactions are initiated (Gaviria and Hernandez, 1994; Spencer *et al.*, 1999b; Spencer and Bazer, 2004a). Moreover, this is an important period of organogenesis, and the time during which implantation occurs. The peri-implantation embryo is undergoing a very active period of development and as a result is vulnerable to the surrounding environment (Dziuk, 1992; Goff, 2002; Spencer *et al.*, 2004a). While it was generally accepted that the embryonic phase of development is "autonomous", it is becoming clear that intrauterine and other external factors can influence the development of the conceptus in the early stages of gestation (Stroband and Van der Lende, 1990; Gluckman, 1997). Recently, it has become accepted that mechanisms of maternal constraint may indeed have a much earlier influence on the growth and the development of the conceptus than previously thought (Hancock *et al.*, 2012; Sharma *et al.*, 2013). Further, influences acting during the peri-conception and pre-implantation stages of gestation may affect and even programme fetal growth and physiology in later gestation (Gluckman, 1997).

There is a complex interplay of maternal, fetal, genetic and environmental factors that have a significant impact on embryo development. These interactions between the embryo and uterus are necessary for normal embryo development and can be either direct or indirect. While it is important to consider the signalling mechanisms that occur between the embryo and uterus at a cellular and genomic level (discussed later in this review), it is equally important to examine the maternal effects that influence embryonic environment. Therefore, the following section aims to discuss specific factors of maternal constraint that act during early embryonic development. Known effects that occur during mid- to late pregnancy will be summarised; however, the discussion will focus on early gestation as related to two main sources of maternal constraint (dam size and litter size) which are relevant to this thesis. Other factors of maternal constraint: dam nutrition, age and parity will first only be summarised in keeping with the scope of this review.

2.3.1 Dam nutrition

The effects of specific maternal factors that influence embryonic and fetal development and growth have been investigated in various studies (reviewed by Dziuk, 1992; Gluckman, 1997; Robinson *et al.*, 1997; Johns *et al.*, 2006). Maternal or dam nutrition is a widely studied factor, particularly because of the current understanding of fetal programming and the developmental origins of adult disease. Barker's (1995;1998) work in human epidemiology has led to the acceptance that under-nutrition during gestation, resulting in constrained fetal growth and development, and subsequent low birth weight, is a definitive factor increasing the risk of cardiovascular, diabetes and other metabolic diseases in later life. In addition, many reports from animal studies demonstrate the effects of maternal under- and over-nutrition during differing stages of gestation to alter fetal growth and

development, regulate gestationally important hormones and alter birth weights (Wallace, 1948; Mellor and Murray, 1982; Rhind *et al.*, 1989; Parr, 1992; Gunn *et al.*, 1995; Harding, 1997; Wu *et al.*, 2004; Oliver *et al.*, 2005; Watkins *et al.*, 2008; Greenwood *et al.*, 2010). The mechanisms of maternal under-nutrition, in mid- to late gestation that lead to fetal growth retardation are complex, but can be simplified to inadequate supply of nutrients to the conceptus. Further discussion is beyond the scope of this review.

Recent studies indicate that maternal nutrition during the pre-implantation period also has a significant effect on fetal growth with subsequent alterations to post-natal health and productivity. This suggests that the maternal environment in later gestation has lesser influence than previously thought (Kwong *et al.*, 2000; Watkins *et al.*, 2008). There is now significant evidence pointing to the fact that nutritional factors acting in the early pregnancy and peri-conceptional period are key to determining fetal growth, as reviewed by Fleming *et al.* (2012) and Bloomfield *et al.* (2013).

2.3.2 Dam Age and Parity

Human epidemiological studies have demonstrated that first-born infants are smaller at birth than second or third offspring (Wilcox *et al.*, 1996; Ong *et al.*, 2002). Similar results were observed in other mammalian species: equine (Wilsher and Allen, 2003), bovine (Kertz *et al.*, 1997) and ovine (Gardner *et al.*, 2007) studies. Similarly, maternal age effects have been demonstrated. Adolescent humans and sheep give birth to smaller offspring than their mature counterparts (Kirchengast and Hartmann, 2003; Loureiro, 2014). It is suggested that the likely cause of these effects is driven by differential nutrient compartmentalization, with age and parity being interconnected (Gluckman and Hanson, 2004). To date the mechanisms underlying these effects have been poorly investigated. No doubt, these dam factors have

been shown to have some influence on embryonic and fetal growth. However, the scope of this review does not allow for further discussion of this factor of maternal constraint.

2.3.3 Dam size

Gluckman and Hanson's (2004) review of maternal constraint states that maternal size is a key factor restricting fetal growth, size at term and post-natal growth. The earliest reports of differential maternal size, the classic study of Walton and Hammond (1938), demonstrated that fetal growth was not solely determined by the fetus' own genotype, but also by maternal genotype. In that study, birth size of foals from reciprocal Shire horse (large) and Shetland pony (small) crosses were similar to that of pure bred foals in which the breed of mare was the same; that is a foal born to Shire dam x Shetland sire was equal in weight to a purebred Shire foal and a foal born to a Shetland dam x Shire sire was equal in weight to a purebred Shetland foal. Similarly, reports in other species following embryo transfer (ET) demonstrated the importance of the maternal environment and the ability of maternal size to restrict fetal growth and development: cattle (Joubert and Hammond, 1958), sheep (Hunter, 1956; Jenkinson *et al.*, 2012b; Sharma *et al.*, 2012a; Sharma *et al.*, 2013), mice (Cowley *et al.*, 1989), horses (Allen *et al.*, 2002; Giussani *et al.*, 2003) and humans (Brooks *et al.*, 1995). In contrast, Dickinson *et al.* (1962) suggested that fetal genotype had a greater impact on birth weight than the maternal environment, although similar results were obtained, i.e. pure-bred large (Lincoln) lambs gestated in pure-bred small (Welsh Mountain) ewes were constrained compared to those gestated in Lincoln ewes; and Welsh Mountain lambs gestated in Lincoln ewes had heavier birth weights than those of Welsh Mountain ewes. Similarly, an examination of fetal vs maternal genotype effect has been examined in humans, from which it was concluded that the fetal influence is

only of consequence when the maternal constraint is significantly diminished (Ounsted *et al.*, 1988).

While it has been demonstrated that genotypically small maternal size results in constraint of fetal growth, it is important to recognise that an embryo that has been transferred to a maternal environment associated with enhanced birth size (large maternal size) leads to increased fetal growth, independent of genotype (Gluckman, 1986). Thus, fetal growth within its usual uterine environment is likely subjected to some degree of natural constraint, and so in this case the true genetic potential of the fetus is not realised. This is of importance as it ensures that fetal growth is therefore matched to maternal size, limiting the risk of complicated births e.g. dystocia (Gluckman and Hanson, 2004).

It has been stated that maternal genotype affects fetal growth through factors that determine uterine size and blood supply, maternal metabolism and placentation (Gluckman, 1986). These factors are generally only considered limiting in mid- to late pregnancy; yet there is evidence that maternal size effects may occur in the early pregnancy and pre-implantation period, well before the physical uterine space could be considered limiting. Our group developed a “dam size” model of maternal constraint using two breeds of dissimilar mature body size sheep: Suffolk (large ~80kg) and Cheviot (small ~60kg). Figure 2.5 illustrates the experimental design used. The effects, following reciprocal ET between these breeds, on offspring weight and size were recorded at day 19, and 90 of gestation and at birth and are summarised in Table 2.2.

Importantly, the results of Sharma *et al.* (2013) study demonstrated that maternal size can restrict embryo size as early as day 19 of gestation i.e. before implantation takes place. Purebred Cheviot embryos, a genotypically small breed of sheep, that were gestated in the

genotypically large breed, Suffolk, were longer than the Cheviot embryos gestated in a Cheviot ewe suggesting that the Suffolk dam provided a luxurious or spacious environment that enhanced the growth of the Cheviot embryo (Table 2.2). In contrast, the Suffolk embryos that were gestated in Cheviot ewes were shorter in length at day 19 than the Suffolk embryos gestated in Suffolk ewes (Sharma *et al.*, 2013), suggesting that the Cheviot dam constrained the growth of the Suffolk embryos. Additionally, Sharma et al (2013) reported that the trophoblast BNC number differed between the breeds of sheep used in the study. BNC play a vital role in histotroph secretion necessary for early embryo development (Guillomot *et al.*, 1993) which may explain the observed embryo effects. Histotroph is made up of the uterine secretions that are the sole support and supply of nutrients to the conceptus prior to implantation (Ashworth, 1995). Therefore it is possible that the genotype differences that occur in the dams of dissimilar size might result in differences in these critical secretions from the uterus in the early preimplantation period to signal and control or determine conceptus growth trajectory. To date little is known about the mechanisms that drive early embryo growth, particularly in relation to the differential embryo growth observed in the dam size model, warranting further investigation.

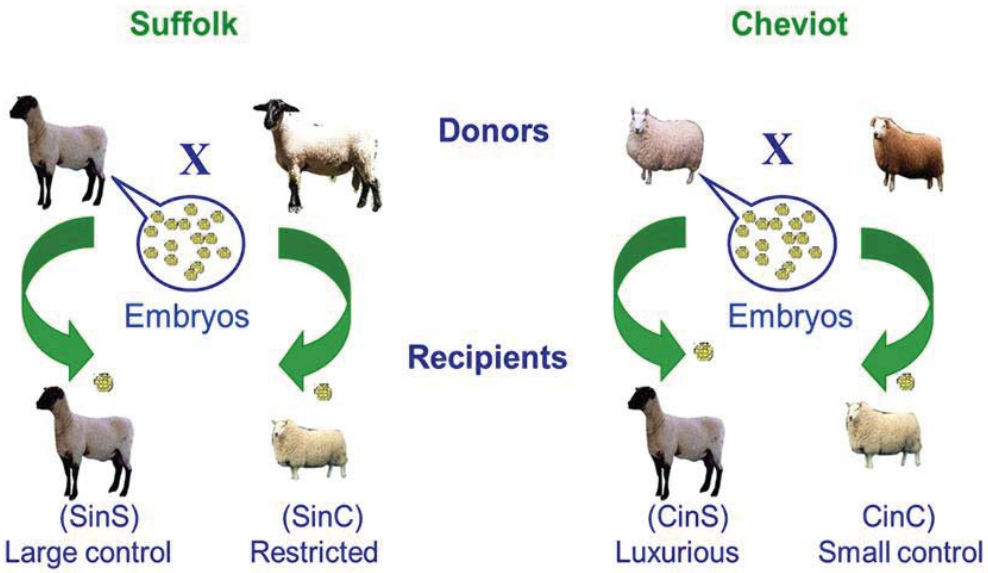

Figure 2.5 Illustration of dam size model experimental design of maternal constraint using reciprocal embryo transfer between large genotype Suffolk and small genotype Cheviot sheep to produce a restricted and a luxurious uterine environment. SinS: Suffolk embryos gestated in Suffolk ewes, SinC: Suffolk embryos gestated in Cheviot ewes, CinS: Cheviot embryos gestated in Suffolk ewes, CinC: Cheviot embryos gestated in Cheviot ewes. (Adapted from Sharma e*t al*., 2010)

Table 2.2 The effect of ewe and embryo genotype combination on offspring weight and size following reciprocal embryo transfer between large genotype Suffolk sheep and small genotype Cheviot sheep at day 19 and 90 of gestation and at birth. CinC (Cheviot in Cheviot =small control), CinS (Cheviot in Suffolk = luxurious environment), SinS (Suffolk in Suffolk = large control), SinC (Suffolk in Cheviot = restricted environment). Table shows least square means (± SEM). Within a row, means with differing superscripts are different from each other ($p<0.05$).

Day of gestation	Parameter measured	Treatment Group				Reference
		CinC	CinS	SinC	SinS	
19	Embryo length (mm)	12.9 ± 0.53^{b}	15.2 ± 0.67^{c}	11.0 ± 0.57^{a}	13.4 ± 0.53^{b}	Sharma et al., 2013
19	Embryo width (mm)	2.2 ± 0.07^{ab}	2.7 ± 0.10^{c}	2.0 ± 0.08^{a}	2.4 ± 0.08^{bc}	Sharma et al., 2013
19	Heart bulge width (mm)	1.7 ± 0.08^{a}	2.0 ± 0.10^{b}	1.5 ± 0.08^{a}	2.0 ± 0.08^{b}	Sharma et al., 2013
90	Fetal body weight (g)	579 ± 27^{a}	600 ± 28^{a}	694 ± 32^{a}	681 ± 27^{b}	Jenkinson et al., 2012
90	Crown-rump length (cm)	27.1 ± 0.2^{a}	27.1 ± 0.2^{a}	27.9 ± 0.3^{b}	27.3 ± 0.2^{ab}	Jenkinson et al., 2012
Birth	Birth weight (g)	5.16 ± 0.22^{a}	5.54 ± 0.23^{ab}	5.04 ± 0.20^{a}	5.94 ± 0.19^{b}	Sharma et al., 2012
Birth	Crown-rump length (cm)	56.18 ± 0.90^{a}	56.89 ± 0.92^{ab}	56.35 ± 0.78^{a}	59.25 ± 0.76^{b}	Sharma et al., 2012

2.3.4 Litter size

Early human studies showed that compared to singletons, twins are born up to three weeks earlier and have lower birth weights (Bleker *et al.*, 1979). In other polycotous species, Gardner *et al.* (2007) stated that "the maternal uterine space is finite in capacity"; thus as litter size increases, individual birth weight decreases. Using highland and lowland breeds of sheep, Gardner *et al.* (2007), showed that the birth weights of twins, triplet and quadruplets were 87%, 75% and 62% of the average singleton birth weight, respectively. Similar evidence of lower birth weight with increasing litter size is observed in many species for example cattle, (Anderson *et al.*, 1982), pigs, (Waldorf *et al.*, 1957) and mice (McLaren, 1965). It appears that the physical capacity of the uterine environment and placental and vascular insufficiencies are the dominant influences acting to restrict fetal growth in multiple pregnancies, similar to the previously described factors of maternal constraint. It has been traditionally accepted that litter size effects have their greatest influence in late gestation, restricting nutrient availability to the fetuses (Bleker *et al.*, 1979; Muhlhausler *et al.*, 2011). However, there is emerging evidence suggesting that growth and developmental trajectories in twins and singletons are set during the periconceptional period (Rattray *et al.*, 1974; Iffy *et al.*, 1983; Hancock *et al.*, 2012).

Using a twin fetal reduction technique at day 42 to 43 of pregnancy in sheep Hancock *et al.* (2012) showed that those that were conceived as twins but were reduced to singleton were of similar size at birth to those that remained twins for the entire period of gestation. This suggests that the fetal growth trajectory of twins may be largely determined during the early gestational period, rather than in response to the physical limitations of late gestation. Hancock *et al.* (2012) also demonstrated that twins and reductions had greater fat mass

than singletons in adulthood; again suggesting that twin conception may result in specific signals between the embryo and dam that result in the programming of many aspects of growth and development. Little is known of what mechanisms may be influenced by litter size or uterine function to drive conceptus growth between gestational days 0 and 42/43, when Hancock *et al.* (2012) performed the reductions of twins to singletons. This warrants further investigation.

There is a clear indication that the intrauterine environment during the very early stages of gestation has an impact on embryonic growth and development which can subsequently affect fetal growth and survival, and potentially also postnatal growth. For this effect to occur there must be some signalling and/or interaction between the embryo and dam. Thus, further investigation of the influence of the changing intrauterine environment is required.

2.4 Uterine adaptions to pregnancy: Structural, secretory and biochemical (cell signalling) function during embryo development

Embryogenesis comprises a series of developmentally timed events (previously described in this literature review), which are controlled by mechanisms that regulate cell proliferation and differentiation to result in the progression of the zygote through to blastocyst then to embryo and finally fetal stages. A successful pregnancy is dependent on the differentiation, organisation and subsequent formation of tissues and organs which are driven by cell signalling and gene functioning (McGeady *et al.*, 2006).

The signals which drive pregnancy are diverse and it is fundamental to the stage of gestation and embryonic location within the uterus that these signals are coordinated for

normal embryogenesis to occur (McGeady *et al.*, 2006). It is well understood that restructuring of the endometrium is essential to the nourishment, development and implantation of the embryo, particularly in early gestation (Spencer and Bazer, 2004b). It is the uterine adaptions and properties at the structural and secretory level during this period that is of interest, as the developmental trajectory of the embryo is driven by endometrial mechanisms that operate at these early stages (Sandra *et al.*, 2011). The following section of this review will examine the structural and signalling changes that occur during early pregnancy within the uterus. For the purpose of this thesis the discussion will focus on the mechanisms that occur in sheep unless stated otherwise.

2.4.1 Structural and secretory adaptations

Most uterine factors that regulate conceptus growth and development are highly related to the ability of the dam to provide nutrition and protection for the developing conceptus. In the ungulate uterus, histotrophic and haemotrophic nutrition are most influential on conceptus development, onset of pregnancy recognition signals and fetal-placental growth (Spencer and Bazer, 2004b). Emerging evidence continues to demonstrate that early gestational factors are important for the development of the embryo during this period and have the potential to programme growth and development throughout the rest of gestation and subsequently in postnatal life. Of these gestational factors nutrition is vital and uterine secretory activity provides the necessary nutrients during the critical time of early development.

Although not much is known about the actual components of histotroph or of the requirements of the developing conceptus, recent data provides some evidence of the secretory products of the endometrium and conceptus (Ashworth, 1995; Robinson *et al.*,

2008). A major secretory product, retinol-binding protein has been shown to be secreted by both the conceptus and endometrium (Trout *et al.*, 1991; Harney *et al.*, 1993; Doré *et al.*, 1994). This protein plays an important role in embryonic development and extra-embryonic membrane differentiation via transport of retinol (vitamin A) to and within the developing conceptus and its secretion appears to be driven by progesterone (Harney *et al.*, 1993). Osteopontin (SPP1) and glycosylated cell adhesion molecule-1 proteins are believed to be involved in regulation of implantation (Johnson *et al.*, 1999b; Spencer *et al.*, 1999a). Other important secretions being examined for their role in embryo development of providing a source of energy, nutrient transport, differentiation of trophectoderm and development of metabolic pathways include glucose, amino acids, glutathione, calcium, sodium, potassium (Moffatt *et al.*, 1987; Gao *et al.*, 2009). Additionally, significant components of uterine secretions are the cytokines and growth factors (Ko *et al.*, 1991; Martal *et al.*, 1997), which are potentially involved in regulation of blastocyst development, uterine receptivity and interferon tau (IFNτ) secretion (Roberts *et al.*, 2003; Imakawa *et al.*, 2004). These may include insulin-like growth factors (IGFs), fibroblast growth factor 1 and 2 (FGF1, FGF2), transforming growth factor (TGF), and α and β epidermal growth factor (EGF).

The source of histotroph is the endometrial glands (Wimsatt, 1950; Amoroso, 1952; Bazer, 1975). Several reviewers have discussed at length the unequivocal role of endometrial gland secretions as regulators of conceptus development and survival, pregnancy recognition signal production, implantation and placentation (Roberts and Bazer, 1988; Gray *et al.*, 2001a). Comprehensive investigation by Gray *et al* (2000, 2001b, 2001c, 2002) using the uterine gland knockout (UGKO) ewe model demonstrated an essential role of endometrial glands and secretions in peri-implantation embryo growth and survival.

Adult UGKO ewes are unable to establish pregnancies both naturally and following ET (Gray *et al.*, 2000; 2001c). Further, on day 14 post-mating, uterine flushes of UGKO ewes contain either no conceptus or an obviously growth constrained tubular conceptus, compared to the morphologically normal blastocysts present on day 6 or 9 post-mating (Gray *et al.*, 2001c). These effects are as a result of decreased secretion of specific components of histotroph products of the glandular epithelium rather than a defect of the luminal epithelium (Gray *et al.*, 2002). Similarly, the importance of these glands has been demonstrated in cows (Bartol *et al.*, 1995) and pigs (Knight *et al.*, 1977).

In the individual animal, morphogenesis of its uterus is initiated during fetal stages of development but is only completed following post-natal differentiation and development (adenogenesis) of the endometrial glands (Wiley *et al.*, 1987; Taylor *et al.*, 2000). In the ovine neonate endometrial gland adenogenesis is considered complete by 56 days postnatally (Taylor *et al.*, 2000). Final maturation and growth of the endometrial glands occurs at puberty. However, endometrial glands must undergo extensive hyperplasia during pregnancy (day 15 to 50), followed by hypertrophy to meet the histotrophic demands of the developing conceptus (Stewart *et al.*, 2000). The growth of the endometrial glands has been demonstrated to be quite significant: four-fold in length and 10-fold in width from day 16 of gestation to term (Wimsatt, 1950). Other important structural changes that occur after implantation are described comprehensively by Wimsatt (1950) including changes to placentomes in caruncular regions of the endometrium and uterine vascularity throughout pregnancy. However, these changes are beyond the scope of this review and will not be discussed further.

The substantial growth and remodelling of uterus occurs from day 15/16 to days 50-60 and is necessary to accommodate conceptus development and growth in the later stages of pregnancy (Wimsatt, 1950). Further, an important aspect of the remodelling of the uterine epithelium of sheep involves the migration and fusion of conceptus trophoblast BNC into the uterine epithelium (described previously) (Boshier, 1969; Wooding, 1980). This process begins at day 16 of pregnancy and is significant as it functions to form the synepithelio-chorionic placenta characteristic of the ovine pregnancy (Wooding, 1992; Spencer *et al.*, 2006). This process is also involved in the synthesis and secretion of proteins and steroid hormones, which are major components of histotroph (Wooding, 1992; Stewart *et al.*, 2000). Histotroph synthesis and secretion, necessary for fetal-placental growth is dependent on this intercaruncular-endometrial remodelling and subsequent differentiation of function (Spencer *et al.*, 1999b; Stewart *et al.*, 2000). It is suggested that this process is mediated by specific factors acting at the conceptus-endometrial interface (Spencer *et al.*, 1999b; 2004b), which will be discussed below.

2.4.2 Cell signalling during embryo development

The remodelling, regulation of endometrial gland morphogenesis and secretory function is driven by sequential exposure of the pregnant ovine uterus to a variety of steroid and protein hormones (Stewart *et al.*, 2000). These hormones act in a paracrine manner to elicit gene expression changes and development of the endometrium to support the growing and developing conceptus (Spencer and Bazer, 2004b). It is important that a combined and integrated paracrine and endocrine system of signals from conceptus, ovary and uterus work together in order to ensure that the uterus is receptive and the embryo is sufficiently advanced for implantation, and to ensure optimal support of conceptus growth and

development for the rest of the pregnancy (Ashworth and Bazer, 1989; Spencer and Bazer, 2004a).

Sequential exposure to oestrogen, progesterone, INFτ, placental lactogen (PL) and placental growth hormone (GH) drives endometrial gland morphogenesis and secretory function in the pregnant ovine uterus (Spencer *et al.*, 1999b; Noel *et al.*, 2003). Endometrial gland morphogenesis, remodelling and secretory activity is a necessary part of the establishment of pregnancy. Without the processes of maternal recognition and receptivity to implantation, of which the endometrial glands play an important role, pregnancy cannot be established (Spencer *et al.*, 2007; Bauersachs and Wolf, 2015). Reciprocal interactions between the conceptus and endometrium are critical to the maintenance of pregnancy. Firstly, in order for pregnancy to be established, the luteolytic process that is characteristic of cycling ewes must be abrogated (McCracken *et al.*, 1984). The endometrial luminal epithelium (LE) and superficial glandular epithelium (sGE) release luteolytic prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in a pulsatile manner during late dioestrus (Gray *et al.*, 2000). These luteolytic pulses are driven by carefully coordinated changes in progesterone and oestrogen and involve oxytocin binding to oxytocin receptors (*OXTR*) on the LE and sGE (Wathes and Hamon, 1993; Stevenson *et al.*, 1994b). Figure 2.6 illustrates the sequential hormone (progesterone, oestrogen and oxytocin) action via their respective receptors in the endometrium. Briefly, in cyclic ewes during oestrus and metoestrus circulating oestrogen from ovarian follicles stimulate *OXTR* on the LE and sGE via action of the oestrogen receptor α (*ESR1*). Progesterone receptors (*PGR*) are also present on the LE and sGE; however, circulating progesterone at this stage is too low to activate these *PGR*s causing subsequent suppression of *ESR1* and *OXTR*. Between days 5 and 11 circulating progesterone

from the maturing corpus luteum (CL) increases. This increased progesterone acts via *PGR* to block expression of *ESR1* and *OXTR* for 8 to 10 days. If there is no embryo present, continuous exposure of the endometrium to progesterone eventually results in loss of *PGR* from the LE and sGE after day 11. This ends the *PGR* block and results in rapid increase in *ESR1* on days 12 to 13 and followed by induction of *OXTR* on day 14 due to increasing oestrogen secretion from the ovarian follicles (Spencer *et al.*, 1995a; Spencer and Bazer, 2004a). Oxytocin secreted from the posterior pituitary and CL then binds to *OXTR* on the LE and sGE inducing the pulsatile release of $PGF_{2\alpha}$ resulting in regression of the CL and a return to cyclicity (Spencer *et al.*, 2006).

In contrast, in pregnant sheep, the conceptus trophectoderm synthesises and secretes INFτ between day 10 to day 21 of gestation, peaking at day 14 to 16 (Roberts *et al.*, 1999) (Figure 2.6). INFτ acts in a paracrine manner on LE and sGE to supress *ESR1* gene expression and consequently, the *OXTR* gene (Spencer and Bazer, 1996). This involves INFτ binding to type 1 INF receptors (*INFAR*) on LE and SGE, thereby inhibiting expression of *ESR1* via an Interferon regulatory factor 2 (*IRF2*) signalling pathway. This prevents oestrogen from inducing *OXTR* via *ESR1*, and results in abrogation of the luteolytic $PGF_{2\alpha}$ pulses being produced by the endometrium, maintained progesterone production by the CL and results in the establishment of pregnancy (Roberts *et al.*, 1984). Additionally, INFτ is associated with induction and expression of several endometrial genes that are thought to play important roles in uterine receptivity and conceptus development (Hansen *et al.*, 1999; Spencer *et al.*, 2004a).

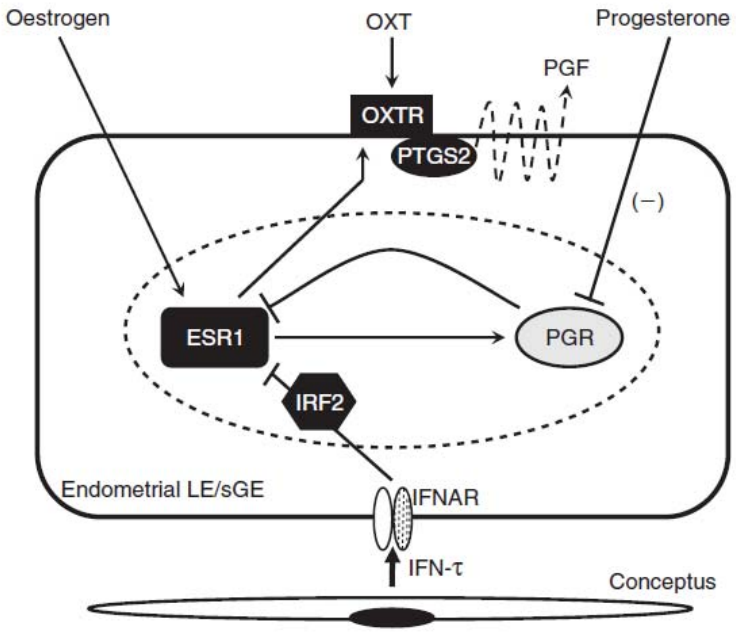

Figure 2.6 Illustration of hormonal regulation and integrated signalling between embryo-uterine inter-face during early pregnancy. (Spencer *et al.*, 2006). In cyclic ewes circulating oestrogen increases expression of oestrogen receptor (*ESR1*) and oxytocin receptor (*OXTR*) present on the luminal epithelium (LE) and superficial glandular epithelium (sGE) during oestrus and metoestrus. At the same time circulating levels of progesterone are inadequate to activate progesterone receptors (*PGR*) to cause the suppression of *ESR1* and *OXTR*. Maturation of the corpus luteum (CL) during early dioestrus increases circulating progesterone, activating PGR with resulting suppression of ESR1 and OXTR for 8 to 10 days, in combination with low oestrogen. Continuous progesterone exposure results in down-regulation of *PGR* in LE and sGE (days 11 and 12) ending the progesterone block of *ESR1* and *OXTR*. This is followed by increased ESR1 and subsequent induction of *OXTR* by oestrogen (day 13 and 14), allowing oxytocin secreted by the pituitary and CL to bind to *OXTR* resulting in luteolytic pulses of $PGF_{2\alpha}$ via a prostaglandin synthase 2 (*PTGS2*) pathways. In pregnant sheep, interferon tau (INF-τ), secreted by the elongating conceptus from day 11 to 25 of pregnancy, binds to type 1 INF receptors (IFNAR) on LE and sGE thereby inhibiting ESR1 via IFN regulatory factor 2 (IRF2) signalling pathway. This prevents ESR1 expression and thereby inhibits the ability of oestrogen to induce OXTR expression and pulsatile release of $PGF_{2\alpha}$, abrogating luteolysis.

Although the presence of a pre-implantation embryo is not necessary for priming the maternal environment for pregnancy and the first steps of uterine remodelling (Cross *et al.*, 1994), successful pregnancy does require embryo recognition and in response various factors that drive uterine receptivity to the developing embryo (Sandra *et al.*, 2011). The presence of the embryo is not necessary to induce changes in the uterus important for implantation (Guillomot *et al.*, 1993). However, the lifespan of these reactions is short-lived. In contrast, embryo development is dependent on specific uterine factors and its presence within the uterus is necessary to ensure specific cross-talk to occur between the maternal and conceptus tissues (Lawson *et al.*, 1983; Gray *et al.*, 2001c; Satterfield *et al.*, 2006). In sheep, blastocyst elongation does not occur *in vitro* and requires presence within the uterus in order for elongation to be induced (Fléchon *et al.*, 1986). It is this elongation that initiates the production of INFτ, the important signal to the maternal tissues to prepare for implantation and pregnancy (Roberts *et al.*, 1984). Following elongation, establishment of conceptus trophoblast tissue occurs, allowing BNC invagination into the maternal tissue to occur.

In the sheep, conceptus trophoblast BNC are associated with PL production as early as day 16 of pregnancy (Wooding *et al.*, 1992). Although the exact role of PL during pregnancy in sheep has not be fully established, it has been demonstrated that embryonic PL is primarily involved in stimulating the endometrial gland and secretion in early pregnancy (Stewart *et al.*, 2000; Noel *et al.*, 2003). In addition ovine PL plays a role in steroidogenesis in the CL, endometrial proliferation, *PGR* expression and protein synthesis and secretion (Spencer *et al.*, 1999b). PL produced by the BNC are also associated with the expression of uterine milk proteins (*UTMP*) (Stewart *et al.*, 2000), and osteopontin (*SPP1*) (Johnson *et al.*,

1999b) markers of uterine secretory activity, by the glandular epithelium. Additionally, placental GH has been shown to be a part of this uterine-conceptus interplay, as increased expression levels are correlated with onset of GE hypertrophy and increases in *UTMP* and *SPP1* (Lacroix *et al.*, 1996). It has been proposed that PL and GH may be secondary signals for maintenance of pregnancy, acting to reinforce the action of INFτ and working with progesterone and estrogen to regulate uterine function during pregnancy (Spencer *et al.*, 1999b).

The mechanisms by which INFτ, PL and GH work to influence GE remodelling are yet to be deciphered. However, it is believed that INFτ may act to induce gene expression within the endometrial glands (Hansen *et al.*, 1999; Rosenfeld *et al.*, 2002). It is necessary that the entire sequence of events involving progesterone and estrogen occur in synchrony, since no change in endometrial gland size or function was observed in sheep infused with PL and GH alone (Noel *et al.*, 2003). Therefore, it can be concluded that endometrial remodelling necessary for histotroph production and uterine receptivity is dependent on specific paracrine-acting factors. These factors act to initiate a complex interplay between the maternal and embryo interface resulting in embryo implantation, maintenance of pregnancy and fetal-placental growth. Of these paracrine factors, progesterone plays an extremely important role both in recognition of pregnancy and in uterine receptivity through various mechanisms, and there is need to further understand the mechanisms by which these processes occur.

2.5 Progesterone regulated embryo maternal interactions

Progesterone, the "pregnancy hormone", stimulates and maintains many uterine endometrial functions that are important for maternal pregnancy recognition, conceptus growth and development, receptivity to implantation, placentation and maintaining the pregnancy to term (Spencer and Bazer, 2002; Spencer *et al.*, 2004b). The source of progesterone in sheep is the CL in early pregnancy; however, the placenta takes over progesterone production once the pregnancy has been established at approximately day 55 of gestation (Harrison and Heap, 1978). In the cyclic ewe, plasma progesterone concentration is usually below 0.4 ng/mL during the first four days of the cycle (Figure 2.7) (Thorburn *et al.*, 1969). Progesterone concentrations then increase between day 4 and 9 to an mean of 1.5-2.5 ng/mL and remain at this concentration for approximately five days before declining on day 14/15 to below 0.4 ng/mL on the day before oestrus (Thorburn *et al.*, 1969). On the day of oestrus progesterone concentration is 0.1 ng/mL (Thorburn *et al.*, 1969). However, in pregnancy, progesterone concentrations rise from day 4 to 15 in a similar manner to the cyclic, non-pregnant ewe (Figure 2.8) (Bassett *et al.*, 1969). Concentrations are then maintained above 2.0 ng/mL for the duration of the pregnancy; 2.0-2.5 ng/mL until approximately day 50, with a steady rise to day 130 (Bassett *et al.*, 1969). Pregnancy loss has been reported in the case of inadequate progesterone and/ or a delayed or advanced rise in progesterone (Henricks *et al.*, 1971; Miller and Moore, 1976; Wilmut *et al.*, 1985; Mann and Lamming, 1995; 1999).

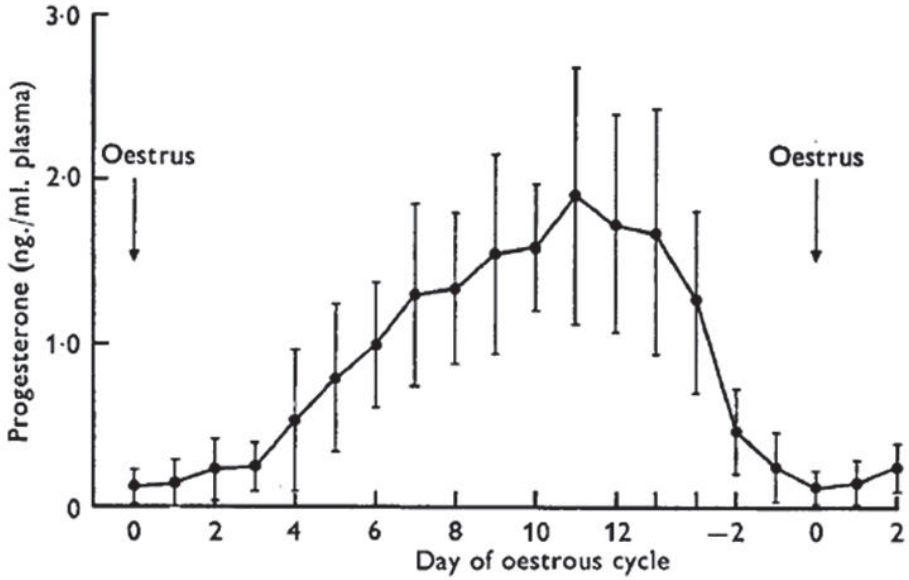

Figure 2.7 Mean progesterone concentration in the peripheral plasma of sheep during the oestrous cycle (Thorburn *et al.*, 1969).

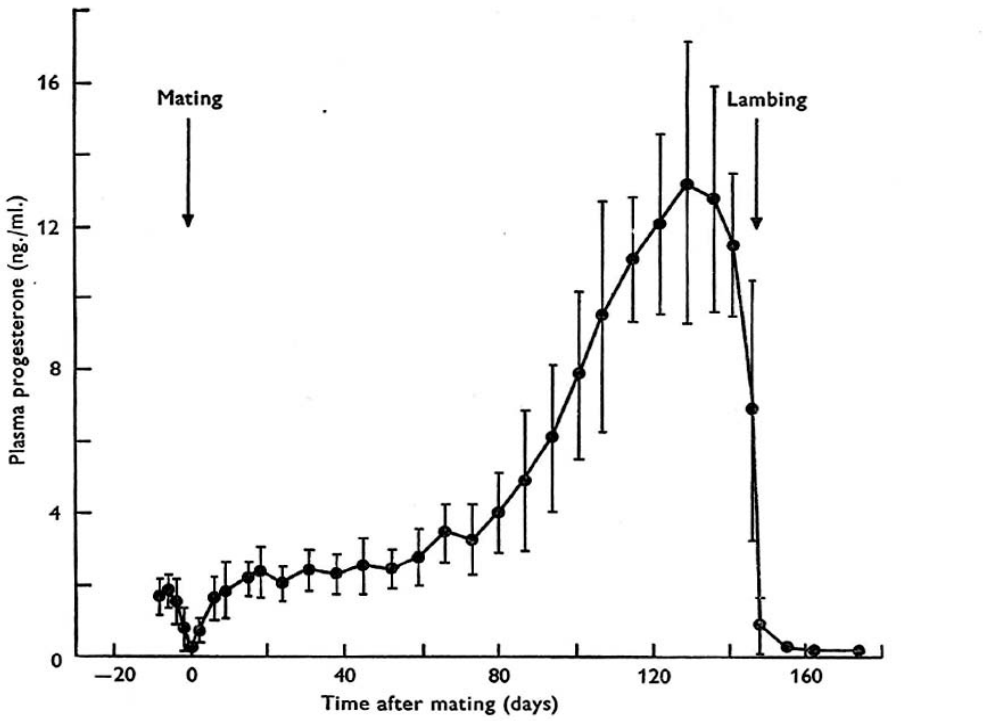

Figure 2.8 Mean progesterone concentration in the peripheral plasma of sheep throughout pregnancy (Bassett *et al.*, 1969).

The actions of progesterone are mediated by the progesterone receptor (PGR) (Spencer and Bazer, 1995). In sheep continuous exposure of the endometrium to progesterone for at least eight days represses *PGR* gene expression in the endometrial epithelium (Spencer *et al.*, 1995b; Gray *et al.*, 2006). In cycling ewes, loss of *PGR* allows for *ESR* and subsequently *OXTR* induction with a resulting luteolytic response (Spencer *et al.*, 2008). In contrast, in pregnant ewes, during the peri-implantation period the conceptus INFτ signal acts to sustain CL progesterone production, essential for recognition of pregnancy leading to implantation and placentation in many species (Bazer *et al.*, 2011). *PGR*s are not detectable in the uterine luminal epithelium and glandular epithelium in sheep after day 11 and 13 of pregnancy, respectively (Spencer and Bazer, 1995). However, *PGR*s are found in the endometrial stroma and myometrium throughout most of gestation (Spencer *et al.*, 2004b).

A paradox of early pregnancy is that it is necessary for *PGR* expression to be reduced in order for progesterone's pivotal role in uterine receptivity and implantation to occur (Spencer *et al.*, 2004a). So while uterine *PGR* gene expression is down-regulated, progesterone simultaneously stimulates expression of genes coded for uterine receptivity, endometrial gland morphogenesis and histotroph secretion that support conceptus survival, growth and development in early pregnancy (Spencer *et al.*, 1999b; Bazer *et al.*, 2011), These include prostaglandin endoperoxide synthase 2 (*PTSG2* or *COX2*) (Charpigny *et al.*, 1997; Gray *et al.*, 2006), cathepsin L (*CTSL*) (Song *et al.*, 2005), galectin 15 (*LGALS15*) (Gray *et al.*, 2006; Satterfield *et al.*, 2006). Secreted phosphoprotein 1 (*SPP1*) (or osteopontin) (Johnson *et al.*, 2000; 2003) and uterine milk proteins (*UTMP* or *SERPINS*) (Stewart *et al.*, 2000). At the same time PGR loss is also associated with a reduction of anti-adhesive mucin glycoprotein 1 (*MUC1*) expression (Bazer *et al.*, 2009). In contrast, continued stromal

expression of *PGR* throughout pregnancy is associated with increased expression of progestomedins, fibroblast growth factors 7 and 10 (*FGF7*, *FGF10*), hepatocyte growth factor (*HGF*) and c-met proto-oncogene (*MET*) which act in a paracrine manner to mediate progesterone effects on endometrial cellular functions, epithelial cell proliferation and differentiation and conceptus development (Chen *et al.*, 2000a; 2000b; Spencer and Bazer, 2002; Satterfield *et al.*, 2008a).

Progesterone plays a critical role via its action through endometrial gene regulation to influence and maintain pregnancy and embryo survival. However, their mechanisms may also be important to not only the survival of the developing conceptus with respect to establishing pregnancy but may also have significant influence on conceptus growth and development. This effect of exogenous administration of progesterone on embryo growth in sheep during the early pregnancy pre-implantation period has long been investigated (Foote *et al.*, 1957; Wintenberger-Torrès *et al.*, 1967; Bindon, 1971a).

Progesterone effects on embryo growth have been well examined in cattle: increasing concentration exogenously in early pregnancy (day 2 to day 5 and day 5 to day 9) resulted in more developmentally advanced embryos compared to controls at day 14 in heifers (Garrett *et al.*, 1988). Similar results were observed in beef-heifers following administration of progesterone from day 3 until slaughter at day 13 and 16 (Carter *et al.*, 2008); and at day 16 in cows following progesterone supplementation from day 5 to day 9 (Mann *et al.*, 2006). Further, poor embryo development, at day 16 post first insemination, was demonstrated in cows with lower (endogenous) progesterone concentrations during the luteal phase than cows with well-developed embryos (Mann and Lamming, 2001). In contrast, progesterone supplementation from day 4.5 to 8 had no effect on conceptus size in treated heifers

compared to controls (Beltman *et al.*, 2009) or in cows supplemented from day 12 to 16 (Mann *et al.*, 2006). These studies concur with work done by Clemente *et al.* (2009) in which similar size increase in conceptus length was observed at day 14 following ET (day 7) into progesterone-primed heifers. This suggests that progesterone has an indirect effect on embryo growth acting via modifications to the uterine environment (Clemente *et al.*, 2009).

In sheep, these above progesterone effects on embryonic and fetal growth have been further examined using ET studies. The work of Wintenberger-Torres *et al*. (1967) demonstrated an effect as early as days 8 to 11 with observed advanced segmentation of fertilised ovum following progesterone supplementation from day 2 to 11. Kleeman *et al*. (1994) were able to demonstrate increased fetal weight and crown-rump length at day 74 following progesterone supplementation in recipient ewes in the first three days of pregnancy compared to the control group. In a follow-up study, they demonstrated increases in not only fetal weights but also fetal organ (brain, kidney, spleen, gut, head and thorax) weights and dimensions in response to recipient progesterone supplementation from day 1 to 3 (Kleemann *et al.*, 2001). These studies have made significant addition to the understanding of how progesterone may influence embryo growth, and are consistent with the work done in cattle by Clemente *et al.* (2009), which suggests progesterone mediates changes within the uterus necessary for the advancement of conceptus development and growth. However, other research suggests however that the enhanced early conceptus growth may be a function of differential cell allocation of trophectoderm to inner-cell mass cells resulting in the possibility of a larger placenta and therefore improved nutrient delivery necessary for growth (Hartwich *et al.*, 1995).

More recent studies have started to unravel the mechanisms that drive these effects on embryo growth. There has been an increased understanding of progesterone's action on uterine gene expression to cause uterine structural changes, endometrial gland morphogenesis and histotroph production which may all act to provide the support and necessary nutrients for embryo growth and development elongation in sheep and cattle, as described above (Satterfield *et al.*, 2006; 2009; Forde *et al.*, 2009). Little evidence exist that progesterone has a direct effect on the growth of the embryo, and it is increasingly apparent that further investigation of progesterone's role in the mechanisms that drive embryo development and growth in the uterine environment, and to further elucidate its function in the embryo maternal dialogue, is needed.

2.6 Summary

Embryo growth is driven by both genetic and environmental factors. To date, it is apparent that environmental effects play a major role in determining embryo growth and development. Maternal size and litter size are key factors that have been shown to enhance or constrain embryo/fetal growth and development. Based on the research of Sharma *et al.* (2013) and Hancock *et al.* (2012) these maternal constraint effects can be detrimental to embryonic development as early as the pre-implantation stages of gestation. The embryo-maternal dialogue and contributing hormones, particularly progesterone, play an important role in orchestrating the effects that have been reported. However, further understanding of maternal constraint effects on embryo growth in early gestation is warranted. Furthermore, the molecular mechanisms driven by progesterone to overcome constraint effects are yet to be elucidated.

Based on previous research it is postulated that:

1. Mechanisms acting in early pregnancy, before implantation, programme or drive early embryo and fetal growth trajectory.
2. Embryonic growth is driven by dam signals controlled by differential gene expression
3. Maternal constraint on embryo development due to dam size can be overcome by administering exogenous progesterone.

Therefore the aims of this thesis are:

a) To examine the effects of litter size (singleton vs twin) on pre-implantation embryo size in early gestation. (Chapter 3)

b) To determine if pre-implantation embryo gene expression differs in embryos that are conceived as either a singleton or twin and how this may be related to differences observed in growth. (Chapter 3)

c) To examine the effects of mature dam size on pre-implantation embryo size and development and establish if morphometric measurements are an accurate representation of the stage of growth and development. (Chapter 4/5)

d) To determine if administration of exogenous progesterone in early pregnancy can overcome the previously observed maternal constraint effects exerted by dam size. (Chapter 6).

e) To determine the mechanisms through which progesterone is involved in maternal signalling via the uterine endometrium to influence embryo growth and development. (Chapter 7/9).

f) To determine the critical period of progesterone administration that elicits effects on embryo growth and survival. (Chapter 8/9).

Foreword to Chapter 3

The study presented in Chapter 3 of this thesis uses the physiologic model of maternal constraint, litter size. The methodology of this chapter includes transcriptomics analysis of embryonic tissue in order to investigate the molecular mechanisms underlying differential embryo growth in singleton and twin pregnancies. Analysis of RNA-seq data is expected to generate a list of genes that are differentially expressed between experimental groups; the data can then be used for further analysis that may determine biological and molecular pathways. It was necessary to perform two RNA-seq analyses on the embryonic tissue that was examined as the initial analysis (RUN1) performed by New Zealand Genomics Limited (NZGL, Dunedin) was found to be unreliable due to a reagent quality error. Therefore, the results section of this chapter reports RNA-seq analysis of the corrected run (RUN2), but qPCR analysis (gold standard for gene expression, used for validation of the RNA-seq data) is based on the ten genes that were chosen from RUN1. The respective runs will be referred to within the text of the chapter as necessary.

3 Comparison of pre-implantation single and twin embryo size and embryonic gene expression at day 21 of gestation

3.1 Abstract

The aim of this study was to examine differences in size and biological function of twin vs singleton embryos at day 21 of pregnancy using morphometric measurements and RNA-seq analysis. It was proposed that differing growth trajectory in singleton and twin pregnancies are determined in the early pre-implantation period and that this is determined by the embryo. Reciprocal embryo transfer (ET) between singleton and twin pregnancies was performed at day 7 of gestation, in which donors also served as recipients to produce the following experimental groups: single embryo transferred into single corpus luteum (CL) ewes (1E1CL), single embryo transferred into two CL ewes (1E2CL), twin embryos transferred into single CL ewes (2E1CL), and twin embryos transferred into two CL ewes (2E2CL). Two control (singleton bearing (Con1E1Cl) and twin bearing (Con2E2CL)) non ET groups were also included in the analysis.

At day 21, 1E1CL embryos were smaller ($p<0.05$) than the other ET and control embryo groups. There were no differences ($p>0.05$) between the other ET and control embryo groups. This suggests the possibility of an embryo transfer effect in this group compared to the singleton bearing control. Approximately 71% of the RNA-seq reads mapped to the ovine genome (OARv3.2). However, there were no differentially expressed genes (FDR $p<0.05$, fold difference >1.5) generated from the comparisons of embryo groups examined.

This is the first study to use RNA-seq to compare gene expression in singleton and twin embryos following reciprocal transfer. The results do not confirm that pre-implantation events (embryonic or maternally driven) are responsible for the differential growth resulting in lighter birth weights reported in twins compared to singletons. Further studies are needed to decipher the mechanisms that are occurring during this period to drive growth

and development in early gestation in twins compared to singletons. The use of other methodologies, such as *in situ* hybridisation or the examination of specific embryonic cell lineages, would be worthwhile to achieving this.

3.2 Introduction

Twin pregnancies represent a natural model of intrauterine growth restriction (IUGR) in comparison to singleton pregnancies (Gootwine *et al.*, 2007). In human and sheep studies, the outcome of this IUGR is lighter birth weights observed in twins (Alexander *et al.*, 1998; Buckler and Green, 2004; Gardner *et al.*, 2007). Further, in humans, lighter birth weights are associated with increased risk of poorer long-term health outcomes, such as cardiovascular disease, obesity and diabetes (Newsome *et al.*, 2003; Simmons, 2008).

It has been postulated that the smaller size at birth and shortened gestation length seen in twins compared to singletons is due to maternal uterine constraint either as a result of limitations of intrauterine space and/or placental nutrient supply in late gestation (Greenwood *et al.*, 2000; Gootwine, 2005; Rhind *et al.*, 2010; Bleker *et al.*, 2014). However, contrasting evidence has led to the proposal that events in early gestation, perhaps as early as the preimplantation period, play a critical role in determining the differences observed in growth trajectory and size at birth in twins compared to singletons (Muhlhausler *et al.*, 2011). More recently, Hancock *et al*. (2012) demonstrated that size at birth and long term effects, particularly adult fat mass in twin-born sheep are determined in early gestation, before day 41. This follows on from evidence produced by unilateral fetectomy of ovine twins at day 50 of gestation (Vatnick *et al.*, 1991). They showed that fetal body weight in twins that were surgically reduced to singletons was intermediate between non-reduced twins and singletons in late gestation (day 136). Further, MacLaughlin *et al.* (2005) showed that maternal peri-conceptional under-nutrition altered the growth trajectory of twins compared to singletons.

Little is known of the early pregnancy mechanisms involved in determining or programming fetal growth trajectory and development. Moreover, it has not yet been established when during the early pregnancy period the differences occur. Whereas, Hancock *et al.* (2012) observed differences in fetal size at day 41 of gestation, in another model of maternal constraint, using mature maternal size, not pregnancy rank, differences were observed in embryo size as early as day 19 as a result of differing maternal size (Sharma *et al.*, 2013). Therefore, it is yet to be determined if there are specific signals or a combination of signals that sense and relay the status of the embryonic environment and thus in response determine the developmental trajectory of the embryo. As such, it is important to investigate how these mechanisms may differ between singleton and twin pregnancies, which will allow better understanding of how growth during this period is programmed, and determine why the differences observed between singletons and twins, occur. The objectives of this study were to determine if differences in the size of twin vs singleton embryos are present in the early pre-implantation period of gestation, and to examine total embryonic gene expression to potentially elucidate any mechanisms involved in driving the developmental trajectory of twins compared to singletons. These objectives were met using reciprocal day 7 embryo transfer (ET), morphological data and transcriptomics of day 21 embryos.

3.3 Materials and Methods

All experimental procedures were carried out with the approval of the Massey University Animal Ethics Committee (MUAEC).

3.3.1 Experimental animals and design

Experimental pregnancy groups were established by reciprocal ET of purebred Romney embryos between singleton bearing ewes with one active corpus luteum (CL) and twin-bearing ewes with two active CLs, such that four experimental groups were established: single embryo transferred into single CL ewe (1E1CL); single embryo transferred into a two CL ewe (1E2CL); twin embryos transferred into single CL ewe (2E1CL); and twin embryos transferred into two CL ewe (2E2CL). Two control groups were also established from non-ET pregnancies, singleton bearing ewes (Con1E1CL), and twin bearing ewes (Con2E2CL). Ewes were multiparous, 3-5 years of age with a total average liveweight of 65.6kg (2012, 59.4 kg; 2013, 71.1 kg). In order to improve the chances of obtaining the required number of pregnancies for each of the four experimental ET groups and two control groups, this study was conducted in 2 consecutive years.

Oestrus synchronisation and Artificial Insemination

Oestrus was synchronised in 180 ewes using a controlled intravaginal progesterone drug releasing device (CIDR) (Eazi-breed CIDR; Pfizer; Auckland, New Zealand) for 13 days. Ewes were put in an indoor shed at 1600h on the day prior to Artificial Insemination (AI) and held overnight. Water and food were withheld. At approximately 0800h AI commenced (day 0). Each ewe was sedated using acetyl chlorpromazine (Acezine 10, Ethical agents Ltd; Auckland, New Zealand; 0.1 mg/kg bodyweight intramuscularly) and secured in a laparoscopic cradle in dorsal recumbency. Laparoscopic AI (using 0.5 mL semen collected from one of four Romney rams) was performed on each ewe following surgical preparation and infusion of local anaesthetic (lignocaine hydrochloride, 20 mg/mL: approximately 4ml) of the abdominal surgical site. Following AI procaine penicillin (Duplocillin LA; Intervet Ltd;

Wellington, New Zealand; 10.5 mg/kg body weight intramuscularly) was administered for prophylaxis. The ewes were then allowed to walk to a nearby paddock and were observed for a minimum of one hour.

Detection of Ovulation Rate, Embryo harvest and Transfer

Trans-rectal ultrasonographic examination of ewes was performed on day 6 to identify single and twin ovulators to be included in the following phase of the trial. Single and twin ovulators were randomly assigned to experimental embryo transfer or control groups. Embryos were recovered on day 7. Donor ewes (who were also recipients) were anaesthetised (5.0 mg/kg thiopentone sodium, (Bomathal, Bomac Laboratories Ltd; Auckland, New Zealand) IV, for induction; and maintained on ~2% halothane). The donor ewes were placed in dorsal recumbency, and restrained in a laparoscopic cradle. Surgical preparation of the site was performed, a midline incision was made and the uterus was exteriorised. The embryos were flushed from the uterus following placement of a Foley catheter into the caudal portion of the uterine horn. New embryos were then inserted into the uterus through a small stab incision in the uterine horn. The uterus was then replaced into the abdomen and the midline incision sutured. Ewes were given prophylactic antibiotics (procaine penicillin; Duplocillin LA; Intervet Ltd; Wellington, New Zealand; 10.5 mg/kg body weight intramuscularly) and analgesia (flunixin meglumine; Flunixin Injection; Norbrook NZ Ltd; Auckland, New Zealand; 2.2 mg/kg body weight intramuscularly) post surgically.

Collected embryos were examined immediately following harvest for viability (fertilised, appropriate transferable stage for age, structurally sound, (Stringfellow and Givens, 2013). They were then re-inserted into the ewes in order to establish the following transfer groups, such that 77 reciprocal transfers over the 2 year period were performed:

1- Singleton embryos (harvested from a ewe with a single CL) transferred into a ewe that was also identified as having a single CL, and a single embryo that was removed, (1E1CL).
2- Twin embryos (harvested from a ewe with two CLs) transferred into a ewe that was also identified as having two CLs, and twin embryos were removed, (2E2CL).
3- Singleton embryos (harvested from a ewe with a single CL) transferred into a ewe that was identified as having two CLs, and twin embryos that were removed, (1E2CL).
4- Twin embryos (harvested from a ewe with two CLs) transferred into a ewe that was identified as having a single CL, and single embryo that was removed, (2E1CL).

In addition, ultrasonic examination was used to assign ewes to one of two control groups (no embryo transfer) of singleton bearing and twin bearing ewes (year 1: twin bearing, n=12; single bearing, n=12; year 2: twin bearing, n=12; singleton bearing, n=0).

Embryo and reproductive tissue harvest –Day21

On day 21 of gestation, only the ewes (n=62; 2012 liveweight 59.6 kg; 2013 liveweight 69.6 kg) that successfully maintained pregnancies, based on non-return rates as detected by a vasectomised ram, were euthanised using captive bolt stunning and exsanguination. Of the 62 euthanised ewes only those that satisfied the requirements of their experimental group, in terms of embryo number and CL number, were included in the analysis (Table 3.1). The entire uterus was immediately removed and placed on ice. Embryos were flushed from the uterus and examined under a microscope and photographed (Leica Mz75 dissecting microscope fitted with a Leica DFC280 camera, running on Leica Firecam software v3.4; Leica Microsystems, Heerbrugg, Switzerland) at a magnification of 1.6x, and calibrated using

a 1 mm scale. The harvested embryos were snap frozen and stored at -80°C for later transcriptomic/ gene expression studies. Left and right ovaries were examined and weighed and CLs were dissected out and weighed.

Table 3.1 Number of ewes that received embryo transfers at day 7, number of pregnant ewes (that did not return to estrus) and were euthanised at day 21 and number of ewes that satisfied embryo and CL number requirement for experimental group allocation and control groups.

		Experimental Group[1]					
		1E1CL	1E2CL	2E1CL	2E2CL	Con1E1CL	Con2E2CL
Year 1	Transferred	9	13	11	12	N/A	N/A
	Pregnant/Euthanised	4	7	7	6	5	8
	Satisfied CL/ embryo number requirement	4	7	3	5	4	5
Year 2	Transferred	2	5	6	19	N/A	N/A
	Pregnant/Euthanised	2	2	3	10	0	8
	Satisfied CL /embryo number requirement	0	2	0	5	0	5
Total	Transferred	11	18	17	31	N/A	N/A
	Pregnant/Euthanised	6	9	10	16	5	16
	Satisfied CL /embryo number requirement	4	9	3	10	4	10

[1]Experimental groups: 1E1CL = ewe that was singleton bearing had a single CL and received a single embryo transfer; 1E2CL = ewe that was twin bearing and had 2CLs and received a single embryo transfer; 2E1CL = ewe that was singleton bearing and had 1CL and received a twin embryo transfer; 2E2CL = ewe that was twin bearing and had 2CLs and received a twin embryos transfer, Con1E1CL = control (no ET) ewe that was singleton bearing; Con2E2CL = control (no ET) ewe that was twin bearing.

3.3.2 Embryo morphometric measurements

Embryo length, width, and heart bulge were measured using ImageJ (U.S. National Institutes of Health, Bethesda, Maryland, USA). Embryo length (EL) was defined as the distance from the medial aspect of the head to the tip of the embryonic tail, following the outer curvature of the embryo. Embryo width (EW) was defined as the distance between the two widest points of the embryo with the line passing just below the heart bulge, including somites. Heart bulge width (HB) was defined as the distance between the two

widest points of the heart bulge with the line passing through the midsection of the heart bulge, excluding the somites (Wales and Cuneo 1989). (Figure 3.1)

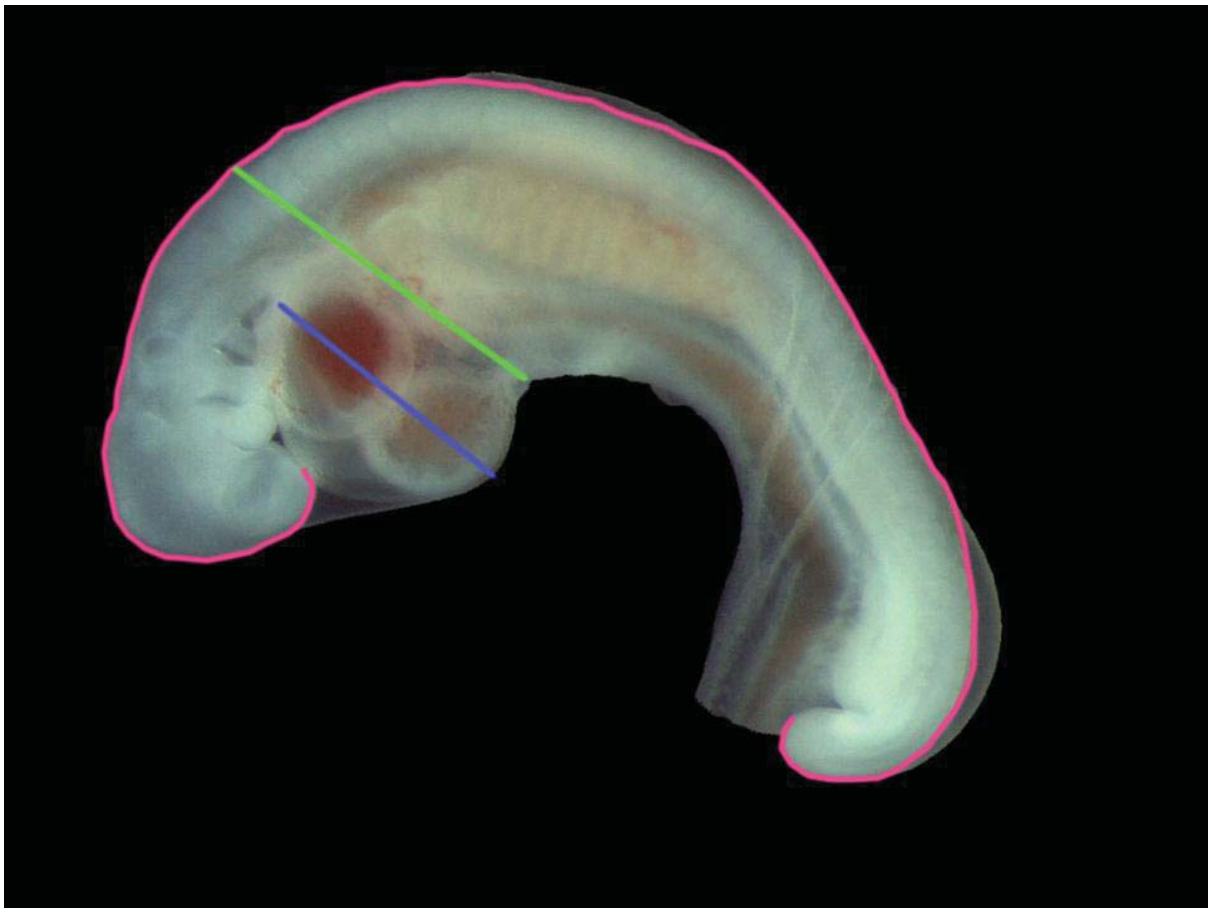

Figure 3.1 Image of day 19 sheep embryo showing the measurements of embryo length, embryo width and heart bulge width. Embryo length (pink) = distance from the medial aspect of the head to the tip of the embryonic tail, following the outer curvature of the embryo. Embryo width (green) = distance between the two widest points of embryos with the line passing just below and not including the heart bulge but including somites. Heart bulge width (blue) = distance between the two widest points of the heart bulge with the line passing through the midsection of the heart bulge and excluding somites.

Statistical analysis

Using the numbers detailed in Table 3.1, PROC GENMOD function was used to compare pregnancy rates (pregnant-euthanised/received ET) in the different embryo transfer groups following logit transformation. The percentage of pregnant ewes that satisfied the criteria of

the allocated group (satisfied criteria/pregnant-euthanised) was compared, after logit transformation, using the PROC GENMOD function.

Embryo measurements were subjected to analysis of variance (ANOVA, mixed model procedure), with respect to the fixed effects of embryo (twin or singleton) and recipient ewe (1CL or 2CL). All statistical procedures were performed using SAS (Version 9.3, SAS Institute Inc., Cary, NC, USA).

3.3.3 Transcriptomic analysis of embryos

RNA preparation

Tissue Grinding

The whole embryos were ground into a fine powder by cryo-grinding using a stainless steel mortar and pestle that was maintained at -196°C during the entire grinding process using liquid nitrogen. This prevented the embryos from thawing and quickened the grinding process. The mortar and pestle was prepared for grinding by baking at 200°C for five hours to inhibit RNase activity. The powdered tissue was stored at -80°C for molecular analysis. The mortar and pestle was washed with DEPC-treated milliQ water, and then wiped with Azowipe disinfectant (Bamford Medical, Auckland, New Zealand), prior to grinding each embryo, to prevent cross-contamination.

RNA Extraction

Total RNA was extracted from the whole embryo using TRIzol® Reagent (Invitrogen™, Life Technologies, Auckland, New Zealand). An RNase free work environment was maintained throughout the process by using baked glassware (200°C, 5 hours), and other labware (polypropylene test-tubes, homogeniser probe and spatula) soaked overnight in 0.1% DEPC

treated milliQ water, and then autoclaved to remove DEPC residue that would affect downstream processing. The workbench was wiped with RNase *Zap*® (Ambion Biosystems, Melbourne, Australia) using Henry Schein® isopropanol wipes.

The steps for RNA extraction were as follows:

1. Homogenisation: This was carried out in the fume hood on ice. The entire sample of powdered embryo was homogenised in 1 mL of cold TRIzol® Reagent using 20 second pulses (10 second break between pulses) at 24,000 rpm using a T 25 Basic Ultra-Turrax powder homogeniser (IKA, Germany). The homogeniser probe was rinsed (DEPC treated milliQ water 75% v/v cold ethanol) and wiped with Henry Schein® isopropanol wipes between each sample to prevent cross-contamination. The rest of the processing steps took place on the bench top.
2. Chloroform step: Homogenised samples were transferred to chloroform (Sigma-Aldrich Inc., New Zealand) at a volume of 200 µL of chloroform per 1 mL of TRIzol® Reagent. The samples were then vortexed for 5 seconds and incubated on ice for 5 minutes, so that separation into two phases: aqueous and organic could occur.
3. Centrifugation: The samples were then centrifuged at 16,000 g at 4°C for 15 minutes. Three distinct phases were achieved post-centrifugation: a lower dark pink phenol-chloroform phase, a light pink interphase and an upper colourless aqueous phase.
4. The upper colourless aqueous phase, containing the RNA was transferred to 200 µL of chloroform and vortexed and centrifuged as in steps 2 and 3 above.
5. The aqueous phase obtained from step 4 was transferred to 500 µL cold isopropanol (Sigma Aldrich Inc., Auckland, New Zealand) per 1mL of TRIzol reagent

containing sample. Samples were mixed gently by inverting and incubated at -20°C for 45 minutes. Samples were centrifuged at 16,000 g for 15 minutes at 4°C producing supernatant and a pellet containing RNA. The supernatant was discarded.

6. Re-suspension: The RNA pellet was re-suspended in 1 mL of sterile 75%v/v molecular grade ethanol (Merck Millipore, Auckland, New Zealand) by vortex (5 seconds) and followed by centrifugation (16,000 g, 4°C, 5 minutes). The resulting supernatant was discarded and the re-suspension process was repeated.
7. Drying: The pellet was then air dried for ~2-5mins at room temperature.
8. Final re-suspension to working RNA sample: The air-dried pellet was re-suspended in ~15-20 µL of nuclease free water (NFW) (Ambion Inc., Applied Biosystems, Melbourne, Australia). The samples were then incubated on ice for 30 minutes to ensure complete suspension and stored at -80°C until further processing.

Quantification

Purity and concentration of extracted RNA was determined using NanoDrop spectrophotometer running NanoDrop 3.1.2 software (ND-1000 spectrophotometer, Biolab Ltd, Auckland, New Zealand). Each RNA sample was diluted (10 fold) using 10mmol/L Tris, pH 7.0 (361). NanoDrop was initialised by loading 2 µL DEPC treated milliQ water and blanked with 2 µL of 10mmol/L Tris. Diluted samples were loaded at a volume of 2 µL and "260/280" and "260/230" absorbance ratios were recorded. The measurement pedestal and sampling arm were rinsed with distilled water and blot dried using Kimwipes (Kimberly-Clark® Professional, Auckland, New Zealand) between samples. Levels of approximately 2.0 for 260/280 nm absorbance were accepted as pure RNA samples i.e. free of protein

contamination; levels of approximately 1.8-2.0 for 260/230 nm were accepted as free of organic solvents (TRIzol and isopropanol). The concentration of RNA per sample (ng/µL) was calculated from the equation A260*40 whereby an absorbance reading at 260nm (A260) of 1.0 unit is equivalent to 40 µg of RNA per mL.

Quality check

RNA integrity was assessed using the Agilent 2100 Bioanalyser and Agilent 6000 Nano Kit according to the manufacturer's instructions. An electropherogram, a gel-like image and a ribosomal ratio (18s to 28s ribosomal subunits) is generated by this method. It provides information on the size and distribution of any fragments that are present, and produces an RNA integrity number (RIN): of 1 to 10 (where 1 is a highly degraded sample, and 10 most intact) (Schroeder *et al.*, 2006). Any samples with a RIN value lower than 7 were discarded.

Sequencing

Transcriptome (RNA-seq) data were generated using high throughput sequencing (HTS) from the extracted RNA using Illumina HiSeq 2000 (Illumina, CA, USA; service provided by New Zealand Genomics Limited, Dunedin). DNA libraries were generated from the RNA which allowed the addition of an adaptor to both ends of each DNA fragment. These DNA fragments were then processed through flow cells where they bind to oligonucleotides and amplified to form clusters of identical sequences. The clusters were sequenced simultaneously through measurement of the florescence of the nucleotide to produce paired-end reads.

Alignment and Mapping

Generated reads were aligned to the *Ovis Aries* genome version 3.2 (OARv3.2): http://www.ncbi.nlm.nih.gov/genome?term=ovis+aries (NCBI) using CLC genomics Workbench software (CLC bio, Denmark). All reads that did not align uniquely to the genome were discarded. The mapping parameters for maximum number of allowed mismatches was set at three, maximum length of fractions and similarity of fractions were both set at 0.8. Maximum number of hits per read was set at 10. These parameters allowed a count (number of reads mapped per gene) to be generated, which was then normalised to account for length of genes to generate a reads per kilobase per million reads (RKPM) statistic.

Determination of differentially expressed genes

Differences in gene expression were determined using CLC Genomics Workbench to perform the Baggerly test using normalised and transformed RPKM values. A Baggerly test is similar to a two sample t-test except that the statistic is weighted in accordance with the number of reads in each sample (Baggerly *et al.*, 2003). Genes with a corrected false discover rate (FDR) p value of 0.05 and a fold change greater than 1.5 were deemed significantly differentially expressed.

Gene ontology

DAVID Bioinformatics Resources 6.7 (National Institute of Allergy and Infectious Diseases (NIAID), NIH) https://david.ncifcrf.gov/ (Huang *et al.*, 2009b; Huang *et al.*, 2009a) was utilised to compare the lists of differentially expressed genes (DEGs) to a background list to

identify enriched gene ontology and/or KEGG terms. For functional annotation clusters an enrichment score (ES) of greater than 1.5 was used as a significant cut off.

The background list to which the DEGs were compared, was created by submitting a list of all the genes detected in the embryos from RNA-seq (RKPM expression values greater than 0). Annotations were based on the mouse genome as many sheep genes have not been assigned functional orthologies. In addition, descriptions of individual gene function were determined using searches via gene data banks and journal articles, in order to ascertain which genes may be involved in the difference in growth between embryo groups, and to identify genes that could be used for the PCR validation process.

3.3.4 Quantitative real time PCR (qPCR) validation of transcriptomics results

The possibility of false positive results exists in RNA-seq data-sets in spite of using multiple testing corrections. Therefore, a subset of genes was chosen from the RNA-seq analysis for validation by qPCR. qPCR is considered the gold standard for determining differential gene expression (Bustin *et al.*, 2009); however, there are limitations to the number of genes that can be feasibly examined. Ten candidate genes were chosen that demonstrated differential expression between at least two comparisons and ranged in expression i.e. high, medium, low transcript abundance, so as to allow for examination of the accuracy of the variation in gene expression that was determined by the RNA-seq analysis. The genes selected for validation were also identified as having some functional role in embryo growth.

First strand cDNA synthesis

DNase treatment

All of the RNA samples were DNase treated with amplification grade deoxyribonuclease I (Invitrogen™, Life technologies, Auckland New Zealand) to degrade any potential carry-over genomic DNA contamination. A 10 µL reaction mix was prepared for each sample in a separate 0.2 mL tube. The mix contained 2.5 µg of RNA, 1 µL of 10X DNase I reaction buffer (Invitrogen™, Life technologies, Auckland New Zealand), 1 µL of amplification grade DNase I (Invitrogen™, Life technologies, Auckland New Zealand) and NFW (Ambion Inc., Applied Biosystems, Melbourne, Australia). An additional standard curve sample, from a control group (2E2CL), was also prepared, to be used for generating standard curves. The no reverse transcriptase (RT) control mix was prepared by adding 1 µL of 10X DNase I Reaction Buffer, 1 µL of amplification grade DNase I and 7 µL of nuclease free water to determine the efficiency of the DNase treatment and to confirm that the samples were free of DNA contamination post DNase treatment. As per manufacturer's instructions all of the tubes were incubated at room temperature for approximately 15 minutes. 1 µL of 25 mmol/L EDTA, pH 8.0 (Invitrogen™, Life technologies, Auckland New Zealand) was added to each tube just before the 15 minute incubation was completed. The contents were then mixed gently and centrifuged for 10 seconds at 4°C. The reaction tubes were then transferred to a Bio-Rad Thermal cycler in which they were further incubated at 65°C for 10 minutes and then at 4°C for 1 minute. After the incubation the tubes were then centrifuged again briefly at 4°C.

cDNA synthesis

cDNA synthesis was performed on the DNase treated samples. Superscript® Vilo™ cDNA synthesis kit (Invitrogen™, Life technologies, Auckland New Zealand) was used according to the manufacturer's protocol. 4 µL of 5X Vilo Reaction Mix, 2 µL of 10X Superscript Enzyme Mix and 3 µL of NFW were added to each of the 11 µL DNase treated samples, including the standard curve sample tube. The no (RT) tube, containing 4 µL of 5X Vilo Reaction Mix and 5 µL of nuclease free water was prepared to determine the efficiency of the DNase treatment and to confirm that the samples were free of DNA contamination post DNase treatment. The tubes containing the reaction mix were centrifuged at 4°C for approximately 10 seconds, and then transferred to Bio-Rad Thermal Cycler. The following cycles were run as per manufacturer's instructions: incubation at 25°C for 10 minutes, followed by a second incubation period at 42°C for 60 minutes, then reaction termination at 85°C for 5 minutes and final incubation at 4°C for 35 minutes. cDNA was then stored at -80°C until use.

Designing of Primers and Probes

Ovine sequences for candidate and reference genes were first searched in the GenBank (NCBI). A 200 base pair (bp) region was selected. Primers and probes (Taqman® Assay) were designed and supplied by Applied Biosystems. All probes are Taqman® MGB probes with a 6-FAM™ reporter at the 5' end and non-fluorescent quencher at the 3' end, and are listed in Table 3.2. These primers and probe were designed to all run at the same qPCR conditions defined by the manufacturer. All primer-probes were of an amplicon length of 50 to150 bp for optimum PCR efficiency, probe length of 13 to 30 bps for conventional Taqman probes, melting temperature of 68°C to 70°C, percent GC content of 30% to 80%. An NCBI primer

blast was performed as an additional check for quality control to ensure that the primers and probes produced were from regions that encode for the target protein only and not from any other homologous region and had an optimal sequence identity for gene expression in ovine species.

Qualitative analysis of the primer products (primer specificity)

Using the standard curve sample at 10 fold dilution, qPCR was run on all candidate and reference gene primers and probes in duplicate. A no (RT) and no template control (also in duplicate) were included for each primer and probe. The PCR amplicons were run on a 2% agarose gel (as per protocol for RNA gels) in order to confirm that the primers and probes were designed appropriately and amplified the gene of interest only, thereby indicating primer specificity. Specificity was determined by the presence of a single band of the expected product size (80-100bp) in the cDNA samples, with no other bands that might indicate the presence of another product; and no bands in the no RT and no template controls.

Reference gene selection

Reference genes are stably expressed across experimental groups and physiological states, and are used to normalise gene expression data generated by qPCR. It is, therefore, important to select the most stable reference gene candidates for the tissue being examined. Three candidate reference genes to be used to normalise the qPCR data in this study were selected from a pool of potential reference genes (18S, ACTB, Cyclophilin A, GAPDH, HPRT1, RPL19, YWHAZ) in accordance with the "BestKeeper index" described by Pfaffl *et al.* (2004). In brief, qPCR was performed in triplicate on 100-fold dilutions of cDNA

from a subsample of each of the experimental ET and control groups (n=2-5, total 21) using ABI Prism 7900HT Sequence Detector (Applied Biosystems, Foster City, CA, USA; as per manufacturer's qPCR conditions, protocol is described in the following section). Threshold of quantification cycle (Ct) values generated by the Prism software was used to calculate the geometric mean, arithmetic mean, standard deviation (STDEV) and coefficient of variance (CV%) for each potential reference gene under examination (Appendix I) Reference genes were ordered from least stably to most stably expressed according to variability observed as determined by the CV% (most stable has the least variation and therefore lowest CV%; Pfaffl *et al.*, 2004). The three most stable reference genes were selected to be used to normalise mRNA expression in this study.

Primer efficiency

Optimal qPCR efficiency should be 2 (100%), however, an efficiency between 1.8 and 2.2 (90% and 110%) is considered acceptable (Bustin *et al.*, 2009). Standard curves for the target and candidate reference genes were generated through a series of serial dilutions of cDNA, using the standard curve sample. Two-fold dilution series (1, 1:2, 1:4, 1:8, 1:16) were used to generate a standard curve for each gene. Primer efficiencies were measured by performing qPCR on an ABI Prism 7900HT Sequence Detector (as per manufacturer's qPCR conditions, protocol is described in the following section) on the dilution series for each gene. Ct values that were generated by the software were plotted on the y axis against the log value of the cDNA dilution factor on the x axis. The slope of the line (E) was calculated using the equation: $E = 10^{-1/slope}$. A slope of between -3.6 and -3.1 is indicative of an efficiency between 90 and 110%. Primer efficiencies are listed in Table 3.2.

Table 3.2 Candidate target and reference genes tested by qPCR. Gene ID, NCBI accession number, forward, reverse primer and probe sequences, amplicon sizes (base pairs, bp) and primer efficiency.

Gene ID[1]		Sequence of Primers and Probes[2]	Amplicon size (bp)	NCBI accession ID	Efficiency
	Forward Primer	CGGTGCCAGCATGTCAAC			
DKK4	Reverse Primer	ACCATTGGCCGTACGTCTTC	86	XM_004021804.2	1.81
	Probe	CTCTGCGTGAACAATG			
	Forward Primer	GGTATCCGAGCTGAAGCAGTTC			
ISG17	Reverse Primer	GGCGCTGCTGGAAAGC	62	AF152103.1	2.10
	Probe	TCGCCCAGAAGATCAA			
	Forward Primer	TGCCTGCCTACCTCAAGTTC			
LAPTM5	Reverse Primer	GTCATCAGTGGGACCTTGGA	70	XM_004005050.2	1.89
	Probe	CACCCGCCTGCTGCT			
	Forward Primer	TGGACCAACTGCACCAAGTG			
LGALS3	Reverse Primer	GTCCAGAAGGGATGCCATAGG	92	XM_004010664.2	2.09
	Probe	CACCTCCTGCCTACCC			
	Forward Primer	GCAGAAGGAAGAGAAAGTGCTTACT			
LGALS15	Reverse Primer	GTATTCCTTCTCCAGCACCAAGA	85	NM_001009238.1	2.28
	Probe	CCAGGCCAGCCATTTG			
	Forward Primer	GCTCATGACACAGTTCGGATTG			
LOC101103603	Reverse Primer	GTCCCGAAATCCGTATTCTGCTA	84	XM_004019572.2	2.02
	Probe	ACGGCTGGTCAATACT			
	Forward Primer	GCAGGTGACTGGATGGTACA			
LOC101117738	Reverse Primer	CCACCAGAACAAGCAATAACCTTTC	68	XM_004022794.2	2.04
	Probe	CATGGACAGCATCTCC			
	Forward Primer	CCTGTAGGAAGGTGGACAAGGA			
LRRC32	Reverse Primer	ACCTCGATGTCCCGTGAGA	85	XM_004016341.2	2.03
	Probe	CCGTGGCCTGCTCCAG			
	Forward Primer	GTGTGGCTACAGGTACCAAGATTAT			
TKDP1	Reverse Primer	TGATTATTTCAGCCAATCCTGTCAGT	69	NM_001009291	1.94
	Probe	TCCGGCTCCCTTCTCA			

	Forward Primer	AGAGCTTCAACCTCTTCTACACAGA			
TP1	Reverse Primer	AGTGCAGAGCTGCTCCAG	74	NM_001123399.1	2.28
	Probe	CTGGGACACCACCCTC			
ACTB	Forward Primer	ACCAGTTCGCCATGGATGATG			
	Reverse Primer	CCGGAGCCGTTGTCAAC	55	NM_001009784.1	2.01
	Probe	ACGAGCGCAGCAATAT			
RPL19	Forward Primer	CAAAAACAAGCGGATTCTCATG			
	Reverse Primer	GCTTCTTGCGAGCCTTGTCT	65	AY158223.1	2.04
	Probe	AACATATCCACAAGCTGAA			
GAPDH	Forward Primer	GGGCTGCTTTTAATACTGGCAAA			
	Reverse Primer	CATGTAGACCATGTAGTGAAGGTCAA	80	NM_001190390	2.05
	Probe	CATCGTTGCCATCAATG			
Cyclophilin A	Forward Primer	GTACTGGTGGCAAGTCCATCT			
	Reverse Primer	CAGGACCTGTATGCTTCAGAATGA	72	AY251270.1	
	Probe	ATGGCGAGAAATTTG			
HPRT1[3]	Forward Primer	AGGTGTTTATTCCTCATGGACTAATTATGG	77	EF078978	
	Reverse Primer	CACCCATCTCCTTCATCACATCTC			
	Probe	ACAGGACCGAACGACTG			
YWHAZ[3]	Forward Primer	GAGGGTCGTCTCCAGTATTGAG			
	Reverse Primer	TTCTCGAGCCATCTGCTGTTTT	67	AY970970.1	
	Probe	CAGCACCTTCCGTCTTT			
18S[3]	Eukaryotic 18S rRNA endogenous VIC/MGB Probe and Primers (Applied Biosystems, Foster City, CA, USA).				

[1]Gene names: *DKK4* = Dickkopf WNT signalling pathway inhibitor 4, I*SG17* = Interferon stimulated gene 17, *LAPTM5* = Lysosomal-associated protein transmembrane 5, *LGALS3* = Lectin galactoside-binding, soluble 3, *LGALS15* = Lectin galactoside-binding soluble 15, *LOC101103603* = Pregnancy associated glycoprotein-4 like, *LOC101117738* = Pregnancy associated glycoprotein-1 like, *LRRC32* = Leucine rich repeat containing 32, *TKDP* = Trophoblast Kunitz domain protein-1, *TP1* = Trophoblast protein 1, *ACTB* = Beta actin, *RPL19* = Ribosomal protein L19, *GAPDH* = Glyceraldehyde-3-phosphate dehydrogenase, *HPRT1* = Hypoxanthine phosphoribosyltransferase 1, *YWHAZ* = Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta.

[2]The fluorescent dye FAM™ reporter is bound to 5' end and minor binding groove (MGB) non-fluorescent quencher is present at the 3'end of the Taqman probe for all the probes except 18s which comes with VIC/MGB reporter.

[3]Efficiencies are not listed for reference genes that were not selected for use to normalise mRNA expression.

Quantitative Real Time PCR

Amplification of first strand cDNA was performed using the Taqman gene expression assay (Applied Biosystems, Melbourne, Australia). PCR amplifications for target genes were carried out in triplicate using a ABI Prism 7900HT Sequence Detector (Applied Biosystems, Foster City, CA, USA). A simplex amplification reaction was carried out in 384-well plates with a total reaction volume per well of 10 µL. Each reaction contained 2 µL cDNA sample and 8 µL of master mix (5 µL of Taqman gene expression assay (Applied Biosystems, Melbourne, Australia), 1 µL forward, reverse primers and probe. In each PCR run, the standard curve for each gene (Figure 3.2) and a no template control were included. Optimal cDNA dilutions were determined from the validation runs performed when primer specificity was tested. cDNA samples were diluted at 1:100 for reference genes, *ACTB, RPL19 and GAPDH* assays; *LGALS15* and *TP1* cDNA were used undiluted and for all other target genes cDNA at a dilution of 1:10 was used for loading the wells. The qPCR programme was as follows:

- Initial cycle of 50°C for 2 minutes
- 95°C for 10 minutes
- 95°C for 15 seconds
- 60°C for 1 minute

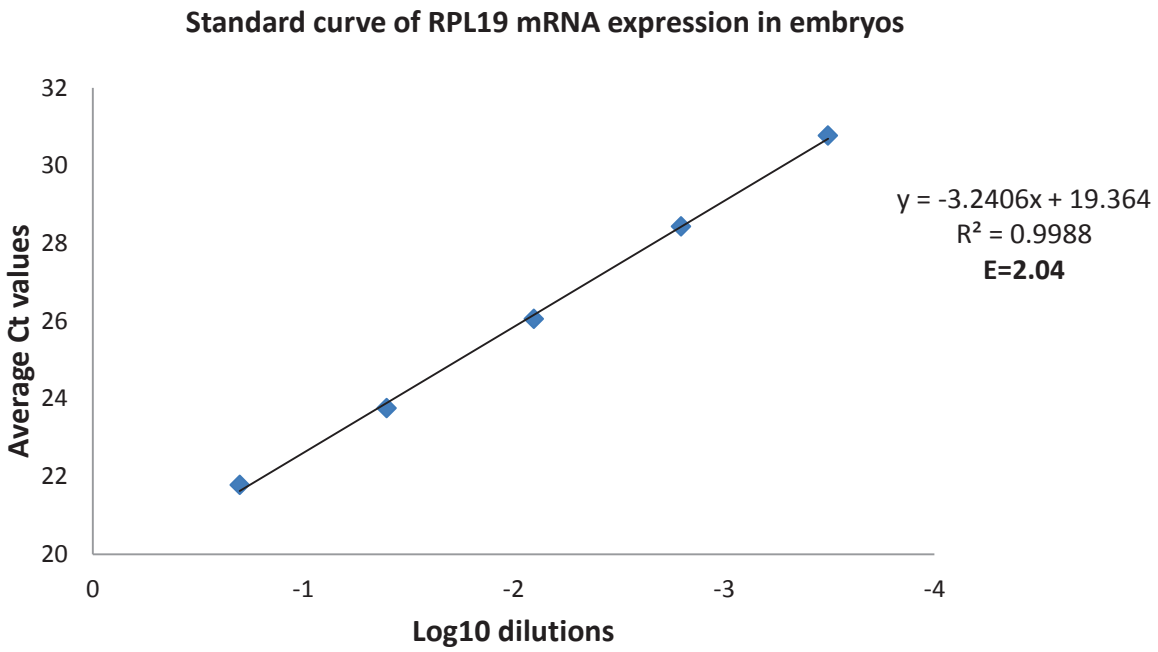

Figure 3.2 Representative standard curve for *RPL19* gene. Cycle threshold (Ct) values for *RPL19* are represented on the Y axis, and log10 values of cDNA dilutions are represented on the X axis. R^2 represents the line of best fit through the points. E is the corresponding real time amplification efficiency.

Statistical analysis of qPCR data and correlation validation of RNA-seq data

The mRNA expression was calculated using the Ct values generated by the ABI Prism 7900HT Sequence Detector software. Differential expression was calculated as a fold change in mRNA expression levels of the target gene in the experimental ET group relative to its expression to the comparator group (control or other experimental ET group). Target gene expression was normalised with the BestKeeper index of three reference genes, selected from a panel of potential reference genes as described earlier (Pfaffl *et al.*, 2004). Differential expression per experimental group was calculated as fold change relative to the comparator group, using (Pfaffl, 2001) relative quantification method:

$$RR = \frac{(E_{target})^{\Delta Ct_{target}\ (\text{experimental ET - comparator})}}{(E_{RG})^{\Delta Ct_{RG}\ (\text{experimental ET - comparator})}}$$

Where RR is the relative ratio of target gene to reference gene; E_{target} is the real time PCR amplification efficiency of the target gene transcript determined from the slope of the standard curve; E_{RG} is the geometric mean of real time PCR amplification efficiency of the reference genes; $\Delta Ct_{target\ (treated - comparator)}$ and $\Delta Ct_{RG\ (treated - comparator)}$ are the Ct differences between the experimental ET group and the comparator group (experimental ET or control) for the target gene and the geometric mean of the reference genes, respectively. Statistical significance of the fold change was calculated as 95% confidence intervals.

A linear regression plot showing the correlation between gene expression measured by RNA-seq and qPCR was generated in SAS (Version 9.4, SAS Institute INC., Cary, NC, USA). Significance of correlation was determined by Spearman's correlation coefficient (R) and associated p-value. The gene expression values were represented as a fold change between comparisons.

3.4 Results

3.4.1 Pregnancy rates

When the ET group ewes were examined together, regardless of year, there were no differences ($p>0.05$) in the total pregnancy rates (Table 3.3). The average pregnancy rate for the ET groups was 53.7%. The 2E1CL group had lowest ($p<0.05$) pregnant rate of ewes that satisfied CL/embryo number for allocated experimental group compared to the other experimental transfer groups (17.6% compared to 1E1CL, 36.4%; 1E2CL, 50.0%, and 2E2CL,

32.3%) Overall, of all the pregnant ewes that were allocated to the 2E1CL group, 30.0% fulfilled embryo/CL number requirements, upon euthanasia. In all the other ET groups greater than 60% of the pregnant ewes satisfied the embryo/CL number requirements of their allocated experimental groups (1E1CL, 66.7%; 2E2CL, 62.5%), and 100% of the ewes satisfying these requirements in the 1E2CL group. There was no difference ($p>0.05$) in the pregnancy rates when the control groups were compared to the respective ET groups.

Table 3.3 Pregnancy rates (%) of embryo transfer groups and percentage of pregnant ewes that satisfied the requirements of the experimental group that they were allocated to.

		Experimental Group[1]				
		1E1CL	1E2CL	2E1CL	2E2CL	
Pregnancy rate of ewes that satisfied criterial of allocated group (satisfied criteria/transferred)	%	36.4^{b}	50.0^{c}	17.6^{a}	32.3^{b}	
	logit (SE)	-0.56 (0.63)	-1.55×10^{-15} (0.47)	-1.54 (0.64)	-0.74 (0.38)	
Total pregnancy rate (euthanised/transferred)	%	54.5	50.0	58.8	51.6	**53.7**
	logit (SE)	0.18 (0.61)	-1.39×10^{-17}(0.47)	0.36 (0.49)	0.65 (0.36)	
Percentage of pregnant ewes that satisfied criteria of allocated group (satisfied criteria/euthanised)	%	66.7^{ab}	100.0^{b}	30.0^{a}	62.5^{ab}	
	logit (SE)	0.69 (0.87)	26.51 ($1.89 \times 10^{5)}$	-0.85 (0.69)	0.51 (0.52)	

[1]Experimental groups: 1E1CL = single embryo transferred to a ewe that was singleton bearing and had a single CL; 1E2CL = single embryo transferred to a ewe that was twin bearing and had 2CLs; 2E1CL = twin embryo that were transferred to a ewe that was singleton bearing and had 1CL; 2E2CL = twin embryo that were transferred to a ewe that was twin bearing and had 2CLs.

[2]Values are expressed as %, values are back transformed logit. Untransformed data values (SE) are included in row below % values

Different superscripts within rows indicate differences between groups

3.4.2 Embryo morphometric data

Single embryos that were transferred to ewes that were singleton bearing and had only one CL (1E1CL) were smaller in length, width and heart bulge width ($p<0.05$) than singleton or twin embryos in any of the other groups (Table 3.4). There were no differences ($P>0.05$) between any of the other experimental ET or control groups in any of the morphometric measurements.

3.4.3 RNA integrity

All samples collected had a RIN number greater than 7 and two distinct bands on the electropherogram indicating 18s and 28s ribosomal units were present and intact (Figure 3.3). Therefore all samples were of high quality, with minimal degradation and acceptable for RNA sequencing (Table 3.5).

Table 3.4 Embryo length, width and heart bulge (HB) width of singleton and twin embryos that were transferred to recipients that either that had either 1 or 2 corpora lutea (CLs) and control singleton and twin embryos that were not transferred. Values are least square means ± standard error of the mean. Different superscripts within columns indicate significant differences ($p<0.05$).

Experimental	Embryo length (mm)		Embryo width(mm)		HB width (mm)	
Group[1]	*n*[2]	Mean	*n*	Mean	*n*	Mean
1E1CL	2	16.44±1.50^{a}	3	2.81±026^{a}	4	1.77±0.14^{a}
1E2CL	7	21.21±0.80^{b}	9	3.50±0.15^{b}	8	2.29±0.10^{b}
2E1CL	4	20.21±1.06^{b}	6	3.47±0.19^{b}	6	2.18±0.12^{b}
2E2CL	15	20.89±0.50^{b}	20	3.49±0.10^{b}	20	2.05±0.06^{ab}
Con1E1CL	4	20.83±1.06^{b}	4	3.63±0.23^{b}	4	2.29±0.14^{b}
Con2E2CL	10	21.78±0.67^{b}	19	3.74±0.11^{b}	19	2.20±0.07^{b}

[1]Experimental groups 1E1CL = single embryo transferred to a ewe that was singleton bearing had a single CL; 1E2CL = single embryo transferred to a ewe that was twin bearing and had 2CLs; 2E1CL = Twin embryo that were transferred to a ewe that was singleton bearing and had 1CL; 2E2CL = twin embryo that was transferred to a ewe that was twin bearing and had 2CLs, Con1E1CL = control (no ET) singleton embryo; Con2E2CL = control (no ET) twin embryo.
[2]number of embryos per group differ between morphometric analysis based on the ability to successfully measure length, width or heart bulge width from photographs.

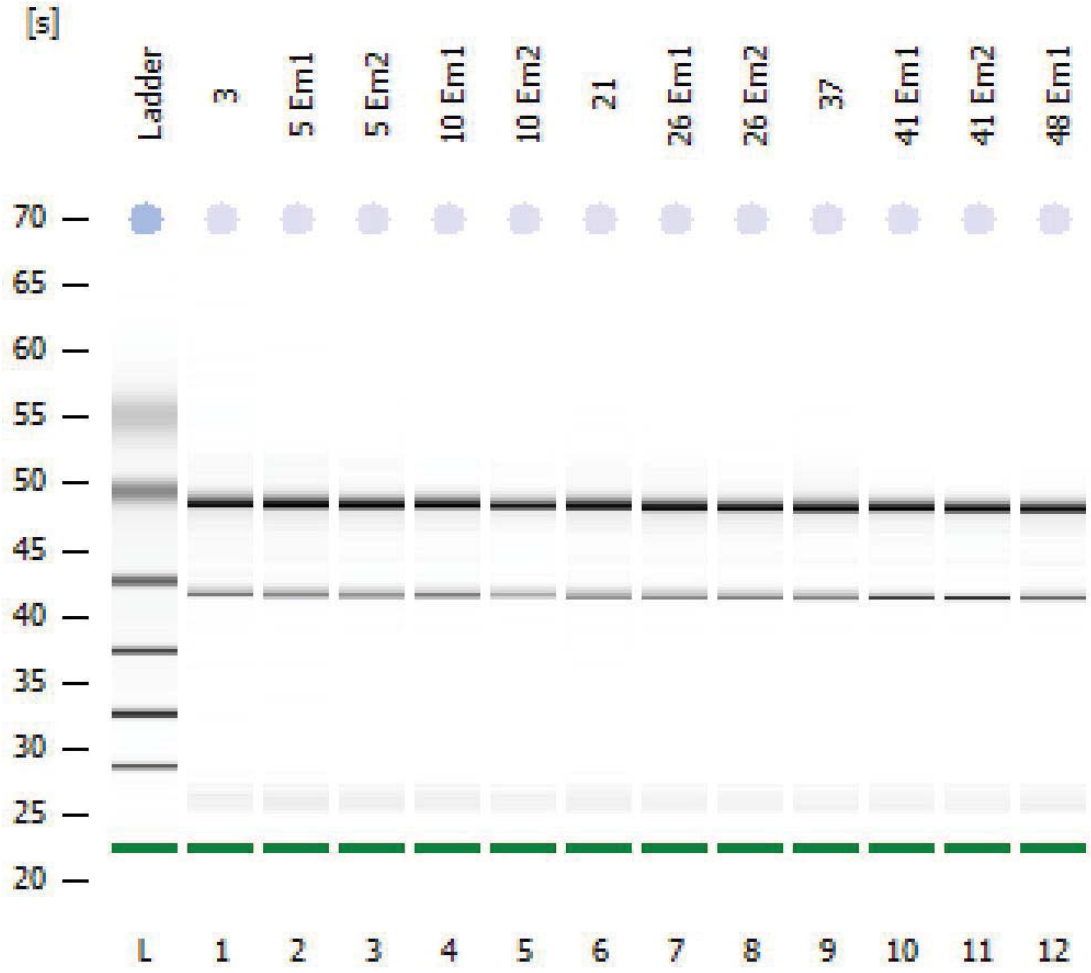

Figure 3.3 Electropherogram illustration of 18S and 28S RNA detected by the Agilent 2100 Bioanalyser in whole singleton and twin embryos. Quantity of 28S and 18S RNA is represented by upper and lower bands respectively. Two intact bands indicate that the RNA is not degraded and is of acceptable quality for RNA sequencing.

Table 3.5 Embryo RNA integrity and RNA concentration using Agilent 2100 bioanalyser using Agilent 6000 Nano Kit and NanoDrop 3.1.2 software (ND-1000 spectrophotometer, Biolab Ltd, Auckland, New Zealand) respectively.

Sample ID			RNA Integrity Number (RIN)[1]	RNA concentration (ng/ml)
Ewe ID	Embryo	Experimental group[2]		
59	Em1	Con1E1CL	10	931.1
63	Em1	Con1E1CL	10	1026.4
131	Em1	Con1E1CL	9.9	1174.1
146	Em1	Con1E1CL	9.9	920.1
3	Em1	1E1CL	10	498.8
37	Em1	1E1CL	10	925.7
110	Em1	1E1CL	10	1130.4
155	Em1	1E1CL	10	1007.5
21	Em1	1E2CL	10	779.2
53	Em1	1E2CL	10	1068.9
84	Em1	1E2CL	10	777.7
99	Em1	1E2CL	10	1002.2
106	Em1	1E2CL	10	741.1
112	Em1	1E2CL	10	1360.6
169	Em1	1E2CL	10	906.8
1642	Em1	1E2CL	10	764.8
1733	Em1	1E2CL	9.8	827.8
41	Em1	Con2E2CL	10	893.4
41	Em2	Con2E2CL	10	1286.5
70	Em1	Con2E2CL	10	1010.2
70	Em2	Con2E2CL	9.8	1023.5
86	Em1	Con2E2CL	10	745.4
86	Em2	Con2E2CL	9.7	921.4
135	Em1	Con2E2CL	10	1485.7
135	Em2	Con2E2CL	10	1486.5
149	Em1	Con2E2CL	10	903.7
149	Em2	Con2E2CL	10	1376.6
10	Em1	2E1CL	10	1009.8
10	Em2	2E1CL	9.7	696.6
95	Em1	2E1CL	10	955.2
95	Em2	2E1CL	10	967.6
100	Em1	2E1CL	10	1099.8
100	Em2	2E1CL	10	727.1
5	Em1	2E2CL	10	754.9
5	Em2	2E2CL	10	1105.5
48	Em1	2E2CL	10	1045.7
48	Em2	2E2CL	10	1042.4
73	Em1	2E2CL	10	1103.7
73	Em2	2E2CL	10	903.7
1656	Em1	2E2CL	9.7	658.2
1656	Em2	2E2CL	9.5	578.6
1679	Em1	2E2CL	9.5	566.9
1679	Em2	2E2CL	9	721.1

[1] A RIN of 7 or greater indicates that samples are not degraded and are acceptable for sequencing

[2]Experimental groups 1E1CL = single embryo transferred to a ewe that was singleton bearing had a single CL; 1E2CL = single embryo transferred to a ewe that was twin bearing and had 2CLs; 2E1CL = Twin embryo that were transferred to a ewe that was singleton bearing and had 1CL; 2E2CL = twin embryo that was transferred to a ewe that was twin bearing and had 2CLs, Con1E1CL = control (no ET) singleton embryo; Con2E2CL = control (no ET) twin embryo.

3.4.4 RNA-seq analysis

The singleton and twin embryos that were examined by RNA-seq (RUN2) produced an average of 4,337,497 paired-end reads per embryo (Appendix II). Approximately 71% of the generated reads mapped back to the ovine genome version 3.2 (OARV3.2). A total of 17,764 gene transcripts were detected in the whole embryo tissue.

Differentially expressed genes: RNA-seq data

There were no differentially expressed genes generated between any of the pair-wise comparisons performed on the RNA-seq data (RUN2) (fold change > 1.5, FDR $p<0.05$). A principle component analysis showed that in all comparisons i.e. between individual embryos, between experimental groups and comparisons of all twin vs all singleton embryos, there was significant variation in gene expression between individual embryos within experimental groups. Significant variation in gene expression between individual embryos was found when the embryo samples were examined as a total group. This may explain the observed lack of differential expression.

3.4.5 qPCR validation of RNA-seq data

The gene expression pattern of the RNA-seq analysis (RUN2) showed a positive correlation to five out of the 11 comparisons between embryo groups, as tested with qPCR (Table 3.6/ Appendix III). The genes that were chosen for qPCR were identified initially as differentially expressed in RUN1 but more importantly were identified as potentially having a functional role in embryo growth. Overall, qPCR partially validates the RNA-seq analysis.

Table 3.6 Correlation of gene expression fold changes determined by RNA-seq and reverse transcription quantitative PCR (qPCR) for embryo group comparisons. Comparisons that showed positive correlation (p<0.05) between methods are in bold.

Comparison	Spearman's R	p-value
1E1CL VS Con1E1CL	0.55	0.16
2E2CL VS Con2E2CL	**0.81**	**0.01**
1E2CL VS 2E1CL	**0.79**	**0.02**
1E1CL VS 1E2CL	0.24	0.57
1E1CL VS 2E2CL	**0.86**	**0.007**
1E1CL VS 2E1CL	0.67	0.07
2E2CLVS 2E1CL	**0.88**	**0.004**
2E2CL VS 1E2CL	0.43	0.29
Con1E1CL VS Con2E2CL	0.24	0.57
All 1E VS All 2E	**0.74**	**0.04**
All 1CL VS All2CL	0.69	0.06

Experimental groups 1E1CL = single embryo transferred to a ewe that was singleton bearing had a single CL; 1E2CL = single embryo transferred to a ewe that was twin bearing and had 2CLs; 2E1CL = Twin embryo that were transferred to a ewe that was singleton bearing and had 1CL; 2E2CL = twin embryo that was transferred to a ewe that was twin bearing and had 2CLs, Con1E1CL = control (no ET) singleton embryo; Con2E2CL = control (no ET) twin embryo.

3.4.6 Differentially expressed genes: qpCR analysis

Differential gene expression was highly variable between pairwise comparisons (Table 3.7 (A) (B) and (C)), with the number of examined genes showing differential expression varying from one to seven per pairwise comparison. In the comparison between control singleton embryos and control twin embryos, *ISG17* was the only gene that was differentially expressed. There were seven differentially expressed genes in the comparison between singleton embryos gestated in 2CL ewes (1E2CL) compared to twin embryos gestated in 1CL ewes. *ISG17* was the only gene that demonstrated differential gene expression in all the comparisons except for the 2E2CL vs 1E2CL comparison.

Gene ontology/functional analysis did not produce any enriched GO clusters and/or KEGG terms. The gene functions for the genes used for validation determined by gene data bank searches are listed in Appendix IV.

Table 3.7 (A): Embryonic mRNA gene expression levels in single embryos transferred to ewes with 1CL (previously singleton bearing) (1E1CL) experimental ET group compared with control group singleton bearing ewes (Con1E1CL), and single embryos transferred to ewes with 2CLs (previously twin bearing)(1E2CL), twin embryos transferred to ewes with 1CL (2E1CL), and twin embryos transferred to ewes with 2CLs (2E2CL) experimental ET groups.

Gene	IE1CL vs Con1E1CL	1E1CL vs 1E2CL	1E1CL vs 2E1CL	1E1Cl vs 2E2CL
DKK4	0.74 (0.1-5.26)	2.20 (0.58-8.35)	0.93 (0.16-5.28)	1.76(0.47-6.56)
ISG17	**0.58 (0.40-0.84)**	**0.53 (0.35-0.80)**	**0.61 (0.4 -0.92)**	**0.47 (0.33-0.67)**
LGALS15	1.27 (0.71-2.26)	**3.25 (2.25-4.69)**	1.36 (0.95-1.95)	**3.72 (2.07-6.68)**
LOC101103603	1.18 (0.15-9.27)	4.30 (0.98-18.76)	1.81 (0.28-11.72)	2.63 (0.56-12.30)
LOC101117738	1.13 (0.12-10.27)	2.64 (0.77-9.03)	1.61 (0.26-5.24)	2.29 (0.58-9.08)
LAPTM5	**1.55 (1.39-1.72)**	1.73 (1.64-1.83)	**1.49 (1.36-1.63)**	**2.07 (1.90-2.25)**
TKDP1	2.29 (0.37-14.29)	3.07 (0.80-11.83)	2.01 (0.26-15.81)	1.93 (0.44-8.33)
LRRC32	**1.42 (1.02-1.98)**	**2.32 (1.83-2.93)**	**1.27 (1.07-1.52)**	**2.53 (1.55-4.14)**
LGALS15	4.42 (0.60-29.79)	4.59 (0.72-29.16)	5.37 (0.75-38.68)	3.66 (0.55-24.52)
TP1	1.71 (0.59-4.95)	1.45 (0.40-5.22)	2.62 (0.58-11.97)	**1.62 (0.51-5.19)**

Data are normalised with reference genes ACTB, GAPDH and RPL19. Data are shown as fold change with 95% confidence interval (given in parenthesis). If confidence intervals do not include "1" then mRNA expression levels are statistically different between groups (**bold**).

Table 3.7 (B): Embryonic mRNA gene expression levels in twin embryos transferred to ewes with 2CLs (previously twin bearing) (2E2CL) experimental ET group compared with control group twin bearing ewe (Con2E2CL), and twin embryos transferred to ewe with 1CL (previously singleton bearing) (2E1CL), single embryos transferred to a ewe with 2CLs (1E2CL), and twin embryo transferred to ewe with 1CLs (2E1CL) experimental ET groups.

Gene	2E2CL vs Con2E2CL	2E2Cl vs 2E1CL	2E2Cl vs 1E2CL	1E2CL vs 2E1CL
DKK4	**0.29 (0.09-0.93)**	0.53 (0.10-2.72)	1.25 (0.38-4.13)	**0.42 (0.18-0.97)**
ISG17	**0.91 (0.84-0.98)**	**1.29 (1.06-1.57)**	1.11 (0.92-1.35)	1.16 (0.86-1.58)
LGALS15	**0.47 (0.23-0.97)**	**0.36 (0.18-0.73)**	0.87 (0.43-1.76)	**0.42 (0.30-0.59)**
LOC101103603	0.29 (0.07-1.18)	0.69 (0.12-3.86)	1.64 (0.45-5.89)	**0.42 (0.18-0.96)**
LOC101117738	**0.28 (0.05-1.52)**	0.70 (0.09-5.37)	1.15 (0.25-5.24)	**0.61 (0.23-1.59)**
LAPTM5	**0.74 (0.66-0.82)**	**0.72 (0.62-0.84)**	**0.84 (0.74-0.95)**	**0.86 (0.83-0.90)**
TKDP1	0.78 (0.27-2.27)	1.04 (0.19-5.83)	1.60 (0.76-3.35)	**0.65 (0.49-0.87)**
LRRC32	0.68 (0.40-1.14)	**0.50 (0.30-0.83)**	0.91 (0.53-1.57)	**0.55 (0.46-0.65)**
LGALS15	1.92 (0.49-7.51)	1.47 (0.42-5.07)	1.25 (0.45-3.52)	1.17 (0.63-2.17)
TP1	0.99 (0.44-2.22)	1.62 (0.51-5.17)	0.89 (0.39-2.06)	1.81 (0.91-3.60)

Data are normalised with reference genes ACTB, GAPDH and RPL19. Data are shown as fold change with 95% confidence interval (given in parenthesis). If confidence intervals do not include "1" then mRNA expression levels are statistically different between groups (**bold**).

Table 3.7 (C): Embryonic mRNA gene expression levels in embryos of control twin bearing ewes (Con2E2CL) compared to control singleton bearing ewes (Con1E1CL); embryos of all 1 embryo experimental groups compared to embryos of all 2 embryo experimental groups; embryos of all 1CL experimental groups compared to embryos of all 2CL experimental groups.

Gene	Con2E2CL vs Con1E1CL	all 1E vs all 2E	all 1CL vs all 2CL
DKK4	1.44 (0.22-9.20)	0.67 (0.28-1.59)	1.39 (0.56-3.43)
ISG17	**1.34 (1.17-1.53)**	**0.77 (0.67-0.89)**	**0.68 (0.60-0.78)**
LGALS3	0.72 (0.36-1.45)	1.12 (0.70-1.79)	**2.26 (1.47-3.46)**
LOC101103603	1.56 (0.22-11.23)	0.66 (0.25-1.75)	1.47 (0.54-4.06)
LOC101117738	1.74 (0.15-19.67)	0.76 (0.25-2.26)	1.22 (0.39-3.82)
LAPTM5	1.02 (0.90-1.16)	**1.15 (1.04-1.27)**	**1.31 (1.19-1.43)**
TKDP1	1.52 (0.33-7.02)	0.80 (0.38-1.69)	1.20 (0.55-2.62)
LRRC32	0.82 (0.58-1.17)	1.10 (0.81-1.49)	**1.76 (1.33-2.33)**
LGALS15	0.60 (0.14-2.53)	1.63 (0.70-3.80)	1.58 (0.65-3.84)
TP1	1.06 (0.54-2.08)	1.31 (0.73-2.36)	0.89 (0.48-1.65)

Data are normalised with reference genes *ACTB, GAPDH* and *RPL19*. Data are shown as fold change with 95% confidence interval (given in parenthesis). If confidence intervals do not include "1" then mRNA expression levels are statistically different between groups (**bold**).

3.5 Discussion

The aim of this study was to identify if singleton and twin embryonic size and gene expression differs in the early pre-implantation period of gestation, with the objective of determining the mechanisms involved in the programming of the developmental trajectory of twins compared to singletons. This is the first study in sheep to use reciprocal ET and RNA-seq to examine gene expression of embryos at day 21, peri-implantation stage of gestation. However, the results are inconclusive.

This investigation was unable to confirm the hypothesis that programmed growth trajectory in singletons compared to twins differs from the early pre-implantation gestation period as suggested by Hancock *et al.*, 2012, nor that it is determined by the embryo. At day 21, all of the embryos examined in the experimental ET and control groups were of a similar size except for the 1E1CL ET group (single embryos that were transferred to ewes with a single CL, and had a single embryo that was removed). These embryos were significantly smaller than all other groups. It is possible that an embryo transfer effect may be an important factor that influenced 1E1CL embryo size in this experiment compared to the singleton control (Con1E1CL). However, a similar effect was not observed in the twin groups, so this seems an unlikely explanation. In addition, when compared to the 1E2CL group, it is possible that additional circulating progesterone as a result of the presence of two CLs had a direct effect on the growth of the 1E2CL early pre-implantation embryos. Early administration of exogenous progesterone has been shown to positively affect embryo growth (Kleemann *et al.*, 1994; 2001) and it is suggested that the effect can be obtained indirectly via the uterus as opposed to a direct effect on the embryo itself (Clemente *et al.*, 2009). In addition, progesterone plays an important role in the maintenance of pregnancy

(Spencer and Bazer, 2002). Three of the successfully maintained pregnancies, of twin embryo transfers to single CL ewes (2E1CL) which were potentially progesterone-deficient, spontaneously reduced to singletons (1E1CL) before day 21. This resulted in a group of only three embryos for examination which resulted in a large standard error, making it difficult to detect any differences in embryo size compared to the other ET twin group (2E2CL n= 10). This highlights the importance of maternal progesterone action on survival and growth of pre-implantation embryos. It is unfortunate that progesterone concentration profiles for the ewes had not been determined as it may further confirm these hypotheses.

Studies in humans and sheep report that twin growth trajectory diverges from singletons early in gestation (Rattray *et al.*, 1974; Iffy *et al.*, 1983). Although Hancock *et al.* (2012) reported that reduced twins appeared to be on the same growth trajectory as non-reduced twins compared to singletons, and deduced that growth trajectory is determined in the pre-implantation period, they reported no difference in size, as measured by ultra-sound, between any of the groups at day 41 (the day before reduction surgeries were performed). It is possible therefore, in spite of not observing any size differences in this study at day 21, that there are underlying embryonic and maternal factors and/or signals at work at this stage of pregnancy that are responsible for the reported differences in size at birth and gestation length. It is also possible that the low number of observations obtained and examined, for each experimental group, makes the analysis difficult. Further investigation is warranted, with the study designed to include a larger pool of recipient ewes that will ensure enough numbers of embryo/offspring to be collected at pregnancy day 21, 41 and at birth.

Many critical processes of organogenesis and rapid cell division occur during the preimplantation period (Dziuk, 1992). It is not surprising that a large number of gene transcripts (17,764) were detected in the whole embryo samples, highlighting the vast number of complex processes and potential gene pathways involved in the regulation of embryo growth and development at this time. There were no differentially expressed genes in the pairwise comparisons examined between embryo experimental and control groups in the RNA-seq analysis. However, there was wide variation in the number of candidate genes that showed significant differential expression in qPCR analysis between pairwise comparisons. Interestingly, the comparison of the control groups to each other, Con1E1CL to Con2E2C, generated only one differentially expressed gene, while the largest number of differentially expressed genes was obtained when 1E2CL group was compared to the 2E1CL group. The correlation of the RNA-seq data and qPCR in five of the group comparisons only partially validated the transcriptome analysis. The lack of correlation between the other pairwise comparisons is likely due to the small number of biological replicates which may have increased the effect of errors in either of the methods. However, even if full validation was obtained and the RNA-seq output deemed to be reliable, it provided little insight into the molecular regulation of embryo growth differences in day 21 twin and singleton embryos.

It is well understood that the fetal genome has a large effect on determining the growth and development of the fetus (Gluckman and Liggins, 1984). However, in this study, there were a relatively small number of biological replicates in some of the experimental groups which may have increased the error of the RNA-seq data and the qPCR analysis. In addition, it is likely that the tissue from these early pre-implantation embryos have significant

heterogeneity, which resulted in the direct inter-embryo variation in early gene expression patterns observed, as suggested by Taylor *et al.*, (2003). This is supported by evidence of variation observed between individual embryos in the principle component analysis of the RNA-seq data and the large 95% confidence intervals of the qPCR data. This analysis indicated that biological variation between replicates within embryo experimental groups was greater than the differences in gene expression due to the group they were allocated. This is not surprising, as individual embryo variation is possible due to differences in time of ovulation and/or fertilisation and differences in rate of cleavage, even if donor/recipient ewes all came into oestrus on the same day (Wilmut *et al.*, 1985), such that each examined embryo may have been at a slightly different stage of development. Further, this heterogeneity may also be considered an important confounding factor that affected the validation of the RNA-seq data. This raises the question as to whether the methods used, morphometric measurements and transcriptomic analysis, are the most suitable methods of investigating embryo growth and development at this stage of gestation.

Although, the pair-wise comparisons on the qPCR data generated small numbers of differentially expressed genes, it is noted that those candidate genes that were chosen for examination play a significant role in embryo maternal interactions. *ISG17*, which is the sole gene to be differentially expressed in all of the comparisons with the exception of 2E2CL vs 1E2CL, is an interferon tau (INFτ) stimulated gene (Wolf *et al.*, 2003; Oliveira *et al.*, 2008). INFτ is recognised as an important pregnancy recognition signal, produced by the conceptus (Martal *et al.*, 1998). Expression of *ISG17* within the ovine embryo, therefore, may be an indication of a possible role of INFτ to influence embryo development and not only act as a signal of its presence in the uterus. The comparison of 1E2CL to 2E1CL experimental ET

groups generated seven out of 10 differentially expressed genes in the qPCR analysis. However, phenotypically there was no corresponding morphometric difference observed between these two groups of embryos. It is possible that although a difference in gene expression is responsible for differential programming of growth-trajectory in twins and singletons during the pre-implantation period, embryonic phenotype detection may not be possible at the developmental stage examined and dissecting the initial pathways that drive embryo growth may be difficult. Patterns of fetal and placental growth are definitely re-programmed in multiple pregnancies (Gootwine *et al.*, 2007), but there exists the likelihood that differences observed at birth by Hancock *et al.* (2012) and at day 136 of gestation by Vatnick *et al.* (1991) are a consequence of an event occurring later than day 21 but earlier than day 42 of gestation. Further, this pair-wise comparison highlights the fact that there may be a more important maternal role that has been overlooked, that may be related to progesterone concentration.

3.6 Summary and conclusions

The mechanisms that induce IUGR in twins compared to singletons are not yet clear. The findings of this study highlight that at day 21 of gestation there are vast and complex interactions at play and the examination of embryos on their own reveals little information due to inter-embryo variation. Therefore, further examination is needed before clear conclusions can be drawn. Other techniques such as *in-situ* hybridisation or examination of specific cell types or lineages may be more valuable to decipher the mechanisms at work in pre-implantation singleton and twin embryos. In addition, the importance of maternally-driven endocrine signals should also be investigated, since there may be a greater

contribution from the uterus to programme growth trajectory of embryos than from the embryos themselves.

Foreword to Chapters 4 and 5

Chapters 4 and 5 of this thesis present work on the genetic model of maternal constraint, dam size. The methodology of Chapter 4 presents morphometric measurements of purebred Cheviot and Suffolk embryos in order to determine if differential size differences between the two breeds are evident at day 19 and 21 of gestation. Ewe hormone profiles of early pregnancy are also presented. In Chapter 5 we developed a method to determine developmental stage based on anatomical milestones of embryogenesis. These methods were applied to the same embryos of Chapter 4 in order to assess the accuracy of using size measurements to determine developmental stage.

4 Morphometric examination of pre-implantation embryos and maternal hormone profiles in Cheviot and Suffolk breeds of sheep

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and

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4.1 Abstract

The aim of this study was to examine embryo size in early gestation (day 19, and day 21) of sheep breeds differing in mature body size: Suffolk, large, and Cheviot, small. Following synchronisation of Suffolk and Cheviot ewes, artificial insemination using semen from rams of the same breed was performed at oestrus (day 0). From day 0 to day 21 jugular venous blood sampling was done every other day on a subsample of each breed of ewe for determination of plasma progesterone (P4), insulin-like growth factor 1 (IGF1), insulin, and adiponectin concentrations. At day 19 and day 21 of gestation a random selection of ewes from each breed, that had not returned to oestrus, were euthanised (day 19: Suffolk, n=5, Cheviot, n=3; day 21: Suffolk, n=4 Cheviot, n=6) and embryos, uteri, ovaries, and ovarian structures were examined and measured. Due to a lack of singleton pregnancies in the Cheviot ewes, only twin embryos were analysed in this study. Twin Suffolk embryos were longer (13.28 ± 1.26) than twin Cheviot embryos (9.25 ± 0.89mm; $p<0.05$) at day 19 of gestation but not at day 21 ($p>0.05$). Uterine weight and size (length, width, length of horns) did not differ ($p>0.05$) between breeds. Mean insulin concentrations were higher ($p<0.001$), and mean adiponectin concentrations were lower ($p<0.05$) in Cheviot ewes than Suffolk ewes for day 0 to day 21. The present study showed that differences in embryo size in Suffolk and Cheviot breeds are evident at day 19 of gestation, even when physical size of the uterus is not limiting, and suggests that this difference may be due to differential embryo-dam interactions in these two breeds of sheep.

4.2 Introduction

Regulation of fetal growth patterns appears to occur during the early stages of pregnancy, a critical time for differentiation and development of the embryo (Dziuk, 1992). This dynamic period is driven by complex interactions between the mother and embryo, during which cell division, implantation and organogenesis occurs (Dziuk, 1992). Previous research has demonstrated that prenatal events that influence conceptus growth and development can affect fetal size and survival before birth (Pomeroy, 1960) as well as postnatal survival and later life production (Huffman *et al.*, 1985; Greenwood *et al.*, 2010).

Gluckman and Hanson (2004) reported that maternal constraint is the major non-genetic factor that determined fetal size at term. The importance of the maternal environment to influence offspring size and development has been demonstrated in the classic study of Walton and Hammond (1938) in which mare size was shown to have a significant influence on foal birth size. Other studies in various animal models also have reported that maternal size has an effect on birth size and postnatal growth: horse (Allen *et al.*, 2002; 2004), sheep (Gardner *et al.*, 2007), cattle (Joubert and Hammond, 1954), mice (Cowley *et al.*, 1989) and humans (Brooks *et al.*, 1995); whereby fetal development and birth weight is reduced in a small mother, while enhanced in a larger mother.

Reciprocal sheep embryo transfer (ET) studies in breeds of dissimilar mature body size have demonstrated differences in lamb birth weight (Hunter, 1956; Dickinson *et al.*, 1962; Sharma *et al.*, 2012a). Interestingly, Sharma et al. (2013) demonstrated that on day 19 of gestation large breed Suffolk (S) embryos gestated in S ewes were longer than those gestated in small breed Cheviot (C) ewes (13.4±0.53mm and 11.0±0.57mm respectively). In contrast, C embryos gestated in S ewes were longer than those gestated in C ewes

(15.2±0.68mm and 12.9±0.53mm respectively). Combined these studies suggest that altered development may be a consequence of differential interactions between the developing conceptus and the dam (Gaviria and Hernandez, 1994; Fligny *et al.*, 2009), which may then have significant influence on subsequent fetal-growth trajectory. The mechanisms by which maternal genotype may act to influence conceptus growth are not fully understood. There is a need to examine the effect of maternal genotype on patterns of embryonic and fetal development.

Hormones that are of importance to the somatotropic axis such as insulin and insulin growth factor 1 (IGF1) are known to play a regulatory role on the reproductive axis at the level of the hypothalamus and peripherally at the gonads. These and other important reproductive hormones have been shown to influence placental, embryonic and fetal development either through direct action on the embryo or via various receptors (Hou and Gorski, 1993; Gluckman, 1997; Matsui *et al.*, 1997; Wathes *et al.*, 1998; Meikle *et al.*, 2004). Progesterone (P4) is necessary for successful implantation and development of the conceptus (Spencer and Bazer, 2004a). Receptors for insulin and IGF1 and IGF2 are found in both the uterus and the embryo (Stevenson *et al.*, 1994a; Watson *et al.*, 1994; Nuttinck *et al.*, 2004) and are known to be critical determinants of uterine function and embryo development (Wathes *et al.*, 1998). Indeed, the action of these hormones appear to be inter-related as IGF1 and insulin have been shown to enhance *in vitro* luteal cell secretion of P4 (Baithalu *et al.*, 2013), while P4 stimulates *IGF1* mRNA and protein expression in the uterus (Simmen *et al.*, 1990). Also of interest is adiponectin, a metabolically active hormone with receptors observed in pig, rabbit and mice embryos and associated with *in vitro* blastocyst development (Chappaz *et al.*, 2008; Čikoš *et al.*, 2010; Fischer *et al.*, 2010). When

examined in a ruminant model, the cow, adiponectin mRNA was undetectable in the embryos examined, and appeared to have little effect on oocyte maturation and pre-implantation embryo development (Maillard *et al.*, 2010). Little is known about the mechanisms by which these and other reproductive hormones act at the endometrial level in ruminants; and less is known about the differences that may be driven by maternal constraint as determined by breed size.

This study aims to examine the relationship of the maternal uterine environment and developing purebred embryos within a "dam size" model using S (large) and C (small) breed sheep; and to examine maternal hormone profiles (P4, insulin, IGF1 and adiponectin) in these breeds during early gestation.

4.3 Materials and Methods

The animals used in this study were managed as one cohort under commercial farming conditions at Massey University's Keeble Farm, Palmerston North, New Zealand. This experiment was approved by the Massey University Animal Ethics Committee.

4.3.1 Experimental Animals and Design

This study was undertaken using Cheviot (C, "small") and Suffolk (S, "large") sheep breeds to provide genotypes with dissimilar mature body size. Two pregnancy groups were established by artificial insemination (day 0) of C and S ewes using semen from rams of same the breed. Ewes were of mixed age (3-6) and parity (2-5). Average body weight of C ewes was 58.1 kg and S ewes was 78.4 kg; with an average body condition score of both breeds of 3.0 (Jefferies, (1961); units 1-5: 1=emaciated, 5=obese). Conceptuses were

recovered from S and C ewes on day 6 (n=3 and n=2 respectively), day 13 (n=5/group), day 16 (n=2 and n=4 respectively), day 19 (n=4 and 6 respectively) and day 21 (n=4 and 10 respectively) of gestation. Day 6, day 13 and day 16 embryos were not analysed due to collection of non-viable embryos (day 6) and poor quality images (day 13, day 16), therefore, this chapter only reports on day 19 and day 21 embryos.

4.3.2 Oestrus synchronisation and artificial insemination of recipients

Oestrus was synchronised in 160 ewes, C (n=80) and S (n=80), using intravaginal progesterone releasing devices (Eazi-breed CIDR; Pharmacia; Auckland, New Zealand) for 13 days. Semen was collected via electro-ejaculation from four rams per breed. Ewes of each breed were artificially inseminated laparoscopically with 0.5 ml of fresh semen from a ram of the same breed, 32 hours after CIDR removal.

4.3.3 Embryo harvest (day 19 and 21)

At day 19 and day 21 of gestation, randomly selected ewes (n=8 and 10 respectively) that included both singleton and twin pregnancies (day 19: S, n=5, C, n=3; and day 21: S, n=5, C, n=5) were euthanized via captive bolt and exsanguination. The uterus was removed and placed on ice. The excess connective tissue was dissected from the uterus and the whole uterus was weighed. Morphometric measurements (uterine horn lengths, uterine body length, and uterine body width) were recorded. Each uterine horn was flushed with 20.0 mL (0.9%) sterile saline for recovery of the embryo. Embryos were examined for viability and then were dissected free of extraembryonic membranes if necessary and preserved in a 5.0 mL vial containing 10% buffered formal saline. Left and right ovaries were dissected and weighed.

A total of 22 (twin) embryos (day 19 n=8: S=4, C=4 and day 21 n=14: S=4, C=10) were examined and used in this study. Singleton S embryos (n= 6) were obtained across collection day 19 and day 21, but no C singleton embryos. Therefore, all singleton embryos were removed from the analysis. Two embryos from the day 19 C group were also removed from the analysis as they were degenerating and therefore non-viable.

4.3.4 Embryo measurements

Day 19 and day 21 embryos were photographed with a stereomicroscope (Leica Mz12 fitted with DFC 320 camera; Leica Microsystems, Heerbrugg, Switzerland) within four hours of collection. The images were then examined using ImageJ (U. S. National Institutes of Health, Bethesda, Maryland, USA). Embryo length and width, and heart bulge width was determined for both day 19 and day 21 embryos. Embryo length (EL) was defined as the distance from the medial aspect of the head to the tip of the embryonic tail, following the outer curvature of the embryo. Embryo width (EW) was defined as the distance between the two widest points of embryos with the line passing just below and not including the heart bulge but including somites. Heart bulge width (HB) was defined as the distance between the two widest points of the heart bulge with the line passing through the midsection of the heart bulge and excluding somites (Figure 4.1).

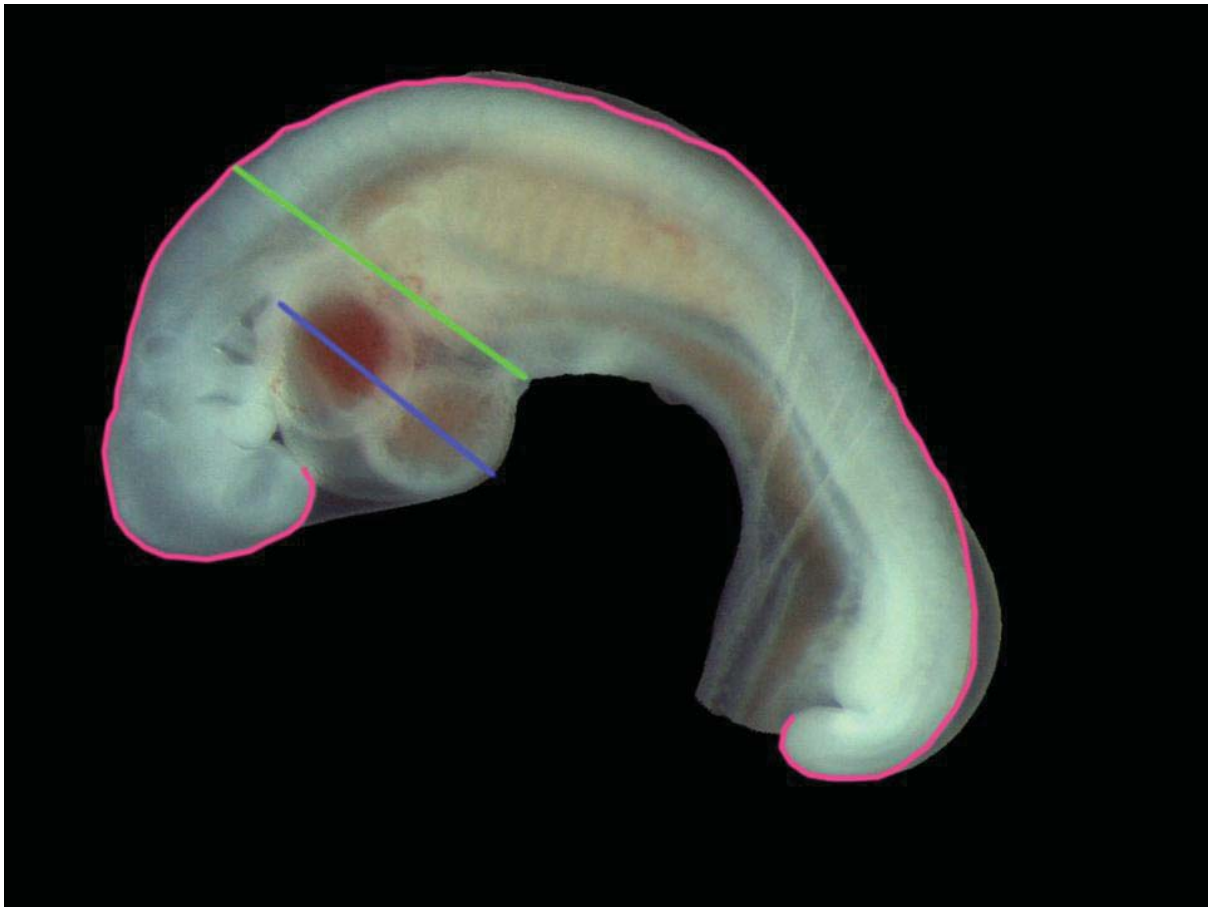

Figure 4.1 Image of day 19 sheep embryo showing the measurements of embryo length, embryo width and heart bulge width. Embryo length (pink) = distance from the medial aspect of the head to the tip of the embryonic tail, following the outer curvature of the embryo. Embryo width (green) = distance between the two widest points of embryos with the line passing just below and not including the heart bulge but including somites. Heart bulge width (blue) = distance between the two widest points of the heart bulge with the line passing through the midsection of the heart bulge and excluding somites.

4.3.5 Blood sampling and hormonal assays

From day 0 to day 21 blood samples were collected every other day via jugular venipuncture (10 mL sodium heparin vacutainer) from a subsample of ewes (12 Suffolk and 12 Cheviot). Plasma was isolated from these blood samples by centrifugation at 3,000 g for 15 minutes. Duplicate samples were stored at -20°C for later hormone assays. Only blood samples for ewes that were twin bearing and whose embryos were examined were used for analysis of hormone concentrations.

Progesterone concentrations were determined using RIA Kit (Diagnostic Product Corporation, Los Angeles, CA, USA). The limit detection of the assay was 0.06 ng/mL and the intra-assay CV for Control I (0.47 ng/mL) and Control II (7.81 ng/mL) were 17% and 3.9%, respectively.

Insulin concentration was determined by an immunoradiometric assay (IRMA) (INS-IRMA; Diasource, Brussels, Belgium) previously used in sheep (de Brun *et al.*, 2014). The limit of detection was 2.8 μUI/mL, and the intra-assay CV for Control I (22.5 μUI/mL) and Control II (91.0 μUI/mL) were 9.3% and 4.5%, respectively.

Concentration of IGF1 was determined using a double antibody RIA, previous described by de Brun *et al.* (2014). The assay limit detection was 0.55 ng/mL and the intra and inter-assay coefficients of variation for Control I (36.8 ng/mL) were 7.9% and 9.4% and for Control II (520.2 ng/mL) were 7.4% and 11%, respectively.

Adiponectin concentrations were determined by a radioimmunoassay (RIA) using a commercial kit (HADP-61 HK, Linco, Millipore) according to (Raddatz *et al.*, 2008). The assay sensitivity was 1.1 ng/mL. The intra-assay CV for Control I (11.9 ng/mL) and Control II (60.5 ng/mL) were 9.3% and 7.0%, respectively.

4.3.6 Statistical analysis

All statistical procedures were performed using SAS (Version 9.4, SAS Institute INC., Cary, NC, USA). Day 19 and day 21 embryo morphometric data was subjected to analysis of variance (ANOVA, mixed model) to determine the effect of ewe breed on embryo size at each time point. Two way interactions (recipient age by breed, and sire by breed) were included in the initial model but were removed if found non-significant ($p>0.05$) and the

model was refitted. When no interactions were significant only the main effect of ewe breed is discussed.

Uterine weight and size measurements and ovarian weight were subjected to ANOVA with respect to ewe breed. Ewe body weight was fitted as a covariate for ovarian weight, and uterine weight, length and width data.

Repeated measure ANOVA with respect to ewe breed, and time was used to analyse the data for plasma P4, insulin, IGF1 and adiponectin concentrations using a two way interaction (breed by time). Where this interaction was not significant the main effect of ewe breed was determined.

4.4 Results

4.4.1 Embryo morphometry

There was no effect ($p>0.05$) of recipient age or sire on the embryo dimensions at day 19 or day 21 (data not shown). Day 19 S embryos were longer ($p<0.05$) than C embryos (Table 4.1). There were no differences ($p>0.05$) in embryo width or heart bulge width at day 19. There were no differences ($p>0.05$) between S and C embryos in any of the morphometric measurements at day 21 (Table 4.1).

4.4.2 Uterine and ovarian weights and morphometric data

There was no difference ($p>0.05$), in uterine weight, uterine body length or body width at day 19 or day 21 when adjusted for body weight (Table 4.2). Day 19 uterine body width and day 21 uterine body length differed ($p<0.05$) between breeds when body weight was not fitted as a covariate (Appendix V). There was no difference ($p>0.05$) in uterine weight. There

was no difference (p>0.05) in uterine horn length (data not shown). The ovaries of S ewes were heavier (p<0.05) than those of C ewes at day 19 (S= 4.6 ± 0.22 g; C= 3.63 ± 0.18 g). Ovaries containing copora lutea (CLs) were heavier (p<0.05) in S ewes compared to C ewes at day 19 (S= 3.5 ± 0.12 g; C= 2.7 ± 0.09 g). There were no differences (p>0.05) in ovarian weights at day 21 (data not shown).

Table 4.1 Suffolk and Cheviot embryo measurements (embryo length, embryo width and heart bulge width) at Day 19 and 21 of gestation.

		Breed	
		Suffolk	Cheviot
		n=4	*n=4*
Day 19	Embryo length (mm)	13.28±1.26[b]	9.54±0.89[a]
	Embryo width (mm)	1.46±0.18	1.11±0.15
	Heart bulge width (mm)	1.56±0.15	1.23±0.12
		n=4	*n=10*
Day 21	Embryo length (mm)	17.20±0.74	17.08±0.53
	Embryo width (mm)	2.70±0.22	2.77±0.14
	Heart bulge width (mm)	1.98±0.14	1.73±0.09

Values are least squares means ± standard error of the mean. Different superscripts within main effects indicate significant differences (p<0.05

Table 4.2 Suffolk and Cheviot uterine weight, uterine body length and body width at day 19 and 21 of gestation.

		Breed	
		Suffolk	Cheviot
		n=2	*n=3*
Day 19	Uterine weight (g)	94.94 ± 16.7	97.31 ± 14.53
	Uterine body length (mm)	49.84 ± 9.19	55.99 ± 7.98
	Uterine body width (mm)	47.84 ± 2.15	35.37 ± 1.86
		n=2	*n=5*
Day 21	Uterine weight (g)	90.54 ± 27.27	96.89 ± 8.28
	Uterine body length (mm)	61.19 ± 12.97	65.19 ± 3.94
	Uterine body width (mm)	52.26 ± 5.38	43.38 ± 1.63

Values are least squares means ± standard error of the mean.

4.4.3 Ewe hormonal concentrations

There were no significant differences ($p>0.05$) in peripheral plasma P4 concentrations due to breed or day (Figure 4.2). Plasma P4 concentration increased initially with time, with the highest concentration for both C and S ewes observed at day 14 (Figure 4.2).

Suffolk ewes tended ($p=0.07$) to have higher IGF1 concentrations on day 0 than Cheviot ewes (Figure 4.3). However, for all other time points there was no effect of breed on IGF1 concentration ($p>0.05$). There was a significant difference ($p<0.05$) in IGF1 concentration between day 0 and all other days for Suffolk ewes, but there was no effect ($p>0.05$) of day on IGF1 concentration from day 2 to day 21.

Day and breed by day interaction had no effect ($p>0.05$) on plasma insulin and adiponectin concentrations (Figure 4.4, Figure 4.5 respectively). There was breed effect seen for both of these hormones. Overall mean concentration of insulin was higher ($p<0.01$) in Cheviot ewes than Suffolk ewes (C= 31.86 ± 5.25 µUI/mL; S= 26.76 ± 5.04 µUI/mL). Overall mean adiponectin concentration was higher ($p<0.001$) in Suffolk than Cheviot ewes (S= 4.19 ± 0.25 ng/mL; C= 2.94 ± 0.22 ng/mL).

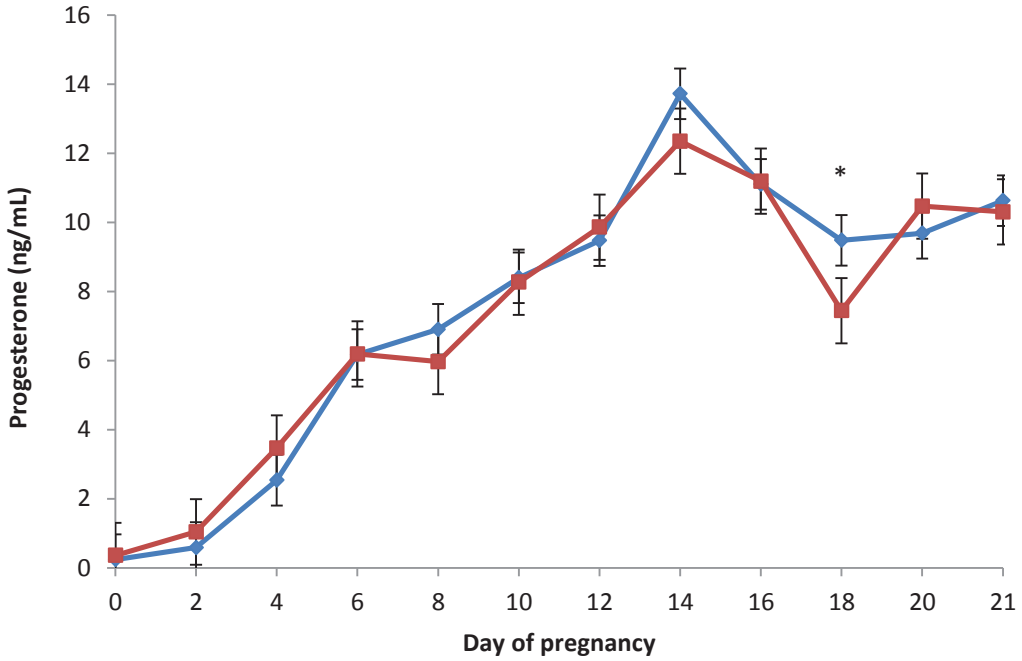

Figure 4.2 Plasma progesterone concentrations in Suffolk (—■—) and Cheviot (—◆—) ewes from day 0 to day 21 of pregnancy. Values are least squares means with standard error of the means. * tends to be different (p<0.1).

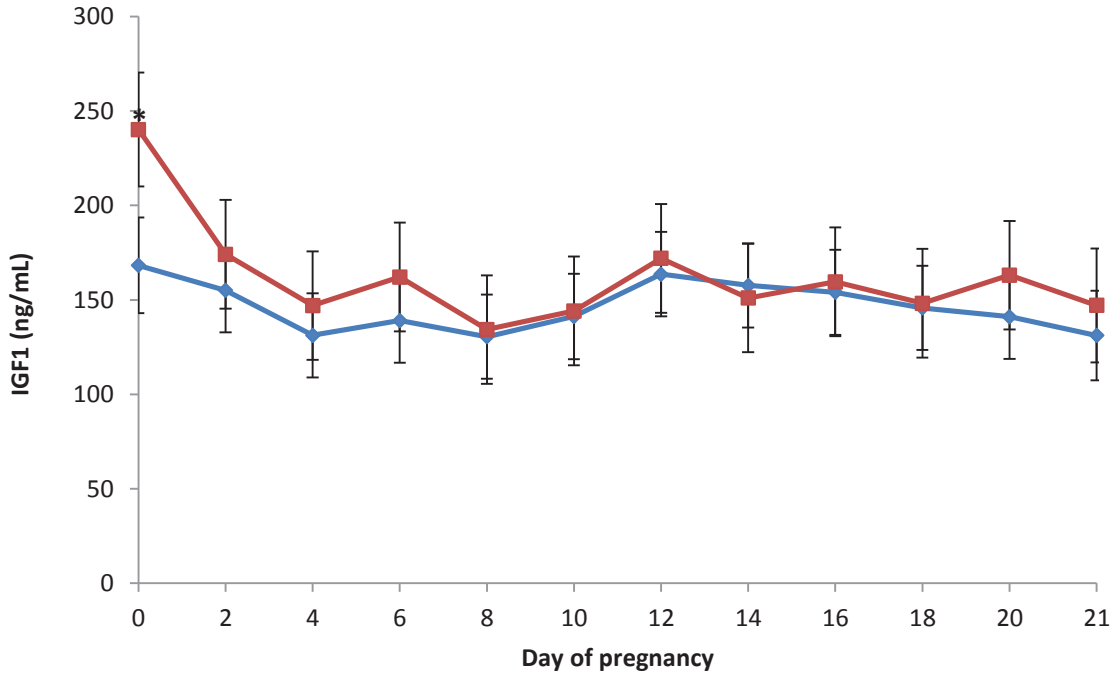

Figure 4.3 Plasma IGF1 concentrations in Suffolk (—■—) and Cheviot (—◆—) ewes from day 0 to day 21 of pregnancy. Values are least squares means with standard error of the means. * tends to be different (p<0.1).

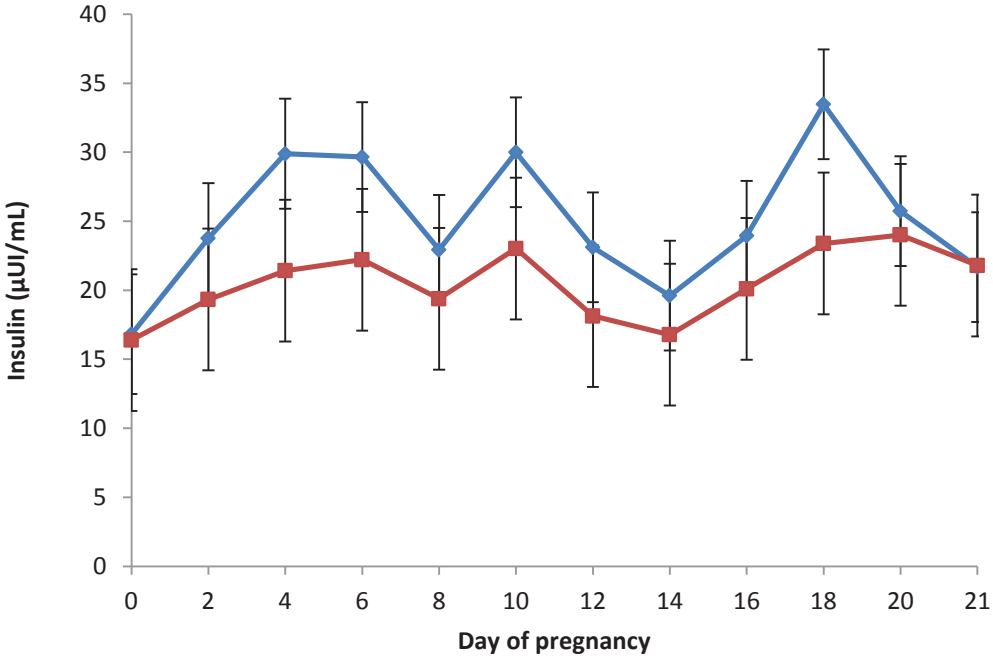

Figure 4.4 Plasma insulin concentrations in Suffolk (—■—) and Cheviot (—◆—) ewes from day 0 to day 21 of pregnancy. Values are least squares means with standard error of the means.

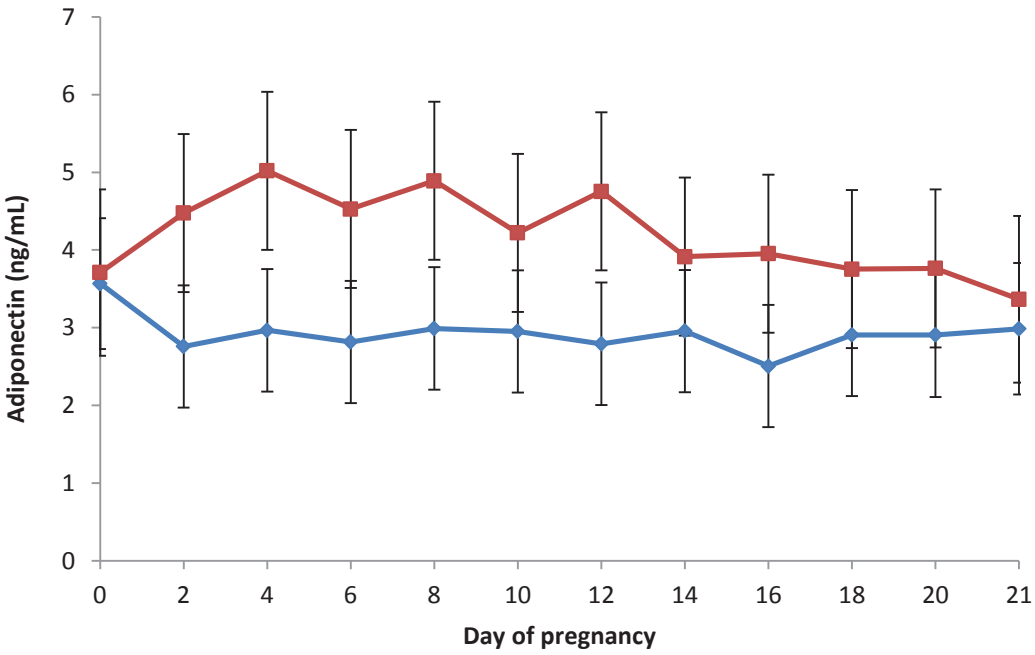

Figure 4.5 Figure 4.5 Plasma adiponectin concentrations in Suffolk (—■—) and Cheviot (—◆—) ewes from day 0 to day 21 of pregnancy. Values are least squares means with standard error of the means.

4.5 Discussion

This study was designed to determine if there were any differences in embryo development and maternal hormonal profiles between Cheviot (C) and Suffolk (S) breeds of sheep in the early pre-implantation period of pregnancy. The present study found that at day 19 of gestation S embryos are longer that C embryos. Similarly, Sharma *et al.* (2013), showed differences of the same magnitude in embryo size at day 19 in an embryo transfer study. This highlights the possibility that embryo transfer does not greatly impact on embryo size at this stage of gestation in this maternal size model. However, in the present study twin embryos were examined, whereas Sharma *et al.* (2013) examined singleton pregnancies. The present results, combined with the findings of Sharma *et al.* (2013) indicates that S embryos developing in S dams grow at a faster rate than C embryos gestating in C dams up to day 19 of gestation. This suggests that the mechanisms involved in embryo development, and the interaction between the developing embryo and dam may differ between these two breeds during day 0 to day 19.

Interestingly, there were no differences observed in embryo size at day 21. This observation may be as a result of the proposed “hourglass model” of growth as previously described (Duboule, 1994). It is characterised firstly by an early period when developmental differences occur, followed by a period of reduced variability or phylotypic stages, lasting to mid gestation, and then ending at birth when progressive divergence in development occurs and phenotypic differences are observed. In support of this concept, similar observations were made through a series of investigations: morphologic differences at day 19 (Sharma *et al.*, 2013), no difference at day 55 (Sharma *et al.*, 2012b), followed by observed phenotypic differences at birth (Sharma *et al.*, 2012a) using the same two breed model. Implantation is

completed by day 21 in sheep (Gaviria and Hernandez, 1994), which further suggest that there are critical mechanisms mediated by the implanting conceptus and/or associated with uterine receptivity that may be responsible for the differences observed at day 19. Further examination is needed to understand the apparent disappearance in size differences observed at day 21.

In this study no differences were detected in uterine morphometric measurements at day 19 and day 21 indicating that physical uterine space does not differ between breeds in early pregnancy. These findings are consonant with the previous findings of Sharma *et al*. (2010). As a consequence, Sharma *et al.* (2013) proposed that in very early pregnancy, when maternal physical uterine space is not a constraint, the apparent differences in embryo size/growth could potentially be due to a number of different mechanisms involving interactions between embryonic trophoblast and maternal uterus. Circulating maternal P4 (Satterfield *et al.*, 2006) in tandem with uterine progesterone receptor (*PGR*) expression (Sequeira *et al*., 2012; using the same C/S model) may offer some explanation to differences in size due to the vital role they play in preparing the uterus for implantation. As a consequence they may also directly or indirectly affect the implanting embryo's development at this stage of gestation. However, in the present study there were no differences observed in circulating ewe P4 concentrations between C and S ewes from day 0 to day 21. These findings precede those of Sharma *et al.* (2013), in terms of gestational age, but not in timing of publication, in which no effect of breed was observed for plasma progesterone in later pregnancy (day 50, day 90, day 120 and day 140).

Plasma P4 peaked at day 14 and then decreased from day 14 to 20 in both breeds. To our knowledge, there are no other reports that discuss a decrease in P4 during early pregnancy.

The period of day 14 to day 20 coincides with the luteal phase of the cyclic ewe and it may be that a slight decrease in P4 concentration occurs during this time as the uterus adjusts to embryo signalling its presence within the uterus. It is understood that once P4 concentrations remain above 2.0 ng/mL, the pregnancy will be maintained (Parr *et al.*, 1987). However, it has been predicted that the timing of progesterone increase from periovulatory to luteal concentration is more important than the absolute concentration (Wilmut and Sales, 1981). The frequency of blood sampling in this study likely enabled detection of this decrease. However, peripheral P4 concentration may not be a true reflection of P4 concentration at the paracrine level within uterus, nor the action of the hormone within this tissue (Spencer and Bazer, 1995; Abecia *et al.*, 1996).

Spicer *et al.* (1993) stated that IGF1 concentrations were under genetic control and that breed would therefore influence concentration, and that the ovary was not a major source of blood IGF1. In this study, however, there was no difference between the S and C ewes examined. It is therefore unlikely that this circulating IGF1 is directly involved in the differences in embryo size that were observed at day 19, since the principle source of circulating IGF1 is the liver (Sjögren *et al.*, 1999). Further, locally produced IGF1 from the ovary, uterus and embryo is more likely to be responsible for paracrine stimulation of early embryo growth (Ko *et al.*, 1991; Wathes *et al.*, 1998).

Overall, insulin and adiponectin concentrations differed between the breeds examined. A large variation in the concentrations of both these hormones between individual ewes at each time point was observed as reflected by the standard error reported here. It is difficult to decipher from these results if peripheral insulin and adiponectin influenced the embryo size at day 19. Insulin and adiponectin receptors have been identified within the uterus of

various species and therefore, may be involved in paracrine driven embryo growth and uterine changes (Korgun *et al.*, 2003; Lord *et al.*, 2005; Takemura *et al.*, 2006; Schmidt *et al.*, 2008; Kim *et al.*, 2011; Smolinska *et al.*, 2014). Adiponectin is associated with insulin sensitivity, reduced gluconeogenesis and hepatic glucose release (Kadowaki *et al.*, 2006). In humans maternal circulating adiponectin concentrations are inversely correlated with fetal growth (Aye *et al.*, 2013). This is contrary to our finding, since the higher concentrations were observed in the Suffolk ewes with the larger embryos at day 19. Although uterine receptors have been identified in some species, to our knowledge there are no reports on adiponectin receptors in the ovine uterus. Further, the action of adiponectin at this level is relatively unknown and further investigation is needed. Placental and fetal availability of glucose, necessary for conceptus growth, is associated with maternal insulin resistance (Owens, 1991), such that the observed embryo size difference at day 19 in the present study may be related to the differential insulin sensitivity between these breeds in early pregnancy. However, it is also important to consider that the differences observed in circulating Insulin and adiponectin between these breeds may have limited direct impact on embryo growth as hormone action is not only dependent on circulating hormone concentration but also on target tissue sensitivity driven by receptor content (Meikle *et al.*, 2004). Further investigation is needed to examine this.

4.6 Summary and conclusions

This study demonstrated that differences in size of purebred embryos, gestated in dams of differing maternal size (C and S), are evident as early as day 19 of gestation but were not apparent soon after. Further, in the case of these twin pregnancies, maternal

concentrations of P4 and IGF1 did not differ between C and S ewes, between days 0 to day 21 of gestation, but concentrations of insulin and adiponectin did. This may suggest that the latter hormones are involved in the size difference observed. However, maternal circulating hormone concentrations may not be an accurate indication of uterine hormone concentrations, nor the action of these hormones within the uterine environment to drive transfer of nutrients, receptivity to implantation, or embryo growth directly. Further investigation is therefore warranted.

5 Assessment of Cheviot and Suffolk embryo size and somite count as indicators of developmental stage and embryo growth

5.1 Abstract

The peri-implantation stage of gestation is a critical time for embryo growth as many of the processes of differentiation and organogenesis are taking place. During this time embryo length increases exponentially. Crown-rump measurements have long been an accepted means of determining stage of development and age of embryos/fetuses. However, numerous other changes associated with growth at this period also take place rapidly. Thus, assignments of length and size may not be an accurate assessment of stage of development. In Chapter 4, morphometric measurements (curved length, width and heart bulge width) were used to determine differences in development between the embryos at day 19 and 21 of gestation in breeds of sheep differing in mature body size (Cheviot and Suffolk). In this study, the same embryos examined in Chapter 4 were assigned a developmental rank (1, 2, or 3) and their somites were counted to determine if the size differences reported were an accurate representation of stage of gestation. At day 19 Suffolk embryos differed ($p<0.05$) in developmental rank compared to Cheviot embryos, but not at day 21. At day 19 Suffolk embryos also had a greater ($p<0.05$) number of somites than Cheviot embryos. Somite number did not differ ($p>0.05$) at day 21. Embryo length was significantly correlated to somite number in day 19 and day 21 embryos, $r = 0.97$ and 0.80, respectively ($p<0.01$). The results of this study suggests that the size of an embryo, as measured as curved crown rump length, can be considered a reliable representation of embryo development at both day 19 and day 21 of gestation in sheep.

5.2 Introduction

Embryo development is an ordered process, beginning with an increase in cell numbers, followed by differentiation and then development of organs and systems (Dziuk, 1992). The peri-implantation stage between days 13 to 19 is a very critical time as this is when differentiation takes precedent (Wales and Cuneo, 1989). Additionally, embryo length has been shown to increase exponentially during this period (Wales and Cuneo, 1989).

Breed is generally considered to influence pre-natal growth and development in the later stages of gestation (Hunter, 1956; Joubert, 1956). However, there is an increasing body of evidence that suggests that embryo/fetal growth may be determined in early gestation (Smith *et al.*, 1998; Watkins *et al.*, 2008; Salomon *et al.*, 2011). Further, the effect of breed on embryo growth during the early pre-implantation period has been examined in sheep using reciprocal embryo transfer between small breed Cheviot (C) and large breed Suffolk (S) (Sharma *et al.*, 2012b; 2013). These studies have demonstrated differences in embryo size as early as day 19 of gestation following reciprocal transfers, yet no differences were observed between those embryos that were transferred to same breed ewes. In Chapter 4 however, pure bred S embryos were observed to be longer than pure bred C embryos at day 19 but not at day 21.

Traditionally crown-rump length is used to estimate age and stage of development of a fetus, and requires little technical experience (Dziuk, 1992). This principle was used to determine differences in size between the embryos of the breeds examined, by measuring the length along the greater curvature from medial aspect of the head to the tip of the embryonic tail, in Chapter 4 and by Sharma *et al.* (2013). However, length measurements may be unreliable predictors of biological development at this stage of gestation (Green and

Winters, 1945). The numerous accompanying processes of differentiation and changes in the relative size and features of various tissues occur so rapidly that assignment of growth milestones based on size may not be significant. Therefore, the aim of this study was to compare the developmental stages of Suffolk and Cheviot embryos, by ranking embryos based on certain developmental milestones, and somite counts, to determine if the size differences reported in Chapter 4 are an accurate representation of differences in growth between the embryos of C and S breeds at days 19 and 21.

5.3 Materials and Methods

The embryos examined in this study were obtained from the experiment described in Chapter 4 (p.106) summarised below.

All experimental procedures were carried out with the approval of the Massey University Animal Ethics Committee.

5.3.1 Experimental animals and design

This study was undertaken using Cheviot (C, "small") and Suffolk (S, "large") sheep breeds to provide genotypes with dissimilar mature body size. Two pregnancy groups were established by artificial insemination (AI) (day 0) of oestrus synchronised C and S ewes using semen from rams of same the breed. Ewes were of mixed age (3-6 years old) and parity (2-5). Average body weight of C ewes was 58.1 kg and S ewes was 78.4 kg; with an average body condition score of both breeds of 3.0 (Jefferies, (1961); units 1-5: 1=emaciated, 5=obese). Conceptuses were recovered from S and C ewes on day 6 (n=3 and n=2 respectively), day 13 (n=5/group), day 16 (n=2 and n=4 respectively), day 19 (n=4 and 6

respectively) and day 21 (n=4 and 10 respectively) of gestation. Day 6, day 13 and day 16 embryos were not analysed due to collection of non-viable embryos (day 6) and poor quality images (day 13, day 16), therefore, this chapter only reports on day 19 and day 21 embryos.

5.3.2 Day 19 and Day 20 embryo harvest

At day 19 and day 21 of gestation, randomly selected ewes (n=8 and 10 respectively) that included both singleton and twin pregnancies (day 19: S, n=5, C, n=3; and day 21: S, n=5, C, n=5) were euthanized via captive bolt and exsanguination. The uterus was removed and placed on ice. The excess connective tissue was dissected from the uterus and the whole uterus was weighed. Each uterine horn was flushed with 20 mL (0.9%) sterile saline for recovery of the embryo. The embryos were dissected free of extraembryonic membranes if necessary and preserved by placing them in serial solutions of 4% paraformaldehyde, followed by 15% sucrose solution and then embedded in Tissue-Tek® OCT Compound (Sakura® Finetek, USA), and stored at -80°C for further analysis.

5.3.3 Analysis of embryo developmental stage

The OCT-imbedded embryos were thawed by placing the cassettes in a petri-dish into a water bath (45°C). Once the OCT had reached liquid consistency the embryos were removed, placed in the petri-dish, flooded with a solution of 10% phosphate buffered saline (PBS). Embryos were then examined under a stereomicroscope (Leica MZ12 fitted with DFC 320 camera; Leica Microsystems, Heerbrugg, Switzerland) at a magnification of 1.6x, and adjusted for light and contrast such that internal structures of the embryo could be visualised, taking advantage of its translucency, to ensure that they were intact. Each embryo was dissected free from any extra-embryonic membranes and then the embryos

were flooded with approximately 2.0 mL of a 0.05% solution of Ponceau S (Sigma Aldrich, St Louis, MO, USA) in order to highlight the somites. Ponceau S is a protein stain of light red colour that can be removed by continued washing (Hayner *et al.*, 1982). It was used since it adequately provided contrast between somites and other embryonic tissue, while allowing the possibility of removal in the event that further examination of the embryos via other methods might be necessary (Appendix VI). The embryos remained in the Ponceau S solution for approximately one minute and then the excess stain was removed by flushing with PBS. Embryos were then given a score based on developmental markers (Table 5.1, Figure 5.1-3) and somites were counted.

A total of 22 (twin) embryos (day 19 n=8: S=4, C=4 and day 21 n=12: S=4, C=10) were examined and used in this study. Singleton S embryos (n= 6) were obtained across collection days day 19 and day 21, but no C singleton embryos. Therefore, all singleton embryos were removed from the analysis. Two embryos from the day 19 group were also removed from the analysis as they were degenerating and therefore non-viable.

5.3.4 Statistical analysis of embryo developmental stages and somite count

All statistical procedures were performed using SAS (Version 9.4, SAS Institute INC., Cary, NC, USA). Day 19 and day 21 embryo somite counts were subjected to analysis of variance (ANOVA) generalised model to determine the effect of ewe breed on somite count at each time point. Non parametric, Wilcoxon-Mann-Whitney analysis was conducted to detect breed differences on developmental rank. PROC COR function was used to calculate Spearman's correlation coefficient (R) and associated p values for somite number and embryo length in day 19 and day 21 embryos.

Table 5.1 Characteristics of embryos used to assign a developmental score.

Developmental score	Characteristics
1	Elongated body First and second brachial arches present
2	Caudal one third of body torsed heart bulge more prominent otic placode visible
3	"C"-shaped body 3 or 4 brachial arches/grooves 1st brachial arch may be dividing into maxillary and mandibular processes prominent heart and liver bulges Fore limb bud Large otic placode

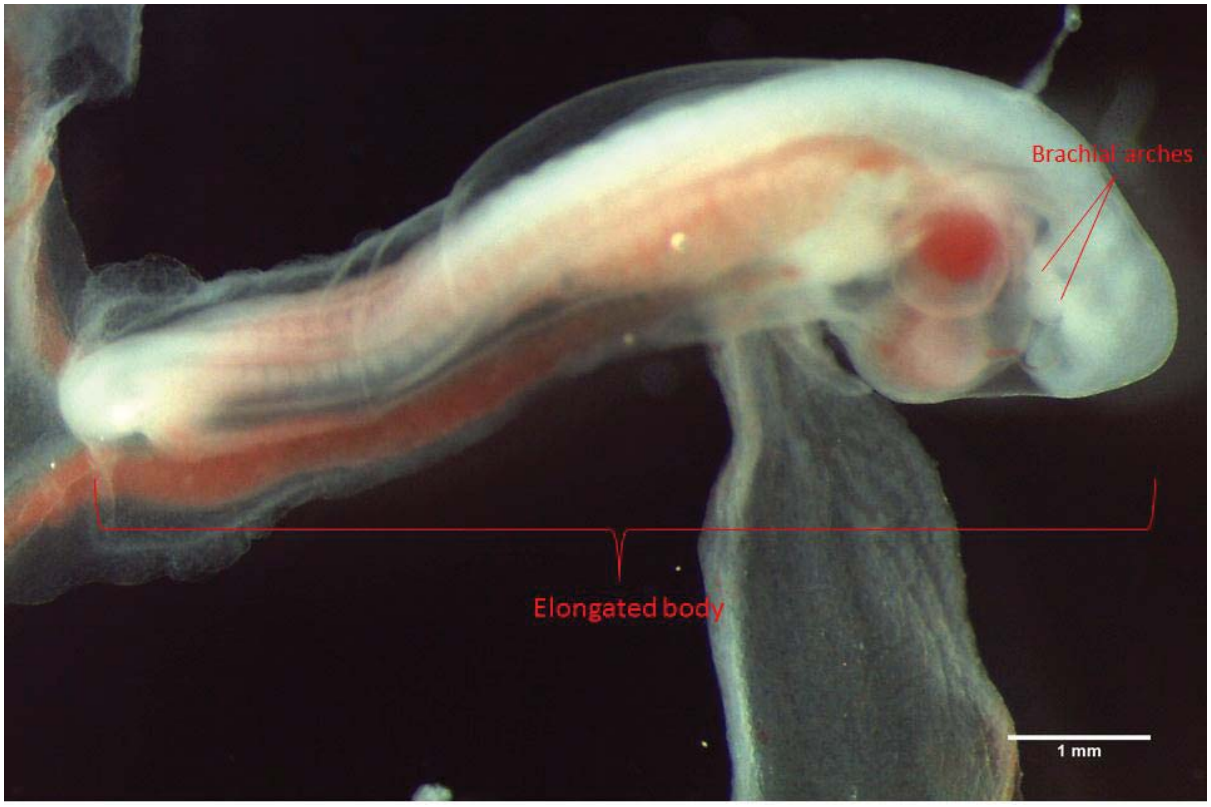

Figure 5.1 Illustration of an embryo at with a developmental score of "1".

Figure 5.2 Illustration of an embryo at with a developmental score of "2".

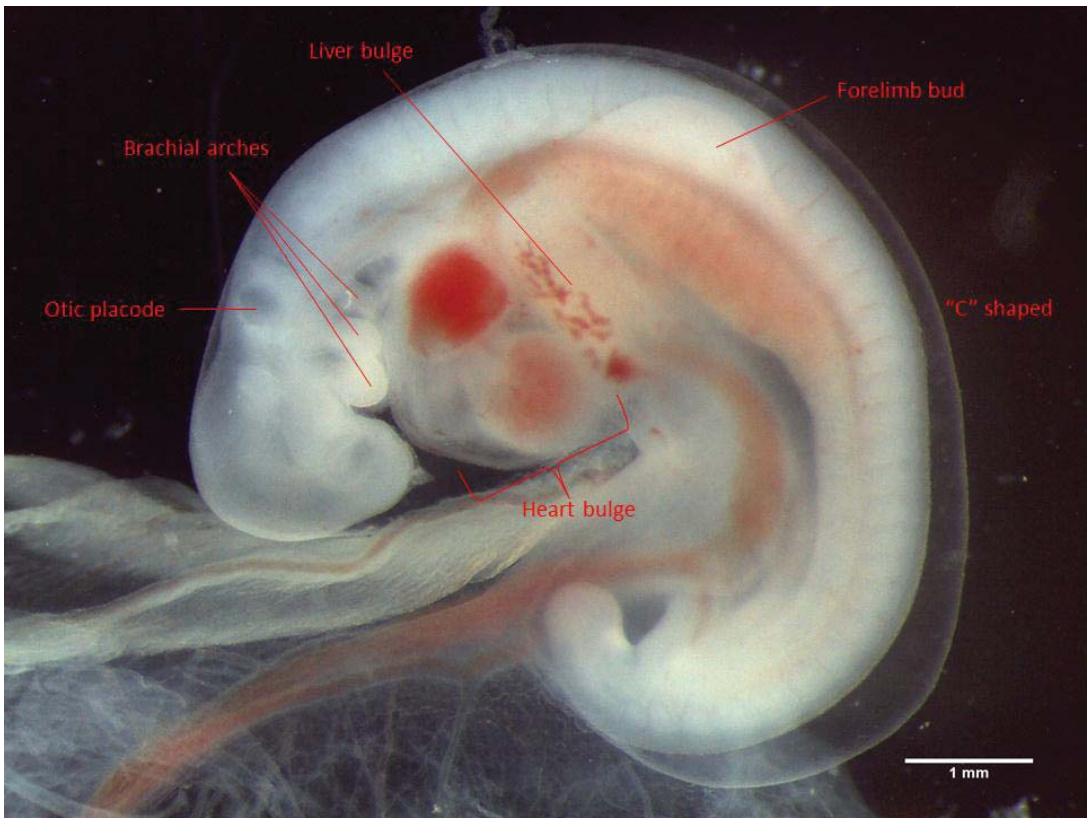

Figure 5.3 Illustration of an embryo at with a developmental score of "3".

5.4 Results

At day 19, Suffolk embryos (median 2.5) were at a later developmental stage ($p<0.05$) than the Cheviot embryos (median 1.5), ($W_s = 25$, $z = 2.13$, $r = -0.75$). No Suffolk embryos were at developmental rank "1" while no Cheviot embryos were ranked "3" at day 19. At day 19, Suffolk embryos had a higher number of somites ($p<0.05$) than Cheviot embryos (30.5 and 27.0 respectively).

Day 21 embryos did not differ in developmental rank ($p>0.05$). All embryos were ranked at "3". Somite number did not differ ($p>0.05$) between breeds at day 21. Mean somites counts were 36.25 and 37.5 for Cheviot and Suffolk embryos respectively.

Day 19 and day 21 embryo length significantly correlated with somite number, $R = 0.97$, and $R= 0.80$ respectively ($p<0.01$) (Figure 5.4).

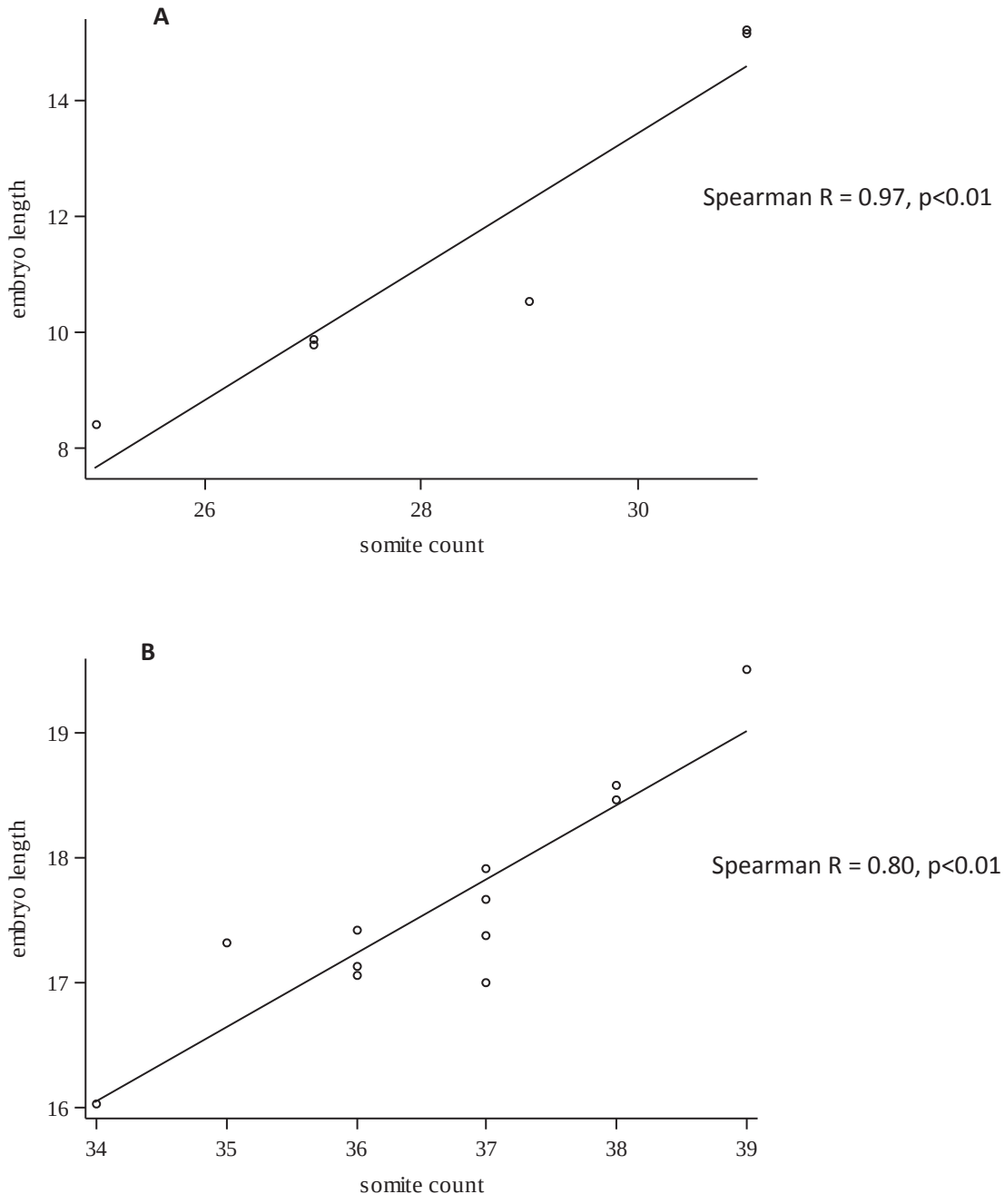

Figure 5.4 Correlation of embryo length (mm) and somite number in A: day 19 and B: day 21 embryos.

5.5 Discussion

The objectives of this study were to examine Cheviot and Suffolk embryos at day 19 and day 21, using a developmental rank system and by comparing the number of somites, to determine if the size differences reported in Chapter 4 could be considered an accurate representation of differential developmental stages of the embryos gestated in these two breeds of sheep. The present study found that Suffolk embryos at day 19 were at a later developmental stage and had more somites than Cheviot embryos, while at day 21 no differences were seen, confirming the findings of Chapter 4.

It was noted that, at day 19, in both the breeds only two of the developmental ranks were represented, whereby Suffolk embryos were only either rank 2 or 3 and Cheviot embryos were only either rank 1 or 2, indicating that the breed specific variation between embryos on a particular day was limited to only the changes that represent the difference between two ranks. Wilmut and Sales (1981) referred to variation in stage of development between embryos as a result of time of ovulation and fertilisation, in spite of synchronisation of the donor ewes in their embryo transfer experiments. It is likely that this would also be an important consideration when synchronisation is followed by AI as is the case in the present study. Similar variation between embryos recovered within hours of each other in spite of having similar times of mating was reported by Green and Winters (1945). Differences in somite numbers were also reported by these authors consistent with changes to other structural features of the embryos they examined. The exception being an embryo recovered at day 15.5 which was thought to be considerably advanced for its gestational age (Green and Winters, 1945). The main limitation of Green and Winters' (1945) study was that they only examined one embryo at each time point. Additionally, this

study examines twin embryos, and on observation of the raw data, it was found that twin embryos differed in somite number by only one or two somites, but were observed to not be any further along in respect to their developmental rank, further giving support to asynchronous fertilisation, not only between ewes but also between ova within the ewe.

Somites, future vertebrae, are special features of the embryo. The present study suggests that the development of somites is consistent with the increase in embryo length reported in Chapter 4. It was considerably difficult to count the somites, particularly in the embryos that were in ranked as developmental score 2 in spite of the benefit of staining. This was due to the torsion of the caudal third making it necessary to manipulate the embryos orientation while counting in order to visualise all of the somites. Thus, this method may not be useful for future studies. Somite numbers in the Suffolk embryos were similar to previous reports for day 19 Suffolk-cross embryos by Bryden *et al.,* (1972). These authors also reported on the relationship of length to somite number; however, their method of measuring length differed from that used in this study, so that a direct comparison of findings was not possible.

Irrespective of breed, day 21 embryos were all ranked 3 according to the characteristics listed in Table 5.1, although there were subtle differences between some of the embryos indicative of developmental variation. These differences included slightly larger limb buds, greater embryo curvature, the presence of a hind limb bud, and what appeared to be the formation of the cervical sinus.

5.6 Summary and conclusions

This study confirms that the differences in development of purebred embryos gestated in dams of differing maternal size (C and S), are evident at day 19 of gestation but are no longer apparent soon after, at day 21. Further, in the case of twin embryos there is some variation between litter-mates in number of somites but not developmental rank consistent with proposed variation between times of ovulation and fertilisation. The results of this study suggests that embryo size, as measured by curved crown rump length can be considered a reliable representation of embryo development at days 19 and 21 of gestation in sheep.

Foreword to Chapters 6 to 9

The following chapters of this thesis present further research in the Cheviot-Suffolk dam size model of maternal constraint. The decision was made focus on this dam size model on the basis of a lack of clear results in the single-twin model of maternal constraint, the promising preliminary results described in Chapters 4 and 5, the success of the model to date (Jenkinson *et al.*, 2012a; Sharma *et al.*, 2012a; Sharma *et al.*, 2013), and the opportunity to examine new aspects of embryo growth, within this model, based on evidence from previous work by the research team at the International Sheep Research Centre. The methodologies of these Chapters present morphometric measurements of embryos and RT-qPCR differential gene expression analysis of maternal uterine tissue in order to determine the effects of progesterone administered to ewes in early pregnancy on embryo growth.

6 Effect of administration of exogenous progesterone on embryo size and maternal plasma hormone concentrations in a dam size model of maternal constraint

6.1 Abstract

Maternal constraint is considered a major factor that limits fetal size at term. Maternal uterine environmental effects may be initiated very early in pregnancy when rapid cell division and embryo development takes place. Progesterone (P4) is known to be crucial to embryo growth acting via its receptor to regulate uterine function and histotroph production. The objective of this study was to examine exogenous P4 effects on embryo size in early pregnancy (up to day 19) using a "dam size" sheep model in which purebred embryos were transferred between ewes of genetically dissimilar mature body size. Suffolk (S, genetically large) embryos were transferred into four recipient groups of Cheviot (C, genetically small) or Suffolk (S) ewes that either had, or had not, been pre-treated with P4 via CIDR between days 0 to 6 of pregnancy, resulting in 25 embryo transfers (ET) into P4 pre-treated S ewes (SinSP4), 24 ET into untreated S ewes (SinSnP4), 31 ET into P4 pre-treated C ewes (SinCP4) and 27 ET into untreated C ewes (SinCnP4). At day 19, S embryos from the SinCP4 ewes were larger ($p<0.05$) than those from the SinCnP4 ewes and similar in size ($p>0.05$) to SinSnP4 embryos. Circulating plasma P4 concentrations on day 3 were higher ($p<0.05$) in SinSP4 and SinCP4 recipient ewes than their untreated controls. This study suggests that P4 has the ability to overcome maternal constraint during early gestation, and the critical time may be prior to or on day 3 of gestation. This has practical implications for improving embryo growth and development.

6.2 Introduction

Embryo development during early pregnancy is a critical time for determining the future growth of the fetus and in consequence its birth weight (Walker *et al.*, 1996). The effects of the maternal uterine environment on fetal growth are initiated very early in pregnancy, a period when rapid cell division and organogenesis takes place (Dziuk, 1992; van Mourik *et al.*, 2009). It is likely that improving embryo growth in the first 20 days after conception, prior to implantation, may improve pregnancy outcomes in sheep, such as lamb birth weight, and ultimately enhance survival (Huffman *et al.*, 1985).

Maternal constraint due to differential maternal size is a major factor that is known to limit fetal growth, size at term and post natal growth (Gluckman and Hanson, 2004). The effect of maternal size on birth size and postnatal growth is well studied. Enhanced embryo/fetal development and growth, in a relatively capacious uterine environment, have been demonstrated in various animal models (Walton and Hammond, 1938; Hunter, 1956; Joubert and Hammond, 1958). In contrast, reduced fetal development and birth weight in response to a constrained maternal uterine environment has been also demonstrated (Allen *et al.*, 2002; Gardner *et al.*, 2007; Sharma *et al.*, 2012a). Differential embryo growth has been demonstrated (Sharma *et al.*, 2013) using a "dam size" model with reciprocal embryo transfer between sheep of dissimilar mature body size (Suffolk ~80kg mature weight vs Cheviot ~60kg mature weight). These differences were observed by day 19 of pregnancy, before the spatial limitations of the *in utero* environment would be expected to constrain embryo growth. Suffolk (S) embryos transferred at day 6 and gestated until day 19 in Cheviot (C) ewes were smaller (reduced length and width) than S embryos in S dams. Further, these differences were observed to extend to differences in birth weight (Sharma

et al., 2012a). Little is known about potential mechanisms that act during this early gestational period to determine fetal growth trajectory. Sharma et al. (2013) suggested that the difference was a consequence of an altered trophoblast binucleate cell composition, and uterine secretory capacity and environment. A parallel study, using the same sheep model, demonstrated that maternal uterine progesterone receptor (PGR) expression tended ($P< 0.1$) to be negatively associated with early embryo growth observed at day 19 (Sequeira *et al.*, 2012).

Progesterone (P4) is known to play a vital role in embryo growth and acts via its receptor to regulate uterine function and histotroph in early pregnancy (Spencer *et al.*, 2004a; Satterfield *et al.*, 2006; Spencer *et al.*, 2008). Enhanced embryonic/fetal growth effects in sheep have been demonstrated following P4 administration in very early pregnancy (Kleemann *et al.*, 1994; 2001; Hartwich *et al.*, 1995). In addition, exogenous P4 administered between day 1.5 and day 12 post-mating induced the expression of endometrial genes that stimulated blastocyst growth and development (Satterfield *et al.*, 2006). To date, no studies have investigated the role of P4 to overcome maternal constraint on embryonic growth and development during the pre-implantation period in the sheep “dam-size” model.

Hormones such as oestradiol, insulin like growth factor 1 (IGF1), and insulin are known to have an influence on embryo, fetal and placental development; acting either directly on the embryo or indirectly via alteration of uterine function and secretions through various receptors (Hou and Gorski, 1993; Gluckman, 1997; Matsui *et al.*, 1997; Wathes *et al.*, 1998; Meikle *et al.*, 2004). It is not known if these hormones differ in the “dam-size” model during the early stages of embryo development, although Sharma et al. (2012a) demonstrated higher plasma IGF1 concentrations on day 90 in S ewes carrying C embryos compared to C

ewes carrying C embryos; but no differences were observed on day 50, 120 or 140 of gestation.

The objectives of this study were, firstly, to confirm previous findings of maternal constraint on embryo size on day 19 in the sheep "dam-size" model when capacity of the uterus is not a limiting factor and to examine the hormone (P4, oestradiol, IGF1, insulin) profile during this period; and secondly, to examine the effects of exogenous maternal P4 on embryo size during the early embryonic period (up to day 19 of gestation). It was postulated that embryos that are transferred to ewes that have been primed with exogenous P4 (from day 0-6) will be larger on day 19 than embryos transferred to ewes whose uteri were not primed with exogenous P4 prior to embryo transfer.

6.3 Materials and Methods

All experimental procedures were carried out with the approval of the Massey University Animal Ethics Committee.

6.3.1 Experimental animals and design

This study was undertaken using ewes of dissimilar mature body size S and C as established in previous studies (Sharma *et al.*, 2013). Experimental pregnancy groups were established by transferring single purebred S (large) embryos into randomly allocated S (large) and C (small) ewe recipient groups which had, or had not, been treated with exogenous P4 between days 0 and 6, such that four experimental groups were established,

(SinCP4[1]: S embryos in C ewes that had been treated with exogenous progesterone day 0-6 of pregnancy, SinSP4: S embryos in S ewes that had been treated with exogenous P4 day 0-6 of pregnancy, SinCnP4: S embryos in untreated C ewes and SinSnP4: S embryos in untreated S ewes).

Twenty one, 4 year old S ewes were used as donors to transfer embryos to 43 S and 51 C recipients. Recipient ewes were mixed age (3 and 5 years old) and parity (2-4), and average body condition score of 2.5 (Jefferies, (1961); units 1-5: 1= emaciated, 5= obese). Recipients were balanced between treatment groups for age and body condition score. Recipients of each breed were randomly divided into two groups: one group receiving exogenous progesterone from day 0 to 6 and the other not. Recipient groups therefore consisted of 25 S ewes with exogenous P4 (SP4), 31 C ewes with exogenous P4 (CP4); 24 S ewes no exogenous P4 (SnP4) and 27 C with no exogenous P4 (CnP4). Single embryos were transferred into the recipients such that a total of 94 transfers were performed. Conceptuses were recovered on day 19.

6.3.2 Donor protocol: Oestrus synchronisation, superovulation, artificial insemination-day 0 and embryo recovery-day 6

Oestrus in the S donors was synchronised by placement of intravaginal progesterone releasing devices (Eazi-breed CIDR; Pfizer; Auckland, New Zealand) for 13 days (Wheaton *et al.*, 1993). Superovulation of donor ewes was achieved by administration of porcine FSH (Follotropin-V; Bioniche Animal Health; Ontario, Canada) in tapering doses (52, 30, 26, 20 mg) over four days starting 60 hours before CIDR removal. At the time of CIDR removal,

[1] Nomenclature "Sin" is used to refer to embryos within the uterine environment they are gestated in i.e. breed of dam and administration of P4. Where it is left out it refers solely to the recipient ewes or their respective tissues.

each donor was injected with 200 IU of serum gonadotropin (Pregnacol; Bioniche Animal Health; NSW, Australia).

Three S rams were electro-ejaculated to collect semen. Each of the donor ewes were laparoscopically inseminated with 0.5 mL of semen randomly selected from one of the three rams, at approximately 36 hours after CIDR removal.

Embryos were recovered from the donors via laparotomy on day 6 after artificial insemination (AI = day 0). General anaesthesia was induced in the donors using intravenous Thiopentone sodium (Bomathal, Bomac Laboratories Ltd; Auckland, New Zealand) at a dose rate of 5.0 mg/kg bodyweight; followed by maintenance on 2% halothane in oxygen via endotracheal tube. Donor ewes were placed in dorsal recumbency within a cradle. The abdomen was clipped and surgically prepared and a small incision was made in the caudal abdomen. The uterus was exteriorised, a Foley catheter was placed in the caudal portion of the uterine horn via a stab incision and a flushing catheter placed into each utero-tubal junction.

Embryos were flushed from each horn using 50.0 mL of commercially prepared flushing media (Complete Ultra, Emcare Flush; ICP Bio Ltd; Henderson, Auckland, New Zealand) and collected in a petri dish. The uterus was replaced into the abdomen and the abdominal incision sutured. Ewes received post-operative procaine penicillin (Duplocillin LA; Intervet Ltd; Wellington, New Zealand; 10.5 mg/kg body weight intramuscularly) and flunixin meglumine (Flunixin Injection; Norbrook NZ Ltd; Auckland, New Zealand; 2.2 mg/kg body weight intramuscularly) as prophylactic antimicrobial and analgesia respectively.

Flushed embryos were immediately examined under light microscopy (x25 magnification). They were categorised morphologically (early morulae, late morulae, early blastocyst, late blastocyst) and for viability (fertilised, appropriate transferable stage for age, structurally sound) prior to transfer to ewes recipient (Stringfellow and Givens, 2013).

To improve the chances of obtaining the required number of pregnancies for each of the six groups following ET, three successive synchronisations, superovulations, and AI and ET sessions were performed over three consecutive days for each of the protocols thus dividing the experimental flock into three cohorts (mixture of all four groups) by day. This allowed the better management of the large number of recipient ewes and multiple protocols required for the experiment.

6.3.3 Recipient Protocol: Oestrus synchronisation, P4 treatment application, embryo transfer- day 6

Oestrus synchronisation in recipients was undertaken using the same protocol as for donors. On day 0, the day on which both donors and recipients were deemed to be oestrus, recipient groups were randomly divided into halves; one half received a new intravaginal CIDR, whilst the other half were left untreated. Thus four recipient ewe groups were established: SP4 (n=25), CP4 (n=31) SnP4 (n=24) and CnP4 (n=27). CIDRs were left in for six days and removed at the time of ET.

On day 6, immediately following embryo collection from donors, single viable embryos were transferred to each recipient via laparotomy. Embryos were only transferred into recipient ewes that had at least one active corpus luteum (CL). Recipients were deemed unsuitable if there was no sign of an active ovulation site, if a corpus albicans was observed

on an ovary, or had lost their treatment CIDR. Recipient ewes were sedated with acepromazine (Acezine 10, Ethical agents Ltd; Auckland, New Zealand; 0.1 mg/kg bodyweight intramuscularly) and restrained in a cradle in dorsal recumbency for laparoscopy. Ovaries were first examined for the presence of viable corpora lutea (CLs), after which an embryo was introduced by stab puncture into the uterine horn, ipsilateral to the ovary with the CL. A total of 94 transfers were undertaken resulting in the establishment of the following groups: SinSP4 (n=22), SinCP4 (n=25), SinSnP4 (n=21) and SinCnP4 (n=26).

The recipient ewes in all the treatment groups were maintained together under farm conditions. Crayon-harnessed vasectomised rams were introduced to recipient ewes on day 15 post AI to detect pregnancy status. All ewes that did not return to oestrus by day 17 post AI were considered to be pregnant and were selected for euthanasia and harvest of embryo and reproductive tissue on day 19.

6.3.4 Embryo Harvest- day 19

On day 19 of gestation, the recipient ewes that were identified as pregnant (SinSP4 (n=5); SinSnP4 (n=15); SinCP4 (n=7); SinCnP4 (n=21)) were euthanised via free bullet stunning and exsanguination. The uterus was immediately removed and placed on ice. Excess connective tissue was dissected from the uterus. The uterus was weighed and dimensions were measured (length of left and right uterine horns, length and width of uterine body). The conceptus was then recovered by flushing the uterine horn ipsilateral to the ovary containing the CL/s with 20 mL of flushing media into the petri dish. This was repeated on the other uterine horn to recover residual trophoblast tissue. Left and right ovaries were weighed and CLs were dissected out and weighed.

The embryos were immediately examined under a stereomicroscope and photographic images of the whole embryo were captured (Leica MZ12 fitted with DFC 320 camera; Leica Microsystems, Heerbrugg, Switzerland) at a magnification of 1.6x, and adjusted for light and contrast such that internal structures of the embryo could be visualised, taking advantage of its translucency at the day 19 stage of development. The software was calibrated using a 1 mm scale. Subsequently, embryo length, width, and heart bulge were measured using ImageJ (U.S. National Institutes of Health, Bethesda, Maryland, USA). Embryo length (EL) was defined as the distance from the medial aspect of the head to the tip of the embryonic tail, following the outer curvature of the embryo. Embryo width (EW) was defined as the distance between the two widest points of the embryo with the line passing just below the heart bulge, including somites. Heart bulge width (HB) was defined as the distance between the two widest points of the heart bulge with the line passing through the midsection of the heart bulge, excluding the somites (Wales and Cuneo, 1989).

Immediately following image capture, embryos were dissected from their extra embryonic membranes, snap frozen in liquid nitrogen and stored in vials at -80°C.

6.3.5 Blood sampling and hormone assays

Blood samples via jugular venipuncture (10 mL sodium heparin vacutainer) were taken from a subsample of 15 ewes from each treatment group (SnP4, SP4, CnP4, CP4) on days 0, 3 and every ewe that had an ET on day 6. Blood samples were also taken from all pregnant ewes prior to euthanasia on day 19. Plasma was separated by centrifugation at 3000xg for 15 minutes. Duplicate samples were stored at -20°C for later hormone assays.

Hormone assays were performed on a subset (n=10 per treatment group) of plasma samples collected on days 0, 3, 6, and from 31 samples collected on day 19 (SP4: 5, CP4: 7, SnP4: 10 and CnP4: 9). Ovine plasma P4 concentrations were determined by electrochemiluminescence immunoassays (ECLIAs) (Elecsys 2010 immunology analyser, Roche) utilizing the Progesterone II assay kits (Roche diagnostics, Mannheim, Germany). The limit of sensitivity was 0.03 ng/mL. The inter-assay coefficient of variation (CV) was 2.7%. Plasma oestradiol concentrations were measured using ECLIA using the Estradiol III assay kit (Roche diagnostics, Indianapolis, IN, USA).The limit of sensitivity was 5 pg/mL and the inter-assay CV was 3.7%.

Insulin and IGF1 plasma concentrations were measured by specific radioimmunoassay (RIA) established and validated for maternal and fetal sheep plasma. Plasma insulin was measured as described by Oliver et al. (1993), except that ovine insulin was used as the standard (Sigma Chemical, St Louis, MO, batch #19254). The minimal detectable concentration of insulin was 0.02 ng/ml and the inter- and intra-assay CVs were 7.4% and 5.3%, respectively. Plasma IGF1 was measured using an IGFBP-blocked RIA (Blum and Breier, 1994; Vickers *et al.*, 1999). The minimal detection limit was 0.7 ng/ml and inter and intra-assay CVs were 13.0% and 9.8% respectively.

6.3.6 Statistical analysis

Pregnancy rate was analysed between ewe groups using a generalised model, following conversion of data to a binomial distribution of pregnant/not pregnant. Embryo morphometric data was subjected to analysis of variance (ANOVA) with respect to breed of recipient ewe, and treatment (administration of exogenous P4). Interactions such as embryo transfer day, recipient age, donor or sire, were included in the initial model but were

removed if found non-significant (p>0.05) and the model was refitted. Breed of recipient ewe by treatment always remained in the model for testing of the hypothesis. When no interactions were significant (p>0.05) only the main effects of breed of recipient ewe and treatment are discussed.

CL weights and uterine weights and morphometric data was subjected to ANOVA with respect to breed of recipient ewe, and administration of exogenous P4 treatment. Number of CLs and recipient ewe body weight (BW) was fitted as a covariate for CL weight and uterine weight, uterine body length and uterine width data analysis respectively. Breed of recipient ewe by treatment always remained in the model for testing of the hypothesis.

Repeated measure ANOVA with respect to breed of ewe, treatment, and time was used to analyse the data for plasma P4, oestradiol, insulin and IGF1 concentrations. Two and three way interactions (breed by time, breed by treatment, treatment by time, and breed by treatment by time) were included in the initial model, but were removed if found non-significant (p>0.05). All statistical procedures were performed using SAS (Version 9.3, SAS Institute Inc., Cary, NC, USA) mixed model procedure.

Plasma P4 concentrations were normalised by log10 transformation prior to analysis. Results are presented as back transformed data. Concentrations in more than 50% of plasma insulin samples from day 0 and day 6 were lower than the minimal detected dose (MDD). The MDD of 0.02 was substituted for all of the samples on all days that were less than the MDD in order to perform the statistical analysis.

6.4 Results

The overall pregnancy rate was 53.9%. There were no differences ($p>0.05$) in pregnancy rate due to breed or age of the recipient ewe, embryo transfer day, number of CLs nor donor (data not shown). There was an effect of treatment on pregnancy rate ($p<0.05$) with both C and S ewes that received exogenous P4 before embryo transfer having lower pregnancy rates than C and S ewes that did not receive P4 (CnP4, 80.8%; SnP4, 71.4%; CP4, 28.0%; SP4, 22.7%).

6.4.1 Embryo dimensions

There was no effect of embryo transfer day, recipient age, donor, or sire on embryo dimensions (data not shown). Day 19 S embryos that were gestated in P4 supplemented C ewes (SinCP4) were larger ($p<0.05$) in both length and width than those from C ewes that did not receive exogenous P4 (SinCnP4) (Table 6.1). SinCP4 embryos were similar in size ($p>0.05$) to embryos gestated in S ewes regardless of whether they received exogenous P4 or not (SinSnP4, SinSP4). However, SinSP4 embryos were longer ($P<0.05$) than SinSnP4 embryos. No difference ($p>0.05$) in heart bulge width was observed between any of the treatment groups.

Table 6.1 The effect of recipient ewe breed, and progesterone (P4) treatment combination on embryo morphometry in sheep

Treatment groups[1]	n	Embryo length (mm)	Embryo width (mm)	Heart bulge width (mm)
SinCnP4	17	$13.45^{a} \pm 0.452$	$2.03^{a} \pm 0.11$	1.75 ± 0.07
SinSnP4	15	$16.11^{b} \pm 0.481$	$2.47^{b} \pm 0.12$	1.85 ± 0.08
SinCP4	6	$17.50^{bc} \pm 0.762$	$2.85^{b} \pm 0.18$	1.88 ± 0.12
SinSP4	5	$18.49^{c} \pm 0.834$	$2.77^{b} \pm 0.20$	1.89 ± 0.13

[1] SinCnP4, Suffolk embryo in Cheviot ewe that did not receive P4; SinSnP4, Suffolk embryo in Suffolk ewe that did not receive P4; SinCP4 Suffolk embryo in P4 primed Cheviot ewe; SinSP4, Suffolk embryo in P4 primed Suffolk ewe.
Values are least squares means ± standard error of the mean.
Different superscripts within main effects indicate significant differences ($p<0.05$)

6.4.2 Uterine and corpus luteum weights and morphometric data

There was no difference ($p>0.05$) in uterine weight (C = 0.122% of BW; S = 0.122% of BW), uterine body length (C = 54.0mm; S = 49.3mm) or width (C = 36.7mm; S = 44.4mm) between C and S ewes, or treatment groups when body weight was fitted as a covariate. There was no difference ($p>0.05$) in length of ipsilateral or contralateral uterine horns. There was no difference ($p>0.05$) in CL weight between C and S ewes or treatment groups when CL number was fitted as a covariate (data not shown).

6.4.3 Ewe hormonal measurements

Progesterone

On day 0, before exogenous P4 was administered to the treatment recipient ewes, plasma P4 concentrations did not differ ($p>0.05$) between ewe groups (Figure 6.1). At day 3, P4 concentration in both CP4 and SP4 recipient ewes were elevated ($P<0.001$) when compared to CnP4 and SnP4 ewes. Concentrations in the treated groups were approximately 5 times higher than in the untreated groups. However on day 3,

concentrations of plasma P4 between un-supplemented groups did not differ (p>0.05); neither did the plasma P4 concentration differ between supplemented groups. There was a significant rise (p<0.05) in plasma P4 concentrations between day 0 to day 3 in both CP4 and SP4, but not in the un-supplemented ewe groups.

On day 6 plasma P4 concentrations of SP4 ewes are higher (p<0.05) than those of CnP4 ewes and SnP4 ewes without supplementation. Plasma P4 concentration in CP4 ewes were intermediate between SP4 ewes and CnP4 and SnP4 ewes. The day 6 P4 concentrations of the un-supplemented groups was a significantly higher (p<0.05) than the concentrations of these groups on day 3. There were no differences (p>0.05) in the plasma P4 concentrations between any of the recipient ewe groups on day 19 (Figure 6.2A).

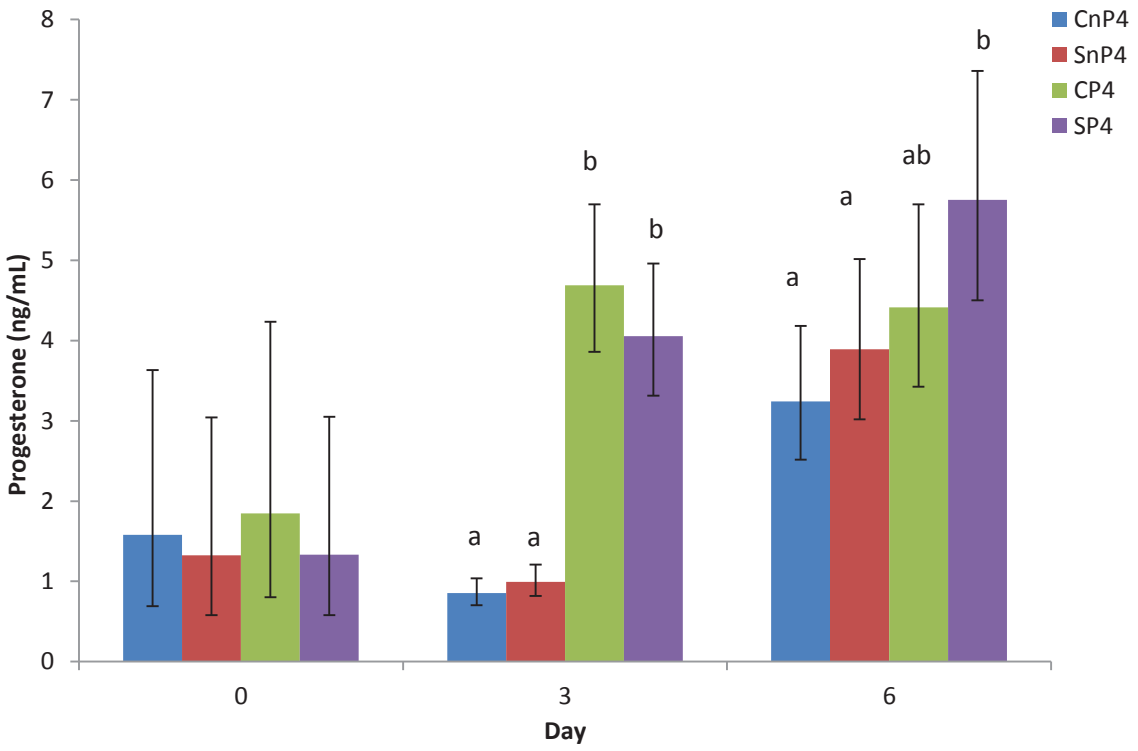

Figure 6.1 Plasma progesterone concentrations (ng/ml) in Cheviot and Suffolk recipient ewes either untreated (CnP4, n=10; SnP4, n=10) or supplemented with progesterone (CP4, n=10; SP4, n=10) via intravaginal CIDR from day 0 to day 6.

Results are expressed as back-transformed least squares means + confidence intervals.
Letters a,b indicate significant differences between groups (day 3, p<0.001; day 6 p<0.05) Data was log10 transformed for analysis.

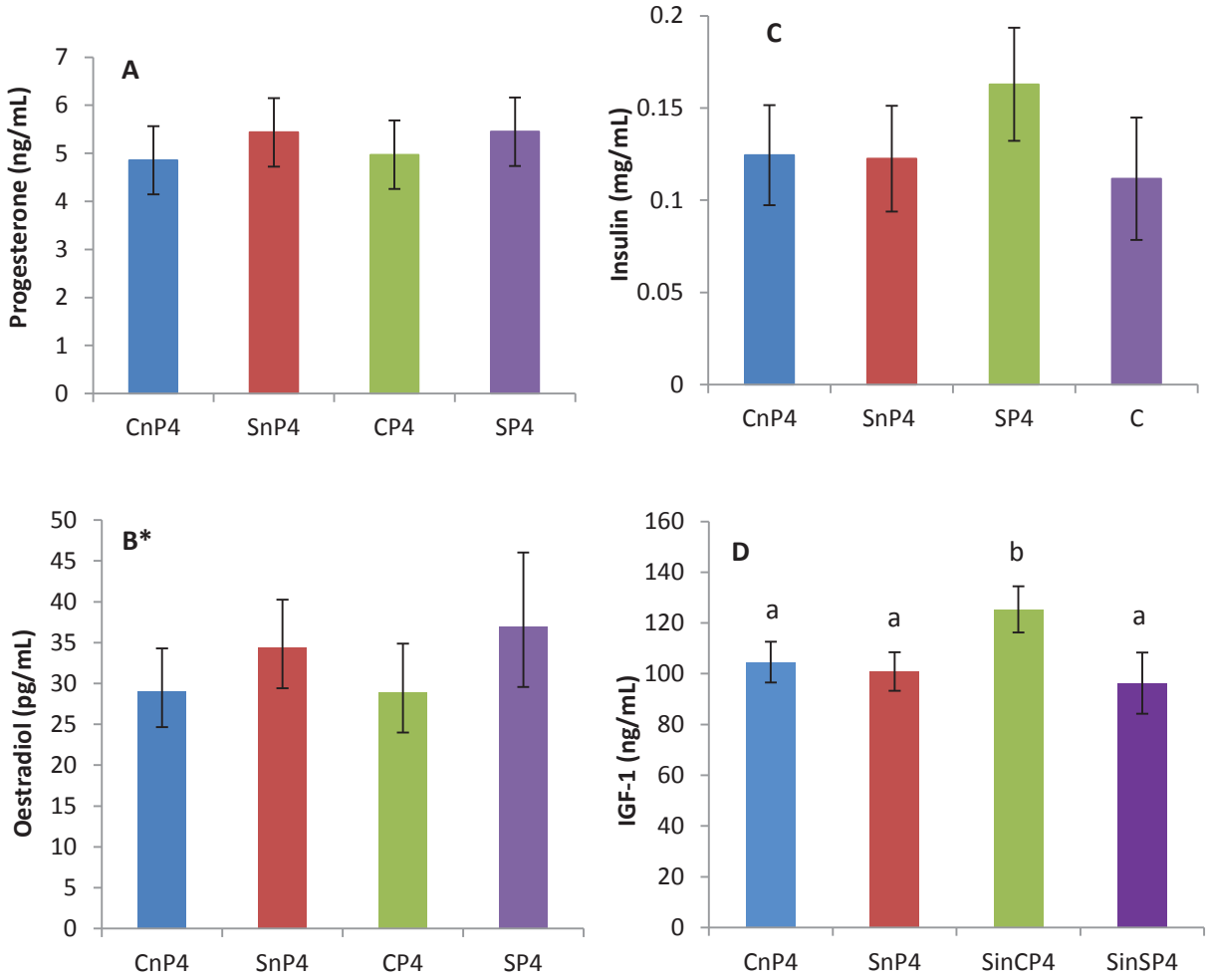

Figure 6.2 Concentrations of A: progesterone (P4 (ng/ml), B: oestradiol (pg/ml), C: insulin (ng/ml and D: IGF1 (ng/ml) in the peripheral plasma of pregnant Cheviot and Suffolk ewes treated with exogenous P4 (CP4, n=7; SP4, n=5) compared to ewes that did not receive P4 (CnP4, n=9; SnP4, n=10) at day 19.

*Values are back-transformed least squares means + confidence intervals. Data was log10 transformed for analysis. All other values are least squares means ± SEM.
Letters a, b indicates significant differences between groups.

Oestradiol

Concentrations of plasma oestradiol on day 0 and 3 did not differ ($p>0.05$) between any of the recipient ewe groups (Figure 6.3). On day 6, SP4 ewes had higher ($p<0.05$) plasma estradiol concentrations than CnP4 ewes. There were no differences between any of the other groups ($p>0.05$). Overall there was a significant decrease ($p<0.05$) in plasma oestradiol concentration in all groups between day 0 and day 3. Plasma oestradiol concentrations also

decreased (p<0.05) between day 3 and day 6 in both C groups (supplemented and non-supplemented with P4) but concentrations did not differ (p>0.05) between those days in the S groups. There were no differences (p>0.05) in the plasma oestradiol concentrations of any of the recipient ewes on day 19, (Figure 6.2B).

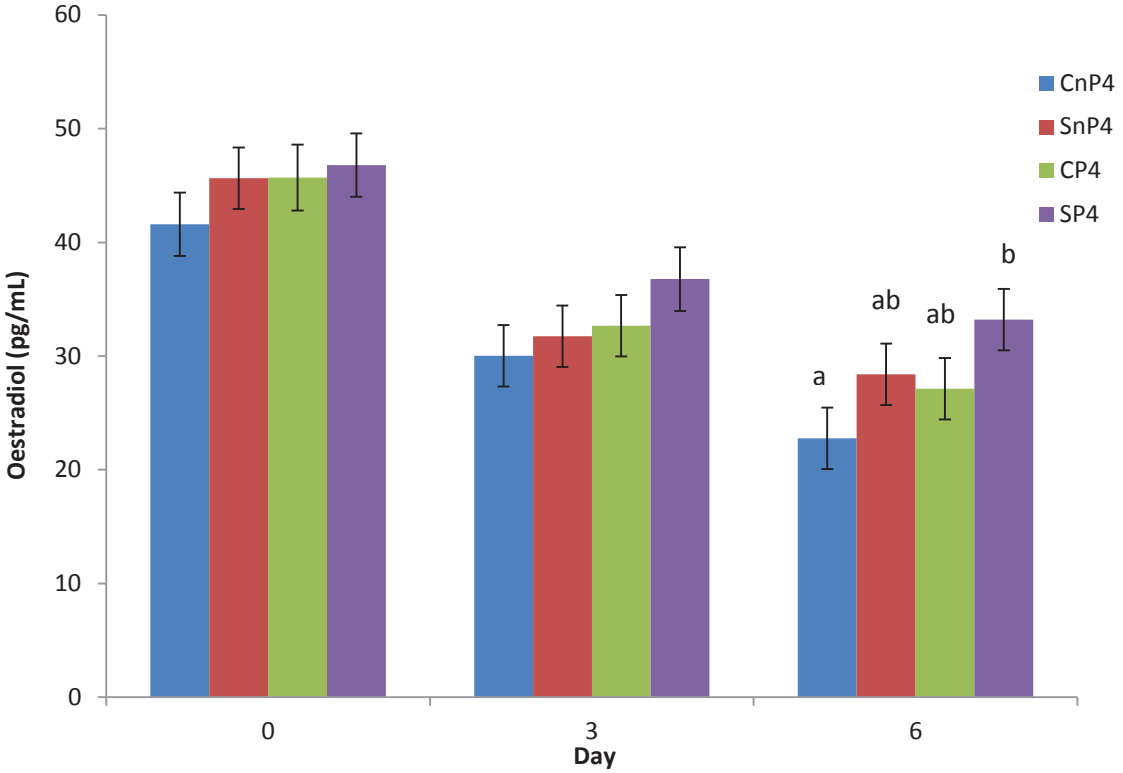

Figure 6.3 Plasma oestradiol concentrations (pg/ml) in Cheviot and Suffolk recipient ewes either untreated (CnP4, n=10; SnP4, n=10) or supplemented with progesterone (CP4, n=10; SP4, n=10) via intravaginal CIDR from day 0 to 6.

Values are least squares means ± SEM.

a b indicates significant differences between groups.

Insulin

Plasma insulin levels did not differ (P>0.05) between breed, between treatment or between day (Table 2), and all interactions were non-significant (p>0.05). There were no significant differences (p>0.05) in the plasma insulin concentrations of any of the recipient ewes on day 19 (Figure 6.2C).

IGF1

Plasma IGF1 concentrations did not differ ($p>0.05$) between breed (Figure 4), although concentrations were lower ($p<0.05$) in ewes that received exogenous P4 (SP4, CP4) than in untreated ewes (Table 2). Concentrations were higher ($P<0.05$) on day 3 than on day 0 and on day 0 than day 6 ($P<0.05$). There were no other differences in plasma IGF1 concentrations between any recipient groups on day 19 (Figure 6.2D).

Table 6.2 Plasma insulin and IGF1 concentrations (ng/ml) in Cheviot (C, n=20) and Suffolk (S, n=20) recipient ewes, in untreated (nP4, n=20) or progesterone supplemented (P4, n=20) recipient ewes, and on days 0, 3 and 6 (n=40).

	Breed		Treatment		Day		
	C	S	nP4	P4	0	3	6
Insulin (ng/ml)	0.082±0.018	0.105±0.017	0.098±0.018	0.089±0.018	0.063±0.012	0.101±0.012	0.116±0.345
IGF1 (ng/ml)	98.60±3.18	105.24±3.20	109.80±3.20[b]	94.052±3.18[a]	102.14±5.04[b]	114.91±3.37[c]	88.72±3.48[a]

Values are least squares means ± SEM.
[a, b] indicates significant differences between groups.

6.5 Discussion

This study confirms the findings of Sharma et al. (2013) such that S embryos that were gestated in C ewes were smaller on day 19 when compared with those gestated in S ewes. However, exogenous P4 supplementation during the peri-conception period (days 0 to 6) increased S embryo size when gestated in C recipients. This is consistent with previous reports that embryo development can be mediated via exposure of the embryo to a P4-primed uterus, as demonstrated in cattle by Clemente et al. (2009), rather than direct effects of progesterone on the embryo per se (Kleemann *et al.*, 1994; 2001). Additionally, it is possible that these effects may be evidence of fetal growth trajectory programming that might result in an increase in birth weight in the treated group, as shown by Sharma et al. (2012a). Further studies in which pregnancies of treatment groups are taken to term are needed to confirm this hypothesis.

This study aligns with previous findings, that at day 19, physical uterine capacity is not a limiting factor (Sharma, 2010). Uterine weight is proportional to mature body weight. This is not surprising as other body organs were similarly proportional to body weight when compared between animals of different mature body size (Butterfield *et al.*, 1983). However, at day 19 the embryo is located only within the uterine horn and in this study absolute length of uterine horns as well as length adjusted for mature body size did not differ between the S and C ewes, as was previously seen in this model (Sharma 2010).

A possible explanation for the increased embryo growth is that the uterus was at a stage resembling that of a more advanced stage of pregnancy due to the exogenously administered P4. In this study baseline P4 levels between breeds and within treatment groups were the same and this is supported by the CL weights and numbers reported.

Administration of exogenous P4 would result in asynchrony between the uterus and the transferred embryo as was suggested by Kleeman et al. (1994). When asynchrony effects were tested using serial transfers of embryos, surviving embryos showed greater crown–rump length and fetal weight than the control fetuses although embryo survival rates were low following two transfers (Wilmut and Sales, 1981; Young *et al.*, 1995). The same phenomenon manifested in the present study as increased size at day 19 in S embryos that were gestated in P4-primed C ewes, alongside the concurrent decrease in pregnancy rates in the SinCP4 and SinSP4 recipient groups.

There is typically considerable variation in the stage of development of transferred embryos, as a result of time of ovulation and fertilisation, in spite of synchronisation, in relation to stage of advancement of the recipient uterus (Wilmut and Sales, 1981). This may account for the reduced pregnancy rates observed in the progesterone supplemented groups in the present study. Thus, Wilmut and Sales (1981, p. 183), suggested that "the way in which progesterone concentration increases apparently plays a major role in determining the stage of development of the uterus" and therefore its receptivity to the transferred embryo. Indeed, it has been shown that P4 regulates expression of various endometrial genes, such as galectin15 (*LGALS15*), secreted phosphoprotein 1 (*SPP1*) and cathepsin L (*CTSL*), that encode for nutritive protein secretion into the uterine lumen and endometrial receptivity to implantation, thus influencing the stage of development (Gray *et al.*, 2006; Satterfield *et al.*, 2006). Furthermore, Parr *et al.* (1987) reported that successful conception required a minimum plasma P4 concentration of 2.0 ng/mL, whilst concentrations higher than 4.0 - 5.0 ng/mL resulted in reduced conception rates. This aligns with the findings in this study in which P4 supplemented groups had plasma P4 levels in excess of 4.0 ng/mL on

days 3 and 6 (range 4.05 - 5.75) compared with the non-supplemented groups (range 0.85 - 3.89). Interestingly, return to service and pregnancy rates were unaffected in studies in which P4 supplementation occurred later than day 3 post fertilisation (McMillan, 1987; Kleemann *et al.*, 1994; 2001; Carter *et al.*, 2008). This suggests that the critical period for a P4 effect on advancing embryo growth may indeed be prior to or on day 3, even though the higher plasma concentrations can have deleterious effect on embryo survival.

The oestradiol concentrations reported here are consistent with previously reported hormonal profiles for early pregnancy (Carnegie and Roberston, 1978). These authors stated that concentrations were below detectable concentrations; this is in accordance with the findings in this study in which more sensitive assays are used and low oestradiol concentrations are reported. Progesterone has long been established to have anti-oestrogenic effects in early pregnancy, via anti-luteolytic action through inhibition of oestrogen receptors to ensure establishment of pregnancy (McCracken *et al.*, 1984). However, in this study, exogenous P4 administration did not affect ewe plasma oestradiol concentrations or profile during the post-ovulatory/peri-conception period. Even on day 19 plasma oestradiol concentrations were still low across all the treatment groups. This was expected as higher concentrations of oestradiol are associated with luteolysis, and loss of pregnancy (McCracken *et al.*, 1984).

It was expected that circulating concentrations of insulin and IGF1 would not differ between recipient ewe groups on days 0, 3, or 6, since P4 is not known to affect plasma concentrations of these hormones, particularly this early in pregnancy. Administration of exogenous P4 did not alter circulating insulin concentrations in the recipient ewes of this study, nor was any difference in insulin between breeds, nor any differences between the

days of sampling. Likewise, there is no difference in circulating IGF1 concentrations between S and C recipient ewes. Interestingly, recipient ewes that were supplemented with exogenous P4 had lower circulating concentrations of IGF1 than did ewes that did not receive exogenous P4. It is unlikely that this circulating plasma IGF1 is involved in directing embryo growth. The principle source of circulating IGF1 is the liver (Sjögren *et al.*, 1999). Therefore, this circulating IGF1 may not represent the uterine IGF1 environment to which the embryo is exposed and further suggests that it is more likely that locally produced IGF1 from the ovary, uterus and embryo is responsible for uterine modification and direct stimulation of early stage embryo growth (Ko *et al.*, 1991; Wathes *et al.*, 1998). Moreover, it is debatable that administration of exogenous P4 was the driver for the lower circulating IGF1 in the supplemented group, as liver IGF1 is predominantly regulated by growth hormone (Wathes *et al.*, 1998). Further, Stevenson *et al.* (1994) suggested that IGF1 is under the direct influence of oestrogen rather than P4 at the uterine level via stimulation of *IGF1* expression in the epithelium in response to increased oestradiol concentrations. Therefore, examination of uterine and/or embryonic *IGF1* mRNA and protein concentration may offer further insight into the significance of IGF1 to mediate conceptus development, particularly at a local level this early in gestation.

6.6 Summary and conclusions

This study confirmed the effects of maternal constraint on a large mature body size S embryo gestated in a small mature body size C ewe. Further, the findings indicated that administration of exogenous P4 during the pre-implantation period, days 0 to 6, enhanced the growth of embryos allowing the embryo to overcome the apparent effects of maternal

constraint up to day 19 of gestation. The findings of this study aligned with previous studies and provided clear evidence to support the hypothesis that during the early peri-conception period, the maternal uterine environment, mediated by P4, advanced embryo growth. These results suggest the need for a deeper examination of the biochemical pathways and mechanisms of progesterone's effect on embryo growth and survival. Future studies should focus on the expression of uterine genes that are involved in uterine development and histotroph secretion associated with stimulation of embryo development. Such knowledge could make a significant difference to improving embryo growth and potentially survival in sheep and other mammals.

7 Comparison of uterine gene expression in a Cheviot-Suffolk model of maternal constraint in response to exogenous administration of progesterone

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7.1 Abstract

Maternal constraint due to differential maternal size has been shown to limit embryo growth in early gestation. Little is known about the mechanisms which drive embryo growth, nor how these mechanisms differ in response to maternal constraint. It has been proposed that administration of progesterone advances the uterine endometrial structure and secretory activity which may result in asynchrony between the uterus and transferred embryo. Such treatment results in advancement of the embryo's development to regulate itself to the uterine environment. The aim of the present study was to examine differential gene expression in ovine endometrial tissue at day 19 of gestation following administration of progesterone in a dam size model of maternal constraint. On day 6, purebred Suffolk embryos were transferred into four recipient (treatment) groups of dissimilar mature body size: small mature body size breed Cheviot, and large mature body size breed Suffolk that were or were not pre-treated with progesterone for six days (day 0 to day 6) prior to embryo transfer. Embryos and endometrial tissues were recovered from all groups on day 19 (embryo size results are reported in Chapter 6). Quantitative RT-PCR was used to determine differential expression of selected uterine genes: cathepsin L (*CTSL*), insulin growth factor 1 (*IGF1)* insulin-like growth factor 1 receptor (*IGF1R),* endometrial galectin 15 (*LAGLS15*), mucin 1 (*MUC1*), progesterone receptor (*PGR*), prostaglandin-endoperoxide synthase 2 (*PTGS2*), ovine uterine serine proteinase inhibitor (*SERPIN*) and radical S-adenosyl methionine domain containing 2 (*RSAD2*) in the respective treatment groups. When combined uterine horns (ipsilateral and contralateral to the ovary containing the CL) were examined, progesterone administration resulted in up-regulation of *PGR, IGF1, MUC1,* and *PTGS2;* and down-regulation of *RSAD2* gene expression in Cheviot ewes compared to

the control group. When the uterine horns ipsilateral to the CL alone were examined, progesterone administration was observed to up-regulate gene expression in *MUC1* and *PTGS2* in Cheviot ewes; and to up-regulate *MUC1, PGR, PTGS2* while down-regulating *CTSL* and *RSAD2* in Suffolk ewes. These results suggest that progesterone administration alters expression of uterine genes that encode for secretion of histotroph and receptivity to implantation. The changes in gene expression enable Suffolk embryos gestated in Cheviot ewes to overcome the limitations of the constrained environment, and to advance to a size similar to their natural potential (when gestated in a Suffolk ewe), as reported in Chapter 6. This study increased the understanding of the mechanisms by which progesterone acts within the ovine uterine endometrium, and added to the knowledge of the maternal-conceptus dialogue, which may lead to manipulations to improve embryo growth and survival.

7.2 Introduction

Various human and animal studies have suggested that the maternal uterine environment affects embryo and fetal growth, which consequently leads to long term effects on health and production (Walton and Hammond, 1938; Cowley *et al.*, 1989; Barker, 1995; Robinson *et al.*, 2000; Godfrey and Barker, 2001; Gluckman and Hanson, 2004; Gardner *et al.*, 2007). Maternal constraint due to differential maternal size is known to limit conceptus growth (Joubert and Hammond, 1958; Allen *et al.*, 2002; Gluckman and Hanson, 2004; Sharma *et al.*, 2012b; 2013). In Chapter 6, the morphological characteristics of day 19 embryos and the hormonal profiles of their dam-ewes were investigated in a "dam-size" model of maternal constraint. The results confirmed previous day 19 findings, that in a restricted uterine environment, Suffolk (S) embryos that were gestated in Cheviot (C) ewes had impaired development when compared to control environment S embryos gestated in S ewes (Sharma *et al.*, 2013). Further, administration of exogenous progesterone (P4) to recipient C ewes (CP4) from day 0 to day 6 of pregnancy resulted in increased embryo size of the S embryos that were gestated in the restricted environment (SinCP4), such that at day 19 these embryos were similar in size to the control group (SinSnP4). This was accompanied by higher circulating plasma P4 concentrations at day 3 in those groups that received exogenous P4, compared to the groups that did not. Previous studies in cattle and sheep suggest that altered embryo development is mediated via exposure of the embryo to a P4-primed uterus, rather than a direct effect of P4 on the embryo itself (Kleemann *et al.*, 1994; 2001; Clemente *et al.*, 2009).

It has been suggested that administration of exogenous P4 causes changes in the uterus that advances the uterine stage of pregnancy, resulting in asynchrony between the uterus

and the transferred embryo (Kleemann *et al.*, 1994). In response, the embryo attempts to adapt to the advanced uterine environment by accelerating its own growth and development (Wilmut *et al.*, 1985).

During early gestation the uterus is an active metabolic organ which secretes histotroph containing various nutrients, hormones, growth factors and cytokines which are important modulators of the maternal-embryo dialogue (Ashworth, 1995). These may have a direct influence on embryo growth during the pre-implantation period. P4 has been shown to regulate uterine structure and function via its receptor (PGR) and stimulate various uterine genes responsible for uterine receptivity to embryo implantation, endometrial gland morphogenesis and histotroph secretion (Gray *et al.*, 2006; Satterfield *et al.*, 2008a; 2009 Forde *et al.*, 2009; Satterfield *et al.*, 2009). Satterfield *et al*. (2006) demonstrated differential expression of cathepsin L (*CTSL*), radical S-adenosyl methionine domain containing 2 (*RSAD2*) and galectin 15 (*LGALS15*) in the endometria of sheep which had or had not received exogenous P4 (between days 1.5 and 12). Additionally, blastocysts were morphologically advanced in the P4 treated groups at both day 9 and day 12.

Although our understanding of the mechanisms that drive embryo growth continues to progress, little is known about how these mechanisms differ in response to maternal constraint. Therefore, examination of uterine gene expression in a maternal constraint model with and without administration of exogenous P4 might help decipher if the observed differing embryo size at day 19 is a result of differential uterine environments, and if P4 administration can advance endometrium development allowing transferred embryos to overcome maternal constraint. This would further our understanding of the maternal-conceptus dialogue in early pregnancy. Therefore, the effect of exogenous P4

administration on uterine gene expression in a dam size model of maternal constraint was investigated in the study reported here.

7.3 Materials and Methods

The uterine tissue that was examined in this study was obtained from the experiment described in Chapter 6 (p.142) summarised below.

All experimental procedures were carried out with the approval of the Massey University Animal Ethics Committee (MUAEC).

7.3.1 Experimental animals and design

This study was undertaken using ewes of dissimilar mature body size S and C as established in previous studies (Sharma *et al.* 2013). Experimental pregnancy groups were established by transferring single purebred S (a 'large' breed) embryos into randomly allocated S (large) and C (a 'small' breed) ewe recipient groups which had, or had not, been treated with exogenous P4 between days 0 and 6, such that four experimental groups were established, (SinCP4: S embryos in C ewes that had been treated with exogenous progesterone day 0-6 of pregnancy, SinSP4: S embryos in S ewes that had been treated with exogenous P4 day 0-6 of pregnancy, SinCnP4: S embryos in untreated C ewes and SinSnP4: S embryos in untreated S ewes).

To establish these four groups twenty-one, 4 year old S ewes, that were laparoscopically inseminated on day 0 (day of oestrus) using semen from three S rams, were used as donors to transfer embryos to 43 S and 51 C recipients on day 6. Recipient ewes were mixed age (3 and 5 years old) and parity (2-4), and average body condition score of 2.5 (Jefferies 1961;

units 1-5: 1= emaciated, 5= obese). Recipients were balanced between treatment groups for age and body condition score. Recipients of each breed were randomly divided into two groups: one group receiving exogenous progesterone from day 0 to 6 and the other not. Recipient groups therefore consisted of 25 S ewes with exogenous P4 (SP4), 31 C ewes with exogenous P4 (CP4); 24 S ewes no exogenous P4 (SnP4) and 27 C with no exogenous P4 (CnP4). Single embryos were transferred into the recipients such that a total of 94 transfers were performed. Conceptuses were recovered on day 19, from all ewes that were identified at pregnant by non-return to estrus using a vasectomised ram (SinSP4[2] (n=5); SinSnP4 (n=15); SinCP4 (n=7); SinCnP4 (n=21)).

7.3.2 Uterine tissue collection- day 19

On day 19 of gestation, recipient ewes were euthanised and the uteri were removed. At the same time that embryos were collected, a sample of tissue from the mid-region of both uterine horns (ipsilateral and contralateral to the CL/embryo) was dissected out, snap frozen and stored at -80°C for gene expression studies. Uterine samples were excluded if the ewe had 3 CLs, or if the embryo was on the horn contralateral to the ovary containing the CL.

7.3.3 RNA preparation

Total RNA was extracted from approximately 100 mg of frozen uterine tissue sample using TRIzol® Reagent (Invitrogen™, Life Technologies, Carlsbad, CA, USA). An RNase free work environment was maintained throughout the process by using RNase free labware. The workbench was wiped with RNase *Zap*® (Ambion Biosystems, Life Technologies, Carlsbad, CA, USA), and 70% isopropanol.

[2] Nomenclature "Sin" is used to refer to embryos within the uterine environment they are gestated in i.e. breed of dam and administration of P4. Where it is left out it refers solely to the recipient ewes.

The steps for RNA extraction were as follows:

(Steps 1-5 were carried out in the fume hood on ice, all other steps were carried out on the bench top)

1. Tissue grinding: Approximately 100 mg of frozen uterine tissue was placed in a 2 mL RNase free polypropylene tube with 100 µL of cold nuclease free water (NFW) (Ambion Inc., Life Technologies, Carlsbad, CA, USA) and a stainless steel bead. The tissue was ground using a bead beater for 30 seconds. The tube was removed and placed in ice.
2. Homogenisation: This was carried out in the fume hood on ice. One mL of cold TRIzol® Reagent was added to the tube containing the entire sample of ground uterine tissue. The sample was then homogenised using the bead-beater for 20 seconds and placed on ice for 2-3 minutes.
3. Chloroform step: The homogenate was transferred to a new tube with chloroform (Univar®, Ajax Finechem, Auckland, New Zealand) at a volume of 200 µL of chloroform per 1 mL of TRIzol® Reagent. The samples were then vortexed for 5 seconds and incubated on ice for 2 -3 minutes.
4. Centrifugation: The samples were centrifuged at 16,000 g at 4°C for 15 minutes. Three distinct phases were achieved post-centrifugation: a lower dark pink phenol-chloroform phase, a light pink interphase and an upper colourless aqueous phase.
5. The aqueous phase, containing the RNA was transferred to 200 µL of chloroform and vortexed and centrifuged as in steps 3 and 4 above.
6. The aqueous phase obtained from step 5 was transferred to 500 µL cold molecular biology grade isopropanol (Fisher Bioreagents, Fisher Scientific, USA) per 1mL of

TRIzol reagent containing sample. Samples were mixed gently by inverting and incubated at -20°C for 45 minutes to allow precipitation. Samples were centrifuged at 16,000 g for 15 minutes at 4°C producing supernatant and a pellet containing RNA. The supernatant was poured off to discard.

7. Re-suspension: The RNA pellet was washed in 1 mL of sterile 75%v/v molecular grade ethanol (Emsure®, Merck Millipore, Auckland, New Zealand) by vortex (5 seconds) and followed by centrifugation (16,000 g at 4°C for 5 minutes). The resulting supernatant was poured off and the wash process was repeated.
8. Drying: The pellet was then air dried for approximately 5-10 minutes at room temperature.
9. Final re-suspension to working RNA sample: The air-dried pellet was re-suspended in approximately 15-20 µL of NFW. The samples were then incubated on ice for 30 minutes to ensure complete suspension and stored at -80°C until further processing.

Quantification

Purity and concentration of extracted RNA was determined using a NanoDrop spectrophotometer (ND-2000 spectrophotometer, Thermoscientific, USA). The NanoDrop was initialised and blanked by loading 1 µL NFW. Samples were loaded at a volume of 1.0 µL and 260/280 and 260/230 absorbance ratios were recorded. The measurement pedestal and sampling arm were wiped using Kimwipes (Kimberly-Clark® Professional, Auckland, New Zealand) between samples. Levels of approximately 2.0 for the 260/280 nm absorbance were accepted as pure RNA samples (that is free of protein contamination). Levels of approximately 1.8-2.0 for 260/230 nm were accepted at free of organic solvents (TRIzol and

isopropanol). Concentration of RNA per sample (ng/µL) was calculated from the equation A260*40 whereby an absorbance reading at 260nm (A260) of 1.0 unit is equivalent to 40 µg of RNA per mL.

DNase Treatment and quality check

Using the RNA concentrations calculated from NanoDrop, all of the samples were diluted to approximately 1.0 µg/µL using NFW. All of the RNA samples were DNase treated with amplification grade deoxyribonuclease to degrade any potential carry-over genomic DNA contamination. A 10 µL reaction mix was prepared for each sample in a separate 0.2 mL tube. The mix contained 2.5 µg of RNA, 1 µL of 10X DNase I reaction buffer, 2.0 µL of amplification grade DNase I (Invitrogen^{TM}, Life technologies, Carlsbad, CA, USA) and NFW (Ambion Inc., Life Technologies, Carlsbad, CA, USA). As per manufacturer's instructions all of the tubes were incubated at room temperature for approximately 15 minutes. EDTA (1 µL of 25 mmol/L, pH 8.0; Invitrogen^{TM}, Life technologies, Auckland New Zealand) was added to each tube just before the 15 minutes incubation was completed. The contents were then mixed gently and centrifuged at 16,000 g for 10 seconds at 4°C. The reaction tubes were then transferred to a Sensoquest labcycler (Göttingen, Germany) in which they were further incubated at 65°C for 10 minutes and then at 4°C for 1 minute.

In order to confirm the integrity of the RNA samples and to confirm that the samples were free of DNA contamination post DNase treatment, a small volume (approximately 2.0 µL) of each of the DNase treated RNA samples was run on a 1% agarose gel for quality control. Agarose gels were made with DNase/RNase free agarose tablets (0.5 g/tablet, Bioline, Meridian Life Sciences, Memphis, TN, USA) in a 0.5X solution of TBE buffer (Ultrapure 10X TBE buffer, Invitrogen^{TM}, Thermo Fisher Scientific, Auckland, New Zealand) to

which ethidium bromide (Invitrogen™, Thermo Fisher Scientific, Auckland, New Zealand, 10 mg/mL) was added at a concentration of approximately 0.03 mg/50 mL of gel solution. Approximately 1.0 µL of loading dye (6X DNA loading dye, bromophenol blue 0.03%, Thermo Scientific, Auckland, New Zealand), was added to each sample and the solution made up to 10.0 µL using NFW. The total solution was loaded into the gel (one sample per well). A DNA ladder (100bp plus Gene Ruler, Thermo Scientific, Auckland, New Zealand) was also loaded into one well of the gel, as a marker to determine the size of the bands and as a control to check the quality of the gel. Gels were run for 60 minutes at 80V and then viewed under ultraviolet light using Geldoc/UVP Multidoc-it digital imaging system (Upland, CA, USA) to check for genomic DNA contamination. The absence of a bright band at the upper third of the lane on the gel indicated that the sample was free of genomic DNA since genomic DNA typically runs much slower through the gel. The concentration of remaining sample was determined using Qubit® 2.0 Fluorometer/Qubit RNA HS Assay kit (Invitrogen, Life Technologies, Carlsbad, CA, USA). Using NFW, the samples were then diluted to 10.0 ng/µL and stored in 15.0 µL aliquots at -80°C until further use.

7.3.4 Designing of primers and probes

Ovine sequences for candidate and reference genes were first searched in NCBI (Geer *et al.*, 2009) and Ensembl genome browser (Cunningham *et al.*, 2015). Using Geneious Ver. 8.1.6 (Kearse *et al.*, 2012) the mRNA sequences from both data banks were aligned to identify the position of introns in the coding sequence. Primers were then designed either side of an intron from a selected region of 200-220 base pairs (bp). Primers between 20-25 bp each were designed, with total amplicon size ranging from 80-190 bps, to optimise efficiency. uMelt℠ (https://www.dna.utah.edu/umelt/um.php; Dwight *et al.*, 2011) was used to

predict melt curves for each of the primer pairs, and to identify the possibility of formation of primer dimers, which reduces reaction efficiency. Only primer pairs that were predicted to produce a single peak were deemed acceptable. An NCBI primer BLAST (Altschul *et al.*, 1990) was performed as an additional quality control check to ensure that the primers produced were from regions that encode for the target protein only, were not from any other homologous region, and had an optimal sequence identity for gene expression in ovine species.

Primers were synthesised by Integrated DNA technologies (IDT, IA, USA) and supplied lyophilised. They were re-suspended with NFW according to the manufacturer's instructions to 100 µM and stored at -80°C. Working solutions were made up at 10 µM for use in quantitative reverse transcriptase PCR (RT-qPCR) reactions. The primers used for this study are listed in Table 7.1.

Table 7.1 Candidate and reference gene ID, accession number, forward and reverse primer sequences, amplicon sizes (base pairs, bp) and primer efficiency tested by reverse-transcription PCR (RT-qPCR).

Gene Identifier[1]	NCBI accession	Forward Primer Sequence	Reverse Primer Sequence	Efficiency (%)	Amplicon Size (bp)
CTSL	XM_004004094.2	ACAGCCAAGGGAAACATAGCT	TCTCCTGCCTTTGAAAGCCA	103	102
IGF1	NM_001009774.3	TCAGCAGTCTTCCAACCCAA	CAAGCACAGGGCCAGATAGA	100	110
IGF1R	XM_012098367.1	GGAATGGGTCATGGACGGAG	GGGCAGAGCAATCATCAGGT	98	89
LGALS15	NM_001009238.1	CGGGAGTTGGCAGAAGGAAG	CAAACTGGCAGATGGGCTTG	106	131
MUC1	XM_012107297.1	ACAGAGGGATTTTCTGGGCC	AGCTGACTGAACTGTGCCTT	92	123
PGR	XM_012095331.1	TGGTATTTGGACTAGGATGGAGA	AAACTCCTGTGGGATCTGCC	97	149
PTGS2	NM_001009432.1	CAGAGCTCTTCCTCCTGTGC	CAGATTTGTGCCCTGGGGAT	103	142
RSAD2	XM_004005669.2	GCAGGCTGGTGAAGTTCTGT	TGCTCGTCAAAGCTGTCACA	103	148
SERPIN	NM_001009304.1	GCTTCCCAAGATTGACCCCA	TCCGTGGTTAAGGCATGCTC	100	138
RPL19	XM_004012836.2	ATGCCCGAGAAGGTAACGTG	GTCTGCCTTCAGCTTGTGGA	101	180
SF1	XM_004019657.1	GAGAGTTGGCTCGCTTGAAT	CCCCTCCACACTTGGTACAC	103	120
TBP	XM_015097549.1	AGAATAAGAGAGCCCCGCAC	GCTCCCCACCATGTTCTGAA	106	177

[1]Gene names: *CTSL* = Cathepsin L, *IGF1* = Insulin-like growth factor 1, *IGF1R* = Insulin-like growth factor 1 receptor, *LGALS15* = Endometrial galectin 15, *MUC1* = Mucin 1, *PGR* = Progesterone receptor, *PTGS2* = Prostaglandin-endoperoxide synthase 2, *RSAD2* = Radical S-adenosyl methionine domain containing 2, *SERPIN* = Ovine uterine serine proteinase inhibitor, *RPL19* = Ribosomal protein L 19, *SF1* = Splicing factor 1, *TBP* = TATA box binding protein.

Primer optimisation

RT-qPCR was used to check the optimum annealing temperature and RNA sample concentration for each primer. Using KAPA SYBR® FAST One step qRT-PCR kit (KAPA Biosystems, MA, USA), 20 µL volume reactions were made up containing 10.0 µL KAPA SYBR® FAST qPCR Master Mix (2X), 0.4 µL KAPA RT Mix (50X), 0.4 µL (0.02mM) of forward primer, 0.4 µL (0.02 mM) of reverse primer, 1.0 µL of DNase treated RNA from a randomly selected sample (either at a 10 fold or 50 fold dilution), and 7.8 µL of NFW as per the manufacturer's instructions. Reactions were run in duplicate on two randomly chosen test samples. Duplicate no template controls, in which 1.0 µL of NFW was substituted for RNA template, were included for each primer pair. The reactions were run on a Rotor Gene Q series (Qiagen, Hilden Germany) using the following protocol:

5 minutes at 42°C

5 minutes at 95°C

10 seconds at 95°C

20 seconds at annealing temp (50°, 56°, 60°C) } Repeated for 40 cycles

60°C to 90°C at 1°C increments (melt curve)

Optimal annealing temperature and dilution was identified when a single peak was obtained for the melt curves of the test samples that matched in peak temperature, and when a default threshold of 0.2 was set, quantification cycle (Ct) values for each sample were greater than 20.

Primer specificity

Using the predetermined sample dilutions and annealing temperature, RT-PCR was used to confirm the specificity of the each primer pair to generate a single PCR product. 20.0 µL volume reactions were made up using KAPA SYBR® FAST One step qRT-PCR kit as described previously. 1.0 µL of DNase treated RNA from two randomly selected samples at the determined dilution were used and a no template control, in which 1.0 µL of NFW was substituted for RNA template, was included for each primer pair. The reactions were run on Sensoquest labcycler (Göttingen, Germany) at the following protocol:

5 minutes at 42°C

5 minutes at 95°C

30 seconds at 95°C

30 seconds at annealing temp (56°, 60°C)

30 seconds at 72°C

} Repeated for 40 cycles

7 minutes at 72°C

4°C for ∞

The amplified products (10.0 µL) from the samples were run on 2% agarose gel (as per protocol) at 60V for 30 minutes and then visualised using Geldoc/UVP Multidoc-it digital imaging system (Upland, CA, USA). Primers were considered specific if a single band of expected product size (80-190 bp) was observed in cDNA sample with no other bands, and no bands were observed in the no template control. Primer pairs that did not conform to this were deemed non-specific and new primers were therefore redesigned for these genes

following the described protocol. PCR products, from the primer pairs that were determined to be specific, were held on ice to be used immediately for cloning.

Cloning/ Plasmid extraction

Cloning is the insertion of the amplified PCR product into a plasmid vector. The use of plasmid vector ensures that the primer pair is identical to the target that is being quantified. Plasmids are highly stable and accurate calculation of copy number of the standard DNA is possible in compliance with MIQE guidelines, increasing the accuracy and reproducibility of the standard curve used for determining the efficiency of RT-qPCR reactions.

The cloning reaction was performed using the Topo® TA Cloning® Kit (Invitrogen, Life Technologies, Auckland New Zealand). A 6.0 µL reaction was made up using 4.0 µL of the fresh PCR product (tested for specificity), 1.0 µL of salt solution and 1.0 µL of pCR™2.1 TOPO® vector. The reaction was mixed gently and incubated at room temperature for 5 minutes to ligate the DNA insert to the vector. The recombinant plasmids were then transformed into a vial of One Shot® TOP 10 chemically competent *Escherichia coli* cells by incubating 2.0 µL of the TOPO® cloning reaction with 50.0 µL of cells on ice for 30 minutes, and then heat shocked for 30 seconds at 42°C and immediately transferred to ice. SOC media (250.0 µL) was added to the transformed cells and incubated at 37°C while being horizontally shaken at 200 rpm for 1 hour. The suspension was then plated, at a volume of 60.0 µL/ plate, onto pre-warmed Luria-Bertani (LB) agar plates (each prepared with 100.0 µL Ampicillin (10.0 mg/mL, resuspended from Ampicillin salt, Life Technologies, Auckland New Zealand), 40.0 µL IPTG (0.1M, Life Technologies, Auckland New Zealand), and 40.0 µL X-Gal

(20.0 mg/mL, Thermoscientific, Auckland, New Zealand). The plates were incubated overnight at 37°C, and screened for blue and white colonies the following day. White colonies were the competent cells with the recombinant plasmid DNA (Figure 7.1). Three to four of the successfully transformed (white colonies) that were in close approximation to blue ones were then inoculated into separate 1.5 mL tubes containing LB broth (1 colony/ 1 mL of broth containing 5.0 mg ampicillin per 10.0 mL of LB broth). The tubes were incubated at 37°C overnight while being shaken horizontally at 200 rpm. The following day 10.0 µL of each of the LB broth cultures was transferred to 1.0 mL of Cryobroth (Ft Richard, Auckland, New Zealand) (10 mg ampicillin/20 mL of cryobroth) incubated overnight at 37°C and then stored at -80°C for future use. The remainder of the culture was used to extract plasmids.

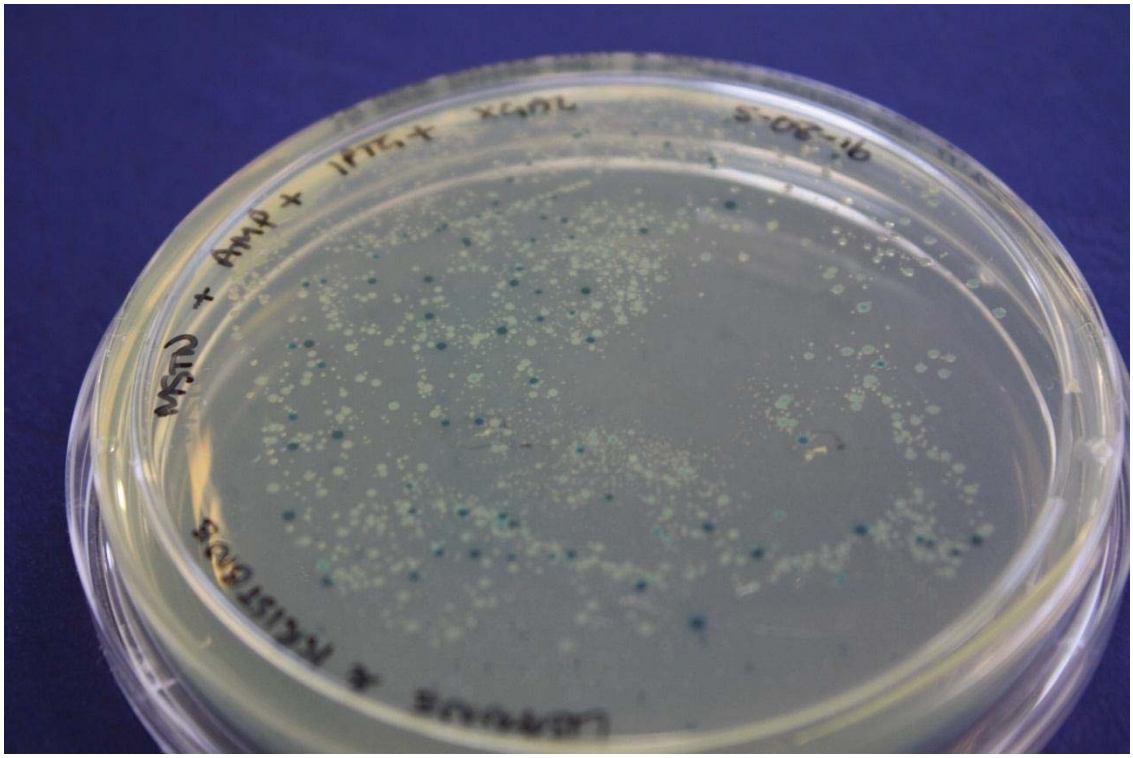

Figure 7.1 LB agar plate of *E.coli*-plasmid suspension. White colonies are the transformed competent cells with the inserted plasmid. Blue colonies are non-transformed cells.

Plasmids were extracted from the LB broth culture of the transformed cells using PureLink® Quick Plasmid DNA Miniprep Kits (Invitrogen, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions:

1. Cells were first harvested by centrifuging the remaining LB broth culture at 12,000 g for 5 minutes to produce a pellet. All of the medium was then removed.
2. The cells (pellet) were then resuspended in the resuspension buffer (250 µL) and vortexed until homogenous.
3. Lysis buffer (250 µL) was added and the tube was mixed gently by inverting and then incubated for 5 minutes at room temperature.
4. Precipitation buffer (350 µL) was added, and the tube was shaken until the content was homogenous. The lysate was then centrifuged at 12000 x g for 10 minutes.
5. The supernatant from step 4 was then loaded into a spin column mounted into a 2 mL wash tube. The column/wash tube was centrifuges at 12,000 g for 1 minute and the flow-through was discarded.
6. Wash buffer (W10) with pre-added ethanol (500 µL) was added to the column and incubated at room temperature for 1 minute. Column and wash-tube was then centrifuged at 12,000 g for 1 minute and the flow-through discarded.
7. Wash buffer (W9) with pre-added ethanol (700 µL) was added to the column and centrifuged at 12,000 g for 1 minute and the flow-through discarded.
8. The spin column was placed into a clean 1.5 mL recovery tube and 75.0 µL of the preheated elution buffer was added to the centre of the column. The column was incubated at room temperature for 1 minute.

9. Plasmid was recovered by centrifugation of the column/recovery tube at 12000 x g for 2 minute. The spin column was discarded and the plasmid was stored at -80°C for further use.

The success of the cloning was checked by running end point PCR using the extracted plasmids. A 20.0 µL PCR reaction was set up using 4 µL 5x HOT FIREpol® Blend Master-mix (Solis Biodyne, Tartu, Estonia), 0.6 µL (0.3 µM) each of forward and reverse primers that matched the gene cloned in the plasmid, 1.0 µL of plasmid template and 13.8 µL of NFW. A *no template control* was included in which 1.0 µL of NFW was substituted for plasmid template. The reaction was run using Sensoquest labcycler (Göttingen, Germany) at the following programme:

95°C for 12 minutes

95°C for 30 seconds

Annealing temp (56°, 60°C) for 30 seconds } Repeated for 40 cycles

72°C for 30 seconds

72°C for 7 minutes

4°C for ∞

The entire 20 µL of PCR product was run on 2% agarose gel (as per protocol) at 60V for 60 minutes and then visualised using Geldoc/UVP Multidoc-it digital imaging system (Upland, CA, USA). Cloning was considered successful (i.e. DNA specific to the gene cloned was inserted into the vector) if a single band of expected product size was observed with no

other bands, and no bands observed in the *no template control.* If no band was observed it was concluded that there was no insert present in the plasmid.

An M13 check PCR was then performed on a single plasmid per gene chosen from the end point PCR. Reactions were made up using 10 µL of 5x HOT FIREpol® Blend Master-mix, 1.5 µL (0.75 µM) each of M13 forward (GTA AAA CGA CGG CCA GT) and M13 reverse (CAG GAA ACA GCT ATG AC) primers, 1.0 µL of plasmid template and 36 µL of NFW. A *no template control* was included in which 1.0 µL of NFW was substituted for plasmid template. The reaction was run using Sensoquest labcycler (Göttingen, Germany) at the following programme:

95°C for 12 minutes

95°C for 30 seconds
50°C for 30 seconds
72°C for 30 seconds
} Repeated for 40 cycles

72°C for 7 minutes

4°C for ∞

The PCR product (5.0 µL) was run on a 1% agarose gel (as per protocol) and cloning was confirmed if a single band was obtained that corresponded to the length of the vector with the DNA insert (290 – 389 bp). The remainder of the M13 PCR product was stored at -80°C and used to make the dilution series for the standard curve.

Primer efficiency

Efficiency of the primers was measured by performing RT-qPCR (as per the protocol that is described in the following section) using serial dilutions of the M13 PCR product. Plasmid (DNA) concentration of the M13 PCR products was determined using Qubit®2.0 Fluorometer/ Qubit dsDNA HS Assay Kit (Invitrogen, Life Technologies, Carlsbad, CA, USA) (as per manufacturer's instructions). The concentrations were used to calculate the number of DNA molecules/µL of the M13 stock solution according to the following formula:

$$\boldsymbol{y} = \frac{x\ (g\mu L^{-1})}{(\#bp \times 660(Da)) \times (6.022 \times 10^{23})}$$

Where y is the number of molecules of DNA/µL of stock solution; *x* is the concentration of the plasmid in the M13 stock solution determined by Qubit®; #bp is the total size of the plasmid plus insert; 660 is the weight in Da per bp; 6.022 x 10^{23} is Avogadro's number, the number of molecules in one mole of substance. The calculated number of molecules was then used to generated serial dilutions (1:100000, 1:10000; 1:1000; 1:100; 1:10) to be used to produce the standard curve.

The efficiency was calculated by Rotor-Gene Q Series software 2.3.1 (Qiagen, Hilden Germany) for each primer tested. Molecules of DNA were plotted on the x axis against the Ct value calculated by the software on the y axis. A slope value of between -3.6 and -3.1 is equivalent to an efficiency between 90 and 110%. Figure 7.2 shows an example of the graphical output produced by the software. Primer efficiencies are listed in Table 7.1.

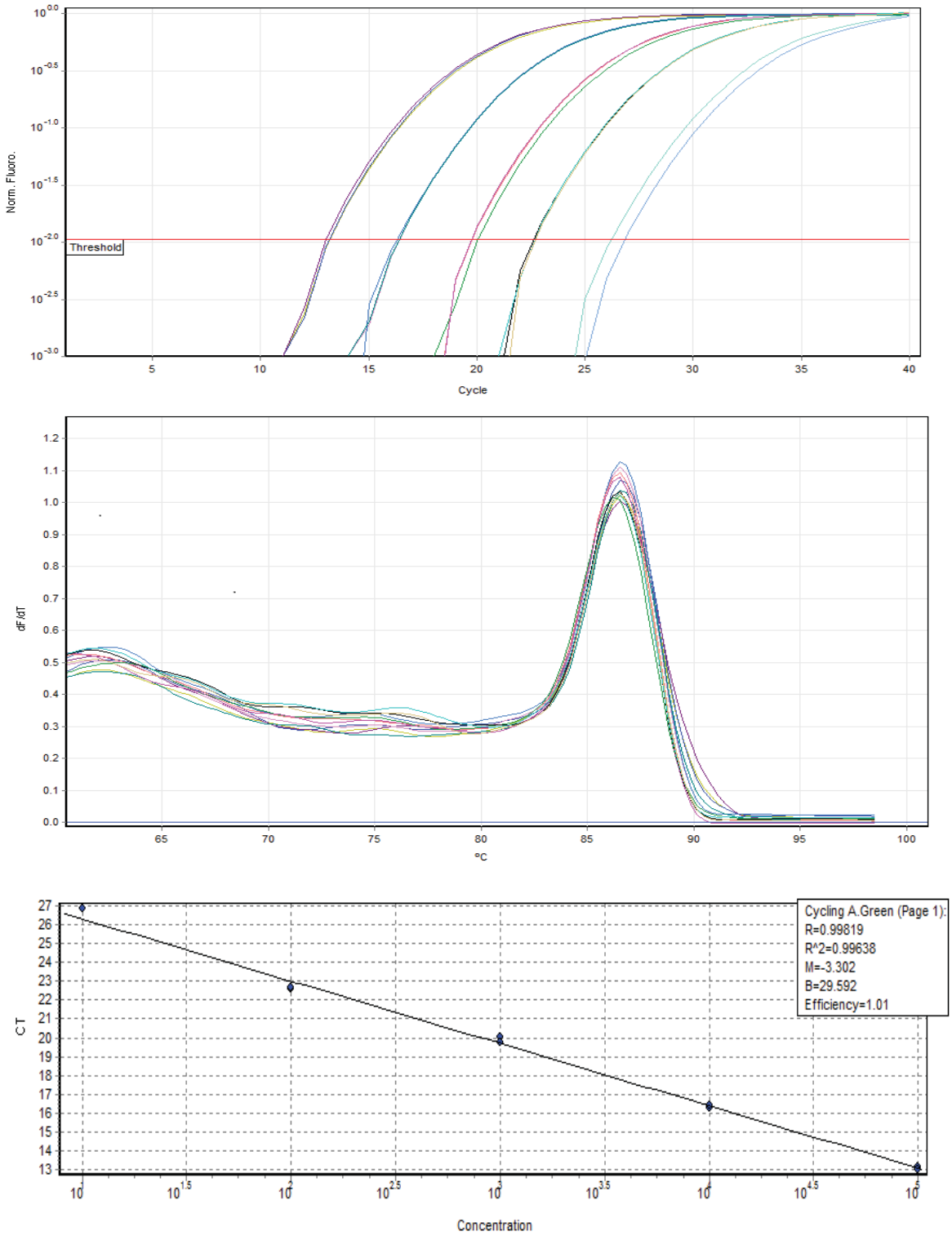

Figure 7.2 Graphical output of the reverse transcriptase quantitative PCR (Rt-qPCR) five point dilution series for measuring primer efficiency. Amplification plot over cycles (top), melt curve (middle) and standard curve (bottom) for Insulin like growth factor 1 (*IGF-1*) using 1:10. 1:100, 1:1000, 1:10000, and 1:100000 diluted plasmid. The efficiency of the standard curve is 1.01 (101%) which is an acceptable level with a strong linear relationship (R^2 = 0.996). There is only one peak in the melt curve indicating good specificity due to only one PCR product being amplified. Therefore the primer is acceptable for use in RT-qPCR to measure gene expression.

7.3.5 Quantitative PCR reactions

RT-qPCR reactions were carried out using Rotor Gene Q series (Qiagen, Hilden Germany). Each reaction contained 10 µL of 2X KAPA SYBR® FAST qPCR Master Mix, 0.4 µL of 50X KAPA RT Mix, 0.4 µL (200nM) of forward primer, 0.4 µL (200nM) of reverse primer, 1 µL of DNase treated RNA (at a concentration of 10 ng/µL), and 7.8 µL of NFW as per the manufacturer's instructions. Reactions were run in triplicate. Reactions were run in Rotor disk®100 well rings (Qiagen, Hilden, Germany). Triplicate *no template controls* and standard curve dilution series were included for each run. The following RT-qPCR protocol was used:

5 minutes at 42°C

5 minutes at 95°C

10 seconds at 95°C

20 seconds at annealing temp (56°, 60°C) } Repeated for 40 cycles

60°C to 90°C at 1°C increments (melt curve)

7.3.6 Statistical analysis of RT-qPCR data

Threshold Ct values (triplicate) for each sample was obtained from Rotor gene Q series software 2.3.1 (Qiagen, Hilden, Germany). The mRNA expression was calculated as fold change, normalised with three reference genes (RPL19, SF1 and TBP), using the following equation (Pfaffl, 2001):

$$\boldsymbol{RR} = \frac{(\boldsymbol{E}_{target})^{\Delta \mathbf{Ct}_{target}\ (\mathbf{treated\ -\ comparator})}}{(\boldsymbol{E}_{RG})^{\Delta \mathbf{Ct}_{RG}\ (\mathbf{treated\ -\ comparator})}}$$

Where RR is the relative ratio of target gene to reference gene; E_{target} is the real time PCR amplification efficiency of the target gene transcript determined from the slope of the standard curve; E_{RG} is the geometric mean of real time PCR amplification efficiency of the reference genes; $\Delta Ct_{target\ (treated\ -\ comparator)}$ and $\Delta Ct_{RG\ (treated\ -\ comparator)}$ are the Ct differences between the treatment group and the comparator (control) group for the target gene and the geometric mean of the reference genes respectively. Statistical significance of the fold change was calculated as 95% confidence intervals.

7.4 Results

Differential expression of uterine horns: ipsilateral vs contralateral

PGR, and MUC1 mRNA expression[3] was lower (0.92 [0.89-0.95], 0.87 [0.83-0.90] respectively) while *CTSL and SERPIN* mRNA expression was higher (1.16 [1.16-1.17], 1.85 [1.42-2.41] respectively) in the uterine horn that were ipsilateral to the ovary containing the CL compared to the contralateral uterine horn regardless of treatment when Suffolk and Cheviot ewes were examined together. mRNA expression did not differ between uterine horns ipsilateral and contralateral to the ovary containing the CL for any of the other genes examined. When horns that were ipsilateral to the ovary containing the CL were compared to horns that were contralateral to the ovary containing the CL for the individual breeds of sheep, mRNA expression was lower in the Suffolk breed for *CTSL* (0.92 [0.90-0.94]), *PGR* (0.78 [0.77-0.79]), *MUC1* (0.72 [0.65-0.80]), and higher for *SERPIN* (2.17 [1.34-3.54]) regardless of treatment. In the Cheviot breed *CTSL* and *SERPIN* mRNA expression was higher

[3] Expression levels are reported as a fold change + 95% confidence interval, where 1.0 = no difference in expression between comparator groups.

(1.43 [1.38-1.48]; 1.66 [1.25-2.19] respectively) in the horn ipsilateral to the CL compared to the contralateral horn.

Differential expression between treatment groups

PGR mRNA expression was increased in all treatment groups (CnP4, CP4 and SP4) when compared to the control (SnP4) for combined uterine horns (Table 7.2). CP4 ewes also had lower *PGR* mRNA expression than CnP4 ewes in combined uterine horns. When only the uterine horn ipsilateral to the CL was examined *PGR* mRNA expression did not differ between the two Cheviot groups, nor did mRNA expression for both Cheviot groups differ from the *PGR* mRNA expression of the control, SnP4. In the contralateral horn *PGR* mRNA expression was lower in CP4 treatment group compared to the CnP4 and SnP4 group (Table 7.3). *IGF1* and *SERPIN* mRNA expression was higher in all the treatment groups compared to the control for combined uterine horns (Table 7.2).

PTGS2 and *MUC1* had higher mRNA expression in the Cheviot groups with and without exogenous P4 compared to the control, however the Suffolk ewes who received exogenous P4 did not differ in mRNA expression compared to SnP4 ewes when combined uterine horns were examined (Table 7.2). In uterine horns ipsilateral to the CL both *PTGS2* and *MUC1* were observed to have higher mRNA expression in the ewes that received P4 (CP4 and SP4) compared to SnP4 and CnP4 When the contralateral horn was examined separately CP4 ewes were higher in *PTGS2* and *MUC1* expression compared to CnP4 ewes (Table 7.3).

Compared to the control group, SnP4, *CTSL* had higher mRNA expression in both Cheviot groups, but lower expression in the Suffolk group that received P4 for combined uterine

horns (Table 7.2). Only in the SP4 group was *CTSL* mRNA expression lower compared to SnP4 in the ipsilateral horn.

LGALS15 mRNA expression was lower in SP4 group compared to the SnP4 group in combined horns, while Cheviot ewes did not differ (Table 7.2). *RSAD2* mRNA expression was lower in all treatment groups compared to SnP4 when examined in the ipsilateral horn, but only lower in the two groups that received exogenous P4 (CnP4 and SnP4) when examined in the combined horns and contralateral horn (Table 7.2, 7.3). The comparison of differential expression between groups for the combined horns and horn ipsilateral to the ovary containing the CL is represented graphically in Appendix VII.

Table 7.2 Pregnancy day 19 uterine horn mRNA expression levels in pregnant Cheviot ewes that were and were not administered exogenous progesterone from day 0-6 (CP4 and CnP4) and pregnant Suffolk ewes that were administered exogenous progesterone from day 0-6 (SP4) for combined ipsilateral and contralateral to CL horns (left) and for horn ipsilateral to the CL only (right). Fold change is expressed relative to levels in control Suffolk ewes that were not administered exogenous progesterone (SnP4, n=18; n=9 for combined and horn ipsilateral to the CL respectively). Data is normalised with *RPL19*, *SF1* and *TBP*. Data is shown as fold change with 95% confidence intervals (given in parenthesis). If confidence intervals do not include 1, then mRNA expression levels are significantly different from SnP4 control (bold). Different superscripts indicate that mRNA expression levels differ between CnP4, CP4 and SP4 treatment groups (p<0.05).

	Combined horns			Horn ipsilateral to CL only		
	CnP4	CP4	SP4	CnP4	CP4	SP4
	Expressed as fold change relative to SnP4			Expressed as fold change relative to SnP4		
n	20	12	10	10	6	5
CTSL	**1.15 (1.04 - 1.28)^{b}**	**1.16 (1.01 - 1.33)^{b}**	**0.81 (0.78 - 0.84)^{a}**	0.80 (0.62 - 1.03)	1.00 (0.77 - 1.30)	**0.76 (0.57 - 0.99)**
IGF1	**1.73 (1.68 - 1.78)^{a}**	**2.25 (2.11 - 2.40)^{b}**	**1.44 (1.22 - 1.71)^{a}**	1.03 (0.89 - 1.20)	**1.55 (1.02 - 2.37)**	1.28 (1.00 - 1.64)
IGF1R	1.11 (0.89 - 1.37)	1.24 (0.95 - 1.63)	0.75 (0.50 - 1.13)	**0.66 (0.47 - 0.94)**	0.98 (0.55 - 1.73)	0.74 (0.46 - 1.18)
LGALS15	1.24 (0.89 - 1.72)	0.95 (0.58 - 1.53)	**0.63 (0.43 - 0.94)**	0.93 (0.56 - 1.56)	0.97 (0.46 - 2.07)	0.96 (0.48 - 1.92)
MUC1	**1.12 (1.12 - 1.13)^{a}**	**2.06 (1.85 - 2.29)^{b}**	1.10 (0.90 - 1.34)^{a}	**0.78 (0.72 - 0.84)^{a}**	**1.60 (1.26 - 2.03)^{c}**	**1.05 (1.01 - 1.09)^{b}**
PGR	**1.63 (1.49 - 1.77)^{b}**	**1.23 (1.22 - 1.25)^{a}**	**1.53 (1.44 - 1.62)^{b}**	1.01 (0.90 - 1.15)^{a}	1.03 (0.87 - 1.22)^{a}	**1.40 (1.39 - 1.41)^{b}**
PTGS2	**1.29 (1.27 - 1.31)^{b}**	**2.07 (2.00 - 2.14)^{c}**	1.06 (0.91 - 1.23)^{a}	**0.79 (0.73 - 0.85)^{a}**	**1.30 (1.29 - 1.31)^{b}**	**1.19 (1.06 - 1.33)^{b}**
RSAD2	1.02 (0.77 - 1.36)^{b}	**0.49 (0.31 - 0.77)^{a}**	**0.39 (0.29 - 0.52)^{a}**	**0.44 (0.27 -0.72)**	**0.29 (0.15 - 0.57)**	**0.22 (0.11 - 0.45)**
SERPIN	**2.61 (1.80 - 3.80)**	**3.04 (1.87 - 4.95)**	**1.95 (1.24 - 3.08)**	**1.63 (1.02 -2.61)**	1.19 (0.52 - 2.72)	1.77 (0.81 - 3.87)

Table 7.3 Pregnancy day 19 uterine horn mRNA expression levels in pregnant Cheviot ewes that were and were not administered exogenous progesterone from day 0-6 (CP4 and CnP4) and pregnant Suffolk ewes that were administered exogenous progesterone from day 0-6 (SP4) for uterine horns contralateral to ovary containing the CL. Fold change is expressed relative to levels in control Suffolk ewes that were not administered exogenous progesterone (SnP4, n=9). Data is normalised with *RPL19*, *SF1* and *TBP.* Data is shown as fold change with 95% confidence intervals (given in parenthesis). If confidence intervals do not include 1, then mRNA expression levels are significantly different from SnP4 control (bold). Different superscripts indicate that mRNA expression levels differ between CnP4, CP4 and SP4 treatment groups (p<0.05).

	Horn contralateral to CL only		
	CnP4	CP4	SP4
	Expressed as fold change relative to SnP4		
n	10	6	5
CTSL	**0.60 (0.54 -0.68)^{a}**	**0.56 (0.45 - 0.70)^{ab}**	0.87 (0.56 - 1.35)^{b}
IGF1	**1.05 (1.03 - 1.07)^{a}**	**1.38 (1.22 - 1.55)^{b}**	**1.62 (1.29 - 2.05)^{c}**
IGF1R	**0.67 (0.46 - 0.97)**	0.67 (1.00 - 0.45)	0.76 (0.36 -1.60)
LGALS15	**0.59 (0.43 - 0.82)^{b}**	**0.39 (0.26 - 0.59)^{a}**	**0.42 (0.28 - 0.63)^{a}**
MUC1	**0.59 (0.47 - 0.73)^{a}**	1.12 (0.83 - 1.52)^{b}	1.15 (0.64 - 2.08)^{b}
PGR	**0.94 (0.91 - 0.98)^{b}**	**0.62 (0.58 - 0.67)^{a}**	**1.66 (1.45 - 1.89)^{c}**
PTGS2	**0.76 (0.72 - 0.80)^{a}**	**1.39 (1.23 - 1.57)^{b}**	0.94 (0.64 - 1.38)^{a}
RSAD2	0.86 (0.71 - 1.05)^{c}	**0.35 (0.22 - 0.54)^{a}**	**0.67 (0.56 - 0.80)^{b}**
SERPIN	**1.51 (1.04 - 2.21)^{a}**	**3.29 (2.55 - 4.23)^{c}**	**2.15 (1.42 - 3.26)^{b}**

7.5 Discussion

The objective of this study was to examine uterine tissue from Cheviot (C) and Suffolk (S) ewes that were and were not administered exogenous P4 from day 0 to day 6 of pregnancy to determine if there were any differences in the expression of selected genes that might account for the differences in embryo size reported in chapter 6.

Findings reported in chapter 6 showed increased size in S embryos gestated in C ewes that were administered exogenous P4 from day 0-6 (SinCP4) as compared to S embryos that were gestated in C ewes which did not received exogenous P4 (SinCnP4). These differences

in size suggest an alleviation of the effects of maternal constraint when gestating a larger breed embryo in a smaller breed ewe. It was proposed that this was a consequence of an indirect effect of P4 acting via the uterus rather than P4 acting directly on the embryo (Clemente *et al.*, 2009).

In the present study CP4 ewes had down-regulated *PGR* expression compared with CnP4 ewes when combined uterine tissue (ipsilateral and contralateral to the ovary containing the CL) was examined. There was however, no differential expression of *PGR* expression between CP4 and CnP4 ewes when only the uterine tissue ipsilateral to the CL was examined. In the pregnant ewe down-regulation of *PGR* in the endometrium after day 11 and 13 of gestation is associated with recognition of pregnancy, and initiation of structural changes to the uterine endometrium necessary for implantation of the developing conceptus (Spencer and Bazer, 1995; Spencer *et al.*, 2004a). The observed differences in PGR expression in combined compared to ipsilateral horns for the CP4 and CnP4 groups suggest that exposure to increased concentrations of P4 due to exogenous administration may have caused a global or endocrine effect on uterine down-regulation of *PGR*, rather than just a localised or paracrine down-regulation limited to the ipsilateral horn due to CL derived P4 (Weems *et al.*, 1989; Lozano *et al.*, 1998). The lower *PGR* expression in the contralateral horn in the CP4 ewes compared to CnP4 supports this argument. It may be postulated that this global effect on the uterus increases the area of implantation and/or sites for attachment, which may be involved in increased embryo growth. Further studies in which CP4 and CnP4 ewes are taken to a later stage of pregnancy/birth, and placentomes/placental measurements are taken would be need to confirm this.

Interestingly, in contrast, both the combined and ipsilateral horns of SP4 ewes had up-regulated PGR expression at day 19 compared to SnP4. However, *PGR* mRNA expression in the horn ipsilateral to the CL was lower than in the contralateral horn. Thus, it is proposed that there may be an embryo effect in the case of the S embryo gestated in P4-primed S ewe. Signalling from the S embryo may act in response to the P4 advanced environment to limit uterine structural and secretory changes via *PGR* in order to signal to its environment that it is attempting to advance its development beyond its natural genetic potential. Further investigation is needed to confirm this.

Whilst *PGR* is down-regulated in response to P4, the expression of other endometrial genes that are involved in structural and secretory changes in the uterine endometrium vary in their response to P4. The IGF system has been implicated in the cellular proliferation and differentiation of the uterus and embryo before and during implantation (Letcher *et al.*, 1989; Simmen *et al.*, 1993; Stevenson *et al.*, 1994a; Reynolds *et al.*, 1997). To date, there is significant between and within species conflict concerning the stimulus that drives *IGF1* endometrial expression. In pigs (Simmen *et al.*, 1990) and mice (Kapur *et al.*, 1992) uterine *IGF1* mRNA levels are positively correlated with P4 plasma concentrations; whereas in cattle, P4 was reported to have no effect on *IGF1* expression (McCarthy *et al.*, 2012). Previous reports in sheep state that the highest *IGF1* expression was reported at estrus associated with circulating estradiol (Stevenson *et al.*, 1994a). Stevenson *et al.* (1994a) reported that while *IGF1* expression decreased when plasma P4 concentrations were high, they could not find a direct correlation to *PGR* expression or plasma P4 concentrations. Generally high expression of levels of *IGF1* in uterine tissue were reported for all species during pregnancy indicating that IGF1 plays an important role in regulation of uterine

secretions involved in driving embryo growth (Wathes *et al.*, 1998). This is supported by reports of increased IGF1 protein in uterine lumen fluid (Ko *et al.*, 1991) and presence of *IGF1R* on the preimplantation embryos (Watson *et al.*, 1992). In the present study there was no difference between P4 treated and non-treated ewes for both breeds when only the ipsilateral horn was examined. In contrast, *IGF1* was up-regulated in the P4 treatment groups compared to the non-treated and control groups in the combined horns and contralateral horn. Thus suggesting, that there may yet be a role of IGF1 in advancing embryo growth, in response to a regulatory effect of P4 on uterine *IGF1* gene expression in the ewe in alignment with the exocrine response of P4 reported earlier. Indeed, Sequeira *et al.* (2016) suggest the possibility of a P4-*PGR* driven stromal mechanism, enhancing stromal *IGF1* mRNA expression and in turn *IGF1R* expression in the luminal epithelium (LE) consistent with their proposed stimulation of embryo growth.

It is suggested that IGF1 is the principle modulator of *IGF1R* expression (Wathes *et al.*, 1998). No difference was observed in the *IGF1R* expression in the combined uterine tissue between any of the groups, nor between P4 treated and non-treated C or S ewes in the ipsilateral or contralateral horn. However, *IGF1R* is reported to have high concentration in uterine endometrial glands compared to other regions of the endometrium (Wathes *et al.*, 1998), which aligns with IGF1's proposed role in regulating uterine secretory activity to provide nutritional support to preimplantation embryos.

LGALS15 is one of the most abundant P4 induced genes in the ovine uterus, particularly involved in the biological processes of cell adhesion, growth and differentiation (Gray *et al.*, 2004; 2005). *LGALS15* is therefore thought to be crucial to the success of implantation (Gray *et al.*, 2005; Farmer *et al.*, 2008), and its protein is thought to be a major component of

histotroph, which promotes blastocyst elongation (Gray *et al.*, 2004; Lewis *et al.*, 2007). *LGALS15* mRNA has previously been shown to be up-regulated in ovine endometria LE and superficial glandular epithelium (sGE) on day 16 and 17 in response to P4 administered from day 5 to 16 (Gray *et al.*, 2004; 2006) but inhibited when a *PGR* antagonist was administered. Likewise, similar results were reported for day 9 and 12 uterine *LGALS15* mRNA expression in response to P4 administered from day 1.5 to 9 or 12 respectively (Satterfield *et al.*, 2006; 2009). Enhanced blastocyst elongation was observed in P4-treated ewes, and early induction of *LGALS15* was hypothesized to be involved in driving the observed effect (Satterfield *et al.*, 2006). Grey *et al*. (2004) reported LGALS15 protein in the uterine fluid of P4-treated ewes that was to be absorbed by conceptus trophectoderm. However, when the uterine epithelia of pregnant ewes were examined, *LGALS15* mRNA expression was shown to be lower at day 18 and 20 compared to day 14 to 16 (Gray *et al.*, 2004). In the present study, *LGALS15* mRNA expression in the horn ipsilateral to the CL did not differ between any of the ewe groups. In the combined uterine tissue the SP4 group was down regulated compared to SnP4. It appears that the effect of *LGALS15* on conceptus growth primarily occurs during day 12 to 16 of gestation stimulated by interferon tau (IFNτ) from the developing conceptus (Satterfield *et al.*, 2006) which is at its maximum on day 14 to 16 (Farin *et al.*, 1989; Roberts *et al.*, 1999). Therefore, it could be postulated that in the present study, day 19 reflects a stage of pregnancy at which *LGALS15* expression has stabilised within the uterine endometrium, having already passed through a transitory increase and exerted its effects during the critical blastocyst stage.

Another novel LE and sGE endometrial gene that is induced by P4 and stimulated by INFτ is *CTSL* (Song *et al.*, 2005). *CTSL* is a cathepsin thought to regulate uterine receptivity for

implantation and trophoblast invasion (Salamonsen, 1999). Song *et al.* (2005) report high expression levels of *CTSL* in LE and sGE at day 18 to 20 of gestation. However, in the present study there was no apparent effect of administration of P4 on *CTSL* expression in C ewes, yet *CTSL* had lower expression in the SP4 ewes compared to SnP4 ewes. It is likely that like *LGALS15*, this INFτ-stimulated gene, under the influence of exogenous P4 has already advanced through a period under which it exerts its effects on implantation, and further strengthens our suggestion of an embryo-signalled counter-response to limit the embryo to its pre-programmed genetic potential.

P4 and INFτ clearly share complex and complementary actions on gene expression within the ovine uterus in the preimplantation stage of gestation. *RSAD2* was observed to be down-regulated in all the comparator groups in the horn ipsilateral to the CL compared to the control group (SnP4); however, there was no difference between the comparator groups in their expression at day 19. Similarly, Grey *et al.* (2006) reported *RSAD2* to be a P4-decreased gene at day 16 in their study, in spite of being categorised as an INFτ-stimulated gene. It should be noted that in the contralateral horn, in the absence of the embryo *RSAD2* is down regulated in the CP4 group compared to CnP4. In other studies, *RSAD2* was shown to be up-regulated in pregnant ewes (Song *et al.*, 2007) and heifers (Forde *et al.*, 2011b) at day 17 and day 16 respectively, in response to supplemented and conceptus produced INFτ, independent of P4. *RSAD2* is suggested to play a biological role in establishing uterine receptivity to implantation and immunity (Song *et al.*, 2007) and its expression fluctuates depending on day of pregnancy and varies between endometrial tissue (Song *et al.*, 2007; Spencer *et al.*, 2008). Of note, is the reported loss of *RSAD2* mRNA in the GE between day 16 and 18 in response to the decreasing secretion of INFτ from the conceptus as trophoblast

implantation is initiated (Song *et al.*, 2007). This response may indeed be amplified by administered P4 in the present study and indicate a more advance uterus and conceptus in these P4 treatment groups. Opportunities for further investigation include understanding the regulation of INFτ stimulated genes by P4.

The expression of *PTGS2* mRNA in the uterus was consistent with observed *PGR* expression since *PTGS2* was up-regulated in the ipsilateral horns of both Cheviot and Suffolk ewes that were administered P4 compared to ewes that were not. P4 administration and loss of *PGR* in endometrial LE and sGE is associated with up-regulation of *PTGS2* (Eggleston *et al.*, 1990; Charpigny *et al.*, 1997). PTGS2, also known as cyclooxygenase 2 (COX2) is a rate-limiting enzyme for prostaglandin secretion (Kim *et al.*, 2003). Whereas luteolytic $PGF2_{\alpha}$ pulses are associated with cyclic ewes, basal PGF2α is enhanced in pregnancy (Charpigny *et al.*, 1997), and mRNA expression of *PTSG2* in the uterine LE and sGE tissues (Eggleston *et al.*, 1990; Simmons *et al.*, 2009) aligns with this secretory pattern. *PTGS2* and prostaglandins are associated with cellular adhesiveness and increased vascular permeability concordant with blastocyst implantation in mice (Charpigny *et al.*, 1997; Kennedy *et al.*, 2007). More recently, they have been shown to be critical determinants of conceptus elongation during the peri-implantation period in sheep (Dorniak *et al.*, 2011). The up-regulation in response to P4 administration reported here is therefore a positive indication of the proposed action of P4 to advance uterine secretions to enhance embryo growth during this preimplantation period.

The presence of *MUC1* on the apical surface of uterine epithelia is associated with blocking of receptors associated with adhesion necessary for the early stages of implantation (Carson *et al.*, 2000; Burghardt *et al.*, 2002). It has been suggested that down-

regulation of *MUC1* expression in the LE is associated with *PGR* loss and allows exposure of other molecules on the endometrium's apical surface, important for adhesion during this critical day 16 to 18 stage of gestation in sheep (Johnson *et al.*, 2001). In contrast, the present study shows P4 administration, although associated with down-regulation of *PGR*, resulted in up-regulation of *MUC1* in the treated ewes of each breed compared to the non-treated ewes of the same breed (ipsilateral horns). To our knowledge, this is the first study that has examined *in vivo MUC1* expression in response to exogenously administered P4 in sheep. In an *in vitro* model using endometrial cell cultures *MUC1* mRNA was shown to be up-regulated in response to P4 (Raheem *et al.*, 2016). It is therefore, possible that higher concentrations of P4 may alter or refine the expression response of this gene, in a similar manner to other species. Total uterine *MUC1* expression increases during the receptive phase of implantation in rabbits and humans (Hey *et al.*, 1994; Hoffman *et al.*, 1998). However, in rabbits, localised reduction of *MUC1* is reported to occur at individual sites of adhesion/implantation in response to signals produced by the blastocyst (Hoffman *et al.*, 1998). Further, it may be postulated that the ovine blastocyst also causes a direct reduction of *MUC1* expression at localised implantation sites (Raheem *et al.*, 2016).

Uterine LE and sGE *PGR* down-regulation, in response to a protracted period of P4 (>10 days) administration, results in abundant expression of *SERPIN* in the endometrial GE of the ovine uterus (Ing *et al.*, 1989; Spencer *et al.*, 1999b). SERPIN (serine peptidase inhibitor, also known as uterine milk protein, UTMP) is an important secretory protein associated with endometrial gland hyperplasia and blastocyst growth (Spencer *et al.*, 2004b). *SERPIN* expression in the ipsilateral horn did not differ between any of the groups examined in the present study. Stewart *et al.* (2000) describes *SERPIN* expression to be non-typical of the

classic *PGR* gene regulation, i.e. requiring a protracted exposure of the uterus to P4, in contrast to other endometrial genes that demonstrate amplified expression as early as day 13 in response to P4-induced PGR down regulation in LE and sGE. Ing *et al.* (1989) reported low levels of *SERPIN* expression following 6 days of P4 administration, with increased *SERPIN* expression as the duration of P4 administration increased to 30 days. It is possible, in the present case, that the typical day 16 onset of *SERPIN* expression was not altered in the P4 treated groups by the early administration of P4 as it was only administered for 7 days.

7.6 Summary and conclusions

The findings of the present study have advanced the understanding of P4's action on the expression of structural and secretory genes involved in histotroph secretion and receptivity to implantation. The main finding of the present study was that exogenous P4 administration from day 0 to day 6 altered uterine gene expression at the critical preimplantation stage of gestation. This is the first time that P4-induced temporal changes in uterine tissue have been examined in a maternal constraint model of differential maternal size. The present study demonstrated that when observed at day 19, Suffolk "large" breed embryos gestated in a restricted uterine environment appear to have overcome the constraint exerted by the small breed maternal environment following day 0 to 6 administration of P4. Further, the analysis demonstrated that P4 administration alters expression of endometrial genes specifically related to endometrial gland morphogenesis *(LGALS15*), contributors to histotroph (*SERPIN*), and receptivity to implantation (*CTSL*, *MUC1*, *PTGS2*). The changes reported at day 19 suggest that in sheep, as in cattle, embryo growth is driven by indirect effects of P4 on the uterus rather than P4 directly acting on the

embryo. Further, it appears that asynchrony between the uterus and the transferred embryo due to P4-advancement of the uterine structure and secretions is responsible for overcoming the effects of maternal constraint as observed in this dam size model. This work provides a foundation for future studies to further examine the role of P4 regulation of embryonic growth and development via endometrial gene expression. In future investigations, it would be prudent to repeat this design but examine specific areas of endometrial tissue, i.e. intercaruncular vs caruncular, or sGE vs LE vs stroma, rather than whole uterine tissue, and include serial collection of tissue at additional critical time points, day 6, 12, and 16, to allow a better examination of temporal and spatial changes in endometrial gene expression.

8 Effect of timing of exogenous progesterone administration on embryo size and maternal progesterone concentration and pregnancy rate in a dam size model of maternal constraint

8.1 Abstract

Progesterone is known to be crucial to embryo growth, acting via its receptor to regulate uterine structural and secretory function. Administration of progesterone in the early pregnancy, post-ovulatory, period has been shown to enhance embryo growth in sheep. In Chapter 6, this action of progesterone, administered from day 0-6, was shown to allow sufficient growth of the embryo to day 19 that it was able to overcome the limiting effects of constraint due to maternal size. However, as in previously reported studies, this accelerated embryo growth was associated with decreased embryo survival in ewes that were administered progesterone in this post-ovulatory period. The objectives of this study therefore, were to examine administration of exogenous progesterone during specific time periods between day 0 and 6, and to determine if there was a critical time point at which advancement of embryo growth could be achieved while maintaining embryo survival. Suffolk embryos were transferred into Cheviot ewes that received exogenous progesterone via CIDR on day 0 to 3, day 0 to 6, day 2 to 4 and day 3 to 6. Two additional experimental groups were established in which Suffolk embryos were transferred into Cheviot and Suffolk ewes that did not receive progesterone. At day 19, Suffolk embryos that were gestated in ewes that received progesterone were longer ($p<0.05$) than those transferred to Cheviot ewes that did not receive progesterone. Additionally, Suffolk embryos that were gestated in ewes that received progesterone from day 2 to 4 were similar in size ($p>0.05$) than those embryos gestated in ewes that received progesterone from day 0 to 3 and 0 to 6, but had higher ($p<0.05$) pregnancy rates than these groups (65.0% compared to 19.23% and 20.0%, respectively). The embryo size differences were accompanied by peak plasma progesterone concentration in the ewes in the day immediately following CIDR insertion. This study

confirms that administration of exogenous progesterone from day 0 to 6 has the ability to allow embryos to overcome maternal constraint during early gestation. Further, these results suggest that day 3 of pregnancy is a critical time in both driving embryo growth and survival to day 19. This has practical implications towards understanding early pregnancy mechanisms that may influence subsequent development, pregnancy survival outcomes and long term production.

8.2 Introduction

Progesterone (P4) is known to be associated with embryo growth via regulation of uterine structure and secretory mechanisms (Spencer *et al.*, 2004b). Administration of exogenous P4 in the post-ovulatory period has been shown to advance conceptus elongation in sheep (Kleemann *et al.*, 1994; 2001; Hartwich *et al.*, 1995) and cattle (Mann *et al.*, 2006; Clemente *et al.*, 2009). In Chapter 6, we demonstrated that administration of exogenous P4 from day 0 to day 6 of pregnancy resulted in a positive effect on embryo size, such that Suffolk (S, large breed) embryos gestated in S ewes that received P4 were longer than those gestated in S ewes that did not receive P4. Moreover, administration of exogenous P4 allowed for S embryos that were gestated in a restricted environment, Cheviot (C, small breed) ewes to overcome the limitations of this environment and restore the growth potential of these embryos (i.e. to a length similar to gestated in S ewes that did not receive P4. These findings align with previous reports of embryonic/fetal growth effects in sheep following peri-conceptional P4 exposure (Kleemann *et al.*, 1994; 2001). Further, Kleeman *et al.* (1994) showed that P4 administration to ewes prior to day 3, rather than day 3 to 6, resulted in increased fetal crown-rump length at day 74 compared to fetuses of ewes that were not exposed to P4. Similar effects of early rather than late progesterone supplementation are reported in cows (Mann *et al.*, 2006; Carter *et al.*, 2008).

In Chapter 6 it was observed that accompanying this increase in embryo length, in response to exogenous P4 administration, the pregnancy rates of the ewes that received P4 was reduced compared to the ewes that did not receive P4. Kleeman *et al.* (1994) suggested that administration of exogenous P4 induced an environment of asynchrony between the uterus and implanted embryos. This resulted in an attempt by the embryo to adapt to the

more advanced uterus by accelerating its growth to correct for the asynchrony (Wilmut and Sales, 1981; Lawson *et al.*, 1983; Young *et al.*, 1995). All of these authors reported lowered embryo survival rates following the asynchronous transfers. However, when P4 treatment was applied later than day 3, fetal growth was enhanced, without the associated decrease in embryo survival in sheep (McMillan, 1987; Kleemann *et al.*, 1994) and cattle (Carter *et al.*, 2008). In contrast, Mann *et al.* (2006) reported no difference in pregnancy rate between early and late supplementation in cattle. (Goff, 2002). It has been suggested that the timing and/or strength of increase in P4 concentration determine the stage of development of the uterus, maintaining synchrony between the embryo and endometrium, controls luteolysis (Lawson and Cahill, 1983; Goff, 2002), and is therefore critical to the development, growth and subsequent survival of the embryo (Wilmut and Sales, 1981; Mann *et al.*, 2006).

This chapter investigates the effect of administration of P4 at different times in early pregnancy on embryo size and survival in a dam size model of maternal constraint. These findings are significant to understanding the mechanisms in early pregnancy that may influence subsequent development, pregnancy survival outcomes and long term production.

8.3 Materials and Methods

The methods for this study are described in detail below, however, they were previously described in chapter 6 (p.142). Changes are limited to the experimental design in the form of inclusion of new treatment groups and number of animals per treatment group.

All experimental procedures were carried out with the approval of the Massey University Animal Ethics Committee.

8.3.1 Experimental animals and design

This study was undertaken using ewes of dissimilar mature body size Suffolk (S) and Cheviot (C) as established in previous studies (Sharma *et al.*, 2013) and Chapters 4, 5, 6, and 7. Experimental pregnancy groups were established by transferring single purebred S (large) embryos into randomly allocated C (small) ewe recipient groups which had or had not been treated with exogenous P4 for differing time periods between days 0 and 6, such that six experimental groups[4] were established, (SinCP4$^{0-3}$: S embryos in C ewes that were treated with exogenous P4 from day 0-3, SinCP4$^{0-6}$: S embryos in C ewes that were treated with exogenous P4 from day 0-6, SinCP4$^{2-4}$: S embryos in C ewes that were treated with exogenous P4 from day 2-4, SinCP4$^{3-6}$: S embryos in C ewes that were treated with exogenous P4 from day 3-6 and SinCnP4: S embryos in untreated C ewes). An additional control group was established of S embryos that were transferred to untreated S ewes (SinSnP4).

Twenty one, mixed aged (3 and 4 year old) S ewes were used as donors for the transfer of embryos into 107 C and 20 S recipients. Recipient ewes were mixed age (2 - 6 years old) and parity (2-4), and average body condition score of 3.0 (Jefferies, (1961); units 1-5: 1= emaciated, 5= obese). Recipients were balanced between treatment groups for age and body condition score. The C recipients were randomly divided into the five treatment groups. Single embryos were transferred into the recipients such that a total of 127 transfers were performed. Conceptuses were recovered on day 19.

[4] Nomenclature “Sin” refers to embryos that were gestated in the respective ewe treatment groups. In cases where “Sin” is omitted it refers to the recipient ewes and/or their tissues.

8.3.2 Donor protocol: Oestrus synchronisation, superovulation, artificial insemination-day 0 and embryo recovery-day 6

Oestrus in the S donors was synchronised by placement of intravaginal progesterone releasing devices (Eazi-breed CIDR; Pfizer; Auckland, New Zealand) for 13 days (Wheaton *et al.*, 1993). Superovulation of donor ewes was achieved by administration of porcine FSH (Follotropin-V; Bioniche Animal Health; Ontario, Canada) in tapering doses (52, 30, 26, 20 mg) over four days starting 60 hours before CIDR removal. At the time of CIDR removal, each donor was injected with 200 IU of serum gonadotropin (Pregnacol; Bioniche Animal Health; NSW, Australia).

Three S rams were electro-ejaculated to collect semen. Each of the donor ewes were laparoscopically inseminated with 0.5 mL of semen randomly selected from one of the three rams, at approximately 36 hours after CIDR removal.

Embryos were recovered from the donors via laparotomy on day 6 after artificial insemination (AI = day 0). General anaesthesia was induced in the donors using intravenous Thiopentone sodium (Bomathal, Bomac Laboratories Ltd; Auckland, New Zealand) at a dose rate of 5 mg/kg bodyweight; followed by maintenance on 2% halothane in oxygen via endotracheal tube. Donor ewes were placed in dorsal recumbency within a cradle. The abdomen was clipped and surgically prepared and a small incision was made in the caudal abdomen. The uterus was exteriorised, a Foley catheter was placed in the caudal portion of the uterine horn via a stab incision and a flushing catheter placed into each utero-tubal junction.

Embryos were flushed from each horn using 50 mL of commercially prepared flushing media (Complete Ultra, Emcare Flush; ICP Bio Ltd; Henderson, Auckland, New Zealand) and

collected in a petri dish. The uterus was replaced into the abdomen and the abdominal incision sutured. Ewes received post-operative procaine penicillin (Duplocillin LA; Intervet Ltd; Wellington, New Zealand; 10.5 mg/kg body weight intramuscularly) and flunixin meglumine (Flunixin Injection; Norbrook NZ Ltd; Auckland, New Zealand; 2.2 mg/kg body weight intramuscularly) as prophylactic antimicrobial and analgesia respectively.

Flushed embryos were immediately examined under light microscopy (x25 magnification). They were categorised morphologically (early morulae, late morulae, early blastocyst, late blastocyst) and for viability (fertilised, appropriate transferable stage for age, structurally sound) prior to transfer to ewes recipient (Stringfellow and Givens, 2013).

To improve the chances of obtaining the required number of pregnancies for each of the six groups following ET, three successive synchronisations, superovulations, and AI and ET sessions were performed over three consecutive days for each of the protocols thus dividing the experimental flock into three cohorts (mixture of all six groups) by day. This allowed the better management of the large number of recipient ewes and multiple protocols required for the experiment.

8.3.3 Recipient Protocol: Oestrus synchronisation, P4 treatment application, embryo transfer- day 6

Oestrus synchronisation in recipients was undertaken using the same protocol as for donors. On day 0, the day on which both donors and recipients were deemed to be oestrus, C recipient groups were randomly divided into five groups. Each groups received a new intravaginal CIDR according to the treatment group assigned. The groups were: $CP4^{0-3}$ (n=31), $CP4^{0-6}$ (n=31), $CP4^{2-4}$ (n=26), $CP4^{3-6}$ (n=21) and CnP4 (n=20). The S recipients (SnP4, n=27) did not receive any treatment CIDRs.

On day 6, immediately following embryo collection from donors, single viable embryos were transferred to each recipient via laparotomy. Embryos were only transferred into recipient ewes that had at least one active corpus luteum (CL) and had successfully completed P4 treatment. Recipients were deemed unsuitable if there was no sign of an active ovulation site, if a corpus albicans was observed on an ovary. Recipient ewes were sedated with acepromazine (Acezine 10, Ethical agents Ltd; Auckland, New Zealand; 0.1 mg/kg bodyweight intramuscularly) and restrained in a cradle in dorsal recumbency for laparoscopy. Embryos were introduced by stab puncture into the uterine horn, ipsilateral to the ovary with the CL. A total of 127 transfers were undertaken resulting in the establishment of the following groups: SinCP4$^{0-3}$ (n=26), SinCP4$^{0-6}$ (n=25), SinCP4$^{2-4}$ (n=20), SinCP4$^{3-6}$ (n=17), SinCnP4 (n=15), and SinSnP4 (n=19).

The recipient ewes in all the treatment groups were maintained together under commercial farming conditions. Crayon-harnessed vasectomised rams were introduced to recipient ewes on day 15 post AI to detect pregnancy status. All ewes that did not return to oestrus by day 17 post AI were considered to be pregnant and were selected for euthanasia and harvest of embryo and reproductive tissue on day 19.

8.3.4 Embryo Harvest- day 19

On day 19 of gestation, the recipient ewes (n=56) that were identified as pregnant (SinCP4$^{0-3}$ (n= 5); SinCP4$^{0-6}$ (n=4); SinCP4$^{2-4}$ (n= 13); SinCP4$^{3-6}$ (n=13); SinCnP4 (n=11); SinSnP4 (n=10)) were euthanised via free bullet stunning and exsanguination. The uterus was immediately removed and placed on ice. Excess connective tissue was dissected from the uterus. The uterus was weighed and dimensions were measured (length of left and right uterine horns, length and width of uterine body). The conceptus was then recovered by

flushing the uterine horn ipsilateral to the ovary containing the CL/s with 20 mL of flushing media into the petri dish. This was repeated on the other uterine horn to recover residual trophoblast tissue. Left and right ovaries were weighed and CLs were dissected out and weighed.

The embryos were immediately examined under a stereomicroscope and photographic images of the whole embryo were captured (Leica MZ12 fitted with DFC 320 camera; Leica Microsystems, Heerbrugg, Switzerland) at a magnification of 1.6x, and adjusted for light and contrast such that internal structures of the embryo could be visualised, taking advantage of its translucency at the day 19 stage of development. The software was calibrated using a 1 mm scale. Subsequently, embryo length, width, and heart bulge were measured using ImageJ (U.S. National Institutes of Health, Bethesda, Maryland, USA). Embryo length (EL) was defined as the distance from the medial aspect of the head to the tip of the embryonic tail, following the outer curvature of the embryo. Embryo width (EW) was defined as the distance between the two widest points of the embryo with the line passing just below the heart bulge, including somites. Heart bulge width (HB) was defined as the distance between the two widest points of the heart bulge with the line passing through the midsection of the heart bulge, excluding the somites (Wales and Cuneo 1989).

Immediately following image capture, embryos were placed in 4% paraformaldehyde, then 15% sucrose solution and then embedded in Tissue-Tek® OCT Compound (Sakura® Finetek, USA), and stored at -80°C.

8.3.5 Blood sampling and hormone assays

Blood samples via jugular venipuncture (10 mL sodium heparin vacutainer) were taken from a subsample of 15 ewes from each treatment group ($CP4^{0-3}$, $CP4^{0-6}$, $CP4^{2-4}$, $CP4^{3-6}$, CnP4, SnP4) on days 0-6 and every ewe that had an ET on day 6. Blood samples were also taken from all ewes prior to euthanasia on day 19. Plasma was separated by centrifugation at 3000xg for 15 minutes. Duplicate samples were stored at -20°C for later hormone assays.

Progesterone hormone assays were performed on a subset (n=15 per treatment group) of plasma samples collected on days 0 to 6, and from 58 samples collected on day 19 ($CP4^{0-3}$: 5, $CP4^{0-6}$: 4, $CP4^{2-4}$: 13, $CP4^{3-6}$: 13, CnP4: 11, SnP4: 11). Ovine plasma P4 concentrations were determined by electrochemiluminescence immunoassays (ECLIAs) (Elecsys 2010 immunology analyser, Roche) utilizing the Progesterone II assay kits (Roche diagnostics, Mannheim, Germany). The limit of sensitivity was 0.03 ng/ml. The inter-assay coefficient of variation (CV) was 1.83%.

8.3.6 Statistical Analysis

Pregnancy rate was analysed between ewe groups using a generalised model, following conversion of data to a binomial distribution of pregnant/not pregnant. Embryo morphometric data was subjected to analysis of variance (ANOVA) with respect to treatment group. Interactions between treatment groups and fixed effects of embryo transfer day, recipient age, donor ewe or sire were included in the initial model but were removed if found non-significant ($p>0.05$) and the model was refitted. When no interactions were significant ($p>0.05$) only the main effect of embryo treatment group is discussed.

CL weights and uterine weights and morphometric data was subjected to ANOVA with respect to treatment group. Number of CLs and recipient ewe body weight (BW) was fitted as covariates for CL weight and uterine weight, uterine body length and uterine width analysis.

Repeated measure ANOVA with respect to treatment and time was used to analyse the data for plasma P4 concentrations. All statistical procedures were performed using SAS (Version 9.3, SAS Institute Inc., Cary, NC, USA) mixed model procedure.

8.4 Results

The overall pregnancy rate was 49.6%. There were no differences ($p>0.05$) in pregnancy rate due to age of recipient ewe, embryo transfer day, number of CLs nor donor (data not shown). There was an effect of time of P4 treatment on pregnancy rate ($p<0.0001$) with Cheviot ewes that received P4 from day 0-3 and day 0-6 having lower ($p<0.05$) pregnancy rates than all the other recipient ewe groups ($CP4^{0-3}$, 19.2%; $CP4^{0-6}$, 20.0%; $CP4^{2-4}$, 65.0%; $CP4^{3-6}$, 76.5%; CnP4, 73.3%; SnP4, 72.2%)

8.4.1 Embryo morphometric measurements

There was no effect ($p>0.05$) of embryo transfer day, recipient age, donor ewe, sire, CL weight or CL number on embryo dimensions (data not shown). Day 19 $SinCP4^{0-3}$, $SinCP4^{0-6}$ $SinCP4^{2-4}$ and $SinCP4^{3-6}$ embryos were longer ($p<0.05$) than SinCnP4 (Table 8.1). There was no difference in length ($p>0.05$) of $SinCP4^{0-3}$, $SinCP4^{0-6}$ and $SinCP4^{3-6}$ embryos compared to SinSnP4 embryos, while $SinCP4^{2-4}$ were longer ($p<0.05$) than SinSnP4. Further, $SinCP4^{0-3}$ and

SinCP4$^{2-4}$ embryos were longer (p<0.05) than SinCP4$^{3-6}$ embryos, and SinCP4$^{0-6}$ embryos tended be longer (p=0.07) than SinCP4$^{3-6}$ embryos.

All SinCP4 embryo widths were greater (p<0.05) than CnP4 embryos regardless of when they were administered P4 (Table 8.1). There were no difference (p>0.05) in width of SinCP4$^{0-3}$, SinCP4$^{0-6}$, SinCP4$^{3-6}$ and SinSnP4 embryos, however SinCP4$^{2-4}$ embryos were wider (p<0.05) than SinSnP4. Further, SinCP4$^{2-4}$ embryos were wider (p<0.05) than SinCP4$^{3-6}$ embryos, but did not differ (p>0.05) in width compared to SinCP4$^{0-3}$ and SinCP4$^{0-6}$ embryos. Heart bulge width did not differ (p>0.05) between treatments groups.

Table 8.1 The effect of time of progesterone (P4) treatment on day 19 embryo morphometry in sheep

Treatment groups[1]	*n*[2]	Embryo length (mm)	Embryo width (mm)	Heart bulge width (mm)
SinCP4$^{0-3}$	5	17.26 ± 0.75^{cd}	2.72 ± 0.14^{bc}	1.84 ± 0.09
SinCP4$^{0-6}$	4	16.72 ± 0.84^{bcd}	2.67 ± 0.16^{bc}	1.81 ± 0.10
SinCP4$^{2-4}$	13	17.39 ± 0.47^{d}	2.77 ± 0.09^{c}	1.78 ± 0.06
SinCP4$^{3-6}$	13	14.91 ± 0.49^{b}	2.40 ± 0.09^{b}	1.74 ± 0.06
SinCnP4	10	13.45 ± 0.53^{a}	2.06 ± 0.10^{a}	1.56 ± 0.07
SinSnP4	8	15.37 ± 0.64^{bc}	2.39 ± 0.12^{b}	1.71 ± 0.08

[1] Treatment groups: SinCP4$^{0-3}$, Suffolk embryo in a Cheviot ewe primed with P4 from day 0-3 of gestation; SinCP4$^{0-6}$, Suffolk embryo in a Cheviot ewe primed with P4 from day 0-6 of gestation; SinCP4$^{2-4}$, Suffolk embryo in a Cheviot ewe primed with P4 from day 2-4 of gestation; SinCP4$^{3-6}$, Suffolk embryo in a Cheviot ewe primed with P4 from day 3-6 of gestation; SinCnP4, Suffolk embryo in a Cheviot ewe that did not receive exogenous P4; SinSnP4, Suffolk embryo in a Suffolk ewe that did not receive exogenous P4.
[2]1 embryo in SinCnP4 and 2 embryos in SinSnP4 group were morphometrically measured due to poor images.

8.4.2 Uterine and corpus luteum weights and morphometric data

There was no difference (p>0.05) in uterine weight, with or without ewe body weight fitted as a covariate. There was no difference (p>0.05) in uterine width or uterine body length between treatment groups when body weight was fitted as a covariate. Uterine body

width of $CP4^{0-6}$ was wider ($p<0.05$) than CnP4, SnP4, $CP4^{2-4}$ and $CP4^{3-6}$ when body weight was not fitted as a covariate (Appendix 5.1). Also, uterine body width of $CP4^{0-3}$ ewes was wider ($p<0.05$) than the uterine body width of CnP4 ewes. The uterine body length of $CP4^{0-6}$, $CP4^{2-4}$, $CP4^{3-6}$ and CnP4 ewes were all longer ($p<0.05$) than the uterine body length of SnP4 ewes (Appendix VIII).

There was no difference ($p>0.05$) in length of horns ipsilateral or contralateral to the ovary containing the CL between treatment groups. There was no difference ($p>0.05$) in CL weight between treatment groups when CL number was or was not fitted as a covariate (data not shown).

8.4.3 Ewe progesterone concentrations

There was an effect ($p<0.001$) of day, treatment and an interaction between day of pregnancy and treatment on plasma P4 concentration (Table 8.2, Figure 8.1). On day 0 before exogenous P4 was administered to the treatment ewes, plasma P4 concentrations in $CP4^{0-3}$ were higher ($p<0.05$) than all other ewe groups except for $CP4^{0-6}$ from which there was no difference ($p>0.05$) (Table 8.2, Figure 8.1). Further, on day 0, $CP4^{0-6}$ plasma P4 concentrations were higher ($p<0.05$) than $CP4^{3-6}$, CnP4 and SnP4. Plasma P4 concentrations did not differ ($p>0.05$) between $CP4^{2-4}$, $CP4^{3-6}$, CnP4 or SnP4.

Day 2 plasma P4 concentrations did not differ ($P>0.05$) between $CP4^{0-3}$ and $Cp4^{0-6}$, but differed ($p<0.05$) between these two groups and all other ewe groups. However, P4 concentrations did not differ ($p>0.05$) between the other ewe groups. On day 3, P4 concentrations between $CP4^{0-3}$, $CP4^{0-6}$, and $CP4^{2-4}$ did not differ ($p>0.05$) but concentrations differed ($p<0.05$) from all other groups. $CP4^{3-6}$ plasma concentration tended to differ

(p=0.05) from CnP4 and SnP4. Day 4 and 5 plasma P4 concentration did not differ (p>0.05) between $CP4^{0-3}$, $CP4^{2-4}$, CnP4 and SnP4. Further, $CP4^{0-6}$ and CP^{3-6} ewes had higher (p<0.05) P4 concentrations than all the other ewe groups, but did not differ (p>0.05) from each other at day 4 and 5. There was no difference (p>0.05) between any of the ewe groups at day 6.

Table 8.2 Day 0-6 plasma progesterone (P4) concentrations (ng/mL) in Cheviot ewes that did and did not receive exogenous P4 and Suffolk ewes that did not receive exogenous P4 via intravaginal CIDR for various time periods from day 0 to day 6.

		Day						
Treatment groups[1]	*n*	0	1	2	3	4	5	6
$CP4^{0-3}$	14	1.55 ± 0.14^{c}	3.55 ± 0.27^{b}	4.56 ± 0.34^{c}	4.00 ± 0.37^{c}	3.17 ± 0.44^{a}	3.98 ± 0.54^{a}	5.30 ± 0.62
$CP4^{0-6}$	12	0.87 ± 0.16^{bc}	3.05 ± 0.30^{b}	4.06 ± 0.36^{c}	4.65 ± 0.40^{c}	5.63 ± 0.47^{b}	5.56 ± 0.59^{b}	4.90 ± 0.67
$CP4^{2-4}$	15	0.55 ± 0.11^{ab}	0.69 ± 0.26^{a}	2.57 ± 0.33^{b}	4.11 ± 0.35^{c}	3.55 ± 0.46^{a}	3.63 ± 0.54^{a}	4.10 ± 0.60
$CP4^{3-6}$	14	0.43 ± 0.12^{a}	0.39 ± 0.27^{a}	0.86 ± 0.34^{a}	2.61 ± 0.41^{b}	6.22 ± 0.45^{b}	6.69 ± 0.59^{b}	5.49 ± 0.63
CnP4	12	0.42 ± 0.13^{a}	0.39 ± 0.30^{a}	0.92 ± 0.36^{a}	1.48 ± 0.40^{a}	2.46 ± 0.47^{a}	3.35 ± 0.59^{a}	5.62 ± 0.67
SnP4	13	0.41 ± 0.12^{a}	0.71 ± 0.28^{a}	0.80 ± 0.35^{a}	1.47 ± 0.38^{a}	2.28 ± 0.46^{a}	2.92 ± 0.56^{a}	4.36 ± 0.64

[1] $CP4^{0-3}$, Cheviot ewes primed with P4 from day 0-3 of gestation; $CP4^{0-6}$, Cheviot ewes primed with P4 from day 0-6 of gestation; $CP4^{2-4}$, Cheviot ewes primed with P4 from day 2-4 of gestation; $CP4^{3-6}$, Cheviot ewe primed with P4 from day 3-6 of gestation; CnP4, Cheviot ewes that did not receive exogenous P4; SnP4, Suffolk ewes that did not receive exogenous P4.

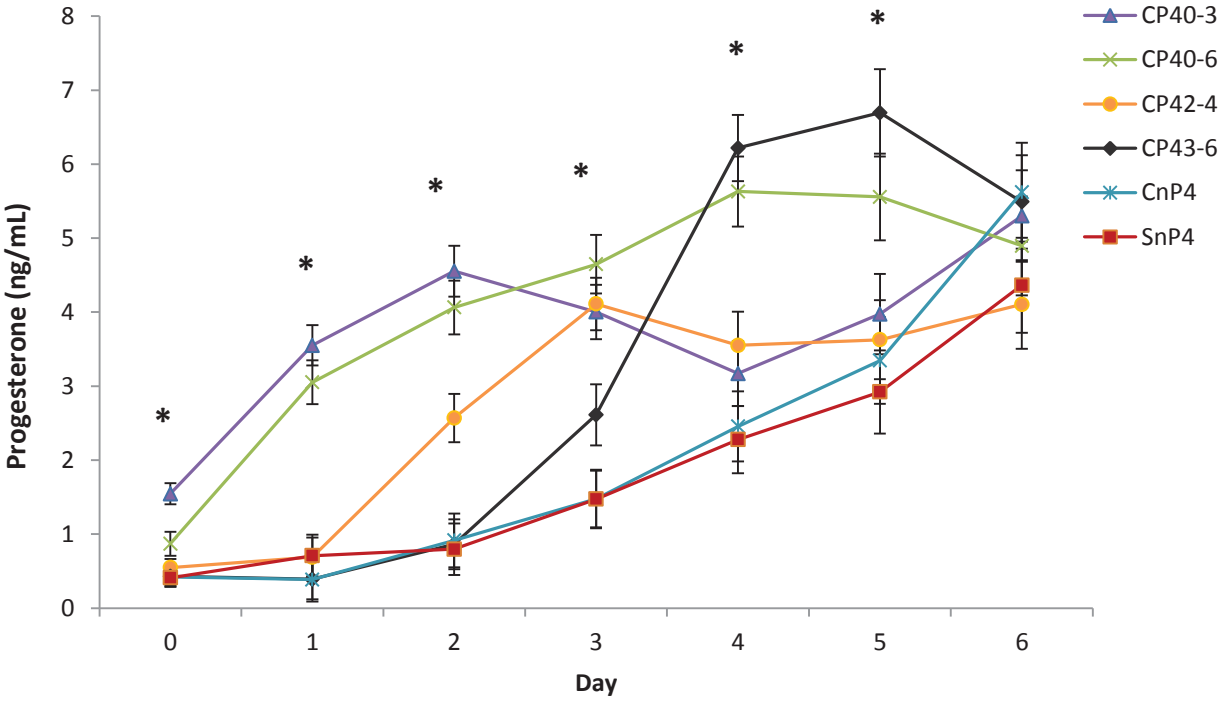

Figure 8.1 Day 0-6 plasma progesterone (P4) concentrations (ng/mL) in Cheviot ewes that did and did not receive exogenous P4 for various time periods from day 0 to day 6 (CP40-3 (n=14), CP40-6 (n=12), CP42-4 (n=15), CP43-6 (n=14) CnP4 (n=12)) and Suffolk ewes that did not receive exogenous P4 (SnP4 (n=13)) via intravaginal CIDR.

*indicates different ($p<0.05$) P4 concentration in one or all ewe groups on that day.

Progesterone concentration in $CP4^{0-3}$ and $CP4^{0-6}$ increased ($p<0.05$) from day 0-2 (Figure 8.1). There was no change ($p>0.05$) in P4 concentration in these two groups from day 2 to 3. P4 concentration in $CP4^{0-3}$ ewes decreased ($p<0.05$) from day 3 to 4 with no difference ($p>0.05$) in concentration between days thereafter. In contrast, P4 concentration continued to increase ($p<0.05$) from day 3 to 4 in $CP4^{0-6}$ ewes, with no difference ($p>0.05$) in concentration in this group between days 4 to 6.

There was no difference ($p>0.05$) in P4 concentration between day 0 and 1 in $CP4^{2-4}$ and $CP4^{3-6}$ ewes (Figure 8.1). P4 concentration then increased ($p<0.05$) in both these groups

from day 1 to 3. From day 3 to 6 plasma P4 concentration did not differ ($p>0.05$) in the $CP4^{2-4}$ ewes, however in $CP4^{3-6}$ ewes there was a further increase ($p<0.05$) in plasma concentration from day 3 to 4, with no difference ($p>0.05$) from day 4 to 6 thereafter. Plasma P4 concentration did not differ ($p>0.05$) from day 0 to 3 in the CnP4 ewes, followed by an increase ($p<0.05$) from day 3 to 4, and further increase from day 5 to 6. Likewise P4 concentration of SnP4 ewes did not differ ($p>0.05$) from day 0 to 5, with an increase ($p<0.05$) from day 5 to 6. A similar pattern in progesterone concentration from day 0 to 6 was observed when only the ewes that were pregnant at day 19 were examined although numbers per cohort were much lower than the total subset sampled (Appendix IX). There was no differences ($p>0.05$) in plasma P4 concentration between any of the groups at day 19 (Figure 8.2).

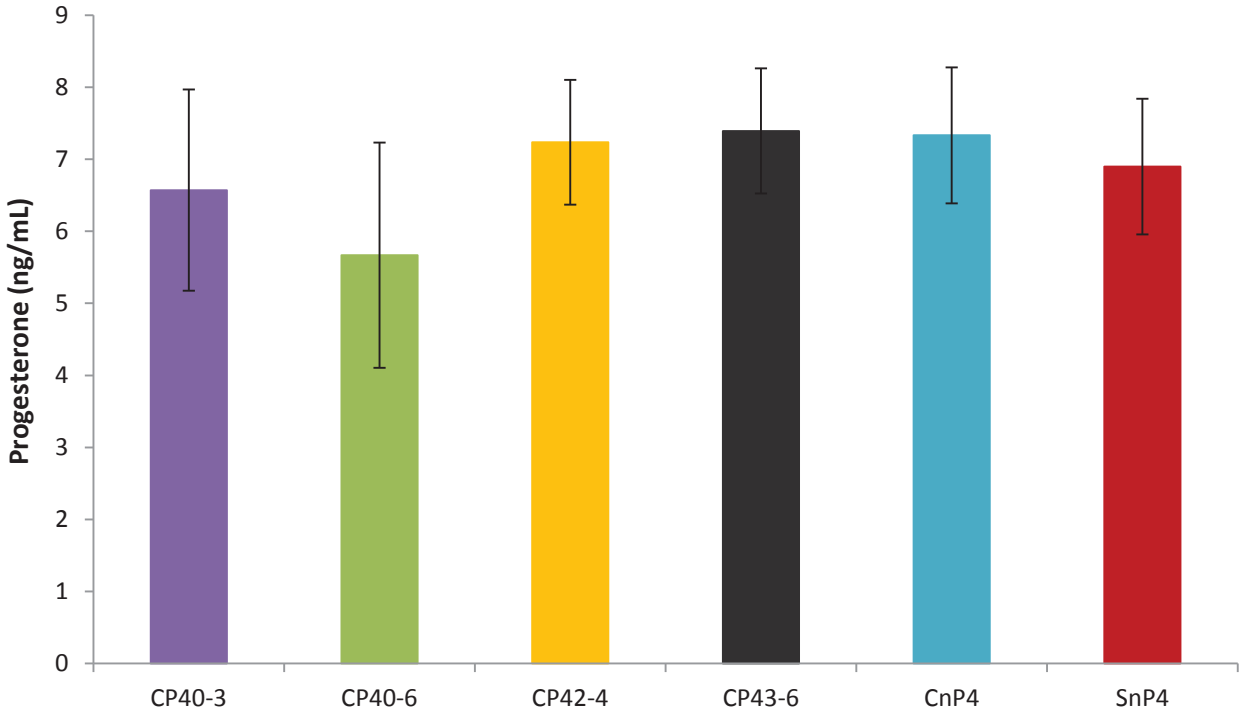

Figure 8.2 Day 19 concentrations of progesterone (ng/mL) in the peripheral plasma of Cheviot ewes that did and did not receive exogenous P4 for various time periods during day 0-6 (CP40-3, n=5; CP40-6, n=4; CP42-4, n=13; CP43-6, n=13; CnP4, n=11) and Suffolk ewes that did not receive P4 (SnP4, n=11). Values are least squares means ± SEM.

8.5 Discussion

This study was designed to examine the effects of different time of administration of P4 on embryo size and survival in a dam size model of maternal constraint. This study confirms the findings reported in Chapter 6 that exogenous P4 administration in early pregnancy increased Suffolk (S) embryo size when gestated in Cheviot (C) ewes, restoring them to a similar size as S embryos gestated in S ewe that did not receive P4. It also extends on Chapter 6 by showing that P4 administered at a range of times between day 0 and 6 can have a positive effect on embryo size. A further finding was that S embryos that were gestated in C ewes that received exogenous P4 from day 2 to 4 not only were able to overcome the apparent maternal constraint, attaining a size similar to SinCP4$^{0-3}$ and SinCP4$^{0-6}$ embryos, but also had higher pregnancy rates at day 19. SinCP4$^{3-6}$ embryos also showed enhanced growth, although less than SinCP4$^{0-3}$ and SinCP4$^{2-4}$ and was accompanied by increased pregnancy rates. This aligns with previous reports that timming of P4 administration is critical to enhancing embryo growth, while ensuring embryo survival (Kleemann *et al.*, 1994; Carter *et al.*, 2008).

In the present study P4 concentrations of CP4$^{0-3}$, CP4$^{0-6}$ were observed to peak at day 2, CP4$^{2-4}$ peaked at day 3 and CP4$^{3-6}$ at day 4. These results align with the previous suggestion that the period surrounding day 3 may be critical day for an effect of post-ovulatory P4 on timing of uterine change and therefore embryo growth (Kleemann *et al.*, 1994). It has been suggested that P4 alters embryo growth by acting indirectly through advancement of the uterus (Gray *et al.*, 2006; Satterfield *et al.*, 2006; 2008b; Clemente *et al.*, 2009; Forde *et al.*, 2009; 2011a). In Chapter 7 the ability of exogenous progesterone to alter expression of uterine genes that encode for structural and secretory function was reported to be

associated with an advancement of the uterus in this model of maternal constraint. Given that increased embryo growth was seen in all of the P4 supplemented groups, it adds weight to this hypothesis.

Exogenous administration of P4 by means of CIDR devices was succesful in increasing peripheral plasma P4 concetrations usually within the following day. Plasma P4 concentration decreased in $CP4^{0-3}$ ewes on removal of the CIDR (day 3 to 4). However, plasma P4 concentratios were the same from day 4 in $CP4^{0-3}$, $CP4^{2-4}$, CnP4 and SnP4 ewes and all treatment groups had similar P4 concentrations at day 6. The unexpected observation in the $CP4^{0-6}$ ewes may have been due to the combined effect of reduced output from the CIDRs and growth of the animals own CL. Importantly, there was no difference in weight of CL tissue between animals at day 19, nor an effect on plasma P4 concentration when CL number or weight was fitted as a covariate, suggesting normal luteal function, and ruling out the likelihood that exogenous P4 administration negatively affected development of the endogenous CL. All ewe groups, had day 0 baseline P4 concentrations consistent with reports for concentration at day of oestrus (Bassett *et al.*, 1969; Thorburn *et al.*, 1969) with the exception of the ewes of $CP4^{0-3}$ and $CP4^{0-6}$ treatment groups. This higher P4 concentration may refelct differences in time of ovulation in spite of synchronisation.

There appears to be an optimal window for greatest embryo survival, and timing and concentration of post-ovulatory P4 is important to both embryo growth and survival as suggested by (Wilmut and Sales, 1981; Mann *et al.*, 2006). However, it appears that a rise in concentration of P4 before day 3 results in not only advancement of uterus but also acceleration of the luteolytic mechanism, as suggested by Wilmut *et al.* (1985). It is possible that the early day 2 rise in P4 results in a uterus this is more asynchronous to the

transferred embryo than the uterus that was exposed from day 3 onwards. Given that P4 induced changes in the uterus advances embryo growth, one would reasonably hypothesise that this would result in improved pregnancy rates due to higher production of the anti-luteolytic hormone INFτ from the conceptus. Lawson and Cahill (1983) demonstrated that P4 treatment from day 0 to 3 in cyclic ewes shortened the cycle length, thereby exhibiting early luteolytic actvity. However, when they transferred day 10 embryos into these ewes at day 6 they were able to maintain pregnancies. This suggest that in the present study, that although P4 administration resulted in accellerated embryo growth in the $CP4^{0-3}$ and CP^{0-6} groups, the uterus was too far advanced or the embryos were unable to sufficiently mature to produce sufficent INFτ to abrogate the luteolytic mechanism as suggested by (Lawson *et al.*, 1983). It is possible that the elevated P4 at day 0 in $CP4^{0-3}$ and $CP4^{0-6}$ ewes may have confounded the survival rates reported here as these ewes may have been further post-ovulatory compared to the other groups and closer to luteolysis, since concentration and timing of increase appears to be critical to embryo survival. Further studies with greater ewe numbers are required to confirm the survival data.

Successful conception is associated with P4 concentrations of approximately 2.0 ng/mL while reduced conception rates have been reported at concentrations higher than 4 - 5 ng/mL (Parr *et al.*, 1987). In the present study peak plasma P4 concentrations were in excess of 4.0 ng/mL on day 2 in $CP4^{0-3}$, $CP4^{0-6}$ treatment groups.

8.6 Summary and conclusions

This study confirmed that administration of P4 in the post-ovulatory period of day 0 to 6, enhanced growth of embryos allowing the embryo to overcome the apparent effects of

maternal constraint up to day 19. This study provided some evidence that the period around day 3 was a critical time for the post-ovulatory rise in P4, that resulted in advanced embryo growth without an associated pregnancy loss. With this evidence it is now appropriate to further examine the maternal-conceptus dialogue, and to examine the changes that occur in expression of uterine genes that encode for structural and secretory function that determine embryo growth and development, to complement the gene expression findings of Chapter 7.

9 Effect of timing of exogenous progesterone administration during the post-ovulatory period of pregnancy on day 6 and day 19 uterine gene expression in a dam size model of maternal constraint

9.1 Abstract

Progesterone, via its receptor, has been shown to advance the uterine environment by altering expression of endometrial genes that encode for structural and secretory activity necessary for histotroph production and receptivity of the uterus to implantation. Administration of exogenous progesterone in the post-ovulatory period of pregnancy has been shown to enhance embryo growth, but is often accompanied by decreased embryo survival rates. Using a dam size model of maternal constraint, exogenous progesterone administered from day 0 to 6 was shown to increase the length of day 19 large breed Suffolk embryos gestated in small breed Cheviot ewes (Chapter 6). Correspondingly, altered endometrial gene expression in response to exogenous progesterone suggesting an advanced uterine environment was reported in Chapter 7. This increase in embryo size was accompanied by a decrease in pregnancy rate in the ewes that were administered progesterone. In Chapter 8, Suffolk embryos that were gestated in Cheviot ewes that received progesterone from day 2 to 4 had increased size at day 19 of gestation similar to that of Suffolk embryos gestated in Cheviot ewes that received the progesterone from day 0 to day 6. However, the ewes that received progesterone from day 2 to 4 had higher pregnancy rates than those ewes that received progesterone on day 0 to 3 or day 0 to 6. The aim of this study was to examine uterine gene expression at day 6 and day 19 using RT-qPCR to decipher if the timing of progesterone administration altered their expression. The observed differential gene expression in day 6 and day 19 uterine tissue confirms that progesterone administration alters the expression of genes such as diacylglycerol-O-acyltransferase (*DGAT2*), hepatocyte growth factor (*HGF*) and prostaglandin endoperoxide synthase 2 (*PTSG2*), endometrial galectin 15 (*LGALS15*), insulin like growth factor 1 (*IGF1*)

and its receptor (*IGF1R*) and mucin glycoprotein 1 (*MUC1*) that encode for endometrial structural and secretory activities necessary for embryo implantation and histotroph production. These changes are likely to be responsible for the enhanced embryo growth seen in progesterone-primed ewes. Additionally, this study highlights that timing of progesterone administration is critical to achieving enhanced growth while not affecting embryo survival, consistent with the results reported in Chapter 8. These are important findings which provide a useful platform for future studies aiming to advance embryo development and manipulate embryo survival. Further, investigation is needed to determine whether the pregnancy loss occurs as a result of the embryo failing to abrogate luteolysis.

9.2 Introduction

It has been suggested that altered embryo development driven by exogenous progesterone (P4) administration is mediated via an indirect effect of P4 acting on the uterus, rather than a direct effect of P4 on the embryo itself (Kleemann *et al.*, 1994; 2001; Clemente *et al.*, 2009). Further, administration of exogenous P4 in the post ovulatory period has been shown to advance conceptus development (Kleemann *et al.*, 1994; 2001; Mann *et al.*, 2006; Clemente *et al.*, 2009), but is associated with a complimentary reduction in embryo survival/pregnancy rate (Kleemann *et al.*, 1991).

In Chapter 6 we demonstrated that exogenous P4 administered to dams from day 0 to 6 advances embryo growth, such that large breed Suffolk (S) embryos transferred to P4-primed uteri of small breed Cheviot (C) ewes were able to overcome the apparent effects of maternal constraint. At day 19, the S embryos gestated in CP4 ewes were observed to be longer than S embryos gestated in C ewes that did not receive P4, and were similar in size to S embryos gestated in S ewes. This was accompanied by significantly higher plasma P4 concentrations at day 3 in the treated ewes and reduced pregnancy rates (C, 80.8% and S, 71.4% versus CP4, 28.0% and SP4, 22.7%; $p<0.05$). Following this, in Chapter 7 we demonstrated that exogenous P4 advances structural and secretory modifications of uterus, in alignment with reports by Grey *et al.* (2006), Satterfield *et al.* (2008a,2009) and Forde *et al.* (2009), which is associated with accelerated embryo growth. These findings align with the suggestion of Kleeman *et al.* (1994) that exogenous P4 administration results in an asynchronous environment between the embryo and the uterus. In turn the embryo is forced to accelerate its growth to adapt to the advanced environment to correct the asynchrony (Wilmut and Sales, 1981; Lawson *et al.*, 1983; Young *et al.*, 1995). Embryo-

uterine synchrony is regulated by the timing of the post-ovulatory rise in plasma P4, which is responsible for both initiating changes to the uterus and control of luteolysis (Lawson and Cahill, 1983). It appears that although the response to a P4-advanced uterine environment is accelerated embryo growth, conceptus maturation to a stage that can produce the appropriate anti-luteolytic signal may not be inherent (Lawson *et al.*, 1983). It has been suggested that development, growth and subsequent survival of the embryo is therefore dependent on the timing and strength of the P4 increase, which determines the stage of the development of the uterus (Wilmut and Sales, 1981).

In Chapter 8 it was shown that administration of P4 to C ewes from day 2 to 4 resulted in a similar size increase of day 19 S embryos as those S embryos gestated in C ewes that were administered P4 from day 0 to 3 and day 0 to 6, while maintaining higher pregnancy rates in these ewes ($CP4^{2-4}$, 65.0% versus $CP4^{0-3}$, 19.2% and $CP4^{0-6}$, 20.0%, respectively). This is comparable to the C ewes that did not receive exogenous P4 (CnP4, 73.3%). These findings align with previous studies in which P4 administration later than day 3 improved fetal growth in sheep and cattle, without an associated decrease in embryo survival (McMillan, 1987; Kleemann *et al.*, 1994; Carter *et al.*, 2008). It is therefore, evident that timing of P4 administration is critical to embryo-maternal interactions and regulates not only growth and development but also luteolysis and survival. Further there is little information on the changes that occur at a molecular level in the post-ovulatory/pre-implantation period in response to P4 and the subsequent effects within a model of maternal constraint. The investigations reported in Chapter 7 begin to unravel some of the mechanisms that are at work at day 19. Thus, the aim of this study was to examine if variation in the timing of P4 administration in C ewes during the post-ovulation period, day 0 to 6, causes a different

patterns of uterine gene expression at day 6 and day 19 of gestation. Further, this study aims to determine if alterations in gene expression are consistent with advanced uterine structural and secretory function that may be responsible for the differences in embryo size and pregnancy rates reported in Chapter 8.

9.3 Materials and Methods

The experimental and molecular biology methods used for this study are previously described in Chapter 8 (p.206) and 7 (p.169) respectively. Changes have been made here to the number of animals per treatment group, to reflect the change in experimental design. Additional genes have been examined in this experiment and details are included in the methods described below.

All experimental procedures were carried out with the approval of the Massey University Animal Ethics Committee.

9.3.1 Experimental animals and design

This study was undertaken using ewes of dissimilar mature body size S and C as established in previous studies (Sharma *et al.* 2013) and Chapters 4 to 8. Experimental pregnancy groups were established by transferring single purebred S ('large' breed) embryos into randomly allocated C ('small' breed) ewe recipient groups which had, or had not, been treated with exogenous P4 between days 0 and 6, and S ewes that had not been treated with exogenous P4, such that six experimental groups were established, (SinCP4$^{0\text{-}3}$: S embryos in C ewes that had been treated with exogenous progesterone day 0 to 3 of pregnancy; SinCP4$^{0\text{-}6}$: S embryos in C ewes that had been treated with exogenous

progesterone day 0 to 6 of pregnancy; SinCP4$^{2-4}$: S embryos in C ewes that had been treated with exogenous progesterone day 2 to 4 of pregnancy; SinCP4$^{3-6}$: S embryos in C ewes that had been treated with exogenous progesterone day 3 to 6 of pregnancy; SinCnP4: S embryos in untreated C ewes and SinSnP4: S embryos in untreated S ewes).

To establish these six groups twenty-one, 4 year old S ewes, were superovulated and laparoscopically inseminated on day 0 (day of oestrus) using semen from three S rams. These ewes were used as donors to provide S embryos which were transferred to 20 S and 107 C recipients on day 6. Recipient ewes were mixed age (2-6 years old) and parity (2-4), and average body condition score of 3.0 (Jefferies 1961; units 1-5: 1= emaciated, 5= obese). Recipients were balanced between treatment groups for age and body condition score. Cheviot recipients were randomly divided into five treatment groups. Recipient groups therefore consisted of 31 C ewes administered exogenous P4 from day 0 to 3 (CP4$^{0-3}$); 31 C ewes administered exogenous P4 from day 0 to 6 (CP4$^{0-6}$), 26 C ewes administered exogenous P4 from day 2 to 4 (CP4$^{2-4}$), 21 C ewes administered exogenous P4 from day 3 to 6 (CP4$^{3-6}$), 20 C ewes that were not administered exogenous P4 (CnP4), and 27 S ewes that were not administered exogenous P4 (SnP4). Single embryos were transferred into the recipients such that a total of 127 transfers were performed. Conceptuses were recovered on day 19, from all ewes that were identified as pregnant by non-return to estrus using a vasectomised, crayon-harnessed ram (SinCP4$^{0-3}$ (n= 5); SinCP4$^{0-6}$ (n=4); SinCP4$^{2-4}$ (n= 13); SinCP4$^{3-6}$ (n=13); SinCnP4 (n=11); SinSnP4 (n=10)[5]).

[5] Nomenclature “Sin” refers to embryos that were gestated in the respective ewe treatment groups. In cases where “Sin” is omitted it refers to the recipient ewes and/or their tissues

9.3.2 Uterine tissue collection- day 6

On day 6, at the same time that the uterus was externalised for embryo transfer via laparotomy, a small uterine biopsy was obtained from the mid-section of the horn that was contralateral to the corpus luteum (CL). A small stab incision was made and the protruding endometrium was clamped to provide haemostasis and a sample of approximately 5.0 mm^2 was removed. The sample was immediately snap frozen and stored at -80°C for gene expression studies. Biopsy samples were obtained from ewes that did and did not receive embryo transfers that had detectable ovulation sites as determined by the presence or absence of a CL.

9.3.3 Uterine tissue collection- day 19

On day 19 of gestation, recipient ewes were euthanised and the uteri were removed. At the same time that embryos were collected, a sample of tissue from the mid-region of both uterine horns (ipsilateral and contralateral to the CL/embryo) was dissected out, snap frozen and stored at -80°C for gene expression studies. Uterine samples were excluded if the ewe had 3 CLs, or if the embryo was on the horn contralateral to the ovary containing the CL.

9.3.4 RNA preparation

Total RNA was extracted from frozen uterine tissue samples (whole day 6 samples and approximately 100 mg of day 19 samples) using TRIzol® Reagent (Invitrogen™, Life Technologies, Carlsbad, CA, USA). An RNase free work environment was maintained throughout the process by using RNase free labware. The workbench was wiped with RNase *Zap*® (Ambion Biosystems, Life Technologies, Carlsbad, CA, USA), and 70% isopropanol.

The steps for RNA extraction were as follows:

(Steps 1-5 were carried out in the fume hood on ice, all other steps were carried out on the bench top)

1. Tissue grinding: Approximately 100 mg of frozen uterine tissue was placed in a 2 mL RNase free polypropylene tube with 100 µL of cold nuclease free water (NFW) (Ambion Inc., Life Technologies, Carlsbad, CA, USA) and a stainless steel bead. The tissue was ground using a bead beater for 30 seconds. The tube was removed and placed in ice.
2. Homogenisation: This was carried out in the fume hood on ice. One mL of cold TRIzol® Reagent was added to the tube containing the entire sample of ground uterine tissue. The sample was homogenised using the bead-beater for 20 seconds and then placed on ice for 2-3 minutes.
3. Chloroform step: The homogenate was transferred to a new tube and chloroform (Univar®, Ajax Finechem, Auckland, New Zealand) was added at a volume of 200 µL of chloroform per 1 mL of TRIzol® Reagent. The samples were then vortexed for 5 seconds and incubated on ice for 2 -3 minutes.
4. Centrifugation: The samples were centrifuged at 16,000 g at 4°C for 15 minutes. Three distinct phases were achieved post-centrifugation: a lower dark pink phenol-chloroform phase, a light pink interphase and an upper colourless aqueous phase.
5. The aqueous phase, containing the RNA was transferred to 200 µL of chloroform and vortexed and centrifuged as in steps 3 and 4 above.
6. The aqueous phase obtained from step 5 was transferred to 500 µL cold molecular biology grade isopropanol (Fisher Bioreagents, Fisher Scientific, USA) per 1mL of

TRIzol reagent containing sample. Samples were mixed gently by inverting and incubated at -20°C for 45 minutes to allow precipitation. Samples were centrifuged at 16,000 g for 15 minutes at 4°C producing supernatant and a pellet containing RNA. The supernatant was poured off and discarded.

7. Re-suspension: The RNA pellet was washed in 1 mL of sterile 75%v/v molecular grade ethanol (Emsure®, Merck Millipore, Auckland, New Zealand) by vortex (5 seconds) and followed by centrifugation (16,000 g, at 4°C for 5 minutes). The resulting supernatant was poured off and the wash process was repeated.
8. Drying: The pellet was then air dried for approximately 5-10 minutes at room temperature.
9. Final re-suspension to working RNA sample: The air-dried pellet was re-suspended in approximately 15-20 µL of NFW. The samples were then incubated on ice for 30 minutes to ensure complete suspension and stored at -80°C until further processing.

Quantification

Purity and concentration of extracted RNA was determined using a NanoDrop spectrophotometer (ND-2000 spectrophotometer, Thermoscientific, USA). The NanoDrop was initialised and blanked by loading 1.0 µL NFW water. Samples were loaded at a volume of 1.0 µL and 260/280 and 260/230 absorbance ratios were recorded. The measurement pedestal and sampling arm were wiped using Kimwipes (Kimberly-Clark® Professional, Auckland, New Zealand) between samples. Levels of approximately 2.0 for the 260/280 nm absorbance were accepted as pure RNA samples (i.e. free of protein contamination). Levels of approximately 1.8-2.0 for 260/230 nm absorbance were accepted as free of organic

solvents (TRIzol and isopropanol). The concentration of RNA per sample (ng/µL) was calculated from the equation A260*40, whereby an absorbance reading at 260nm (A260) of 1.0 unit is equivalent to 40 µg of RNA per mL.

DNase Treatment and quality check

Using the RNA concentrations calculated from NanoDrop, all of the samples were diluted to approximately 1.0 µg/µL using NFW. All of the RNA samples were DNase treated with amplification grade deoxyribonuclease to degrade any potential carry-over genomic DNA contamination. A 10 µL reaction mix was prepared for each sample in a separate 0.2 mL tube. The mix contained 2.5 µg of RNA, 1.0 µL of 10X DNase I reaction buffer, 2.0 µL of amplification grade DNase I (Invitrogen^{TM}, Life technologies, Carlsbad, CA, USA) and NFW (Ambion Inc., Life Technologies, Carlsbad, CA, USA). As per manufacturer's instructions all of the tubes were incubated at room temperature for approximately 15 minutes. EDTA (1.0 µL of 25 mmol/L, pH 8.0, Invitrogen^{TM}, Life technologies, Auckland New Zealand) was added to each tube just before the 15 minutes incubation was completed. The contents were then mixed gently and centrifuged at 16000 g for 10 seconds at 4°C. The reaction tubes were then transferred to a Sensoquest labcycler (Göttingen, Germany) in which they were further incubated at 65°C for 10 minutes and then at 4°C for 1 minute.

In order to confirm the integrity of the RNA samples and to confirm that the samples were free of DNA contamination post DNase treatment, a small volume (approximately 2.0 µL) of each of the DNase treated RNA samples was run on a 1% agarose gel for quality control. Agarose gels were made with DNase/RNase free agarose tablets (0.5 g/tablet, Bioline, Meridian Life Sciences, Memphis, TN, USA) in a 0.5X solution of TBE buffer (Ultrapure 10X TBE buffer, Invitrogen^{TM}, Thermo Fisher Scientific, Auckland, New Zealand) to

which ethidium bromide (Invitrogen™, Thermo Fisher Scientific, Auckland, New Zealand, 10 mg/mL) was added at a concentration of approximately 0.03 mg/50 mL of gel solution. Approximately 1.0 µL of loading dye (6X DNA loading dye, bromophenol blue 0.03%, Thermo Scientific, Auckland, New Zealand), was added to each sample and the solution made up to 10 µL using NFW. The total solution was loaded into the gel (one sample per well). A DNA ladder (100 bp plus Gene Ruler, Thermo Scientific, Auckland, New Zealand) was also loaded into one well of the gel, as a marker to determine the size of the bands and as a control to check the quality of the gel. Gels were run for 60 minutes at 80V, and then viewed under ultraviolet light using Geldoc/UVP Multidoc-it digital imaging system (Upland, CA, USA) to check for genomic DNA contamination. The absence of a bright band at the upper third of the lane on the gel indicated that the sample was free of genomic DNA since genomic DNA typically runs much slower through the gel. The concentration of remaining sample was determined using Qubit® 2.0 Fluorometer/Qubit RNA HS Assay kit (Invitrogen, Life Technologies, Carlsbad, CA, USA). Using NFW, the samples were then diluted to 10 ng/µL and stored in 15.0 µL aliquots at -80°C until further use.

9.3.5 Designing of primers and probes

Ovine sequences for candidate and reference genes were first searched in NCBI (Geer *et al.*, 2009) and Ensembl genome browser (Cunningham *et al.*, 2015). Using Geneious Ver. 8.1.6 (Kearse *et al.*, 2012) the mRNA sequences from both data banks were aligned to identify the position of introns in the coding sequence. Primers were then designed either side of an intron from a selected region of 200-220 base pairs (bp). Primers between 20-25 bp each were designed, with total amplicon size ranging from 80-190 bps, to optimise efficiency. uMelt℠ (https://www.dna.utah.edu/umelt/um.php, Dwight *et al.*, 2011)) was

used to predict melt curves for each of the primer pairs, and to identify the possibility of formation of primer dimers, which reduces reaction efficiency. Only primer pairs that were predicted to produce a single peak were deemed acceptable. An NCBI primer BLAST (Altschul *et al.*, 1990) was performed as an additional quality control check to ensure that the primers produced were from regions that encoded for the target protein only, were not from any other homologous region, and had an optimal sequence identity for gene expression in ovine species.

Primers were synthesised by Integrated DNA technologies (IDT, IA, USA) and supplied lyophilised. They were re-suspended with NFW according to the manufacturer's instructions to 100 µM and stored at -80°C. Working solutions were made up at 10 µM for use in quantitative reverse transcriptase PCR (RT-qPCR) reactions. The primers used for this study are listed in Table 9.1.

Table 9.1 Candidate and reference gene ID, accession number, forward and reverse primer sequences, amplicon sizes (base pairs, bp) and primer efficiency tested by reverse-transcription PCR (RT-qPCR).

	Gene Identifier[1]	NCBI accession	Forward Primer Sequence	Reverse Primer Sequence	Efficiency (%)	Amplicon Size (bp)	References[2]
Day 6	*DGAT2*	XM_012096077.1	CCCTCCAGGACCTGTTTTCC	TGAGCCAGCAATCGGTACAG	97	159	
	FGF7	XM_012180603	ATCAGGACAGTGGCTGTTGG	CCGCTGTGTGTCCATTTAGC	97	182	
	FGF10	NM_001009230.1	TTGAGAACGGGAAGGTCAGC	AGCAACAACTCCGATTTCCAC	104	89	
	HGF	XM_012176562.1	TGGTGTTTCACAAGCAATCCA	AGGTCATGCATTCAACTTCTGAAC	97	79	Satterfield *et al.* (2006)
	IGF1	NM_001009774.3	TCAGCAGTCTTCCAACCCAA	CAAGCACAGGGCCAGATAGA	105	110	
	PGR	XM_012095331.1	TGGTATTTGGACTAGGATGGAGA	AAACTCCTGTGGGATCTGCC	108	149	
	PTGS2	NM_001009432.1	CAGAGCTCTTCCTCCTGTGC	CAGATTTGTGCCCTGGGGAT	99	142	
	MSTN	NM_001009428.1	GATCTTGCTGTAACCTTCCC	GTGGAGTGCTCATCACAATC	99	125	Jeanplong *et al.* (2013)
Day19	*CTSL*	XM_004004094.2	ACAGCCAAGGGAAACATAGCT	TCTCCTGCCTTTGAAAGCCA	102	102	
	IGF1	NM_001009774.3	TCAGCAGTCTTCCAACCCAA	CAAGCACAGGGCCAGATAGA	105	110	
	IGF1R	XM_012098367.1	GGAATGGGTCATGGACGGAG	GGGCAGAGCAATCATCAGGT	99	89	
	LGALS15	NM_001009238.1	CGGGAGTTGGCAGAAGGAAG	CAAACTGGCAGATGGGCTTG	100	131	
	MUC1	XM_012107297.1	ACAGAGGGATTTTCTGGGCC	AGCTGACTGAACTGTGCCTT	100	123	
	PGR	XM_012095331.1	TGGTATTTGGACTAGGATGGAGA	AAACTCCTGTGGGATCTGCC	108	149	
	PTGS2	NM_001009432.1	CAGAGCTCTTCCTCCTGTGC	CAGATTTGTGCCCTGGGGAT	99	142	
	RSAD2	XM_004005669.2	GCAGGCTGGTGAAGTTCTGT	TGCTCGTCAAAGCTGTCACA	103	148	
	SERPIN	NM_001009304.1	GCTTCCCAAGATTGACCCCA	TCCGTGGTTAAGGCATGCTC	97	138	
	SPP1	NM_001009224.1	TGCAGTGATTTGCTTCTGCC	GCTTCTGAGATGGGTCAGGC	107	146	
Reference genes	*RPL19*	XM_004012836.2	ATGCCCGAGAAGGTAACGTG	GTCTGCCTTCAGCTTGTGGA	101	180	
	SF1	XM_004019657.1	GAGAGTTGGCTCGCTTGAAT	CCCCTCCACACTTGGTACAC	101	120	Paten *et al.* (2014)
	TBP	XM_015097549.1	AGAATAAGAGAGCCCCGCAC	GCTCCCCACCATGTTCTGAA	101	177	

[1]Gene names: *CTSL* = Cathepsin L, *DGAT2* = Diacylglycerol-O- acyltransferase 2, *FGF7* = Fibroblast growth factor 7, *FGF10* = Fibroblast growth factor 10, *HGF* = Hepatocyte growth factor, *IGF1* = Insulin like growth factor 1, *IGF1R* = Insulin like growth factor 1 receptor, *LGALS15* = Endometrial galectin 15, *MUC1* = Mucin 1, *MSTN* = Myostatin, *PGR* = Progesterone receptor, *PTGS2* = Prostaglandin-endoperoxide synthase 2, *RSAD2* = Radical S-adenosyl methionine domain containing 2, *SERPIN* = Ovine uterine serine proteinase inhibitor, SPP1 = Secreted phosphoprotein 1, *RPL19* = Ribosomal protein L 19, *SF1* = Splicing factor 1, *TBP* = TATA box binding protein.

[2]Forward and reverse primer sequences were used from these references where indicated, but checked against Geneious and NCBI primer BLAST as outlined in the text.

Primer optimisation

RT-qPCR was used to check the optimum annealing temperature and RNA sample concentration for each primer. Using KAPA SYBR® FAST One step qRT-PCR kit (KAPA Biosystems, MA, USA), 20.0 µL volume reactions were made up containing 10.0 µL KAPA SYBR® FAST qPCR Master Mix (2X), 0.4 µL KAPA RT Mix (50X), 0.4 µL (0.02 mM) of forward primer, 0.4 µL (0.02mM) of reverse primer, 1.0 µL of DNase treated RNA from a randomly selected sample (either at a 10 fold or 50 fold dilution), and 7.8 µL of NFW as per the manufacturer's instructions. Reactions were run in duplicate on two randomly chosen test samples. Duplicate no template controls, in which 1.0 µL of NFW was substituted for RNA template, were included for each primer pair. The reactions were run on a Rotor Gene Q series (Qiagen, Hilden Germany) using the following protocol:

5 minutes at 42°C

5 minutes at 95°C

10 seconds at 95°C

20 seconds at annealing temp (50°, 56°, 60°C) } Repeated for 40 cycles

60°C to 90°C at 1°C increments (melt curve)

Optimal annealing temperature and dilution was identified when a single peak was obtained for the melt curves of the test samples that matched in peak temperature, and when a default threshold of 0.2 was set, the quantification cycle (Ct) values for each sample was greater than 20.

Primer specificity

Using the predetermined sample dilutions and annealing temperature, RT-PCR was used to confirm the specificity of the each primer pair to generate a single PCR product. 20.0 µL volume reactions were made up using KAPA SYBR® FAST One step qRT-PCR kit as described previously. 1.0 µL of DNase treated RNA from two randomly selected samples at the determined dilution were used and a no template control, in which 1.0 µL of NFW was substituted for RNA template, was included for each primer pair. The reactions were run on Sensoquest labcycler (Göttingen, Germany) at the following protocol:

5 minutes at 42°C

5 minutes at 95°C

30 seconds at 95°C

30 seconds at annealing temp (56°, 60°C)

30 seconds at 72°C

} Repeated for 40 cycles

7 minutes at 72°C

4°C for ∞

The amplified products (10 µL) from the samples were run on 2% agarose gel (as per protocol) at 60V for 30 minutes and then visualised using Geldoc/UVP Multidoc-it digital imaging system (Upland, CA, USA). Primers were considered specific if a single band of expected product size (80-190 bp) was observed in cDNA sample with no other bands, and no bands were observed in the no template control. Primer pairs that did not conform to this were deemed un-specific and new primers were therefore redesigned for these genes

following the described protocol. PCR products, from the primer pairs that were determined to be specific, were held on ice to be used immediately for cloning.

Cloning/ Plasmid extraction

Cloning is the insertion of the amplified PCR product into a plasmid vector. The use of plasmid vector ensures that the primer pair is identical to the target that is being quantified. Plasmids are highly stable and accurate calculation of copy number of the standard DNA is possible in compliance with MIQE guidelines, increasing the accuracy and reproducibility of the standard curve used for determining the efficiency of RT-qPCR reactions.

The cloning reaction was performed using the Topo® TA Cloning® Kit (Invitrogen, Life Technologies, Auckland New Zealand). A 6.0 µL reaction was made up using 4.0 µL of the fresh PCR product (tested for specificity), 1.0 µL of salt solution and 1.0 µL of pCR™2.1 TOPO® vector. The reaction was mixed gently and incubated at room temperature for 5 minutes to ligate the DNA insert to the vector. The recombinant plasmids were then transformed into a vial of One Shot® TOP 10 chemically competent *Escherichia coli* cells by incubating 2.0 µL of the TOPO® cloning reaction with 50.0 µL of cells on ice for 30 minutes, and then heat shocked for 30 seconds at 42°C and immediately transferred to ice. SOC media (250 µL) was added to the transformed cells and incubated at 37°C while being horizontally shaken at 200 rpm for 1 hour. The suspension was then plated, at a volume of 60 µL/ plate, onto pre-warmed Luria-Bertani (LB) agar plates (each prepared with 100.0 µL Ampicillin (10 mg/mL, resuspended from Ampicillin salt, Life Technologies, Auckland New Zealand), 40 µL IPTG (0.1M, Life Technologies, Auckland New Zealand), and 40.0 µL X-Gal

(20.0 mg/mL, Thermoscientific, Auckland, New Zealand). The plates were incubated overnight at 37°C, and screened for blue and white colonies the following day. White colonies were the competent cells with the recombinant plasmid DNA. Three to four of the successfully transformed (white colonies) that were in close approximation to blue ones were then inoculated into separate 1.5 mL tubes containing LB broth (1 colony/1 mL of broth containing 5.0 mg ampicillin per 10.0 mL of LB broth). The tubes were incubated at 37°C overnight while being shaken horizontally at 200 rpm. The following day 10 µL of each of the LB broth cultures was transferred to 1.0 mL of Cryobroth (Ft Richard, Auckland, New Zealand) (10 mg ampicillin/20 mL of cryobroth) incubated overnight at 37°C and then stored at -80°C for future use. The remainder of the culture was used to extract plasmids.

Plasmids were extracted from the LB broth culture of the transformed cells using PureLink® Quick Plasmid DNA Miniprep Kits (Invitrogen, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions:

1. Cells were first harvested by centrifuging the remaining LB broth culture at 12,000 g for 5 minutes to produce a pellet. The medium was poured off to discard.
2. The cells (pellet) were then resuspended in the resuspension buffer (250 µL) and vortexed until homogenous.
3. Lysis buffer (250 µL) was added and the tube was mixed gently by inverting and then incubated for 5 minutes at room temperature.
4. Precipitation buffer (350 µL) was then added, and the tube was shaken until the content was homogenous. The lysate was then centrifuged at 12,000 g for 10 minutes.

5. The supernatant from step 4 was then loaded into a spin column mounted into a 2 mL wash tube. The column/wash tube was centrifuges at 12,000 g for 1 minute and the flow-through was discarded.
6. Wash buffer (W10) with pre-added ethanol (500 µL) was added to the column and incubated at room temperature for 1 minute. Column and wash-tube was then centrifuged at 12000 g for 1 minute and the flow-through discarded.
7. Wash buffer (W9) with pre-added ethanol (700 µL) was added to the column and centrifuged at 12,000 g for 1 minute and the flow-through discarded.
8. The spin column was placed into a clean 1.5 mL recovery tube and 75 µL of the preheated elution buffer was added to the centre of the column. The column was incubated at room temperature for 1 minute.
9. Plasmid was recovered by centrifugation of the column/recovery tube at 12,000 g for 2 minute. The spin column was discarded and the plasmid was stored at -80°C for further use.

The success of the cloning was checked by running end point PCR using the extracted plasmids. A 20.0 µL PCR reaction was set up using 4 µL 5x HOT FIREpol® Blend Master-mix (Solis Biodyne, Tartu, Estonia), 0.6 µL (0.3 µM) each of forward and reverse primers that matched the gene cloned in the plasmid, 1.0 µL of plasmid template and 13.8 µL of NFW. A no template control was included in which 1.0 µL of NFW was substituted for plasmid template. The reaction was run using Sensoquest labcycler (Göttingen, Germany) at the following programme:

95°C for 12 minutes

95°C for 30 seconds

Annealing temp (56°, 60°C) for 30 seconds } Repeated for 40 cycles

72°C for 30 seconds

72°C for 7 minutes

4°C for ∞

The entire 20 µL of PCR product was run on 2% agarose gel (as per protocol) at 60V for 60 minutes and then visualised using Geldoc/UVP Multidoc-it digital imaging system (Upland, CA, USA). Cloning was considered successful (i.e. DNA specific to the gene cloned was inserted into the vector) if a single band of expected product size was observed with no other bands, and no bands observed in the no template control. If no band was observed it was concluded that there was no insert present in the plasmid.

An M13 check PCR was then performed on a single plasmid per gene chosen from the end point PCR. Reactions were made up using 10.0 µL of 5x HOT FIREpol® Blend Master-mix, 1.5 µL (0.75 µM) each of M13 forward (GTA AAA CGA CGG CCA GT) and M13 reverse (CAG GAA ACA GCT ATG AC) primers, 1.0 µL of plasmid template and 36.0 µL of NFW. A no template control was included in which 1.0 µL of NFW was substituted for plasmid template. The reaction was run using Sensoquest labcycler (Göttingen, Germany) at the following programme:

95°C for 12 minutes

95°C for 30 seconds
50°C for 30 seconds
72°C for 30 seconds
} Repeated for 40 cycles

72°C for 7 minutes

4°C for ∞

The PCR product (5.0 µL) was run on a 1% agarose gel (as per protocol) and cloning was confirmed if a single band was obtained that corresponded to the length of the vector with the DNA insert (290 – 389 bp). The remainder of the M13 PCR product was stored at -80°C and used to make the dilution series for the standard curve.

Primer efficiency

Efficiency of the primers was measured by performing RT-qPCR (as per the protocol that is described in the following section) using serial dilutions of the M13 PCR product. Plasmid (DNA) concentration of the M13 PCR products was determined using Qubit®2.0 Fluorometer/ Qubit dsDNA HS Assay Kit (Invitrogen, Life Technologies, Carlsbad, CA, USA) (as per manufacturer's instructions). The concentrations were used to calculate the number of DNA molecules/µL of the M13 stock solution according to the following formula:

$$\boldsymbol{y} = \frac{x\ (g\mu L^{-1})}{(\#bp \times 660(Da)) \times (6.022 \times 10^{23})}$$

Where y is the number of molecules of DNA/µL of stock solution; *x* is the concentration of the plasmid in the M13 stock solution determined by Qubit®; #bp is the total size of the plasmid plus insert; 660 is the weight in Da per bp; 6.022 x 10^{23} is Avogadro's number, the number of molecules in one mole of substance. The calculated number of molecules was then used to generated serial dilutions (1:100000, 1:10000; 1:1000; 1:100; 1:10) to be used to produce the standard curve.

The efficiency was calculated by Rotor-Gene Q Series software 2.3.1 (Qiagen, Hilden Germany) for each primer tested. Molecules of DNA were plotted on the x axis against the Ct value calculated by the software on the y axis. A slope value of between -3.6 and -3.1 is equivalent to an efficiency between 90 and 110%. Primer efficiencies are listed in Table 9.1.

9.3.6 Quantitative PCR reactions

RT-qPCR reactions were carried out using Rotor Gene Q series (Qiagen, Hilden Germany). Each reaction contained 10.0 µL of 2X KAPA SYBR® FAST qPCR Master Mix, 0.4 µL of 50X KAPA RT Mix, 0.4 µL (200 nM) of forward primer, 0.4 µL (200nM) of reverse primer, 1.0 µL of DNase treated RNA (at a concentration of 10.0 ng/µL), and 7.8 µL of NFW as per the manufacturer's instructions. Reactions were run in triplicate. Reactions were run in Rotor disk®100 well rings (Qiagen, Hilden, Germany). Triplicate *no template controls* and standard curve dilution series were included for each run. The following RT-qPCR protocol was used:

5 minutes at 42°C

5 minutes at 95°C

10 seconds at 95°C

20 seconds at annealing temp (56°, 60°C) } Repeated for 40 cycles

60°C to 90°C at 1°C increments (melt curve)

9.3.7 Statistical Analysis of RT-qPCR data

Threshold Ct values (triplicate) for each sample were obtained from Rotor gene Q series software 2.3.1 (Qiagen, Hilden, Germany). The mRNA expression was calculated as fold change, normalised with three reference genes[6] (RPL19, SF1 and TBP), using the following equation (Pfaffl, 2001):

$$\boldsymbol{RR} = \frac{(\boldsymbol{E}_{target})^{\Delta \mathbf{Ct}_{target}\ (\mathbf{treated\ -\ comparator})}}{(\boldsymbol{E}_{RG})^{\Delta \mathbf{Ct}_{RG}\ (\mathbf{treated\ -\ comparator})}}$$

Where RR is the relative ratio of target gene to reference gene; E_{target} is the real time PCR amplification efficiency of the target gene transcript determined from the slope of the standard curve; E_{RG} is the geometric mean of real time PCR amplification efficiency of the reference genes; $\Delta Ct_{target\ (treated\ -\ comparator)}$ and $\Delta Ct_{RG\ (treated\ -\ comparator)}$ are the Ct differences between the treatment group and the comparator (control) group for the target gene and the geometric mean of the reference genes respectively. Statistical significance of the fold change was calculated as 95% confidence intervals. Ewes that contained a CL on both left and right ovaries were included in the analysis of combined horns (ipsilateral and

[6] Reference genes were selected from a panel of potential reference genes *RPL19*, *SF1*, *TBP*, *ACTB*, *18S*, *HMBS* with respect to stability of expression (i.e. low CV% according to the BestKeeper index (Pfaffl *et al.*, 2004)

contralateral together), but were excluded when ipsilateral horns or contralateral horns were examined individually.

9.4 Results

9.4.1 Differential gene expression of Day 6 uterine endometrium

PGR mRNA expression in CnP4, $CP4^{0-3}$, $CP4^{3-6}$ ewes did not differ from SnP4 ewes at day 6 (Table 9.2). $CP4^{n}$ $CP4^{0-3}$, $CP4^{0-6}$ and $CP4^{3-6}$ *PGR* mRNA expression did not differ from each other. $CP4^{2-4}$ PGR mRNA did not differ from CnP4 and $CP4^{3-6}$, but differed from $CP4^{0-3}$ and $CP4^{0-6}$.

DGAT2 mRNA expression was lower in CnP4 ewes compared to SnP4 on day 6 (Table 9.2). In all CP4 groups, *DGAT2* mRNA was up-regulated compared to both CnP4 and SnP4.

FGF7 mRNA expression in CnP4, $CP4^{0-3}$, $CP4^{0-6}$ and $CP4^{3-6}$ ewes did not differ from SnP4 ewes (Table 9.2). Compared to CnP4 ewes, *FGF7* mRNA was up-regulated in $CP4^{0-3}$ and $CP4^{2-4}$ ewes, with $CP4^{2-4}$ ewes having higher *FGF7* expression levels than all other groups. *FGF7* mRNA expression levels in $CP4^{0-6}$ ewes was lower compared to CnP4 ewes, but did not differ from expression levels in $CP4^{3-6}$ ewes on day 6.

CnP4 and $CP4^{3-6}$ ewes had lower *FGF10* mRNA expression levels than SnP4 ewes, while $CP4^{0-3}$ and $CP4^{0-6}$ ewes did not differ in *FGF10* mRNA expression from SnP4 ewes (Table 9.2). All of the CP4 ewe groups had higher *FGF10* mRNA expression levels than CnP4 ewe, with $CP4^{0-3}$, $CP4^{0-6}$ and $CP4^{2-4}$ *FGF10* mRNA expression also higher than $CP4^{3-6}$. *FGF10* mRNA expression did not differ between $CP4^{0-3}$ and $CP4^{0-6}$ nor between $CP4^{0-6}$ and $CP4^{2-4}$, but it did differ between $CP4^{0-3}$ and $CP4^{2-4}$.

HGF mRNA expression in CnP4, $CP4^{0-6}$, $CP4^{2-4}$ and $CP4^{3-6}$ ewes was higher than SnP4 ewes (Table 9.2). $CP4^{0-6}$, $CP4^{2-4}$ and $CP4^{3-6}$ ewes had higher *HGF* mRNA expression than CnP4 and $CP4^{0-3}$ ewes. mRNA expression of $CP4^{2-4}$ ewes did not differ from $CP4^{0-6}$ or CP^{3-6}, however, $CP4^{0-6}$ and $CP4^{3-6}$ mRNA *HGF* mRNA expression differed.

All cheviot ewes had lower *IGF1* mRNA expression than $SP4^{n}$ ewes, regardless of if they received P4 or not, at day 6 (Table 9.2). $CP4^{0-3}$ ewes had lower *IGF1* mRNA expression levels than all the other C ewe groups (CnP4, $CP4^{0-6}$, $CP4^{2-4}$, and $CP4^{3-6}$) which did not differ in *IGF1* expression levels.

CnP4 and $CP4^{3-6}$ ewes *PTGS2* mRNA expression did not differ from SnP4 ewes (Table 9.2). $CP4^{0-3}$ ewes had the highest *PTGS2* mRNA expression levels compared to all other groups. $CP4^{0-6}$ and $CP4^{2-4}$ ewes *PTGS2* mRNA expression did not differ from each other and were both higher than CnP4 and $CP4^{3-6}$ ewes. $CP4^{3-6}$ ewes had lower PTGS2 mRNA expression than CnP4 ewes at day 6.

MSTN appeared to be generally very lowly expressed in the day 6 tissue and there was wide variation even within triplicates samples being run in the PCR. Attempts were made to increase the overall expression by using 100 ng/µL concentrations of the samples; however, this did not improve the output. Due to this the results of the RT-qPCR analysis on *MSTN* was omitted as the output generated was considered un-reliable. The comparison of differential expression between groups for day 6 uterine horn tissue is represented graphically in Appendix X.

Table 9.2 Pregnancy day 6 uterine horn mRNA expression levels in pregnant Cheviot ewes that were and were not administered exogenous progesterone for various time periods from day 0-6 (CP40-3, CP40-6 CP42-4, CP43-6 and CnP4) for horns contralateral to ovary containing the CL. Fold change is expressed relative to levels in control Suffolk ewes that were not administered exogenous progesterone (SnP4, n=7). Data is normalised with RPL19, SF1 and TBP. Data is shown as fold change with 95% confidence intervals (given in parenthesis). If confidence intervals do not include 1, then mRNA expression levels are significantly different from SnP4 control (bold). Different superscripts indicate that mRNA expression levels differ between CP40-3, CP40-6 CP42-4, CP43-6 and CnP4 treatment groups (p<0.05).

	CnP4	$CP4^{0-3}$	$CP4^{0-6}$	$CP4^{2-4}$	$CP4^{3-6}$
	Expressed as fold change relative to SnP4				
n	6	6	7	6	5
DGAT2	**0.71 (0.63 - 0.80)^{a}**	**3.57 (2.72 - 4.68)^{d}**	**2.53 (2.20 - 2.92)^{cd}**	**1.60 (1.05 - 2.45)^{bc}**	**1.41 (1.36 - 1.46)^{b}**
FGF7	1.02 (0.98 - 1.06)^{b}	1.10 (0.82 - 1.48)^{c}	0.88 (0.75 - 1.02)^{a}	**1.37 (1.19 - 1.59)^{d}**	0.94 (0.86 - 1.03)^{ab}
FGF10	**0.68 (0.64 - 0.72)^{a}**	1.04 (0.90 - 1.21)^{c}	1.04 (0.93 - 1.15)^{cd}	**1.09 (1.08 - 1.11)^{d}**	**0.81 (0.72 - 0.91)^{b}**
HGF	**1.13 (1.09 - 1.17)^{b}**	0.99 (0.90 - 1.09)^{a}	**1.71 (1.71 - 1.72)^{d}**	**1.53 (1.41 - 1.65)^{cd}**	**1.42 (1.49 - 1.35)^{c}**
IGF1	**0.86 (0.85 - 0.87)^{b}**	**0.69 (0.68 - 0.70)^{a}**	**0.86 (0.78 - 0.96)^{b}**	**0.90 (0.82 - 0.99)^{b}**	**0.94 (0.92 - 0.96)^{b}**
PGR	1.00 (0.84 - 1.20)^{ab}	0.82 (0.63 - 1.07)^{a}	**0.81 (0.70 - 0.94)^{a}**	**1.35 (1.03 -1.76)^{b}**	1.16 (0.84 -1.60)^{ab}
PTGS2	1.01 (0.84 - 1.22)^{b}	**2.55 (2.55 - 2.55)^{d}**	**1.42 (1.14 - 1.76)^{c}**	**1.44 (1.13 - 1.83)^{c}**	0.89 (0.78 - 1.02)^{a}
MSTN	0.98 (0.96 - 1.00)	**0.77 (0.68 - 0.88)**	1.50 (0.26 - 8.45)	0.44 (0.18 - 1.12)	0.90 (0.58 - 1.38)

Gene names: *DGAT2* = Diacylglycerol-O- acyltransferase 2, *FGF7* = Fibroblast growth factor 7, *FGF10* = Fibroblast growth factor 10, *HGF* = Hepatocyte growth factor, *IGF1* = Insulin like growth factor 1, *MSTN* = Myostatin, *PGR* = Progesterone receptor, *PTGS2* = Prostaglandin-endoperoxide synthase 2, *RPL19* = Ribosomal protein L 19, *SF1* = Splicing factor 1, *TBP* = TATA box binding protein.

9.4.2 Differential expression of Day 19 uterine horns

Differential expression of Day 19 uterine horns: ipsilateral vs contralateral

When Cheviot and Suffolk ewes were examined together, *PGR*, *CTSL*, *MUC1*, *SERPIN* and *SPP1* mRNA expression[7] was higher (1.12 [1.01 -1.24], 1.18 [1.09 – 1.27], 1.09 [1.03 – 1.16], 2.12 [1.57 – 2.87], 1.48 [1.11 – 1.96] respectively) and IGF1R mRNA expression was lower (0.88 [0.82 – 0.94]) in the uterine horn that was ipsilateral to the ovary containing the CL compared to the contralateral uterine horn regardless of treatment. mRNA expression did not differ between uterine horns that were ipsilateral and contralateral to the ovary containing the CL for the remaining genes when both breeds were examined together. When C ewes were examined alone, mRNA expression was higher in the uterine horn ipsilateral to the ovary compared to the contralateral horn for *PGR* (1.15 [1.01 - 1.30]), *CTSL* (1.20 [1.08 – 1.33]), *RSAD2* (1.28 [1.07 – 1.52]) and *SERPIN* (1.93 [1.35 – 2.75]) regardless of treatment. *IGF1R* mRNA expression was lower (0.89 [0.83-0.95]) in the horn ipsilateral to the CL compared to the contralateral horn.

Day 19 uterine tissue differential expression between treatment groups

PGR expression was higher in CnP4 ewes when compared to SnP4 ewes for both combined and ipsilateral uterine horns (Table 9.3 and 9.4). In combined horns mRNA expression in all CP4 ewes did not differ from SnP4 ewes. When only the ipsilateral horn was examined the $CP4^{0-3}$, $CP4^{2-4}$ and $CP4^{3-6}$ group was lower in *PGR* mRNA expression compared to $CP4^{n}$ ewes. $CP4^{0-3}$ ewes had lower *PGR* expression levels than $CP4^{2-4}$ and $CP4^{3-6}$ ewes when examined in the ipsilateral horn alone. However, in the combined horns $CP4^{0-3}$, $CP4^{0-6}$,

[7] Expression levels are reported as a fold change + 95% confidence interval, where 1.0 = no difference in expression between comparator groups.

$CP4^{2-4}$ and $CP4^{3-6}$ did not differ in their *PGR* mRNA expression levels; however, only $CP4^{0-3}$ and $CP4^{2-4}$ PGR expression was down-regulated compared to CnP4. In the contralateral horn $CP4^{0-3}$, $CP4^{2-4}$ and $CP4^{3-6}$ were all down-regulated compared to CnP4 (Table 9.5).

IGF1 and *IGF1R* have similar patterns of mRNA expression between treatment groups in both the combined horns and the ipsilateral horn alone. In the combined horns CnP4 *IGF1* mRNA expression is lower compared to the control SnP4; however, there is no difference in *IGF1R* mRNA expression between these two ewe groups (Table 9.3). $CP4^{0-3}$ *IGF1* and *IGF1R* expression is higher than that of CnP4, and does not differ from $CP4^{0-6}$ in both combined and ipsilateral horns (Table 9.3 and 9.4). $CP4^{2-4}$ *IGF1* and *IGF1R* mRNA expression is also higher than CnP4 expression in combined and ipsilateral horns. However, *IGF1* mRNA expression in $CP4^{2-4}$ ewes was higher than $CP4^{0-3}$ in both the combined horns and the ipsilateral horn. *IGF1R* mRNA expression in $CP4^{2-4}$ ewes is higher compared to $CP4^{0-3}$ and $CP4^{3-6}$ ewes in both the combined horns and the ipsilateral horn. In the contralateral horn $CP4^{2-4}$ has higher *IGF* and *IGF1R* mRNA expression than CnP4, $CP4^{0-3}$ and $CP4^{3-6}$ ewe groups (Table 9.5).

In combined horns *PTGS2* mRNA expression did not differ between CnP4 $CP4^{0-3}$ and $CP4^{0-6}$ ewes and did not differ between each of these groups and the control SnP4 (Table 9.3). $CP4^{2-4}$ and $CP4^{3-6}$ ewes did not differ from each other in *PTGS2* mRNA expression, and had higher mRNA expression when compared to CnP4 and SnP4 ewes in the combined horns. In the ipsilateral horn $CP4^{2-4}$ had higher *PTGS2* mRNA expression than all the CnP4, $CP4^{0-3}$ and $CP4^{3-6}$ ewe groups (Table 9.4).

Table 9.3 Pregnancy day 19 uterine horn mRNA expression levels in in pregnant Cheviot (C) ewes that were and were not administered exogenous progesterone (P40 for various time periods from day 0-6 ($CP4^{0-3}$, $CP4^{0-6}$ $CP4^{2-4}$, $CP4^{3-6}$ and CnP4) for combined uterine horns. Fold change is expressed relative to levels in control Suffolk (S) ewes that were not administered exogenous progesterone (SnP4, n=14). Data is normalised with *RPL19*, *SF1* and *TBP.* Data is shown as fold change with 95% confidence intervals (given in parenthesis). If confidence intervals do not include 1, then mRNA expression levels are significantly different from SnP4 control (bold). Different superscripts indicate that mRNA expression levels differ between $CP4^{0-3}$, $CP4^{0-6}$ $CP4^{2-4}$, $CP4^{3-6}$ and CnP4 treatment groups (p<0.05).

	Combined horns: ipsilateral and contralateral to CL				
	CnP4	$CP4^{0-3}$	$CP4^{0-6}$	$CP4^{2-4}$	$CP4^{3-6}$
	Expressed as fold change relative to SnP4				
n	18	10	6	26	24
CTSL	**0.85 (0.74 - 0.98)^{c}**	0.84 (0.63 - 1.12)^{bc}	**0.42 (0.40 - 0.44)^{a}**	1.01 (1.00 - 1.02)^{b}	1.03 (0.99 - 1.08)^{bc}
IGF1	**0.89 (0.84 - 0.93)^{a}**	**1.10 (1.09 - 1.11)^{b}**	1.03 (0.68 - 1.56)^{abc}	**1.18 (1.07 - 1.30)^{c}**	**1.13 (1.07 - 1.19)^{bc}**
IGF1R	0.91 (0.82 - 1.01)^{a}	**1.15 (1.04 - 1.27)^{b}**	0.89 (0.64 - 1.25)^{ab}	**1.24 (1.07 - 1.43)^{c}**	1.01 (0.92 - 1.11)^{b}
LGALS15	**1.26 (1.11 - 1.44)^{c}**	**0.68 (0.50 - 0.92)^{b}**	**0.47 (0.37 - 0.59)^{a}**	0.91 (0.80 - 1.05)^{b}	**0.85 (0.75 - 0.97)^{b}**
MUC1	**1.05 (1.05 - 1.06)^{ab}**	**1.42 (1.19 - 1.69)^{acd}**	1.00 (0.93 - 1.07)^{b}	**1.58 (1.50 - 1.67)^{c}**	**1.39 (1.37 - 1.41)^{d}**
PGR	**1.27 (1.13 - 1.42)^{b}**	0.91 (0.74 - 1.12)^{a}	1.14 (0.81 - 1.60)^{ab}	1.02 (0.88 - 1.18)^{a}	1.12 (0.99 - 1.28)^{ab}
PTGS2	1.02 (0.89 - 1.16)^{a}	0.99 (0.75 - 1.31)^{ab}	1.09 (0.82 - 1.46)^{abc}	**1.27 (1.17 - 1.38)^{c}**	**1.24 (1.08 - 1.43)^{bc}**
RSAD2	0.95 (0.72 - 1.25)^{b}	0.85 (0.66 - 1.11)^{b}		1.21 (0.98 - 1.48)^{c}	1.02 (0.79 - 1.31)^{bc}
SERPIN	0.69 (0.42 - 1.12)^{a}	1.38 (0.63 - 3.03)^{ab}	0.73 (0.29 - 1.83)^{ab}	1.02 (0.69 - 1.50)^{ab}	1.20 (0.79 - 1.81)^{b}
SPP1	**2.32 (1.59 - 3.39)^{b}**	**2.12 (1.29 - 3.48)^{ab}**	0.93 (0.39 - 2.23)^{a}	**1.50 (1.07 - 2.10)^{a}**	**2.66 (1.89 - 3.74)^{b}**

Gene names: *CTSL* = Cathepsin L, *IGF1* = Insulin like growth factor 1, *IGF1R* = Insulin like growth factor 1 receptor, *LGALS15* = Endometrial galectin 15, *MUC1* = Mucin 1, *PGR* = Progesterone receptor, *PTGS2* = Prostaglandin-endoperoxide synthase 2, *RSAD2* = Radical S-adenosyl methionine domain containing 2, *SERPIN* = Ovine uterine serine proteinase inhibitor, SPP1 = Secreted phosphoprotein 1, *RPL19* = Ribosomal protein L 19, *SF1* = Splicing factor 1, *TBP* = TATA box binding protein

Table 9.4 Pregnancy day 19 uterine horn mRNA expression levels in pregnant Cheviot (C) ewes that were and were not administered exogenous progesterone (P4) for various time periods from day 0-6 ($CP4^{0-3}$, $CP4^{0-6}$ $CP4^{2-4}$, $CP4^{3-6}$ and CnP4) for horns ipsilateral to the ovary containing the CL. Fold change is expressed relative to levels in control Suffolk (S) ewes that were not administered exogenous progesterone (SnP4, n=6). Data is normalised with *RPL19*, *SF1* and *TBP*. Data is shown as fold change with 95% confidence intervals (given in parenthesis). If confidence intervals do not include 1, then mRNA expression levels are significantly different from SnP4 control (bold). Different superscripts indicate that mRNA expression levels differ between $CP4^{0-3}$, $CP4^{0-6}$ $CP4^{2-4}$, $CP4^{3-6}$ and CnP4 treatment groups ($p<0.05$).

	Horn ipsilateral to CL only				
	CnP4	$CP4^{0-3}$	$CP4^{0-6}$	$CP4^{2-4}$	$CP4^{3-6}$
	Expressed as fold change relative to SnP4				
n	6	4	2	10	6
CTSL	**1.07 (1.03 - 1.11)^{a}**	**1.19 (1.10 - 1.28)^{b}**	0.87 (0.26 – 2.96)^{abc}	**1.18 (1.01 - 1.37)^{ab}**	1.45 (1.38 - 1.53)^{c}
IGF1	0.90 (0.83 – 0.98)^{a}	**1.50 (1.26 - 1.79)^{c}**	1.05 (0.20 - 5.53)^{abc}	**1.41 (1.40 - 1.41)^{b}**	**1.25 (1.23 - 1.27)^{b}**
IGF1R	**0.89 (0.86 – 0.93)^{a}**	**1.17 (1.14 - 1.20)^{c}**	0.87 (0.17 – 4.35)^{abc}	**1.26 (1.18 - 1.35)^{d}**	1.05 (0.97 - 1.14)^{b}
LGALS15	1.57 (0.98 - 2.50)^{b}	0.73 (0.50 - 1.05)^{a}	0.77 (0.57 - 1.04)^{a}	1.06 (0.64 - 1.77)^{ab}	1.41 (1.00 - 1.99)^{b}
MUC1	1.06 (0.86 - 1.32)^{a}	**1.74 (1.60 - 1.90)^{b}**	1.00 (0.73 - 1.37)^{a}	**1.58 (1.34 - 1.86)^{b}**	**2.04 (1.99 – 2.10)^{c}**
PGR	**1.49 (1.24 - 1.79)^{c}**	**0.75 (0.73 - 0.77)^{a}**	1.15 (0.39 – 3.43)^{ab}	1.12 (0.91 - 1.38)^{b}	0.95 (0.75 - 1.20)^{b}
PTGS2	0.95 (0.69 - 1.31)^{a}	0.94 (0.56 - 1.58)^{ac}	1.34 (0.69 – 2.60)^{abcd}	**1.46 (1.15 - 1.85)^{d}**	**1.58 (1.32 - 1.88)^{b}**
RSAD2	2.37 (0.93 – 6.09)^{b}	0.72 (0.21 - 2.44)^{a}		2.25 (0.91 - 5.55)^{b}	1.88 (0.63 – 5.55)^{b}
SERPIN	**0.44 (0.30 - 0.65)^{a}**	1.56 (0.94 - 2.57)^{b}	0.16 (0.01 - 2.31)^{ab}	0.68 (0.40 - 1.15)^{a}	1.08 (0.82 - 1.43)^{b}
SPP1	**1.87 (1.29 - 2.71)^{bc}**	1.18 (0.92 - 1.52)^{a}	0.29 (0.04 - 2.26)^{ab}	1.30 (0.75 – 2.28)^{a}	**2.59 (1.69 – 3.98)^{c}**

Gene names: *CTSL* = Cathepsin L, *IGF1* = Insulin like growth factor 1, *IGF1R* = Insulin like growth factor 1 receptor, *LGALS15* = Endometrial galectin 15, *MUC1* = Mucin 1, *PGR* = Progesterone receptor, *PTGS2* = Prostaglandin-endoperoxide synthase 2, *RSAD2* = Radical S-adenosyl methionine domain containing 2, *SERPIN* = Ovine uterine serine proteinase inhibitor, SPP1 = Secreted phosphoprotein 1, *RPL19* = Ribosomal protein L 19, *SF1* = Splicing factor 1, *TBP* = TATA box binding protein.

Table 9.5 Pregnancy day 19 uterine horn mRNA expression levels in pregnant Cheviot (C) ewes that were and were not administered exogenous progesterone (P4) for various time periods from day 0-6 ($CP4^{0-3}$, $CP4^{0-6}$ $CP4^{2-4}$, $CP4^{3-6}$ and CnP4) for horns contralateral to the ovary containing the CL. Fold change is expressed relative to levels in control Suffolk (S) ewes that were not administered exogenous progesterone ($SP4^{n}$, n=6). Data is normalised with *RPL19*, *SF1* and *TBP*. Data is shown as fold change with 95% confidence intervals (given in parenthesis). If confidence intervals do not include 1, then mRNA expression levels are significantly different from SnP4 control (bold). Different superscripts indicate that mRNA expression levels differ between $CP4^{0-3}$, $CP4^{0-6}$ $CP4^{2-4}$, $CP4^{3-6}$ and CnP4 treatment groups ($p<0.05$).

	Horn contralateral to CL only				
	CnP4	$CP4^{0-3}$	$CP4^{0-6}$	$CP4^{2-4}$	$CP4^{3-6}$
	Expressed as fold change relative to SnP4				
n	6	4	2	10	6
CTSL	**1.24 (1.10 - 1.41)^{b}**	0.91 (0.42 - 1.96)^{bc}	**0.21 (0.06 - 0.77)^{a}**	**1.40 (1.26 - 1.57)^{c}**	**1.46 (1.26 - 1.69)^{bc}**
IGF1	1.01 (0.85 - 1.20)^{a}	**1.20 (1.02 - 1.41)^{c}**	1.39 (0.61 - 3.20)^{abc}	**1.62 (1.27 - 2.07)^{d}**	1.16 (0.87 - 1.55)^{b}
IGF1R	0.81 (0.56 - 1.19)^{a}	1.23 (0.99 - 1.53)^{b}	1.02 (0.72 - 1.45)^{ab}	**1.61 (1.11 - 2.34)^{c}**	0.91 (0.59 - 1.40)^{a}
LGALS15	0.91 (0.81 - 1.02)^{b}	**0.49 (0.24 - 0.99)^{b}**	**0.15 (0.10 - 0.23)^{a}**	**0.78 (0.76 - 0.81)^{b}**	0.62 (0.35 - 1.10)^{b}
MUC1	1.13 (0.96 - 1.33)^{a}	**1.89 (1.09 - 3.25)^{ab}**	1.07 (0.86 - 1.35)^{a}	**2.15 (1.65 - 2.79)^{b}**	**1.37 (1.13 - 1.67)^{a}**
PGR	**1.25 (1.07 - 1.47)^{b}**	**0.73 (0.59 - 0.90)^{a}**	1.08 (0.47 - 2.46)^{ab}	**0.78 (0.62 - 0.98)^{a}**	**0.72 (0.58 - 0.89)^{a}**
PTGS2	1.30 (0.93 - 1.83)	1.10 (0.58 - 2.09)	1.67 (0.49 - 5.71)	**1.38 (1.16 - 1.64)**	1.26 (0.85 - 1.86)
RSAD2	**0.52 (0.37 - 0.73)^{ab}**	0.82 (0.47 - 1.44)^{bc}		**0.89 (0.86 - 0.92)^{c}**	0.88 (0.68 - 1.14)^{bc}
SERPIN	0.71 (0.35 -1.44)^{a}	1.48 (0.35 - 6.28)^{ab}	2.19 (0.59 - 8.12)^{ab}	**1.83 (1.24 - 2.69)^{b}**	2.15 (0.88 - 5.24)^{ab}
SPP1	**2.58 (1.41 - 4.74)^{b}**	**4.45 (1.25 - 15.84)^{b}**	0.92 (0.72 - 1.18)^{a}	**2.25 (1.58 - 3.21)^{b}**	**4.78 (2.27 - 10.04)^{b}**

Gene names: *CTSL* = Cathepsin L, *IGF1* = Insulin like growth factor 1, *IGF1R* = Insulin like growth factor 1 receptor, *LGALS15* = Endometrial galectin 15, *MUC1* = Mucin 1, *PGR* = Progesterone receptor, *PTGS2* = Prostaglandin-endoperoxide synthase 2, *RSAD2* = Radical S-adenosyl methionine domain containing 2, *SERPIN* = Ovine uterine serine proteinase inhibitor, SPP1 = Secreted phosphoprotein 1, *RPL19* = Ribosomal protein L 19, *SF1* = Splicing factor 1, *TBP* = TATA box binding protein.

CTSL mRNA expression of the $CP4^{0-6}$ ewes was lower than all the other C ewe groups and SnP4 control when examined in the combined horns (Table 9.3). In the ipsilateral horn $CP4^{0-3}$ and $CP4^{2-4}$ *CTSL* mRNA did not differ in expression levels, and $CP4^{3-6}$ had the highest CTSL expression levels compared to CnP4, $CP4^{0-3}$ and $CP4^{2-4}$ (Table 9.4). In the combined horn $CP4^{0-3}$, $CP4^{2-4}$ and $CP4^{3-6}$ did not differ in *CTSL* mRNA expression from each other or from SnP4 while CnP4 ewes had lower *CTSL* mRNA expression than $CP4^{2-4}$ and SnP4 ewes.

CnP4 *LGALS15* mRNA expression was higher than all the C ewe treatment groups and the SnP4 control ewes in the combined horns (Table 9.3); however, *LGALS15* MRNA expression in this group did not differ from $CP4^{2-4}$ and $CP4^{3-6}$ ewes when the ipsilateral horn was examined (Table 9.4). When the combined horn was examined $CP4^{0-6}$ ewes had the lowest *LGALS15* expression compared to all the C ewe treatment groups. $CP4^{0-3}$, $CP4^{2-4}$ and $CP4^{3-6}$ did not differ in their *LGALS15* mRNA expression in the combined horn. When the ipsilateral horn was examined on its own $CP4^{0-3}$ and $CP4^{0-6}$ did not differ from $CP4^{2-4}$ in *LGALS15* mRNA expression, and $CP4^{2-4}$ and $CP4^{3-6}$ did not differ in *LGALS15* mRNA expression.

MUC1 mRNA expression was higher in CP^{0-3}, $CP4^{2-4}$ and $CP4^{3-6}$ ewes compared to $CP4^{0-6}$, CnP4 and SnP4 ewes in both combined and ipsilateral horns (Table 9.3 and Table 9.4). When the ipsilateral horn was examined on its own $CP4^{3-6}$ *MUC1* mRNA was higher than CP^{0-3}, $CP4^{2-4}$, and *MUC1* mRNA did not differ between $CP4^{0-3}$ and $CP4^{2-4}$ ewe groups.

In the combined horns, all of the C ewe groups except for $CP4^{0-6}$ had higher *SPP1* mRNA expression levels than the control $SP4^{n}$ (Table 9.3). In the ipsilateral horn only, CnP4 and $CP4^{0-6}$ ewes had higher *SPP1* expression levels than SnP4 (Table 9.4), and these ewe groups did not differ in their expression of *SPP1* between themselves. $CP4^{3-6}$ had the highest *SPP1* mRNA expression compared to all the C ewe groups. $CP4^{0-3}$, $CP4^{0-6}$ and CP^{2-4} ewes did not

differ in *SPP1* expression in the ipsilateral horn. In the contralateral horn $CP4^{0-3}$, $CP4^{2-4}$ and $CP4^{3-6}$ ewes all had higher *SPP1* expression levels than $CP4^{0-6}$ ewes (Table 9.5).

SERPIN mRNA expression in all the C ewe groups did not differ from the control SnP4 in the combined horn (Table 9.3). CnP4 *SERPIN* mRNA expression was lower than $CP4^{3-6}$ expression, but did not differ from the other C ewe treatment groups in the combined horns. In the ipsilateral horn $CP4^{0-3}$ ewes had higher *SERPIN* mRNA expression than CnP4 and $CP4^{2-4}$ ewes (Table 9.4).

Expression of *RSAD2* mRNA in $CP4^{0-6}$ group was omitted from this study as there were not enough biological replicates (combined horns, n=2; ipsilateral horn, n=1) to reliably examined expression levels when outliers were removed from this group. In the combined horns all other C ewe groups do not differ in *RSAD2* mRNA expression from the control $SP4^{n}$ (Table 9.3). $CP4^{2-4}$ ewes have higher *RSAD2* mRNA expression than all C ewe groups except $CP4^{3-6}$, which do not differ between themselves in the combined horns. When the ipsilateral horn was examined on its own, $CP4^{0-3}$ ewes had lowest *RSAD2* mRNA expression compared to all the other C ewe groups. The comparison of differential expression between groups for combined horns and the horn ipsilateral to the ovary containing the Cl is represented graphically in Appendix XI

9.5 Discussion

The aim of this study was to examine if the timing of administration of P4 in Cheviot (C) ewes during the post-ovulation period, day 0 to 6, caused a difference in uterine gene

expression at day 6 and day 19 consistent with advanced uterine structural and secretory function that may be responsible for the variation in embryo size reported in Chapter 8.

In Chapter 8 it was shown that administration of P4 from day 2 to 4 of pregnancy in C ewes resulted in an increased size of Suffolk (S) embryos, similar to the S embryos gestated in C ewes that were administered exogenous P4 from day 0 to 3 and day 0 to 6. S embryos from C ewes that received P4 were longer than S embryos gestated in C or S ewes that did not receive exogenous P4. This enhanced size was accompanied by higher a pregnancy rate in the $CP4^{2-4}$ group at day 19 compared to the $CP4^{0-3}$ and $CP4^{0-6}$ groups.

Findings reported in Chapter 7 showed exogenous P4 stimulated changes in expression of uterine genes at day 19 of pregnancy that are responsible for structural and secretory activities necessary for receptivity to implantation and embryo growth. It is likely that these changes were responsible for the observed increase in size of S embryos from P4 treated C ewes allowing them to overcome the natural constraint exerted by being gestated in the C ewe. Although similar changes were seen in response to administration of exogenous P4 in the day 19 tissue examined in this chapter, definitive confirmation of the results of Chapter 7 were not possible in this study, since only two ewes out of a possible four were included in the analysis of individual ipsilateral and contralateral horns for the $CP4^{0-6}$ group. The other two ewes of this group contained a CL on both left and right ovaries. All four of these ewes were included in the combined horn analysis. The findings from the examination of day 6 endometrial tissue support the proposed P4 effect of advancing embryo growth through changes in uterine gene expression. To our knowledge, this is the first study to administer exogenous P4 in differing durations during the day 0 to 6 post-ovulatory period in sheep,

and these results provide the first evidence of exogenous P4 alterations of post-ovulatory, day 6, endometrial tissue.

Day 6 uterine gene expression

In the present study *PGR* mRNA expression was up-regulated in the $CP4^{2-4}$ ewe group compared to $CP4^{0-3}$ and $CP4^{0-6}$ groups. It is well established that down-regulation of *PGR* in response to continuous exposure of the endometrium to P4 is necessary for driving many of alterations of structural and secretory mechanism important for conceptus implantation and histotroph production (Spencer and Bazer, 1995; Spencer *et al.*, 2004a). It is has also been suggested that the timing at which P4 concentrations achieve luteolytic levels determines embryo survival rates. The day 6 findings reported here align with the plasma progesterone levels reported in Chapter 8. Combined these results support the proposal that administration of exogenous P4 from day 0 results in an early increase of P4 to luteolytic levels, corresponding down regulation of PGR in the endometrium, and subsequent increased asynchrony between the uterus and the transferred embryo to which the embryo cannot adapt. This may explain the low pregnancy rates in these two groups compared to the other P4 treated groups.

In the present study, *IGF1* mRNA expression was lower in all the C ewe groups compared to the S group at day 6, which aligns with the previously reported difference in *IGF1* expression between these two breeds at day 19 that may account for the difference in size of their natural offspring (Sequeira *et al.*, 2016). Satterfield *et al.* (2008) reports no difference in *IGF1* expression in ovine endometria in response to exogenous P4 administration from day 1.5 to day 9 or to day 12; as does McCarthy *et al.* (2012) in heifers in response to P4 administration from day 3 to 5, 7, 13 or 16. In the present study, there

were no differences in *IGF1* mRNA expression between the C ewe groups with the exception of the $CP4^{0-3}$ ewes in which *IGF1* mRNA expression was lower. This finding adds strength to our proposal that day 3 is a critical period for P4-endometrial driven effects, since the removal of exogenous P4 at day 3 in this group resulted in the observed lower *IGF1* expression levels at day 6.

Day 6 *PTGS2* mRNA expression was up-regulated in $CP4^{0-3}$, $CP4^{0-6}$ and $CP4^{2-4}$ ewes compared to CnP4 ewes, while in $CP4^{3-6}$ ewes *PTGS2* mRNA was down-regulated compared to CnP4 ewes. These results add strength to the proposal that day 3 is a critical period related to the timing of exogenous P4 administration to advance the uterus encouraging accelerated embryo growth while ensuring embryo survival. Up-regulation of *PTGS2* is associated with loss of *PGR* in endometrial LE and sGE (Charpigny *et al.*, 1997). In the present study $CP4^{0-3}$ had the highest expression of *PTGS2* compared to all the groups, which may be related to the removal of exogenous P4 at day 3 since the $CP4^{0-6}$ ewes showed similar *PTGS2* mRNA expression as the $CP4^{2-4}$ group. This suggests that a short exposure to P4 in combination with starting administration at day 0 may result in amplified uterine gene expression effects and result in a uterus advanced beyond a stage to which the implanted embryo can adapt.

In the present study mRNA expression levels of *FGF7*, *FGF10* and *HGF* increased in response to P4 administration. These factors are referred to as “progestomedins” and are thought to mediate the action of P4 to regulate uterine epithelial function during the peri-implantation period since paradoxically *PGR* loss is associated with epithelial changes conducive to implantation (Spencer and Bazer, 1995). Endometrial stroma remains PGR-positive throughout pregnancy and express *FGF7*, *FGF10* and *HGF* (Rubin *et al.*, 1995; Chen

et al., 2000a; 2000b). Presumably, these progestomedins act on the *PGR*-negative epithelium to induce the structural and secretory changes (Chen *et al.*, 2000b; Satterfield *et al.*, 2008a; 2009). In support of this, in the present study administration of P4 resulted in up-regulation of *FGF10* in all of the P4-treated ewes. *HGF* was up-regulated in $CP4^{0-6}$, $CP4^{2-4}$ and $CP4^{3-6}$ and down-regulated in $CP4^{0-3}$. This is the earliest day of pregnancy that these genes have been examined in response to exogenous administration of P4; Satterfield *et al.* (2008) examined day 9 and 12 uterine tissue. These results support our proposed critical day 3 timing of P4 administration on embryo growth and survival, since, in particular, increases in *HGF* expression appear to be optimal in the groups that were administered exogenous P4 around or after day 3 of pregnancy.

DGAT2 mRNA expression was higher in all of the CP4 ewe groups compared to both CnP4 and SnP4 groups at day 6. To our knowledge, this is the first reported examination of *DGAT2* in the ovine endometrium. Previous reports indicate that *DGAT2* mRNA expression in bovine endometrium is stimulated by P4: endogenous (Bauersachs *et al.*, 2005) and exogenous (Forde *et al.*, 2009). *DGAT2* catalyses the terminal step in the synthesis of triacylglyceride (Cases *et al.*, 2001). It is likely that a P4-driven increase in *DGAT2* may increase the availability of this energy source for the developing blastocysts in sheep as has been proposed for cattle (Forde *et al.*, 2009; Ledgard *et al.*, 2012). Thus, the findings reported here align with the proposed importance of timing of P4 increase since the ewes that were administered P4 from day 0 to 3 have the highest *DGAT2* mRNA expression levels at day 6, suggesting that these ewes may have a uterus that is well advanced but to which the transferred embryos cannot adapt.

Day 19 uterine gene expression

When day 19 uterine tissue was examined in the present study *PGR* mRNA expression was down-regulated in $CP4^{0-3}$ and $CP4^{2-4}$ ewes compared to the CnP4 ewes when the combined horns were analysed. $CP4^{0-6}$ *PGR* mRNA expression did not differ in either the combined horns or the ipsilateral or contralateral horns compared to CnP4 ewes. In Chapter 7, the *PGR* mRNA expression did not differ between CP4 ewes (administered from day to 6) and CnP4 ewes in the ipsilateral horn, but was down regulated in the contralateral horn. This suggests an endocrine P4 effect that amplifies the localised paracrine P4 effect in the horn ipsilateral to the P4 secreting CL resulting in the overall down-regulation reported for the combined horns in that study. In the present study, a similar finding was observed as $CP4^{0-3}$, $CP4^{2-4}$ and $CP4^{3-6}$ ewes had down-regulated *PGR* expression in ipsilateral and contralateral horns. There was no difference in *PGR* expression between the $CP4^{0-3}$, $CP4^{2-4}$ and $CP4^{3-6}$ ewes in the combined horns and contralateral horns at day 19 which aligns with an overall stage specific effect of *PGR* to P4 as reported by Spencer and Bazer (1995). In addition, embryo size did not differ between $CP4^{0-3}$, $CP4^{0-6}$, and $CP4^{2-4}$ groups yet importantly they were longer than CnP4 embryos. In the ipsilateral horn $CP4^{0-3}$ ewes had lower *PGR* mRNA expression compared to $CP4^{2-4}$ and $CP4^{3-6}$ ewes. This may be indicative of an effect of the timing of administration of P4, whereby these embryos may have required a greater localised stimulus to ensure survival compared to the $CP4^{2-4}$ and $CP4^{3-6}$ groups.

In the present study, *IGF1* mRNA expression was up-regulated in $CP4^{0-3}$, $CP4^{2-4}$ and $CP4^{3-6}$ ewes in combined, ipsilateral and contralateral horns when compared to CnP4 ewes. Similarly, *IGF1R* was up-regulated in $CP4^{0-3}$, $CP4^{2-4}$ and $CP4^{3-6}$ ewes in combined and ipsilateral horn, but only up-regulated in the contralateral horn of $CP4^{0-3}$ and $CP4^{2-4}$ ewes

compared to CnP4 ewes. IGF1 is known to play an important role in regulation of uterine secretions necessary for driving embryo growth and modulates IGF1R expression in the uterine endometrial glands (Wathes *et al.*, 1998). These findings also support the proposal that exogenous P4 administration advances the uterine environment, resulting in larger embryo size observed at day 19. The higher *IGF1* expression levels of the ipsilateral horn in $CP4^{0-3}$ ewes compared to $CP4^{2-4}$ and $CP4^{3-6}$ ewes are consistent with the findings reported for *PGR* expression in this horn. This adds strength to the proposal that the embryos in this group may have required a greater stimulus to ensure survival and adapt to the uterine environment established in response to earlier rise in P4 concentration.

PTGS2 mRNA expression was up-regulated in $CP4^{2-4}$ and $CP4^{3-6}$ in combined and ipsilateral horns when compared to CnP4 ewes. Additionally, $CP4^{2-4}$ and $CP4^{3-6}$ ewes had higher *PTGS2* mRNA expression in the ipsilateral horn compared to $CP4^{0-3}$. *PTGS2* and prostaglandins increase vascular permeability and cellular adhesiveness (Charpigny *et al.*, 1997). These findings indicate that the timing of P4 administration may have an effect on uterine receptivity to implantation, such that later administration of exogenous P4 (post day 2) and higher levels of *PTGS2* are associated with higher pregnancy rates and increased embryo size in these groups compared to $CP4^{0-3}$ group.

Similar to the findings reported in Chapter 7, *LGALS15* mRNA expression was down-regulated in all the C ewes that received P4 compared to CnP4 ewes in the combined horn. In the ipsilateral horn, only the $CP4^{0-3}$ and $CP4^{0-6}$ ewes had down-regulated *LGALS15* mRNA compared to CnP4 ewes. These findings are similar to the reported decrease at day 18 and 20 compared to 14 to 16 by Grey *et al.* (2004). Previous reports suggested that *LGALS15* exerts its effect on conceptus growth primarily during day 12 to 16 of gestation

stimulated by interferon tau (INFt) (Satterfield *et al.*, 2006). Therefore, these findings give strength to the proposal that at day 19 *LGALS15* expression has stabilised within the uterine endometrium, having passed through a transitory increase that coincides with blastocyst elongation as was suggested in Chapter 7.

MUC1 expression was up-regulated in, $CP4^{2-4}$ and $CP4^{3-6}$ compared to CnP4 in both combined and ipsilateral horns. In the ipsilateral horn $CP4^{0-3}$ *MUC1* mRNA expression was also up-regulated. Again, it appears that timing of P4 administration may have an effect on uterine changes necessary for receptivity. MUC1 is an anti-adhesive protein (Carson *et al.*, 2000; Burghardt *et al.*, 2002), and it is proposed that *MUC1* must be down regulated to allow adhesion during implantation. In Chapter 7, *MUC1* was shown to be up-regulated in response to exogenously administered P4 and it was proposed that, in the sheep, exogenous P4 resulted in an increase in total *MUC1* but localised reduction at implantation sites, similar to the response seen in rabbits (Hoffman *et al.*, 1998). In the present study, this proposal might further be refined to state that timing of administration of P4 may alter *MUC1* expression since the ewes that received P4 from day 3 to 6 had higher *MUC1* expression levels compared to the $CP4^{0-3}$ and $CP4^{2-4}$ ewes in the ipsilateral horn. It is difficult however to conclude that the effects observed here are related to the higher embryo pregnancy rates in the $CP4^{2-4}$ and $CP4^{3-6}$ groups.

In the present study *CTSL* expression was up-regulated only in $CP4^{2-4}$ ewes in the combined and contralateral horns compared to CnP4 ewes. There was no difference in *CTSL* expression between $CP4^{0-3}$ and $CP4^{2-4}$ when the ipsilateral horn was examined. *CTSL* is an INFτ stimulated gene that is induced by P4 (Song *et al.*, 2005). It is proposed that *CTSL* regulates receptivity to implantation (Salamonsen, 1999). Therefore, the higher expression

levels of *CTSL* in the P4 administered groups are no surprise, since it is likely that the larger embryos produce greater quantities of INFτ and subsequently induced higher expression levels compared to the CnP4 group. The higher *CTSL* expression in $CP4^{3-6}$ group, further justifies the theory of differential effects due to timing of P4 administration. *CTSL* expression however, may parallel the INFτ secretion pattern of the elongating conceptus (Song *et al.*, 2005), such that the later administration of P4 in the $CP4^{3-6}$ group coincides with a delayed elongation and thus delayed INFτ secretion. Since the mechanisms by which INFτ, in association with P4, act to moderate the epithelial expression of *CTSL* are not yet understood, it may be bold to suggest that the larger $CP4^{0-3}$ and $CP4^{2-4}$ embryos would be at a further stage of implantation and therefore have decreasing INFτ secretion coincident with the lower *CTSL* in these groups compared to $CP4^{3-6}$ ewes. It may also be possible that these embryos are attempting to halt the advancement of their uterine environment to ensure that they do not grow beyond their natural genetic potential, with detrimental consequences later in pregnancy. Future studies are needed to examine the P4 regulation of INFτ stimulated genes.

RSAD2, another INFτ stimulated gene associated with establishing uterine receptivity (Song *et al.*, 2007) had the highest mRNA expression levels in $CP4^{2-4}$ ewes compared to CnP4 and $CP4^{0-3}$ ewes in the combined horn. In the ipsilateral horn of CnP4, $CP4^{2-4}$, $CP4^{3-6}$ ewes *RSAD2* mRNA expression levels did not differ between each other but were higher than $CP4^{0-3}$. It is likely that the timing of administration of P4 affected the expression of this gene with lower *RSAD2* in the ipsilateral horn of $CP4^{0-3}$ ewes likely associated with earlier embryo implantation in this group, and therefore decreased INFτ and localised *RSAD2* as suggested

by Song et al., (2007). The lower *RSAD2* expression levels in these ewes may also be related to the low pregnancy rates in this group.

SERPIN is a secretory protein important to blastocyst growth, but is thought to be expressed in response to long periods of P4 exposure (Stewart *et al.*, 2000). Whilst expression levels of *SERPIN* mRNA were similar in the all the P4 groups compared to CnP4, with the exception of $CP4^{3-6}$ which was higher when the combined horns were examined, expression levels varied between groups in the ipsilateral horn. $CP4^{0-3}$ ewes had higher *SERPIN* mRNA expression compared to CnP4 and $CP4^{2-4}$, but did not differ from $CP4^{3-6}$ or $CP4^{0-6}$ ewes. In the contralateral horn $CP4^{2-4}$ was the only group that showed up-regulated *SERPIN* compared to CnP4 ewes. In Chapter 7, it was proposed that exogenous P4 administration did not alter *SERPIN* expression at day 19 since it was only administered for 7 days. Similarly, in the present study, differences in the timing of P4 administration does not appear to have affected *SERPIN* expression to the point where a definitive conclusion could be made about the changes reported here and how these may relate to observed embryo size or survival.

SPP1 (also known as osteopontin) is another important secretory product of the glandular epithelium. It is major component of histotroph and is thought to play an important role in uterine implantation (Johnson *et al.*, 2003). Johnson *et al.* (2000) demonstrated increased *SPP1* expression in ovine uterine endometria at day 25 in response to exogenous P4 administered from day 5 to 24. In contrast, in the present study, $CP4^{0-6}$ and $CP4^{2-4}$ ewes had down-regulated *SPP1* mRNA when combined horns were examined compared to CnP4 ewes, while $CP4^{0-3}$ and $CP4^{2-4}$ *SPP1* mRNA were down-regulated in the ipsilateral horn. *SPP1* was observed to have no difference in expression levels in CnP4 and

$CP4^{3-6}$ ewes. It is possible that the embryo itself may play a role in regulating the effects of *SPP1* via INFτ or placental lactogen (PL), secreted from day 16 (Johnson *et al.*, 1999a; Johnson *et al.*, 2000). It is therefore possible that in the present study the implanting embryo may limit expression of *SPP1* in the $CP4^{0-3}$ and $CP4^{2-4}$ groups following P4-induced accelerated growth to ensure that they do not advance beyond their genetic potential. Whereas, being smaller in size, $CP4^{3-6}$ embryos may not elicit this feedback to control uterine *SPP1* expression.

9.6 Summary and conclusion

The findings of this study support the idea that exogenous P4 administration causes structural and secretory changes consistent with an advanced physiological state of the uterine endometrium and subsequent acceleration of embryo growth to day 19. These endometrial changes, for example up-regulation of *DGAT2*, *HGF* and *PTGS2* are induced in the uterus in the immediate post-ovulatory period (day 6) in the absence of the embryo. Observed differences at day 19 include up-regulation of *IGF1*, *IGF1R* and *MUC1* in the ipsilateral horn, *PTGS2* in contralateral horn and down-regulation of *LGALS15* in the combined horns, suggesting that the effects of exogenous P4 are continued to day 19 of pregnancy. Day 3 appears to be a critical stage before which administration of P4 results in an asynchronous uterine environment, that whilst encouraging embryo growth is apparently hostile to embryo survival. Investigation is warranted into whether pregnancy losses occur due to luteolysis as a result of failure of the embryo to advance to match the stage of pregnancy reflected in the uterus and produce sufficient quantities of INFτ to abrogate this process. Additionally, examination of embryonic/conceptus signalling mechanisms are

needed to confirm the hypothesis that the embryo/conceptus can reciprocally regulate the uterine environment to ensure it is not forced to grow beyond its natural genetic potential resulting in complications or losses later in pregnancy. Overall, the results of the present study, allow for further development of a model in which early exogenous P4 administration can be used to advance embryo development and overcome the effects of early pregnancy maternal constraint.

10 General Discussion

10.1 Overview of thesis

Previous research has demonstrated that maternal constraint during early gestation exerts profound effects on the development and growth trajectory of embryos with consequences to birth size and survival (Hancock *et al.*, 2012; Sharma *et al.*, 2012a; 2012b; 2013). Specifically, large breed Suffolk (S) embryos were smaller in size at birth, when gestated in small breed Cheviot (C) ewes compared to control S ewes (Sharma *et al.*, 2012a), with these differences in size being observed as early as day 19 of gestation (Sharma *et al.*, 2013). Similarly, Hancock *et al.,* (2012) showed that the smaller size of twin-born lambs compared to singletons may be determined by events occurring in early gestation, before day 41. The aim of this thesis was to build on these previous findings and further examine the effects of genetically and physiologically restricted uterine environments on embryo development. Specific objectives were to examine the embryo transcriptome to determine the embryo's role in driving its growth trajectory and to investigate expression of uterine genes that code for hormone and growth factors that relate to altered uterine secretory and structural function. Studies were limited to the early pre-implantation period of gestation, so as to determine maternal involvement in control of embryo growth, while also examining a possible method of improving embryo growth and survival.

Briefly, chapter 3 investigated differences in size and transcriptome of singleton and twin embryos at day 21 of gestation. Chapter 4 and 5 compared size and developmental characteristics of embryos of C and S breeds and plasma hormone concentrations of C and S ewes at day 19 and day 21 of pregnancy. Chapter 6 examined the effects of administration of exogenous progesterone (P4) from day 0 to 6 to alleviate the effects of constraint due to maternal size in reciprocally embryo transferred (ET) C and S embryos. Chapter 7 examined

uterine gene expression changes in C and S ewes in response to administration of P4 to determine mechanisms that might account for the differing embryo sizes observed in Chapter 6. Chapter 8 examined if altering timing and length of P4 administration during the immediate post-ovulatory period, day 0 to 6, might achieve a similar increase in embryo size as seen in chapter 6 without the associated decrease in embryo survival. Chapter 9 examined gene expression of uterine endometrial tissue at day 6 and day 19 to determine if the effect of differing time of P4 administration could be related to differentially advanced and asynchronous uterine environments thereby explaining the embryo size differences and pregnancy rates of Chapter 8.

The following sections will focus on the general outcomes, results, conclusions and implications of the experiments of this thesis. Identified limitations and weaknesses of the research will also be discussed, followed by recommendations for future research. Finally, the main conclusions of this research will be summarised.

10.2 Summary of main findings and conclusions drawn

10.2.1 Embryo size and development in the peri-implantation period

Embryo size differences were observed at day 19 and 21 in response to the differential environments of both models of maternal constraint. In the dam size model it was determined that differential size observed at day 19 occurs between pure-bred C and S embryos gestated in ewes of their respective breed, similar to the findings of Sharma *et al.*, (2013) (Chapter 4). Differences in embryo size were also seen when reciprocal ETs were performed between the C and S ewes (Chapters 6 and 8). Although, in the singleton vs twin

model only one embryo group showed a difference in size at day 21 compared to the other groups (the singleton embryos that were transferred to ewes that had one corpus luteum (CL), 1E1CL). This did not rule out the possibility that growth trajectory is determined in early gestation in this model (Chapter 3). Further, it was determined that size differences could be used as an accurate indication of developmental stage, as assessed by both developmental rank and somite number. This indicates that physical embryo size is consistent with anatomical development (Chapter 5). These findings supported previous suggestions that growth and development are programmed in the early pre-implantation period (Smith *et al.*, 1998; Salomon *et al.*, 2011) and more importantly that maternal constraint effects are evident during this early pregnancy period as suggested by Hancock *et al.* (2012) and Sharma *et al.* (2013).

10.2.2 Embryonic gene expression

In Chapter 3, it was determined that day 21 embryos have many complex biological processes occurring, represented by the large number of gene transcripts that were detected in the whole-embryo samples examined. No detectable differences were found between embryo groups in the differential expression of genes by RNA-seq analysis. Further, the analysis indicated that the biological variation between individual embryos within the experimental group was greater than the differences in gene expression as a result of the group to which they were assigned. This may be associated with heterogeneity of the embryonic tissue as suggested by Taylor *et al.*, (2003), possibly due to differences in time of ovulation and fertilisation even though donor ewes were oestrous synchronised. Quantitative real time PCR (qPCR) of the same embryo samples only partially validated the RNA-seq findings, and although some differential expression was observed between the

pair-wise comparisons using this method, it provided little information about the embryo's role in molecular regulation of embryo growth in day 21 singletons and twins. Overall, the results of this study are inconclusive.

10.2.3 Effects of progesterone on embryo growth

Progesterone (P4) administered from day 0 to 6 of pregnancy overcame the apparent constraint of maternal size up to day 19 in a C/S sheep model (Chapter 6, 7, 8 and 9). The studies in these chapters added strength to the suggestion that P4 acts via indirect stimulation of the uterus, rather than via a direct effect on the embryo itself (Clemente *et al.*, 2009; Forde *et al.*, 2009). Further, these studies confirm the reports that the embryo need not be present within the uterus at the time of administration of exogenous P4 (Clemente *et al.*, 2009).

Corresponding examination of maternal plasma P4 concentrations demonstrated that day 3 of pregnancy appears to be the critical period for both determining development and survival. If P4 concentration rises to luteolytic levels before day 3 then embryo survival is reduced; although, an effect of increased embryo growth is possible, as evidenced by the experimental groups that received P4 from day 0 to 3 and day 0 to 6 in Chapters 6 and 8. Administration of P4 from day 2 to 4 and day 3 to 6 resulted in enhanced embryo size at day 19 without the associated pregnancy loss in these treatment groups (Chapter 8).

Day 19 uterine gene expression of the pregnant ewes added strength to previous studies, confirming that P4 advances uterine secretory and structural activity via up- or down-regulation of the genes that encode for these processes (Gray *et al.*, 2006; Satterfield *et al.*, 2006;2009; Forde *et al.*, 2009) (Chapters 7 and 9). Indeed, it appears that this P4-advanced

uterine environment resulted in asynchrony between embryos and the maternal environment, which induced the embryo to accelerate its growth in order to adapt. This resulted in the observed size differences and the ability to overcome maternal constraint at this stage of gestation. This advanced uterine environment is evident even before the embryo is transferred at day 6 (Chapter 9). It should be acknowledged, however, that results of these studies also highlighted the role of the embryo in this important maternal-conceptus dialogue, whereby, it appeared that gene regulation may also have been "checked" by the accelerated embryo in order to limit growth in an environment that potentially could drive it beyond its natural genetic potential. This was particularly evident by the differences in gene expression of P4-induced, interferon tau (INFτ) stimulated genes such as endometrial galectin 15 (LGALS15) and cathepsin L (CTSL) (Chapter 7 and 9).

10.3 Methodological considerations

Sufficient numbers of biological replicates per treatment group are needed to ensure accuracy of size effects. However, the study reported in Chapter 3 was limited by the number of embryos that were successfully obtained in some of the experimental groups. An attempt was made to achieve sufficient numbers by conducting the experiment over two years. However, in year two the number of single CL ovulators was much lower than twin ovulators which resulted in the lowered embryo numbers in these groups. If the experiment were to be repeated it would be worthwhile to include a larger pool of recipient ewes to ensure sufficient numbers of embryos are obtained. Additionally, the accuracy of identifying the number of ovulations during ET via laparotomy, which allowed visualisation of the ovaries and the CLs present, should be considered. In some of the twin ovulators, the

operator reported differing quality between the CL, indicating questionable viability of one or both CLs, in spite of the presence of corresponding twin embryos. This may have contributed to the losses between transfer and euthanasia in the 2CL ewe groups, since CL-secreted P4 is vital to maintaining pregnancy. This was also a consideration for the 2E1CL transfer group, and raises the question of whether the reduced number of embryos between ET and euthanasia, as well as reductions to 1E1CL, were an effect of limited CL-produced P4 by the recipient ewe. Another consideration is that the twin embryos may have been obtained from a ewe which had a compromised CL such that the embryo was already disadvantaged before transfer to the recipient ewe. The study reported here lacked an examination of maternal plasma P4 concentrations which would have added insight to an important aspect of the differences between single- and twin-bearing ewes, but may have confirmed the reasons offered here as an explanation for pregnancy losses and reductions.

In Chapter 4, the investigation had to be confined to a comparison of twin C and S embryos. In this study it was intended that an adequate number of ewes carrying singleton embryos would be obtained allowing a more comprehensive evaluation of differences in embryo size in early per-implantation between the two breeds. A major drawback in this study was that only twin pregnancies were obtained from the C cohort restricting the examination to twins in both Chapters 4 and 5. It would be worthwhile to increase the number of C ewes in the cohort to allow for a greater chance of obtaining both singleton and twin pregnancies if the experiment were to be repeated.

The main limitation of Chapters 6 and 7 was that there were low pregnancy rates for the treatment groups, C and S ewes that received P4 from day 0 to 6. However, this effect of P4 appeared to be consistent with administration from day 0 and the effect was investigated

with a positive outcome in Chapters 8 and 9, when P4 was administered at different time points. These findings were consistent with the suggestion that if time of luteolysis was advanced by administration of P4, such that the embryo was not able to abrogate the impending luteolysis, in spite of accelerated growth, then loss of pregnancy is imminent. This early induction of luteolysis and associated pregnancy losses in the recipient ewes administered P4 from day 0 to 6 (Chapter 6) and day 0 to 3 (Chapter 8) may also have been amplified by higher concentrations of plasma P4 reported at day 0 in these groups (>1.0 ng/mL), before insertion of the treatment CIDRs. The higher P4 concentration may be indicative of asynchrony between the individual animals used in the study with respect to time of ovulation in spite of synchronisation. It should be noted that in Chapter 6 all ewe groups had mean plasma P4 concentrations greater than 1.0 ng/mL at day 0; however, many of the ewes that were sampled for hormone analysis did not receive embryos. In the study in Chapter 6, the hormonal analysis was merely to test if exogenous P4 altered plasma concentrations over the time period and to examine the hormone profile.

The variation in time of ovulation and fertilisation must also be considered with respect to asynchrony between donors and recipients. Taylor *et al.* (2003) suggested an effect of superovulation procedures on age of individual embryos and synchrony between donors. In this study, efforts were made to transfer embryos that were at a similar stage of development, appropriate for transfer (late morula, early blastocyst and blastocyst) and balanced between treatment groups. These steps should have minimised the potential errors due to the effects of asynchrony as best could be achieved with current techniques.

The day 19 samples examined in Chapters 7 and 9 consisted of mixed uterine tissue comprising myometrium and endothelium. Genes were chosen for differential expression

examination based on their function in altering uterine endometrial secretory and structural activity. However, the expression levels have been reported to differ significantly within different areas of the endometrium (stroma, epithelium: luminal and superficial glandular, caruncular and inter-caruncular). Therefore, although the results of Chapter 7 and 9 provide a global view of the total endometrial expression levels at day 19, variations between areas, especially in cases where a gene may be up-regulated in one area but down-regulated in another may have obscured the true picture of what is occurring at a molecular level in the endometrial tissue. To determine these differences a molecular method that is tailored to assess the differences in expression in specific areas within the uterine endometrium would have to be used.

10.4 Recommendations for future research

The experiments in this thesis focused on developmental differences of peri-implantation embryos. Specifically, in Chapter 3 the aim was to examine molecular differences between twin and singleton embryos at day 21. It was proven that RNA-seq analysis of whole embryos does not provide this information because of the heterogeneity of embryonic tissue at this point. Repeating RNA-seq using cell or site specific samples may be worthwhile to determining the differential expression of genes within the embryo transcriptome. Use of another method such as *in situ* hybridisation would allow for a molecular biological examination of these early gestational stage embryos if the experiment were to be repeated. There is still much to be deciphered about how singleton and twin embryos regulate their growth, resulting in the differences reported at birth. The focus of the experiment reported here was to examine the embryo's role in driving the differential

growth reported previously. However, there may be an important maternal aspect that has been neglected and so examination of the uterine endometrial tissue by gene expression and immune-histochemical methods and maternal plasma P4 concentrations is warranted. If the mechanisms that are at work are known this could be of benefit to both the livestock industry and human obstetrics by allowing opportunities to ensure the survival of twins.

The results of this thesis indicate that embryos may have a potentially important role of controlling the environment they are in, to ensure they do not exceed their genetic limitations. It would be interesting therefore, to include an *in situ* hybridisation examination of the stored embryos from the studies conducted in Chapters 6 and 8 in order to examine this further. Such a study would provide valuable insight into the embryo-maternal dialogue.

Further investigation of uterine tissue, using immuno-histochemistry or *in situ* hybridisation methods, would allow determination of which areas of the endometrium are actively involved in advancement of the uterus, and how this may be related to the maternal constraint effects and P4's alleviation of these effects. Future studies could consider determination of the concentration of specific proteins secreted into the uterine lumen. For example examination of osteopontin (SPP1), uterine milk proteins (SERPIN) and INFτ concentrations would go a long way toward determining if the gene expression responses to P4 administration are translated into changes to the histotrophic nutrition of the embryo which would help explain the accelerated growth. In addition, studies investigating *PGR* functionality may also add valuable insight into the action of P4 at this endometrial level to drive structural and secretory changes.

The model of constraint due to maternal size was restricted in this study to peri-implantation events, only examining day 19 differences. Previous studies using the same

dam size model of maternal constraint reported differences in size at birth but not at day 55 of pregnancy (Jenkinson *et al.*, 2007; Sharma *et al.*, 2012a; 2012b). Knowing what the long-term effects of P4 supplementation as it relates to overcoming constraint would be invaluable to the livestock industry. Thus, it would be worthwhile to repeat the study such that examination of conceptus size and development, and uterine changes are extended to serial time points between days 19 through to birth. This should include measurements of placentomes and the area of uterus that is occupied by the placenta. This would confirm the proposed theory that exogenous P4 administration indirectly allows for an increased number of implantation sites on both ipsilateral and contralateral horns of the uterus. A larger pool of ewes (approximately 800) would be needed to conduct an experiment on this scale. However, limiting the treatment groups to three ($CP4^{0-3}$, $CP4^{2-4}$ and $CP4^{3-6}$) and a control (CnP4) would reduce this number, but also allow larger cohorts within treatment groups to confirm the embryo survival effects. There may also be merit in investigating the reproductive performance of ewes with naturally higher P4 concentrations during early pregnancy to determine if they have improved embryo growth, survival and overall reproductive success.

Equally as important would be to expand the examination of early P4 supplementation on pregnancy rates. Measuring plasma concentrations of P4 daily from day 0 to 21, would determine if a difference truly existed in the timing and concentration of luteolytic P4 in ewes that received P4 from day 0 to 3 and day 0 to 6 compared to those that received P4 from day 2 to 4 and day 3 to 6; Recovery of embryos at day 14 or 16 with examination of uterine tissue would also give greater insight into when uterine changes are initiated,

identify when the actual loss of transferred embryo occurs and what molecular mechanisms may be driving the failure of maternal recognition of pregnancy.

10.5 Practical implications

The results of the study on maternal dam size constraint and progesterone's effect within this model offers exciting and interesting prospects for improving growth and survival of offspring. Progesterone can possibly be used as a tool for advancement of growth in early pregnancy, and although yet to be confirmed possibly extend to birth. However, before this can be implemented, the question "Is P4 supplementation of "at risk" ewes an option?" should be addressed. "At risk" ewes may be those that are identified as producing small lambs from previous lambings, are smaller breeds of sheep or highly fecund sheep producing larger litter sizes such as triplets. Firstly, it must be ensured that pregnancy rates are acceptable or else the cost will out-weigh the benefit. Secondly, confirmation is needed that the effects reported here at day 19 are carried to birth with increased birth weight and survival of offspring in supplemented ewes, and long-term productivity with economic benefit to the livestock industry. Thirdly, important consideration of the need of some constraint is necessary to avoid complications at birth for example dystocia. Once all of these issues can be managed, and with the increasing possibilities of artificial reproductive techniques, this opens the door to increasingly sophisticated manipulations that may have incredible benefits to livestock productivity and human obstetrics. Administration of exogenous progesterone may potentially have an important role in improving the success of ET and *in vitro* fertilisation programmes, whereby giving progesterone to control the time of changes of the uterus, thereby strategically regulating the uterine environment may allow for flexibility and control of ET. The findings of this study also suggest that there may be

merit in selecting ewes with naturally higher P4 concentrations, and in so doing improve embryo survival, growth and overall reproductive success.

10.6 Overall summary and conclusions

This thesis examined the effects of genetically and physiologically restricted uterine environments on embryonic development. The findings of the studies presented herein have led to the following conclusions:

- Examination of the embryo transcriptome via extraction of RNA from whole embryos is difficult because of the inter-embryo variation due to mixed tissue and the complex and multiple processes of differentiation and development that are occurring throughout the embryo
- Embryo development can be accurately assessed by embryo length, developmental rank or somite count
- Maternal constraint occurs in early pregnancy when limitations of space are not of consequence.
- Progesterone plays a role, not only in maternal recognition of pregnancy but also in driving the growth and development of the embryo acting through the differential expression of genes in the uterus/endometrium.
- Day 3 is a critical time-point for progesterone action, allowing for maximising the opportunity for improved pregnancy and embryo growth with minimal embryo loss.

This thesis has added to the knowledge of mechanisms that act during early pregnancy to determine embryo growth and development. Overall, these are important findings that can

be used to develop manipulations which may improve productivity in the livestock industry by influencing embryo survival and growth. Moreover, these findings may be extrapolated to human obstetrical practice as it gives insight into important mechanisms for growth and development of embryos.

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Appendices

Appendix I

Arithmetic mean of individual samples, total arithmetic mean, average standard deviation (STDEV) and coefficient of variation (CV%) of potential references genes assessed for stability for selection to be used to normalise qPCR validation of embryo gene expression. The gene data is listed in order of most stable to least stable left to right. *ACTB*, *RPL19* and *GAPDH* (**bold**) were most stable (lowest CV%) therefore chosen as candidate reference genes.

			Reference Genes						
Sample ID	**Experimental group**[1]	**Embryo**	***ACTB***	***RPL19***	***GAPDH***	*Cyclophilin A*	*18S*	*YWHAZ*	*HPRT1*
59	Con1E1CL	Em1	24.23131	25.06921	24.52581	24.16812	12.92968	25.02286	29.72628
63	Con1E1CL	Em1	24.37485	25.16254	25.22227	24.81520	13.41793	25.75251	29.99195
3	1E1CL	Em1	24.56363	25.06628	24.58616	24.44825	13.19820	25.21137	29.25613
37	1E1CL	Em1	24.68798	24.98608	24.52207	24.53392	13.09772	26.45356	30.79399
21	1E2CL	Em1	24.90271	24.97925	24.99679	24.88772	13.38062	25.35070	29.56271
53	1E2CL	Em1	24.81498	25.12684	25.06240	24.68076	13.09341	25.16991	29.97198
84	1E2CL	Em1	24.58758	25.05516	24.95745	24.55650	12.99123	25.11223	29.69819
99	1E2CL	Em1	24.71713	25.41029	25.04889	24.83154	13.66267	25.28228	29.97840
41	Con2E2CL	Em1	24.61943	25.07446	25.08852	24.47731	13.08820	24.75997	29.31752
41	Con2E2CL	Em2	24.46667	25.40330	25.14916	24.55176	13.00591	24.96084	30.22168
70	Con2E2CL	Em1	24.90452	25.64462	25.72079	25.39855	12.95386	26.57232	31.08328
70	Con2E2CL	Em2	25.17707	25.50452	25.32675	25.26294	13.62053	27.23314	32.30086
86	Con2E2CL	Em1	25.15251	25.83656	26.00791	25.25891	13.25068	25.70214	30.33917
10	2E1CL	Em1	24.66647	25.21577	24.98054	24.74926	13.26470	25.56032	30.04094
10	2E1CL	Em2	25.01728	25.48461	25.25225	25.23476	13.02837	26.83422	32.30295
95	2E1CL	Em1	24.66317	25.46392	24.98311	24.53629	13.10199	25.11243	29.50369
5	2E2CL	Em1	24.94498	25.12720	25.07326	24.59212	13.03215	25.05139	28.93529
5	2E2CL	Em2	24.78025	25.16047	25.37750	24.81148	13.47965	24.93755	28.94995
48	2E2CL	Em1	24.63523	24.84046	24.48617	24.13308	12.66870	24.47758	29.08278
48	2E2CL	Em2	25.07769	25.63617	25.23084	25.19498	13.26292	25.14859	29.86684
73	2E2CL	Em1	24.74371	24.89751	24.82055	24.58327	12.96799	25.43255	29.45833
	Arithmetic mean		**24.74901**	**25.24501**	**25.06758**	24.74794	13.16653	25.48278	30.01823
	Average STDEV		**0.193421**	**0.230848**	**0.264136**	0.282472	0.194203	0.545592	0.662403
	CV%		**0.781531**	**0.914431**	**1.053697**	1.141395	1.474974	2.141021	2.206669

[1]Experimental groups: 1E1CL = single embryo transferred to a ewe that was singleton bearing with a single CL; 1E2CL = single embryo transferred to a ewe that was twin bearing and had two CLs; 2E1CL = twin embryo that were transferred to a ewe that was singleton bearing and had a single CL; 2E2CL = twin embryo that was transferred to a ewe that was twin bearing and had two CLs, Con1E1CL = control (no ET) singleton embryo, recovered from a ewe with a single CL ; Con2E2CL = control (no ET) twin embryo recovered from a ewe with two CL.

Appendix II Reads and mapping statistics of RNA-seq data for each embryo samples.
Samples were generated from whole embryo RNA. Embryos were harvested at day 19 of gestation. Average and percent mapped for each experimental group is shown in bold.

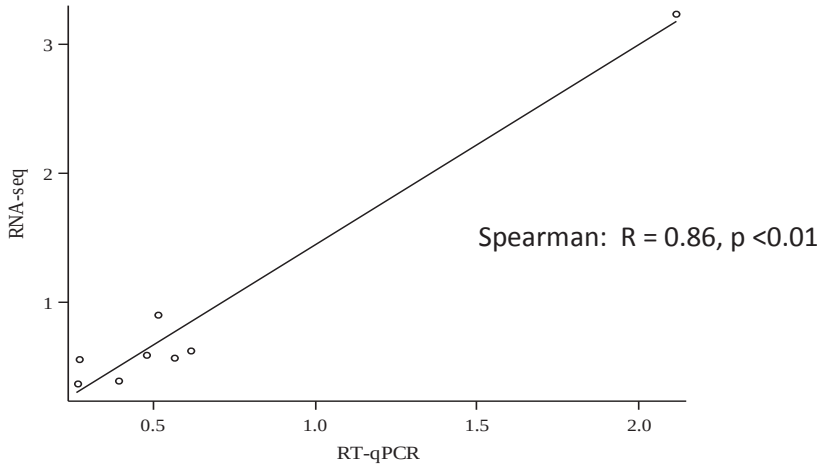
Sample ID			Total no. paired end reads	% reads mapped to the
Ewe ID	Embryo	Group[1]	(mapped and unmapped)	genome (OARv3.2)
59	Em1	C1E1CL	3,948,806	74.63
63	Em1	C1E1CL	8,455,052	74.06
131	Em1	C1E1CL	8,010,498	75.59
146	Em1	C1E1CL	3,589,952	69.73
		Mean	**6,001,077**	**73.50**
3	Em1	1E1CL	4,413,664	71.21
37	Em1	1E1CL	4,391,224	65.88
110	Em1	1E1CL	3,580,180	71.85
155	Em1	1E1CL	3,367,012	71.51
		Mean	**3,779,472**	**70.11**
21	Em1	1E2CL	9,063,960	74.81
53	Em1	1E2CL	3,515,048	71.25
84	Em1	1E2CL	2,611,946	66.99
99	Em1	1E2CL	3,207,752	71.53
106	Em1	1E2CL	2,664,480	69.62
112	Em1	1E2CL	3,946,162	73.25
169	Em1	1E2CL	4,579,754	75.76
1642	Em1	1E2CL	7,302,560	75.98
1733	Em1	1E2CL	2,460,008	69.09
		Mean	**4,372,408**	**72.03**
41	Em1	C2E2CL	3,858,382	73.94
41	Em2	C2E2CL	2,946,752	71.37
70	Em1	C2E2CL	2,637,568	65.08
70	Em2	C2E2CL	2,907,028	67.37
86	Em1	C2E2CL	4,113,216	65.43
86	Em2	C2E2CL	4,407,096	71.39
135	Em1	C2E2CL	3,883,696	68.93
135	Em2	C2E2CL	3,038,896	72.77
149	Em1	C2E2CL	4,954,998	74.78
149	Em2	C2E2CL	2,080,754	70.38
		Mean	**3,482,839**	**70.14**
10	Em1	2E1CL	4,061,228	72.82
10	Em2	2E1CL	5,461,542	71.32
95	Em1	2E1CL	7,441,626	69.81
95	Em2	2E1CL	4,332,060	63.60
100	Em1	2E1CL	4,045,790	71.80
100	Em2	2E1CL	5,453,912	75.87
		Mean	**5,132,693**	**70.87**
5	Em1	2E2CL	5,754,230	74.79
5	Em2	2E2CL	2,936,302	72.65
48	Em1	2E2CL	5,360,732	73.37
48	Em2	2E2CL	2,248,858	72.52
73	Em1	2E2CL	4,335,752	70.25
73	Em2	2E2CL	6,091,936	70.43
1656	Em1	2E2CL	3,337,924	68.69
1656	Em2	2E2CL	2,629,788	71.17
1679	Em1	2E2CL	4,203,444	69.56
1679	Em2	2E2CL	4,880,790	75.10
		Mean	**4,177,976**	**71.85**
	Total Mean		**4,337,497**	**71.35**

[1]Experimental groups: 1E1CL = single embryo transferred to a ewe that was singleton bearing with a single CL; 1E2CL = single embryo transferred to a ewe that was twin bearing and had two CLs; 2E1CL = twin embryo that were transferred to a ewe that was singleton bearing and had a single CL; 2E2CL = twin embryo that was transferred to a ewe that was twin bearing and had two CLs, Con1E1CL = control (no ET) singleton embryo, recovered from a ewe with a single CL ; Con2E2CL = control (no ET) twin embryo recovered from a ewe with two CL.

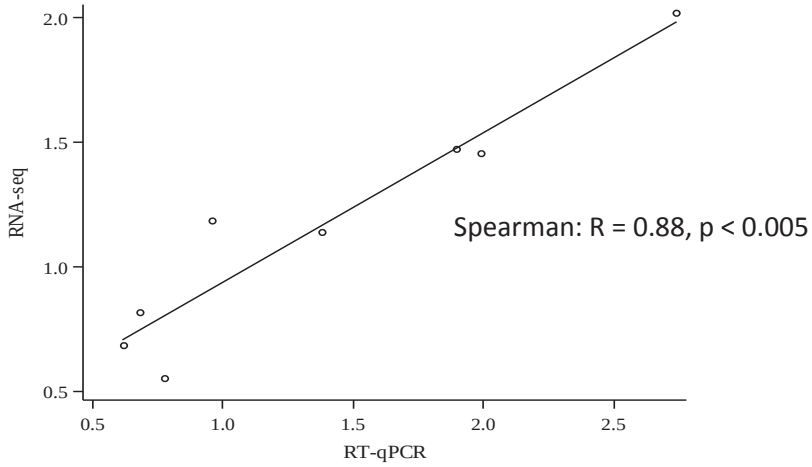
Appendix III

Graphical illustration of correlation of gene expression fold change determined by RNA-seq and reverse transcriptase quantitative PCR (qPCR) for all embryo group comparisons.

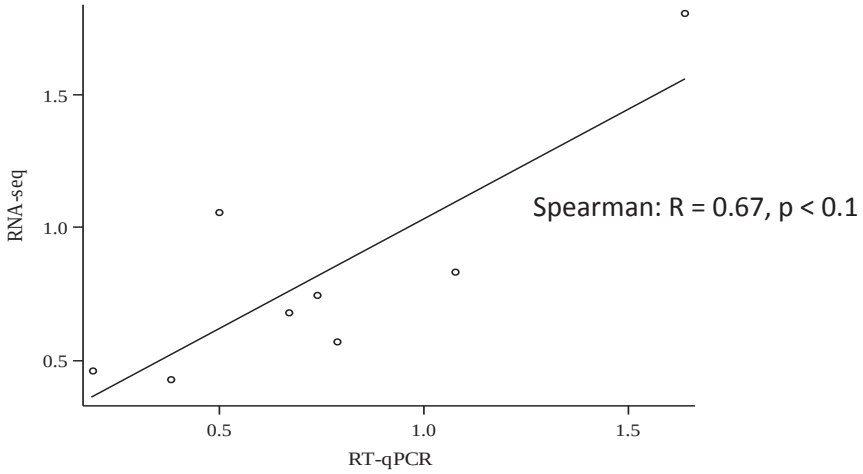
A. 1E1CL vs 2E2CL



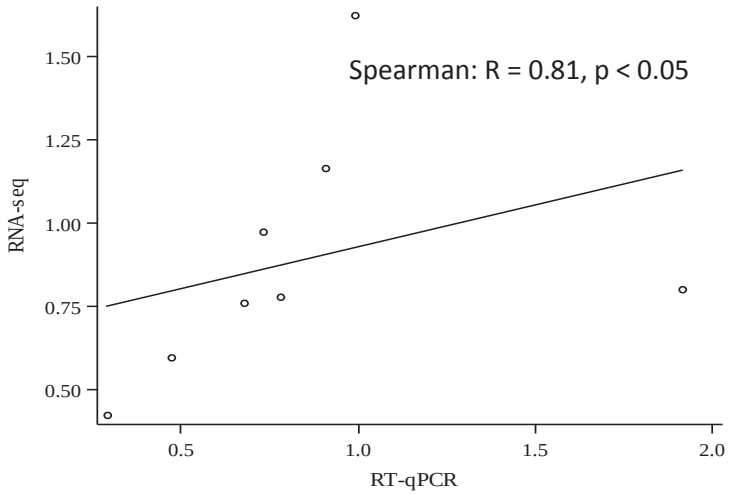
B. 2E2CL vs 2E1CL



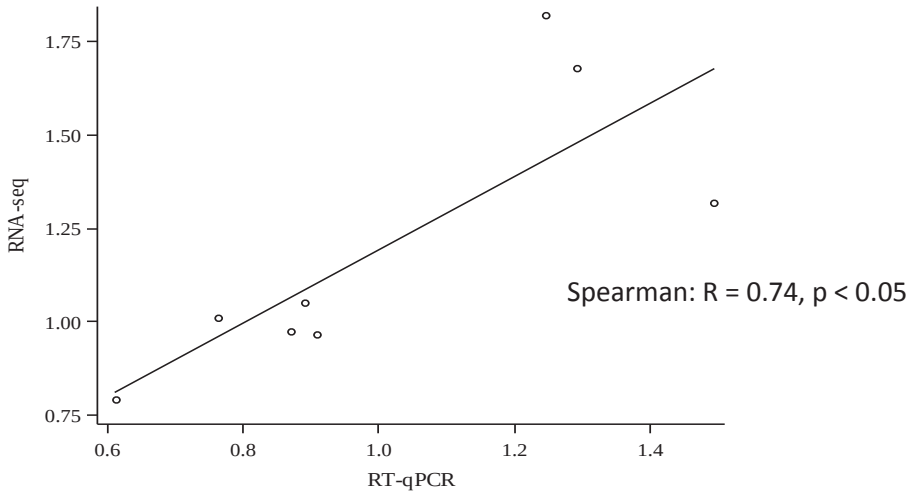
C. 1E2CL vs 2E1CL



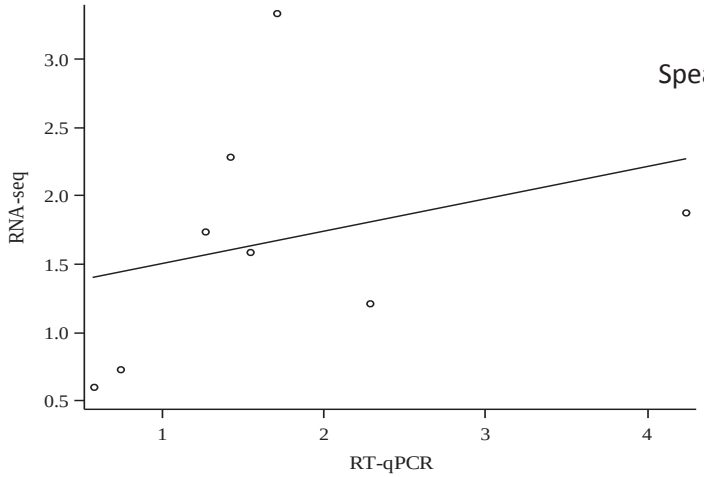
D. 2E2CL vs Con2E2CL



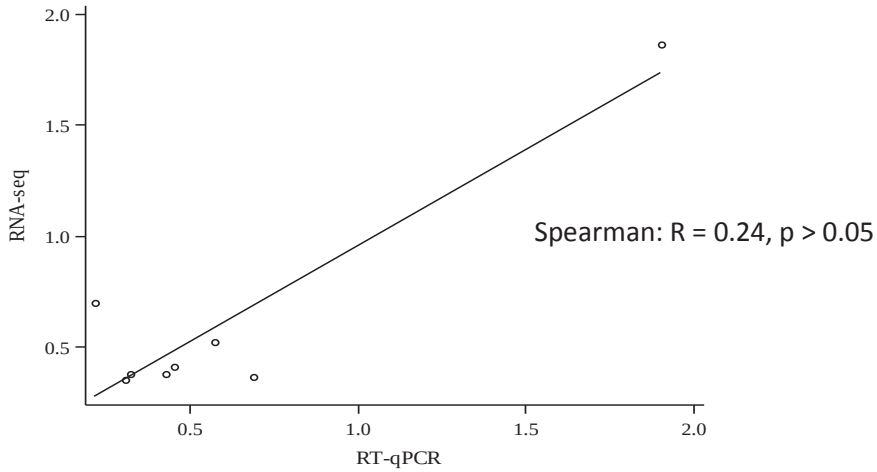
E. all 1E vs all 2E



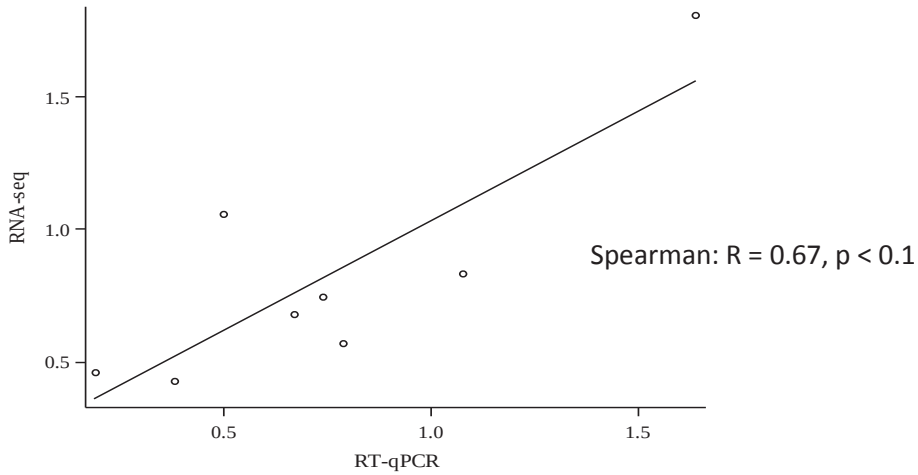
F. 1E1CL vs Con 1E1CL



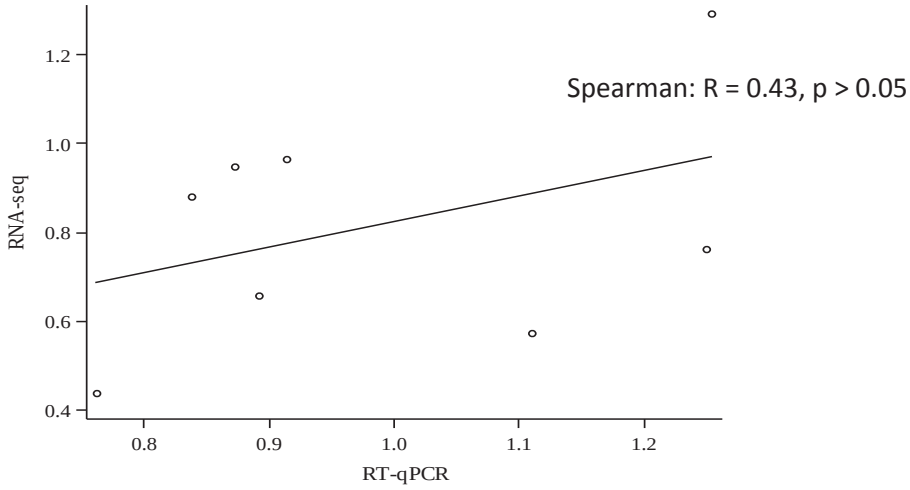
G. 1E1CL vs 1E2CL



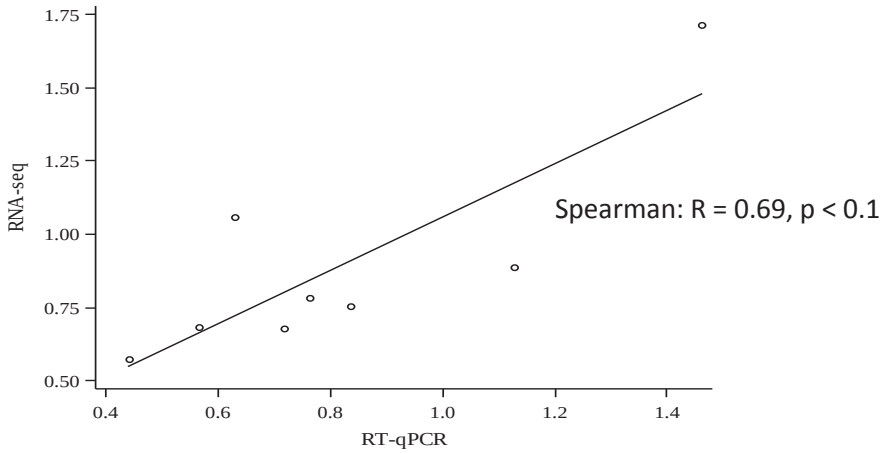
H. 1E1CL vs 2E1CL



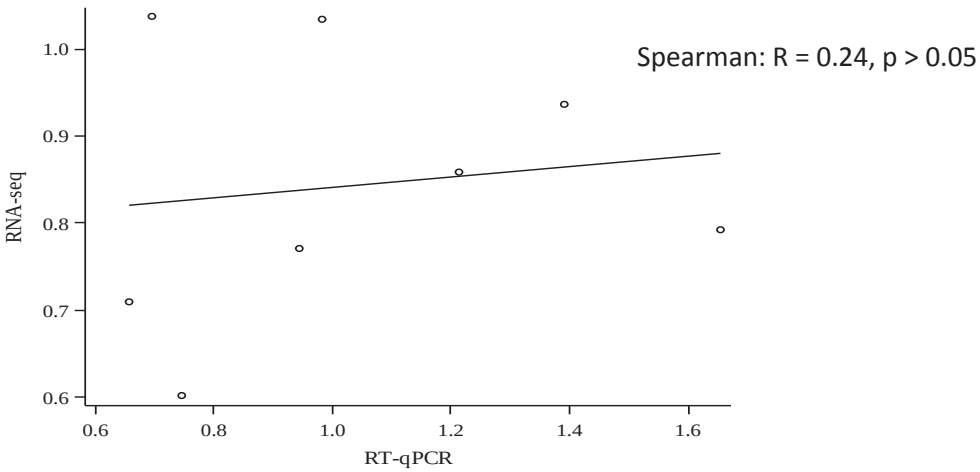
I. 2E2CL vs 1E2CL



J. all 1CL vs all 2CL



K. Con2E2CL vs Con1E1CL



Appendix IV

Gene names and description of function of the ten genes that were examined by qPCR to determine differential gene expression in singleton and twin embryos reciprocally transferred into ewes with 1 or 2 corpus luteum/corpora lutea (CL/s). These genes were used for validation of RNA-seq data.

Gene ID	Gene name	Description of function
DKK4	Dickkopf Wnt signaling pathway inhibitor 4	Involved in embryonic development through its interactions with the Wnt signaling pathway
ISG17	Interferon stimulated gene 17	Up-regulated in the ruminant uterus in response to interferon-tau (IFNτ) during early pregnancy
LAPTM5	Lysosomal-associated protein transmembrane 5	May have a special functional role during embryogenesis and in adult hematopoietic cells
LGALS3	Lectin, galactose binding, soluble 3	Required for terminal differentiation of columnar epithelial cells during early embryogenesis
LGALS15	Lectin galactoside-binding soluable 15	Biological role in cell adhesion and migration, in sheep expressed in the luminal and superficial glandular endothelium of uterus. It is associated with blastocysts elongation (attachment factor).
LOC101103603	**Predicted:** Pregnancy-associated glycoprotein 4-like	Aspartic-type endopeptidase activity
LOC101117738	**Uncharacterised:** Pregnancy-associated glycoprotein 1-like	Aspartic-type endopeptidase activity
LRRC32	Leucine rich repeat containing 32	Expressed in various areas in the mid-gestation developing embryo, including skin, lens fibre cells, nasal cavity, smooth and skeletal muscles, lung and megakaryocytes of the fetal liver
TKDP1	Trophoblast Kunitz domain protein 1	May play a role in mediating maternal-conceptus interactions in the immediate preimplantation period
TP-1	Trophoblast protein 1	May play a role in mediating maternal-conceptus interactions in the immediate preimplantation period

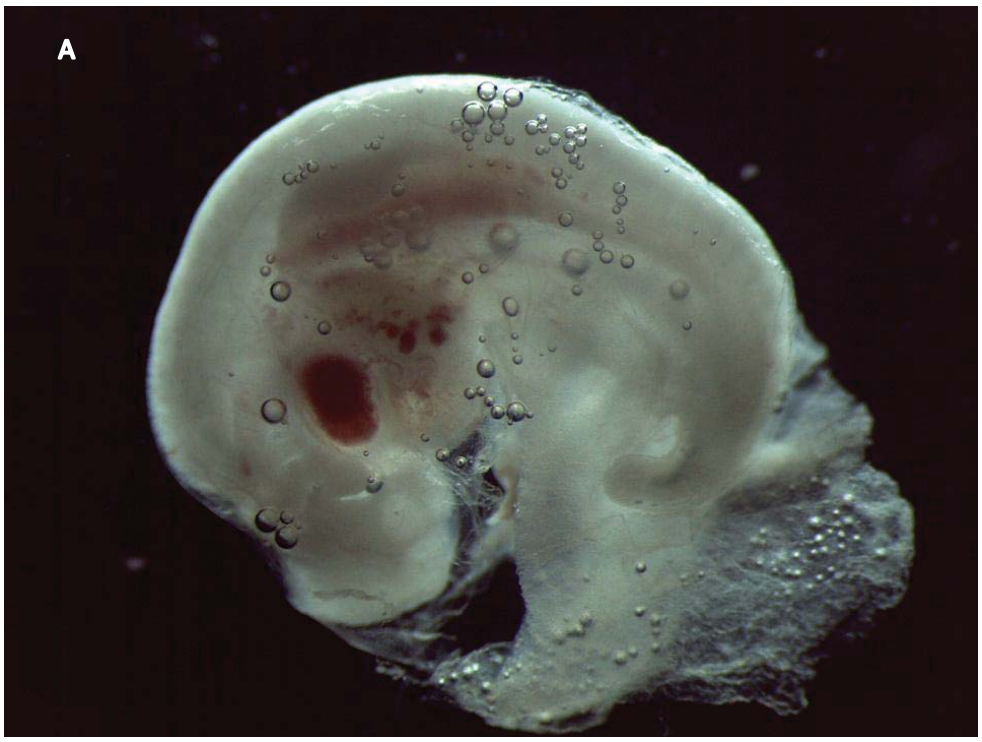
Appendix V

Suffolk and Cheviot uterine weight, uterine body length and body width at day 19 and 21 of gestation when body weight was not fitted as a covariate

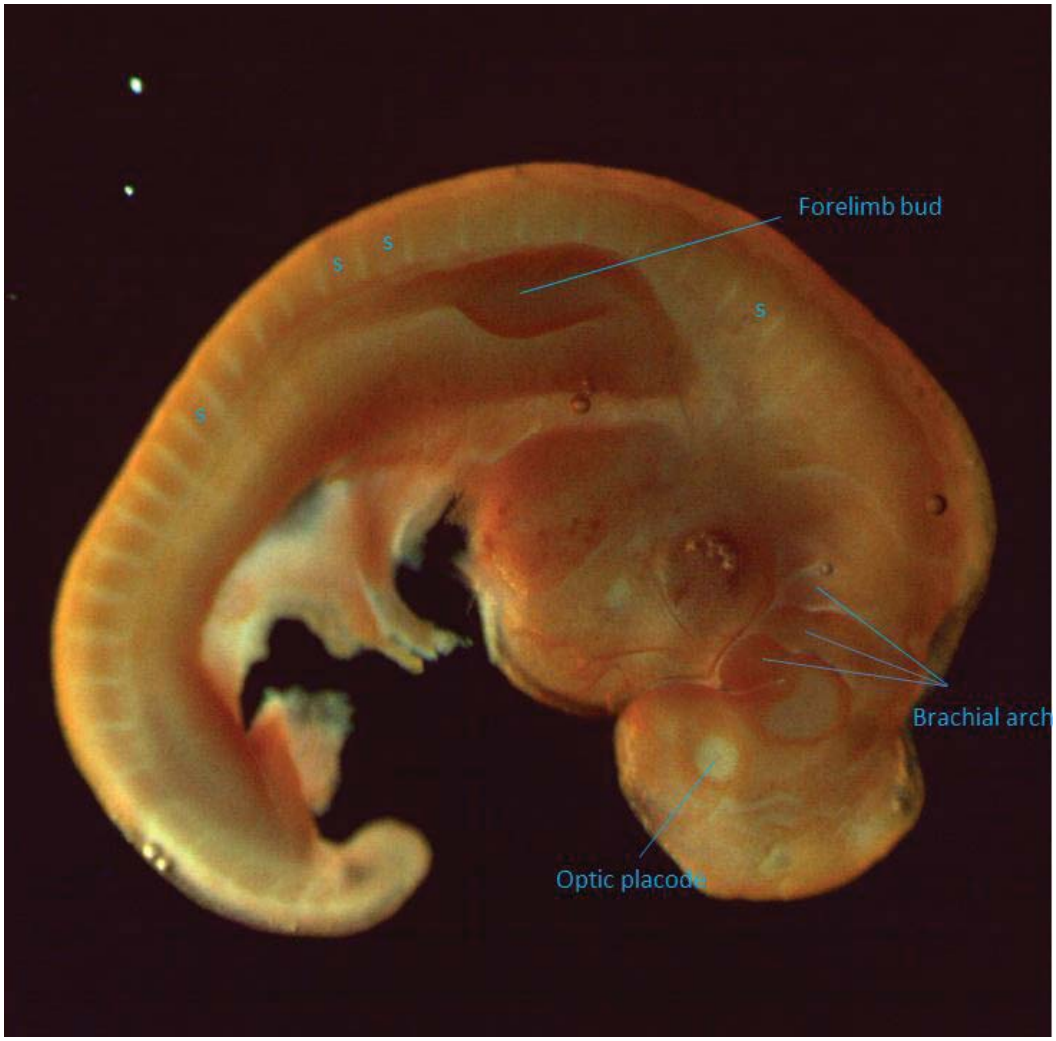
		Breed	
		Suffolk	Cheviot
day19		*n=4*	*n=6*
	Uterine weight (g)	92.61 ± 11.08	84.72 ± 9.04
	Uterine body length (mm)	47.50 ± 6.25	63.0 ± 5.10
	Uterine body width (mm)	45.50 ± 1.78[b]	36.00 ± 1.43[a]
day 21		*n=4*	*n=10*
	Uterine weight (g)	98.48 ± 7.36	91.05 ± 4.65
	Uterine body length (mm)	56.50 ± 3.43[a]	67.60 ± 2.17[b]
	Uterine body width (mm)	46.00 ± 1.70	42.20 ± 1.07

Values are least squares means ± standard error of the mean. Different superscripts within main effects indicate significant differences ($p<0.05$).

Appendix VI



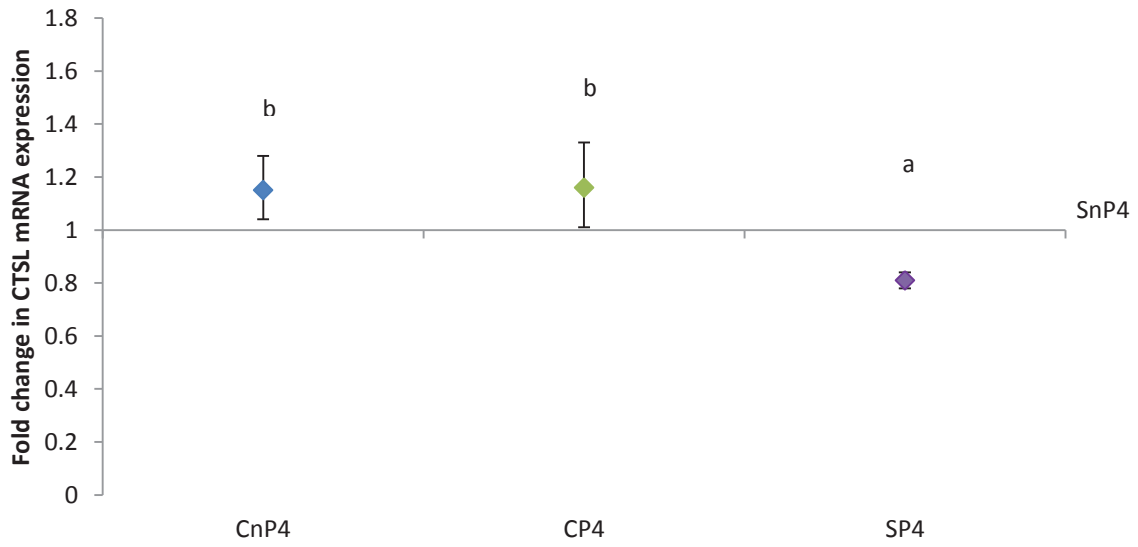
Appendix VI Figure 1 Developmental Stage “3” embryo thawed from OCT (A) and dissected of extra-embryonic membranes (B)



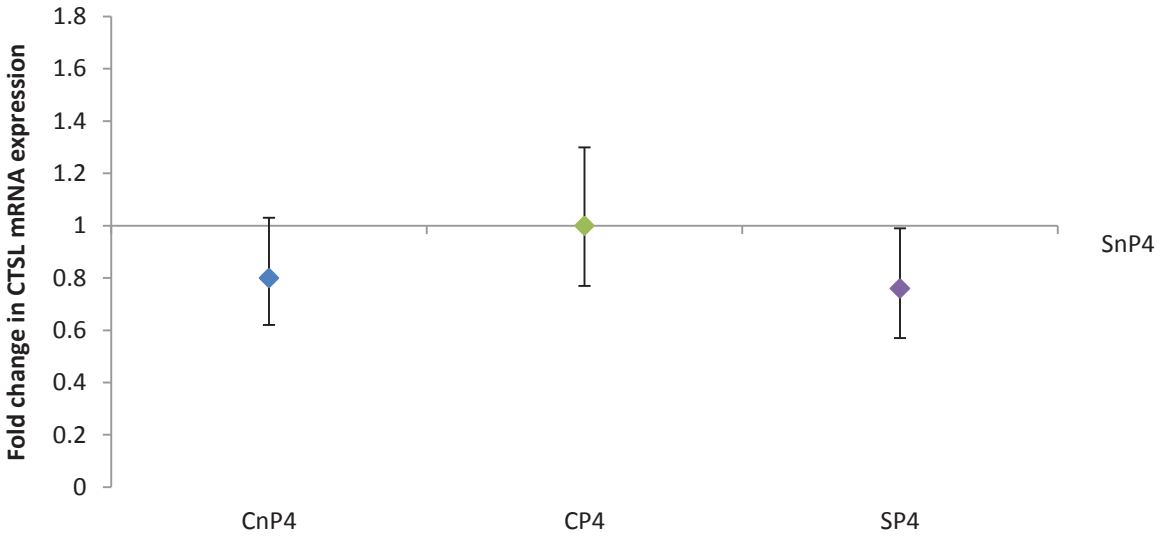
Appendix VI Figure 2 Developmental stage “3” embryo stained with Ponceau S highlighting somites (s) along the greater curvature.

Appendix VII

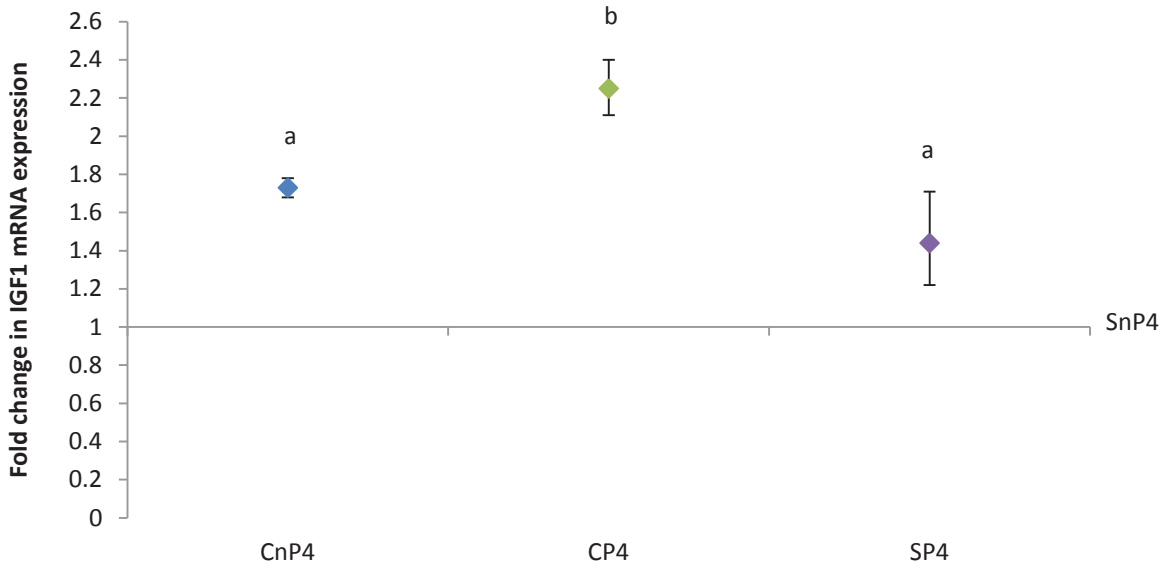
Graphical representations of day 19 uterine horn mRNA expression of candidate genes in Cheviot and Suffolk ewes that were or were not administered exogenous progesterone.



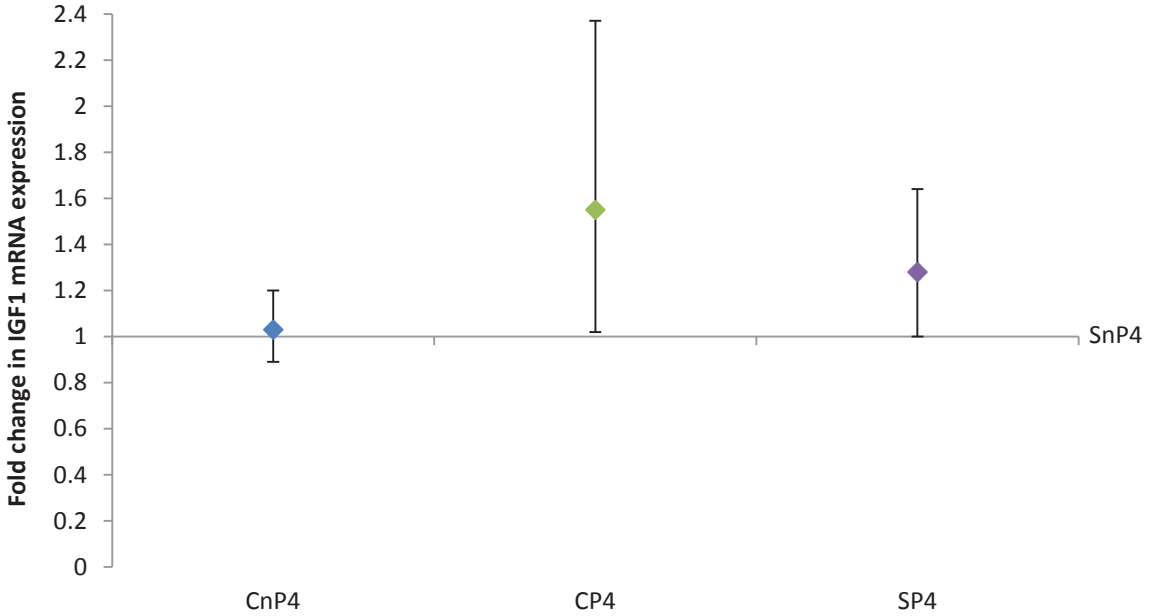
Appendix VII Figure 1: Differential mRNA expression levels of *CTSL* in day 19 in combined uterine horn tissue of pregnant Cheviot and Suffolk ewes that were or were not administered exogenous progesterone (CP4, CnP4, SP4) compared to control group pregnant Suffolk ewes that were not administered exogenous progesterone (SnP4). Data is shown as a fold change with 95% confidence interval. If the confidence intervals cross the x axis then mRNA expression differs from the control group. Different letters indicate significant difference between groups.



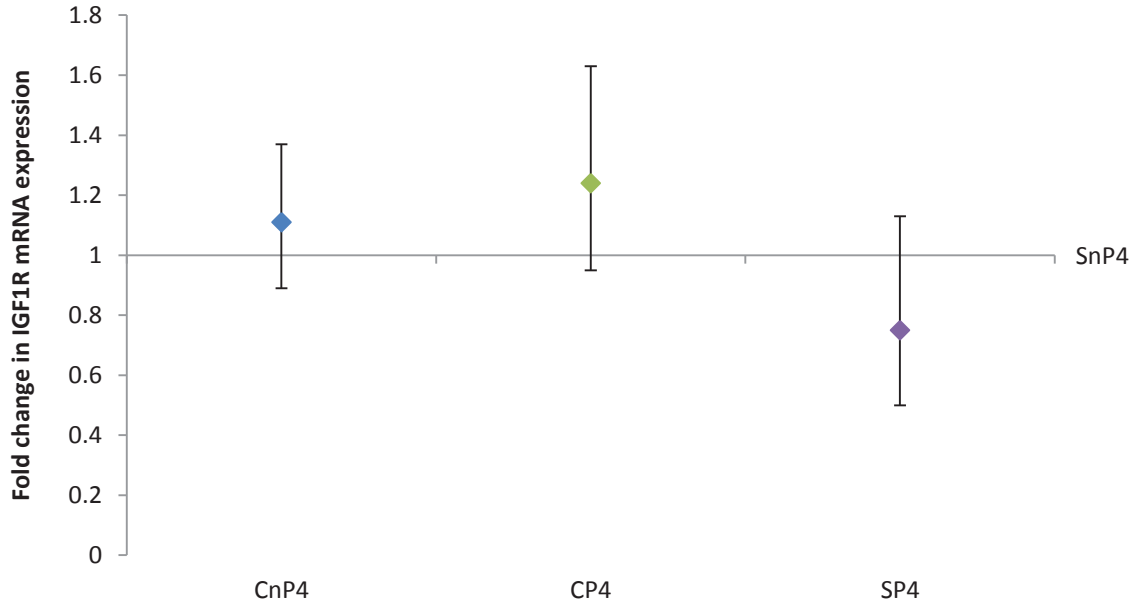
Appendix VII Figure 2: Differential mRNA expression levels of *CTSL* in day 19 uterine horns tissue ipsilateral to the CL of pregnant Cheviot and Suffolk ewes that were or were not administered exogenous progesterone (CP4, CnP4, SP4) compared to control group pregnant Suffolk ewes that were not administered exogenous progesterone (SnP4). Data is shown as a fold change with 95% confidence interval. If the confidence intervals cross the x axis then mRNA expression differs from the control group. Different letters indicate significant difference between groups.



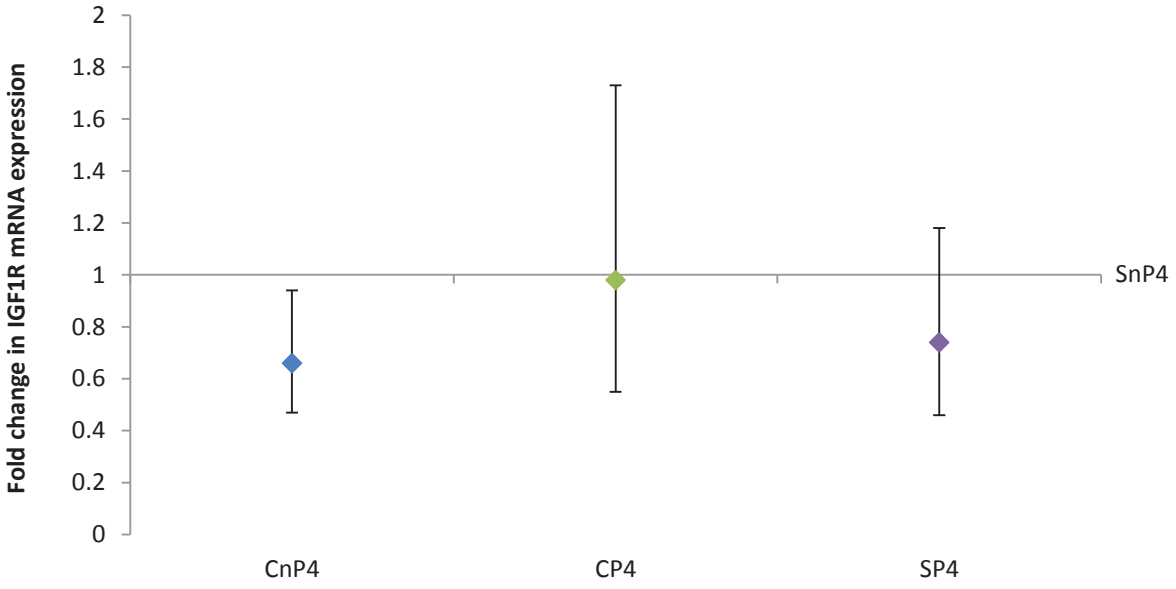
Appendix VII Figure 3: Differential mRNA expression levels of *IGF1* in day 19 in combined uterine horn tissue of pregnant Cheviot and Suffolk ewes that were or were not administered exogenous progesterone (CP4, CnP4, SP4) compared to control group pregnant Suffolk ewes that were not administered exogenous progesterone (SnP4). Data is shown as a fold change with 95% confidence interval. If the confidence intervals cross the x axis then mRNA expression differs from the control group. Different letters indicate significant difference between groups.



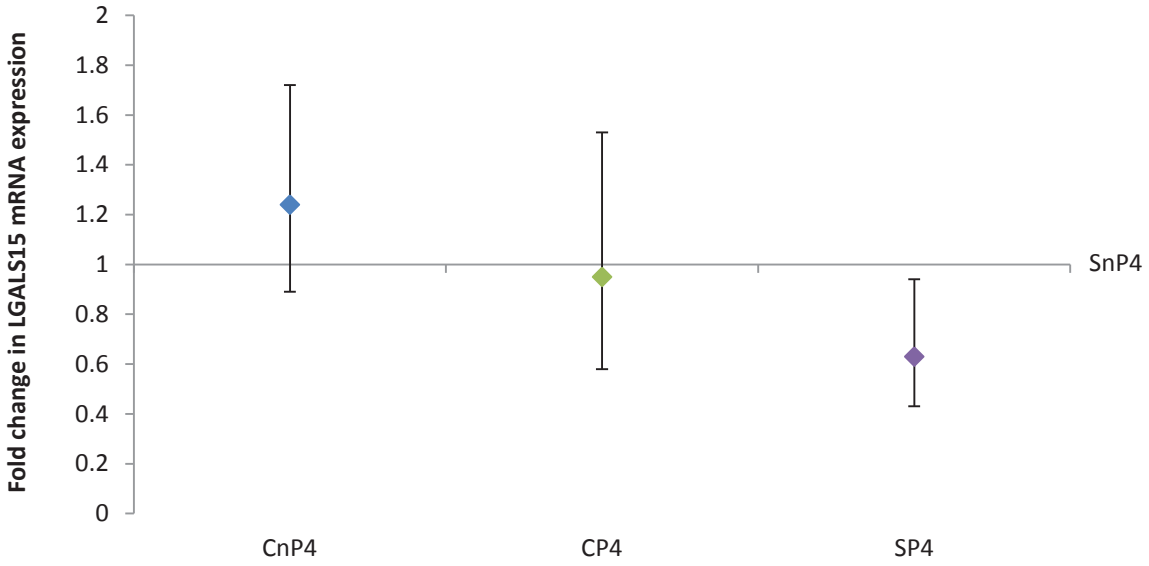
Appendix VII Figure 4: Differential mRNA expression levels of *IGF1* in day 19 uterine horns tissue ipsilateral to the CL of pregnant Cheviot and Suffolk ewes that were or were not administered exogenous progesterone (CP4, CnP4, SP4) compared to control group pregnant Suffolk ewes that were not administered exogenous progesterone (SnP4). Data is shown as a fold change with 95% confidence interval. If the confidence intervals cross the x axis then mRNA expression differs from the control group. Different letters indicate significant difference between groups.



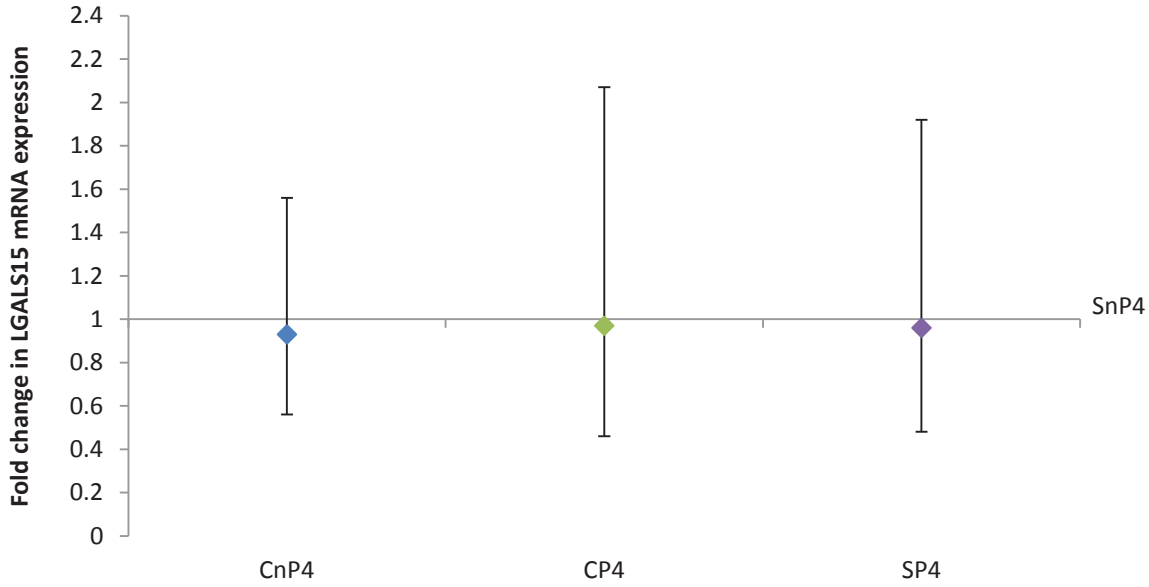
Appendix VII Figure 5: Differential mRNA expression levels of *IGF1R* in day 19 in combined uterine horn tissue of pregnant Cheviot and Suffolk ewes that were or were not administered exogenous progesterone (CP4, CnP4, SP4) compared to control group pregnant Suffolk ewes that were not administered exogenous progesterone (SnP4). Data is shown as a fold change with 95% confidence interval. If the confidence intervals cross the x axis then mRNA expression differs from the control group. Different letters indicate significant difference between groups.



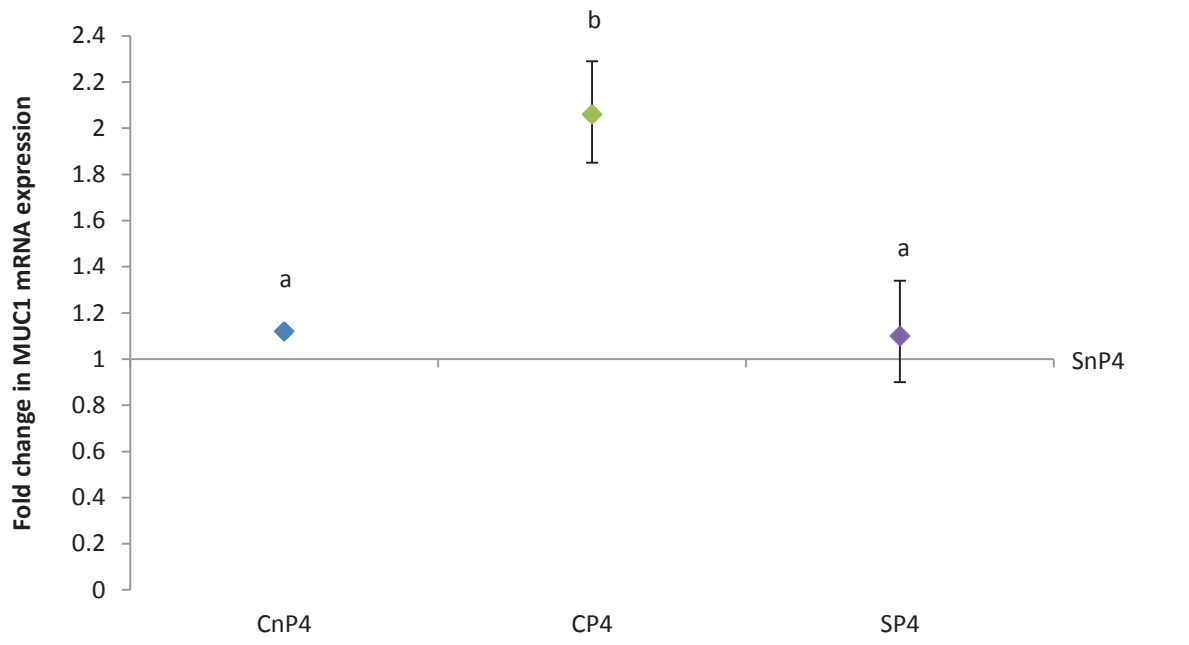
Appendix VII Figure 6: Differential mRNA expression levels of *IGF1R* in day 19 uterine horns tissue ipsilateral to the CL of pregnant Cheviot and Suffolk ewes that were or were not administered exogenous progesterone (CP4, CnP4, SP4) compared to control group pregnant Suffolk ewes that were not administered exogenous progesterone (SnP4). Data is shown as a fold change with 95% confidence interval. If the confidence intervals cross the x axis then mRNA expression differs from the control group. Different letters indicate significant difference between groups.



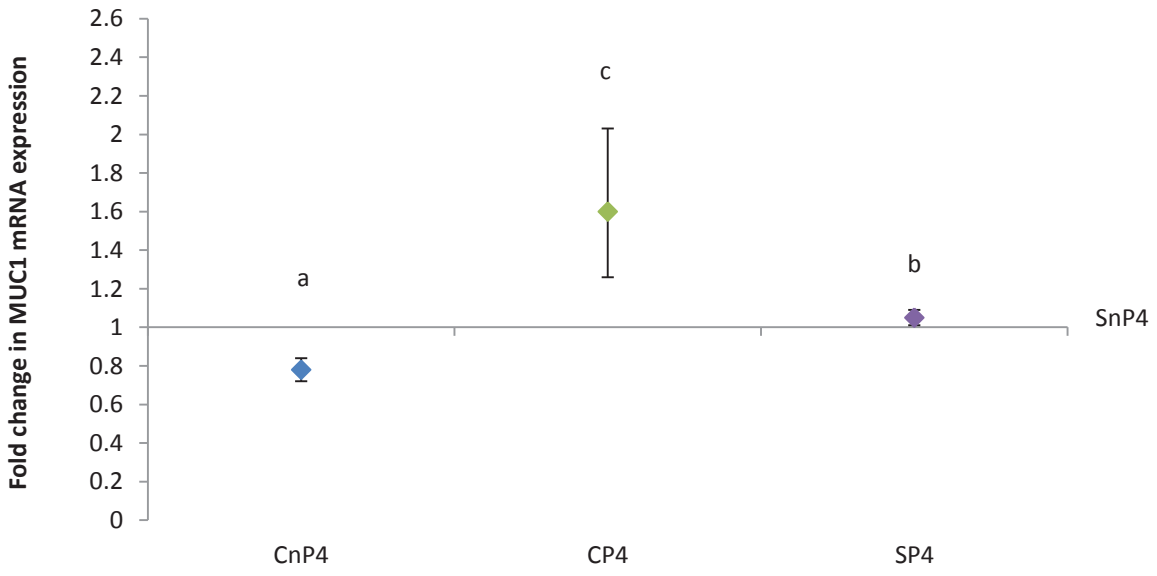
Appendix VII Figure 7: Differential mRNA expression levels of *LGALS15* in day 19 in combined uterine horn tissue of pregnant Cheviot and Suffolk ewes that were or were not administered exogenous progesterone (CP4, CnP4, SP4) compared to control group pregnant Suffolk ewes that were not administered exogenous progesterone (SnP4). Data is shown as a fold change with 95% confidence interval. If the confidence intervals cross the x axis then mRNA expression differs from the control group. Different letters indicate significant difference between groups.



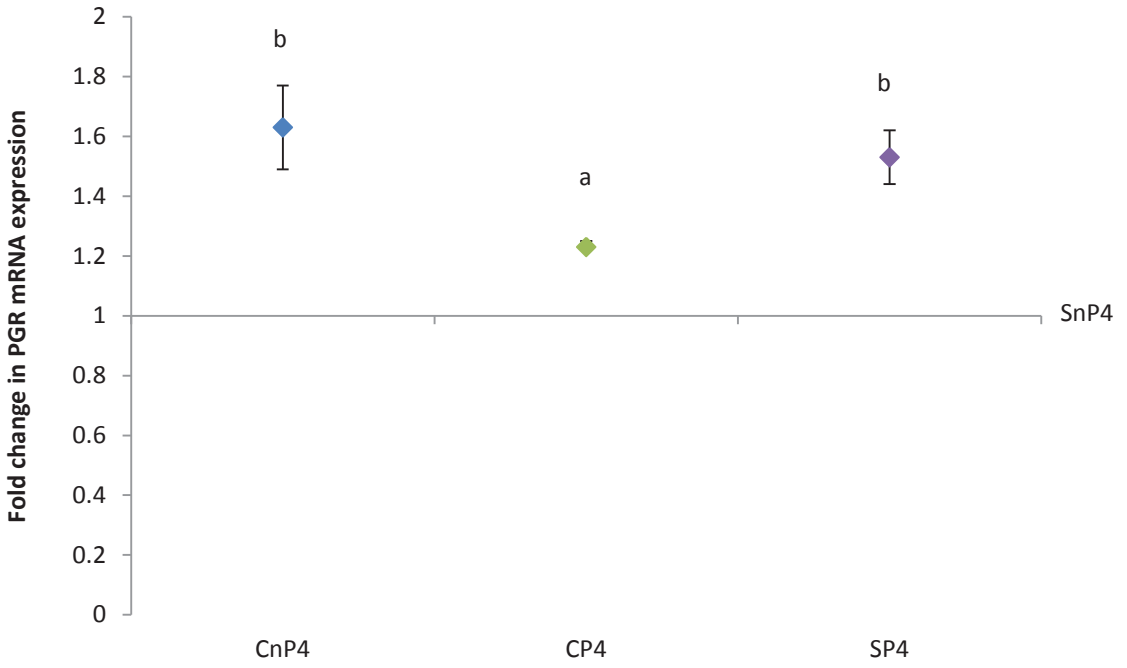
Appendix VII Figure 8: Differential mRNA expression levels of *LGALS15* in day 19 uterine horns tissue ipsilateral to the CL of pregnant Cheviot and Suffolk ewes that were or were not administered exogenous progesterone (CP4, CnP4, SP4) compared to control group pregnant Suffolk ewes that were not administered exogenous progesterone (SnP4). Data is shown as a fold change with 95% confidence interval. If the confidence intervals cross the x axis then mRNA expression differs from the control group. Different letters indicate significant difference between groups.



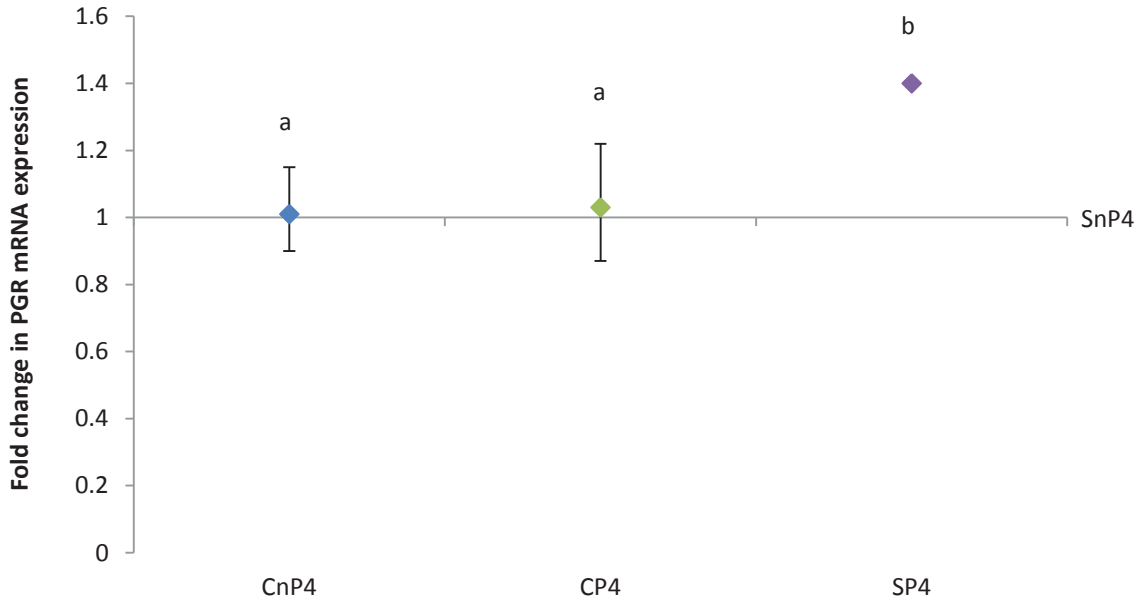
Appendix VII Figure 9: Differential mRNA expression levels of *MUC1* in day 19 in combined uterine horn tissue of pregnant Cheviot and Suffolk ewes that were or were not administered exogenous progesterone (CP4, CnP4, SP4) compared to control group pregnant Suffolk ewes that were not administered exogenous progesterone (SnP4). Data is shown as a fold change with 95% confidence interval. If the confidence intervals cross the x axis then mRNA expression differs from the control group. Different letters indicate significant difference between groups.



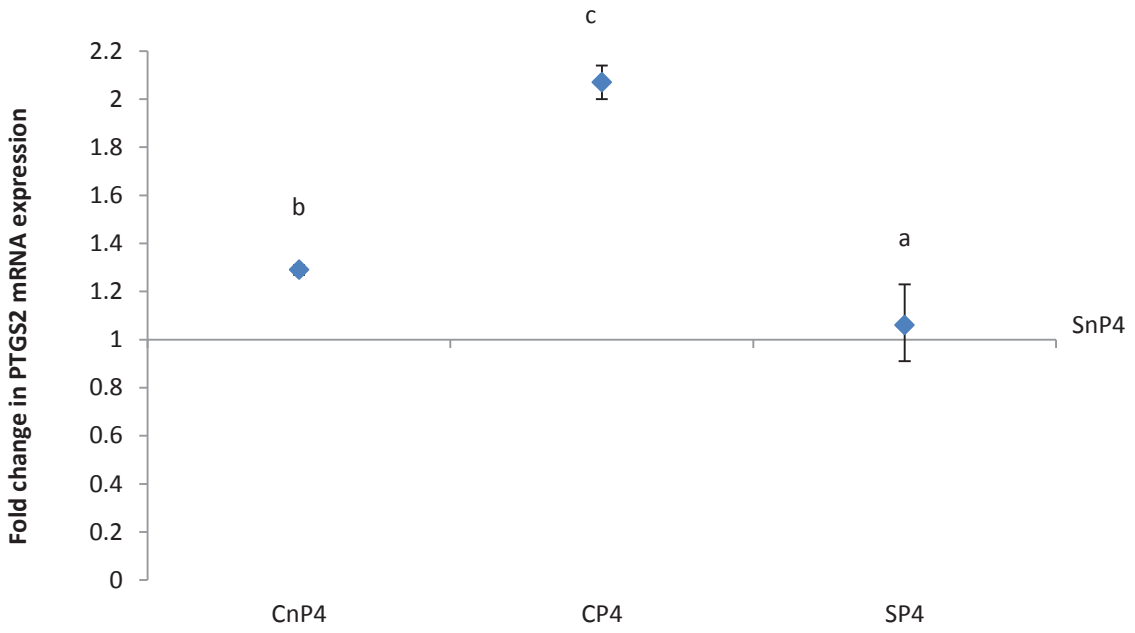
Appendix VII Figure 10: Differential mRNA expression levels of *MUC1* in day 19 uterine horns tissue ipsilateral to the CL of pregnant Cheviot and Suffolk ewes that were or were not administered exogenous progesterone (CP4, CnP4, SP4) compared to control group pregnant Suffolk ewes that were not administered exogenous progesterone (SnP4). Data is shown as a fold change with 95% confidence interval. If the confidence intervals cross the x axis then mRNA expression differs from the control group. Different letters indicate significant difference between groups.



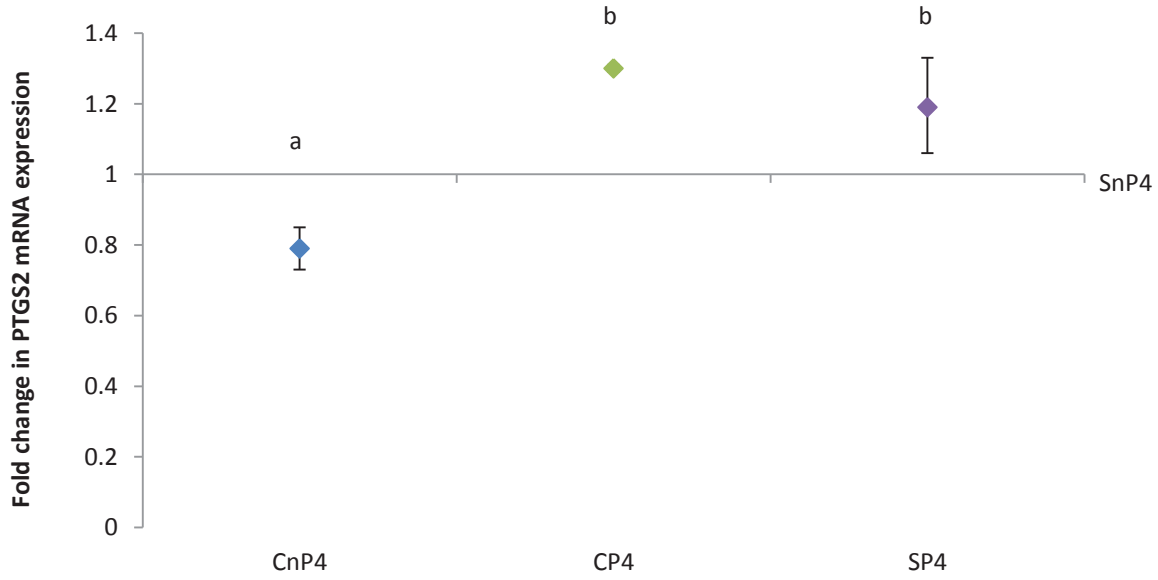
Appendix VII Figure 11: Differential mRNA expression levels of *PGR* in day 19 in combined uterine horn tissue of pregnant Cheviot and Suffolk ewes that were or were not administered exogenous progesterone (CP4, CnP4, SP4) compared to control group pregnant Suffolk ewes that were not administered exogenous progesterone (SnP4). Data is shown as a fold change with 95% confidence interval. If the confidence intervals cross the x axis then mRNA expression differs from the control group. Different letters indicate significant difference between groups.



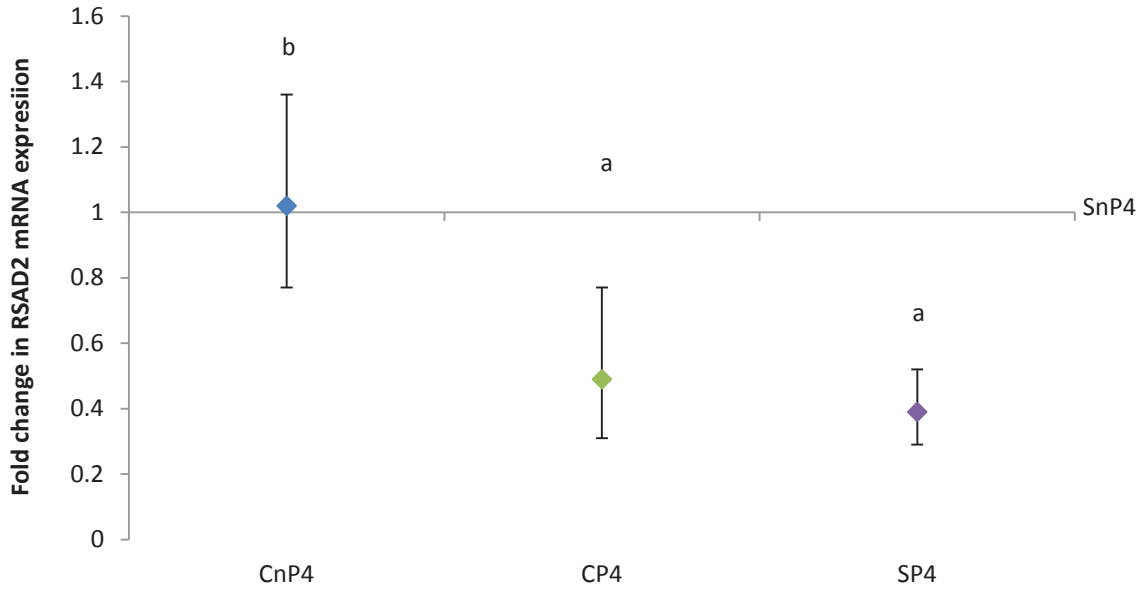
Appendix VII Figure 12: Differential mRNA expression levels of *PGR* in day 19 uterine horns tissue ipsilateral to the CL of pregnant Cheviot and Suffolk ewes that were or were not administered exogenous progesterone (CP4, CnP4, SP4) compared to control group pregnant Suffolk ewes that were not administered exogenous progesterone (SnP4). Data is shown as a fold change with 95% confidence interval. If the confidence intervals cross the x axis then mRNA expression differs from the control group. Different letters indicate significant difference between groups.



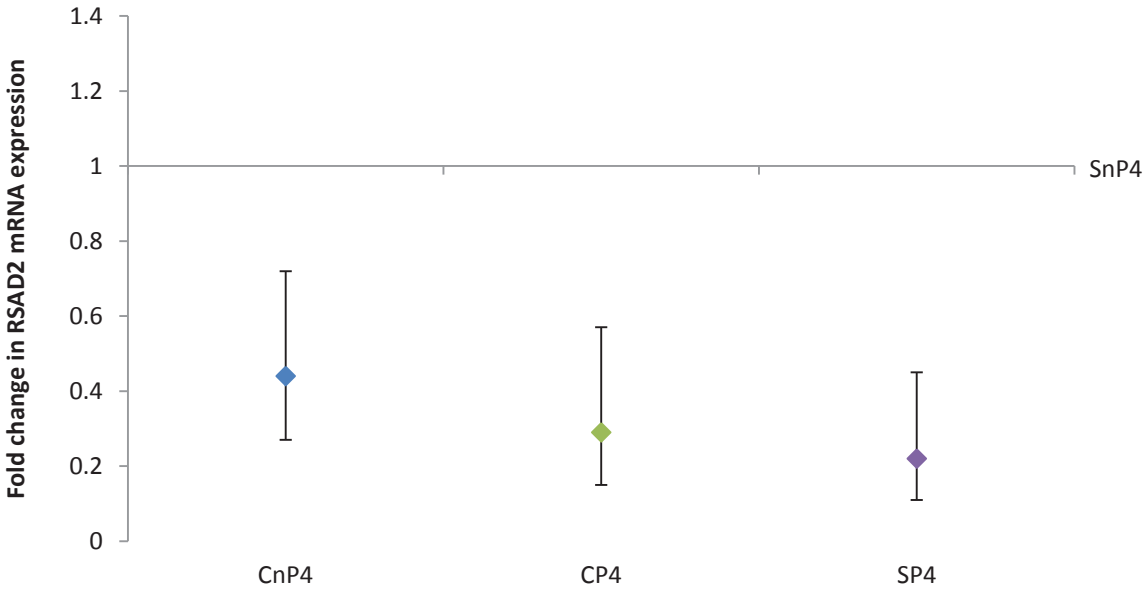
Appendix VII Figure 13: Differential mRNA expression levels of *PTGS2* in day 19 in combined uterine horn tissue of pregnant Cheviot and Suffolk ewes that were or were not administered exogenous progesterone (CP4, CnP4, SP4) compared to control group pregnant Suffolk ewes that were not administered exogenous progesterone (SnP4). Data is shown as a fold change with 95% confidence interval. If the confidence intervals cross the x axis then mRNA expression differs from the control group. Different letters indicate significant difference between groups.



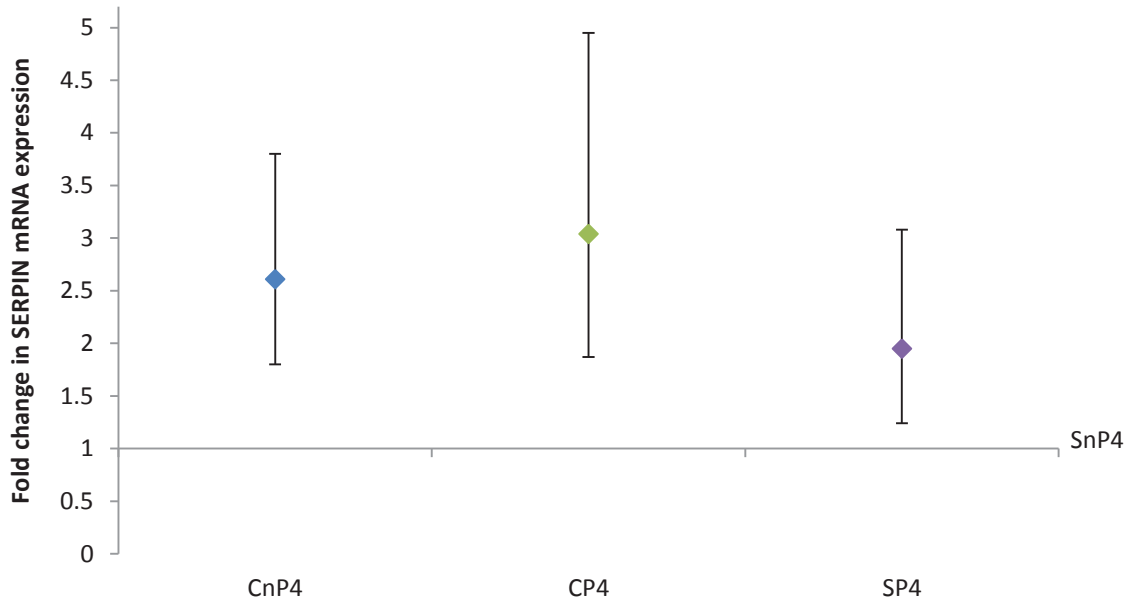
Appendix VII Figure 14: Differential mRNA expression levels of *PTGS2* in day 19 uterine horns tissue ipsilateral to the CL of pregnant Cheviot and Suffolk ewes that were or were not administered exogenous progesterone (CP4, CnP4, SP4) compared to control group pregnant Suffolk ewes that were not administered exogenous progesterone (SnP4). Data is shown as a fold change with 95% confidence interval. If the confidence intervals cross the x axis then mRNA expression differs from the control group. Different letters indicate significant difference between groups.



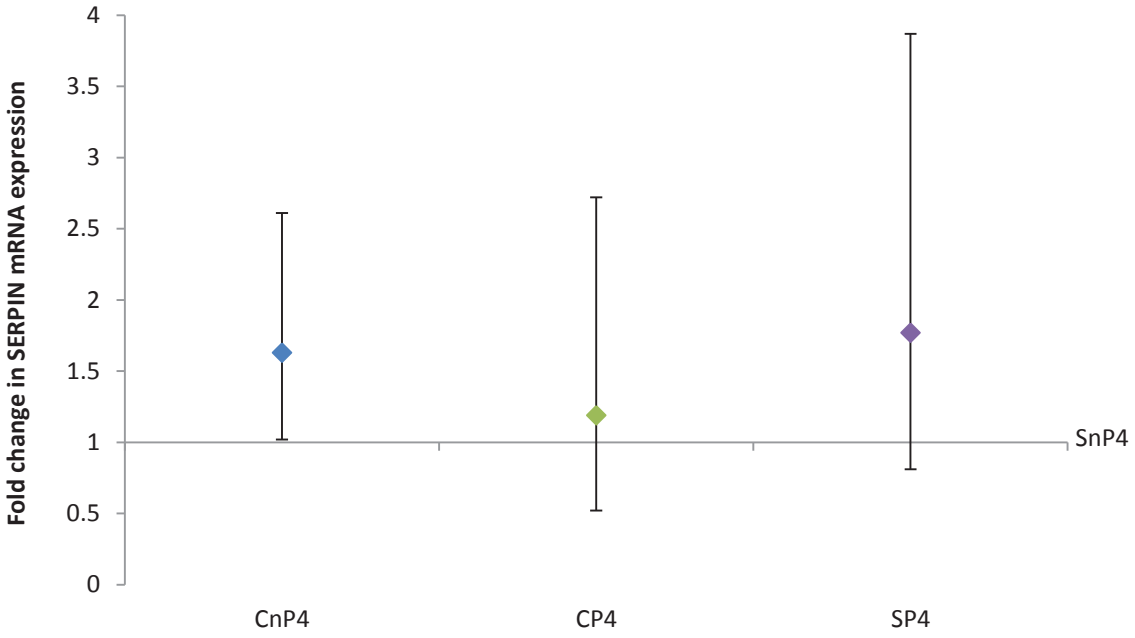
Appendix VII Figure 15: Differential mRNA expression levels of *RSAD2* in day 19 in combined uterine horn tissue of pregnant Cheviot and Suffolk ewes that were or were not administered exogenous progesterone (CP4, CnP4, SP4) compared to control group pregnant Suffolk ewes that were not administered exogenous progesterone (SnP4). Data is shown as a fold change with 95% confidence interval. If the confidence intervals cross the x axis then mRNA expression differs from the control group. Different letters indicate significant difference between groups.



Appendix VII Figure 16: Differential mRNA expression levels of *RSAD2* in day 19 uterine horns tissue ipsilateral to the CL of pregnant Cheviot and Suffolk ewes that were or were not administered exogenous progesterone (CP4, CnP4, SP4) compared to control group pregnant Suffolk ewes that were not administered exogenous progesterone (SnP4). Data is shown as a fold change with 95% confidence interval. If the confidence intervals cross the x axis then mRNA expression differs from the control group. Different letters indicate significant difference between groups.



Appendix VII Figure 17: Differential mRNA expression levels of *SERPIN* in day 19 in combined uterine horn tissue of pregnant Cheviot and Suffolk ewes that were or were not administered exogenous progesterone (CP4, CnP4, SP4) compared to control group pregnant Suffolk ewes that were not administered exogenous progesterone (SnP4). Data is shown as a fold change with 95% confidence interval. If the confidence intervals cross the x axis then mRNA expression differs from the control group. Different letters indicate significant difference between groups.



Appendix VII Figure 18: Differential mRNA expression levels of *SERPIN* in day 19 uterine horns tissue ipsilateral to the CL of pregnant Cheviot and Suffolk ewes that were or were not administered exogenous progesterone (CP4, CnP4, SP4) compared to control group pregnant Suffolk ewes that were not administered exogenous progesterone (SnP4). Data is shown as a fold change with 95% confidence interval. If the confidence intervals cross the x axis then mRNA expression differs from the control group. Different letters indicate significant difference between groups.

Appendix VIII

Ewe uterine weights and morphometry at day 19 of gestation.

Treatment groups[1]	*n*	Uterine weight (g)	Uterine body width (cm)	Uterine body length (cm)
$CP4^{0-3}$	5	73.80 ± 7.09	3.98 ± 0.21^{cb}	6.12 ± 0.37^{ab}
$CP4^{0-6}$	4	82.71 ± 7.93	4.50 ± 0.24^{c}	6.63 ± 0.41^{b}
$CP4^{2-4}$	13	66.67 ± 4.40	3.68 ± 0.13^{ab}	6.66 ± 0.23^{b}
$CP4^{3-6}$	13	62.44 ± 4.40	3.51 ± 0.14^{ab}	6.57 ± 0.24^{b}
CnP4	11	63.74 ± 4.78	3.44 ± 0.14^{a}	6.43 ± 0.25^{b}
SnP4	10	65.76 ± 4.58	3.56 ± 0.15^{ab}	5.36 ± 0.26^{a}

[1] $CP4^{0-3}$, Cheviot ewes primed with P4 from day 0-3 of gestation; $CP4^{0-6}$, Cheviot ewes primed with P4 from day 0-6 of gestation; $CP4^{2-4}$, Cheviot ewes primed with P4 from day 2-4 of gestation; $CP4^{3-6}$, Cheviot ewe primed with P4 from day 3-6 of gestation; CnP4, Cheviot ewes that did not receive exogenous P4; SnP4, Suffolk ewes that did not receive exogenous P4.

Values are least squares means ± SE of the mean. Different superscripts within main effects indicate significant differences ($p<0.05$).

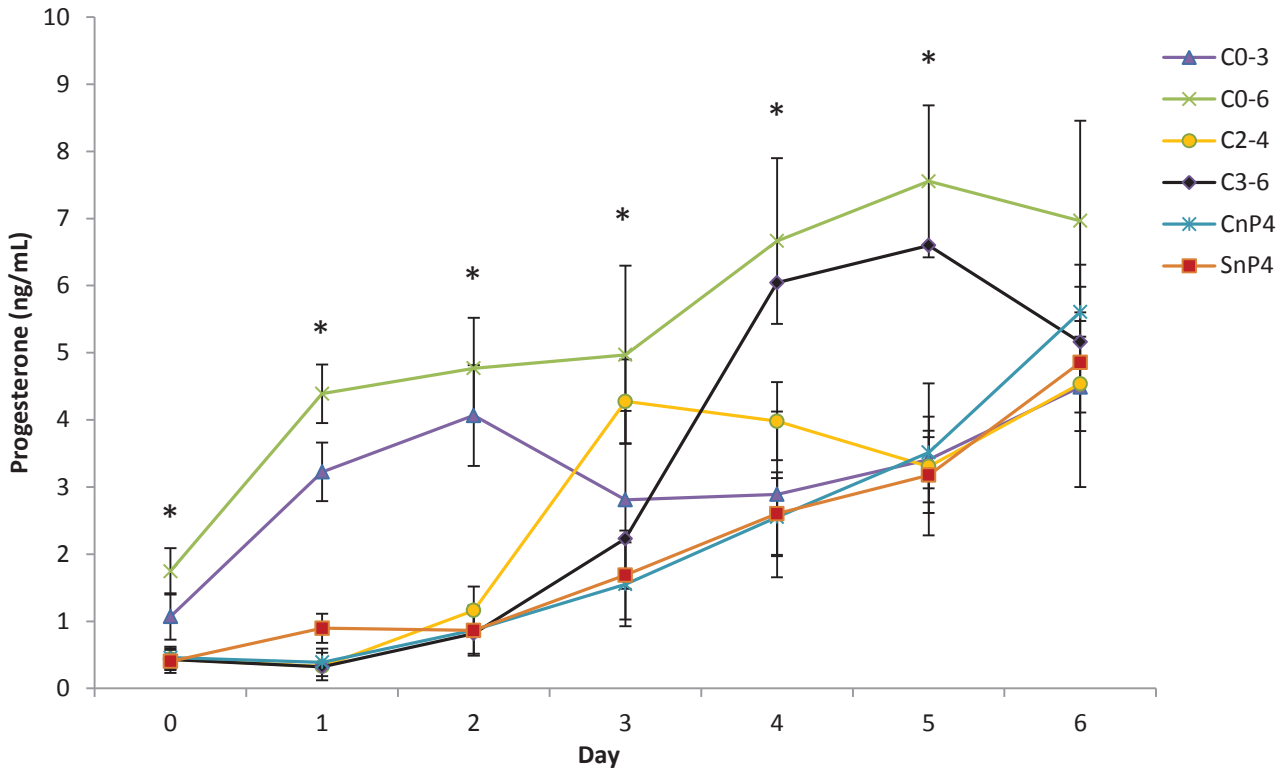
Appendix IX

Appendix IX Table 1: Day 0-6 plasma progesterone (P4) concentrations (ng/mL) in Cheviot ewes that did or did not receive exogenous P4 and Suffolk ewes that did not receive exogenous P4 via intravaginal CIDR for various time periods from day 0 to day 6 in only the ewes that were pregnant at day 19.

		Day						
Treatment groups[1]	*n*	0	1	2	3	4	5	6
$CP4^{0-3}$	2	1.07 ± 0.34^{a}	3.23 ± 0.44^{b}	4.07 ± 0.75^{b}	2.81 ± 1.33^{ab}	2.89 ± 1.23^{ac}	3.41 ± 1.13^{a}	4.49 ± 1.49
$CP4^{0-6}$	2	1.75 ± 0.34^{b}	4.39 ± 0.44^{b}	4.77 ± 0.75^{b}	4.97 ± 1.32^{b}	6.67 ± 1.23^{b}	7.56 ± 1.13^{b}	6.97 ± 1.49
$CP4^{2-4}$	9	0.44 ± 0.16^{a}	0.33 ± 0.21^{a}	1.16 ± 0.35^{a}	4.28 ± 0.62^{b}	3.98 ± 0.58^{c}	3.31 ± 0.53^{a}	4.54 ± 0.70
$CP4^{3-6}$	9	0.43 ± 0.15^{a}	0.32 ± 0.19^{a}	0.82 ± 0.34^{a}	2.23 ± 0.59^{a}	6.04 ± 0.55^{b}	6.60 ± 0.51^{b}	5.16 ± 0.67
CnP4	8	0.46 ± 0.16^{a}	0.39 ± 0.21^{a}	0.87 ± 0.35^{a}	1.55 ± 0.62^{a}	2.55 ± 0.58^{a}	3.52 ± 0.53^{a}	5.61 ± 0.70
SnP4	8	0.40 ± 0.17^{a}	0.90 ± 0.22^{a}	0.86 ± 0.37^{a}	1.69 ± 0.66^{a}	2.60 ± 0.62^{a}	3.18 ± 0.57^{a}	4.85 ± 0.75

Values are least square means ± SEM.

[1]$CP4^{0-3}$, Cheviot ewes primed with P4 from day 0-3 of gestation; $CP4^{0-6}$, Cheviot ewes primed with P4 from day 0-6 of gestation; $CP4^{2-4}$, Cheviot ewes primed with P4 from day 2-4 of gestation; $CP4^{3-6}$, Cheviot ewe primed with P4 from day 3-6 of gestation; CnP4, Cheviot ewes that did not receive exogenous P4; SnP4, Suffolk ewes that did not receive exogenous P4.



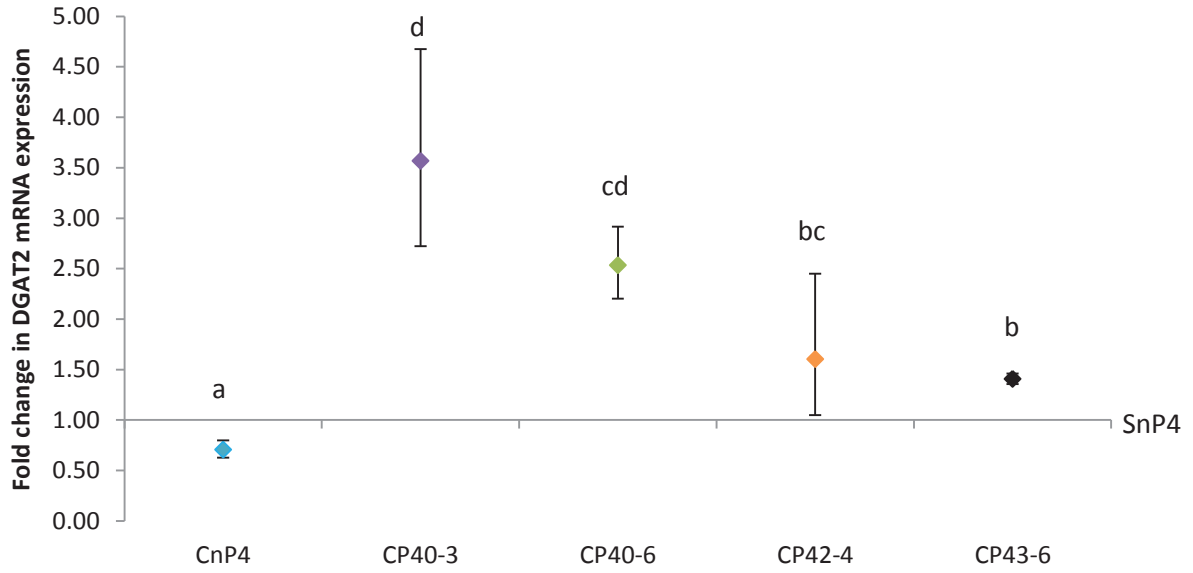
Appendix IX Figure 1 Day 0-6 plasma progesterone (P4) concentrations (ng/mL) in Cheviot ewes that did and did not receive exogenous P4 and Suffolk ewes that did not receive exogenous P4 via intravaginal CIDR for various time periods from day 0 to day 6 in only the ewes that were pregnant at day 19.

Values are least squares means ± SEM.

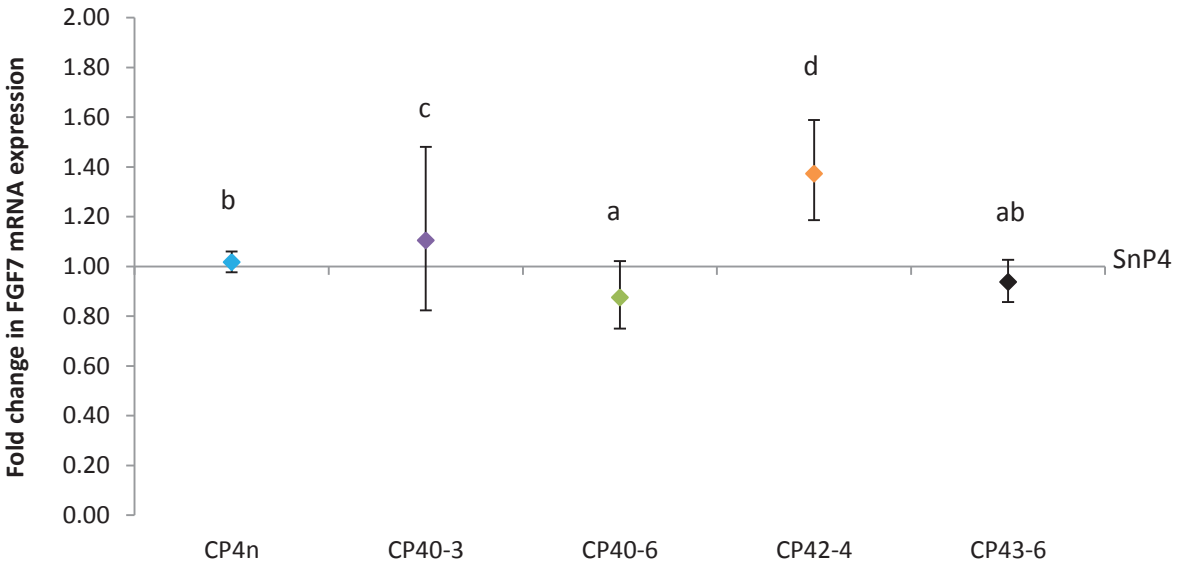
*indicates different ($p<0.05$) P4 concentration between two or all ewe groups on that day.

Appendix X

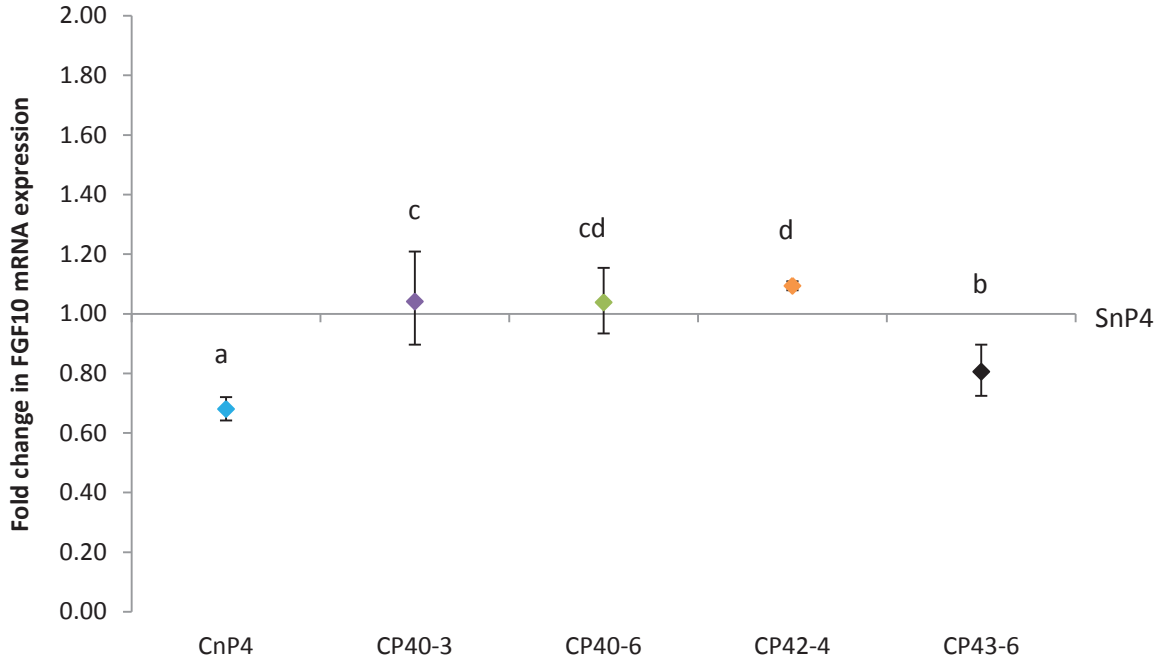
Graphical representation of day 6 uterine horn mRNA expression of candidate genes in Cheviot ewes administered exogenous progesterone at different time periods from day 0-6 and Suffolk ewes without exogenous progesterone



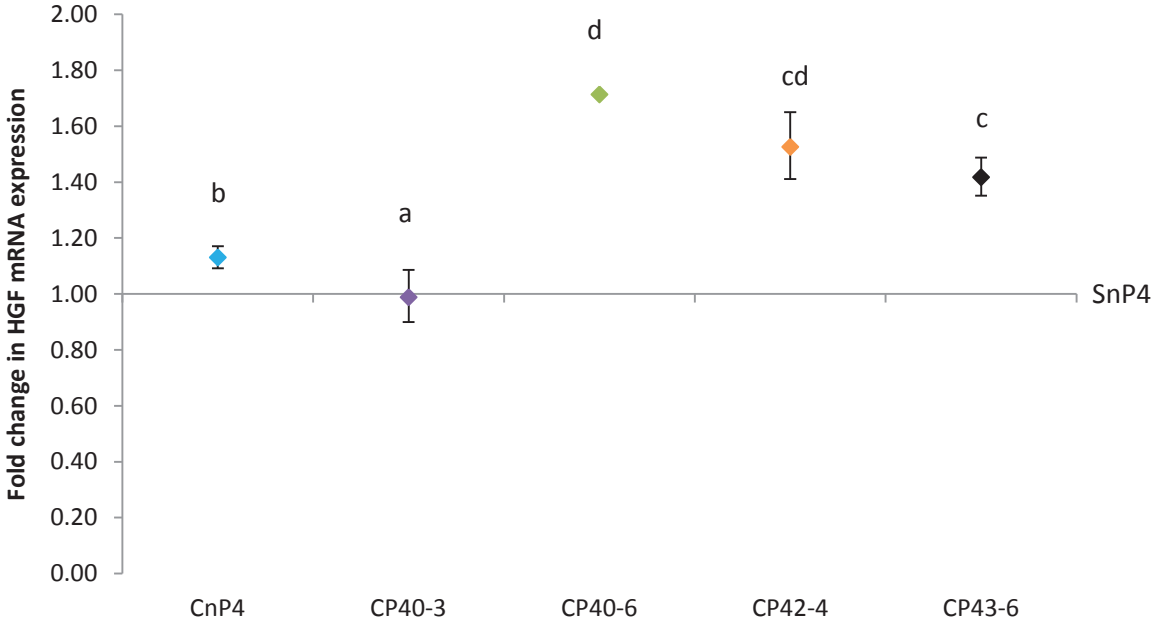
Appendix X Figure 1: Differential mRNA expression levels of *DGAT2* in day 6 uterine horns ipsilateral to the CL of pregnant Cheviot ewes that were and were not administered exogenous progesterone at different time periods from day 0-6 ($CP4^{0-3}$, $CP4^{0-6}$, $CP4^{2-4}$, $CP4^{3-6}$, CnP4) compared to control group pregnant Suffolk ewes that were not administered exogenous progesterone (SnP4) Data is shown as a fold change with 95% confidence interval. If the confidence intervals cross the x axis then mRNA expression differs from the control group. Different letters indicate significant difference between groups.



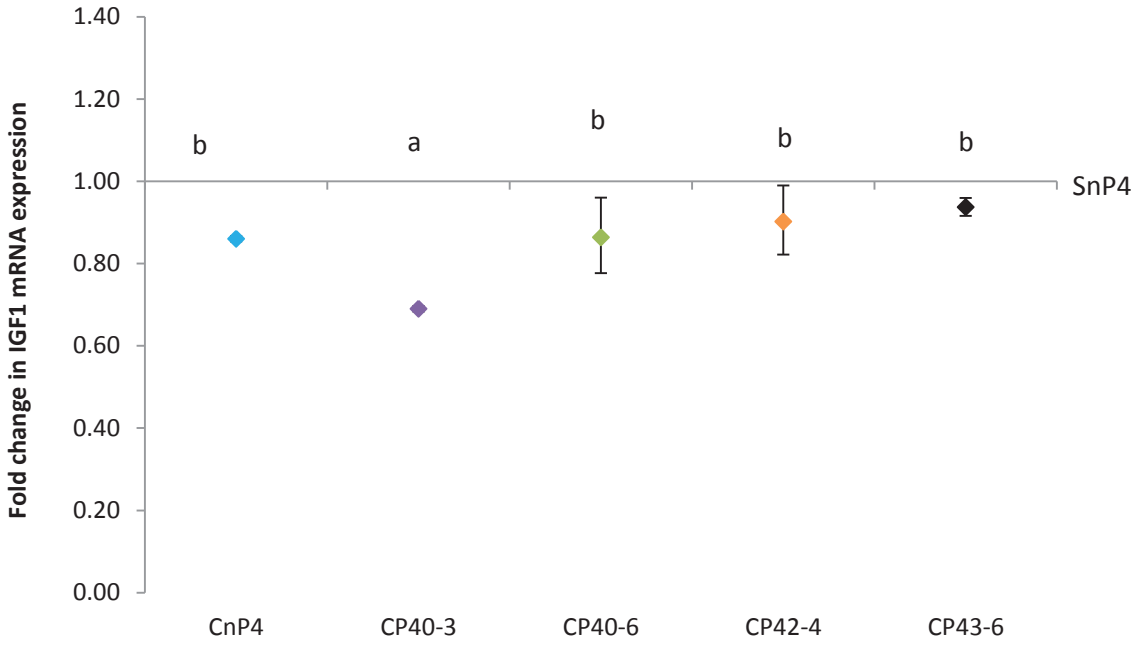
Appendix X Figure 2: Differential mRNA expression levels of *FGF7* in day 6 uterine horns ipsilateral to the CL of pregnant Cheviot ewes that were and were not administered exogenous progesterone at different time periods from day 0-6 ($CP4^{0-3}$, $CP4^{0-6}$, $CP4^{2-4}$, $CP4^{3-6}$, CnP4) compared to control group pregnant Suffolk ewes that were not administered exogenous progesterone (SnP4) Data is shown as a fold change with 95% confidence interval. If the confidence intervals cross the x axis then mRNA expression differs from the control group. Different letters indicate significant difference between groups.



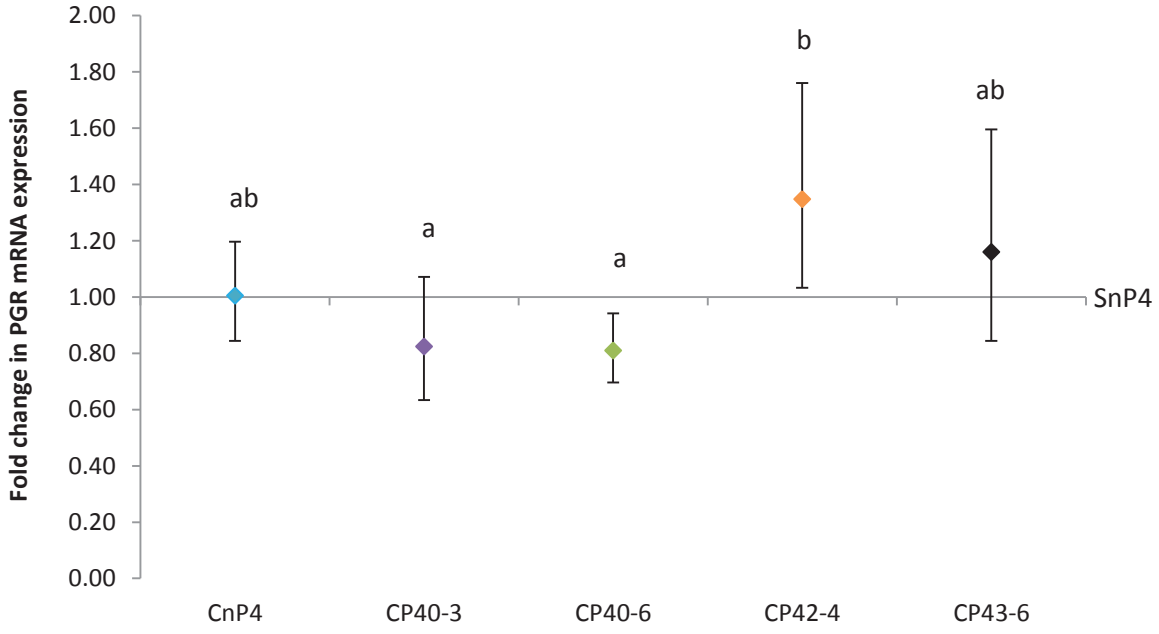
Appendix X Figure 3: Differential mRNA expression levels of *FGF10* in day 6 uterine horns ipsilateral to the CL of pregnant Cheviot ewes that were and were not administered exogenous progesterone at different time periods from day 0-6 ($CP4^{0-3}$, $CP4^{0-6}$, $CP4^{2-4}$, $CP4^{3-6}$, CnP4) compared to control group pregnant Suffolk ewes that were not administered exogenous progesterone (SnP4) Data is shown as a fold change with 95% confidence interval. If the confidence intervals cross the x axis then mRNA expression differs from the control group. Different letters indicate significant difference between groups.



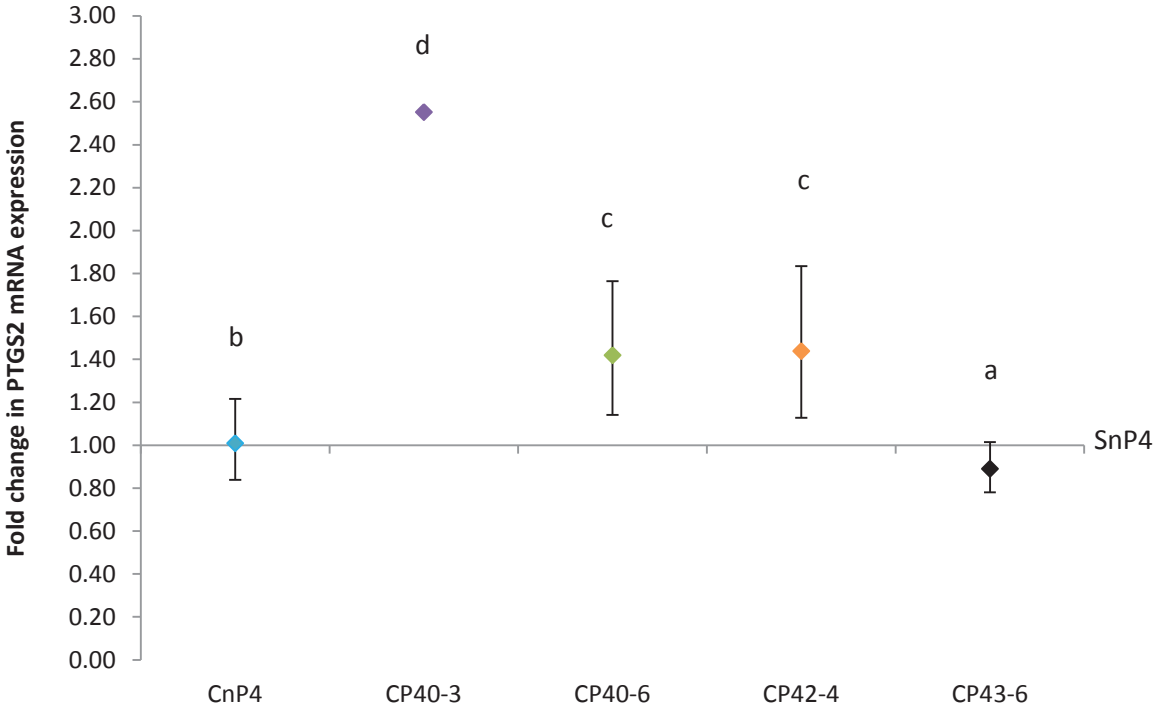
Appendix X Figure 4: Differential mRNA expression levels of *HGF* in day 6 uterine horns ipsilateral to the CL of pregnant Cheviot ewes that were and were not administered exogenous progesterone at different time periods from day 0-6 ($CP4^{0-3}$, $CP4^{0-6}$, $CP4^{2-4}$, $CP4^{3-6}$, CnP4) compared to control group pregnant Suffolk ewes that were not administered exogenous progesterone (SnP4) Data is shown as a fold change with 95% confidence interval. If the confidence intervals cross the x axis then mRNA expression differs from the control group. Different letters indicate significant difference between groups.



Appendix X Figure 5: Differential mRNA expression levels of *IGF1* in day 6 uterine horns ipsilateral to the CL of pregnant Cheviot ewes that were and were not administered exogenous progesterone at different time periods from day 0-6 ($CP4^{0-3}$, $CP4^{0-6}$, $CP4^{2-4}$, $CP4^{3-6}$, CnP4) compared to control group pregnant Suffolk ewes that were not administered exogenous progesterone (SnP4) Data is shown as a fold change with 95% confidence interval. If the confidence intervals cross the x axis then mRNA expression differs from the control group. Different letters indicate significant difference between groups.



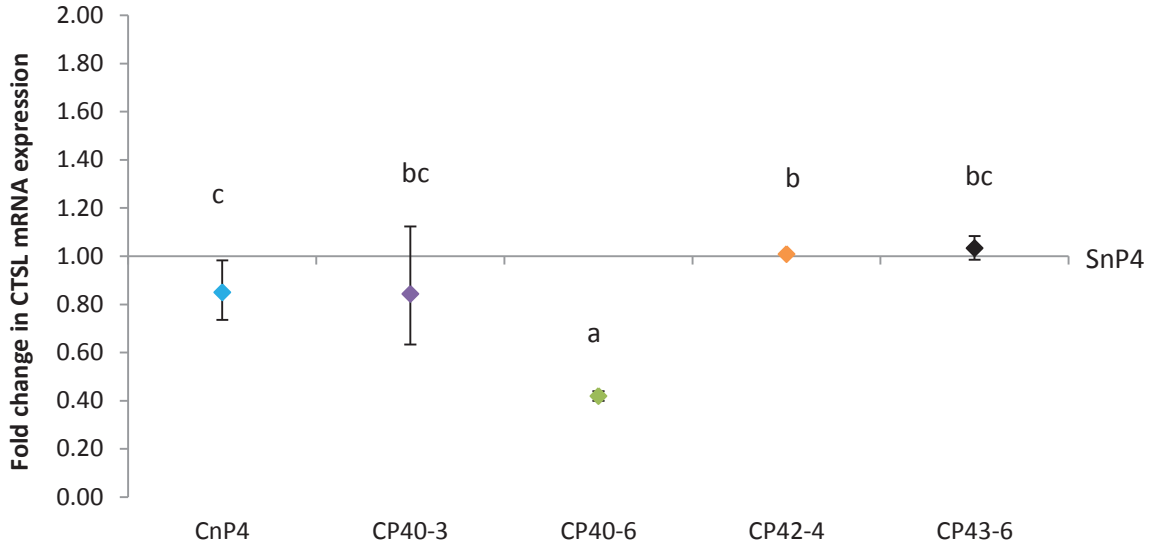
Appendix X Figure 6: Differential mRNA expression levels of *PGR* in day 6 uterine horns ipsilateral to the CL of pregnant Cheviot ewes that were and were not administered exogenous progesterone at different time periods from day 0-6 ($CP4^{0-3}$, $CP4^{0-6}$, $CP4^{2-4}$, $CP4^{3-6}$, CnP4) compared to control group pregnant Suffolk ewes that were not administered exogenous progesterone (SnP4) Data is shown as a fold change with 95% confidence interval. If the confidence intervals cross the x axis then mRNA expression differs from the control group. Different letters indicate significant difference between groups.



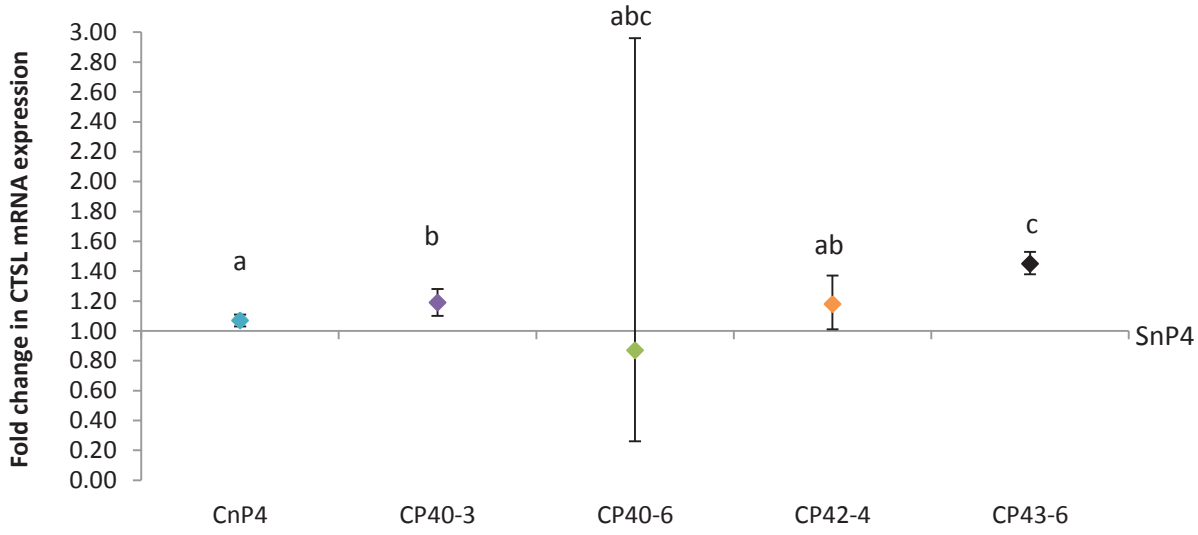
Appendix X Figure 7: Differential mRNA expression levels of *PTGS2* in day 6 uterine horns ipsilateral to the CL of pregnant Cheviot ewes that were and were not administered exogenous progesterone at different time periods from day 0-6 ($CP4^{0-3}$, $CP4^{0-6}$, $CP4^{2-4}$, $CP4^{3-6}$, CnP4) compared to control group pregnant Suffolk ewes that were not administered exogenous progesterone (SnP4) Data is shown as a fold change with 95% confidence interval. If the confidence intervals cross the x axis then mRNA expression differs from the control group. Different letters indicate significant difference between groups.

Appendix XI

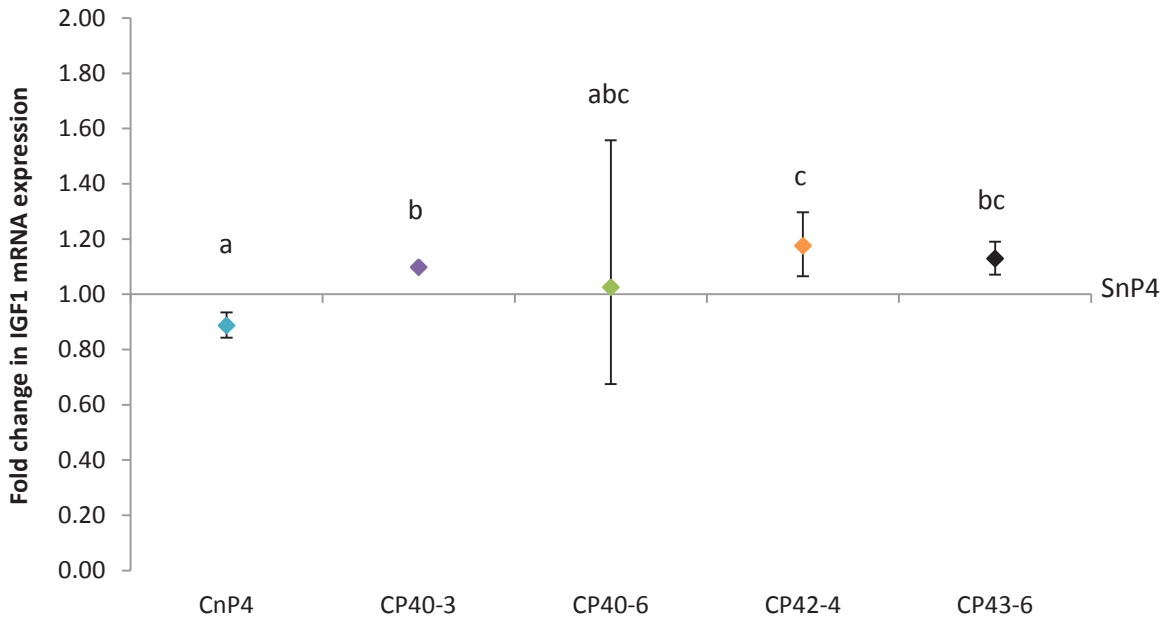
Graphical representation of day 19 uterine horn mRNA expression of candidate genes in Cheviot ewes with and without exogenous progesterone and Suffolk ewes without exogenous progesterone



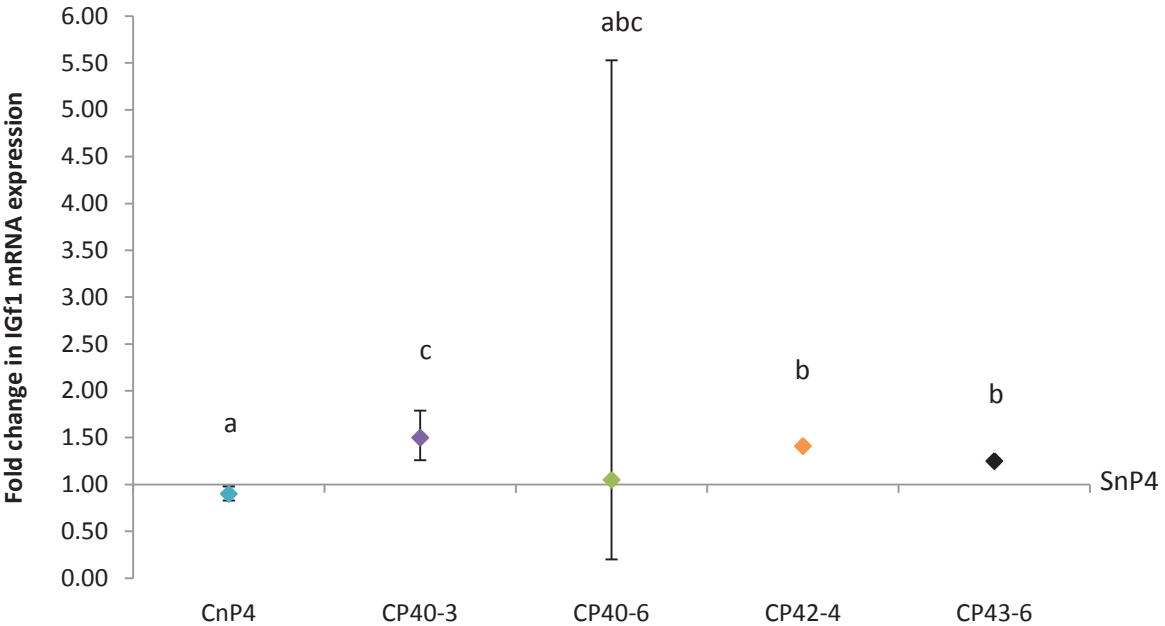
Appendix XI Figure 1 Differential mRNA expression levels of *CTSL* in day 19 combined uterine horns of pregnant Cheviot ewes that were and were not administered exogenous progesterone at different time periods from day 0-6 ($CP4^{0-3}$, $CP4^{0-6}$, $CP4^{2-4}$, $CP4^{3-6}$, CnP4) compared to control group pregnant Suffolk ewes that were not administered exogenous progesterone (SnP4). Data is shown as a fold change with 95% confidence interval. If the confidence intervals cross the x axis then mRNA expression differs from the control group. Different letters indicate significant difference between groups.



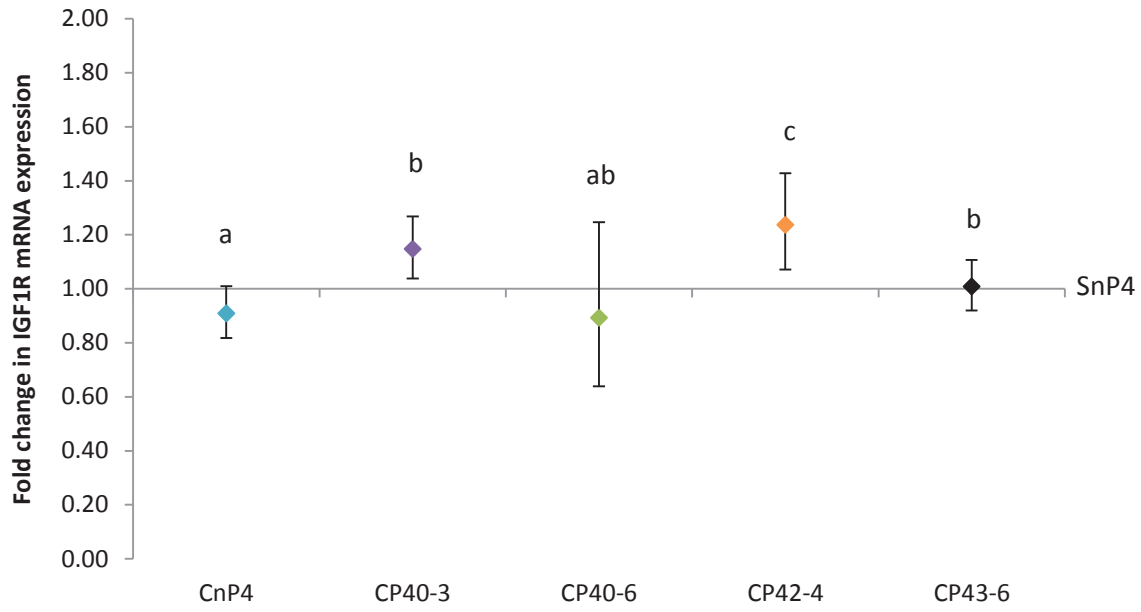
Appendix XI Figure 2 Differential mRNA expression levels of *CTSL* in day 19 uterine horns ipsilateral to the CL of pregnant Cheviot ewes that were and were not administered exogenous progesterone at different time periods from day 0-6 ($CP4^{0-3}$, $CP4^{0-6}$, $CP4^{2-4}$, $CP4^{3-6}$, CnP4) compared to control group pregnant Suffolk ewes that were not administered exogenous progesterone (SnP4). Data is shown as a fold change with 95% confidence interval. If the confidence intervals cross the x axis then mRNA expression differs from the control group. Different letters indicate significant difference between groups.



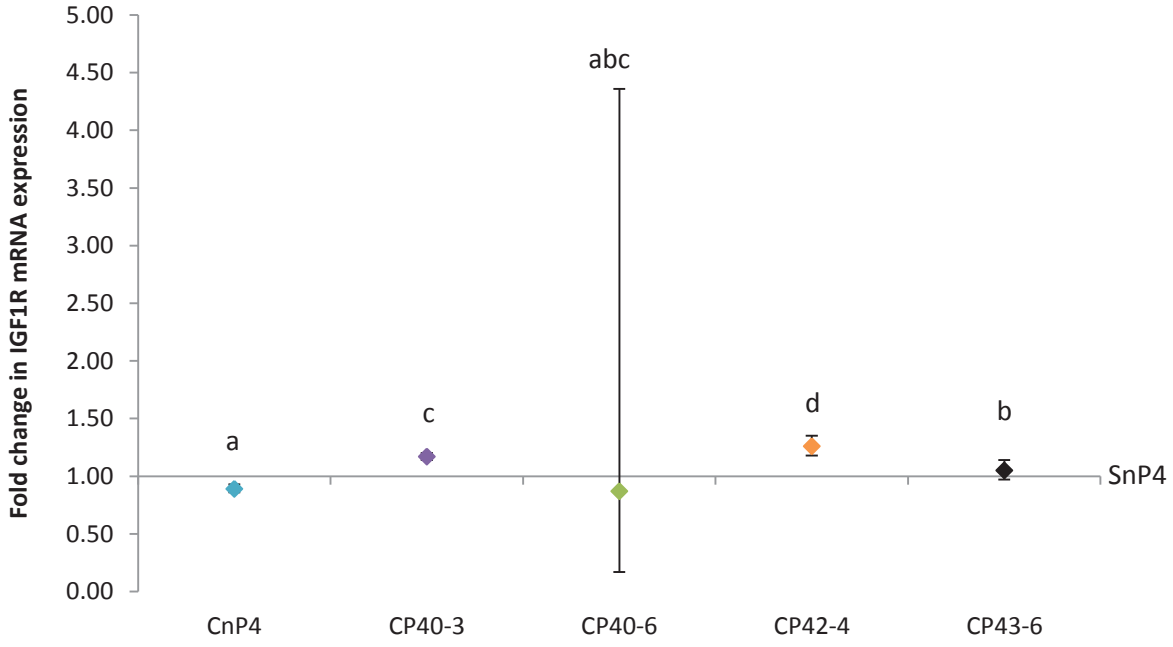
Appendix XI Figure 3 Differential mRNA expression levels of *IGF1* in day 19 combined uterine horns of pregnant Cheviot ewes that were and were not administered exogenous progesterone at different time periods from day 0-6 ($CP4^{0-3}$, $CP4^{0-6}$, $CP4^{2-4}$, $CP4^{3-6}$, CnP4) compared to control group pregnant Suffolk ewes that were not administered exogenous progesterone (SnP4). Data is shown as a fold change with 95% confidence interval. If the confidence intervals cross the x axis then mRNA expression differs from the control group. Different letters indicate significant difference between groups.



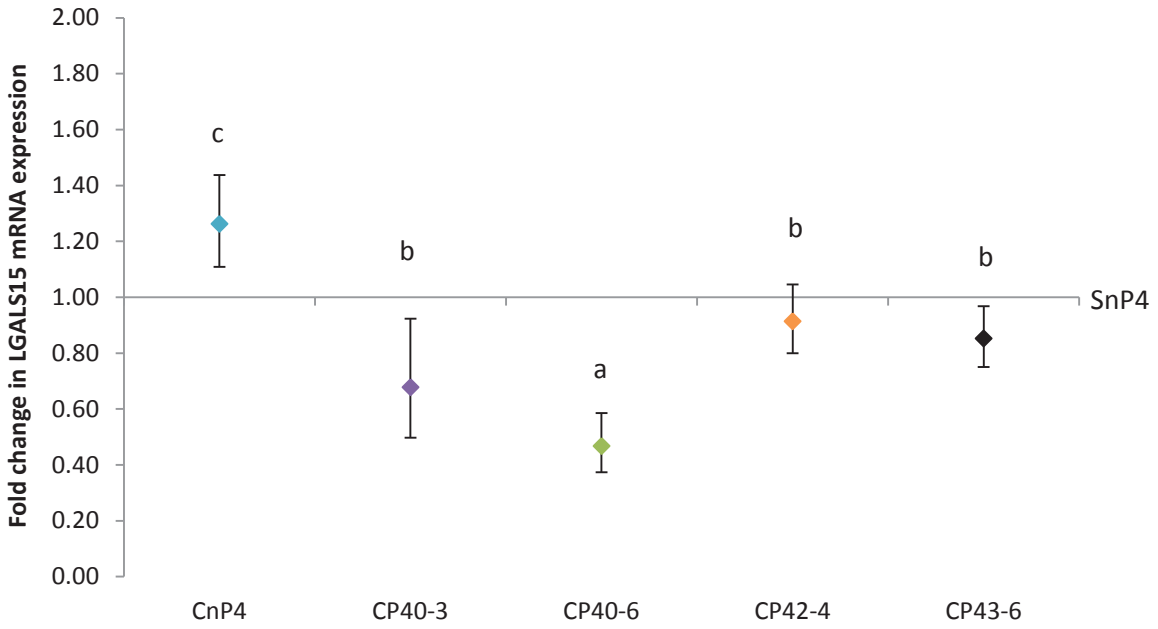
Appendix XI Figure 4 Differential mRNA expression levels of *IGF1* in day 19 uterine horns ipsilateral to the CL of pregnant Cheviot ewes that were and were not administered exogenous progesterone at different time periods from day 0-6 ($CP4^{0-3}$, $CP4^{0-6}$, $CP4^{2-4}$, $CP4^{3-6}$, CnP4) compared to control group pregnant Suffolk ewes that were not administered exogenous progesterone (SnP4). Data is shown as a fold change with 95% confidence interval. If the confidence intervals cross the x axis then mRNA expression differs from the control group. Different letters indicate significant difference between groups.



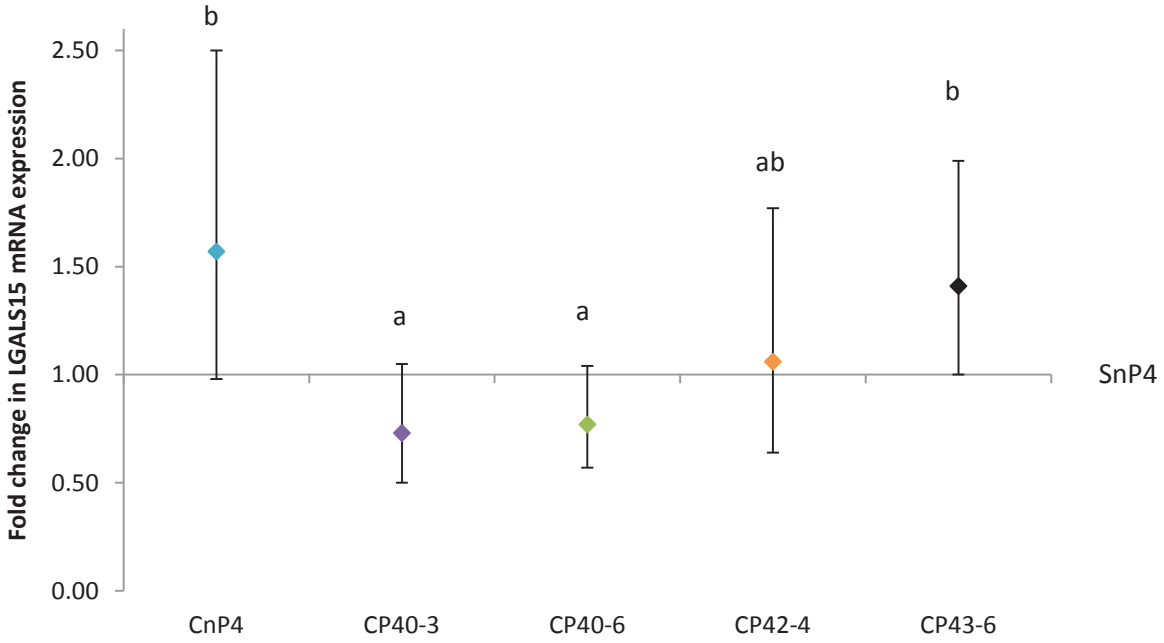
Appendix XI Figure 5 Differential mRNA expression levels of *IGF1R* in day 19 combined uterine horns of pregnant Cheviot ewes that were and were not administered exogenous progesterone at different time periods from day 0-6 ($CP4^{0-3}$, $CP4^{0-6}$, $CP4^{2-4}$, $CP4^{3-6}$, CnP4) compared to control group pregnant Suffolk ewes that were not administered exogenous progesterone (SnP4). Data is shown as a fold change with 95% confidence interval. If the confidence intervals cross the x axis then mRNA expression differs from the control group. Different letters indicate significant difference between groups.



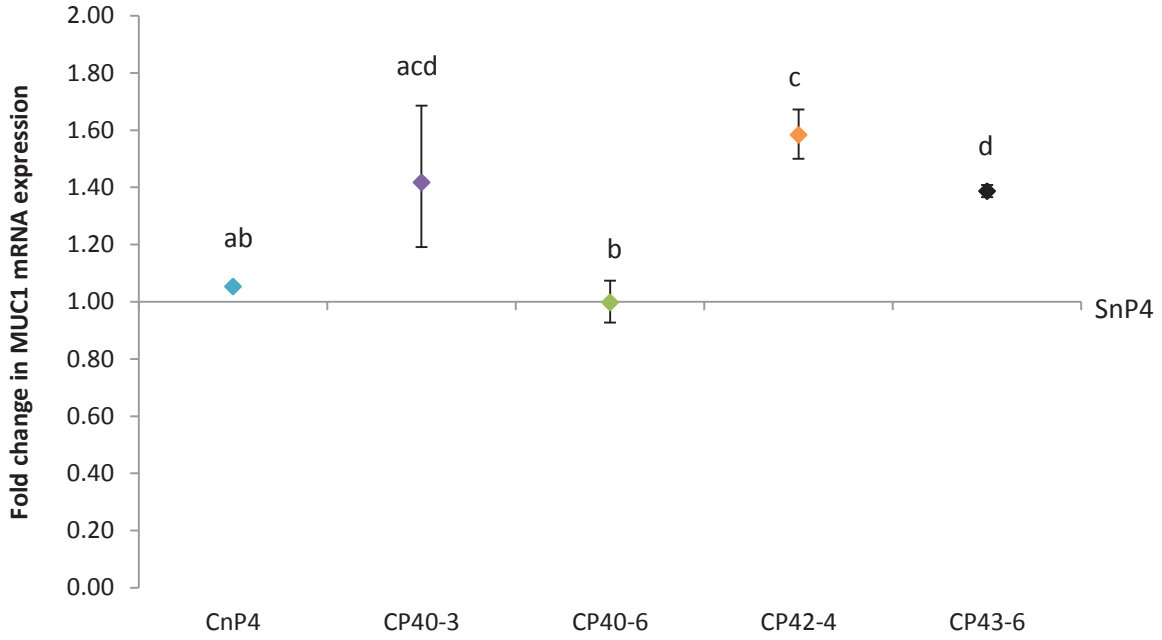
Appendix XI Figure 6 Differential mRNA expression levels of *IGF1R* in day 19 uterine horns ipsilateral to the CL of pregnant Cheviot ewes that were and were not administered exogenous progesterone at different time periods from day 0-6 ($CP4^{0-3}$, $CP4^{0-6}$, $CP4^{2-4}$, $CP4^{3-6}$, CnP4) compared to control group pregnant Suffolk ewes that were not administered exogenous progesterone (SnP4). Data is shown as a fold change with 95% confidence interval. If the confidence intervals cross the x axis then mRNA expression differs from the control group. Different letters indicate significant difference between groups.



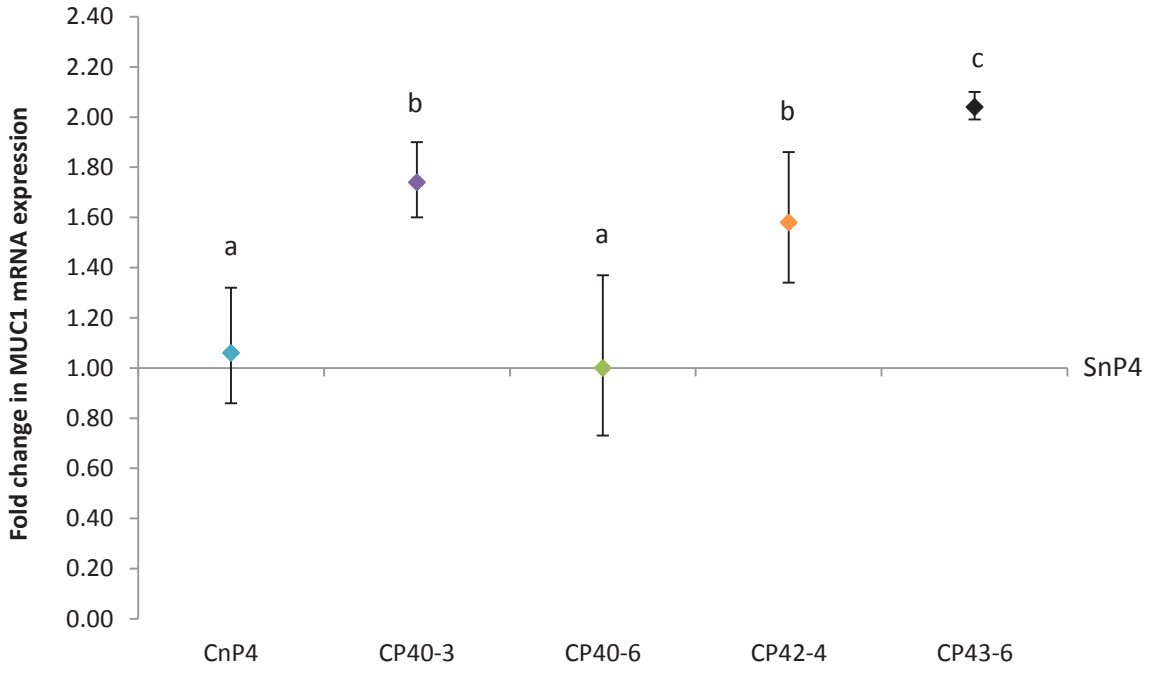
Appendix XI Figure 7 Differential mRNA expression levels of *LGALS15* in day 19 combined uterine horns of pregnant Cheviot ewes that were and were not administered exogenous progesterone at different time periods from day 0-6 ($CP4^{0-3}$, $CP4^{0-6}$, $CP4^{2-4}$, $CP4^{3-6}$, CnP4) compared to control group pregnant Suffolk ewes that were not administered exogenous progesterone (SnP4). Data is shown as a fold change with 95% confidence interval. If the confidence intervals cross the x axis then mRNA expression differs from the control group. Different letters indicate significant difference between groups.



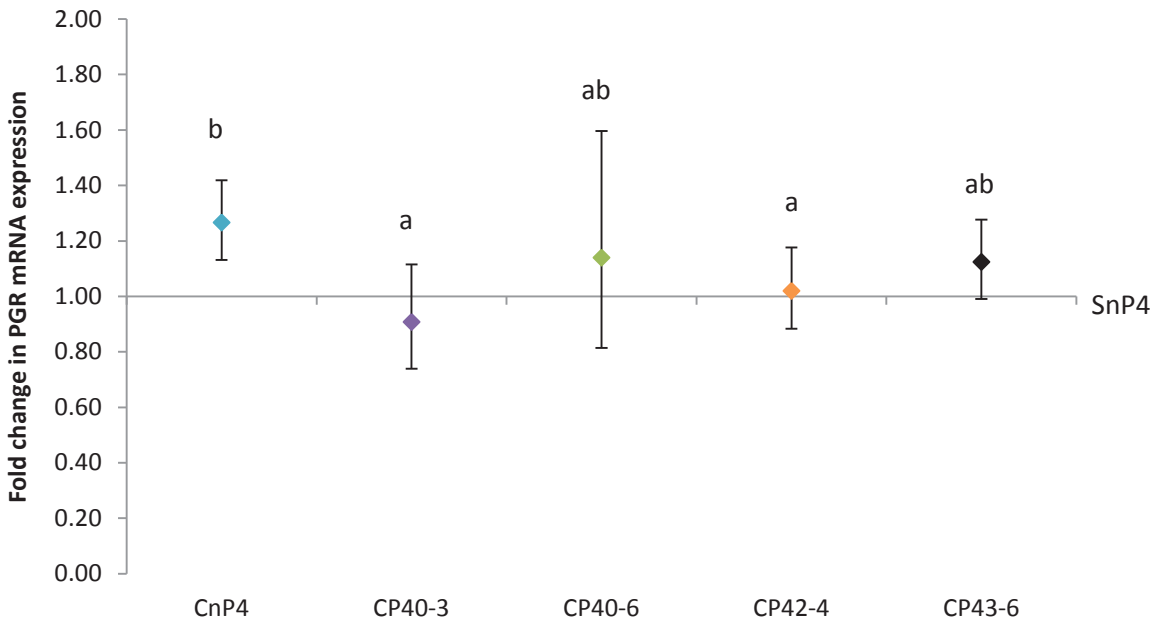
Appendix XI Figure 8 Differential mRNA expression levels of *LGALS15* in day 19 uterine horns ipsilateral to the CL of pregnant Cheviot ewes that were and were not administered exogenous progesterone at different time periods from day 0-6 ($CP4^{0-3}$, $CP4^{0-6}$, $CP4^{2-4}$, $CP4^{3-6}$, CnP4) compared to control group pregnant Suffolk ewes that were not administered exogenous progesterone (SnP4). Data is shown as a fold change with 95% confidence interval. If the confidence intervals cross the x axis then mRNA expression differs from the control group. Different letters indicate significant difference between groups.



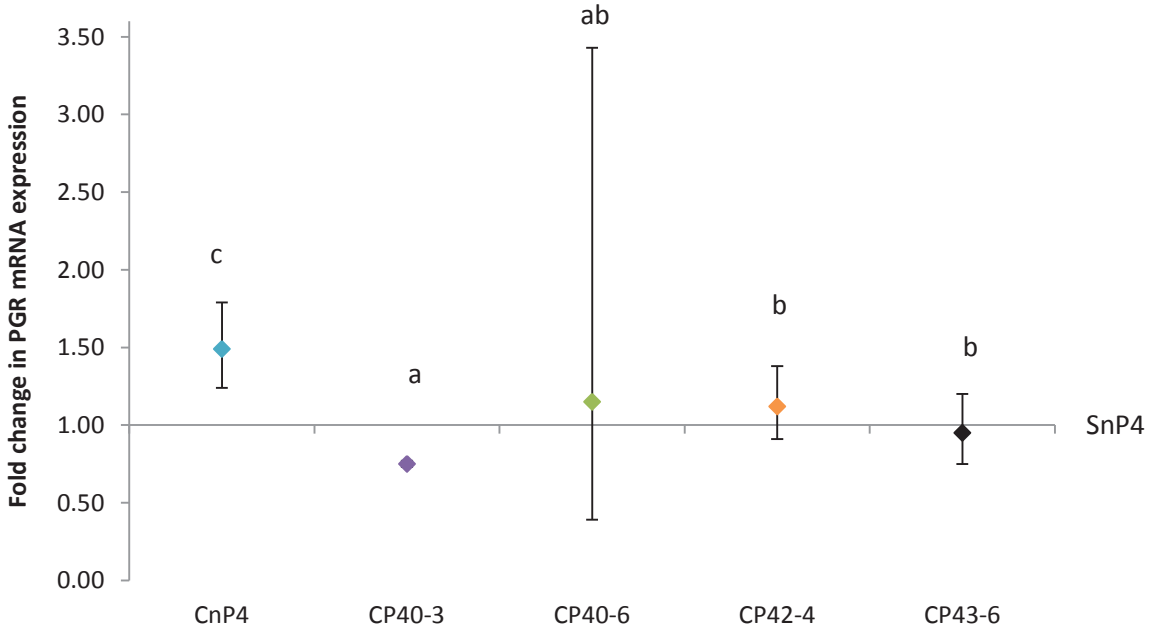
Appendix XI Figure 9 Differential mRNA expression levels of *MUC1* in day 19 combined uterine horns of pregnant Cheviot ewes that were and were not administered exogenous progesterone at different time periods from day 0-6 ($CP4^{0-3}$, $CP4^{0-6}$, $CP4^{2-4}$, $CP4^{3-6}$, CnP4) compared to control group pregnant Suffolk ewes that were not administered exogenous progesterone (SnP4). Data is shown as a fold change with 95% confidence interval. If the confidence intervals cross the x axis then mRNA expression differs from the control group. Different letters indicate significant difference between groups.



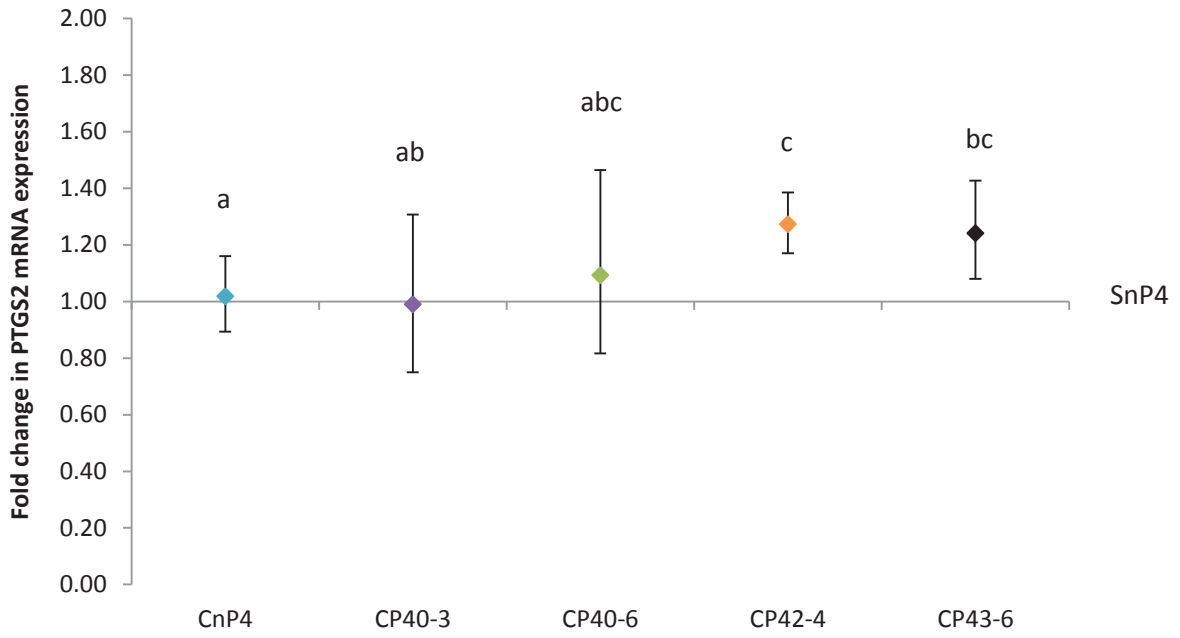
Appendix XI Figure 10 Differential mRNA expression levels of *MUC1* in day 19 uterine horns ipsilateral to the CL of pregnant Cheviot ewes that were and were not administered exogenous progesterone at different time periods from day 0-6 ($CP4^{0-3}$, $CP4^{0-6}$, $CP4^{2-4}$, $CP4^{3-6}$, CnP4) compared to control group pregnant Suffolk ewes that were not administered exogenous progesterone (SnP4). Data is shown as a fold change with 95% confidence interval. If the confidence intervals cross the x axis then mRNA expression differs from the control group. Different letters indicate significant difference between groups.



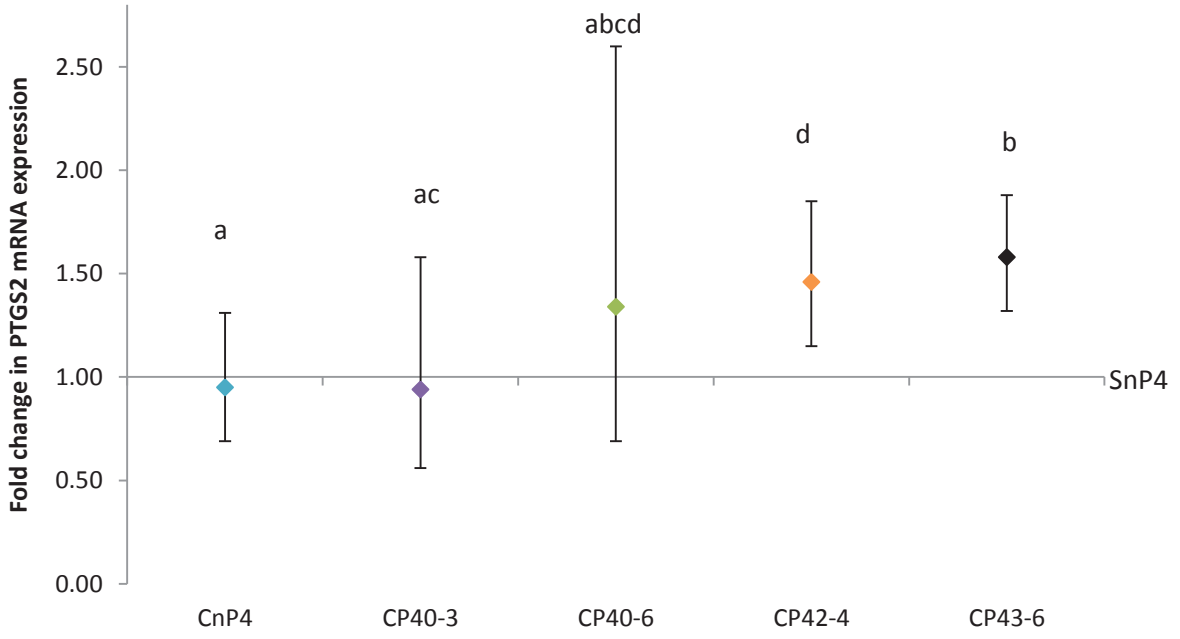
Appendix XI Figure 11 Differential mRNA expression levels of *PGR* in day 19 combined uterine horns of pregnant Cheviot ewes that were and were not administered exogenous progesterone at different time periods from day 0-6 ($CP4^{0-3}$, $CP4^{0-6}$, $CP4^{2-4}$, $CP4^{3-6}$, CnP4) compared to control group pregnant Suffolk ewes that were not administered exogenous progesterone (SnP4). Data is shown as a fold change with 95% confidence interval. If the confidence intervals cross the x axis then mRNA expression differs from the control group. Different letters indicate significant difference between groups.



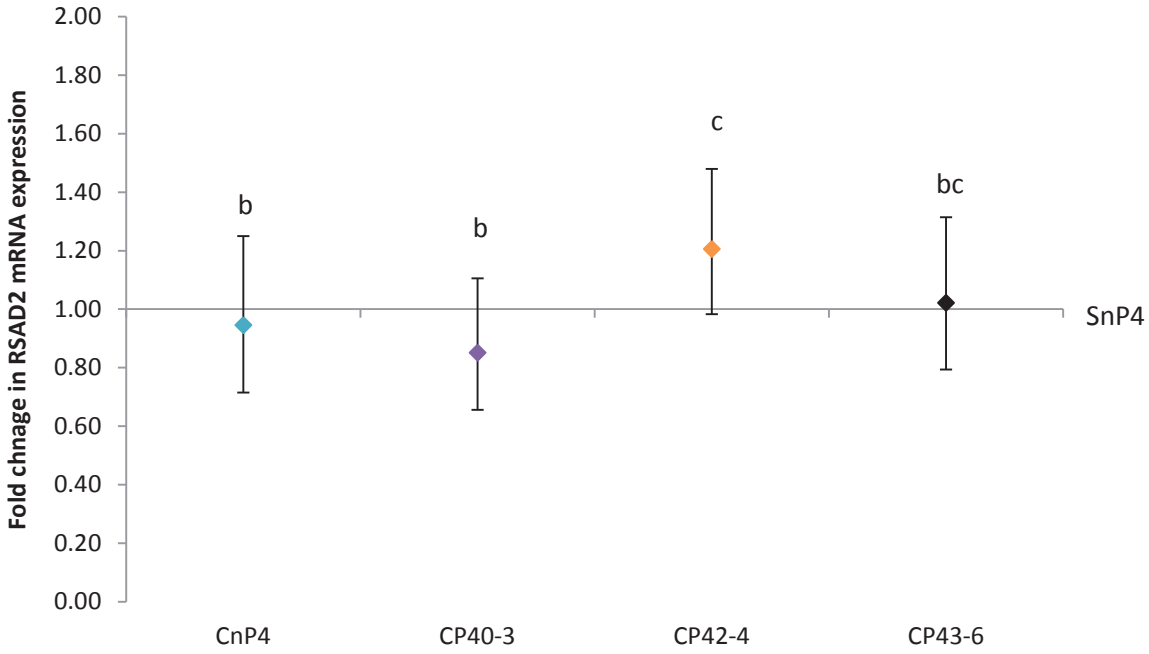
Appendix XI Figure 12 Differential mRNA expression levels of *PGR* in day 19 uterine horns ipsilateral to the CL of pregnant Cheviot ewes that were and were not administered exogenous progesterone at different time periods from day 0-6 ($CP4^{0-3}$, $CP4^{0-6}$, $CP4^{2-4}$, $CP4^{3-6}$, CnP4) compared to control group pregnant Suffolk ewes that were not administered exogenous progesterone (SnP4). Data is shown as a fold change with 95% confidence interval. If the confidence intervals cross the x axis then mRNA expression differs from the control group. Different letters indicate significant difference between groups.



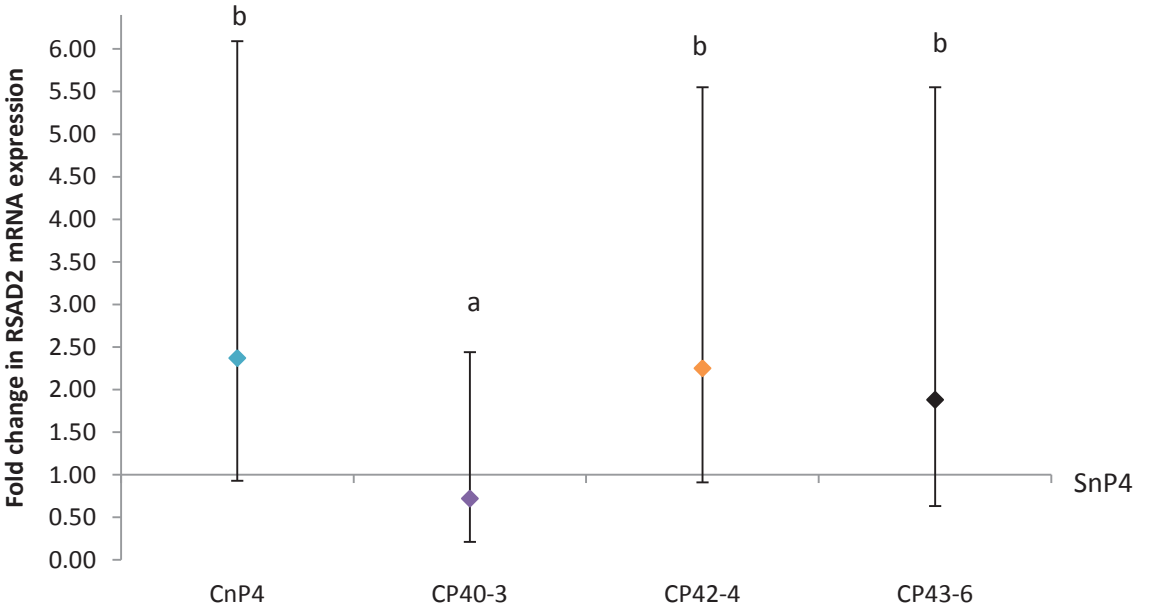
Appendix XI Figure 13 Differential mRNA expression levels of *PTGS2* in day 19 combined uterine horns of pregnant Cheviot ewes that were and were not administered exogenous progesterone at different time periods from day 0-6 ($CP4^{0-3}$, $CP4^{0-6}$, $CP4^{2-4}$, $CP4^{3-6}$, CnP4) compared to control group pregnant Suffolk ewes that were not administered exogenous progesterone (SnP4). Data is shown as a fold change with 95% confidence interval. If the confidence intervals cross the x axis then mRNA expression differs from the control group. Different letters indicate significant difference between groups.



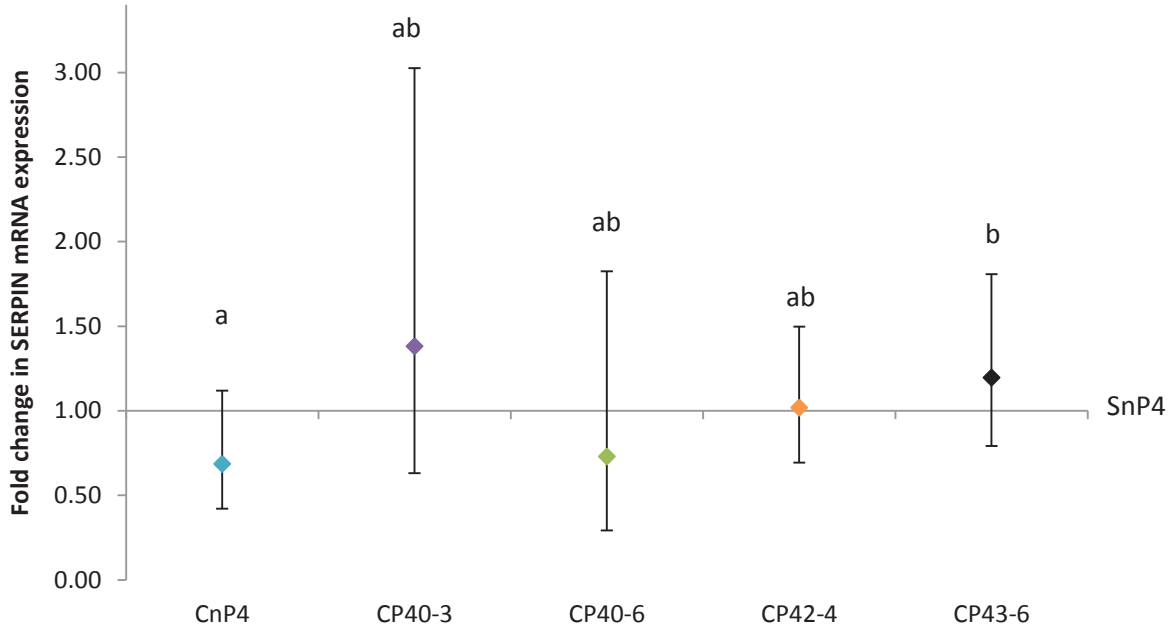
Appendix XI Figure 14 Differential mRNA expression levels of *PTGS2* in day 19 uterine horns ipsilateral to the CL of pregnant Cheviot ewes that were and were not administered exogenous progesterone at different time periods from day 0-6 ($CP4^{0-3}$, $CP4^{0-6}$, $CP4^{2-4}$, $CP4^{3-6}$, CnP4) compared to control group pregnant Suffolk ewes that were not administered exogenous progesterone (SnP4). Data is shown as a fold change with 95% confidence interval. If the confidence intervals cross the x axis then mRNA expression differs from the control group. Different letters indicate significant difference between groups.



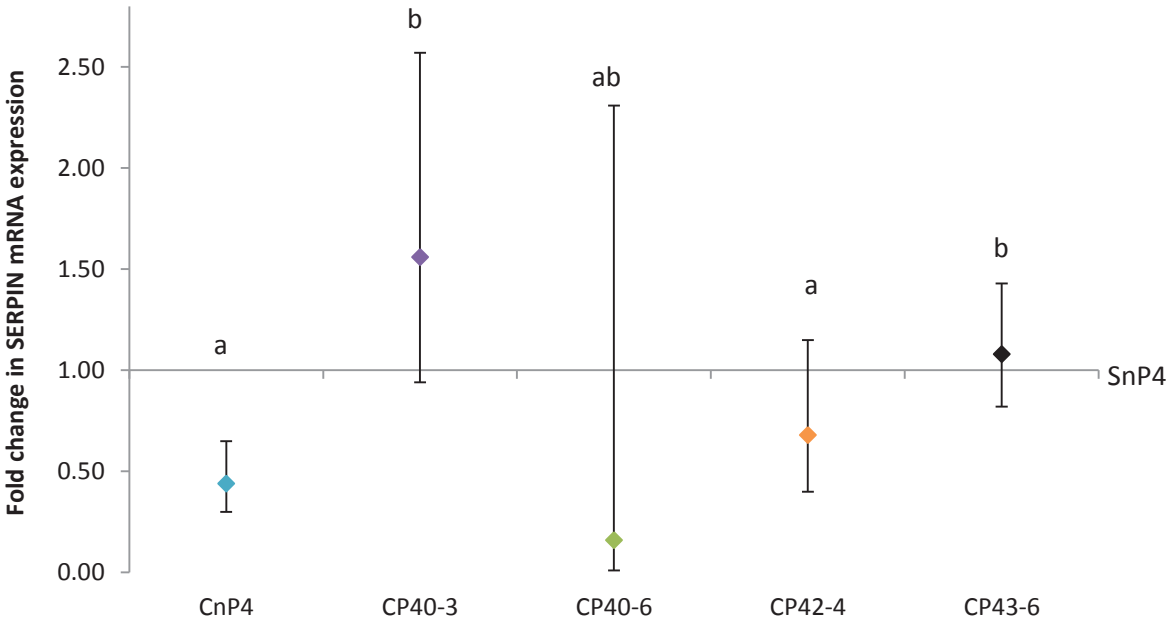
Appendix XI Figure 15 Differential mRNA expression levels of *RSAD2* in day 19 combined uterine horns of pregnant Cheviot ewes that were and were not administered exogenous progesterone at different time periods from day 0-6 ($CP4^{0-3}$, $CP4^{0-6}$, $CP4^{2-4}$, $CP4^{3-6}$, CnP4) compared to control group pregnant Suffolk ewes that were not administered exogenous progesterone (SnP4). Data is shown as a fold change with 95% confidence interval. If the confidence intervals cross the x axis then mRNA expression differs from the control group. Different letters indicate significant difference between groups.



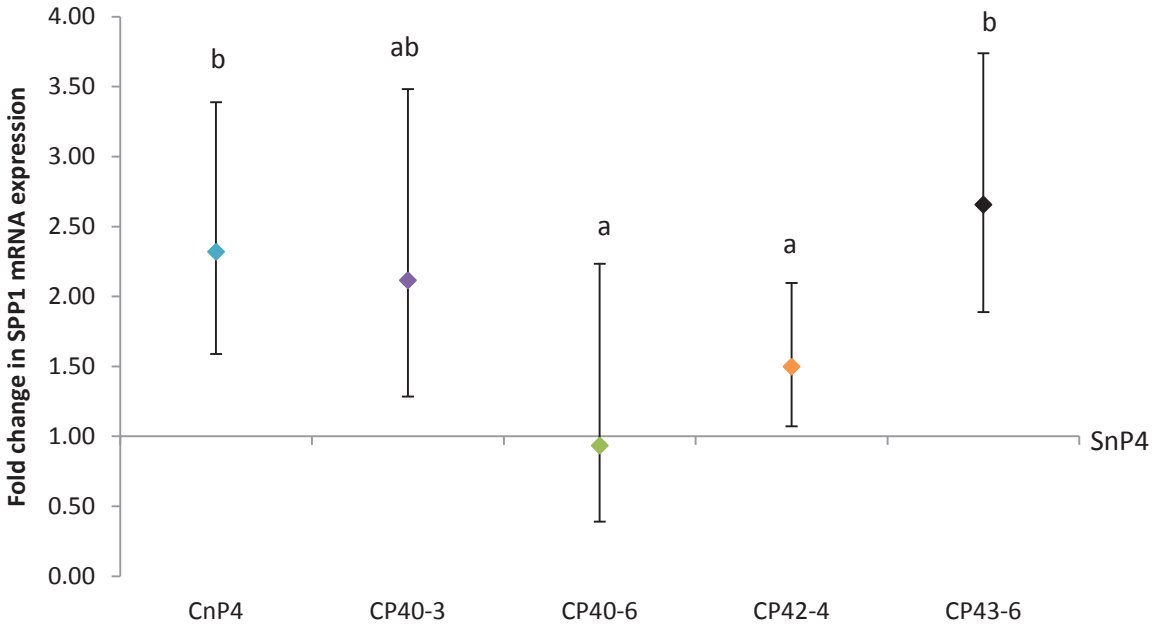
Appendix XI Figure 16 Differential mRNA expression levels of *RSAD2* in day 19 uterine horns ipsilateral to the CL of pregnant Cheviot ewes that were and were not administered exogenous progesterone at different time periods from day 0-6 ($CP4^{0-3}$, $CP4^{0-6}$, $CP4^{2-4}$, $CP4^{3-6}$, CnP4) compared to control group pregnant Suffolk ewes that were not administered exogenous progesterone (SnP4). Data is shown as a fold change with 95% confidence interval. If the confidence intervals cross the x axis then mRNA expression differs from the control group. Different letters indicate significant difference between groups.



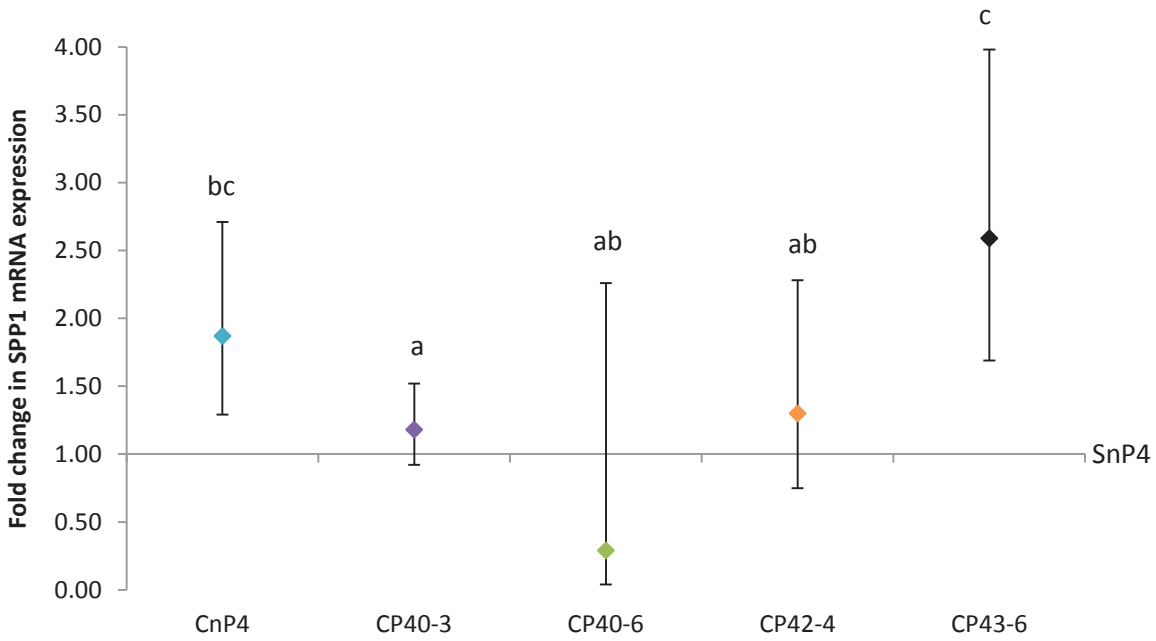
Appendix XI Figure 17 Differential mRNA expression levels of *SERPIN* in day 19 combined uterine horns of pregnant Cheviot ewes that were and were not administered exogenous progesterone at different time periods from day 0-6 ($CP4^{0-3}$, $CP4^{0-6}$, $CP4^{2-4}$, $CP4^{3-6}$, CnP4) compared to control group pregnant Suffolk ewes that were not administered exogenous progesterone (SnP4). Data is shown as a fold change with 95% confidence interval. If the confidence intervals cross the x axis then mRNA expression differs from the control group. Different letters indicate significant difference between groups.



Appendix XI Figure 18 Differential mRNA expression levels of *SERPIN* in day 19 uterine horns ipsilateral to the CL of pregnant Cheviot ewes that were and were not administered exogenous progesterone at different time periods from day 0-6 ($CP4^{0-3}$, $CP4^{0-6}$, $CP4^{2-4}$, $CP4^{3-6}$, CnP4) compared to control group pregnant Suffolk ewes that were not administered exogenous progesterone (SnP4). Data is shown as a fold change with 95% confidence interval. If the confidence intervals cross the x axis then mRNA expression differs from the control group. Different letters indicate significant difference between groups.



Appendix XI Figure 19 Differential mRNA expression levels of *SPP1* in day 19 combined uterine horns of pregnant Cheviot ewes that were and were not administered exogenous progesterone at different time periods from day 0-6 ($CP4^{0-3}$, $CP4^{0-6}$, $CP4^{2-4}$, $CP4^{3-6}$, CnP4) compared to control group pregnant Suffolk ewes that were not administered exogenous progesterone (SnP4). Data is shown as a fold change with 95% confidence interval. If the confidence intervals cross the x axis then mRNA expression differs from the control group. Different letters indicate significant difference between groups.



Appendix XI Figure 20 Differential mRNA expression levels of *SPP1* in day 19 uterine horns ipsilateral to the CL of pregnant Cheviot ewes that were and were not administered exogenous progesterone at different time periods from day 0-6 ($CP4^{0-3}$, $CP4^{0-6}$, $CP4^{2-4}$, $CP4^{3-6}$, CnP4) compared to control group pregnant Suffolk ewes that were not administered exogenous progesterone (SnP4). Data is shown as a fold change with 95% confidence interval. If the confidence intervals cross the x axis then mRNA expression differs from the control group. Different letters indicate significant difference between groups.

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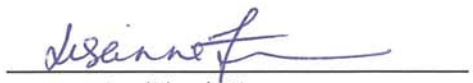

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Fermin, L. M., Pain, S. J., Gedye, K. R., Kenyon, P. R., Blair, H. T. 2016. Progesterone regulation of endometrial genes: their function in pre-implantation embryo growth and development in an ovine model of maternal constraint. 18th International Congress on animal reproduction (ICAR) Tours, France. Poster presentation.

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