



**RESTRICTED IMPLANTATION AND UNDERNUTRITION ALTER
DEVELOPMENT AND GROWTH OF THE OVINE PLACENTA**

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For my son Undi Andrei, my husband Tamanda
and my parents Mr . George and Mrs. Grace Kachilele Chiyenda

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Date 19/10/94

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ABSTRACT

The major sites of substrate exchange between mother and fetus in the ovine placenta are the placentomes, which are composed of the maternal crypts and fetal cotyledons. The maternal crypts develop from specialised folds of endometrium, termed caruncles. In late gestation, the individual placentomes of the ovine placenta can vary greatly in size and macroscopic appearance. Some exhibit an everted shape macroscopically, in which fetal tissue appears to "overgrow" maternal tissue. In animals with a limited area for implantation, due to multiple pregnancy or pre-pregnancy surgical reduction in the number of caruncles, individual placentomes are larger and the proportion of placentomes exhibiting an everted morphology is increased. The cellular and structural basis of these changes in the ovine placenta, the timing of their onset and their consequences for placental function are unknown.

The aims of this study were to characterise the normal ontogeny of the cellular composition and structure of placentomes in sheep, their relationship to the macroscopic parameters of placentome size and morphology, and the effect of experimental and natural restriction of implantation on the growth and development of placentomes between mid and late gestation.

Growth and development were characterised macroscopically and by morphometric analysis of the cellular composition and structure of placentomes using point and linear intercept counting on tissue sections stained with Masson Trichrome.

Measurements of placental weight confirmed that in singleton-bearing ewes, placental weight is maximal by mid gestation. There was no change in placental weight between 90 and 130 days, but a decrease of 26% was evident by 140 days gestation. There was no change in placentome number between mid and late gestation. The proportion of everted placentomes did not change (range: 9-24%) between 90 and 120 days gestation, but did increase to 82% by 130 days and eversion was complete by 140 days gestation. At 90 days gestation, placentomes were composed of 50% fetal tissue (chorionic membrane and its epithelial layer, 14%; fetal villi, 36%) and 38% maternal tissue (maternal crypts, 28%; placentome capsule,

8% and maternal tissue within the haemophagous zone, 2%). Between 90 and 120 days, the proportion of the placentome that consisted of chorionic epithelium and of fetal trophoderm increased by 77% and 44% respectively, whereas the connective tissue core of the fetal villi decreased by 43%. This resulted in the absolute volume of trophoderm being increased by 46%, while that of the connective tissue core of the fetal villi was decreased by 41%, between mid and late gestation. Surface densities of the trophoderm and fetomaternal syncytium also increased between mid and late gestation, such that placental surface area increased by 70% from 5.9 ± 0.4 to $10.1 \pm 0.8 \text{ m}^2$. In addition, the arithmetic mean barrier thicknesses of the two epithelial cell layers at the fetomaternal interface: trophoderm and the fetomaternal syncytium, decreased by 60% and 36% respectively over the same period. In general, placentome cellular composition and structure did not vary with placentome weight, but eversion was characterised by an increased abundance of trophoderm at mid (+61%) and late gestation (+33%).

Restriction of implantation due to pre-pregnancy excision of most placentomes, reduced the number of placentomes formed (-59 %) and total placental weight (-38%), but increased the mean weight of the individual placentomes (+53 %) at mid-gestation. In late gestation, restriction of implantation reduced both placentome numbers and total placental weight by 60% and 45% respectively, while mean placentome weight increased by 36%. In addition, restriction of implantation increased the proportion of everted placentomes at mid (+38 %) and late gestation (+50 %). At mid gestation, restricted implantation increased the volume densities of trophoderm (+51%), maternal crypts (+15%), and fetomaternal syncytium (+35%) and reduced that of the connective tissue core of fetal villi (-18%). The surface density of trophoderm was also increased by 33%. These compensatory changes were sufficient to maintain total volumes of various functional components in the placenta and the surface area of the fetomaternal interface, despite the reduction in placental weight, and hence fetal weight at mid gestation. By late gestation, despite the fact that the placentomes of restricted placentas had greater volume (+23%) and surface (+21%) densities of trophoderm, the total volumes of functional components and surface area could not be maintained. Consequently, fetal weight was reduced following restricted implantation. Thus fetal weight correlated closely with placental weight in late gestation.

Moderate maternal undernutrition, prior to and during the first half of pregnancy (16% reduction in maternal live weight; 25 % reduction in maternal condition score), limited the compensatory increases in weight of individual placentomes and in surface density of the epithelial cell layers, which occurs following restricted implantation at mid gestation. However, undernutrition with restricted implantation did not concomitantly alter fetal weight in these ewes.

Natural restriction of implantation in ewes carrying twins, increased the volume density of trophoctoderm (+71%) and feto-maternal syncytium (+46%), which maintained the total volumes of these cellular components at mid gestation. In the placentomes of twins, the surface densities of the two epithelial cell layers, trophoctoderm and feto-maternal syncytium increased (+37%) , such that total surface area for exchange was similar to that of the placentas of singletons. In placentas of twins, the barrier thickness of fetal trophoctoderm increased (+15%) and that of feto-maternal syncytium decreased (-20%). These changes were generally similar to those which occurred following experimental restriction of implantation in singletons. Placentas of triplets were similar to those of twins with respect to both the nature and extent of changes in macroscopic and microscopic placental structure. Placentas of multiple conceptuses which were also restricted experimentally, by the pre-pregnancy surgical removal of most caruncles, demonstrated similar compensatory growth responses, but of reduced magnitude, to those observed in experimentally restricted placentas of singletons. Thus there are limits to the extent to which compensatory increases in the abundance and surface area of the exchange epithelia can be induced by restriction of implantation.

To summarise, in this study, ontogenic increases in the volume of fetal trophoctoderm and the surface area of the feto-maternal interface between mid and late gestation have been demonstrated in the ovine placenta. Concomitantly, ontogenic decreases in arithmetic mean barrier thicknesses of these surface epithelia and in the volume of connective tissue in fetal villi occur. These developmental changes in surface area and barrier thickness influence exchange and transfer of substances and are largely determined by the thickness of the epithelial layers and connective tissue of fetal villi and maternal crypts. These changes will

substantially increase placental functional capacity between mid and late gestation in normal singletons. In addition, compensatory enhancement of many of these developmental changes has been shown to occur following natural and experimental restricted implantation. These are sufficient to maintain placental function at mid but not in late gestation, as indicated by fetal weight.

The stimulus to these ontogenic and compensatory adaptations in placental growth and development remains to be determined. The increase in the volume and surface density of trophoctoderm common to all perturbations studied here implicate the trophoctoderm as the major target of any stimulus that induces placental compensatory growth, and that these signals may act via a factor that is common to restricted implantation and maternal undernutrition.

PUBLICATIONS ARISING FROM THIS WORK

ABSTRACTS

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LIST OF ABBREVIATIONS AND ACRONYMS USED IN THIS THESIS

A	non-everted placentome morphology
ACTH	Adrenocorticotrophic hormone
AIB	α -aminobutyric acid
3 β HSD	3 β -hydroxysteroid dehydrogenase
11 β -OHS	11 β -hydroxysteroid dehydrogenase
B, C, D	three variations of everted placentome morphology
Bf	Bouin's fixative
BNC	Trophectodermal binucleate cell
CEP	Chorionic epithelium
CM	Chorionic membrane
cm	Centi metre
CO ₂	Carbon dioxide
Control or C ewe	Ewe in which pregnancy was allowed to proceed normally without interference with carunclectomy
Cot	Cotyledon (Placentome or placental functional unit)
CSF	Colony stimulating factor
CT	Connective tissue core of fetal villi
DNA	Deoxyribonucleic acid
EPC	Ectoplacental cone
F	10% neutral buffered formalin
FGF	Fibroblast growth factor
FMS	Feto-maternal syncytium
FV	Fetal villi
GM-CSF	Granulocyte and macrophage-colony stimulating factor
GTs	Glucose transporters
GT1 or GLUT1	the first isoform of glucose transporters with molecular weight 50 kDa
H&E	Hematoxylin and eosin
HMZ	Haemophagous zone

HPA axis	Hypothalamo-pituitary-adrenal axis
IGF-I	Insulin-like growth factor-i
IGF-II	Insulin-like growth factor-ii
IGFBP	Insulin-like growth factor binding protein
IL-1	Interleukin-1
IUGR	Intra-uterine growth retardation
kDa	Kilo dalton
LBW	Low birth weight
LDL	Low density lipoprotein
LM	Light microscopy
LSMeans	Least squares means
m ²	Metres squared
MAB	Masson trichrome counterstained with alanine blue
MC	Maternal crypts
MLG	Masson trichrome counterstained with light green
mm	Milli metres
mRNA	Messenger ribonucleic acid
MT	Maternal tissue in HMZ
N	Number of ewes, fetuses or placentas studied
n	Number of placentomes morphometrically analysed
O ₂	Oxygen
OPL	Ovine placental lactogen
p	Probability value
P ₅₀	Hemoglobin half saturation pressure for oxygen
PC	Placentome capsule
pH	Power of hydrogen
PI	Ponderal index (fetal weight × 100/length (cm) ³)
PM	Post-mortem
Restricted or R ewe	Ewe which underwent surgery to remove most caruncles (carunclectomy) prior to pregnancy in order to restrict implantation in the subsequent pregnancy

r	Correlation coefficient
SD	Standard deviation of mean
SE	Standard error of mean
SGA	Small for gestational age
SKG	Shrinkage spaces
System A	Transporter system for amino acids with short, polar or linear side-chains
System ASC	Transporter system for alanine, serine, cysteine
System L	Transporter system for large apolar branched-chain and aromatic amino acids
T	Trophectoderm
TGF- β	Transforming growth factor beta
TNF- α	Tumor necrosis factor-alpha
μm	Micro metre
VEGF	Vascular endothelial growth factor
VLDL	Very low density lipoprotein
Vol	Volume

CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION

The placenta is an organ that is characteristic of mammalian pregnancy which is formed through the apposition of uterine and trophoblastic vascularised tissues. It functions to join the developing offspring and the mother, allows selective exchange between mother and fetus of soluble blood-borne substrates and respiratory gases essential for normal fetal growth and development, and secretes hormones essential for the maintenance of pregnancy (Mossman, 1937; Amoroso, 1952; Davies and Wimsatt, 1966; Carter *et al.*, 1991). The placenta also maintains an immunological barrier to protect the fetus against maternal immunosuppressive functions (King *et al.*, 1982; Faber and Thornburgh, 1983; Bassett, 1991). The function of this organ is therefore a major determinant of an individual's development *in utero*, and of birth outcome (Baur, 1971; Alexander, 1974; Mellor, 1983; Boyd, 1987) and health in later life (Barker, 1990). Consistent with this essential role in early development, the placenta has been shown to be capable of substantial and usually successful compensatory changes in growth in response to endocrine, nutritional and surgical disturbances, especially in the first half of pregnancy. Yet the nature of compensatory placental growth, the mechanisms involved and the consequences for placental function are poorly understood. These issues are the central to the investigations described in this thesis.

1.2 MAMMALIAN PLACENTAS

1.2.1 Summary of determinants of placental substrate transfer

Placental substrate transfer function has several important determinants (Faber and Thornburg, 1983; Owens and Robinson, 1988; Hay, 1991; Bassett, 1991; Kelly, 1992; Eaton and Contractor, 1993). These include

a) placental structure:

- i) the number and thickness of the cell layers separating maternal and fetal blood, which are determined by the extent of chorionic invasion of the uterine mucosa that occurs at implantation,
- ii) the surface area for exchange at the feto-maternal interface,
- iii) the abundance, size and location of maternal and fetal tissue vasculature,

- iv) placental mass,
- b) placental perfusion:
 - i) uterine and umbilical blood flow rates
 - ii) the geometrical arrangement of the maternal and fetal vasculature, which determines the relationship of the directions of fetal and maternal blood flow (countercurrent, concurrent, or crosscurrent),
- c) inherent placental transport characteristics such as molecular structures of the membrane (for example, glucose and amino acid transporter proteins) and permeability characteristics,
- d) placental metabolism of substrates and
- e) developmental influences on these structural, perfusion, molecular and metabolic characteristics of the placenta.

The available evidence suggests that the structural characteristics of the placenta are a major influence on placental function. For example, in the human placenta, the thickness of the barrier, which constitutes all cell layers separating fetal and maternal blood, is reported to contribute between 60 and 90% of placental resistance to substrate diffusion between maternal and fetal blood (Mayhew *et al.*, 1984, 1990; Jackson *et al.*, 1985, 1986). It is noteworthy that while placental structure varies between species, critical elements are common or invariant. However, it is those characteristics which vary that have served as a basis for placental classification (Amoroso, 1952). These differences largely originate in the mode and extent of chorionic invasion of the uterine tissues at implantation (Amoroso, 1952).

1.2.2 Classification by placental morphology

Mammalian placentas can be classified on the basis of their macroscopic appearance into:

- a) *placenta diffusa*: in which the surface enlarging factors or chorionic villi are distributed uniformly on the whole outer surface of the chorionic sac (Amoroso, 1952; Baur, 1977). In animals exhibiting such placentation, attachment between fetal and maternal tissues is achieved through microvillous interdigitations of the epithelial surfaces at the interface (King *et al.*, 1982). Hence diffuse placentas are non-invasive, because there is no

penetration of fetal tissues into maternal tissue, such that the uterine lining remains entirely maternal (King *et al.*, 1982; Roberts and Bazer, 1988). A classical example of this type of placentation is the porcine placenta.

- b) *placenta cotyledonaria or multiplex*: in which the villous areas are restricted to form discrete scattered cotyledons such as in the bovine, caprine, and ovine placentas (Amoroso, 1952).
- c) *placenta zonaria*: in which the placenta appears like a girdle-like band (for example the canine placenta) (Amoroso, 1952).
- d) *placenta discoidalis*: in which the placenta assumes a disc-shape (for example the human placenta) (Amoroso, 1952).

In contrast to diffuse placentas, the placental types (b) through (d) can collectively be described as "compact" placentas since they all have surface enlarging factors that are grouped in certain areas of the chorionic sac in the form of compact, macroscopically distinct features (Baur, 1977). Compact placentas differ from diffuse placentas in having a larger surface area for a given volume and in having a weight per unit volume (relative surface area) averaging about seven fold greater than that of diffuse placentas. This suggests there are physiological or functional differences between the compact and diffuse placental types (Baur, 1977, 1981).

1.2.3 Classification by placental histology

Mammalian placentas have also been categorised according to the number of cell layers that separate maternal blood and fetal (chorionic) epithelium. "In the simplest forms, an intact mucosa is apposed to the chorion and the maternal and foetal bloods are separated by maternal endothelium, maternal connective tissue, maternal uterine epithelium, foetal (chorionic) epithelium, connective tissue and endothelium. In the more complex types, however, these layers, especially the maternal constituents are successively broken down, until the chorionic epithelium, which according to Grosser is always intact, although perhaps modified, comes into

Grosser (1909, 1927; cited in Amoroso, 1952) have been modified. However, three major placental types are still recognised, epitheliochorial, endotheliochorial and hemochorial (Faber and Thornburg 1983):

- a) *Epitheliochorial placenta*: in which the trophoblast does not invade the uterine epithelium but attaches to it by interdigitating microvilli; hence the uterine epithelium remains an integral part of the placental barrier throughout pregnancy. Six tissue layers separate fetal and maternal blood systems (Table 1.1) (Amoroso, 1952; Björkman, 1965; Steven, 1975; Mitchell, 1982; Faber and Thornburg, 1983; King, 1989; Hay and Wilkening, 1994). Examples of this type of placentation include those of the equine, porcine, caprine, ovine and bovine species. However, electron microscope evidence suggests that the formation of the caprine and ovine placentas involves a modification of the uterine epithelium into a hybrid feto-maternal syncytium. This syncytium arises from the migration of fetal binucleate cells from the uninucleate trophoblast to fuse with the uterine epithelial cells (Davies and Wimsatt, 1966; Lawn *et al.*, 1969; Steven *et al.*, 1978; Wooding, 1982; Wooding and Morgan, 1989). The evidence supporting this origin of the feto-maternal syncytium has been reviewed recently by Wooding (1992), who suggested that the caprine and ovine placentas may be more appropriately classified as *synepitheliochorial*; where the prefix "syn" emphasizes the contribution of both maternal and fetal cellular components to the uterine epithelial lining.
- b) *Endotheliochorial placenta*: in which both the uterine epithelium and stroma are eroded away, leaving maternal endothelium in contact with chorionic epithelium, as in placentas of the family *Carnivora* (Amoroso, 1952; Faber and Thornburg, 1983; Hay and Wilkening, 1994).
- c) *Haemochorial placenta*: in which the erosion of maternal tissues includes the endothelium, such that chorionic placental villi are bathed directly in maternal blood. Hence, the placenta is composed solely of fetal tissues (Table 1.1). Examples are placentas of rodents, as well as humans and other primates (Amoroso, 1952; Faber and Thornburg, 1983; Hay and Wilkening, 1994).

as well as humans and other primates (Amoroso, 1952; Faber and Thornburg, 1983; Hay and Wilkening, 1994).

In this study, the sheep, a species with compact placentation, has been used to test hypotheses related to normal and compensatory placental growth. Cotyledonary placentas, in particular that of sheep, offer additional indices of altered growth and development, exhibiting variability in size and morphology of individual placental functional units (placentomes). In addition, the sheep has been preferred to other ruminants with a similar placental structure, because it is inexpensive and readily available, tolerates experimental manipulation and its physiology has been better characterised.

1.3 PLACENTAL FUNCTION IN THE OVINE PLACENTA

1.3.1 Structural elements of the ovine placental barrier

In this section, the elements of the placental barrier within the zona intima, the region of the placentome in which maternal and fetal tissues are in intimate association, are described in sequence from maternal to fetal blood, in the mature placenta in late gestation. These structural elements are presumably actively involved in substrate transfer across the placental membrane. For example membrane transport carriers for glucose have been identified on luminal and basal membranes of the syncytiotrophoblast in the placenta of the guinea pig (Yudilevich *et al.*, 1979), rat (Takata *et al.*, 1990) and human (Jonhson and Smith, 1980; Takata *et al.*, 1992).

Maternal stroma

The maternal stroma forms part of the expanded specific implantation site (caruncle) many of which are available in the non-pregnant uterus. The maternal stroma interdigitates with the fetal villi while housing and supporting them (Fig. 1.1). Under the electron microscope, the maternal stroma is composed of a network of basement membrane-like material interspaced with collagenous fibres, fibroblasts and cellular debris (Björkman, 1965; Davies and Wimsatt, 1966; Mitchell, 1982). Each maternal septum contains a central blood vessel (Mitchell, 1982).

of limited size, formed from the fusion of about 20 to 25 fetal binucleate cells with one maternal epithelial cell (Wooding, 1982), which are separated from each other by plasma membranes (Björkman, 1965). The lateral plasma membranes of the syncytial plaques unite at the luminal surface by way of junctional complexes, including tight junctions (Steven and Samuel, 1979). The basement membrane is not well-defined (Davies and Wimsatt, 1966), due to numerous basal processes that arise from this tissue and pierce the maternal stroma in the direction of the blood vessel (Mitchell, 1982). Other features of the syncytium include a height comparable to low columnar epithelium, centrally located irregularly shaped nuclei that lack nucleoli and the absence of mitotic activity (Björkman, 1965; Mitchell, 1982). The nuclei of the syncytium also show a characteristic clumping of the chromatin similar to that in the binucleate cells (Davies and Wimsatt, 1966). The syncytium also contains cytoplasm which resembles that of the binucleate cells of the trophoblast, consistent with the syncytium being derived from the binucleate cells (Davies and Wimsatt, 1966). In addition, the syncytium exhibits a random distribution of thinner areas and aggregates of nuclei, possibly analogous to the vasculosyncytial membranes and syncytial knots in human placentas (Fox, 1967). Mitchell (1982) also described a system of small channels or canaliculi present within the cytoplasm of the syncytium within the ovine placenta. On the apical surface, the plasma membrane of the syncytium interdigitates with the trophoctoderm that borders fetal villi, generally with tall regular microvilli, although occasionally flat apposed surfaces of the two epithelial tissues are evident (Mitchell, 1982). These surfaces probably correspond to areas where the feto-maternal syncytium is attenuated or degenerated thus substantially narrowing the placental barrier due to the reduction in maternal stroma (Davies and Wimsatt, 1966).

Trophoblast bordering the fetal villi

a) Uninucleate trophoctoderm

The uninucleate trophoctoderm consists mainly of cells described as being predominantly large, cuboidal and pale with a centrally-located vesicular nucleus and a single nucleolus (Björkman, 1965; Mitchell, 1982). The tissue exhibits distinct cell boundaries and rests on a well-defined, multi layered basement membrane, that separates it from the mesenchyme (Björkman, 1965; Davies and Wimsatt, 1966; Mitchell, 1982). The lateral membranes unite at the lumen via junctional complexes, some of which are tight junctions (Steven and Samuel; 1979). On the luminal side, the cells exhibit long microvilli, which interdigitate with the feto-maternal syncytium (Mitchell, 1982; King *et al.*, 1982).

b) Trophoctodermal binucleate cells

Binucleate cells constitute about 15 to 20% of all trophoctoderm cells from the beginning of implantation to a few days before term (Mitchell, 1982; Wooding, 1982). They are giant polygonal cells containing two visible nuclei, that are much larger than those of the uninucleate cells, and vacuolated cytoplasm (Björkman, 1965; Mitchell, 1982). The nuclei exhibit a characteristic clumping similar to that in feto-maternal syncytium (Davies and Wimsatt, 1966). They appear detached from the basement membrane due to the uninucleated cells which completely envelope them (Mitchell, 1982).

The arrangement of the intracellular contents of binucleate cells appears to display polarity; such that granules congregate towards the basal end of the cell and rough endoplasmic reticulum towards the luminal side. Rarely, pseudopodial processes are evident into the brush border zone, where the two epithelial cell layers interdigitate at the feto-maternal interface (Davies and Wimsatt, 1966; Mitchell, 1982; Wooding, 1982).

Connective tissue core of the fetal villi

Observations by Mitchell (1982) revealed the presence of vascularised fine connective tissue containing fibroblast cells in the core of fetal villi. In wider villi, an additional histiocyte cell, containing lysosomes and peripheral filiform processes, was also present. Björkman (1965)

commented that in late gestation, the mesenchyme may play a subordinate role as a component of the placental barrier, since many of the capillaries were intimately related to the trophoblast, from which they were separated only by the basement membrane.

1.3.2 Vascularity of the fetal and maternal tissues of the ovine placenta

Maternal vasculature

Each of the maternal septa exhibits a centrally located blood vessel. Two types of microvessels are present. The first type has a thick and pale endothelial lining in which the cells adhere to each other via lateral junctional complexes (Björkman, 1965; Mitchell, 1982). Narrow cytoplasmic processes extend from the cell surface into the lumen of the capillary. The second type of blood vessels are devoid of a basement membrane and appear to be supported by large smooth muscle cells, that are often evident at the base of the endothelial cells forming the blood vessel walls (Mitchell, 1982).

Chorionic vasculature

In the first half of gestation, blood vessels are found embedded within the mesenchyme connective tissue at the cores of the fetal villi (Davies and Wimsatt, 1966). However, from about 100 days gestation onwards, the capillaries of the fetal villi are typically intraepithelial. The capillaries invade the trophoblast by indenting its basal surface, such that the capillaries and the trophoblast are separated only by their merged basement membranes (Björkman, 1965; Davies and Wimsatt, 1966; Mitchell, 1982). The endothelial lining of the capillaries is thin and fenestrated and the endothelial cells adhere together by junctional complexes. Irregular luminal folds are also evident (Björkman, 1965; Mitchell, 1982).

1.4 ONTOGENY OF PLACENTAL GROWTH AND STRUCTURAL DEVELOPMENT IN SHEEP

1.4.1 Introduction

In sheep the non-gravid uterus contains about 60 to 150 circular elevations (about 5mm in diameter) of the endometrium that are aglandular in nature (King *et al.*, 1982; Wooding and

Morgan, 1989). They are divided approximately equally and are arranged in four vertical rows in each of the two horns of the uterus (Cloete, 1939). These elevations are called caruncles and they are predetermined or potential sites of trophoblastic implantation (Figure 1.2). Their numbers tend to vary between breeds, strains, and individuals of the same species (Alexander, 1964a; Stegeman, 1974).

Following trophoblastic attachment to the caruncles, placental development begins by the formation of an elaborate chorionic villous system only in those areas of the recruited caruncles. The resultant fetal cotyledons, together with the endometrial caruncles, constitute the placental units called placentomes. This arrangement gives rise to a "cotyledonary" placenta (Fig. 1.3) according to Grosser's classification (1927; cited in Amoroso, 1952).

1.4.2 Implantation

Following fertilisation in sheep, embryonic development proceeds to the blastocyst stage by approximately the fifth day after oestrous (Rowson and Moor, 1966). Between the fifth and ninth days, the blastocyst hatches out of the zona pellucida. By the eleventh day, the blastocyst has expanded and adopted a collapsed and somewhat wrinkled form, probably due to a faster proliferation rate of the trophectoderm relative to the rate of fluid accumulation within the blastocyst (Rowson and Moor, 1966). Between days 12 to 15 after mating, the ovine blastocyst elongates extensively from a size of about 1mm diameter to an average length of approximately 102 mm. Due to this expansion, the conceptus fills the entire uterine lumen (Rowson and Moor, 1966; Alexander, 1978; Wooding and Morgan, 1989). This process is soon followed by implantation, in which the elongated blastocyst attaches to the uterine mucosa between days 15 to 18 of gestation (Davies and Wimsatt, 1966; Guillomot *et al.*, 1981, 1982), usually near the middle of the horn initially (King *et al.*, 1982; Gaviria and Hernandez, 1994). The implantation process in the ewe ends about day 23 to 24 (Boshier, 1969; Wooding and Morgan, 1989) when trophoblastic attachment to the uterine lumen is complete (Gaviria and Hernandez, 1994).

According to Wooding and Morgan (1989), implantation in ruminants can be defined by the initiation of binucleate cell migration to and fusion with the uterine epithelium, forming a fet-

maternal syncytium, which replaces the uterine epithelium. This process begins about days 15 to 16 after fertilisation in sheep (Boshier, 1969), day 19 in goat and day 20 in the cow.

Binucleate cells are specialised cells, which differentiate from and within the uninucleate trophoctoderm (Davies and Wimsatt, 1966; Lawn *et al.*, 1969; Steven *et al.*, 1978; Wooding, 1982). The binucleate cells first appear at the time the trophoctodermal microvilli come into close apposition to those of the uterine epithelium, a day or two prior to the start of implantation in sheep. In both goats and cows, binucleate cell migration begins about 3 days before implantation (Wooding and Morgan; 1989).

It is clear that a variable number of caruncles are recruited during the implantation process to contribute towards placental formation with some caruncles always remaining unrecruited. The selection mechanism which recruits some caruncles and not others is unknown. However, it appears that the proportion of the caruncles that are recruited tends to correlate with the number and sex of conceptuses in any one pregnancy. On average, 60 to 70% of the caruncles are recruited in female or singleton placentas and about 80% in singleton male and in twin placentas (Alexander, 1964b; Stegeman, 1974). Also the number of attached or recruited caruncles declines with increasing age and parity of the ewe (Alexander, 1974).

1.4.3 Development of chorionic villi

The completion of implantation is marked by the appearance of rudimentary villi, ensuring that the chorionic sac is more closely attached to the caruncles. The villi develop as buds of the fetal ectoderm, which are then invaded by cores of mesoderm containing branches of allantoic vessels (Amoroso, 1952). These villi have been reported to first appear between 21 and 31 days of gestation (Wooding and Morgan, 1989; Wimsatt, 1950; Boshier, 1969; Amoroso, 1952). The villi soon begin to elongate and branch, giving rise to first order and second order branches by the 32nd day of gestation (Wimsatt, 1950). By day 36, the villi are about 1.5 to 2mm long. Between days 36 and 44 the villi lengthen by an extra 2 to 2.5 mm and then produce 3rd order branches. By day 52 they are about 5mm long and show no evidence of further branching thereafter. From this time, the villi elongate further and also increase in girth, due to the accumulation of mesenchymal connective tissue. The villi attain a maximum length of about

10mm and a width of 2 to 3mm by day 74 (Wimsatt, 1950). Individual placentomes reach maximum weight and dimensions between 70 and 100 days of gestation (Cloete, 1939; Barcroft, 1946; Wimsatt, 1950; Amoroso, 1952; Bell, 1984; Kelly *et al.*, 1987; Kelly and Newnham, 1990; Meschia, 1990; Mc Crabb, 1990). Placental DNA analyses suggest that maximum cellularity may be achieved by day 75 (Vatnick and Bell, 1991; 1992; Kulhanek *et al.*, 1974). The phase of villi formation and branching is an important aspect of placental development, since increased branching would increase the surface area for exchange and hence functional potential (Stegeman, 1974). It is not known what factors control this process.

1.4.4 Phase of functional maturation

Introduction

In sheep, placental growth proceeds rapidly from the onset of implantation until the time at which maximum weight is attained, in mid gestation. In the last two months of gestation, placental cellularity; as measured by total number of placental nuclei (Teasedale, 1976) and total placental DNA content, remains constant (Kulhanek *et al.*, 1974; Vatnick *et al.*, 1991).

Although the ovine placenta does not increase in mass during this time, it appears to meet the increasing demands of the fast growing fetus through extensive modification of its structure and other characteristics which presumably enhance function.

Reduction of barrier thickness

As gestation progresses, there is remodelling of the cellular constituents of the fetal villi and maternal crypts to reduce the overall barrier thickness of the tissues separating the maternal and fetal blood systems and enhance substrate transfer. Previous studies in sheep have demonstrated an ontogenic reduction of the amount of the maternal stroma and the attenuation or degradation of the feto-maternal syncytial lining of the crypts within the second half of gestation (Wimsatt, 1950; Stegeman, 1974; Mitchell, 1982). On the fetal side, the villi undergo shrinkage due to the disappearance or loss of the connective tissue at the core of the villi from mid-gestation (Barcroft and Barron, 1946). According to Wimsatt (1950), the thinning of the villi continues until near term (day 141). The loss of the connective tissue and thinning of the fetal villi should raise the relative density of blood vessels and trophoblast cells within the correspondingly

thinner villi. These changes together with the colonisation of the trophoctodermal lining by chorio-allantoic intraepithelial vessels after day 100 of gestation (Davies and Wimsatt, 1966; Mitchell, 1982), and the presence of regional variations in the distribution of the feto-maternal syncytium (Mitchell, 1982) (analogous to vasculosyncytial membranes in the human (Fox, 1967; Jackson *et al.*, 1985; 1988)), should reduce the diffusion distance and hence bring fetal blood in closer proximity to maternal blood. Measurements of the total barrier thickness of all cell layers separating maternal and fetal blood systems in sheep revealed a significant decrease from 22.14 μ m at 120 days to 16.14 μ m at 140 days (Mitchell; 1982). The remodelling of the cellular components of the placental barrier as gestation progresses, indicates that the actual number of cell layers between maternal and fetal blood systems alone may not be a true index of diffusional resistance (Faber and Thornburg, 1983).

Angiogenesis

Angiogenesis is the formation of new blood vessels (West *et al.*, 1985). Blood vessel formation involves capillary proliferation which results in the formation of a new microcirculatory bed consisting of arterioles, venules and capillaries (Folkman and Shing, 1992). In sheep, vascularity of the maternal side of the placenta continues to increase tremendously during the last 60 days of pregnancy (Barcroft, 1946; Barcroft and Barron, 1946; Stegeman, 1974). Concomitantly, the invasion of columnar trophoblast by chorio-allantoic capillaries after day 100 of gestation has also been reported (Davies and Wimsatt, 1966). Stereological studies have demonstrated increased numbers of fetal endothelial cells in the ovine placenta in the second half of gestation (Teasedale, 1976). Morphometric analysis of ovine placentas of twins indicated that vascularisation of the villi increases constantly from about 11 weeks gestation to term (gestation age determined indirectly by fetal crown to rump length) (Stegeman, 1974). In addition, *in vitro* studies showed that ovine fetal cotyledonary tissue contains angiogenic activity in late gestation, since media conditioned with explants of such tissue obtained at 120 days gestation stimulated endothelial cell proliferation in culture (Millaway *et al.*, 1989). However, other studies in the ovine placenta suggest that the maternal component of the placentome is the primary source of placental angiogenic activity at all stages of gestation in sheep, whereas the fetal component secretes activity which inhibits proliferation and migration of endothelial cells required for angiogenesis (Reynolds and Redmer, 1988).

multiplying and migrating (Frazer *et al.*, 1989). Partial degradation products of hyaluronic acid following its catalysis by hyaluronidase have been shown to induce angiogenesis on chick chorioallantoic membrane (West *et al.*, 1985). The mechanism by which capillarisation is stimulated may also involve the destabilisation of the extracellular or collagenous matrix (West *et al.*, 1985), enabling endothelial cell migration into the cores of the villi and maternal crypts.

There is also evidence suggesting a stimulatory role for progesterone in placental angiogenesis. Culture medium, which was conditioned with caruncular tissue explants from ovariectomized ewes treated with exogenous progesterone, induced significantly greater endothelial cell mitogenic activity than that obtained using material from non-progesterone treated ewes (Reynolds and Redmer, 1988). In the porcine endometrium, protein synthesis is high when the ratio of progesterone to oestrogen is at a maximum and is markedly reduced with increasing oestrogen concentrations (Roberts and Bazer, 1988). Porcine uterine flushings revealed variations of concentrations of both hyaluronic acid and hyaluronidase throughout the luteal cycle, low levels of hyaluronic acid and hyaluronidase were detected on days 0 and 6, while peak concentrations were present on days 12 and 15 of the cycle. In cyclic gilts, the levels of the protein and the enzyme decreased by day 18, but remained high in pregnant pigs (Ashworth *et al.*, 1989). These studies indicated the possible stimulatory effect of the hormone progesterone in the secretion of hyaluronidase and hyaluronic acid. Both hyaluronic acid and hyaluronidase are associated with the stimulation of angiogenesis in the pig uterus (Ashworth *et al.*, 1989).

Growth of the surface area for exchange at the feto-maternal interface

For the placenta to effectively function as an organ for respiration and nutrition of the fetus, the fetal and maternal blood streams must be brought into close association over a large area (Aherne and Dunhill, 1966). Typically, fetal mass exceeds that of the placenta after mid-gestation and by term, fetal weight can be 10 fold greater than that of the placenta in sheep (Bell, 1984).

To accommodate fetal demand, placental development must achieve large expansions of surface area for exchange throughout gestation. Studies in several species have demonstrated ontogenic increases in surface area (Baur, 1977), despite a plateau or slowing of the rate of increase in placental weight and DNA content in late gestation (Stegeman, 1974; Kulhanek *et al.*, 1974; Teasedale, 1980; Battaglia and Hay, 1984; Vatnick *et al.*, 1991).

The ontogeny of placental surface area has not been adequately characterised in sheep, hence evidence from studies involving other species is mainly reviewed here. The only study of the growth of surface area in sheep was carried out by Stegeman (1974), who morphometrically determined villous branching in placentas of twin conceptuses using a point counting method. The product of the degree of branching and the total weight of the placenta was used as a reflection of total surface area for exchange. Stegeman's study showed that in the placentas of twins, the villi attain their maximum surface area by means of branching at approximately day 105 to 112 of gestation. However, in this study, gestational age was determined by crown rump length, fetal weight and external dimensions of the fetus, while villous branching and surface area were only analysed using arbitrary and relative indices. Baur (1977) studied the growth of surface area in several species including pig and horse (diffuse placentas), cow (cotyledonary placenta) and rat and human (haemochorial placentas). The rate of growth of the total villous surface in all placental types accelerates as gestation progresses, and at different rates in different species. The cow with a similar placental type to sheep, demonstrated the most rapid growth rate in surface area, when placental surface area was plotted against gestational age. Thus, the actual surface area present at term also varies greatly from one species to another, for example an area of 12m^2 was present in the human placenta, compared to 130m^2 in that of the cow (Baur, 1977). Other studies have also revealed large increases in villous surface area of the human placenta from about 1 to 1.5m^2 at 100 days to about 11 to 14m^2 at term (Wilkin, 1960; cited in Aherne and Dunhill, 1966, Jackson *et al.*, 1992). A 3.7 fold increase in villous surface area, from 3.4m^2 at 28 weeks, to 12.6m^2 at term, has also been reported within the third trimester in the human placenta (Aherne and Dunhill, 1966).

Wooding (1982) showed that in the ruminant placenta, the BNCs constitute 15 to 20 per cent of the trophoctodermal epithelial cells lining the surface of the fetal villi from the time of implantation to about a couple of days before parturition, when their numbers decrease rapidly. Quantitative studies revealed that about 15 to 20 percent of all BNCs are constantly migrating from the trophoctodermal layer to fuse with the uterine epithelium. In the ovine placenta, the feto-maternal syncytial layer persists until term due to continued BNC migration (Wooding, 1982; Wooding and Morgan, 1989). Therefore the migration of BNCs is essential for placental establishment and support of the tremendous increase in surface area for substrate exchange (Wooding, 1992). What

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Variability in gross placentome size and morphology during placental development

At any given gestational age, the ovine placenta is composed of placentomes of various sizes (Fig. 1.4). Previous qualitative reports suggest that the location of the placentome in the uterus may determine individual placentome size. Andersen (1926) noted that in bovine placentas, large placentomes tended to be located in the centre of the uterine horn. A similar observation was made in the ovine placenta by Cloete (1939), who also noted that in the pregnant horn, the larger placentomes were those closest to the implantation site. Cloete (1939) also noted that in the non-pregnant horn, larger placentomes were also more commonly present towards the middle of the horn. While placental weight is maximal at mid gestation then declining, variations in the ontogeny of the growth of individual placentomes occur. In the non-pregnant horn, placentomes tended to reach maximum size later than those located in the pregnant horn (Cloete, 1939). Slower development of placentomes in the non-pregnant horn may be related to delayed increases in the vascularity of the deep caruncular tissues (Reynolds and Redmer; 1992). In the non-pregnant horn, increased vascularity of the deep caruncular tissues occurs on day 30 as opposed to day 24 of gestation in the pregnant horn (Reynolds and Redmer; 1992). In addition, there is a delay in the increase in uterine blood flow to the non-pregnant horn, which remains less than that of the gravid horn throughout gestation (Greiss and Anderson, 1970; Reynolds *et al.*, 1984). However, slower development of placentomes in the non-pregnant horn may also occur due to the delay in the contact of the caruncles with the blastocyst causing a time lag in placentome formation.

The hypothesis as to the relationship between placentome size and uterine location remain untested. However, observations in bovine and ovine pregnancy around the implantation stage indicate that there is preferential location of the conceptus near the middle of the horn at implantation, although the chorion subsequently extends to fill the whole uterine lumen (King *et al.*, 1982; Gaviria and Hernandez, 1994). Consequently within the pregnant horn, attachment to the uterine mucosa and subsequent modification of the uterine epithelial lining is more advanced within the vicinity of the fetus (Gaviria and Hernandez, 1994). This suggests that within the pregnant horn, placentomes closer to the fetus are more likely to be more advanced in development compared to those further from it. Alternatively, the tendency for placentomes located in the middle of either pregnant or non-pregnant horn being larger than those further from it may be related to regional variations in maternal blood supply throughout the uterus. Perry and Rowell (1969) observed that in pig, the largest blood vessels supplying the uterus traversed the middle part of the mesometrium and extended towards the fetuses located in the middle of the uterine horn. Thus the feto-placental units in this region may have superior substrate supply, which would probably deteriorate in relation to the distance from the middle of the horn. Wigglesworth (1964b) commented that a number of placentas supplied in series from the same uterine artery may cause a significant drop in local blood pressure in the more distant placentas with a subsequent decrease in oxygen supply. In sheep, larger placentomes in the middle of the horn may suggest a similar type of blood supply to that in pig, such that the middle of the horn is better perfused than distant parts of the horn.

Other studies carried out in late gestation in sheep (114 and 145 days), using radioactive microspheres, have revealed non-random uneven distribution of uterine and umbilical blood flows within the placenta (Rankin *et al.*, 1970). Maternal blood flow exceeded fetal blood flow in placentomes near the tips of the horn whereas fetal blood flow was dominant in the middle of the horn. Uneven distribution of maternal and fetal blood flows may lead to uneven delivery and clearance of progesterone to placentome tissue early in gestation. This may create a situation whereby placentomes in a certain location of the uterus would be more likely to become everted.

Individual placentomes can vary not only in size but in macroscopic morphology as well. The gross morphology of placentomes alters with the progression of pregnancy (Fig. 1.5). Early in gestation, a fully formed placentome normally exhibits a concave shape (morphology "A") where the maternal tissue surrounds the fetal tissue (Wimsatt, 1950) until about day 137 (Cloete, 1939) or day 140 (Alexander, 1964b) when most placentomes adopt a flat shape (morphologies "B" or "C") or become everted; attaining a convex shape (morphology D) which is the reverse of the early or initial morphology (A). In the everted shape, fetal tissue appears to overgrow and surround maternal tissue, an apparently extended haemophagous zone also covers the whole external surface of the placentome.

Increased eversion placentomes were observed in early gestation (day 60), when ovariectomised ewes were infused with doses of progesterone greater than that needed for maintenance of pregnancy, (Alexander and Williams 1966). In addition, McNeill (1991) found that the incidence of everted placentomes observed in late gestation correlated positively with increasing numbers of corpora lutea. These studies suggest progesterone abundance in early gestation influences placentome eversion.

The everted morphology is typical of bovine placentomes and has been associated with a higher ratio of fetal to maternal tissue in placentomes of this species (Andersen, 1926). In sheep, non-everted placentomes have more fetal than maternal tissue, based on analysis of the placentome components following morphometric analysis (Mitchell and Stratford, 1987) as well as following manual separation of the placentomes into fetal and maternal components (Stegeman, 1974; Caton *et al.*, 1984). Therefore by analogy with the cow, eversion of the ovine placentome may be associated with a further increase in the ratio of fetal to maternal tissue, until the external morphology of the placentome is altered. This further growth of fetal tissue, which is responsible for reversing gross morphology, may largely involve cellular hypertrophy to a greater extent, since a greater proportion of it occurs after the end of the proliferative phase of placental growth (Kulhanek *et al.*, 1974; Vatnick and Bell, 1991).

1.4.5 Placental growth, development and function: Relationship to fetal growth

Placental Size and Function

In general, placental size has been regarded as reflecting function. The extent to which a reduction in placental size and hence function may influence fetal growth, is dependent upon the margin of safety between placental supply of critical substrates and fetal demand (Thomson *et al.*, 1969).

Flexner *et al.*, (1948) demonstrated that sodium transport by the placenta supplies 1,100 times more sodium than the fetus can incorporate; suggesting that as far as sodium transport is concerned placental size is less likely to be an important factor. In contrast, Walker and Turnbull (1953) suggested that the placenta has a small margin of adequacy in relation to oxygen transport.

Experimental restriction of implantation in sheep (by the removal of most implantation sites before mating) restricts placental growth and reduces the margin of safety for oxygen and glucose between delivery to the fetus and consumption by the placenta (Robinson *et al.*, 1979; Owens *et al.*, 1987a, b; Owens *et al.*, 1989). Following experimental restriction of implantation in sheep, the clearance of antipyrine and methylglucose as well as that of urea, decreased with placental weight (Owens *et al.*, 1987b). Hence small placental size limits substrate transfer function. Human placentas of SGA babies revealed substantial reductions in trophoblast mass or cellular content between 38 and 40 weeks of gestation (Teasedale, 1984). This would significantly reduce metabolic and secretory activities of the placenta. *In vitro* studies involving sheep and goat BNCs, demonstrated that the production of progesterone by the BNCs is proportional to the number of secretory cells (Wango *et al.*, 1991). In addition, *in vivo* studies in sheep demonstrated the reduced capacity of OPL secretion (34% of controls) in placentas following experimental restriction of implantation (Falconer *et al.*, 1985). In rhesus monkey, *in vivo* studies also demonstrated that concentrations of the hormone monkey chorionic somatomammotropin secreted by the placenta were proportional to the total placental DNA content (Novy *et al.*, 1981). Chronic heat exposure of pregnant ewes reduces placental weight and is associated with reductions of maternal plasma concentrations of progesterone and placental lactogen in late gestation (Bell *et al.*, 1989). Thus small placental size also limits hormonal synthesis and secretion.

Placental size and fetal growth

The relationship between the size and weight of the placenta and the birth weight of the fetus has been extensively studied both in man and experimental animals. In most of these studies, wet weight of the placenta has been used as a measure of its function. A strong positive association between placental weight and total villous surface area has also been reported during the third trimester in humans (Aherne and Dunill, 1966). In addition, villous surface area is positively associated with the total volume of placenta and fetus in a whole series of animals with compact or diffuse placentas such as in rat, cat, guinea pig, pig, cow, zebra and camel (Battaglia and Meschia, 1986).

In many species, a close positive correlation between fetal and placental weight is evident throughout gestation and at parturition (ranging from $r^2=0.25$ to $r^2=0.81$) (Kelly; 1992; Alexander, 1964; 1974; Thompson *et al.*, 1969; Stegeman, 1974; Jones and Parer, 1983), suggesting that placental size influences fetal growth (Molteni, 1984; Owens *et al.*, 1989). In humans, when placental tissue is adequately processed by trimming off the membranes and cord and a blood-free placental weight calculated, there is a strong positive association between fetal and placental weight or size and the weight-for-dates of the baby (Garrow and Hawes, 1971). In sheep, the association between placental and fetal weight normally emerges in late gestation (Stegeman, 1974; Caton *et al.*, 1984; Harding *et al.*, 1985). Significant positive associations between placental weight and birth weight have also been reported for other species, including the guinea pig (Eckstein *et al.*, 1955), rabbit (Hammond, 1935) and rat (Rosso, 1980). These associations between placental weight, surface area and fetal weight or birth weight show that a substantial proportion of the variation in fetal size may be attributable to variation in placental size and function.

The association between fetal and placental weight may reflect placental influences on fetal growth or vice versa or control of both by a common factor. However, experimental restriction of placental growth reduces fetal growth in sheep suggesting that much of the relationship between the two is causative in nature (Alexander, 1964b; Robinson *et al.*, 1979; Harding *et al.*, 1985; Mellor *et al.*, 1987).

The relationship between fetal and placental size emerges earlier than normal in gestation or is stronger when there is experimental restriction of placental growth in sheep (Alexander, 1964b; Robinson *et al.*, 1979; Mellor and Murray, 1982; Harding *et al.*, 1985), rats (Wigglesworth, 1964) and guinea pigs (Jones, 1989). Low placental weight is usually associated with the slowing of fetal growth at any stage in gestation (Fay and Ellwood, 1993) thus inducing intrauterine growth retardation (IUGR) (Robinson, 1989). Two types of IUGR are now recognised (Fay and Ellwood, 1993):

- i) primary IUGR in which infants are of low birth weight (<10th centile) and small for gestational age, with symmetrically small organs in proportion to their weights (Fay and Ellwood, 1993) and
- ii) secondary IUGR in which infants are thin and relatively long for their weights (i.e with asymmetrical parameters). Such infants are said to exhibit a low ponderal index (PI) ($\text{weight} \times 100/\text{length (cm}^3\text{)}$) (Fay and Ellwood, 1993).

Although this classification of IUGR is recent, other investigators have previously recognised the two types of IUGR. For example, Gruenwald (1963) first referred to primary and secondary IUGR as chronic fetal distress (birthweight more than 2 SDs below mean) and subacute fetal distress (significantly reduced fat without significant reduction in longitudinal growth) respectively. Campbell (1974) also described the two types of IUGR as "overall symmetrical reduction of organ size" and "asymmetrical brain-sparing retardation paralleled by high perinatal loss from hypoxia and prone to hypoglycaemia" respectively.

IUGR has generally been associated with increased rates of intrauterine deaths, perinatal morbidity and mortality (Alexander, 1974; Villar and Belizan, 1982). However, recent studies have indicated that in contrast to primary IUGR, secondary IUGR is associated with significant increases in intrauterine growth problems (Fay *et al.*, 1991), low Apgar scores, hypoglycaemia and perinatal asphyxia (Villar *et al.*, 1990). In addition, higher neonatal morbidity is also associated with low PI, even among infants of normal birth weight (Villar *et al.*, 1990). Low birth weight (fetal weight <2500g at birth) is associated with the onset of a variety of diseases in adults in humans including hypertension (Barker *et al.*, 1987, 1990, 1991, 1992, Wilki, 1993) especially when accompanied by high placental weights, and low postnatal growth rate. Men

and women, who were small babies with large placentas (low fetal:placental weight ratio) at birth have highest blood pressures and worst lung function in adulthood. In guinea pigs, IUGR induced by uterine artery ligation was associated with development of elevated blood pressure in experimental animals, when the animals attained adult body weight and sexual maturation (Swenne *et al.*, 1987; Persson and Jansson, 1992).

A small placenta is common for full term and pre-term babies that have experienced IUGR (in humans; babies weighing <2500g, with a gestational age of less than 37 completed weeks; WHO, 1969; Villar and Belizan, 1982). In sheep, restricted placental size is associated with reduced uterine and umbilical blood flows, reduced substrate supply to the fetoplacental unit resulting in fetal IUGR. IUGR is characterised by variable degrees of oxygen and glucose deficit in many species including sheep and humans (Robinson *et al.*, 1979; Harding *et al.*, 1985; Owens *et al.*, 1987a, b; Owens *et al.*, 1989; Soothill *et al.*, 1987). In sheep, small placentas which are associated with fetal IUGR, have low values of placental permeability of urea per unit fetal weight (Kulhanek *et al.*, 1974). In humans, placentas of small-for-gestational age (SGA) infants (those weighing below the 10th centile for their gestational age) were 45% smaller than those of their appropriately grown counterparts at term (Molteni *et al.*, 1978; Teasedale, 1984). Histological analyses of small placentas associated with SGA infants, revealed significant reductions in the total surface area for exchange (Aherne and Dunhill, 1966; Teasedale, 1984). Similar observations were reported by Boyd and Scott (1985), who found consistently reduced villous surface area in placentas of pregnancies complicated by pre eclampsia and those of SGA infants throughout gestation. Therefore, since small placental size usually indicates reduced surface area for exchange and also reduced blood flow, small placental size may result in insufficient substrate transfer, across the placental fetomaternal interface thus inducing low fetal arterial oxygen tension and hypoxaemia (Robinson *et al.*, 1979). Hypoxaemia and hypoglycaemia are the two factors that commonly characterise impaired fetal growth (Owens *et al.*, 1987 a, b; Owens *et al.*, 1989; Harding and Charlton, 1989; Soothill *et al.*, 1987).

Occasionally, an apparently normal-sized placenta may be present in IUGR (Becker, 1974; cited in Baur, 1981). In the majority of such cases, these placentas have characteristics that reduce their functional efficiency such as infarcts, lesions, abnormally small villi, loss of tertiary villi and

their functional efficiency such as infarcts, lesions, abnormally small villi, loss of tertiary villi and increased numbers of immature villi, excessive syncytial knots, presence of large cytotrophoblast rather than syncytium, increased fibrin deposition within and between villi (Adair *et al.*, 1925; McKeown and Record, 1953; Wigglesworth, 1964a; Stegeman, 1974; Boyd, 1987; Naeye, 1989) and also smaller than normal surface area (Becker, 1974; cited in Baur, 1981).

1.5 ONTOGENY OF TRANSFER FUNCTION IN THE OVINE PLACENTA

1.5.1 Placental Blood Flow

Tremendous increases in blood flow within the utero-placental unit occur throughout pregnancy which is consistent with increasing delivery of large quantities of substrates to meet the growing demand of the fetus and placenta as gestation proceeds. Morphometric observations by Stegeman (1974) revealed that in sheep, the villous blood volume of twin placentas increases continuously from early gestation to term. Studies by Rosenfeld *et al.*, (1974) showed that maternal placental blood flow increases from about 30 ml/ kg/ min at day 40 to 300ml/ kg/ min by day 90. At this time, placental blood flow accounts for 63% of uterine blood flow (300 ml/ kg / min). However, in the last 50 days of pregnancy, total blood flow supplying the placentomes increases to about 1500ml/ kg/ min (Rosenfeld *et al.*, 1974) or 2 litres/ kg /min near term, which accounts for 80% to 90% of total uterine blood flow (Power *et al.*, 1967; Makowski *et al.*, 1968; Meschia, 1990; Longo, 1981). Overall, a seven fold increase in maternal placental blood flow after the placenta reaches its maximum weight has been reported in sheep (Rosenfeld, 1989). These observations are consistent with the increase in endothelial cell nuclei in the maternal tissue component of the ovine placenta during the third trimester (Teasedale, 1976).

Experimental restriction of utero-placental blood flow

The dependence of normal fetal growth on adequate placental perfusion is demonstrated by the consequences of reducing or limiting the normal ontogenic increases in maternal placental perfusion. The rate of oxygen (O₂) exchange between fetal and maternal blood is flow

dependent (Longo, 1981; Meschia, 1990), therefore decreasing uterine blood flow such as occurs due to uterine artery ligation reduces O₂ delivery to the fetus and raises the concentration of carbon dioxide (CO₂). This is followed by intense peripheral vasoconstriction and fetal hypoxaemia (Rudolph, 1989). In sheep, short and long term hypoxaemia increases concentrations of adrenocorticotrophic hormone (ACTH) in fetal plasma (Challis *et al.*, 1989; Jacobs, 1987). This elevates cortisol concentrations in fetal plasma, which in the long term may be responsible for the premature maturation of adrenal function, reduced fetal cell proliferation (hence growth) and subsequent preterm delivery (Challis *et al.*, 1989; Jacobs, 1987). Placental perfusion has been indirectly reduced by the ligation of the uterine artery and causing fetal growth retardation in rats (Wigglesworth, 1964b; Kaminsky *et al.*, 1991) and in guinea pigs (Jones and Parer, 1983; Jones, 1989; Persson and Jansson, 1992; Carter, 1993). Occlusion of the maternal internal common iliac artery induces fetal hypoxaemia and retards fetal growth in sheep (Challis *et al.*, 1989; Clark *et al.*, 1993). In sheep, pre-pregnancy excision of endometrial caruncles reduces uterine and umbilical blood flows in addition to reducing placental weight (Owens *et al.*, 1989) hence inducing chronic fetal hypoglycaemia and hypoxaemia (Robinson *et al.*, 1979; Owens *et al.*, 1989; 1994a).

1.5.2 Placental Permeability

Urea permeability per gram of DNA increases five-fold between mid and late gestation in sheep (Kulhanek *et al.*, 1974). In the same study it was observed that at any given gestational age, placental urea permeability was proportional to total placental DNA. Since maximum DNA content is attained in mid-pregnancy (Kulhanek *et al.*, 1974; Vatnick *et al.*, 1991), it appears that placental permeability in late gestation may be determined partly by factors that have earlier determined maximum placental cell number and partly by those that influence the ontogenic increase that subsequently occurs.

1.5.3 Substrate transfer

The surface epithelia at the feto-maternal interface of the placenta must serve as a placental barrier separating fetal and maternal blood systems and at the same time permit substrate

transfer and exchange between these two systems. Some substrates such as respiratory gases and small hydrophilic molecules like urea, readily diffuse through the placental membranes (Table 1.2). However, other blood-borne nutrients must be transported by specific systems which involve membrane-bound carriers or transport systems (Table 1.2) (Faber and Thornburgh, 1983; Eaton and Contractor, 1993). In this section the mechanisms by which oxygen, glucose, amino and fatty acids and large molecular substances are transported across the placenta are briefly described. Wherever there is paucity of information in sheep, data available in other species is reviewed.

Table 1.2 Examples of substrates and their methods of transfer by the placenta

Substrate type	Substrate Structure	Transfer mechanism
micronutrients e.g. vitamin C	hydrophilic	active transport
micronutrients e.g. iron	hydrophilic	active transport
macronutrient e.g. glucose	hydrophobic	facilitated diffusion
macronutrient e.g. fatty acids	hydrophobic	diffusion
macronutrient e.g. amino acids	hydrophobic	active transport

Determinants of oxygen transport

1. In sheep, the average oxygen pressure gradient between maternal arterial and umbilical venous blood samples is about 60 torr (Comline and Silver, 1970; Faber and Thornburgh, 1983; Rudolph 1989). In addition, the oxygen pressure in fetal arterial blood which ranges between 20 and 30 torr (Comline and Silver, 1970; Faber and Thornburgh, 1983) is considerably lower than the postnatal arterial oxygen pressure of about 100 torr in newborn lambs (Rudolph 1989).

There is experimental evidence in sheep that oxygen transfer across the placenta and its delivery to the fetal sheep is determined by the rates of both uterine (Meschia *et al.*, 1967) and umbilical blood flow (Dawes and Mott, 1964; Itskovitz *et al.*, 1983) such that reduction of these flows reduces oxygen transfer and delivery to the lamb. Experiments involving perfused isolated placental cotyledons in both sheep and rabbits also demonstrated increased

oxygen transfer with increasing flow rate. In pregnant sheep, increases in uterine oxygen uptake observed *in vivo* near term, when compared to lower values in mid-gestation, are partly related to increases in uterine blood flow (Hay and Wilkening, 1994). Also, spinal anaesthesia induced a 65% fall in uterine blood flow in sheep which was associated with a 14% reduction in oxygen transfer and a 9% reduction in umbilical oxygen tension (Lucas *et al.*, 1965).

2. Comparison of maternal and fetal oxyhemoglobin dissociation curves shows that the sheep exhibits one of the largest differences in the haemoglobin half-saturation pressure (P_{50}) between maternal and fetal blood. The P_{50} of sheep fetal haemoglobin is about 26 torr compared to maternal P_{50} of 35 torr. Thus fetal lamb blood has a higher affinity for oxygen than maternal blood (Faber and Thornburgh, 1983; Rudolph; 1989). This facilitates oxygen transfer from maternal to fetal blood (Faber and Hart, 1966; Faber and Thornburg, 1983; Itskovitz *et al.*, 1984).
3. Examination of structure and location of capillaries by electron microscopy suggests that changes in fetal rather than maternal vessels enhance substrate diffusion. The invasion of the trophoblast by capillaries after 100 days gestation (Davies and Wimsatt, 1966; Mitchell, 1982) and the fenestrated nature of the fetal capillary walls may be compatible with gaseous exchange compared with the centrally located maternal vessels lined by thick endothelium (Mitchell, 1982). In addition, in late gestation, the distance between the maternal capillary lumen and the luminal microvillous membrane of the feto-maternal syncytium is twice that of the comparable distance in the fetal villi (Mitchell, 1982). The reduction of intercapillary distance coupled with the increase in blood flow should enhance the diffusion of substances across the placental membrane in late gestation.

Macronutrient Transfer

Glucose Transfer

Glucose is the major nonnitrogenous nutrient required by the developing fetus (Mellor, 1983; Eaton and Carpenter, 1993). Glucose transport occurs by a carrier-mediated mechanism (Widdas, 1952), known as facilitated diffusion. Specific membrane protein molecules operate as carriers and

enhance the rate of glucose transport which occurs down the concentration gradient from maternal to fetal blood (Simmons *et al.*, 1979; Hay, 1991; Eaton and Carpenter, 1993). Thus there is a direct relationship between the rate of glucose transfer and the concentration of glucose in the maternal plasma, the uptake of glucose by the utero-placental unit and the maternal-fetal gradient (Hay *et al.*, 1984; 1990).

Glucose transporters have been identified on both the luminal and basal membranes of the syncytiotrophoblast in the placentas of the guinea pig (Yudilevich *et al.*, 1979), rat (Takata *et al.*, 1990) and the human (Johnson and Smith, 1980; Takata *et al.*, 1992). Further characterisation has shown a molecule with a size range of 43 to 55 kDa (Wadzinski *et al.*, 1988) in human. Subsequently, investigation on human placental membranes using antibody detection methods revealed the presence of one isoform of glucose transporters (GLUT) called GLUT1 (molecular weight 50 kDa) in the syncytiotrophoblast, cytotrophoblast and the endothelial cells (Takata *et al.*, 1992). Within the syncytiotrophoblast, GLUT1 was present in a high concentration at both the microvillous apical and basal plasma membranes, suggesting that it may be essential for the entry and exit of glucose into and out of the syncytiotrophoblast, across to the stroma of the placental villi and finally to the fetal blood vessels (Takata *et al.*, 1992). GLUT1 is also abundant in sheep placental membranes (Ehrhardt *et al.*, 1994, FASEB J 8:A177). In Sheep, GLUT1 abundance is greatest in late gestation and parallels increase in glucose transport capacity *in vitro* (Ehrhardt *et al.* 1994) and *in vivo* (Molina *et al.*, 1991).

In the rat placenta, *in situ* hybridisation methods have enabled the detection of glucose transporter (GT1) mRNA localised in the placental growth plate where it was observed to be most abundant during the phase of rapid placental growth and decreased at term. This was matched by the highest placental glucose metabolism during the phase of rapid placental growth in mid-gestation followed by a significant decrease at term (Zhou and Bondy, 1993). These findings appear to suggest the involvement of GT1 in supplying glucose for use as placental, as opposed to fetal, fuel (Zhou and Bondy, 1992).

The carriers allow for the bi directional transport of glucose across the placenta and the uptake of glucose by the trophoblast from both the maternal and fetal glucose pools (Stacey *et al.*, 1978). The

bi-directional transport is such that glucose transfer capacity of the barrier is greater on the fetal side (Hay, 1991).

In sheep, although placental weight declines by about 20% during the second half of gestation, placental glucose transport capacity increases by about ten-fold during this time (Molina *et al.*, 1991). The growth in placental transfer capacity occurs to meet the increasing energy demands of the fast growing fetus which exhibits progressive increases in the absolute rate of glucose utilisation (Hay, 1994, in press). About 40% of the increase in the placental glucose transport capacity occurs due to an increase in the transplacental glucose gradient which is created by reduced glucose concentration which occurs progressively in the fetus as gestation progresses (Molina *et al.*, 1991; Hay, 1991). The rate of glucose transfer from the maternal circulation to the fetal circulation also increases markedly during the last days of pregnancy in the rat (Rosso, 1975). The net effect is an increase in placental glucose transport of 10 fold while transfer capacity increases five fold (Molina *et al.*, 1991).

In the studies performed by Johnson and Smith (1980) down regulation by steroids, estrone and progesterone, of the monosaccharide carrier systems in the human placenta, was evident, suggesting hormonal regulation of glucose transport capacity.

Amino acid transfer

Most amino acids are present in higher concentrations in fetal than maternal plasma and in even higher concentrations in placental tissue than maternal plasma (Lemons, 1979; Phillips *et al.*, 1978; Larry and Johnson, 1988; Eaton and Contractor, 1993). The ratio of fetal to maternal concentrations is about 2:1 in humans (Lemons, 1979), rabbits and sheep (Reynolds and Young, 1971) and about 5:1 in the guinea pig (Reynolds and Young, 1971). The presence of higher concentrations of amino acids in the cells of the placental membranes compared to maternal plasma, and the fact that amino acids have been observed not to bind to plasma proteins, indicates that the transport of amino acids occurs by active transport across the placental membranes (Faber and Thornburgh, 1983).

The observation of much higher concentrations of most amino acids within the cells of the placenta compared to either fetal or maternal plasma in human term placentas (Phillips *et al.*, 1978; Lemons, 1979) suggested an initial active transport process of amino acids into placental cells from the maternal plasma, followed by a passive diffusion down a concentration gradient into the fetal plasma (Faber and Thornburg, 1983; Eaton and Contractor, 1993).

Several transport systems are involved in the transfer of neutral, acidic and basic amino acids in animal cells (Yudilevich and Swiery, 1985). For example, six transport systems for the transfer of neutral amino acids have been identified (Yudilevich and Swiery, 1985). These include these three major transport systems;

- i) system A which is mainly involved with the transfer of amino acids having short, polar, or linear side-chains such as alanine, glycine and α -aminoisobutyric acid.
- ii) system L which is mainly involved in transport of large apolar branched-chain and aromatic amino acids, such as leucine, isoleucine, tyrosine and valine, and
- iii) system ASC which is restricted to the transportation of alanine, serine and cysteine at normal physiological pH.

However, not all systems need to be present (Yudilevich and Swiery, 1985). Thus the three systems listed above have been identified in human placental membranes (Enders *et al.*, 1976) while in the guinea pig placenta, only system ASC may be present (Eaton and Yudilevich, 1981; Eaton *et al.*, 1982). The amino acid transport systems have been observed to demonstrate some degree of selectivity and overlapping specificity for certain amino acids (Enders *et al.*, 1976; Yudilevich and Swiery, 1985).

In vitro evidence has shown that amino acid transport systems are induced by reduced availability of amino acids, a process that can be blocked by inhibitors of protein synthesis or by low concentrations of amino acids in the incubation medium (Smith *et al.*, 1973). *In vivo* studies in rats showed that fetal amino acid concentrations were maintained in spite of

prolonged hypoaminoacidaemia in the mother, thus enhancement of the amino acid transport system can occur *in vivo* (Domenech *et al.*, 1986).

In the rat placenta, the ability to concentrate the amino acid α -aminoisobutyric acid increases markedly during the last week of pregnancy (Rosso, 1974). This is paralleled by a 10-fold increase in the concentration of the α -amino isobutyric acid in fetal tissues which corresponds to approximately a 100-fold increase in the number of molecules transferred into the fetus across the placenta per gram of placental tissue (Rosso, 1974). During this period, placental weight increases only three-fold (Rosso, 1974). Hence ontogenic enhancement of amino acid transport may occur by as yet unknown mechanisms.

Fatty acid transfer

Fatty acids are required by both the placenta and the fetus for the synthesis of complex lipids and for oxidation (Coleman, 1986). Although fetal tissues readily synthesise fatty acids, studies in non-ruminant species have revealed that about 50% of the fetal requirements for fatty acid are met through placental transport of maternally derived fatty acids (Coleman, 1986).

The extent of placental transfer of lipid varies between species. Animals with epitheliochorial placentation, such as sheep, cow and pig, tend to exhibit relatively low levels of lipid transfer compared to those observed in hemochorial placentas such as in primates and rodents, (Battaglia and Meschia, 1988). Fetal body fat accumulation tends to reflect the levels of placental lipid transfer such that the species with epitheliochorial placentation have fetuses with relatively little body fat compared to human fetuses (Battaglia and Meschia, 1988).

Lipids may be transported across the placenta by one of the following ways:

- i) direct transfer by carrier-mediated transport as observed in human placental membranes (Coleman, 1986; Goldstein *et al.*, 1979, Hull, 1979)

- ii) synthesis within the placenta and subsequent release into the fetal circulation (Hull, 1979; Coleman, 1986) as demonstrated in cultured human placental trophoblast (Coleman and Haynes, 1987).
- iii) hydrolysis of complex lipids derived mainly from the maternal circulation and to a lesser extent from the fetal circulation (Coleman, 1986; Coleman and Haynes, 1987). For example, in human, rabbit and guinea pig, complex lipids are broken down and then released into the fetal circulation as free fatty acids (Elphick *et al.*, 1978, Elphick and Hull, 1977; Thomas and Lowy, 1987). The action of placental lipoprotein lipase, which in the guinea pig is present mainly on the maternal side of the placenta (Thomas *et al.*, 1984), is responsible for the release of fatty acids from complex lipids (Coleman, 1986). Conversely, the lengthening of carbon chains into larger lipid molecules also occurs in the placenta. An example is the conversion of linolenic acid into arachdonic acid (Crawford *et al.*, 1976).

There is evidence showing that the placental capacity to transport fatty acids increases with gestational age. For example in rats, between 15 and 19 days gestation, 10% of fetal fatty acids are of placental origin. By day 21 or term, this proportion increases to 50% (Hummel *et al.*, 1983). Also in rabbits, primates and guinea pigs, large quantities of free fatty acids, bound to albumin, cross the placenta in late gestation (Hull and Ephick, 1979; Jones and Rolph, 1985). Increased placental release of fatty acids in late gestation may also in part result from increased activity of lipoprotein lipase activity, which in the guinea pig increases 11-fold in late gestation (Thomas *et al.*, 1984). In addition, maternal serum very low density lipoprotein (VLDL) triacylglycerol, which acts as a source of free fatty acids for the fetus, (Hummel *et al.*, 1976) increases during pregnancy in the rat (Scow *et al.*, 1964).

Receptor-mediated endocytosis of macromolecules by human placental trophoblast and other mammalian cells

Receptor-mediated endocytosis is a process by which macromolecules are able to enter the cell first binding to specific receptors on the cell membrane, followed by the clustering of receptor-ligand complexes on specific areas of the cell membrane that contain the protein clathrin called "coated pits" which then invaginate to form coated vesicles (Goldstein *et al.*, 1979; Eaton and

Contractor, 1993). Subsequent events induce the loss of the coats from individual vesicles which then fuse to form endosomes within which the dissociation of the ligand-receptor complexes occur.

The products of the dissociation of the complexes may differ. Some, for example the low density lipoprotein (LDL) receptor which transports cholesterol, recycle back to the cell membrane after directing their ligand to a lysosome (Goldstein *et al.*, 1979). Some receptors do not direct their ligands to a lysosome but do recycle, as in the uptake of iron by mammalian cells (Octave *et al.*, 1981). Other receptors and their ligands are both broken down by lysosomes, hence a down regulation of the receptor occurs and new receptors have to be continuously synthesized, as in the transport of insulin (Kasuga *et al.*, 1981). Some receptors are involved in transcytosis, the polar transportation of molecules such as IgA from one side of the cell to another as demonstrated in rabbit (Kuhn and Kraehenbuhl, 1979).

Receptor-mediated endocytosis may play an important role in the modification of placental function (Eaton and Contractor, 1993). For example, human placental trophoblast cells take up LDL, by this mechanism, which then provides cholesterol for progesterone synthesis within the trophoblast cells (Winkel *et al.*, 1980) or possibly for transportation to the fetus (Coleman, 1986).

1.5.4 Placental metabolism

The exchange of nutrients between the placenta and the fetus is partly determined by the extent of placental consumption of those nutrients as well as placental conversion of nutrient substrates to alternative forms (Hay, 1991).

Electron microscope observations in late gestation have revealed the presence of a moderate number of mitochondria in the feto-maternal syncytium and trophoblast of the sheep placenta (Björkman, 1965, Mitchell, 1982); which are supportive of the observation that the placenta is a very active metabolic organ (Villiee, 1958). The placenta is one of the organs that demonstrates highest rates of oxygen consumption (Hay and Wilkening, 1994). Measurements of the oxygen consumption by the uteroplacental tissues in sheep indicated more than 80% of the oxygen

taken up by the pregnant uterus is consumed by the uteroplacental tissues in mid-gestation (Bell *et al.*, 1986). Measurements done within the last two weeks of gestation in sheep showed that the fetus receives about 55% of the entire uterine oxygen uptake, thus 45% is consumed by the utero-placental unit (Meschia *et al.*, 1980; Hay and Wilkening, 1994). This oxygen consumption also includes that which is consumed by the foetal membranes of the placenta which are highly vascularised and appear to receive an increased blood flow as gestation progresses (Barcroft and Barron; 1946). However, a relatively high uterine blood flow rate maintains high oxygen pressure in the maternal capillaries of the sheep placenta and counteracts the negative effect of placental oxygen consumption on oxygen transfer (Meschia *et al.*, 1980).

In mid-gestation (71 to 81 days) in sheep, uteroplacental tissues consume more than 80% of the glucose taken up by the pregnant uterus (Bell *et al.*, 1986). In late gestation, the ovine utero-placental unit utilises two thirds of the entire uterine glucose uptake from the maternal circulation whereas the fetal sheep receives the remaining third (Simmons *et al.*, 1979; Meschia *et al.*, 1980).

Measurements of rates of consumption by uteroplacental tissues in sheep demonstrated that in mid-gestation rates were only 50 and 30% of those in late gestation for oxygen and glucose respectively (Bell *et al.*, 1986). In addition, the mid-gestation net lactate production by these tissues was only 25% of that measured in late gestation (Bell *et al.*, 1986). This may be consistent with significant increases in placental dry matter content between mid and late gestation in sheep (Vatnick and Bell, 1992).

High consumption of oxygen and glucose by uteroplacental tissues indicates that utero-placental consumption of these two substrates limits their transfer to the fetus (Campbell *et al.*, 1966; Hay, 1994, in press). In addition, high glucose consumption by the uteroplacental tissues is a major contributor towards the transplacental glucose gradient (Simmons *et al.*, 1979, Hay and Wilkening, 1994). Analysis of maternal and fetal plasma glucose concentrations in ewes showed that without placental glucose consumption, fetal arterial glucose concentration would more than double. Thus with placental glucose consumption the plasma glucose concentration in the fetus was less than 25% of that of the mother (Comline and Silver, 1970). Within the

placenta most of the glucose is probably either oxidised to provide for the energy cost of hormone production, active transfer of substrates and conversion to lactate which is then excreted into both maternal and fetal circulations (Burd *et al.*, 1975; Sparks *et al.*, 1982). High rates of glucose metabolism have also been reported for cow (Comline and Silver; 1976) and horse epitheliochorial placentas (Silver and Comline; 1975).

It appears that utero-placental glucose consumption is primarily regulated by fetal glucose concentration (Hay *et al.*, 1990). When fetal and maternal glucose concentrations were identical, 80% of glucose metabolised by the utero-placenta was derived from the fetal pool in the umbilical circulation (Simmons *et al.*, 1979). It appears that the system functions to ensure direct supply of glucose to the fetus from the maternal circulation, initially bypassing the placenta. Then fetal consumption determines the amount of glucose that is to be delivered to the placenta for metabolism. Therefore, the system operates in a manner to ensure glucose supply to the fetus which in turn regulates the supply to the placenta. At the same time supply for the mother is protected from direct utero-placental consumption of uterine arterial glucose (Hay, 1991). In addition, the net placental glucose consumption contributes towards the physiological hypoglycaemia of the fetus and helps to establish a glucose gradient that enables both placenta and fetus to compete for glucose molecules (Hay and Wilkening, 1994).

In contrast to the large glucose consumption of the utero-placental unit, the placental amino acid consumption measured within the last month of gestation, is negligible in sheep (Meschia *et al.*, 1980). This is indicated by the equal uterine and umbilical uptakes of neutral and basic amino acids (Meschia *et al.*, 1980). In general, placental amino acid consumption rates are too small to provide enough carbon for even a small fraction of placental oxidation, with the exception of glutamate, which in sheep, is taken up by the placenta from the fetus and oxidised significantly by the placental tissues (Hay and Wilkening, 1994). Thus in normal pregnancy sheep, amino acid utilisation by the conceptus occurs largely within the fetus (Meschia *et al.*, 1980). In the pregnant cow however, the uteroplacental tissues have been observed to consume about 60% of uterine α -amino nitrogen uptake in mid and late gestation (Ferrell and Ford, 1980, Ferrell *et al.*, 1983)

1.6 FACTORS INFLUENCING PLACENTAL GROWTH AND DEVELOPMENT

1.6.1 Introduction

Factors of parental (maternal) origin that are known to influence placental growth and development include environmental factors present at the location of the pregnant mother and those that are present within her body, which in turn have an impact on the uterine environment of the developing feto-placental unit. Examples of external environmental factors are temperature, maternal nutrition (Alexander, 1974; Bell, 1984; Bell et al., 1989) and oxygenation (Jackson et al., 1987b). In addition, at the molecular level, information is accumulating concerning the involvement of peptide growth factors in the regulation of placental growth and function. The possible roles played by the peptide cytokines and insulin-like growth factors (IGFs) in placental growth, development and function is presented. Finally, the roles of progesterone, insulin and glucocorticoids on placental growth, development and function are also considered.

1.6.2 Environmental Factors

Temperature

Prolonged exposure of pregnant ewes to high temperature during the second and/or third trimester of pregnancy reduces placental weight, and the proportions of both fetal and maternal components of the placenta are reduced to similar extents (Alexander and Williams; 1971). Chronic heat exposure of pregnant ewes to temperatures between 38 and 40°C between 45 and 120 days of gestation (Bell *et al.*, 1987) and exposure to 30 or 40°C between 64 and 136 to 141 days gestation (Bell *et al.*, 1989) reduced placental weight by 51% and 54% respectively. In other experiments, six ewes heat stressed from 39 to 125 days showed a 58% reduction in placental weight at 135 days gestation (Thureen *et al.*, 1992). It has been suggested that the placenta shrinks (Alexander; 1974) or fails to grow when heated, but the mechanism by which heat exposure reduces placental weight is not clear. It has also been suggested that the death of placental cells may be a possible explanation for the reduction in placental size during heat stress in pregnancy (M.J. Edwards personal communication cited in Alexander, 1974). More

recently, Vatnick *et al.*, (1991) observed that chronic heating of pregnant ewes between 50 and 75 days gestation caused a 19% reduction in placental weight coupled with a significant reduction in total placental DNA. Thus heating during the first half of pregnancy retards placental growth by reducing cellular proliferation (Vatnick *et al.*, 1991). Similar observations have also been reported by Vatnick and Bell (1991) following chronic heating of pregnant ewes throughout mid to late gestation. In these studies significant reductions in placentome size were paralleled by reduced capacity for protein synthesis. In addition, in vitro studies demonstrated reduced oxygen consumption by the fetal component of the placentome which was partly due to a lower Na^+ , K^+ ATPase-dependent oxygen consumption due to heating (Vatnick and Bell, 1991). Earlier studies by Bell *et al.*, (1987) also showed a significantly smaller uptake of oxygen (-38%) by the pregnant uterus in late gestation, of ewes heated chronically between 45 and 120 days gestation.

Several observations also suggest that negative effects of heating on fetal growth may be manifested through perturbed blood flow within the utero-placental unit. Chronic heating of pregnant ewes for several weeks reduces blood flow to the maternal component of the placentomes (unpublished observations by G. Alexander and J.R.S. Hales; cited in Bell, 1984). In late gestation in sheep, heat stress has also been observed to induce a rapid and sustained reduction in maternal cotyledonary blood flow (Alexander *et al.*, 1987). Also, heating of pregnant ewes from 50 to 120 days gestation, reduced perfusion of the fetal component of the placentome and placental size similarly and to a greater extent greater than the reduction in fetal size (G. Meschia and A.W. Bell; unpublished observations; cited in Bell, 1984). Observations made by Bell *et al.* (1987) revealed that heat stress of pregnant ewes between 45 and 120 days gestation significantly reduced uterine and umbilical blood flows. In addition, a 37% reduction in the placental capacity for glucose transfer was observed (Bell *et al.*, 1987). Mean umbilical uptake of glucose per unit fetal weight and mean fetal glucose/oxygen quotient were reduced but not significantly (Bell *et al.*, 1987). However, other observations in late gestation in sheep showed that chronic heat exposure of pregnant ewes between 39 and 125 days gestation significantly reduced umbilical glucose uptake, which could not be explained by differences in placental perfusion or glucose consumption (Thureen *et al.*, 1992). Thus it appears that heat stress primarily reduces placental size by limiting rates of cellular proliferation and the extent of

hypertrophy, which may occur as a result of the reduced capacity for protein synthesis which occurs due to heat exposure (Bell, 1991). Subsequently, the heat exposed placentas exhibit reduced blood flow and substrate transfer; factors which are associated with reduced fetal growth (Bell *et al.*, 1987).

Altitude

Hypobaric hypoxia is a major stress to which highland inhabitants are constantly exposed (Jackson *et al.*, 1987b). Variations in gross morphology and in histological composition of placenta at high altitude compared to those at sea level have been demonstrated (Adair and Thelander, 1925; Shanklin, 1958; Chabes *et al.*, 1968a; Kruger and Arias-Stella, 1970; Jackson *et al.*, 1987a). The observed differences in structure are consistent with placental efforts to enhance oxygen diffusive conductance (Jackson *et al.*, 1987b). Kruger and Arias-Stella (1970) noted that at high altitude human placentas were heavier but of similar volume to those at sea level. This suggested a more dense distribution of some histological structures per unit placental volume. In addition, placental weight to fetal weight ratios were higher at high altitude than those at sea level; suggesting that a unit fetal weight is supported by more placental mass at high altitude when compared to a similar relationship of these parameters at sea level. These observations suggested the presence of compensatory mechanisms to enhance substrate supply to the fetus at high altitude (Kruger and Arias-Stella 1970). In other studies of the human placenta, Chabes *et al.*, (1968a) reported a three-fold increase in the existence of placental shapes (triangular, rectangular, heart and kidney shaped) in the highland population, other than the round or oval shapes that predominate in general populations (Adair and Thelander, 1925; Shanklin, 1958). The other shapes were associated with higher levels of cord haemoglobin at high altitude. Subsequent studies demonstrated increased numbers and area of terminal villi in placenta at high altitude (Chabes *et al.*, (1968b).

Extensive studies of placentas at high (3600m) and low (400m) altitude in Bolivia revealed several significant microscopic differences in the internal structures of the placentas at the two localities (Jackson *et al.*, 1987a). In the highland placenta, the inter villous space was 40% greater than that of the lowland placenta but the overall villous volume (120cm^3 compared to 160cm^3) and the volume of non-parenchymal tissue was lower in the former when compared to

the latter. Decreases of components responsible for the overall reduction of the villous volume appeared to be proportional since component percentages of the villous volume (trophoblast, fetal capillaries and stroma) were not altered. The reduced villous volume at high altitude was paralleled by reduced surface area for exchange (5.6m^2 compared to 6.9m^2 at low altitude) thus suggesting a decrease in the overall length of the villous branches associated with the reduced volume (Jackson *et al.*, 1987b). The highland placenta also exhibited low volume of fibrin (Jackson *et al.*, 1987a).

Therefore, it appears that at high altitude the placenta adapts by reducing the effective distance for diffusion across the villous membrane. Reduced trophoblast thickness and increased incidence of vasculosyncytial membranes responsible for lowering harmonic mean thickness were found in highland placenta (Jackson *et al.*, 1988). In addition, an increase in the relative length of smaller diameter villi at high altitude has been noted (Jackson *et al.*, 1987b). Despite the structural remodelling in the highland placentas, which should enhance function, birthweight still remains below normal (Kruger and Arias-Stella, 1970; Mayhew *et al.*, 1990) at high altitude compared to sea level. Notable that the Bolivian studies revealed that at high altitude, Amerindians have higher birthweights than other racial groups whereas apparent differences were not significant amongst lowlanders (Jackson *et al.*, 1987a). In addition, Amerindian placentas tended to contain more trophoblast and villous stroma than placentas of non-indians (Jackson *et al.*, 1987a). Thus other factors may influence the effect of altitude on placental parameters. However, other studies in sheep have demonstrated the potential of the placental compensatory mechanisms that enhance oxygen diffusion to meet fetal demand since despite low maternal alveolar hypoxia and low maternal arterial blood, umbilical venous blood was observed to be as well oxygenated as that of fetuses at sea level (Metcalf *et al.*, 1959).

Maternal Nutrition

The effects of maternal undernutrition on the growth and development of the placenta and fetus depend on several factors which include the timing and severity of the exposure to undernutrition (Owens *et al.*, 1989) and also the liveweight and condition score of the ewe at joining (Beck and Davies, 1988; McCrabb *et al.*, 1987). In addition to condition score at joining, maternal body weight in mid pregnancy may also determine total placental weight at that period

of gestation as well as at term. In sheep, maternal body weight at about day 90 of gestation was found to have a significant effect on total placental weight from mid-pregnancy to term ; such that total placental weight for ewes on a high plane of nutrition and with a high body weight was higher than that for those on a low-plane of nutrition and with a low body weight (Wallace, 1948).

Studies conducted in pregnant ewes to test the effects of dietary restrictions on fetal and placental growth at different times during gestation including days 30 to 100 (period of maximal placental growth) and beyond this period have yielded conflicting results. The controversy in the some of the findings appears to be partly related to the variable effects of differing levels of nutrition at different stages of gestation (Robinson, 1990). For example, there is evidence that high plane of feeding which stimulates ovulation rates in ewes (Parr *et al.*, 1987) and in gilts (Ashworth, 1991) may have detrimental effects on embryo survival in these species.

Sub-optimal nutrition from day 30 to not more than 100 days gestation has been observed to induce compensatory growth within the placenta (Faichney and White, 1987; Faichney, 1981, McCrabb *et al.*, 1987, 1991). Some authors have also reported compensatory growth in response to undernutrition, even after day 100 of gestation (Faichney and White, 1987; McCrabb *et al.*, 1991; Foote *et al.*, 1959). On the other hand, in sheep, reduced placental growth due to sub-optimal maternal nutrition between 30 and 96 days (McCrabb *et al.*, 1986; Everitt, 1964) and in the second trimester (Alexander, 1978) or in those ewes that at some point or throughout in pregnancy were poorly fed (Davies *et al.*, 1981; Mellor and Murray, 1981; 1982) has also been reported. There is also evidence for reduced placental growth as a result of maternal undernutrition in rhesus monkey (Novy *et al.*, 1981) and in humans (Villar and Belizan, 1982; Papoz *et al.*, 1981; Papiernik *et al.*, 1981). Both placental and fetal growth are more responsive to nutritional restriction during the phases of maximal growth (Everitt, 1964; Owens *et al.*, 1989). Whereas the placenta is more responsive to maternal undernutrition during the first half of gestation, the fetus is more so in mid to late gestation (Everitt, 1964; Owens *et al.*, 1989).

Growth is the increase in size of an animal or organ (Winick, 1971). Growth can be divided into the phase of rapid cellular proliferation; when the increase in size is due to the production of new cells, and the phase of cellular hypertrophy when there is largely increases in cell size (Winick, 1971). Similarly, placental growth in sheep can conveniently be divided into two separate phases; that of maximal growth; when there is rapid increases in weight, and the phase of curtailed growth; when there is a plateau or even a decrease in weight. Developmental and growth related events such as implantation, villi formation and branching occur at specific times during the rapid cellular proliferation phase (Wimsatt, 1950). It would therefore appear that if levels of certain substrates which are crucial to the occurrence of any of these stages of development and growth are low, the effect of undernutrition on the placenta will depend on its timing and severity (Winick, 1971). Evidence for the importance of the timing of nutritional variation has been provided by McCrabb *et al.*, (1992) such that placental weight was lower ($p < 0.05$) when ewes were feed restricted between 30 to 96 days of gestation but not when feed restriction was imposed between days 50 and 96 or 75 and 96. The results indicate that placental growth is more sensitive to maternal undernutrition in the earlier days of pregnancy; between the 30th and the 50th day of gestation. This period of greater sensitivity to maternal undernutrition encompasses the time of implantation (section 1.3.2) which is completed between the 24th and the 30th day and the development of chorionic villi which follows implantation and proceeds until the villi attain maximal branching by the 45th day (Wimsatt, 1950). Hence the period between 30 and 50 days of gestation is a critical period for the establishment of the placental structure and its subsequent growth to mid-gestation to attain maximum weight and dimensions and cellularity (McCrabb, 1990; Kelly *et al.*, 1987; Kelly and Newnham, 1990; Kulhanek *et al.*, 1974; Vatnick and Bell, 1991).

Maternal undernutrition does not always induce compensatory placental growth which may suggest the presence of other factor(s) with which nutritional levels interact to determine total placental growth and its ultimate weight. Good body condition at mating is reported to interact with mild undernutrition in mid pregnancy to enhance placental growth i.e. to induce compensatory growth (Faichney and White, 1987). Other studies demonstrated compensatory growth of the maternal component of the placenta at day 40 of gestation followed by compensatory growth of the fetal component in late gestation at 140 days gestation ewes

switched from full to limited feed at mating compared to those on full feed throughout gestation (Foote *et al.*, 1959). In contrast, mild undernutrition has not been observed to induce compensatory placental growth in young ewes and in those ewes with poor condition score at mating (Bennett *et al.*, 1964; Russel *et al.*, 1981) probably due to a low state of bodily development and somatic maturity may not to be ideal for pregnancy (Foote *et al.*, 1959). Thus it is likely that pre-pregnancy nutrition establishes some yet unknown parameters which interact with undernutrition to enhance placental growth.

1.6.3 Peptide growth factors: Cytokines and insulin-like growth factors (IGFs)

Introduction

Evidence is accumulating suggesting the involvement of peptide growth factors in placental growth, development and function. Peptide growth factors may be defined as proteins of less than 30kDa molecular weight, which are widely synthesised, and exert a regulatory action on tissue growth, in a paracrine or autocrine manner (Sporn and Roberts, 1988; Han and Fowden, 1994). Thus peptides with stimulatory or inhibitory effects on proliferation of either epithelial and mesenchymal cells have been characterised. Many of these appear to exert diverse effects on each of their several target cells. The effects appear to depend on the presence or absence of other growth factors or the state of differentiation of the target cell (Sporn and Roberts, 1988). This section, briefly considers evidence implicating the involvement of cytokines and IGFs in placental development, growth and function.

Cytokines

These are families of polypeptides that facilitate intercellular communications (Hunt, 1989). Both cytokines and their receptors have been identified in the uteroplacental unit (Hunt, 1989). In normal pregnancy, growth promoting cytokines may include the colony stimulating factors (CSFs) which are derived from macrophages (Hunt, 1989) and other cell types in the uteroplacental unit including the uterine luminal epithelium (Pollard *et al.*, 1987) and trophoblast cells (Sharkey *et al.*, 1993). Another growth promoting cytokine is interleukin-1 (IL-1) which is produced by both human and murine placental macrophages (Flynn *et al.*, 1982) and also by human trophoblast cells (Main *et al.*, 1987) and for which one of the target cells is also the

trophoblast cell. Thus cytokines may exert their effects in an autocrine or paracrine way. The growth promoting cytokines act by enhancing proliferation. For example the CSF-1 for granulocytes and macrophages (GM-CSF) has been shown to enhance murine placental cell proliferation and phagocytosis (Athanasakis *et al.*, 1987).

Cytokines may also play a role in promoting the differentiation of trophoblast cells from one form to another. For example it appears that, both CSF-1 and GM-CSF may be involved in the differentiation of the first trimester cytotrophoblast* to syncytiotrophoblast types in the human placenta with subsequent induction of hormonal secretion (Saito *et al.*, 1993; Guilbert *et al.*, unpublished observations cited by Craine *et al.*, 1990).

Some of the growth factors are known to promote angiogenesis; the growth of new blood vessels from pre-existing vessels and neovascularisation; the growth of new blood vessels. These have also been identified within the tissues of the reproductive tract including the placenta in humans (Smith, 1993). Such substances include fibroblast growth factors (FGFs); a family of mitogenic peptides which stimulate the growth of various cell types including endothelial cells and smooth muscle cells (Burgess and Maciag, 1989; Mc Laren, 1993) that are necessary for angiogenesis. In the human placenta FGFs are expressed on cytotrophoblast and also in syncytiotrophoblast in late pregnancy (Smith, 1993). Another growth factor which promotes proliferation, migration and tube formation of endothelial cells is the vascular endothelial growth factor (VEGF). This growth factor has also been identified on human trophoblast on which it may have a similar effect, especially related to the migration of the trophoblast that forms the trophoblast shell (Loke, 1993).

In addition to enhancing growth, differentiation and function of placental cells the inhibition of trophoblast cell proliferation and migration as well as initiation of cell death may also be a consequence of exposure to cytokines. An example of such cytokines is the tumour necrosis factor-alpha (TNF- α), which is also produced by macrophages (Beutler *et al.*, 1985). This cytokine has been demonstrated to limit trophoblast cell DNA synthesis in the rat (Hunt *et al.*, 1989). The identification of TNF- α receptors on homogenates of human placenta (Eades *et al.*, 1988) may suggest the likelihood of trophoblast cells binding the cytokine. The synthesis of

DNA by rat trophoblast has also been shown to be inhibited by another cytokine called transforming growth factor beta (TGF- β) while promoting the growth of mesenchymal cells (Hunt *et al.*, 1989). This demonstrates that a particular cytokine may have differing effects on different types of target cells.

Thus cytokines appear to exert a wide range of effects in the placenta which include enhancement of proliferation of both trophoblast and mesenchyme, differentiation of cytotrophoblast into syncytiotrophoblast cell types, limiting trophoblast growth and migration which may protect the mother from excessive invasion by trophoblast, enhancement of angiogenesis as well as induction of hormone production and initiation of cell death.

Insulin-like Growth Factors

The insulin-like growth factors (IGF-I and IGF-II) are the two major forms of IGF (Sara and Hall, 1990). They are single chain polypeptides whose amino acid sequences share approximately 43% and 41% homology respectively, with proinsulin (Sara and Hall, 1990). The IGFs also share a tertiary structural similarity with insulin; being single chain polypeptides with three disulphide bonds (Blundell *et al.*, 1978; Sara and Hall, 1990). IGF-I shares approximately 62% amino acid sequence homology with IGF-II (Chard, 1994). The IGFs have been shown to exert similar biological effects to those of insulin on some cells (Zapf *et al.*, 1986). The biological effects of the IGFs include the regulation of cellular proliferation and differentiation of various cell types (Lowe, 1991). Thus these substances have been included in the insulin family of peptides (Wang *et al.*, 1988). The actions of the IGFs are mediated through cell surface receptors. The type 1 IGF receptor is an $\alpha_2\beta_2$ transmembrane glycoprotein with structural similarity to the insulin receptor through which most biological actions of IGF-I and IGF-II are mediated (Cohick and Clemmons, 1993). The type 1 IGF receptor has the highest affinity for IGF-I and the least affinity for insulin (Cohick and Clemmons, 1993). Another receptor through which some actions of IGFs may be mediated is the type 2 IGF receptor. This receptor is identical to the mannose-6-phosphate (M6P) receptor and its known to bind IGF-II (Cohick and Clemmons, 1993). However, its growth promoting activity is not well understood (Cohick and Clemmons, 1993). In the placenta, 70% of the receptors are estimated to be hybrids; these are receptors with 2 beta-subunits with different molecular weight

and having a higher affinity for IGF-I than for insulin (Moxham *et al.*, 1989; Soos *et al.*, 1990). *In vivo*, IGFs are found to be associated with up to six higher molecular weight binding proteins (IGF-BPs) to which they are tightly bound (Sara and Hall, 1990; Cohick and Clemmons, 1993). The IGF-BPs serve to regulate the physiological actions of the IGFs (Sara and Hall, 1990; Cohick and Clemmons, 1993). Messenger RNAs for IGFs, their receptors and IGF-BPs have been identified in placental tissues of several species including humans and rats (Fant *et al.*, 1986; Zhou and Bondy, 1992).

In a recent *in vitro* study in mice, insulin like growth factors; IGF-I and IGF-II, have been shown to be involved in placental development. Kanai-Azuma *et al.*, (1993) found that IGF-I was responsible for stimulating proliferation and migration of 10.5 day ectoplacental cone (EPC) cells while both IGF-II and crude placental extract enhanced the transformation of the EPC cells to giant cells. The role of IGF-II in the regulation of glycoprotein synthesis for the maintenance of pregnancy by trophoblastic giant cells has also been implicated in mice (Kanai-Azuma *et al.*, 1993).

In mice, IGF-II and unidentified receptor (XR) are thought to play a stimulatory role for placental growth since IGF-II gene deletion reduces placental growth (Baker *et al.*, 1993). In addition, transgenic mice with reduced IGF-II expression have small placentas and fetuses (De Chiara *et al.*, 1990; 1991). In rats receptors for both IGF-I and IGF-II have been identified by the use of competitive binding studies; the majority being of the type 2 IGF receptor (Pilistine *et al.*, 1984). Messenger-RNA for IGF-II was highly abundant in trophoblast derived elements of the placenta from implantation to maturity (Zhou and Chin, 1991; Zhou and Bondy, 1992). In contrast, IGF binding protein-2 (IGFBP-2) mRNA was barely detected in early gestation but increased significantly towards term, and the binding protein was most abundant in the junctional zone (Zhou and Bondy; 1992). Therefore it is possible that in rats there is IGF-II driven trophoblast development and that IGFBP-2 controls its autocrine action (Zhou and Bondy, 1992).

There is also evidence implicating the involvement of IGFs in the regulation of growth, differentiation, and function of the human placenta. Both IGF-I and IGF-II mRNA are

expressed in syncytiotrophoblasts and fibroblasts respectively (Wang *et al.*, 1988). However, the expression of IGF-I mRNA is much greater during the first and second trimesters compared to that at term. Also IGF-II is expressed more in the second than that in the first trimester in human (Shen *et al.*, 1986) and sheep (Bassett and Challis, 1991). Thus there may be developmental changes involved in the regulation of the relative amounts of the growth factors (Wang *et al.*, 1988).

The induction of IGF-II mRNA steady state levels in human trophoblasts has been observed to occur following implantation and to correlate with the formation and expansion of the cytotrophoblastic shell (Ohlsson *et al.*, 1989b). In addition, the IGF-II gene is mostly active in the highly proliferative cytotrophoblasts which comprise the cytotrophoblastic shell and less so in mesenchyme of villi and stroma (Ohlsson *et al.*, 1989a). Thus IGF-II may participate in the formation and maintenance of the cytotrophoblastic shell, hence promoting growth within the human placenta (Ohlsson *et al.*, 1989a). Other studies in sheep (Lok *et al.*, 1993; Scarpantoni *et al.*, 1993; Owens *et al.*, 1994) have also indicated the possibility of the involvement of IGF-I on placental growth and structural remodelling. Infusion of IGF-I has been observed to increase the proportion of larger placentomes (Lok *et al.*, 1993) and the surface density of trophoblast and fetal-maternal syncytium but reducing the barrier thickness of trophoblast and the volume density of the connective tissue at the core of the fetal villi (Scarpantoni *et al.*, 1993; Owens *et al.*, 1994).

In vitro evidence suggests a possible role for the IGFs in the regulation of metabolism in the fetal-placental unit. Both IGFs-I and -II stimulated amino acid uptake in early (<20 weeks) and late gestation (38-42 weeks) placenta (Fant *et al.*, 1986; Guyda, 1991). In addition, the stimulatory effect of IGF-I on the release of human placental lactogen by trophoblast cells at 10 and 40 weeks of gestation has been demonstrated (Iwashita *et al.*, 1990). However, the term placenta was observed to be more responsive to the stimulatory effects of IGF-I due to increased receptor number but not receptor affinity (Iwashita *et al.*, 1990).

Other studies showed a stimulatory effect of IGF-I and II on the activity of the enzyme 3 β -hydroxysteroid within isolated human cytotrophoblasts. Thus IGFs may play a role in the maintenance of placental progesterone production in the human placenta (Nestler, 1989, 1990).

The co-localisation of IGFs-I and II and IGFBPs-1 and 2 has been demonstrated in cytotrophoblast cells and feto-maternal syncytium in sheep (Bassett and Challis, 1991) and human placentas which suggests a role for the BPs in determining the localisation of the IGFs for actions on target tissues (Hill *et al.*, 1993). In addition, the co-distribution of ligands and receptors suggests that IGFs may act in an autocrine/paracrine manner (Ohlsson *et al.*, 1989a). The observation of two distinct species of IGF-I receptors with different cross-reactivities with insulin and different binding affinities for IGF-I appears to indicate that these could mediate different biological actions of IGF-I on target cells (Jonas and Harrison, 1985).

Recent studies have shown that undernutrition reduces circulating levels of both fetal and maternal IGF-I and fetal IGF-II in guinea pigs (Dwyer and Stickland, 1992). Fetal and maternal IGF-I were lower in response to starvation in ewes (Oliver *et al.*, 1992). Therefore, maternal undernutrition may also have an indirect effect on placental growth and function mediated via its effect on levels of the IGFs.

1.6.4 Endocrine Factors

Progesterone

Progesterone has a permissive role in the establishment and maintenance of pregnancy. Ashworth and Bazer (1989) found that 10 of the 30 endometrial proteins they studied early in pregnancy in the ewe increased as a consequence of exogenous progesterone administration. The 10 proteins plus those from the trophoblast form a biochemical dialogue between mother and fetus that is essential for embryo survival. Also it has been shown in ewes (Parr *et al.*, 1987) and in gilts (Ashworth; 1991) that the decline in pregnancy rate which occurred in response to overfeeding prior to pregnancy could be reversed by progesterone supplementation. Merino ewes were given replacement progesterone treatment following ovariectomy on the 21st to 23rd day of pregnancy. The ewes that received the lowest dose of 0.05 mg/kg were no longer

pregnant by 60 days gestation (Alexander and Williams, 1966). Similarly, in goats, ovariectomy terminates pregnancy since the corpus luteum is the only source of adequate progesterone levels from the time of implantation to term (Sheldrick *et al.*, 1981).

To investigate the role of progesterone on placental growth, Alexander and Williams (1966) found that between 60 and 61 days gestation, placentome mean weights were closely similar in ovariectomized ewes which received different treatment levels of progesterone ranging from 0.10 mg/kg to 1.35 mg/kg. However, progesterone treatment increased the incidence of placentome eversion at this time in gestation but did not enhance placental growth. More recently, Ogle *et al.*, (1989) demonstrated the trophic effects of progesterone on the growth of the decidua basalis and the junctional zone in the rat placenta. In these experiments, ovariectomy caused more than 99% reduction in placental protein synthesis. Following progesterone but not estrogen treatment, protein and DNA content as well as cellular differentiation were restored to control levels. These results suggest that in rat, progesterone is essential for placental growth, cellular differentiation and for histological integrity (Ogle *et al.*, 1989).

Among the factors that control circulating levels of progesterone is maternal undernutrition, which has been observed to raise the levels of the hormone in gilts (Ashworth, 1991) and in ewes (Cumming *et al.*, 1971; Oddy and Holst, 1991). It is therefore possible that the effects of maternal feed restriction on placental growth may be partly mediated via its effect on progesterone levels. Robinson (1990) suggested the possibility of the levels of feeding which operate through a shift in progesterone doing so by modifying the production of either the trophoblastic proteins and/ or the endometrial secretory proteins. It is interesting to note that experimental restriction of implantation by the surgical removal of caruncles is also known to raise progesterone levels in the ewe during the cycle (Robinson *et al.*, 1976). Analyses of common elements of compensatory placental growth in undernourished ewes and in those with restricted implantation should help elucidate the role of progesterone in the process.

In sheep, the placenta is the major source of the hormone progesterone that is necessary for the maintenance of pregnancy from mid- to late gestation. In late gestation, the ovine placenta

secretes five times the amount of progesterone secreted by the ovary (Linzell and Heap; 1968). Not surprisingly, the removal of the ovine placenta causes abortion or parturition, which may be delayed by exogenous progesterone (Casida and Warwick, 1945; Wooding, 1992). Evidence from *in vitro* studies revealed that BNCs isolated from sheep or cow induce progesterone production from endogenous sources (Reimers *et al.*, 1985, Ullman and Reimers, 1989). Ovine BNCs converted added labelled pregnenolone to progesterone as did ovine luteal cells whereas caprine BNCs produced 5 β -pregnanediol (Wango *et al.*, 1991). This agrees with a report that the placenta of sheep secretes enough progesterone to maintain pregnancy in the absence of ovaries from day 50 of gestation (Casida and Warwick, 1945; Flint *et al.*, 1975). Since the goat profile for 5 β -pregnanediol secretion is similar to that for progesterone by the ovine placenta, it is believed that reductive metabolism of progesterone before it is secreted may account for the inability of the caprine placenta to maintain pregnancy after ovariectomy (Sheldrick *et al.*, 1981). However the feto-maternal syncytium has been suggested as a possible source of progesterone in sheep due to the presence of mitochondria with tubular cristae, smooth endoplasmic reticulum and lipid droplets; structures that are compatible with secretory functions (Mitchell; 1982).

Another function of BNCs is to produce the protein hormone; ovine placental lactogen (Dubois *et al.*, 1976) and deliver it to the maternal circulation as the giant cells fuse with the uterine epithelium (Steven *et al.*, 1978). Around implantation time, this process is one of the earliest cellular interactions and may serve to establish metabolic and immunological dialogue between maternal and fetal systems (Wooding, 1992).

Insulin

A possible role for insulin in promoting placental growth was demonstrated when Fletcher *et al.*, (1982) found a significant positive correlation between fetal plasma insulin concentration and placental weight during the last third of gestation in the rabbit. Also, administered insulin increased fetal and placental weight in rabbits (Susa *et al.*, 1979; Fletcher and Bassett, 1986). In addition, insulin has been reported to enhance protein synthetic rates in ovine placentomes (Horn *et al.*, 1983). These effects may be mediated indirectly via the stimulatory effects of insulin on the cellular uptake of glucose (Hay and Mezmarich, 1988; DiGiacomo and Hay, 1989)

and amino acids (Guyda, 1991). Insulin is also involved in the regulation of human cytotrophoblastic 3 β -hydroxysteroid dehydrogenase (3 β HSD) activity in the synthesis of progesterone (Nestler, 1989).

Glucocorticoids

In recent studies by Wintour *et al.*, (1994), treatment of pregnant ewes with dexamethasone at 0.4 gestation (64 ± 0.4 days) increased the incidence of placentome eversion 48 hours later. This result suggests the possible involvement of increased levels of maternal glucocorticoids in the mechanism of placentome eversion. Studies in rats have revealed that the activity of placental 11 β -hydroxysteroid dehydrogenase (11 β -OHSD) which converts physiological glucocorticoids to inactive products (Low *et al.*, 1993; Benediktsson *et al.*, 1993; Seckl, 1993), correlates positively with birthweight and negatively with placental weight. Thus, fetuses with low birthweight and high placental weight had the lowest 11 β -OHSD activity which appeared to suggest their likelihood for the greatest exposure to maternal glucocorticoids. These observations suggest that high levels of glucocorticoids may be involved in the mechanism inducing disproportionate placental enlargement in the rat (Benediktsson *et al.*, 1993). It is unlikely that the effects of high levels of glucocorticoids on the placenta may also be indirectly related to progesterone withdrawal (within the last week of pregnancy) and the rise in oestrogen levels (within 24 to 48 hours prior to parturition) (Thorburn *et al.*, 1972) since in sheep, these hormonal changes occur after the completion of placentome eversion (Chapter 3).

1.7 COMPENSATORY PLACENTAL GROWTH

Compensatory placental growth can be defined as a process which occurs within the placenta to enhance reduced placental size and/or function in order that the placenta can sustain itself and also support the fetus with a normal drive to growth. This type of growth may be induced by the experimental or natural restriction of placental size or functional tissue in the placenta or by substrate deficiency within the feto-placental unit. It may also occur following placental damage. Most observations suggest that it occurs following perturbation during the first half of pregnancy. The phenomenon has been observed in placental tissue of several species including humans.

1.7.1 Compensatory Placental Growth in Sheep

Following Natural Restriction of Implantation in Multiple Pregnancy

Multiple pregnancy is a natural form of restricted implantation. In a singleton pregnancy in sheep about 70% of uterine caruncles are recruited to form placentomes, while in twin pregnancies more (80%) are recruited (Alexander, 1974). For each individual conceptus in a multiple pregnancy, placental size in terms of numbers of placentomes is severely restricted when compared to that of singletons (Vatnick *et al.*, 1991). Such placentas usually have few but very large placentomes suggesting that compensatory growth has occurred (Alexander, 1964a; Stegeman, 1974). However, despite the increased size of individual placentomes in the ovine twin placentas, placental weight for a single twin is less than that of a singleton, although total placental weight for both twins exceeds the placental weight for a singleton (Davies *et al.*, 1981); which is an indication of partial compensatory placental growth in twins (Alexander, 1964b; 1974). In twins individual placentomes are generally 30% heavier compared to that in singletons (Alexander, 1974). Manual separation of placentomes into fetal and maternal tissues revealed that placentomes of twins had 45% more fetal tissue than those of singletons (Davies *et al.*, 1981). In addition, placentomes of twins have a higher average DNA content than those of singletons (Kulhanek *et al.*, 1974). Also Vatnick *et al.*, (1991) observed that placentas of twins have similar placental concentrations of DNA to those of singletons; hence these placentas exhibit greater cellularity per unit weight of placental tissue. This suggests that in sheep placentas of multiple conceptuses undergo compensatory hyperplasia during the proliferative phase of placental growth during the first half of pregnancy (Vatnick *et al.*, 1991).

Following Experimental restriction of implantation

In sheep, restricted implantation may be induced experimentally by the surgical removal of most caruncles before pregnancy. This reduces the potential area for trophoblastic implantation during any subsequent pregnancy. If an adequate number of caruncles is removed, restricted implantation is induced such that only a few placentomes develop hence forming a smaller than normal weight placenta (Alexander, 1964b). Previous studies in late gestation in sheep have shown that when the number of placentomes is low, there is a compensatory increase in

individual placentome weight and more placentomes are everted compared to controls (Alexander, 1964b; Robinson *et al.*, 1979). Increases in placentome sizes were also obtained following the ligation of one uterine horn (hemihysterectomy) on day 5 of gestation, which halved the number of placentomes formed. The individual placentome weights increased, such that at both 120 and 140 days gestation placental weight was similar in control and restricted ewes (Caton *et al.*, 1984).

Compensatory growth observed in carunclectomized ewes has been considered incomplete because although individual placentome weights increase, total placental weight still remains significantly below normal. In addition, the process is sometimes absent in some ewes such that some small lambs with very small placentas are produced (Alexander, 1964b). Alexander also noted that placentomes from the restricted placentas had more fetal tissue than maternal tissue. This was analysed by a simple physical separation of the fetal from maternal tissue in the placentome about 8 hours after the lambs were delivered. However, manual separation of ovine placentome tissues introduces unmeasurable inaccuracies due to tearing of tissues. Alexander (1964b) noted that upon manual separation of placentomes into fetal and maternal components, the trophoctodermal lining of the fetal cotyledon was not cleanly separated from the maternal crypt surface. Moreover, there is electron microscope evidence which shows that at parturition in sheep, the normal separation of fetal and maternal tissues is such that the trophoctodermal lining of the fetal villi remains attached to the feto-maternal syncytium (Steven *et al.*, 1978). This suggests that following manual separation, estimates of fetal tissues are likely to be lower while maternal tissue volumes are overestimated. Due to this method of analysis the cellular components of the chorionic tissue that were involved in the compensatory growth could not be determined.

Mechanisms by which individual placentomes of restricted placentas achieve increases in weight and how they change morphology are not clear. The extent to which these compensatory growth mechanisms enhance overall placental function have not been documented. Very little is known about the mechanism of the compensatory growth process, and the stimulus(i) involved, and whether or not the process may be manipulated if necessary. Due to considerable variations in the correlation between placental weight and its function (as determined grossly by

fetal weight) as observed in cases of IUGR with normal size placentas, microscopic analyses should be used in the study of placental function to determine the cellular capacity for function. Also if the relationship between placentome size and morphology with function is established, proper sampling procedures for placental tissue in sheep can be designed.

The number of placentomes observed at post mortem of those ewes that have undergone surgery to remove most caruncles prior to pregnancy often exceeds the number of obvious caruncles remaining following carunclectomy (Alexander, 1964, Robinson *et al.*, 1979, Harding *et al.*, 1985). It is not clear whether this is simply due to the caruncles which are present in surgically inaccessible areas i.e. the utero-tubal junction and cervical region (which would not normally be involved in placental establishment) being recruited to form placentomes or the generation of new caruncles to replace the ones that were removed as part of the compensatory process.

Compensatory placental growth in sheep following other manipulations

Hemi-hysterectomy

Compensatory placental growth has also been demonstrated in sheep following other experimental manipulations. Caton *et al.*, (1984) ligated one of the uterine horns on the fifth day of pregnancy prior to the migration of the trophoblast into the uterine horn. This had the consequence of halving the numbers of placentomes formed. Examination of the uterine contents at 120 and 140 days gestation showed that placental and fetal weights of the ligated ewes were similar to those of controls. At 120 days, the average total weight of the fetal component of the placentome in treated placentas was significantly greater than that in control placentas; suggesting compensatory growth of the fetal tissues. In addition, the rate of uterine blood flow to the pregnant horn in the ligated animals and the concentration of peripheral progesterone in maternal blood was significantly greater in the experimental animals. Thus placental function was also enhanced following the hemi-hysterectomy. Similar experiments were carried out by Cefalo *et al.*, (1977) but hemi-hysterectomy was carried out by a surgical removal of one uterine horn prior to pregnancy. It was also found that halving of the potential implantation sites was compatible with the production of term fetuses. In the experimental animals, total placental weight exceeded that of controls (815g compared to 601g) and average

placentome weight was also significantly greater in the experimental animals (15g compared to 8.7g in controls). Cefalo and his associates suggested improved efficiency of implantation in the experimental animals, since the average number of placentomes that formed were significantly more than expected following the removal of one uterine horn. Thus following hemihysterectomy, the embryo could have attached to caruncles which it would not normally do so when both horns are available (Cefalo *et al.*, 1977).

Maternal undernutrition: Pregnancy toxemia

Some of the evidence for compensatory placental growth induced by restriction of maternal nutrition in sheep has already been discussed in an earlier section. In addition, severe reduction of quality and quantity of feed is associated with a condition known as pregnancy toxemia in sheep. This condition was successfully induced in sheep by Mitchell (1982) and placental studies in the toxemic animals demonstrated some aspects of adaptational tissue remodelling and enhanced placental maturation. Some severely toxemic ewes delivered stillborn fetuses and necrotic placentas. However, at 120 days gestation, in the placentomes of severely toxemic ewes the maternal component occupied more than 50% of the volume of the zona intima as opposed to the reverse in normal ewes. In contrast, the relative villous volume decreased mainly due to a decrease of the fetal connective tissue. However, in the mildly toxemic ewes, fetal villi were immature by exhibiting large volumes of connective tissue although volumes of both fetal and maternal capillaries and also the feto-maternal syncytium (but not the trophoblast) had increased. Overall, pregnancy toxemia was associated with the following characteristics:

- i) increases of the relative capillary volume of the septal portion of the placenta and the maternal luminal capillary surface
- ii) increases of the relative volume of degenerate trophoblast but similar or slight increases of the fetal capillary lumina surface when compared to controls
- iii) reduction of the barrier thickness such that at 120 days gestation, the distance between maternal and fetal bloods was 16.14 μ m in toxemic animals compared to 22.14 μ m in

controls. The former figure was similar to the size of the barrier thickness measured in normal animals at 140 days gestation.

Enhanced placental maturity could have been related to marked increases in cortisol in the toxaemic animals. The premature elevated levels in fetal cortisol prior to 125 days gestation could have occurred due to a chronic uterine vascular insult similar to that induced by repetitive embolization of the ovine uterine vasculature that affects the placental tissues (Clapp *et al.*, 1982). However, in the toxaemic animals, normal fetal crown-rump length appeared to indicate that the morphological adaptations in the placenta were advantageous in maintaining fetal well-being prior to the onset of the maternal symptoms of ovine toxemia. (Mitchell, 1982).

1.7.2 Evidence for compensatory growth in other species

Compensatory placental growth has also been reported in other species. For example, placentas from women whose pregnancies were complicated by pre-eclampsia showed significantly more syncytiotrophoblast nuclei per unit placental volume than the normal placenta at 40 weeks in gestation (Teasdale, 1985). Placentas from women at high and low altitude demonstrated compensatory growth of trophoblast and stroma of the placental villi at high altitude (Jackson *et al.*, 1987a) in which the villous tree possesses a more extensive and superior vascular system (Jackson *et al.*, 1987b). In guinea pigs, chronic exposure to hypoxic conditions i.e. 12% oxygen throughout pregnancy, resulted into a significant increase in placental exchange area and diffusion capacity (Bacon *et al.*, 1984). When placental fetal villous circulation was interrupted in mid-pregnancy by the ligation of placental bridging vessels in rhesus monkeys the secondary placental disc atrophied and the primary disc hypertrophied as a result of compensatory hyperplasia mainly of trophoblast cells (Novy *et al.*, 1981). This evidence demonstrates that placental tissue responds to restriction of its size and/or function or to the shortage of essential substrates such as low oxygen by undergoing compensatory growth mechanisms.

Specific Aims

Specifically, the following aims were formulated to test the above hypotheses:

1. To determine in both relative and absolute terms, the cellular and structural changes associated with variations in placentome size and morphology
 - a) normally, and
 - b) when implantation is restricted either naturally or experimentally and
 - c) the ontogeny of placental growth in (a) and (b).
2. To determine the order in which components change (i.e. the sequence of events) and attempt to pinpoint the initial targets for these mechanisms.
3. To determine the extent to which these structural alterations proceed by investigating if there is a limit beyond which they will not occur for example when placental size is reduced further
 - a) due to natural increases in numbers of conceptuses from singletons to triplets or
 - b) due to further experimental restriction of implantation in twin and triplet-bearing ewes.
4. To compare the nature of experimentally induced compensatory placental growth with that which occurs naturally in placentas of multiple conceptuses.
5. To determine if maternal low body weight at mating and continued feed restriction throughout the first half of pregnancy limit the extent of compensatory placental growth which occurs following experimental induction of restricted implantation by investigating whether
 - a) the stimulus is a nutritional deficiency which would be enhanced by maternal undernutrition or
 - b) the stimulus and/or process is dependent on adequate nutrition and so would be reduced or limited by maternal undernutrition.

Specific Aims

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 - a) normally, and
 - b) when implantation is restricted either naturally or experimentally and
 - c) the ontogeny of placental growth in (a) and (b).
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 - b) the stimulus and/or process is dependent on adequate nutrition and so would be reduced or limited by maternal undernutrition.

TABLES AND FIGURES FOR CHAPTER 1

Table 1.1 Cell layers separating fetal and maternal blood

PLACENTAL TYPE	CELL LAYERS							Example	Gross shape
	maternal	maternal	uterine	hybrid	trophoblast	fetal	fetal		
	capillary endothelium	connective tissue	epithelium	feto-maternal syncytium		connective tissue	capillary endothelium		
Epitheliochorial	yes	yes	yes		yes	yes	yes	pig	diffuse
<i>synepitheliochorial</i>	yes	yes		yes	yes	yes	yes	sheep, goat	cotyledonary
Endotheliochorial	yes				yes	yes	yes	cat, dog	zonary , discoid
Haemochorial					yes	yes	yes	human	discoid

Modified from Amoroso (1952) and Faber and Thornburg (1983).

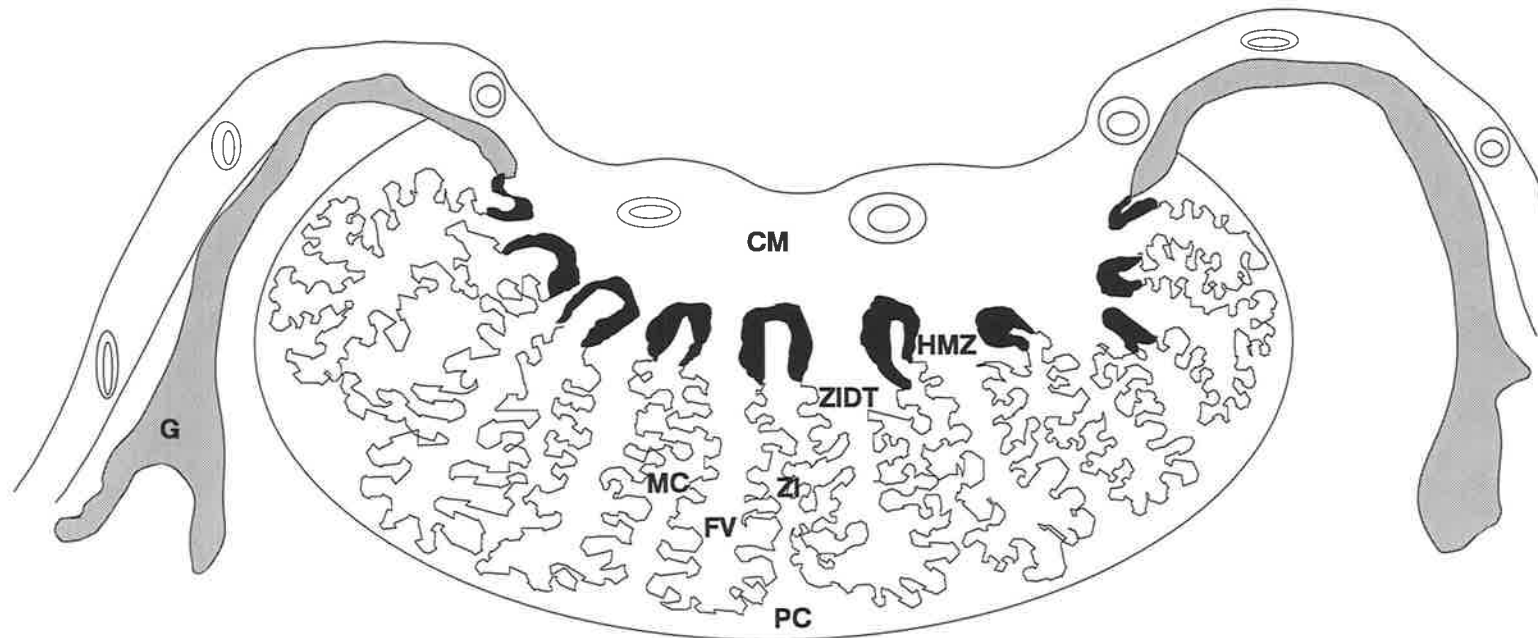


Figure 1.1 Diagram of a vertical section through the centre of a placentome showing the chorionic membrane (CM) and large blood vessel within it, the haemophagous zone (HMZ); where the distances between fetal and maternal tissues are largest, then the zona intermedia (ZIDT) merges with the zona intima (ZI); where there is intimate association between fetal villi (FV) and maternal crypts (MC). The MC stem from the placentome capsule (PC); the expanded caruncle. Extending from the placentome is uterine glandular tissue (G).

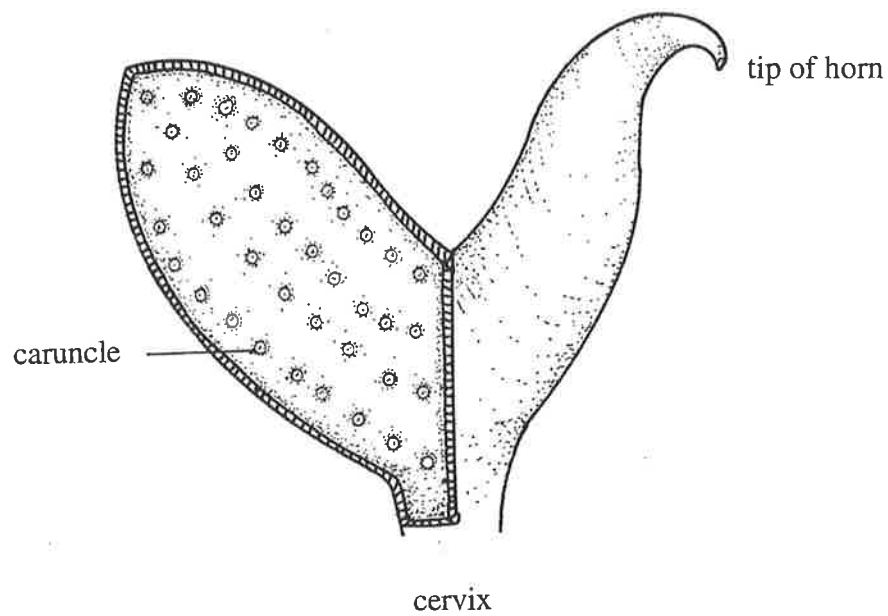


Figure 1.2 Diagram of the non-gravid uterus of the sheep. One uterine horn is open to illustrate the arrangement of the implantation sites (endometrial caruncles) on the internal surface of the uterus.

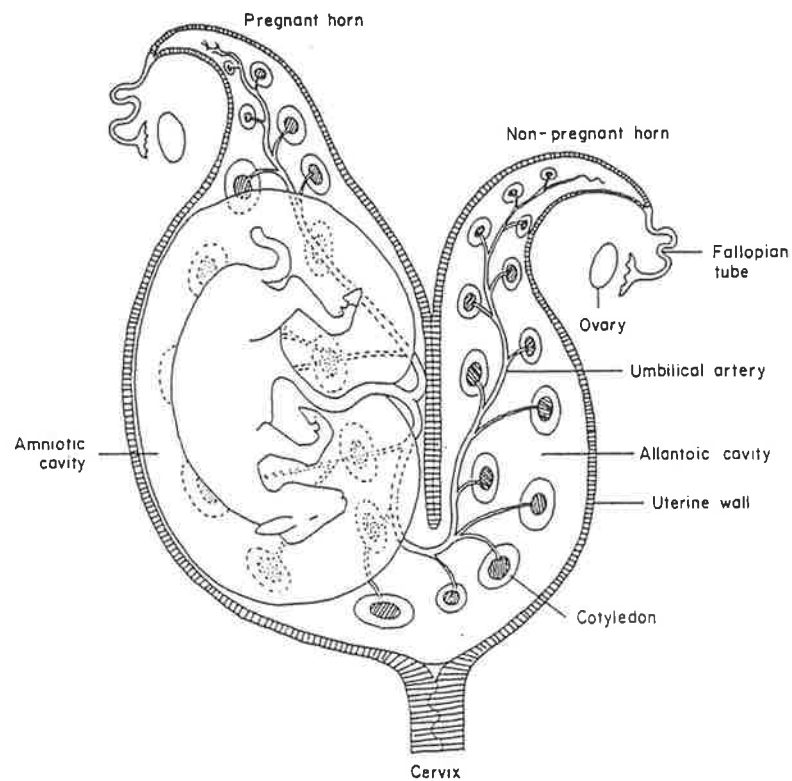


Figure 1.3 Diagram of the cotyledonary placenta of sheep, illustrating the arrangement of placentomes and the fetus connected to the placental mass (Adapted from Steven et al., 1975)



Figure 1.4 Variations of placentome sizes from one placenta, showing non-everted placentomes (morphology A) (Vatnick *et al.*, 1991).

Figures 1.5a and b Variations in shapes of placentomes; showing morphology A (non-everted) and B, C and D (three forms of the everted shape) as classified by Vatnick *et al.* (1991). The dorsal surface of the placentomes represents fetal surface and the ventral surface represents maternal surface. On the fetal surface, the black areas represent translucent chorionic membrane covering large areas of extravasated maternal blood which forms the haemophagous zone. In Figure 1.5b, the placentomes are bisected to illustrate more clearly the variation in the shapes of the placentomes and the position of the haemophagous zone which in morphology D covers the whole external surface of the placentome which assumes a convex shape.

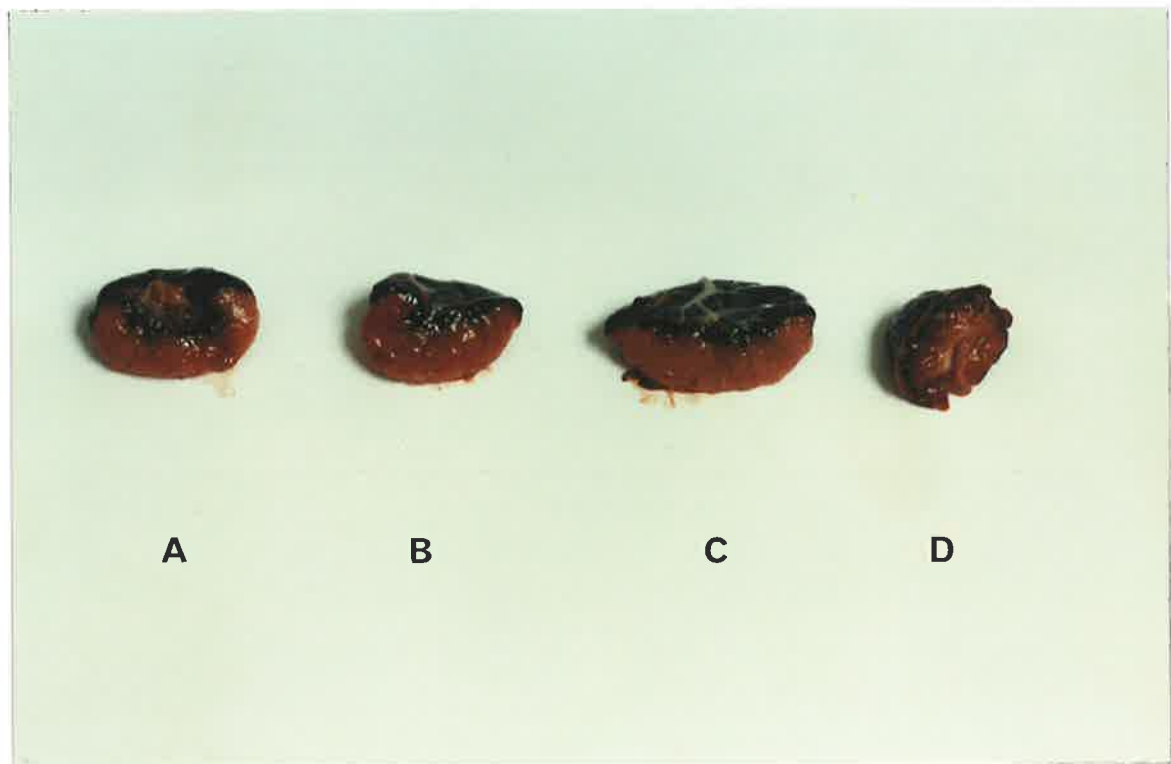
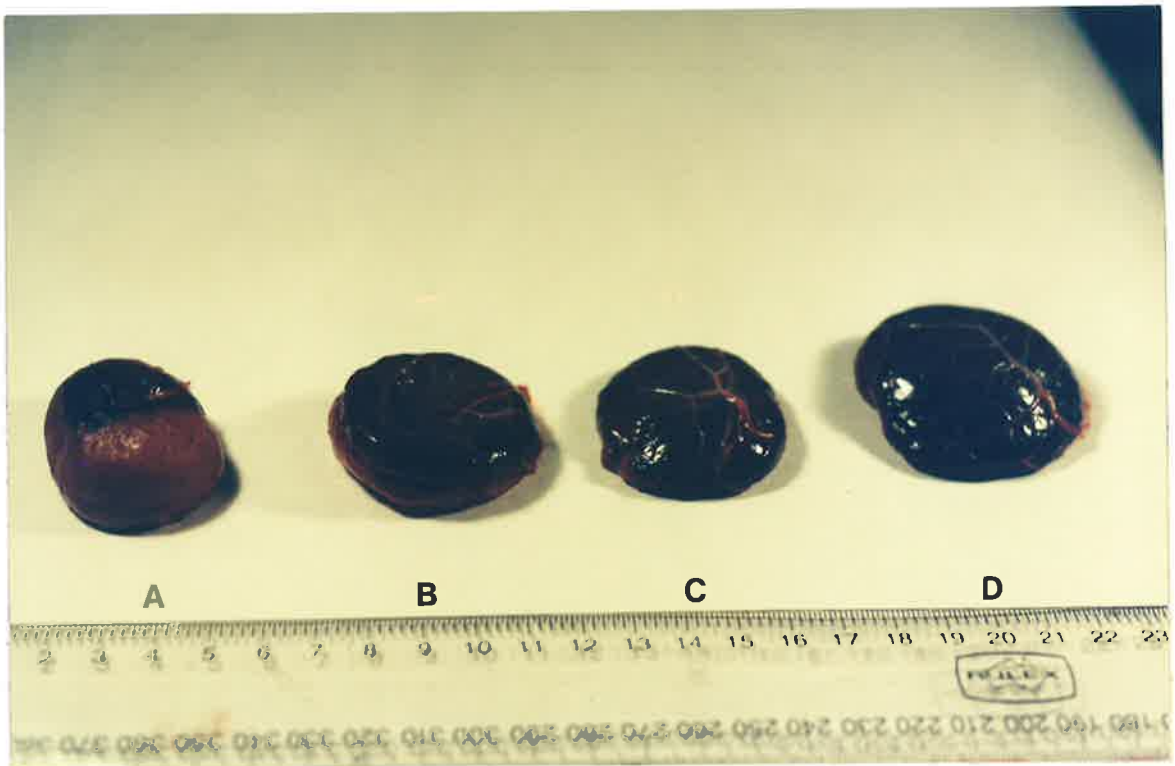


Figure 1.5c A series of placentome sections of morphologies A, B, C, and D at 120 days gestation. Moving from A to D, the haemophagous zone (containing maternal erythrocytes; stained red) appears to reduce as it gradually extends to occupy the external surface of the placentome which flattens (morphology C) before assuming the convex shape (morphology D). The connective tissue within the fetal villi (clear, unstained areas) also reduces from morphology A to C. However, morphology D appears to contain more connective tissue of the fetal villi than the other two varieties of everted morphology. Overall, fetal tissue appears to overgrow maternal tissue with the progression of the reversal of placentome morphology from A (concave shape of the non-everted placentome) to D (convex shape of the everted placentome).

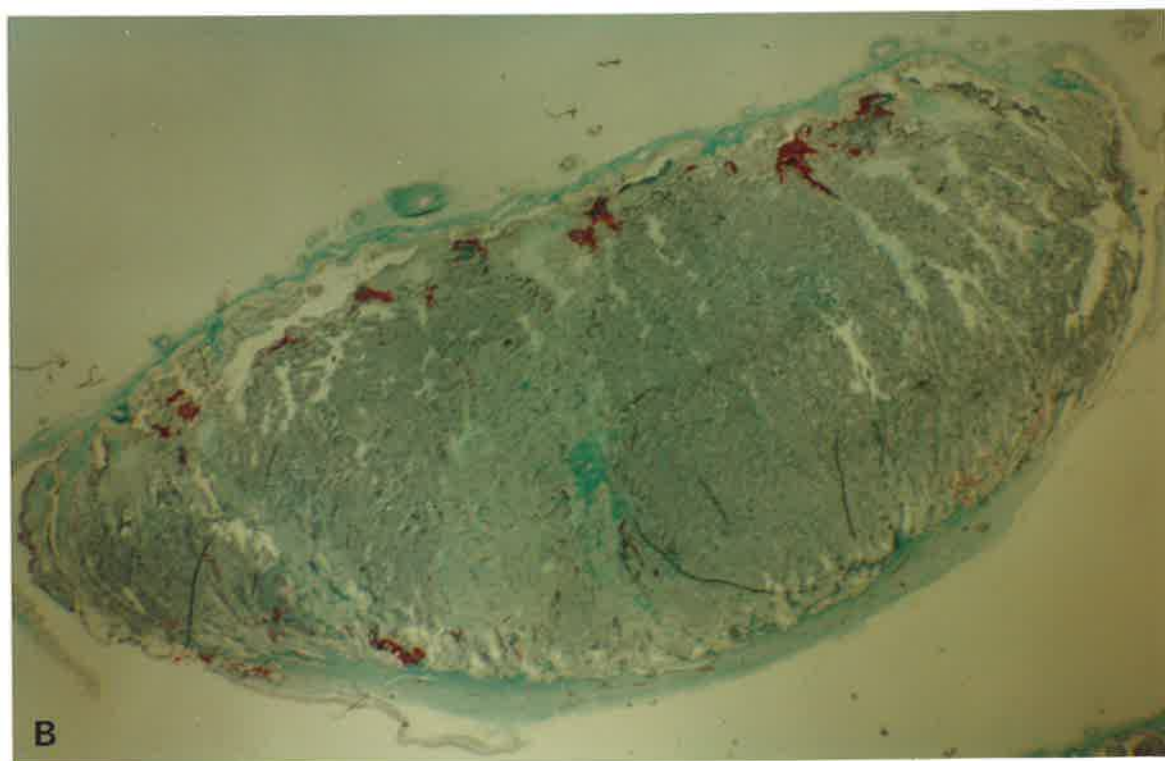
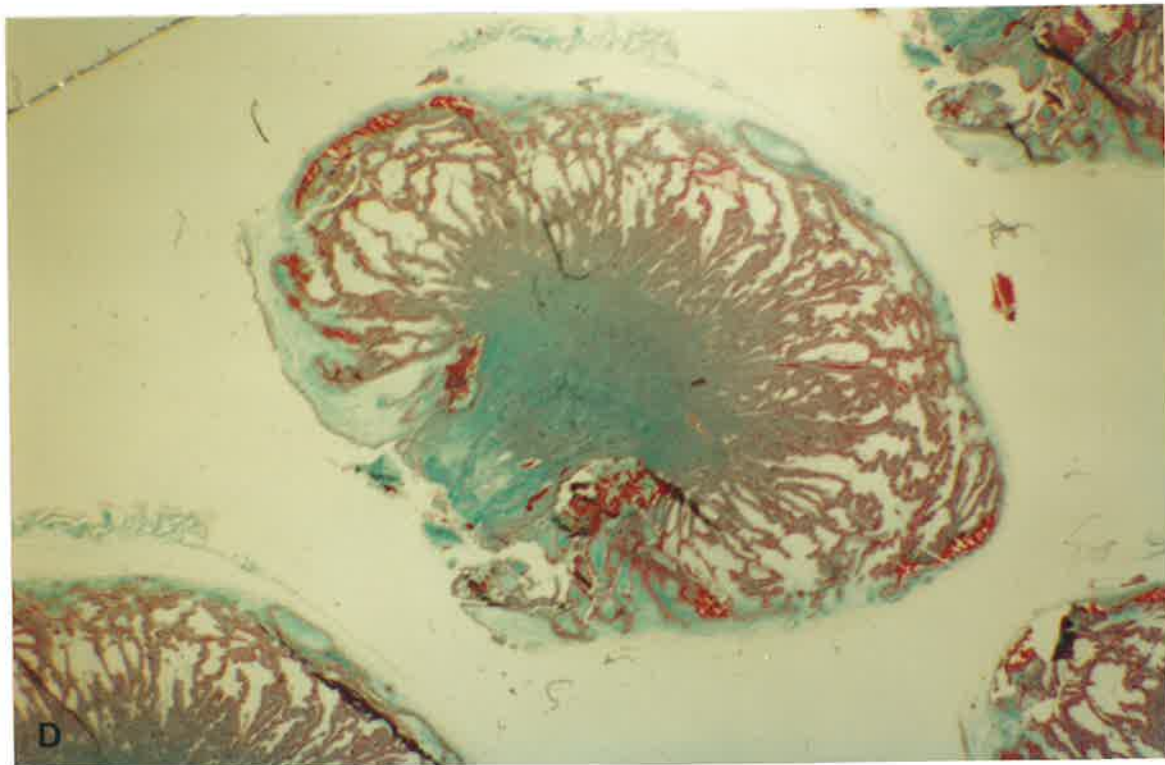
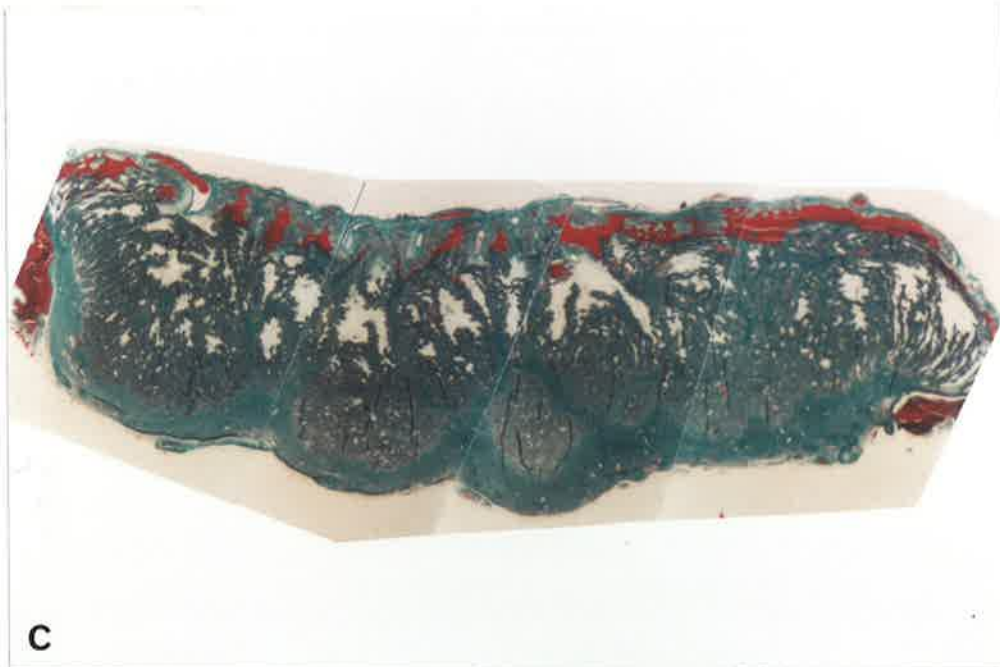


Figure 1.5c Continued; showing the internal structure of placentome morphologies C and D.



CHAPTER 2

MATERIALS AND METHODS

2.1 ANIMALS

Border-Leicester × Merino crossbred ewes were used in morphometric studies of the placenta of singletons and twins at 90 and 120 days gestation and of triplets at 90 days only. In addition, Australian Merino ewes were used in the study of the normal ontogeny of macroscopic placental parameters. These strains have a gestational length of approximately 150 days (Cloete, 1939; Davies and Wimsatt, 1966; Alexander, 1964b).

The initial aim was to study a minimum of six animals in each treatment group (Mayhew 1991), in order to reliably detect differences. However, a high incidence of multiple pregnancies in ewes subjected to experimental restriction of implantation resulted in a maximum of four animals in some treatment groups. The operational sampling used in these studies is outlined in Figure 2.1 and will be described in detail subsequently in this chapter.

2.2 TREATMENT OF ANIMALS

2.2.1 Mating

Ewes were allowed to run with a ram wearing a run harness. Day zero of pregnancy was taken to be the date of marking of the rump of the ewe by pigment smeared on the brisket of the rams. Pregnancy was confirmed by ultrasound scanning about 45 days after mating.

2.2.2 Nutrition

Animals were maintained on pasture with free access to feed lucerne hay supplements and water. In addition, animals which had permanent indwelling catheters inserted into fetal and maternal blood vessels to determine fetal metabolic and endocrine states, were placed in pens at least a week before the surgery and were accustomed to hand feeding. Subsequently, the ewes were kept individually in metabolism cages and fed lucerne hay, a pelleted ration and water *ad libitum*.

2.2.3 Ontogeny

The normal ontogeny of macroscopic parameters of placental growth and morphology was studied using two strains of sheep carrying singletons, Border-Leicester × Merino cross-bred and Australian Merino ewes, at various gestational ages (Table 2.1). All ewes were well-fed.

In addition, ontogenic changes in cellular components and structure of placentomes were analysed in Border-Leicester x Merino ewes at 90 and 120 days gestation (Table 2.1).

2.2.4 Restriction of implantation

The effect of restricted implantation on placental development was studied in Border-Leicester x Merino ewes with experimental and natural restriction of implantation.

Experimental restriction of implantation (carunclectomy)

To restrict implantation, ewes underwent surgery prior to pregnancy to remove most of the caruncles or implantation sites in the uterus (restricted ewes) Alexander (1964b). This technique reduces the number of sites at which blastocyst attachment can occur in the subsequent pregnancy.

All operations were performed with strict attention to asepsis and antisepsis. Anaesthesia was induced with an intravenous injection of thiopentone (1g) and after intubation, maintained with halothane (Fluothane, May and Baker, Dagenham, Essex) and oxygen in a closed circuit.

An abdominal ventral mid-line incision exposed the uterine horns, which were opened individually using a scalpel blade along the antimesometrial border. The total number of visible caruncles from the central region of the uterus ranged from 60 to 104. Up to 80% of these (46 to 89) were excised leaving about 11 to 20 visible caruncles in total with about half of these in each horn. The operation did not involve the utero-tubal junction and the cervical region. The operation was completed on one uterine horn before opening the other one.

The incisions on the uterine horns were closed with sutures of 3/0 chromic catgut with a Taper SH 26mm needle (Ethicon, Johnson & Johnson Medical Products, Pty Ltd, Sydney). The abdomen was closed in two layers with coated vicryl absorbable sutures with a cutting CP-1 36mm needle (Ethicon, Johnson & Johnson Medical Products, Pty Ltd, Sydney). The first layer included the peritoneum and rectus sheath and the second included the skin and subcutaneous tissues.

Upon completion of surgery, ewes were given 1g streptomycin (Glaxo, Australia Pty Ltd) plus 600mg benzylpenicillin (Commonwealth Serum Laboratories, Melbourne, Australia) intramuscularly. They were housed individually in pens for about 2 days to recover from the operation, then returned to pasture. After a minimum period of 4 weeks, the ewes were deemed recovered from the operation and allowed to mate.

Restricted and control ewes were killed at 90 and 120 days gestation (Table 2.2) and placentas of control singleton fetuses were compared with those of singleton fetuses from restricted ewes.

Natural restriction of implantation

The effects of natural restriction of implantation on placental development were studied by comparing placentas of ewes carrying twins or triplets with those of singletons. In addition, some ewes, in which most caruncles were excised to induce restricted implantation, carried twins or triplets. These were included in this study to investigate the effects of further limitation of implantation on placental development in multiple pregnancy. Table 2.3 summarises the details of the design of this study.

Restriction of maternal nutrition

The effect of maternal undernutrition on the placental response to restricted implantation was investigated in control and restricted Border-Leicester x Merino ewes carrying singletons. Ewes were randomly divided into two groups and their live weights and condition scores (on a scale of 0 (very thin) to 5 (overfat), (Russel *et al.*, 1969) were monitored weekly. From 60 days before mating to 90 days gestation one group was well-fed, while the other was underfed. The nutritional intake of the ewes was controlled by pasture management, such that the well-fed group was subjected to low stocking rate and supplementation of clover-grass three times per week, while the under-fed group was subjected to a high stocking rate without supplementation. At mating, the average live weight of the well-fed ewes was 61kg (mean condition score, 4) while that of the underfed ewes was 51kg (16% lower than for the well-fed ewes) (mean condition score, 3). By day 90 of gestation, the average live weights of the well-fed and underfed ewes were 63 ± 2 kg (mean condition score, 4.4 ± 0.2) and 44 ± 1.6 kg (mean condition score, 2 ± 0.3). Table 2.4 summarises the design of this study.

2.2.5 Catheterisation of fetal and maternal blood vessels

The ewes from which placental tissue was collected at 120, 130 and 140 days gestation and their fetuses had vascular catheters implanted in umbilical veins, femoral artery, fetal tarsal vein, maternal jugular vein and carotid artery, at 108 to 110 days gestation to enable blood sampling for the purposes of other studies. On the day of surgery, and daily for three days thereafter, both mother and fetus were given antibiotics. The fetus received 300mg benzylpenicillin (Commonwealth Serum Laboratories, Melbourne, Australia) via the fetal tarsal vein, whereas the mother received 600mg benzylpenicillin and 80mg gentamycin (Delta West Incorporated, West Australia) intramuscularly.

2.2.6 Post-mortem

The ewes used in the studies designed to test the effect of variable maternal nutrition (Chapter 6) and natural restriction of implantation (multiple pregnancy) (Chapter 5) on placental development were killed by captive bolt, while all other ewes were killed by intravenous injection of 20ml of phenobarbitone (Lethobarb, Arnolds of Reading Pty. Ltd. Peakhurst, NSW 2210). The drug quickly crosses the placenta to the fetus, which also receives a lethal dose. Using a scalpel blade, an incision was then made to open the abdomen and the intact uterus and its contents were removed for examination and processing.

2.3 PLACENTAL TISSUE COLLECTION AND MACROSCOPIC MORPHOMETRY AT POST-MORTEM

2.3.1 Fetus

The fetus was removed from the uterus, the umbilical cord tied and severed, and the fetus was blotted dry with tissue paper. Fetal weight and crown-rump length were measured and recorded before the fetus was dissected for examination of individual organs and skeleton.

2.3.2 Placenta

The sampling procedure used in these studies of ovine placental growth and development is outlined in Figure 2.1. For each placenta, placentomes were dissected and trimmed of the membranes, blotted dry with tissue paper and then weighed individually. The aggregate weight of all placentomes was recorded as total placental weight. Morphology of all

placentomes was noted as A (non-everted), B, C, or D (different grades of eversion), (Vatnick *et al.*, 1991) (Figure 1.5).

The uterine position of each placentome was not noted for all animals due to insufficient resources. Since the examination of the relationship between structure and individual placentome size and morphology was a major aim of this work, placentomes of different sizes and morphological appearance were sampled from each placenta as far as possible.

Sampling of tissue

Each placentome was classed as "small" (0-0.49g), "average" (0.5-9.9g), or "large" (≥ 10 g) based on the placentome weight distribution in placentas of control ewes at 120 days gestation in the present work (Figures 1.4 and 2.2), and the placentome weight distribution in late gestation, described in previous studies (Robinson *et al.*, 1979). For all treatment groups, both "average" and "large" placentomes were randomly selected for morphometric analysis. "Small" placentomes were not obtained from all ewes. In addition, the morphology of each of the placentomes was noted as "A"; non-everted or "B", "C" or "D"; everted (Vatnick *et al.*, 1991). Thus, where possible, a placentome of each morphology type within each size class was selected for morphometric analysis in all ewes in all treatment groups at post-mortem.

Placentomes selected for morphometric analysis were bisected vertically and immersion fixed in 10% neutral buffered formalin.

2.4 FIXATION, PROCESSING AND HISTOCHEMISTRY OF OVINE PLACENTAL TISSUE

2.4.1 Optimisation of Tissue Fixation procedure

To determine the best fixation procedure for the placental tissue, each of four placentomes was bisected. One half was fixed in 10% neutral buffered formalin (Stegeman, 1974; Jones and Fox, 1976; Van Der Veen and Fox 1982) for seven to ten days (Drury *et al.*, 1976). The other half was fixed in Bouin's fixative (B_f) for 24 hours, then transferred into 70% alcohol until processed (Bancroft and Stevens, 1977). Some placentomes were fixed in formalin for a short or long period (i.e. days or months), post-fixed in Bouin's for 24 hours (Baur, 1977), then

transferred into 70% alcohol until processed. Post-fixation with Bouin's of tissue pre-fixed with formalin was carried out to test if this fixation procedure would provide better quality of staining than fixation in either formalin or Bouin's alone (Table 2.5). Placentomes were fixed in formalin for one to six months.

Placentome volumes were measured at postmortem and after fixation (prior to embedding) using the displacement method, to investigate if fixation caused macroscopically detectable shrinkage (Table 2.6). No significant difference was evident between pre and post fixation placentome volumes at 120 days gestation ($p=0.17$; paired two sample t-test, one tail). Similar results were also obtained when placentomes at 90 days gestation were analysed. Thus the shrinkage that was evident microscopically (see below) did not significantly alter macroscopic dimensions of the placentomes.

The examination of tissue sections at the microscopic level revealed that formalin produced little shrinkage of tissue components in contrast to Bouin's which produced many artifactual shrinkage spaces between maternal crypts and fetal villi. Also in some cases breakage of maternal or fetal tissues was evident following fixation with Bouin's, suggesting that excessive hardening and drying of the tissue had occurred. This was more common in the young placentas of mid gestation (75 and 90 days) (Figure 2.3). Placentas of late gestation (120 days) appeared to be more resistant to the effects of fixation with Bouin's and fixation with formalin followed by post fixation with Bouin's (Figure 2.4). This indicated that the presence of substantial amount of mesenchymal hydrophilic connective tissue within the fetal villi (Stegeman, 1974), made younger placentas more susceptible to shrinkage when fixed with Bouin's. All morphometric studies were therefore performed on formalin-fixed tissues.

2.4.2 Tissue Embedding for Light Microscopy

Due to the large variations in sizes of individual placentomes, with widths of about 0.5cm to 5cm and depths ranging from about 0.5cm to about 2.5 cm, processing was performed over several days. Basically, tissues were processed following routine procedures as outlined by Drury *et al.*, (1967), but during manual processing, the tissues were left in each of the

solutions overnight due to their large sizes. Prior to processing, a vertical slice of tissue from the exposed surface of the placentome was trimmed to the required size.

Most of the tissues were processed using automatic embedding equipment, which differed depending on the size of blocks, as indicated below. For this procedure, trimmed blocks of tissue were divided into two categories; one containing blocks from "small" and "average" size placentomes and the other containing blocks from "large" placentomes and processed separately.

Tissue blocks from "large" placentomes were processed using a "Tissuetex-VIP" embedder (Miles Scientific, Canada). The dehydration process was carried out through 70%, 80%, and 90%, followed by five changes of absolute alcohol, two changes of xylene and finally three changes of wax (56°C) (Appendix 1). The whole procedure lasted a total of 57 hours.

Tissue blocks from "small" and "average" placentomes were processed using a "Shandon hypercentre command" embedder (Tylor Electronic, Calgary, Alberta, Canada). Dehydration was carried out through 50%, 70%, and 80%, then five changes of absolute alcohol, followed by two changes of xylene, and finally two changes of wax. The whole procedure lasted a total of 40 hours (Appendix 2). Finally, the tissue was embedded in wax on embedding cassettes, and left on a cold plate for the wax to harden.

2.4.3 Optimisation of Staining Procedure

The following stains were tested in order to determine which stain enabled fetal and maternal components of the placentome to be most readily distinguished:

- a) Hematoxylin and eosin (H & E) Drury *et al.*, 1967.
- b) Masson trichrome using Allum (Myers) or Weigerts hematoxylin as nuclear stains counterstained with (i) alanine blue or (ii) light green (Drury *et al.*, 1967).
- c) Alcian blue-Periodic Acid Schiff (PAS) plus a combined Masson-Goldner stain (Stegeman, 1974).

Using a Leitz Wetzlar microtome, a ribbon of 7 μ m thick serial sections was cut. Sections were separated and flattened on a warm tissue preparation flotation waterbath. Sections were placed on slides and left in an oven at 37°C overnight for the specimen to dry before the staining process.

Prior to staining, the sections were dewaxed in xylol or histoclear and hydrated through decreasing concentrations of alcohols to water as described by Drury *et al.*, (1967). For all three stains, staining was carried out using routine procedures (Drury *et al.*, 1967).

At the end of the staining procedure, sections were mounted in PIX (Appendix 3) and then left in an oven at 37°C for at least 48 hours to dry. Prior to microscopic observation, the edges of the coverslips were cleaned using window cleaning detergent or ammonia alcohol to remove excess mounting medium.

Following staining with H & E, various tissue components in placentas from mid gestation could be readily identified, since the fetal villi have a large mesenchyme connective tissue content (Figure 2.5). By 120 days gestation, the fetal villi have lost most of the connective tissue, a process said to start by the 80th day (Wimsat, 1950) and they are almost as compact as the maternal tissue component of placentomes or crypts (Figure 2.6). This makes it more difficult to reliably distinguish fetal villi from maternal crypts.

The Masson trichrome stain, with either of the haematoxylin nuclear stains and counterstained with light green or alanine blue, distinctively differentially stained maternal and fetal tissue components (Figures 2.7 and 2.8). However, the combination of celestine blue-alum haematoxylin and light green was superior to that of either of the haematoxylin with alanine blue. This staining technique gave good results, since different tissue components clearly stained differentially: the maternal tissue component staining a deep green and the fetal tissue, a pale-green to grey. Also against this background, it was easy to identify erythrocytes, even those within the small capillaries, which stained a bright orange red. The Masson trichrome with alanine blue as counterstain and the Masson trichrome modification as described by Stegeman (1974) did not give as good results and were not used any further.

From this study it was concluded that the Hematoxylin and Eosin stain can be used with reasonable success in the study of placental tissue earlier in gestation, but that the Masson trichrome, with allum haematoxylin as nuclear stain and light green to stain collagen, is the superior stain for the ovine placenta at all stages, especially in late gestation.

2.5 STEREOLOGY AND MICROSCOPIC MORPHOMETRY OF THE OVINE PLACENTA

2.5.1 Definitions

"Stereology is a body of mathematical methods relating three-dimensional parameters defining the structure, to two-dimensional measurements obtainable on sections of the structure" (Weibel, 1979). Stereological methods enable quantitative data on structures, particularly those related to function, to be obtained. "The clarification of the pattern of organisation of the components of structures, their relative size and degree of interconnections, is the purpose of stereology" (adapted from Weibel, 1979). In this study of the ovine placenta, the structural quantities of primary interest were the volumes of various components that are known to be actively involved in or influences on exchange functions, their surface areas and barrier thicknesses, as well as related components. The stereological terms which describe these quantitative properties of the structure in relation to the structural space are volume density and surface density (Table 2.7). Volume density is the relative volume fraction of a component in a structure and surface density is the surface area of the component per unit volume of the structure. These two parameters are used to obtain the arithmetic mean barrier thickness (Table 2.7), which estimates the tissue mass making the barrier.

While several methods for estimating structural parameters according to stereological principles can be used, the greatest efficiency is offered by point counting methods, using coherent test systems (Weibel, 1979). Tissue sectioning is the sampling process used in stereology. The tissue section can be defined as a plane intersecting the structure and some of or all of its components. The section or sample can be further probed by applying geometric probes, such as test point sets and test lines. For example, the "density of points" (per unit point set) falling onto the component will be related to the volume density of the component

in the structure. Thus all stereological measurements are obtained as relative measurements, or the ratio of at least two concomitant measurements: one relating to the component of interest, the other to the structure as a whole, referred to as the "reference system"(p 4-5, Weibel, 1979). Stereological principles establish the precise relationship between ratios measured on sections and the corresponding ratios in the spatial structure. The methods used here are summarised below:

Volume density, V_V

$$V_V = P_P = P_a/P_T \quad (1)$$

Thus the volume density V_V of a component can be estimated from the fraction of test points, P_P , lying on the component. This is determined from the ratio of the number of points falling on the component, P_a , to the total number of test points applied, P_T .

Surface density, S_V

$$S_V = 2I_L = 2 \times I_a/L_T \quad (2)$$

The surface density of a component, S_V , expresses the amount of surface area of the component that is contained in the unit containing tissue volume V . It can be shown mathematically that this is related to the number of intersections I_a , that the boundary of the component makes with the length of test lines applied, L_T , or the intersection density of the component surface, I_L .

Arithmetic mean barrier thickness:

$$\tau = V_V/S_V \quad (3)$$

A barrier is a layer of tissue separating two spaces, which is characteristically thin, compared to its extension in the two other dimensions, and essentially, separate the two spaces completely. The arithmetic mean barrier thickness or τ , can be estimated from point counts

using a coherent test system of lines and points. It is defined as the volume of the barrier divided by one of its two bounding surfaces.

2.5.2 Multiple stage sampling

Figure 2.1 shows the operational hierarchy of sampling used in this work which leads from the whole animal and organ to the point counts on tissue sections at the microscopic level. The points in the hierarchy at which the data presented in this thesis were obtained are indicated.

2.5.3 Determination of the relationship between placentome wet weight and volume at post mortem.

Because weight of placentomes could be measured more readily than volume at post mortem, the relationship between placentome wet weight and volume was determined to enable calculation of absolute quantities using wet weight and relative measures of structural parameters. Four placentomes were selected from each of the placentas of nine ewes (five control and four restricted ewes) at different gestational ages. The placentomes were dissected out, and blotted dry with tissue paper and separately weighed. Volumes of the selected placentomes were measured by displacement in physiological saline.

Combined analysis of placentomes from control and restricted placentas at 120 days gestation revealed a high correlation between placentome weight and volume ($r=0.99$) with a slope, 0.993 (not significantly different from 1) and intercept, 0.02 (not significantly different from zero). A similar relationship was observed when placentomes from control and restricted placentas obtained at 120 days gestation were analysed separately. At 90 days gestation, a similar analysis also revealed a high correlation between placentome weight and volume ($r=0.98$) with a slope, 0.943 (not significantly different from 1) and intercept=0.56 not significantly different from zero). Thus placentome volume and weight measurements have a 1:1 relationship and placental volume measured by displacement method is equivalent to its weight.

2.5.4 Selection and optimisation of point-counting methods for microscopic morphometric analysis of the ovine placenta

Structural components of the ovine placentome

Eleven major structural components which were readily identified upon microscopic examination of placentome sections, including those thought to be determinants of transfer function, were studied (Table 2.8 and diagrammatically illustrated in Figure 1.1). The vasculature was not studied because identification by the presence of red blood cells and/or lumen space was not regarded as sufficiently reliable, especially in late gestation when many vessels appear to be collapsed (Stegeman, 1974). Artificial spaces were also quantified as an indicator of the standard and uniformity of the tissue response to processing. The selection and optimisation of procedures for the measurements of volume densities, V_V , of all eleven components and as well as the surface densities, S_V , and arithmetic mean barrier thicknesses, B_T , of the two epithelial cell layers at the fetomaternal interface: trophoblast and fetomaternal syncytium, was undertaken as outlined by Weibel (1979).

Selection of Test Lattice

The placenta, like tissues such as intestinal mucosa and lung, exhibits a degree of directional arrangement of its tissue components or anisotropy. This is in contrast to other tissues, such as liver, where various components are randomly distributed throughout the tissue (i.e. isotropic tissues). In the ovine placentome, fetal and maternal surfaces are separated by a region with semi-regular inter digitation of maternal crypts and fetal villi (Amoroso, 1952). This type of tissue arrangement with preferred orientation of the boundaries between fetal and maternal tissue poses limitations on the test systems that can be used to reliably quantitate structures within the placenta.

Therefore, an isotropic curvilinear test system, the "Merz" test lattice (Figure 2.9) which has been used for morphometric analysis of anisotropic tissues was used. This test system contains a combination of test lines and semi-circles lying in a square lattice, (Aherne and Dunhill, 1982; Weibel, 1979). The key element of the Merz test system is a semicircle, whose diameter (d) corresponds to the spacing of its test points so that the system is coherent as follows:

Test points: P_T

Test point length : $L_T = P_T \times \pi/2 \times d$

Test area: $P_T = P_T \times d^2$

The " Merz" test lattice has been previously used for morphometric analysis of the placenta by Aherne and Dunhill (1966) and Stegeman (1974) and others and was adopted in the present study.

Selection of test system.

The optimal test system, including test point number required to determine various parameters with sufficient precision and optimal magnification was arrived at by following the procedures described by Weibel (1979 Chapter 4). Placentome sections were selected from placentas at 90 and 120 days gestation, and examined on the Wild Heerbrugg sampling microscope at magnifications of 100×, 200×, and 400× to assess the optimum magnification for examining the various components of the placentome at the different gestational ages.

Two criteria were used to determine the optimal magnification. Firstly, that the identification of various structures must be accurate. Secondly, test points should be spaced so that no more than one point can fall on the same profile.

Due to the less compact nature of the tissue in the ovine placenta of day 75 and 90, all components except blood capillaries could easily be identified at 100× and 200× magnification. The blood capillaries were more readily visualised at 400×, which also proved the best for identifying all other placental structures at 120 days, due to its more compact nature at that time. The major structural components, particularly fetal trophoctoderm and feto-maternal syncytium and others, were of relatively similar abundance, therefore the same test system was adequate for measurement of volume densities: the 36 point Merz test lattice.

Because the optimal magnification for identification of various tissue components at both mid and late gestation was 400 x, the test point number to be applied per field was assessed at this magnification. A Merz grid was used to obtain initial estimates of the volume densities of various components considered to be important structural determinants of function, including

fetal trophoctoderm and connective tissue within fetal villi. The Merz grid used contained 36 points with point spacing $d=1.9$ cm and was applied 72 times in a systematic random manner in non-overlapping fields to one section from a single placentome at each gestational age. The estimated volume densities of components are shown in Table 2.9.

A nomogram relating test point number to V_v and expected relative standard error (in % mean) (Weibel 1979) was used to determine the minimum number of test points required for a sample to determine the volume density of various components within a relative standard error of 5% or of 10% of the mean (Table 2.9).

The number of test points required to achieve relative standard errors of the mean of 5 or 10% for various components are shown in Table 2.9. The total number of test points to be applied was based on that needed to estimate the volume density of that component, which was the least abundant of potentially important structural determinants of function at 90 days gestation, with a relative standard error of 5%. This component was the feto-maternal syncytium. Thus between 120 to 180 fields of 36 points or 4320 to 6480 test points were applied to a sample. Since an evaluation of the relationship of structure to individual placentome size and morphology was a major aim of this study, this total number of test points was applied to each placentome studied.

The length of test line needed to be applied to estimate S_v or surface density of the epithelial cell layers bordering fetal villi and maternal crypts with a relative standard error of 5% was also determined, using a nomogram related to S_v and relative standard error (Weibel 1979, page 119). The L36 Merz test lattice used at 400 x had a test line length of 107.44 cm on the optical screen (0.2686cm on the section). The surface density of fetal trophoctoderm and feto-maternal syncytium ranged from $\sim 1 \cdot 10^{-1}$ cm to 200^{-1} cm between mid and late gestation; thus requiring a minimum total test length of between 8 and 14cm or 30 to 52 fields to be applied to each sample to achieve a relative standard error of 5%. The actual test line length applied (120-180 fields per sample or placentome) was an average 40.4cm (32-49cm) resulting in expected relative standard errors of 2.4 to 3%.

In one study (Chapter 4), surface density estimates were calculated for both trophoctoderm and feto-maternal syncytium. This was done in order to investigate if the shrinkage which had occurred in some samples would introduce significant differences between the surface density estimates measured for either of the two apposed epithelial cell layers. Table 2.10 summarises the results obtained for control and restricted Border-Leicester x Merino ewes at 120 days gestation. It was found that the surface density estimates for trophoctoderm and feto-maternal syncytium were not significantly different. Therefore, only the surface density of trophoctoderm was determined in all other studies.

Sampling of tissue for Microscopic Morphometric Section Orientation.

To obtain unbiased estimates of stereological parameters, random sampling of the tissue, then of the sample itself is necessary (Chapter 3, Weibel 1979). Ordered or anisotropic structures also pose additional problems if bias is to be avoided. Volume density measurements of components in anisotropic tissues are generally independent of sectioning angle (Chapter 6, Weibel 1979). In contrast, estimates of surface density of highly ordered structures are influenced by sectioning angle. This can be overcome by the use of isotropic arrangements of curved test lines (Merz lattice), provided the section planes themselves are randomly selected (Mayhew, 1991). The influence of section plane and position within the placentome on estimates of volume density for various components was evaluated.

Two placentomes from the placenta of a restricted ewe at 120 days gestation were bisected vertically and embedded in paraffin wax. One half of the first placentome was sectioned vertically and the other half was sectioned at a randomly selected angle. One half of the second placentome was also completely sectioned vertically, and the second half horizontally. Alternate sections of 7 μ m at 1mm distances (1001 μ m) were cut.

Using the sampling microscope, about 60 fields were sampled using the L36 Merz test lattice from each of the sections and volume densities were calculated for all components (Table 2.11).

The volume densities of various components estimated from vertical sections were not significantly different to those obtained from randomly oriented sections, with percentage differences ranging from -25% for the volume density of the maternal tissue within the haemophagous zone, to +17% for the volume density of the chorionic epithelium (Table 2.11). Similarly, volume densities of various components obtained from vertical sections did not differ significantly from those obtained from horizontal sections (Table 2.11).

Greater variation in estimates of volume densities was observed with random and horizontal sections compared to vertical sections from placentome 2. For example, the volume density of fetal villi obtained from vertical sections from placentome number 2 (0.42 ± 0.02), (Table 2.11 and Figure 2.10) had a lower coefficient of variation of 4.8% than that obtained from horizontal sections (0.44 ± 0.04) or 8.9%.

Therefore, vertical sections through the centre of placentomes were used to avoid the bias of peripheral sections which may not include all components and to achieve a constant angle of orientation to the semi-ordered structures within the placentomes. Thus the degree of bias in estimates of surface density may be biased, but should be consistent between treatment groups.

The sections themselves were then subjected to systematic random sampling using a programmable stepping motor to drive the mechanical stage of a Wild Heerburgg 501 microscope. The dimensions of the placentome section were measured then the distance between fields calculated so as to allow 40 to 60 non-overlapping fields to be sampled per section.

2.5.5 Calculation of absolute stereological parameters: components of volumes and surface areas

In the calculation of absolute volumes and surface areas of various components, any significant influences of placentome size or morphology on the volume or surface densities of the component detected were taken into account.

Mid-gestation

At 90 days gestation, placentome size was not associated with significant changes in the volume densities of any components in both control and restricted placentas. However, placentome morphology did significantly influence the volume density of trophoctoderm (Chapters 3 and 4). Therefore for each component except trophoctoderm, the average volume density of the component was calculated using individual volume density values obtained for each placentome analysed in a placenta, regardless of their size or morphology, then multiplied by placental weight to obtain the volume of the component. For trophoctoderm, the volume density or average volume density obtained in everted placentome(s) was multiplied by the aggregate weight of all everted placentomes in the placenta, and this volume added to that obtained by multiplying the volume density obtained in non-everted placentome(s) by the aggregate weight of non-everted placentomes. Thus the total placental volume for trophoctoderm was obtained by summing the placental volumes of everted and non-everted placentomes. Placental morphology also significantly influenced the volume density of maternal crypts and shrinkage spaces in restricted placentas. Therefore, similar calculations were used to determine the absolute volumes of these components in restricted placentas.

Late-gestation

At 120 days gestation, placentome size influenced the volume densities of fetal villi, trophoctoderm and the connective tissue core of the villi. These effects were different in control and restricted placentas.

In control placentas, placentomes of "average" size tended to have lower volumes of fetal villi and trophoctoderm than in "large" placentomes. Therefore the volume of fetal villi and trophoctoderm was calculated from their volume density or average volume density in "average" size placentomes multiplied by the aggregate weight of "average" (plus "small") placentomes in the placenta, together with that calculated similarly for "large" placentomes. Insufficient number of "small" size placentomes were sampled from control placentas for morphometric analysis, hence these were included with "average" size placentomes. However, "average" and "large" size placentomes account for >95% of control placentome

weight. In restricted placentas, "small" size placentomes had significantly increased volume densities of fetal trophoctoderm and decreases of connective tissue core of fetal villi compared to "average" and "large" size. Therefore, the absolute volumes of these two components was calculated using the volume densities in "small" placentomes and their aggregate weight and the mean volume density in "average" and "large" placentomes and their total combined weight. Thus the volume calculated for "small" placentomes was added to that for the "average" plus "large" placentomes to obtain the placental volume for the component.

In late gestation, placentome morphology influenced the volume densities of fetal villi, trophoctoderm, haemophagous zone and the maternal tissue associated with the haemophagus zone. Therefore to calculate the absolute volume for these components, placentome morphology was taken into account as described above.

2.6 STATISTICAL ANALYSIS AND PRESENTATION OF RESULTS

The normality of distribution of data was assessed using Proc Univariate (Statistical Analysis System, SAS). Analysis of variance was used to assess the effect of between factors, experimental or natural restriction of implantation and maternal undernutrition and of within factors, placentome size and morphology and their interactions (Proc GLM, SAS). Specific contrasts for between factors were carried out using least squares means (LS Means) and using linear contrasts for within or repeated measures factors. The associations between parameters were assessed by correlation analysis (Proc Corr, SAS), and linear regression analysis (Microsoft Excel-4). Tables and figures will be found at the end of each chapter.

TABLES AND FIGURES FOR CHAPTER 2

TABLE 2.1 Design of ontogenic study of placental development in well-fed ewes carrying singletons

STRAIN OF SHEEP	GESTATIONAL AGE (days)	NUMBER OF EWES STUDIED
Border-Leicester × Merino	30	6
	45	2
	60	2
	75	1
	90	9
	120	6
Australian Merino	95	4
	130	9
	140	9

(Data for Border-Leicester x Merino sheep between days 30 and 75 of gestation were provided by Ms. T. De Barro, Department of Obstetrics and Gynaecology, University of Adelaide).

TABLE 2.2 Design of study of effects of experimental restriction of implantation on placental development in well-fed ewes carrying singletons

Gestational age	NUMBER OF EWES STUDIED	
	Control	Restricted
90 days	9	4
120 days	6	11

TABLE 2.3 Design of study of effect of multiple pregnancy on placental development in well-fed Border-Leicester Merino Ewes at 90 days gestation

Fetus Number	NUMBER OF EWES STUDIED	
	Control	Restricted
1	9	4
2	6	2
3	6	8

TABLE 2.4 Design of study to determine effect of maternal undernutrition on placental response, by 90 days gestation, to experimental restriction of implantation

LEVEL OF MATERNAL		NUMBER OF EWES
NUTRITION	TREATMENT	STUDIED
Underfed	Control	6
Underfed	Restricted	4
Well-fed	Control	9
Well-fed	Restricted	4

TABLE 2.5 Fixation regimens of placentomes from ewes at different gestation ages

SHEEP NUMBER	GESTATION (days)	TREATMENT (R/C)	PM DATE	COTYLEDON NUMBER	FIXATIVE USED
246	75	R	14.6.90	1a-4a	F
				1b-4b	B _f
				1a	F+B _f
250	75	C	14.6.90	1a-4a	F
				1b-4b	B _f
				1a	F+B _f
245	90	R	4.6.90	1a-4a	B _f
				1b-4b	B _f
				5a	F+B _f
90	90	C	12.6.90	1a-4a	F
				1b-4b	B _f
93	90	C	18.6.90	1a-4a	F
				1b-4b	B _f
220	120	R	12.8.89	2a	F
				2b	F+B _f
203	120	R	3.4.90	3	F
				2	F+B _f
92	120	C	20.6.90	2a, 3a	F
				2b, 3b	B _f
248	120	C	10.10.89	3a	F
				3b	F+B _f

Data is expressed as sheep number, gestational age, treatment (C= control or R= restricted), date of postmortem (PM), placentome number (cotyledon number) and fixative used (F= formalin, B_f= Bouin's, F+B_f= pre-fixed in formalin and post-fixed in Bouin's).

TABLE 2.6 Placentome volumes before and after fixation

SHEEP NUMBER	GESTATIONAL AGE (days)	PLACENTOME NUMBER	VOLUME AT PM (ml)	VOLUME POST- FIXATION (ml)
279	90	1	16	15
		2	4	6
		3	10	10
		4	28	27
		mean $\pm$ SE	14.5 $\pm$ 5.1	14.5 $\pm$ 4.6
237	120	1	30	30
		2	22	21
		3	22	21
		4	32	27
91	120	1	10	10
		2	7	6
		3	7	8
		4	6	6
233	120	1	18	18
		2	10	10
		3	16	16
		4	16	16
		mean $\pm$ SE	16.3 $\pm$ 2.6	15.8 $\pm$ 2.3

Values are placentome volumes at post-mortem and post-fixation for individual placentomes, and means and standard errors (SE) of the volumes for all placentomes selected at each gestational age.

TABLE 2.7 List of morphometric symbols (p6; Weibel; 1979)

DEFINITION	SYMBOL	DIMENSION
<i>1. PARAMETERS OF STRUCTURE</i>		
Volume (of component or structure)	V	cm ³
Surface area (of component or structure)	S	cm ²
<i>2. PARAMETERS ON TEST LINES</i>		
Length (total) of profiles on test line	L	cm
Intersections with surface boundary	I	cm ⁰
<i>3. PARAMETERS ON TEST POINT SETS</i>		
Point number on profiles or structure	P	cm ⁰
<i>4. PARAMETERS OF TEST SETS</i>		
Test volume	V _T	cm ³
Test line length	L _T	cm
Test point number	P _T	cm ⁰
<i>5. DENSITIES IN SPACE</i>		
Volume density	V _V	cm ⁰
Surface density	S _V	cm ⁻¹
<i>7. OTHER PARAMETERS</i>		
Arithmetic mean barrier thickness	τ	cm

Table 2.8 Placentome components

COMPONENT

NUMBER	NAME	ABBREVIATION
1	Chorionic membrane (excluding epithelial layer)	CM
2	Chorionic epithelium	CEP
3	Haemophagous zone	HMZ
4	Maternal tissue in haemophagous zone	MT
5	Fetal villi including T and CT	FV
6	Epithelial trophoctoderm bordering fetal villi	T
7	Connective tissue core of fetal villi	CT
8	Maternal crypts including FMS	MC
9	Feto-Maternal syncytium	FMS
10	Placentome capsule	PC
11	Shrinkage space	SkG

TABLE 2.9. Optimisation of sample size for estimating volume densities of components at 90 and 120 days gestation, with precisions of 10% or 5% relative standard errors, using L-36 Merz lattice

(a)

OPTIMUM MAGNIFICATION 400 x	GESTATIONAL AGE 90 DAYS ROUGH ESTIMATE OF V_v	REQUIRED NUMBER OF TEST POINTS	REQUIRED NUMBER OF FIELDS			
			<i>Relative</i>		<i>standard</i>	
			<i>errors</i>	<i>errors</i>	<i>errors</i>	<i>errors</i>
			10%	5%	10%	5%
<i>PLACENTOME COMPONENT</i>						
Fetal villi	0.210 ± 0.002	410	1700	11	47	
Trophectoderm	0.093 ± 0.014	1100	4200	31	117	
Connective tissue core of fetal villi	0.097 ± 0.011	1200	4000	33	111	
Maternal crypts	0.180 ± 0.011	450	1950	13	54	
Feto-maternal syncytium*	0.074 ± 0.004	1375	5500	38	153	

(*based on subsequent analyses)

(b)

GESTATIONAL AGE 120 DAYS

OPTIMUM MAGNIFICATION 400 x	ROUGH ESTIMATE OF V _v	REQUIRED		REQUIRED	
		NUMBER OF		NUMBER OF	
		TEST POINTS		FIELDS	
		<i>Relative</i>	<i>standard</i>	<i>errors</i>	
		10%	5%	10%	5%
<i>PLACENTOME COMPONENT</i>					
Fetal villi	0.236 ± 0.025	360	1400	10	39
Trophectoderm	0.163 ± 0.036	560	2300	16	64
Connective tissue core of fetal villi	0.063 ± 0.006	1650	7000	46	194
Maternal crypts	0.185 ± 0.016	500	1800	14	50
Feto-maternal syncytium*	0.086 ± 0.005	1100	4250	31	118

(*based on subsequent analyses)

TABLE 2.10 Comparison of trophoctoderm and maternal crypt surface densities and arithmetic mean barrier thicknesses in control and restricted placentas of well-fed singletons at 120 days gestation

TREATMENT:	CONTROL (N=6)		RESTRICTED (N=11)	
<i>PLACENTOME</i>				
Size	<i>"Average-size"</i>	<i>"Large"</i>	<i>"Average-size"</i>	<i>"Large"</i>
Weight (g)	7.4 $\pm$ 0.2	14.8 $\pm$ 0.4	7.6 $\pm$ 0.4	21.4 $\pm$ 1.4
	(n=6)	(n=6)	(n=8)	(n=11)
<i>PARAMETER</i>				
Surface density of trophoctoderm (cm ⁻¹)	170 $\pm$ 8	173 $\pm$ 6	214 $\pm$ 16	219 $\pm$ 7
Surface density of feto-maternal syncytium (cm ⁻¹)	175 $\pm$ 8	174 $\pm$ 6	222 $\pm$ 11	215 $\pm$ 6
ANOVA	ns	ns	ns	ns

Values are expressed as mean $\pm$ SE; N= Number of ewes studied, n= Number of placentomes morphometrically analysed, ANOVA; LSM means specific comparisons; ns=no significant difference

TABLE 2.11 Volume Densities of Components Determined in Sections Cut at Various Planes

		COMPONENT ANALYSED									
PLANE OF PLACENTOME											
SECTIONING		CM	CEP	HMZ	MT	FV	T	CT	MC	PC	SkG
Vertical sections COT 1 (n=5)		0.077	0.035	0.046	0.005	0.444	0.213	0.092	0.26	0.034	0.08
Random sections COT 1 (n=6)		0.084	0.042	0.05	0.004	0.399	0.21	0.092	0.257	0.03	0.094
% difference $\frac{(R-V)}{R} \times 100$		8.33	17	8	-25	-11.28	-1.43	0	-1.17	-13.33	15
Vertical sections COT 2 (n=6)		0.09	0.04	0.02	0.02	0.42	0.22	0.09	0.28	0.05	0.09
Horizontal sections COT 2 (n=8)		0.07	0.03	0.05	0.01	0.44	0.24	0.11	0.26	0.06	0.08
% difference $\frac{(H-V)}{H} \times 100$		-28.57	-33.33	60	-100.	4.55	8.33	18.18	-7.69	16.67	-12.5

Values are volume densities of placentome components estimated from sections cut in various planes of sectioning (vertical and random sections for placentome 1; COT 1, and vertical and horizontal sections for placentome 2; COT 2) and the % difference of the volume densities for each of the components estimated from sections cut in different planes for each placentome.

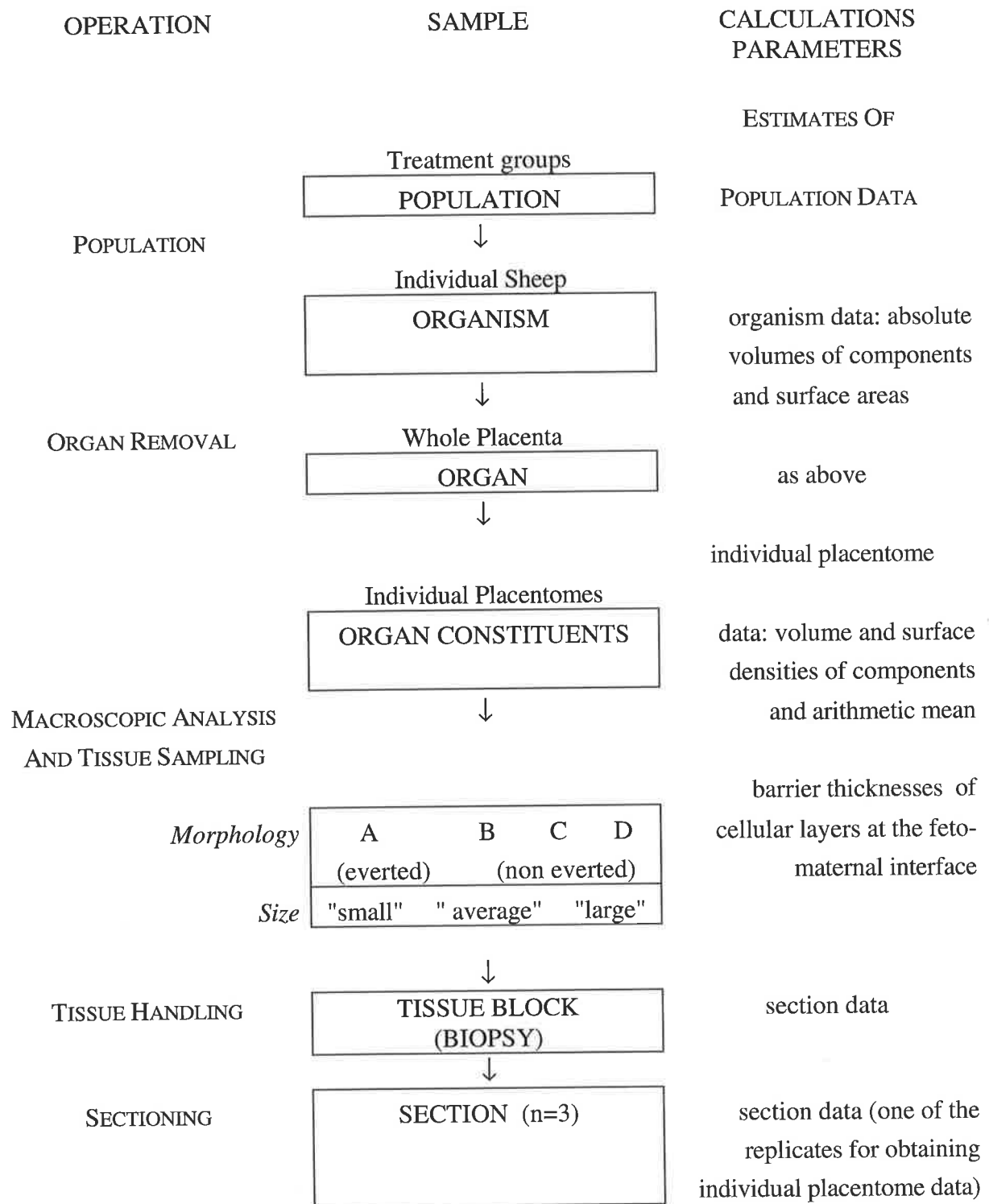


Figure 2.1 The operational sampling hierarchy used in this study (adapted from Weibel, 1979)

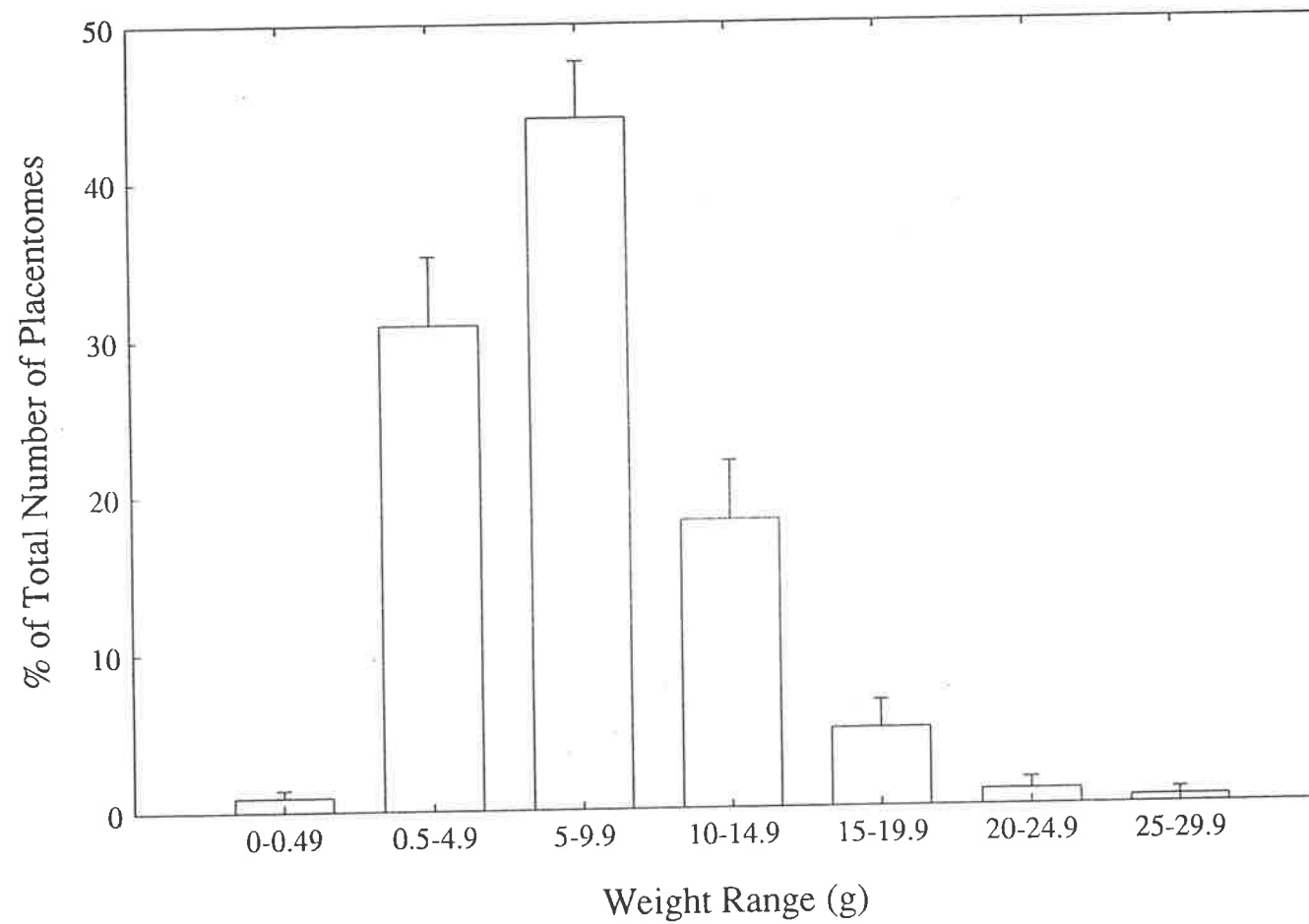


Figure 2.2 The distribution of placentomes in different weight range categories in Border-Leicester X Merino control ewes (N=6) at 120 days gestation.

Figure 2.3 Excessive shrinkage in placental tissue at 90 days gestation due to post-fixation in Bouin's fluid. Fetal villi (FV), Maternal crypts (MC). (H&E; $\times 200$).

Figure 2.4 Moderate shrinkage in placental tissue at 120 days gestation due to post-fixation in Bouin's fluid (H&E; $\times 200$).

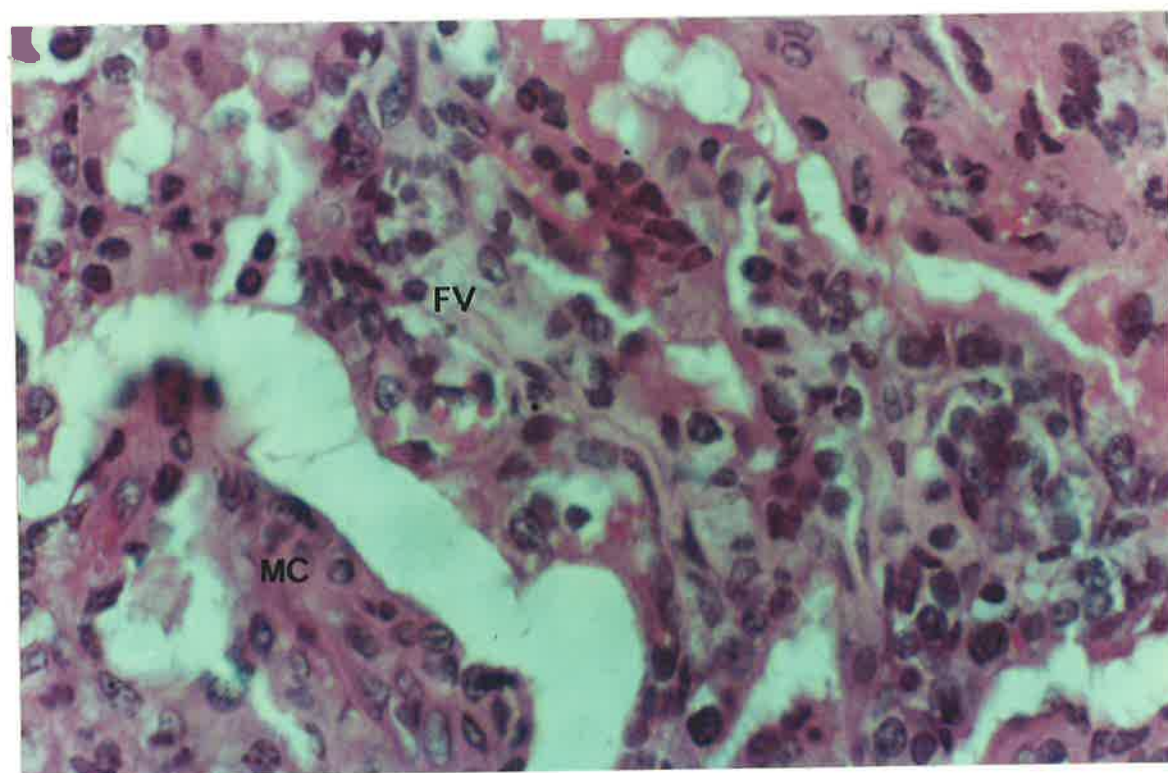
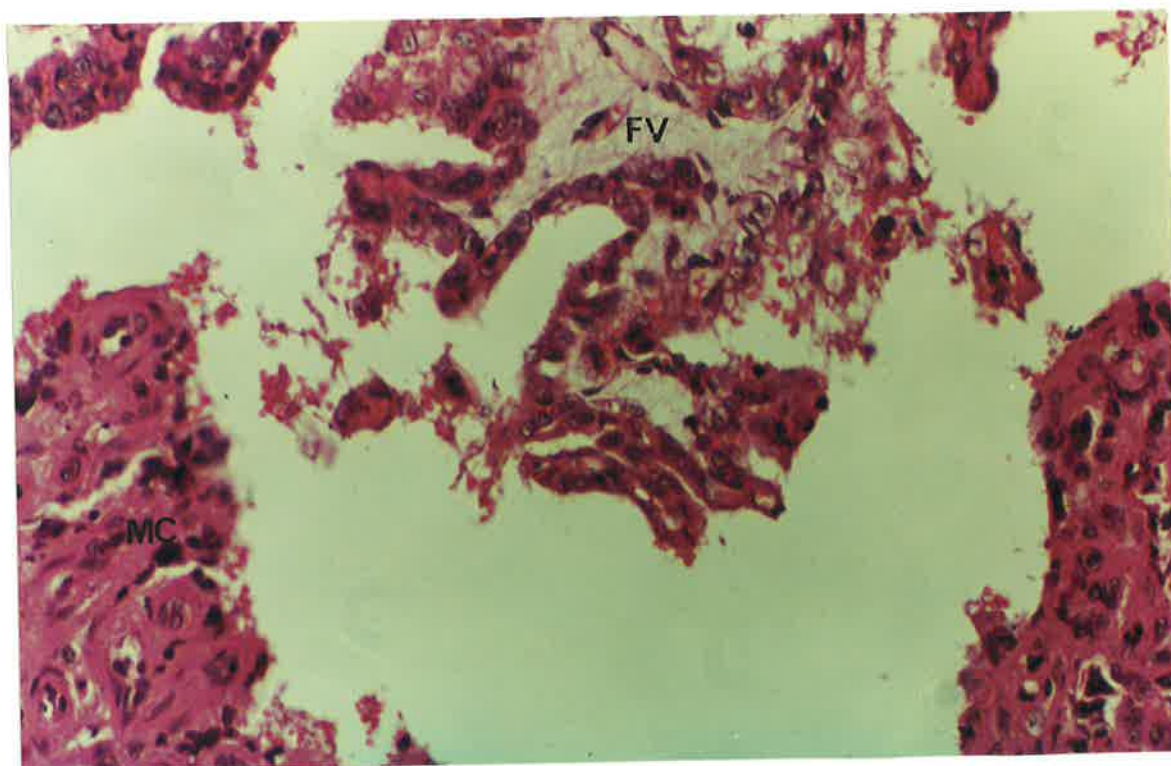


Figure 2.5 A section of a placentome at 90 days gestation showing fetal villi (FV) mostly occupied by mesenchyme connective tissue (CT). (H&E; $\times 200$) (Fixative: 10% neutral buffered formalin).

Figure 2.6 A section from a placentome at 120 days gestation illustrating the apparent similarity between fetal villi (FV) and maternal crypts (MC) following the marked loss of mesenchyme connective tissue from the chore of the FV. (H&E; $\times 100$) (Fixative: 10% neutral buffered formalin).

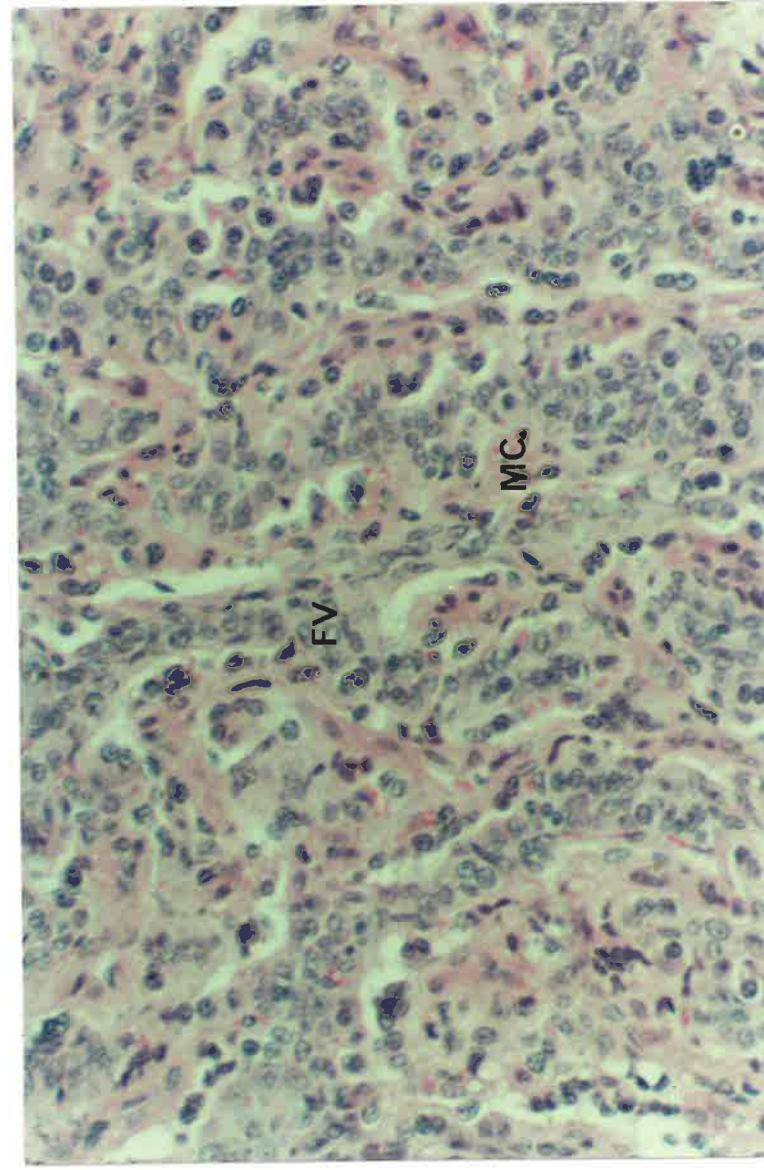
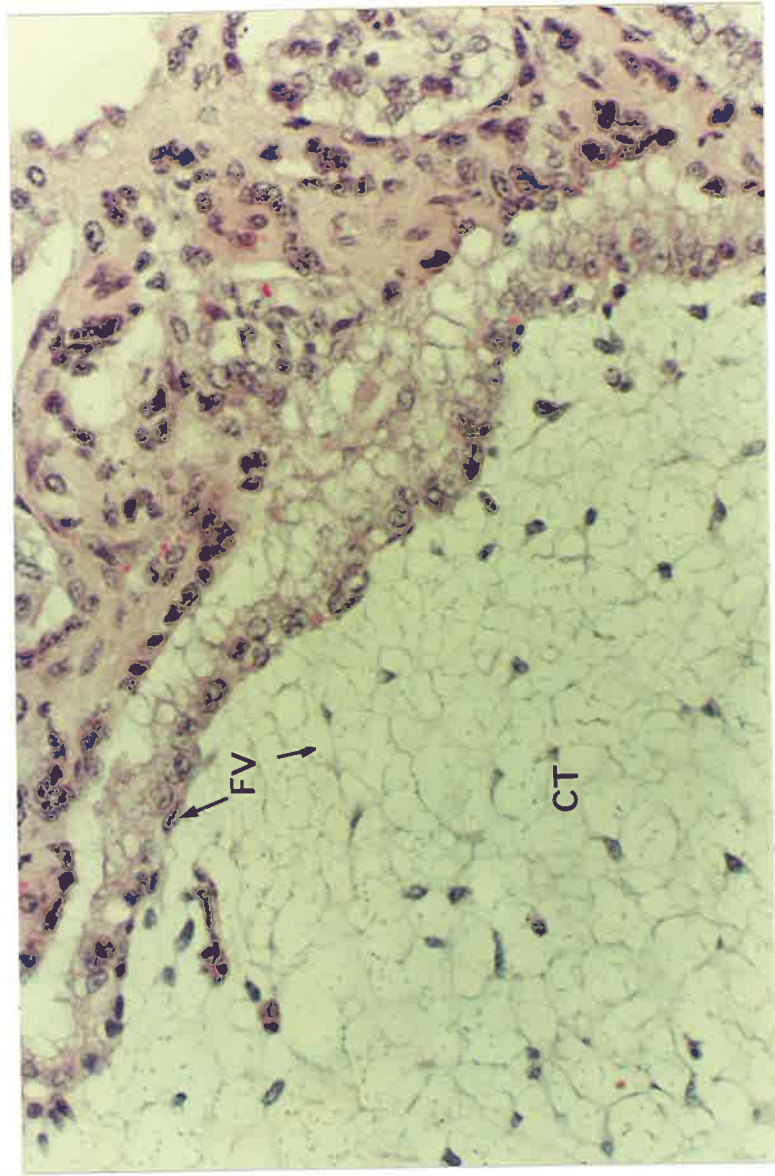
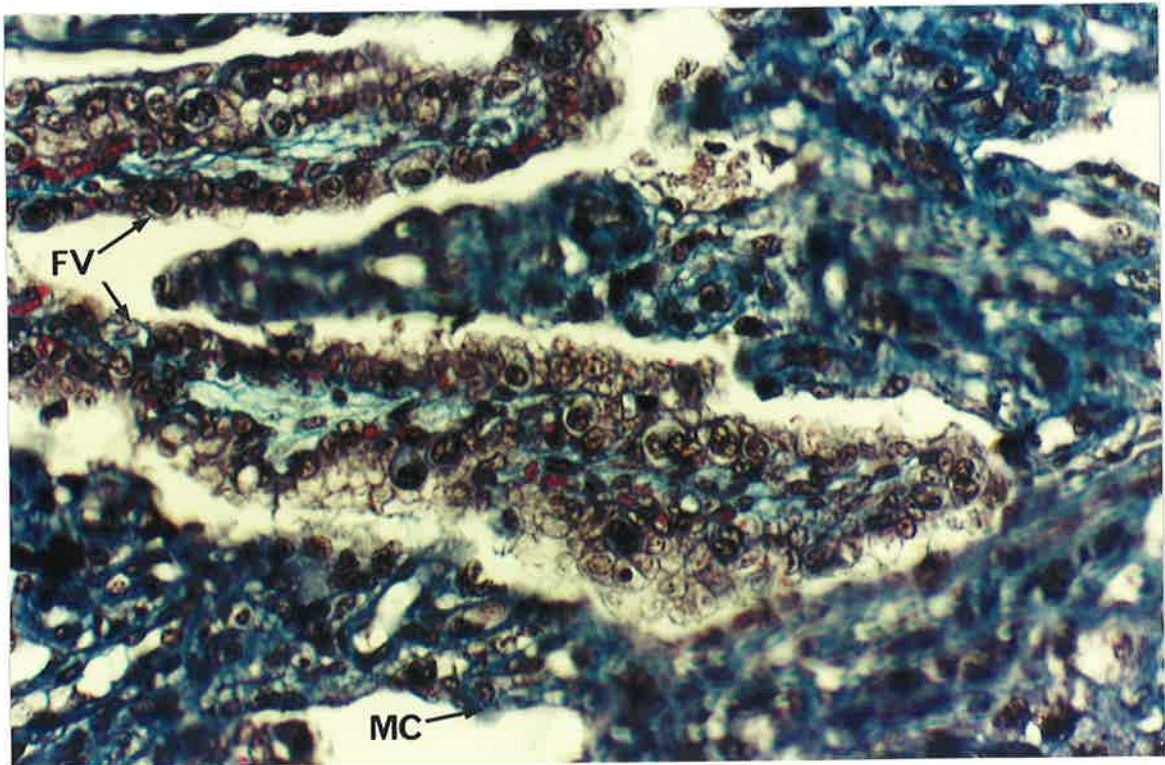
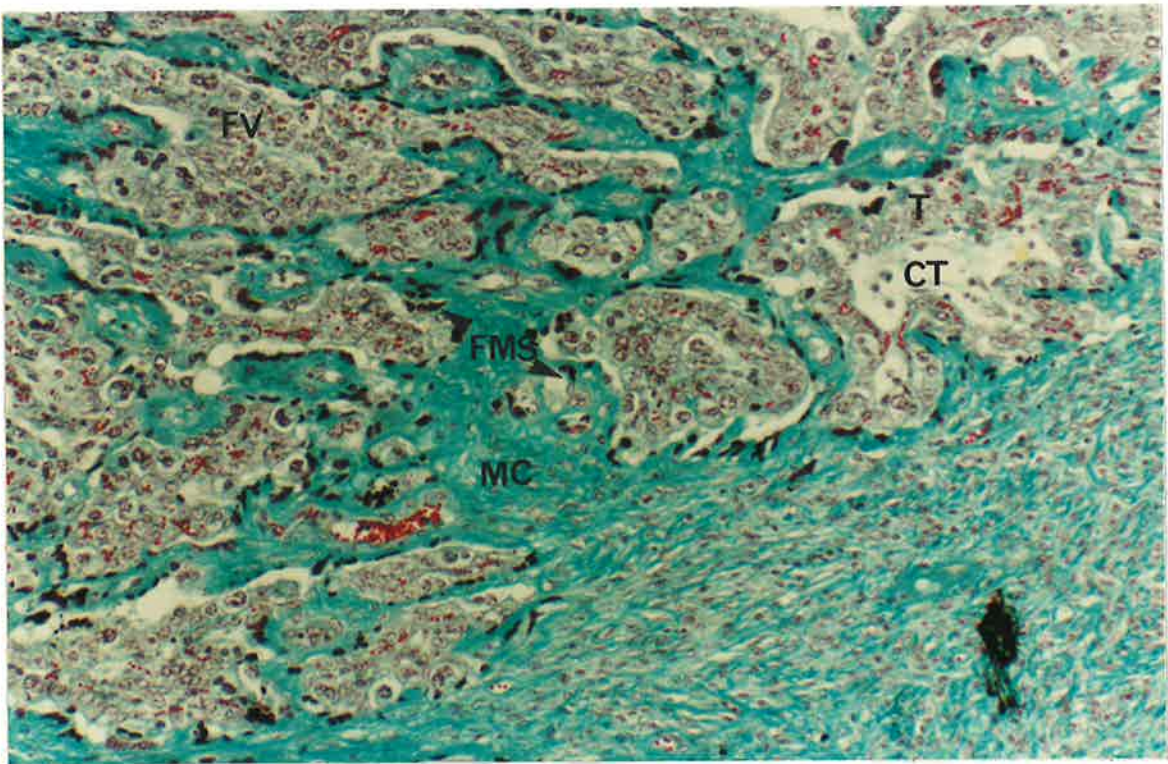


Figure 2.7 A section from a placentome at 120 days gestation illustrating differential staining of fetal (FV) and maternal crypts (MC) with Masson Trichrome counterstained with light green. Making up the FV is mesenchyme connective tissue (CT) at the core, which is bordered by trophoderm (T). Binucleate cells are occasionally observed in fetal trophoderm. Blood vessels containing erythrocytes are also clearly visible within fetal villi and maternal crypts. In addition, nuclei (stained black) belonging to the feto-maternal syncytium (FMS) lining the maternal crypts can be seen. (Fixative: 10% neutral buffered formalin; $\times 100$).

Figure 2.8 A section from a placentome at 90 days gestation illustrating differential staining of fetal villi (FV) and maternal crypts (MC) with Masson Trichrome counterstained with alanine blue. The components of the FV can readily be seen, but the FMS lining the MC is not distinguishable. (Fixative: Bouin's fluid; $\times 200$)



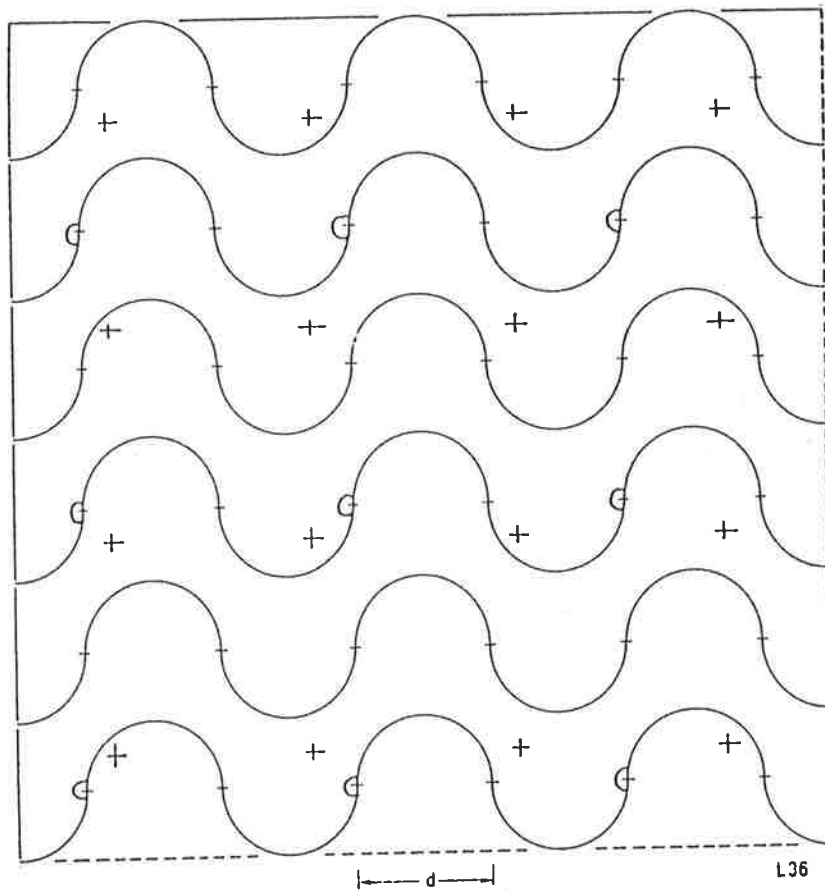
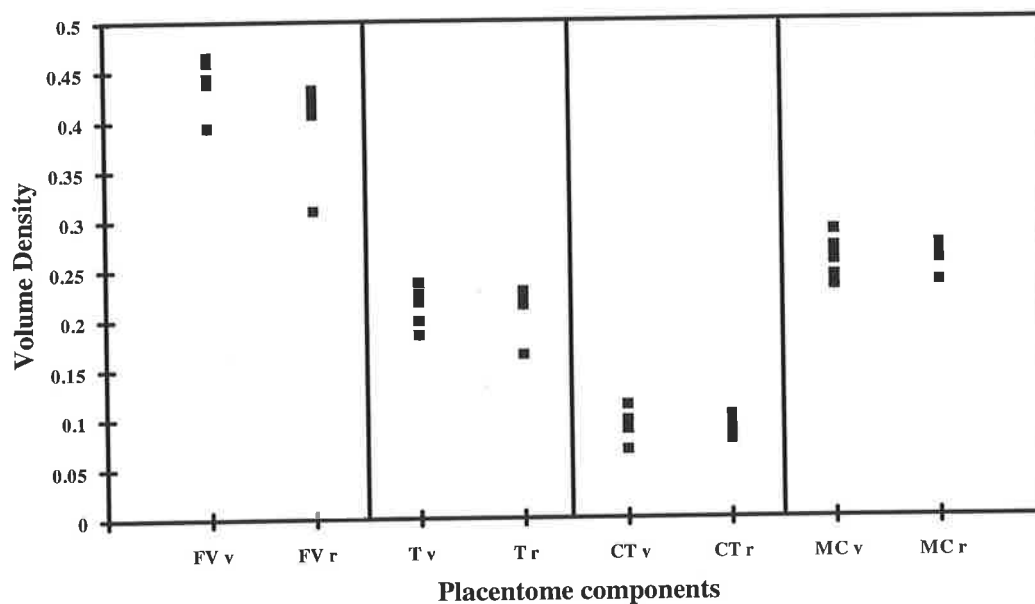


Figure 2.9 A modified test lattice used in the present work. The lattice contains two sets of test points (P_T). The volume densities of most placentome components were analysed using $n=36$ test points, designated as (-; 1.9cm apart). The lattice was modified to include $n=16$ test points, designated as (+; 2.7cm apart), and used to analyse the volume densities of fetal villi, trophoctoderm, maternal crypts and feto-maternal syncytium. The test lattice also contains 6 semi-circular test lines (total line length=107.44cm) which were used to measure surface densities of the epithelial cells by intersection counting (After Merz, 1967; cited in Weibel, 1979).

(a) Placentome 1



(b) Placentome 2

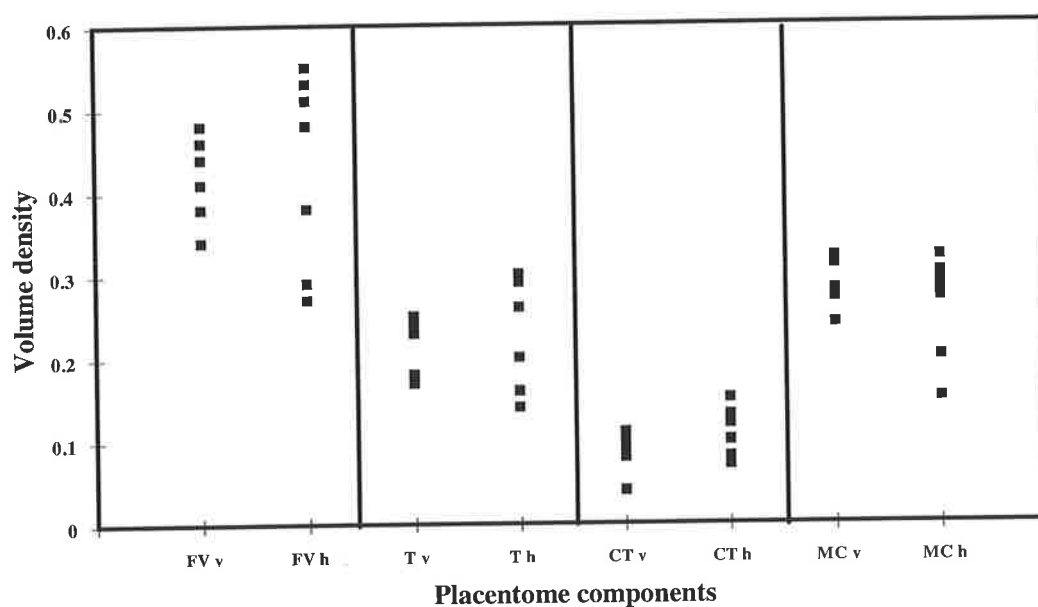


Figure 2.10 Comparison of volume density of placentome components estimated from (a) vertical and random sections and (b) vertical and horizontal sections. Components are fetal villi (FV), trophoctoderm (T), connective tissue core of fetal villi (CT) and maternal crypts (MC). The planes of sectioning are vertical (v), horizontal (h) and random (r). (* $p=0.03$: t-test: paired two sample for means)

APPENDICES FOR CHAPTER 2

Appendix 1

Procedure for Processing Tissue Blocks From "Large" Placentomes for Light Microscopy

Solution	Duration (Hours)
1. 70% alcohol	8
2. 80% alcohol	5
3. 90% alcohol	5
4. 100% alcohol	5
5. 100% alcohol	5
6. 100% alcohol	5
7. 100% alcohol	3
8. 100% alcohol	3
9. xylene	5
10. xylene	4
11. wax	3
12. wax	3
13. wax	3

Appendix 2

Procedure for Processing Tissue Blocks from "Small" and "average" Placentomes for Light Microscopy

Solution	Duration (Hours)
1. 50% alcohol	7
2. 70% alcohol	3
3. 80% alcohol	3
4. 100% alcohol	3
5. Repeat 4 (four times)	
6. Xylene	3
7. Xylene	3
8. Wax	3
9. Wax	3

Appendix 3a: **Fixatives for Light Microscopy Work**

1. Bouin's fluid.

Picric acid (saturated aqueous solution) 75ml

Formalin (40% formaldehyde) 25ml

Glacial acetic acid 5ml

2. 10% Neutral Buffered Formalin

This was either supplied already made up by Delta West Limited, Western Australia or prepared as follows:

- a) Prepare phosphate buffer by dissolving 8.952gms $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ and 3.904gms $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ to make up a litre of solution.
- b) Adjust pH to 7.2 with orthophosphoric acid solution.
- c) To make 10% formalin solution: to 900mls of buffer add 100mls formalin (40% formaldehyde, ACE Chemical Company).

Appendix 3b: **Mounting Medium**

Composition of PIX.

Quantity	Chemical
200gms	Lustrex PIX
100mls	dibutyl phthalate
700mls	xylol (histoclear)

CHAPTER 3

ONTOGENY OF OVINE PLACENTAL

STRUCTURE AND FUNCTION

3.1 INTRODUCTION

Placental growth and development following implantation in sheep has been described quantitatively in terms of macroscopic parameters, such as total weight and physical dimensions of individual placentomes (refer Chapter 1). While the variations that occur in gross morphology and in size of individual placentomes have long been recognised, they have been mostly described in anecdotal terms, as have their possible origins (Cloete, 1939, Alexander 1964a). Structural remodelling of the parenchyma within the ovine placenta throughout gestation has also been described extensively, both qualitatively and semi-quantitatively (Barcroft and Barron, 1946; Björkman, 1965; Stegeman, 1974; Steven *et al.*, 1976, 1981; Mitchell, 1982). However, these studies have examined few animals and the most extensive investigation was largely of twin pregnancies, in which gestational age was determined from fetal weight, crown-rump length, and external dimensions (Stegeman, 1974). While Mitchell (1982) studied qualitative and quantitative aspects of ovine placental development, only a few animals ($n=1$ to 3) were analysed at different stages in late gestation. In addition, Stegeman (1974) reported only relative parameters of placental structure, which can lead to confusion if the reference space or mean volume of the organ under study varies between the different states being compared, as indeed occurs for placental weight and volume during pregnancy (Mayhew, 1991). The ontogeny of a number of major structural determinants of placental transfer function, such as the abundance, surface area and barrier thicknesses of the epithelial cell layers responsible for exchange in the ovine placenta, has not been described previously.

Therefore, the aim of the study described in this chapter was to more fully characterise the morphological and structural development of the ovine placenta between mid and late gestation. In particular, it is over this period of gestation, that the placental weight or mass plateaus and further development involves extensive internal remodelling, and that fetal weight normally begins to correlate with placental weight. In addition, the stage chosen for study in late gestation was at 120 days (term ~150 days), prior to the onset of activation of the

fetal hypothalamo-pituitary-adrenal (HPA) axis (Flint *et al.*, 1975, McMillen *et al.*, 1987). The fetal HPA axis may influence placental development in the last two weeks of pregnancy via the actions of circulating cortisol on fetal trophoblast. Binucleate cell numbers normally decrease in the last week of pregnancy (Wooding, 1983), when fetal cortisol levels are high (Flint *et al.*, 1975, McMillen *et al.*, 1987, McIsaac *et al.*, 1989). This phenomenon can be induced experimentally by dexamethasone treatment (Wooding, 1983).

Specifically, the ontogeny of macroscopic parameters of placentome size and morphology and of microscopic parameters of structure was characterised. The relationship between macroscopic and microscopic parameters of placental development was examined and in particular, the structural basis of placentome eversion was investigated. In addition, the relationship of fetal growth to various structural parameters of placental growth and development was evaluated to determine which appeared to be most critical in limiting or determining fetal growth. The extent to which structural determinants of placental function correlate with more readily obtainable indices of placental growth and development, such as total placental weight, was also assessed.

3.2 MATERIALS AND METHODS

The experimental design and materials and methods used have been described in detail in Chapter 2. Briefly, macroscopic parameters of normal placental growth and development were studied in singleton-bearing Border Leicester $\times$ Merino and Australian Merino ewes between 30 and 140 days gestation (Table 2.1). Microscopic parameters of placental growth and development were measured in the Border Leicester $\times$ Merino cross bred ewes at 90 (N=9) and 120 (N=6) days gestation. All ewes were well fed and their placentas were collected, processed and analysed morphometrically as previously described (Chapter 2).

3.3 RESULTS

3.3.1 Ontogeny of Macroscopic Placental Growth and Development from mid to late gestation

Placental Weight

Placental weight reached a maximum at about 75 days gestation (Figure 3.1). Placental weight plateaued between day 75 and 130, then decreased by 26% ($p < 0.05$) at day 140. It should be noted that observations at 95, 130 and 140 days were made in Australian Merino ewes and the remainder in Border-Leicester $\times$ Merino crossbred ewes. However, when studied at the same gestational age (90 to 95 days gestation), placental weight was not significantly different between the two breeds.

Numbers of Placentomes and Mean Placentome Weights

Placentome numbers and mean placentome weight did not change between 90 and 140 days gestation (Figure 3.2, Table 3.1).

Placentome Weight Distribution

Between mid and late gestation, placentome weights ranged from 0.1g to 40g. Between 90 and 140 days, the majority of placentomes (between ~67 at 90 days and ~85% at 140 days) were distributed equally in the 0.5 - 4.9g weight range and the 5 - 9.9g weight range (Figure 3.3). In contrast, the proportion of placentomes in each of the other five weight range categories was significantly less than that in the 5 - 9.9g weight range ($p < 0.05$) (Figure 3.3).

The proportion of placentomes in the 5-9.9g weight range appeared to increase and that in the higher weight range categories to decrease (Figure 3.3) with increasing gestational age.

When placentomes weighing 10g or more (classified as "large") were included in an extended upper weight range of 10 - 40g, an apparent shift in placentomes from the upper weight range to the 5 - 9.9g or "average" weight range with increasing gestational age could be seen (Figure 3.4).

Gross Placentome Morphology

In mid gestation, about 10% of all placentomes had undergone eversion, where the fetal component of the placentome appears to have grown to surround the maternal component, rather than vice versa (Figure 1.5, Figure 3.5). However, by 130 days about 80% of placentomes were everted and by 140 days almost all placentomes had everted (Figure 3.5; Table 3.1). Similar estimates of the proportion of the placenta that had everted, were obtained whether calculated using the numbers of placentomes that were everted or using the total weight of all everted placentomes (Table 3.1).

Incidence of Eversion in Different Placentome Weight Ranges

Between 90 and 140 days, everted placentomes were equally distributed across the various placentome weight ranges, from 0.5 to 25g, with the exception of 130 days gestation (Figure 3.6). At 130 days, everted placentomes appeared in all but one higher weight range category (30 - 34.9g). While the incidence of everted placentomes appeared to be lower at the extremes of the placentome weight range, this did not reach statistical significance (Figure 3.6).

Variations in Placentome Morphology from Mid to Late Gestation.

The majority of everted placentomes (60-80%) were of the B morphology between mid and late gestation (Figure 3.7). The remaining of everted placentomes were of the C or D morphologies. There was no evidence of an ontogenic progression from B through C to the D type morphology. While the proportion of C placentomes appeared to increase, that of D placentomes decreased with increasing gestational age but did not reach statistical significance (Figure 3.7).

3.3.2 Ontogeny of placental cellular and structural development between 90 and 120 days gestation

Volume Density of Placentome Components

A placentome of "average" size at mid-gestation consisted of about 50% fetal tissue (chorionic membrane and its epithelial cell layer: 14%; fetal villi: 36%) and 38% maternal tissue (maternal crypts: 28%; placentome capsule: 8%; maternal tissue within haemophagous zone:

2%) (Table 3.2). The haemophagous zone occupied 7% and shrinkage spaces, 4%, of the placentome volume. At 120 days gestation, the placentomes of "average" size were of similar composition to those of 90 day gestation placentomes: 52% being comprised of fetal tissue and 36% of maternal tissue (Table 3.2). The haemophagous zone and shrinkage spaces also accounted for similar proportions, 6% and 3% of the placentome volume respectively, in late gestation.

There was no significant effect of placentome size on the volume densities of any components at mid gestation (Table 3.2). However, at 120 days gestation, "average-size" placentomes had significantly smaller volume densities of fetal villi (-10%) and trophoctoderm (-10%) than did "large" placentomes (Table 3.2). In contrast, placentome morphology did influence placentome composition in that eversion was characterised by an increased volume density of trophoctoderm (Table 3.3; Figure 3.8). At 90 days gestation, only one everted placentome was analysed morphometrically, and had a higher trophoctoderm volume density (+61%) than that of the non-everted placentomes (Table 3.3). At 120 days gestation, everted placentomes also had significantly more trophoctoderm (+33%; $p=0.02$) than non-everted placentomes at mid-gestation (Table 3.3).

Between 90 and 120 days gestation, the volume density of chorionic epithelium (+77%; $p = 0.0008$) and trophoctoderm (+44%; $p = 0.002$) increased while that of the connective tissue core of the fetal villi decreased (-43%; $p = 0.0004$) (Table 3.4 and Figure 3.9). Ontogenic changes in the volume densities of other components within placentomes were also observed (Table 3.4, Figure 3.9).

Surface Density and Arithmetic Mean Barrier Thicknesses of Trophoctoderm and Feto-Maternal Syncytium

There was no significant effect of either placentome size or morphology on surface density or on arithmetic mean barrier thickness of the fetal trophoctoderm or the feto-maternal syncytium at either gestational age (Table 3.5).

The surface density of trophoctoderm increased significantly between 90 and 120 days gestation (+ 60%; $p=0.0001$) (Table 3.6 and Figure 3.10). Conversely, the arithmetic mean barrier thicknesses of the fetal epithelial cell layers bordering fetal villi and maternal crypts decreased with increasing gestational age. The arithmetic mean barrier thickness of trophoctoderm decreased by 15%, from $16.4 \pm 0.6\mu\text{m}$ at 90 days to $14.3 \pm 1.7\mu\text{m}$ at 120 days ($p = 0.0001$) and that of feto-maternal syncytium by 36% from $8.5 \pm 0.5\mu\text{m}$ at 90 days to $5.5 \pm 0.5\mu\text{m}$ at 120 days ($p = 0.0002$) (Table 3.6 and Figure 3.11).

Total Placental Volumes of Components, Surface Area and Relative Surface Area at 90 and 120 days Gestation

The volumes of most placental components did not change between mid and late gestation. However, the volume of chorionic epithelium doubled by 120 days (+ 91%; $p = 0.003$), while that of trophoctoderm increased almost 1.5 times (+46%; $p = 0.02$) between mid and late gestation (Table 3.7 and Figure 3.12). These increases in trophoctoderm volume were paralleled by a 70% increase in placental surface area between 90 and 120 days gestation (from 5.9 ± 0.4 to $10.1 \pm 0.8\text{ m}^2$, $p=0.0003$) (Table 3.7 and Figure 3.13). However, since placental weight did not change while fetal weight increased significantly (3.7 times) between 90 and 120 days gestation (Figure 3.1 and Table 3.1), relative surface area of the placenta, (the surface area supplying a gram of fetal and placental tissue) decreased (-35%; $p = 0.0002$) (Table 3.7).

3.3.3 Relationship of structural determinants of placental to fetal growth

Fetal Weight and Fetal/Placental Weight Ratio

While placental weight plateaued then decreased between 90 and 140 days gestation, fetal weight increased 7-fold (Table 3.1). Consequently the ratio of fetal to placental weight increased substantially between mid and late gestation, by approximately 9-fold by 140 days.

Correlation of Fetal Weight and Placental Parameters at 90 and 120 days Gestation

At mid-gestation, fetal weight did not correlate significantly with placental weight ($r=-0.61$; $p=0.08$). However, fetal weight did correlate negatively with the volume of the connective

tissue core (including blood vessels) of the fetal villi at mid gestation ($r=-0.67$, $p=0.05$, $N=9$). Fetal weight did not correlate significantly with any other parameter of placental growth and development at this stage of gestation (Table 3.8).

By 120 days gestation, fetal weight did not correlate significantly with placental weight ($r=0.8$, $p=0.08$). However, fetal weight was positively correlated with the volume of trophoctoderm ($r = 0.82$, $p = 0.05$, $N=6$) and placental surface area ($r = 0.81$, $p = 0.05$, $N=6$) (Table 3.8).

Correlation of Placental Weight and Placental Functional Components

At both mid and late gestation, placental weight correlated positively with the volumes of two placental parameters *viz* fetal villi ($r = 0.85$, $p = 0.004$, $N=9$ at 90 days; $r = 0.86$, $p = 0.03$, $N=6$ at 120 days), the connective tissue core of fetal villi ($r = 0.83$, $p = 0.006$, $N=9$ at 90 days, $r = 0.89$, $p = 0.02$, $N=6$ at 120 days), and with placental surface area ($r = 0.70$, $p = 0.04$, $N=9$ at 90 days, $r = 0.92$, $p = 0.01$, $N=6$ at 120 days) (Table 3.9). Despite the fewer numbers of animals studied in late gestation, the association between placental weight and these structural parameters generally strengthened with increasing gestational age (Table 3.9).

3.3.4 Discussion

Ovine placental growth progressed rapidly from the time by which implantation is largely complete (day 30 of gestation) and maximum placental weight was attained by mid gestation (day 75). Placental weight then plateaued until day 130 of gestation, when a substantial drop in placental weight occurred between day 130 and 140 of gestation. Previous reports indicated that maximum placental weight is attained about day 90 and starts to decline after 100 days gestation (Cloete, 1939; Stegeman, 1974; Bell, 1984; Meschia, 1990). Other studies have previously shown that placental weight shows a decline towards term in sheep (Cloete, 1939; Barcroft and Barron, 1946; Alexander, 1964a; Davies and Wimsatt, 1966; Kulhanek *et al.*, 1974; Rosenfeld *et al.*, 1974; Stegeman, 1974; Bell, 1984; Harding *et al.*, 1985). However, more recent studies indicated that in well-fed ewes, maximal placental weight was achieved at about day 70 and remained unchanged until 100 days, then fell by 140 days (McCrabb *et al.*, 1990). Unfortunately, no data was presented for the period between 100 and

140 days in that study. The current study confirms and extends these findings, suggesting that the decline in placental weight is coincident with the onset of the activation of the fetal HPA-axis. Placental weight also decreases towards term in mice (McLaren, 1965). In contrast, in rats placental weight continues to increase until a day before or at term (Winick and Noble, 1966; Rosso, 1975). Studies in rhesus monkeys indicate that placental weight plateaus at day 145 (term ~167) (Novy *et al.*, 1981). Other studies indicate that placental weight in rhesus monkeys (Kerr *et al.*, 1974) and in humans (McKeown and Record, 1953; Molteni *et al.*, 1978; Teasedale, 1980) continues to increase until term, with a slowing down of growth in the final weeks of pregnancy. Thus in general, a slowing in the rate of increase or a decrease in placental weight in late gestation appears common to many species

The ontogeny of placental growth in terms of size as indicated by placentome dimensions, has been explored previously in both longitudinal and cross-sectional studies. Cloete (1939) found that placentome diameter and weight were maximal by the third and fourth month of gestation respectively. Ultrasound monitoring of placentome diameter suggested that it was maximal between day 70 and day 80, then declined thereafter (Kelly *et al.*, 1987).

Placentome diameter and weight were correlated significantly at mid and late gestation ($r=0.92 \pm 0.01$ at 94 days and $r=0.83 \pm 0.03$ at 141 days of gestation) (Kelly *et al.*, 1987). However, the lines of best fit describing this relationship had significantly different slopes and constants between mid and late gestation. This suggested that ontogenic changes in placentome weight are not accompanied by exactly similar changes in placentome diameter (Kelly *et al.*, 1987).

Placentome numbers and mean placentome weights did not alter between mid and late gestation. Rather, the decrease in placental weight between day 130 and 140 of gestation appeared to be due to parallel trends to reduced placentome number and weight at the later age.

Between mid and late gestation, most placentomes were within the 5-9.9g range and the number and proportion of placentomes present within this range increased as gestation progressed. This is consistent with reports that most placentomes fell into the 5-9.9g range in

ewes studied between 123 to 144 days (Robinson *et al.*, 1979). The parallel reduction in the proportion and number of placentomes in higher weight ranges suggest that these lose mass and shift from higher to lower weight range categories with increasing gestation. Although growth or increase in the mass of placentomes in the lowest weight range may also occur, their contribution to the redistribution of placentomes between weight ranges would be limited due to their low numbers.

The nature of factors influencing the ontogenic variations of placentome size and morphology has not been fully characterised and yet these should determine placental size and function. In sheep, placentome size has been noted previously to vary with location in the uterine horn, with larger placentomes located in the centre of the uterine horn closest to the site of implantation (Cloete, 1939) and smaller ones towards the tips of the horn in placenta of the cow (Andersen, 1926) and sheep (Cloete, 1939). Although this pattern of distribution of placentome size appears to be similar in both pregnant and non-pregnant horns, other observations have suggested slower development of placentomes in the non-pregnant horn compared to the pregnant horn (Cloete, 1939). Delayed increases in the microvascular volume of deep caruncular tissues in the non-pregnant compared to pregnant horn have also been reported (Reynolds and Redmer, 1992). Consistent with these observations, uterine blood flow in the non-pregnant horn is lower than that of the pregnant horn throughout gestation, which may cause or reflect slower placentome development in the former (Greiss and Anderson, 1970; Reynolds *et al.*, 1984). Rankin *et al.*, (1970) demonstrated a higher ratio of maternal to fetal blood flow within the placentomes located towards the tips of the horn which are generally smaller than those in the middle of the horn, where a higher umbilical than maternal blood flow was found in late gestation. It is possible that placentome location within the uterus may determine the size of the placentome and the rate and timing of eversion. Placentome size and gross external morphology may be linked to regional variations in the ratios of maternal to umbilical blood flows and hence the delivery and clearance of substrates as well as hormonal substances such as progesterone and glucocorticoids to specific areas within the placental mass.

Between mid gestation and early in late gestation (day 120), a minor proportion of placentomes (10 to 24%) were everted. Between 120 and 130 days, this proportion had risen to over 80% and at 140 days, almost all placentomes were everted. This is consistent with previous observations that between 95 and 114 days gestation, 10% of all placentomes were everted, increasing to 57% at 137 days (Alexander, 1964a). Thus limited eversion occurs in early gestation, followed by a later period of rapid and more marked eversion (Alexander, 1964a, Cloete, 1939). This phenomenon coincides with the onset of activation of the fetal hypothalamic-pituitary adrenal axis (McMillen *et al.*, 1987). Everted placentomes have increased abundance of trophoblast suggesting that it is an earlier or primary target of this process. Recently, the treatment of pregnant ewes at 0.4 gestation (64 ± 0.4 days) with dexamethasone increased the incidence of placentome eversion 48 hours later (Wintour *et al.*, 1994). Thus there appears to be a role for increased levels of glucocorticoids in the mechanism of placentome eversion. However, it is unlikely that the reduction in progesterone levels and the rise in the levels of oestrogen which occur within the last week of pregnancy (Thorburn, 1972), following the activation of the fetal HPA axis, would have an impact on placentome eversion, which is largely complete by the time the changes in the levels of progesterone and oestrogen occur (Chapter 3).

A role for progesterone in influencing placentome eversion during the first half of gestation is suggested by several findings. When pregnant ewes were ovariectomised between 21 and 23 days after mating, and pregnancy maintained with different levels of progesterone, high doses of the hormone (0.45 to 1.35 mg/kg) resulted in more everted placentomes ($p=0.002$) at day 60 than the maintenance or lower than maintenance doses (0.15 mg/kg) (Alexander and Williams, 1966). Also, Rickets and Flint (1980) reported increased placentome eversion at days 70 and 114 in ovariectomised ewes in which pregnancy was maintained by a weekly treatment with medroxyprogesterone acetate. In addition, McNeil (1991) reported a strong positive correlation between the proportion of everted placentomes in a placenta in late gestation at day 145, and the number of corpora lutea. Ewes with two corpora lutea and hence higher levels of circulating progesterone in early gestation had more everted placentomes than those with one corpus luteum. Thus the extent of placentome eversion up until 120 days gestation may be largely determined by progesterone abundance from early pregnancy. The

coincidence in timing between the late onset and rapid completion of placentome eversion and the onset of activation of the fetal hypothalamo-pituitary adrenal axis suggest a role for fetal cortisol in this later process. Whether glucocorticoids of maternal or fetal origin in mid and late gestation influence placentome eversion directly, via the glucocorticoid receptor or by acting through the progesterone receptor warrants investigation.

The "average" size placentome is composed of about 50% fetal tissue and 36% maternal tissue; with most of the remainder accounted for by the haemophagous zone, 7%. This predominance of fetal tissue over maternal tissue agrees with an earlier morphometric study, which also found that there is more fetal tissue than maternal tissue in the ovine placentome in late gestation (Stegeman, 1974). In contrast, manual separation of placentomes into fetal and maternal components following delivery suggested that on average the placentome consists of 40% fetal tissue and 60% maternal tissue at term (Alexander, 1964a). However, fetal trophoctoderm tissue appears to remain attached to the feto-maternal syncytial surface of the maternal crypts following separation of the placenta following parturition, (Steven *et al.*, 1981). Thus analysis of ovine placental tissues following manual separation, in which tearing of tissues is likely, may underestimate the volume of fetal tissues and overestimate that of maternal tissues.

No ontogenic changes in the proportions of the placentome occupied by the total fetal and total maternal components were observed within the period between 90 and 120 days gestation. However, remodelling of their main constituent tissues occurred, such that by 120 days, placentomes had higher volume densities of fetal trophoctoderm bordering fetal villi and also higher volume densities of chorionic epithelium. In contrast, there was a significant reduction in the volume density of the connective tissue core (including blood vessels) of the fetal villi. Hence fetal villi became thinner as gestation progressed. This is consistent with earlier qualitative descriptions of Stegeman (1974), who also found a decrease of fetal mesenchymal tissue between 11 and 14 weeks of gestation. Teasedale (1976) also reported cellular changes in the ovine placenta consistent with reduced fetal mesenchymal tissue as gestation progressed. The total number of fetal mesenchyme cells (28×10^9) was highest at

82 days gestation, then declined continuously to about 8×10^9 at 143 days gestation (Teasedale, 1976).

While the volume density of trophoctoderm increased between mid and late gestation, its surface density increased even more so, resulting in a decrease in its arithmetic mean barrier thickness to about 86% of that at mid-gestation. Over the same period, the barrier thickness of the fetomaternal syncytium also decreased to about 68% of that at mid-gestation. These ontogenic events suggest that remodelling of the internal structure of placentomes occurs in such a manner so as to increase placentome functional efficiency: increasing the surface area of the two apposing epithelia on the fetomaternal interface and reducing the distance across which substrates are transported. The loss of connective tissue within fetal villi between mid and late gestation will further reduce the barrier to diffusion of substances between fetal and maternal blood by allowing fetal capillaries to be closer to trophoctoderm.

The increased abundance of chorionic epithelium and trophoctoderm between mid and late gestation approximately doubled their absolute volumes, despite no change in placental weight over this time. This increase in trophoctoderm may largely explain the ontogenic increases in placental metabolism between mid and late gestation with oxygen and glucose consumption increasing by 100 and 300% respectively (Bell, 1986). The total surface area of the exchange epithelia in the ovine placenta almost doubles between mid and late gestation, (from about 6m^2 to 10m^2).

Comparison of the ontogenic growth of surface area in sheep as determined in the present study, and that previously characterised in the cow (Baur, 1981) which also has a cotyledonary placenta, revealed that while the surface density of the ovine placenta increased from 116 to 185cm^{-1} (+59%), between 0.6 and 0.8 of gestation, the surface density of the bovine placenta decreased slightly (-5%) from 201 at 0.6 gestation (24 weeks) to 190cm^{-1} at 0.8 gestation (32 weeks) (Table 3.10). This suggests that placental development differs temporally between species. However, in the ovine placenta, total villous surface area increased by 70% (from about $5.9 \pm 0.4\text{m}^2$) compared to a 156% increase in the bovine placenta (from 25 to 53m^2) over this time. The massive difference in total villous surface area present between the ovine

and bovine placentas largely reflects the great difference in the absolute placental masses or sizes. The placental surface area supplying a unit weight of fetal and placental tissue (relative surface area) decreased by 35%, from 48 to 31 cm⁻¹ in the ovine placenta, between 0.6 and 0.8 gestation. In comparison, the relative surface area in the bovine placenta only decreased by 9%, from 35 to 32 cm⁻¹ (Table 3.10). Thus relative surface area decreases between mid and late gestation in the sheep and the cow to achieve comparable values, 31 cm⁻¹ and 32 cm⁻¹ respectively at 0.8 gestation.

Molina observed a 5-fold increase in glucose transport capacity between days 76 and 132. Surface area increased -70% between days 90 and 120 (present results). Despite discrepant time comparisons, this suggests a major change in density of glucose transporters, as observed by Ehrhardt *et al.*, 1994. In sheep, there is no increase in placental weight during the second half of gestation. Thus the increase in surface area, as demonstrated in this study, could largely account for the observed ontogenic increase in glucose transport capacity. Therefore, internal changes in placental structure between mid and late gestation may partly explain how fetal weight and hence demand can increase over threefold while placental weight does not change over this time; rather, the increase in fetal weight is more closely paralleled by the doubling of placental surface area over this period.

At mid gestation, fetal weight did not correlate with placental weight or with various structural parameters of placental development. By late gestation (120 days), fetal weight still did not correlate significantly with placental weight, but with the volume of trophoctoderm and also with placental surface area. This suggests that trophoctoderm and surface area are better indices of placental function than placental weight in late gestation and that placental limitation of fetal growth normally begins to be evidenced between mid gestation and 120 days gestation. These results are consistent with previous reports (Stegeman, 1974, Caton *et al.*, 1984 and Harding *et al.*, 1985). In both mid and late gestation, placental weight correlated positively with the volumes of fetal villi, the connective tissue core of the fetal villi and surface area of the exchange epithelia. Thus placental weight is a good index of major structural determinants of placental function which support fetal growth.

3.3.5 Summary

This study appears to agree that placental weight in sheep may be maximal by day 75, remains unchanged until day 130 then declines by 26% by day 140. Between mid and late gestation (120 days) a significant but minor proportion of placentomes are everted (11.3 ± 9.9 and $24.2 \pm 13.8\%$) with the remainder (~80%) everting later, between day 120 and 140 of gestation.

Between mid and late gestation, the volume of fetal trophoctoderm increased and the arithmetic mean barrier thickness of both fetal trophoctoderm and feto-maternal syncytium decreased. Together with the loss of the connective tissue core of the fetal villi, these changes will reduce the barrier to diffusion. The surface area of the exchange epithelia in the ovine placenta has been determined for the first time and found to double over this period. As observed in another species with a cotyledonary placenta, fetal weight increased more rapidly than placental surface area, thus relative surface area decreased between mid and late gestation.

TABLES AND FIGURES FOR CHAPTER 3

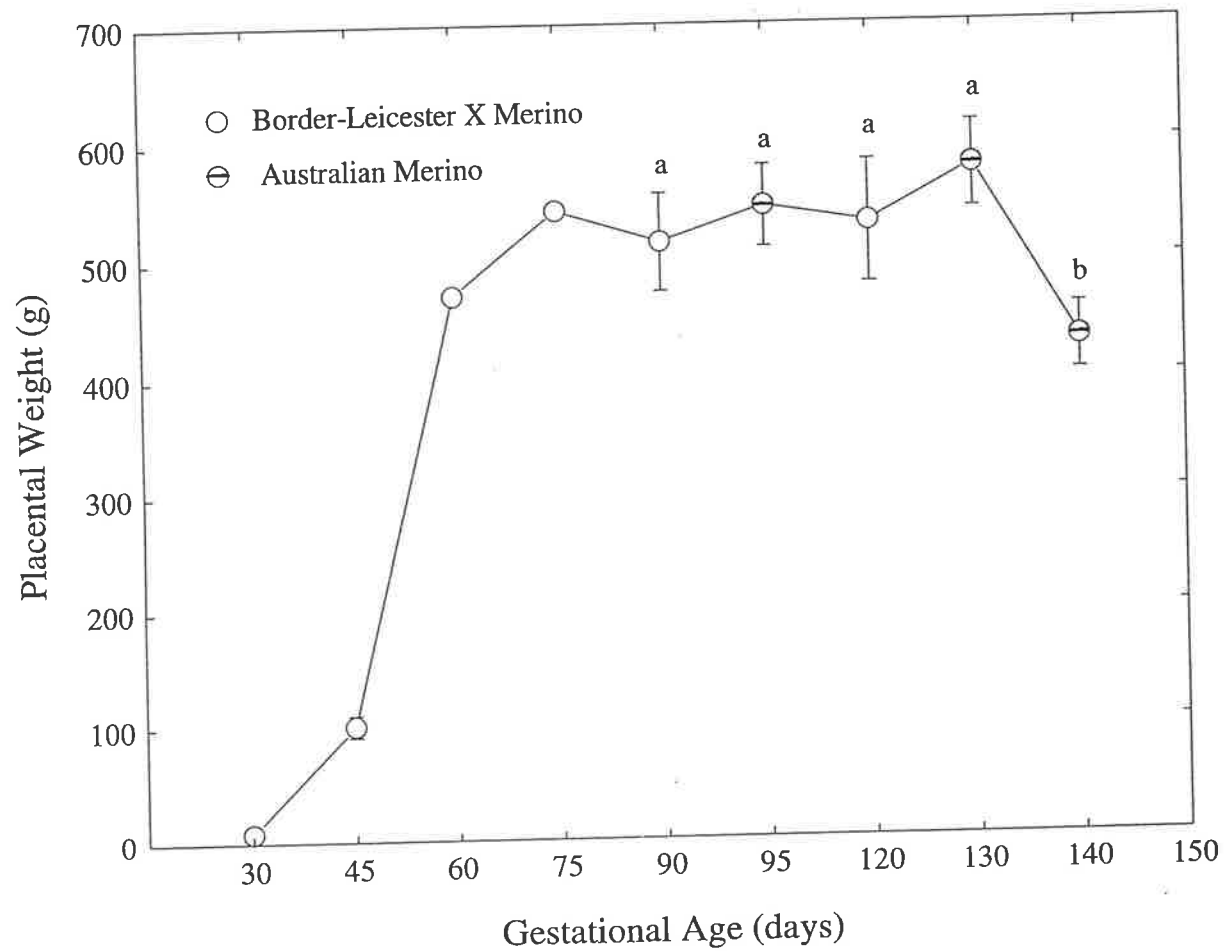


Figure 3.1 The ontogeny of placental growth in Border-Leicester X Merino (○) and Australian Merino ewes (⊖). Values with the same letter are not significantly different ($p < 0.05$; ANOVA; L S Means $\pm$ SE, Specific Contrasts).

TABLE 3.1 Macroscopic parameters of placental growth and development in mid and late gestation

GESTATIONAL AGE (DAYS)	NUMBER OF PLACENTOMES FORMED	MEAN PLACENTOME WEIGHT (g)	TOTAL PLACENTAL WEIGHT (g)	% TOTAL PLACENTOME NUMBER EVERTED	% TOTAL PLACENTAL WEIGHT EVERTED	FETAL WEIGHT (g)	F:P RATIO
<i>Border-Leicester × Merino</i>							
90 (N=9)	74 ± 6 ^a	7.5 ± 1.0 ^a	515 ± 42 ^a	11.3 ± 9.9 ^a	12.9 ± 12.5 ^a	716 ± 33 ^a	1.48 ± 0.16 ^a
120 (N=6)	74 ± 2 ^a	7.4 ± 0.8 ^a	545 ± 35 ^a	24.2 ± 13.8 ^a	25.7 ± 14.8 ^a	2654 ± 77 ^b	4.94 ± 0.25 ^b
<i>Australian Merino</i>							
95 (N=4)	63 ± 6 ^a	8.9 ± 1.1 ^a	531 ± 51 ^a	29.04 ± 13.3 ^a	24.6 ± 13.3 ^a	NA	NA
130 (N=9)	72 ± 3 ^a	8.2 ± 0.7 ^a	579 ± 37 ^a	81.5 ± 10 ^b	82.9 ± 9.5 ^b	3478 ± 119 ^c	6.17 ± 0.37 ^b
140 (N=9)	65 ± 3 ^a	6.7 ± 0.4 ^a	430 ± 28 ^b	99 ± 0.5 ^b	99.3 ± 0.4 ^b	4783 ± 334 ^d (N=4)	12.75 ± 1.84 ^c (N=4)

Values are expressed as mean ± SE; means with different letters are significantly different,

p<0.05; ANOVA: LS Means specific contrasts

NA= data not available

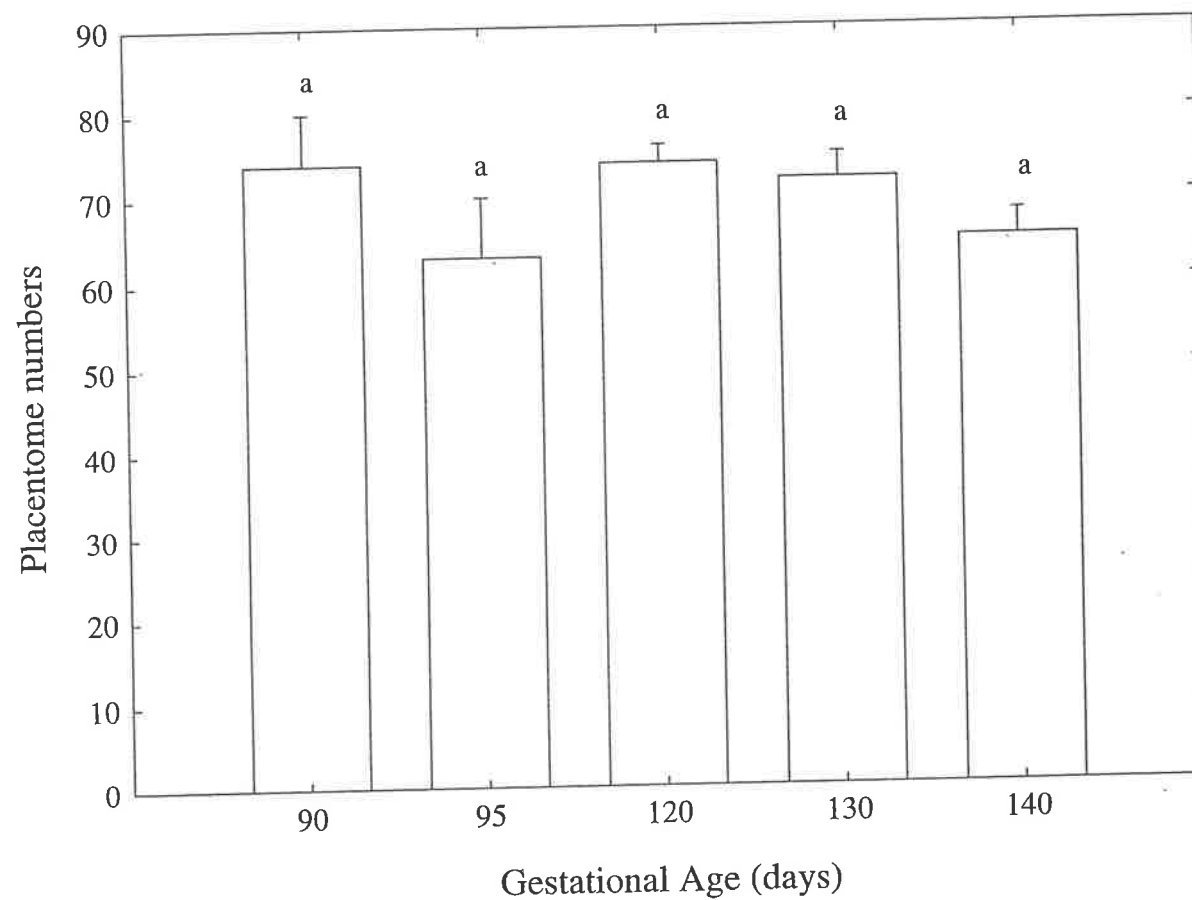


Figure 3.2 Placentome numbers at different gestational ages in Border-Leicester Merino Cross-Bred ewes (90 and 120 days) and Australian Merino Ewes (95, 130 and 140 days). Values with the same letter are not significantly different (ANOVA; L S Means $\pm$ SE, Specific Contrasts).

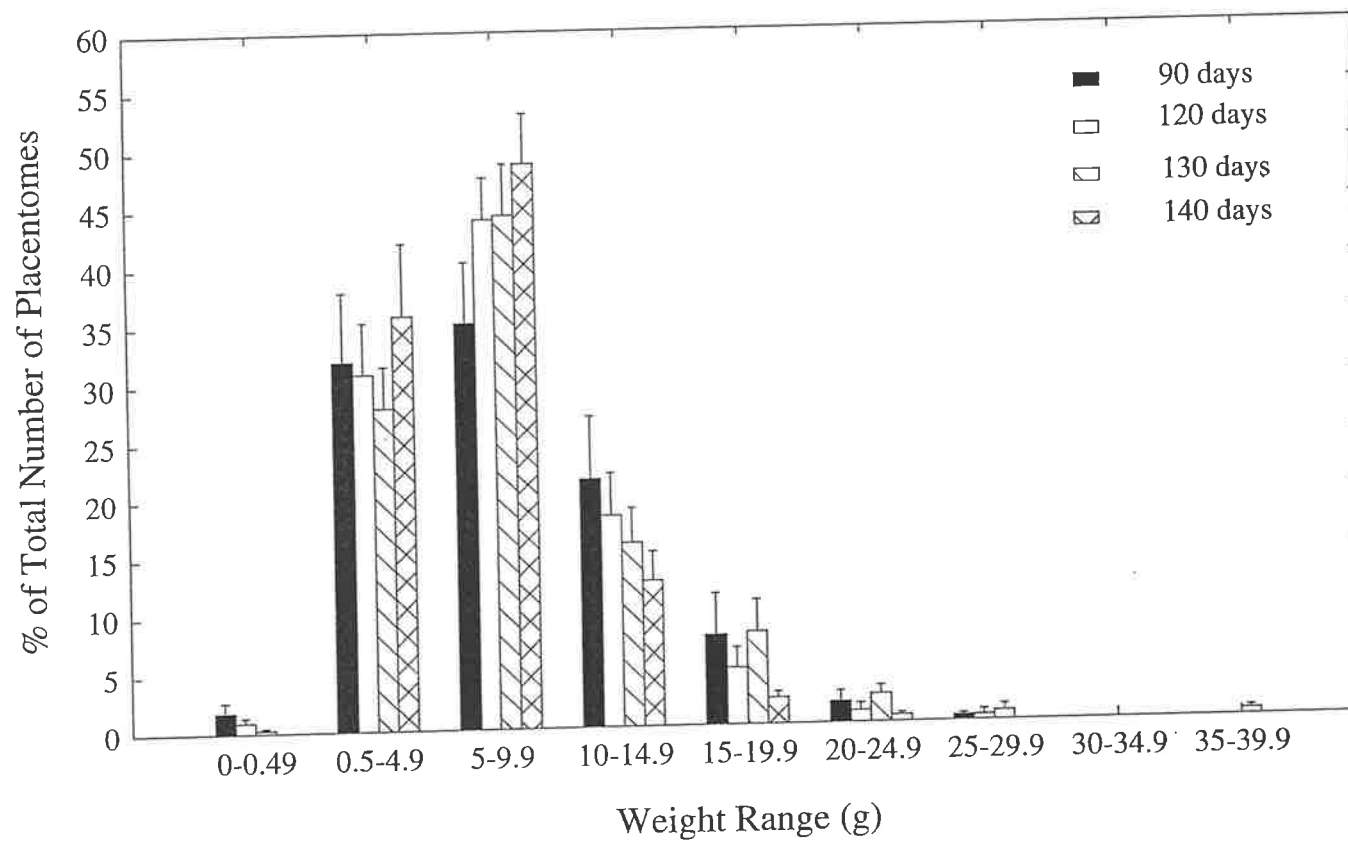


Figure 3.3 The ontogeny of placentome weight distribution within particular weight range categories from mid to late gestation in placentas of Border-Leicester X Merino (90 and 120 days) and Australian Merino ewes (130 and 140 days) (Mean $\pm$ SE).

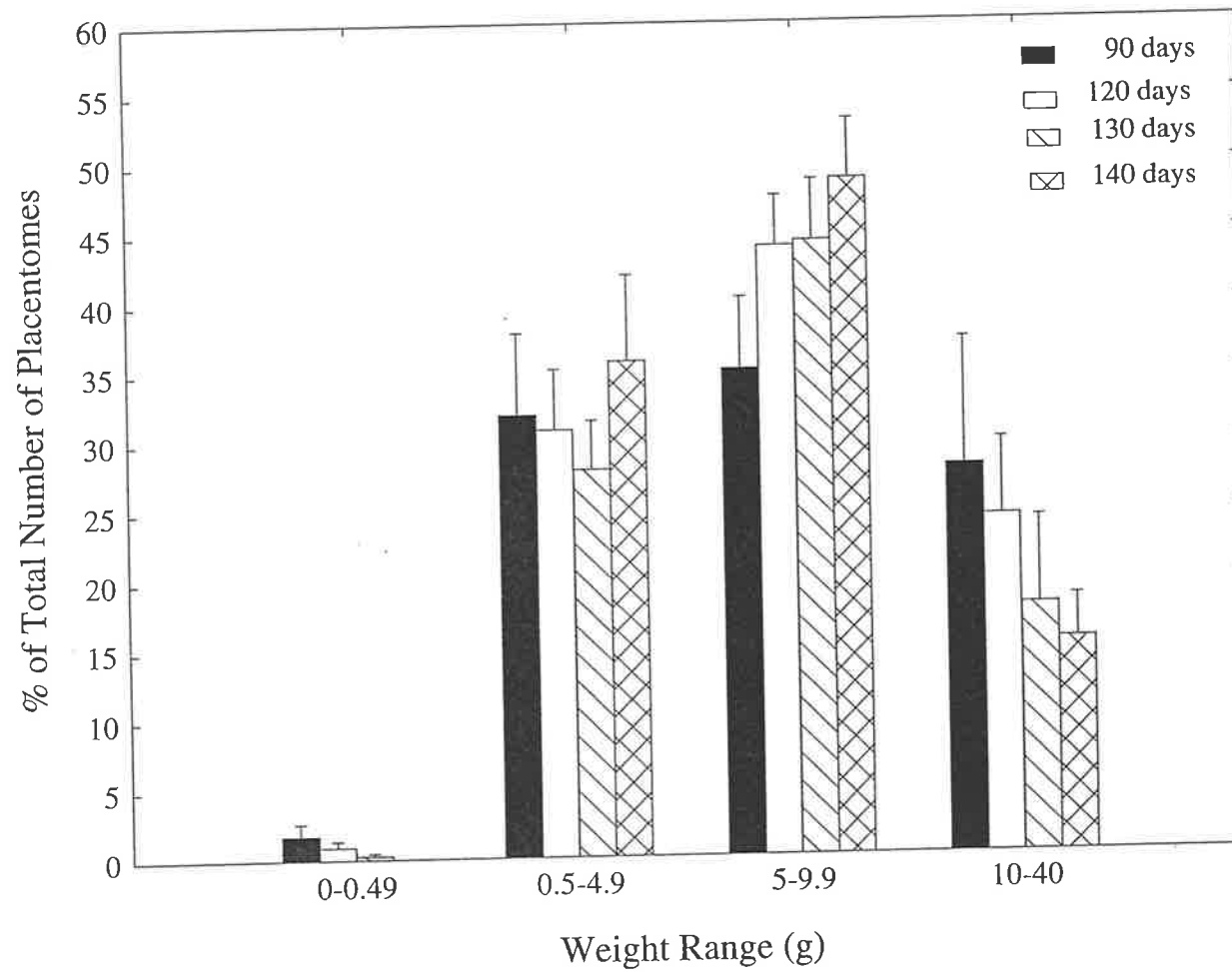


Figure 3.4 The ontogeny of placentome weight distribution from mid to late gestation in placentas of Border-Leicester X Merino (90 and 120 days) and Australian Merino ewes. (130 and 140 days) (Mean $\pm$ SE).

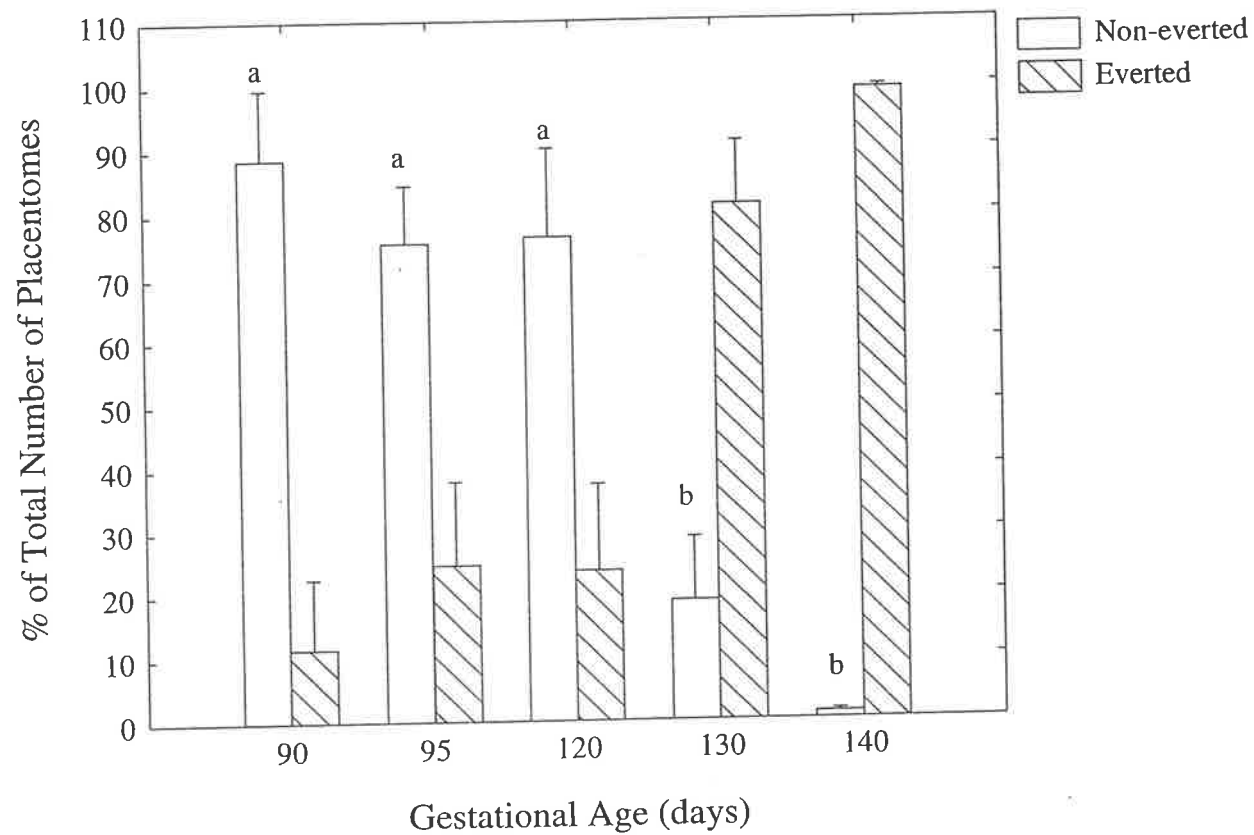


Figure 3.5 The proportion of everted versus non-everted placentomes at different gestational ages in Border-Leicester X Merino (90 and 120 days) and Australian Merino ewes (95, 130 and 140 days). Values with the same letter are not significantly different ($p < 0.0001$, ANOVA; L S Means $\pm$ SE, Specific Contrasts).

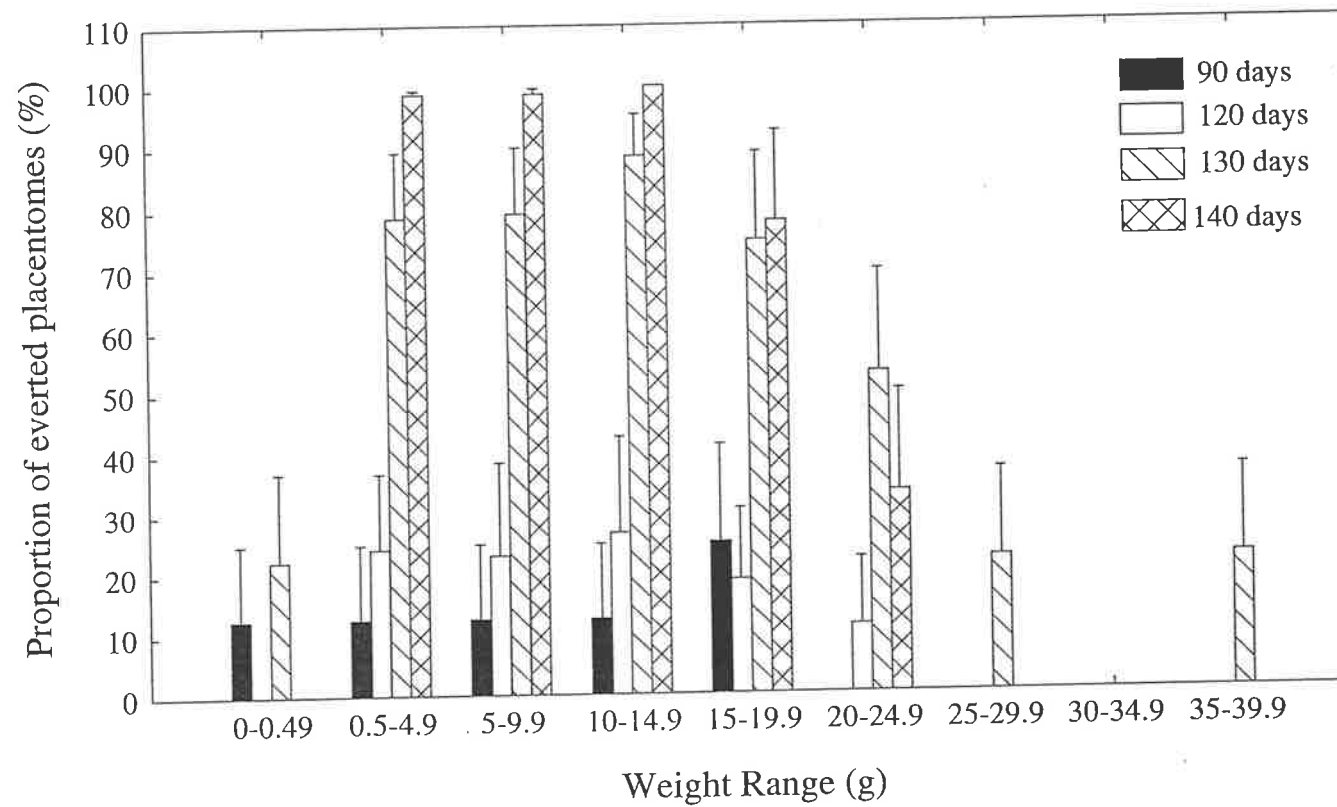


Figure 3.6 The ontogeny of the proportion of placentomes within each weight range which were everted (expressed as a percentage of the total number of placentomes within the weight range) (Mean $\pm$ SE).

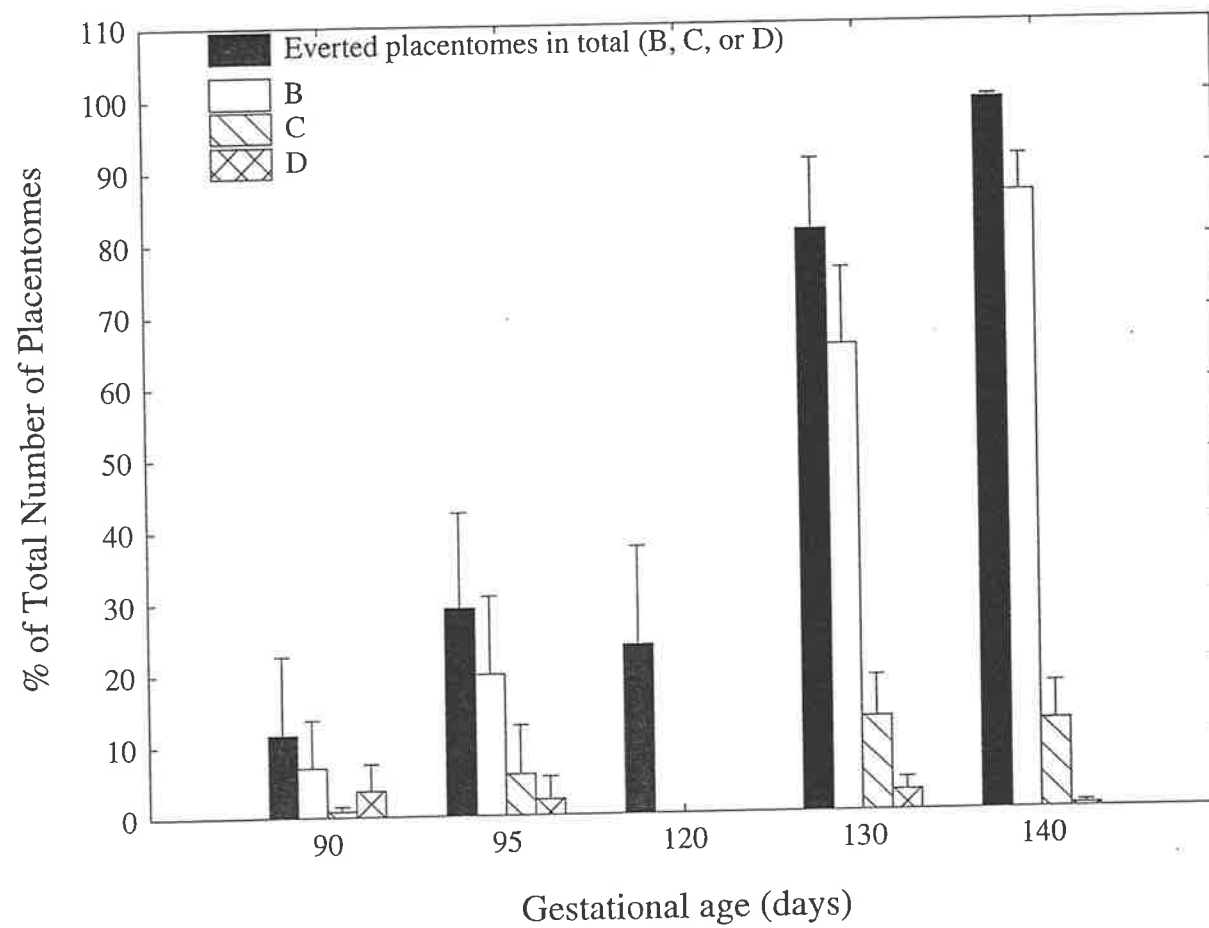


Figure 3.7 The proportion of placentomes which were everted (■) and that of everted placentomes exhibiting one of the three types of everted morphology: morphology B (□), C (▨), or D (▩) in Border-Leicester X Merino (90days) and Australian Merino ewes (95, 130 and 140 days) (Mean $\pm$ SE).

TABLE 3.2. Volume densities of placentome components at 90 and 120 days gestation

GESTATION		90 DAYS (N=9)			120 DAYS (N=6)			ANOVA SUMMARY
PLACENTOME SIZE		"Small"	"Average-size"	"Large"	"Small"	"Average-size"	"Large"	Factor
Weight (g)		2.9 ± 0.8 (n=2)	8.2 ± 0.5 (n=8)	16.3 ± 1.4 (n=7)	4.6 ± 0.6 (n=2)	7.4 ± 0.4 (n=6)	14.8 ± 0.4 (n=6)	GESTATIONAL AGE
COMPONENT								(p=)
Chorionic membrane		0.107 ± 0.005	0.107 ± 0.011	0.115 ± 0.013	0.098 ± 0.017	0.137 ± 0.016	0.131 ± 0.019	ns
Chorionic epithelium		0.024 ± 0.011	0.031 ± 0.004	0.022 ± 0.005	0.033 ± 0.002	0.053 ± 0.007	0.043 ± 0.006	0.0008
Haemophagous zone (HMZ)		0.018 ± 0.010	0.069 ± 0.015	0.050 ± 0.017	0.058 ± 0.029	0.066 ± 0.009	0.051 ± 0.013	ns
Maternal tissue in HMZ		0.007 ± 0.001	0.022 ± 0.004	0.021 ± 0.008	0.015 ± 0.006	0.02 ± 0.004	0.011 ± 0.002	ns
Fetal villi		0.450 ± 0.027	0.363 ± 0.020	0.395 ± 0.032	0.329 ± 0.038	0.332 ± 0.022	0.365 ± 0.037	ns
Trophectoderm		0.200 ± 0.046	0.162 ± 0.014	0.142 ± 0.025	0.231 ± 0.018	0.206 ± 0.019	0.229 ± 0.025	0.002
Connective tissue		0.256 ± 0.074	0.199 ± 0.026	0.236 ± 0.034	0.089 ± 0.016	0.125 ± 0.009	0.127 ± 0.013	0.0004
Maternal crypts		0.284 ± 0.022	0.278 ± 0.021	0.271 ± 0.023	0.353 ± 0.01	0.295 ± 0.018	0.284 ± 0.017	ns
Feto-maternal syncytium		0.081 ± 0.004	0.077 ± 0.006	0.072 ± 0.008	0.122 ± 0.024	0.086 ± 0.007	0.084 ± 0.007	ns
Placentome capsule		0.098 ± 0.010	0.082 ± 0.011	0.091 ± 0.008	0.102 ± 0.007	0.063 ± 0.01	0.083 ± 0.017	ns
Shrinkage spaces		0.022 ± 0.003	0.042 ± 0.012	0.039 ± 0.010	0.008 ± 0.004	0.032 ± 0.009	0.029 ± 0.007	ns

Values are expressed as mean ± SE; ns=no significant difference; ANOVA; LS Means specific comparisons, testing effect of gestational age excluding small placentomes;
Volume density= the fraction of the placentome composed by a particular component.

TABLE 3.3 Gross placentome morphology and volume density of trophoctoderm at 90 and 120 days gestation

GESTATIONAL AGE (days)		90 (N=9)		120 (N=6)		ANOVA SUMMARY (LS Means Comparisons)		
PLACENTOME MORPHOLOGY		<i>Non-everted</i>	<i>Everted</i>	<i>Non-everted</i>	<i>Everted</i>	AGE	PLACMOR	AGE*PLACMOR
Weight (g)		11.5 ± 1.3 (n=16)	2.1 (n=1)	10.1 ± 1.4 (n=10)	10.4 ± 2.2 (n=4)			
Volume density of trophoctoderm		0.153 ± 0.013	0.247	0.203 ± 0.105	0.261 ± 0.016 *	0.002	0.01	ns

Values are expressed as mean ± SE; N=number of ewes; n=number of placentomes analysed morphometrically; ANOVA; LS Means Comparisons, overall effect of gestational age (AGE), placentome morphology (PLACMOR), and their interaction on the volume density of trophoctoderm. There were no significant effects of placentome morphology or its interaction with gestational age on all the other components. At 120 days gestation, non-everted versus everted placentomes had significantly different volume densities of trophoctoderm (p=0.05; lsmeans specific comparisons). Everted (120 days) versus non-everted (90 days) placentomes were significantly different (* p=0.0004; lsmeans specific comparisons).

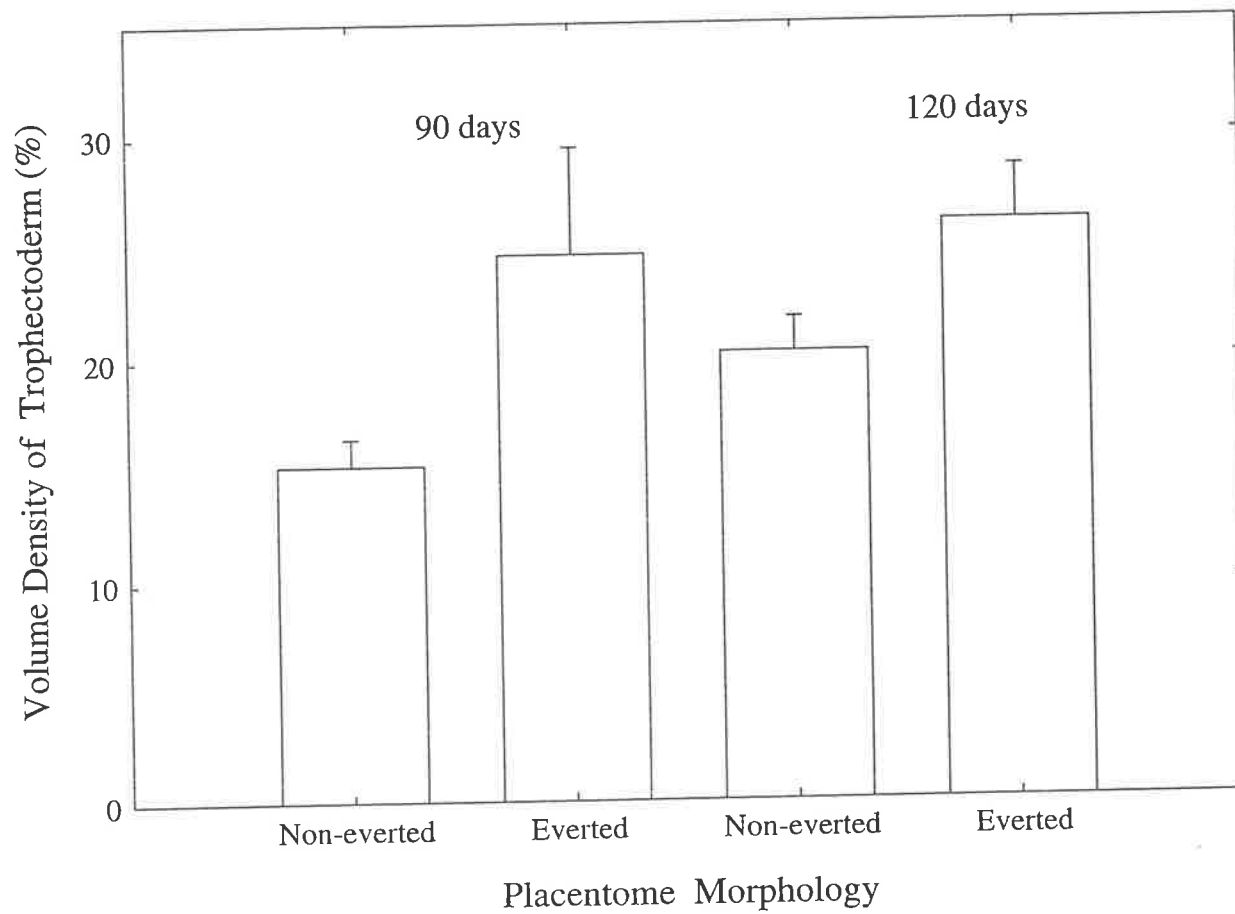


Figure 3.8 Comparison of trophoderm volume density in non-everted and everted placentomes in placentas of singletons at 90 and 120 days gestation.

(Overall effect of placentome morphology; $p=0.01$; ANOVA; L S Means comparisons).

TABLE 3.4 Volume densities of placentome components in mid and late gestation

GESTATIONAL AGE (days)	90 (N=9)		120 (N=6)		ANOVA SUMMARY
PLACENTOME SIZE	"Average-size"	"Large"	"Average-size"	"Large"	
Weight (g)	8.2 ± 0.5 (n=8)	16.3 ± 1.4 (n=7)	7.1 ± 0.4 (n=7)	14.8 ± 0.4 (n=6)	Factor GESTATIONAL AGE (p=)
COMPONENT					
Chorionic membrane	0.111 ± 0.009		0.132 ± 0.010		ns
Chorionic epithelium	0.027 ± 0.004		0.047 ± 0.004		0.0008
Haemophagous zone (HMZ)	0.060 ± 0.009		0.056 ± 0.010		ns
Maternal tissue in HMZ	0.021 ± 0.003		0.015 ± 0.004		ns
Fetal villi	0.378 ± 0.018		0.350 ± 0.019		ns
Trophectoderm	0.152 ± 0.014		0.220 ± 0.015		0.002
Connective tissue	0.216 ± 0.016		0.124 ± 0.017		0.0004
Maternal crypts	0.275 ± 0.013		0.294 ± 0.014		ns
Feto-maternal syncytium	0.074 ± 0.004		0.086 ± 0.005		ns
Placentome capsule	0.086 ± 0.008		0.075 ± 0.008		ns
Shrinkage spaces	0.040 ± 0.007		0.029 ± 0.007		ns
Fetal villi/ maternal crypts	1.443 ± 0.098		1.212 ± 0.105		ns
Total fetal tissue/ Total maternal tissue	1.106 ± 0.074		1.055 ± 0.079		ns

Values are expressed as least squares means ± SE, excepting placentome weight (mean ± SE); ns=no significant difference; ANOVA; LS means comparisons, testing effect of gestational age excluding small placentomes. Volume density= the fraction of the placentome composed by a particular component. Total fetal tissue= fetal villi + chorionic epithelium (excluding chorionic membrane). Total maternal tissue= maternal tissue in haemophagous zone + maternal crypts + placentome capsule.

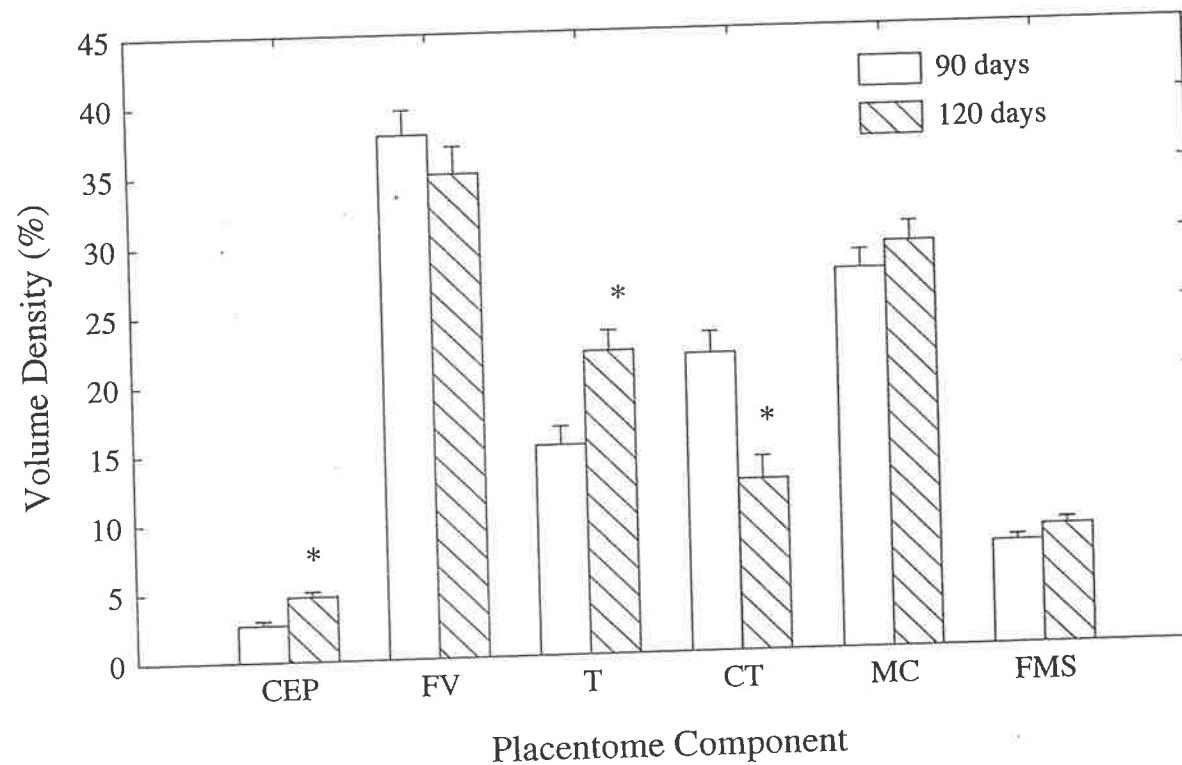


Figure 3.9 Comparison of volume densities of components in placentomes of singleton placentas at 90 (□) and 120 (▨) days gestation: Chorionic epithelium (CEP), Fetal villi (FV), Trophoctoderm (T), Connective tissue core of fetal villi (CT), Maternal crypts (MC), and Feto-maternal syncytium (FMS). (ANOVA; L S Means Comparisons; * $p < 0.01$).

TABLE 3.5 Surface densities and arithmetic mean barrier thicknesses in placentomes at 90 and 120 days gestation

GESTATIONAL AGE (days)		90 (N=9)			120 (N=6)			ANOVA SUMMARY (LSMeans comparisons) Factor: Gestational Age (p)
PLACENTOME SIZE		"Small"	"Average-size"	"Large"	"Small"	"Average-size"	"Large"	
Weight (g)		2.9 ± 0.8 (n=2)	8.2 ± 0.5 (n=8)	16.3 ± 1.4 (n=7)	4.0 (n=1)	7.1 ± 0.4 (n=7)	14.8 ± 0.4 (n=6)	
STRUCTURAL PARAMETER								
Surface density of Trophoctoderm (cm ⁻¹)		121 ± 16	122 ± 10	108 ± 6	166	185 ± 7	185 ± 5	0.0001
Arithmetic mean barrier thickness								0.02
Trophoctoderm (µm)		18.23 ± 2.32	15.88 ± 0.87	18.83 ± 1.10	12.84	13.92 ± 0.76	14.62 ± 0.99	
Arithmetic mean barrier thickness								0.0002
Feto-maternal syncytium (µm)		7.34 ± 0.57	8.44 ± 0.79	8.58 ± 0.93	8.80	5.72 ± 0.48	5.18 ± 0.39	

Values are expressed as mean ± SE; ANOVA; LSMeans comparisons testing effect of gestational age, analysis excluding small placentomes; N=number of ewes; n=number of placentomes morphometrically analysed

TABLE 3.6 Surface densities and arithmetic mean barrier thicknesses in placentomes in mid and late gestation

GESTATIONAL AGE (days)	90 (N=9)		120 (N=6)		ANOVA SUMMARY
<i>PLACENTOME SIZE</i>	<i>"Average-size"</i>	<i>"Large"</i>	<i>"Average-size"</i>	<i>"Large"</i>	(LSMeans
Weight (g)*	8.2 ± 0.5	16.3 ± 1.4	7.1 ± 0.4	14.8 ± 0.4	comparisons)
	(n=8)	(n=7)	(n=7)	(n=6)	Factor: Gestational Age
<i>COMPONENT</i>					(p)
Surface density of trophoctoderm (cm ⁻¹)	116 ± 5		185 ± 6		0.0001
Arithmetic mean barrier thickness Trophoctoderm (µm)	16.37 ± 0.62		14.15 ± 0.69		0.02
Arithmetic mean barrier thickness Feto-maternal syncytium (µm)	8.40 ± 0.45		5.70 ± 0.50		0.0004

Values are least squares means ± SE; * Values are mean ± SE; ANOVA; LS means comparisons testing effect of gestational age, analysis excluding small placentomes; N=number of ewes; n=number of placentomes morphometrically analysed

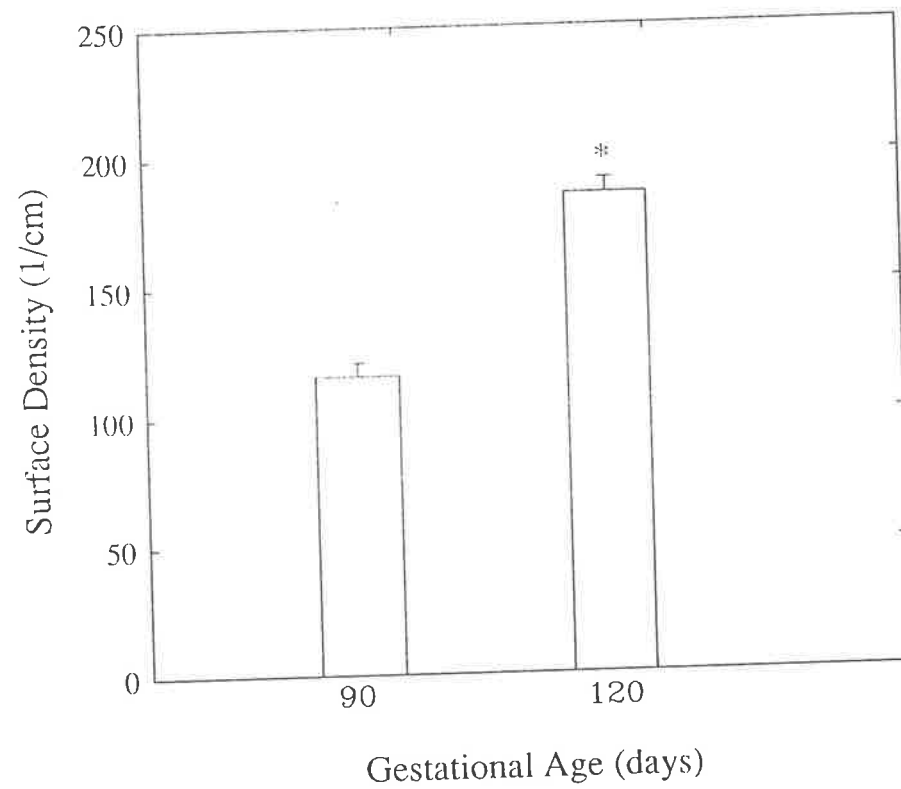


Figure 3.10 Comparison of surface density of trophoblast at 90 and 120 days gestation. (ANOVA; L S Means comparisons; * $p = 0.0001$).

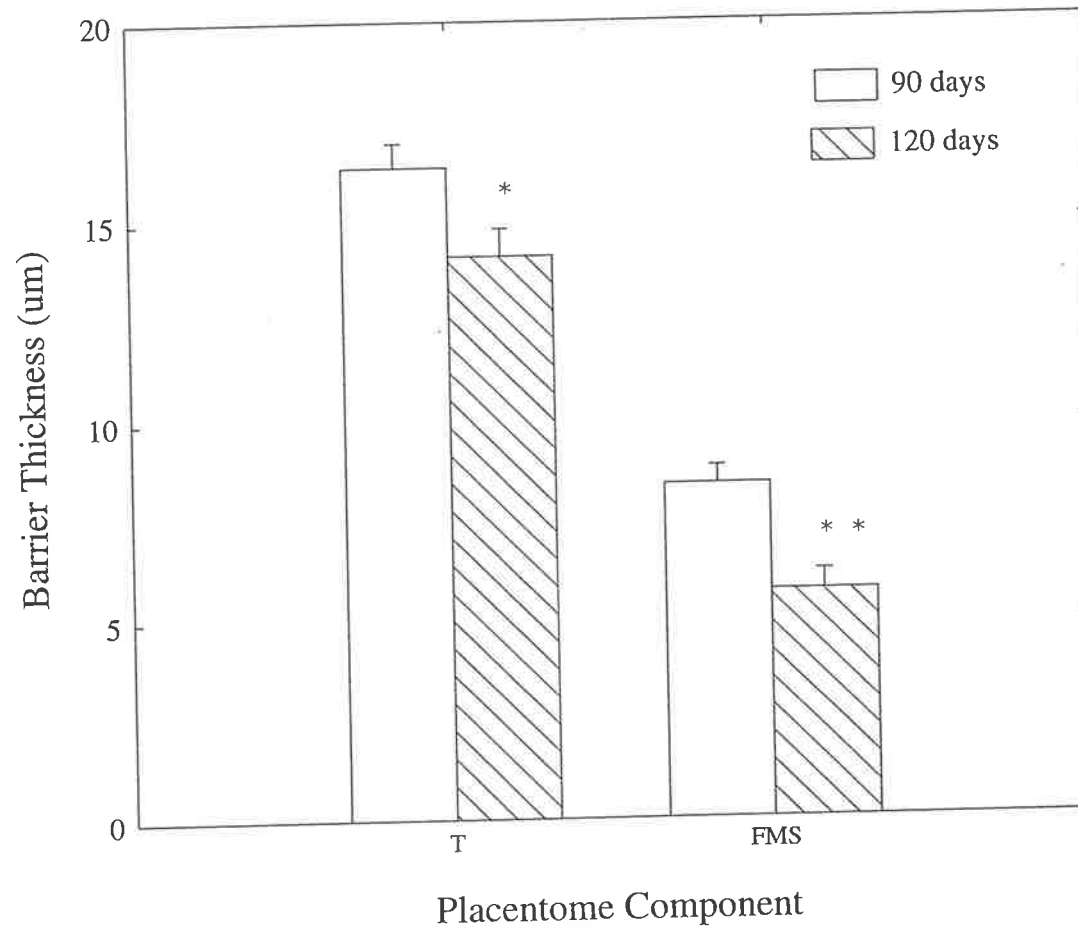


Figure 3.11 Arithmetic mean barrier thickness of the epithelial cell layers at the feto-maternal interface in placentas of singletons at 90 (□) and 120 (▨) days gestation: Trophectoderm (T) and Feto-maternal syncytium (FMS). (ANOVA; L S Means Comparisons * $p < 0.05$; ** $p < 0.01$).

TABLE 3.7 Ontogeny of total placental volumes of components, surface area, and relative surface area between 90 and 120 days gestation

			ANOVA SUMMARY
			Factor: Gestational Age
GESTATIONAL AGE (days)	90 (N=9)	120 (N=6)	(p=)
Placental weight (g)	515 ± 42	545 ± 35	ns
<i>COMPONENT</i>			
Chorionic membrane	57.62 ± 8.67	70.31 ± 6.7	ns
Chorionic epithelium	13.09 ± 2.21	24.95 ± 2.03	0.003
Haemophagous zone (HMZ)	28.29 ± 7.31	31.01 ± 5.04	ns
Maternal tissue in HMZ	10.48 ± 2.83	8.08 ± 1.43	ns
Fetal villi	200.58 ± 20.97	193.23 ± 24.77	ns
Trophectoderm	83.51 ± 4.31	122.25 ± 16.05	0.02
Connective tissue	116.94 ± 16.00	69.32 ± 9.57	ns
Maternal crypts	141.65 ± 12.3	159.55 ± 14.55	ns
Feto-maternal syncytium	38.21 ± 3.01	47.09 ± 4.83	ns
Shrinkage spaces	17.73 ± 4.08	17.08 ± 5.14	ns
Placentome capsule	45.43 ± 5.09	39.02 ± 6.3	ns
Surface area (m ²)	5.93 ± 0.44	10.07 ± 0.81	0.0003
Relative surface area (cm ⁻¹)	47.79 ± 2.32	31.26 ± 1.64	0.0002

Values are expressed as mean ± SE; Relative surface area = Placental surface area/ (fetal weight + placental weight);
Component Volume = Volume density x placental weight

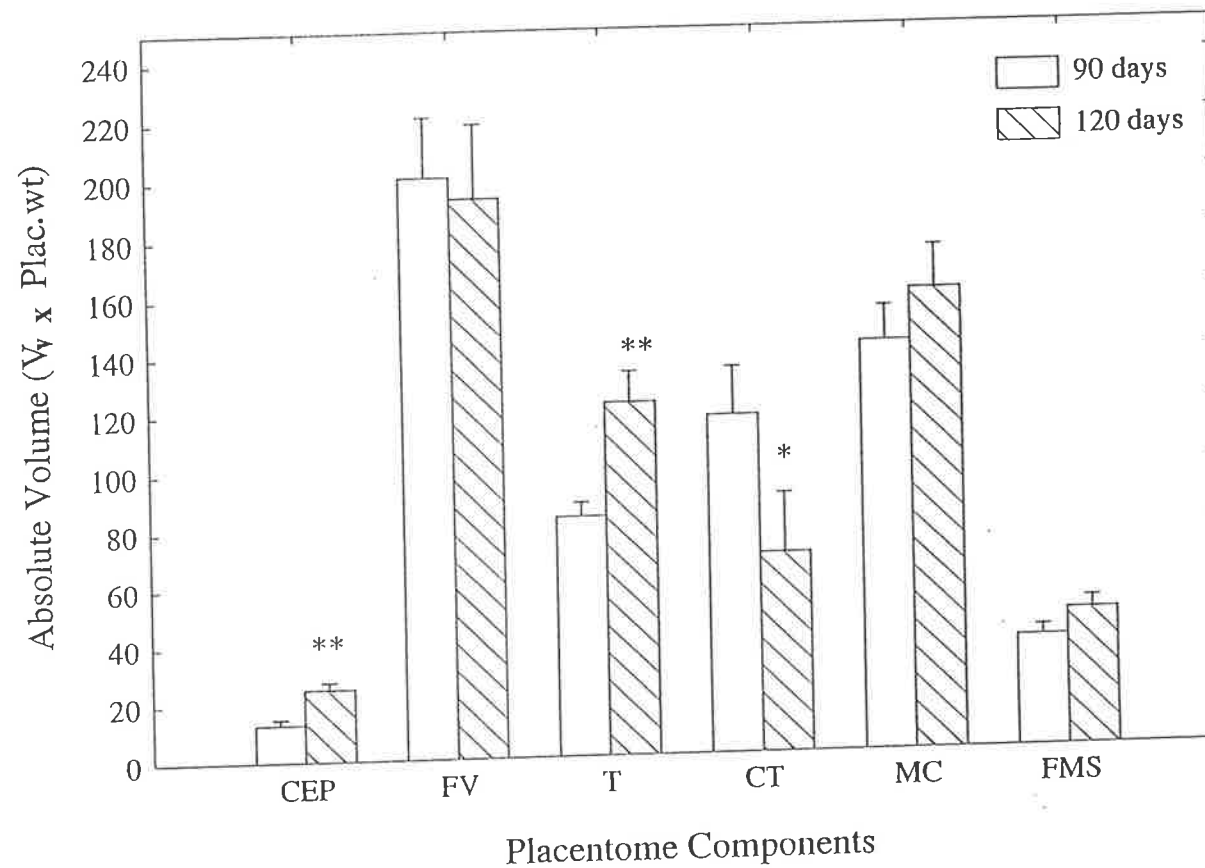


Figure 3.12 Comparison of total placental volumes of components in placentas of singletons at 90 (□) and 120 (▨) days gestation. (ANOVA; L S Means Comparisons ** $p < 0.02$, * $p = 0.06$).
 Absolute volume of component = volume density (V_v) $\times$ placental weight

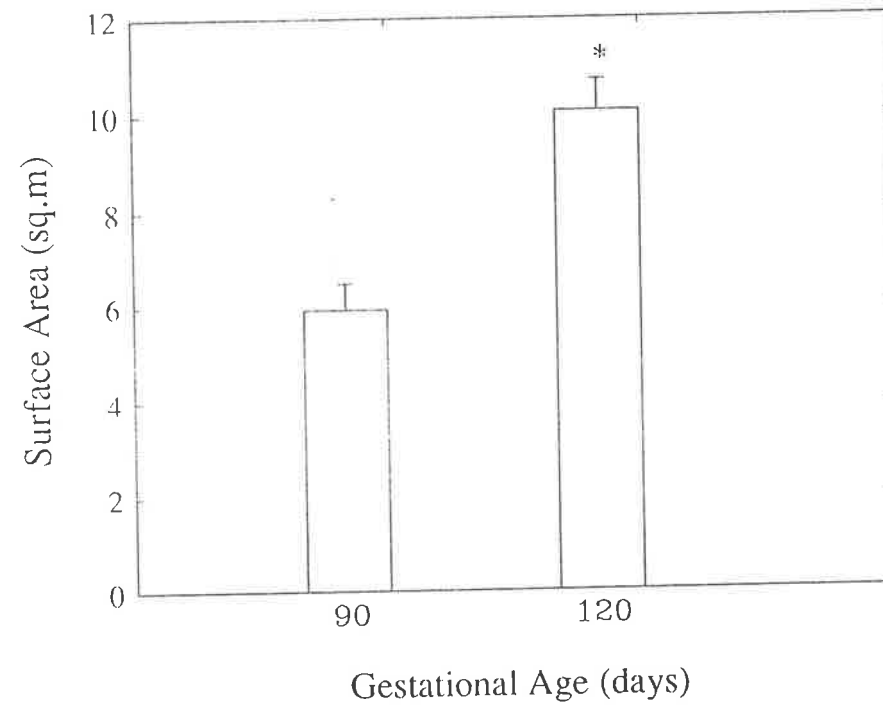


Figure 3.13 Ontogeny of total placental surface area in the ovine placenta (singletons). (ANOVA; L S Means Comparisons; * $p = 0.0003$)

TABLE 3.8 Relationship between fetal weight and placental structural parameters from mid to late gestation

GESTATIONAL AGE (days)			PLACENTAL PARAMETERS		
			PLACENTAL WEIGHT (g)	TROPHECTODERM	SURFACE AREA (m ²)
				CONNECTIVE TISSUE CORE OF FETAL VILLI	
90 (N=9)	Fetal Weight (g)	<i>r</i>	-0.61		-0.67
		<i>p</i>	0.08		0.05
120 (N=6)	Fetal Weight (g)	<i>r</i>	0.8	0.82	0.81
		<i>p</i>	0.08	0.05	0.05

r= correlation coefficient, *p*= coefficient probability

Trophectoderm and connective tissue core of fetal villi expressed as total placental volume= volume density x placental weight

N= number of ewes studied

TABLE 3.9 Relationship between placental weight and placental structural components at mid and late gestation

GESTATIONAL AGE (days)		PLACENTAL PARAMETERS		
		FETAL VILLI	CONNECTIVE TISSUE CORE OF FETAL VILLI	SURFACE AREA (m ²)
90 (N=9)	<i>r</i>	0.85	0.83	0.70
	<i>p</i>	0.004		0.04
120 (N=6)	<i>r</i>	0.86	0.89	0.92
	<i>p</i>	0.03		0.01

r= correlation coefficient, *p*= coefficient probability

Fetal villi and connective tissue core of fetal villi expressed as total placental volume= volume density x placental weight

N= number of ewes studied

TABLE 3.10 Comparison of ontogenic changes in surface density, surface area and relative surface area in ovine and bovine placentas

	GESTATIONAL AGE (FRACTION)	SHEEP	COW
Surface Density of Trophoctoderm (cm ⁻¹)	0.6	116 ± 5	201
	0.8	185 ± 6	190
Total Villous Surface Area (m ²)	0.6	5.93 ± 0.44	25
	0.8	10.07 ± 0.81	64
Relative Surface Area (cm ⁻¹)	0.6	47.79 ± 2.32	35
	0.8	31.26 ± 1.64	32

Data for bovine species (Baur, 1981)

Villous surface area = surface density × placental weight

Relative villous surface area = total villous surface area / (fetal weight + placental weight)

CHAPTER 4

EFFECT OF EXPERIMENTAL RESTRICTION OF IMPLANTATION ON PLACENTAL DEVELOPMENT

4.1 INTRODUCTION

Restriction of implantation in sheep induced by the surgical removal of most implantation sites before pregnancy (Alexander, 1964b; Robinson *et al.*, 1979; Harding *et al.*, 1985; Owens *et al.*, 1989) or by uterine horn ligation (Caton *et al.*, 1984) induces an adaptive increase in the size of individual placentomes, many of which also become everted in shape. Several observations suggest that these adaptive changes may enhance or be accompanied by enhanced placental function.

Experimental restriction of implantation is generally associated with increased fetal weight/placental weight (f:p) ratio in late gestation (Alexander, 1964b; Robinson *et al.*, 1979; Owens *et al.*, 1989). *In vivo* studies in ewes in which implantation was experimentally restricted, showed that the resultant small placenta, has a reduced rate of substrate consumption, but an increased transfer capacity per gram of tissue for both oxygen and glucose when compared to the normally grown placenta (Owens *et al.*, 1989). Other direct measurements of the transfer capacity of the restricted placentas indicate that the predominantly flow-determined transfer of antipyrine and the facilitated-diffusion of glucose, as well as the clearance of urea decrease, in absolute terms, as placental weight decreases (Owens *et al.*, 1987b). Although compensatory placental growth following restricted implantation has been well described in terms of macroscopic parameters, such as placentome size and morphology, there have been no histological studies of the restricted placenta to define the cellular and structural characteristics of the adaptive growth process, which may contribute to the observed metabolic and functional changes.

In sheep, placental restraint of fetal growth normally emerges in late gestation, between 125 and 140 days, as evidenced by the appearance of significant positive correlations between fetal and placental weight (Alexander, 1964a; Caton *et al.*, 1984; Harding *et al.*, 1985). Experimental restriction of implantation accelerates the development of this relationship, such that it is evident as early as 90 to 120 days (Alexander, 1964; Harding *et al.*, 1985).

The consequences of restricted implantation for placental structure at the cellular level, particularly that relating to function, are unknown. In addition, the times of onset of both macroscopic and any microscopic structural adaptations by the placenta to restricted implantation have not been determined. Therefore, the effect of experimental restriction of implantation on placental major structural development in sheep was characterised in mid and late gestation. To determine whether any major structural characteristics of the placenta related to function can be identified as responsible for the apparent placental restraint on fetal growth, the ontogeny of the relationship between fetal weight and various parameters of placental structural development following experimental restriction of implantation were also characterised.

4.2 MATERIALS AND METHODS

Animals, sample collection, morphometric methods.

This study was carried out in Border Leicester $\times$ Merino cross-bred ewes well-fed prior to and throughout gestation. Animals were killed either at 90 or 120 days of gestation for the examination of uterine contents. Fetal and placental parameters were recorded at post-mortem, and placental samples were selected and processed for morphometric analysis as outlined in Chapter 2.

Volume densities of placentome components and surface densities of trophoctoderm were determined as previously described and used to calculate total volumes of placental components and surface areas and barrier thicknesses of the exchange epithelia.

Statistical analysis

Effects of between factors, treatment (two levels: control or experimental restriction of implantation), gestational age (two levels: 90 and 120 days) and their interaction and of within factors (placentome size and morphology) were assessed by analysis of variance (Proc GLM, SAS).

4.3 RESULTS

4.3.1. Experimental restriction of implantation: placental structure in ewes bearing singletons.

Macroscopic parameters of placental development at mid gestation

Experimental restriction of implantation reduced the number of placentomes formed (-59%, $p=0.002$) and total placental weight (-38%, $p=0.0001$) but increased the average weight of individual placentomes (+53%; $p=0.03$) at mid gestation (Table 4.1). In the restricted placentas, a greater proportion of placentomes weighed more than 25g ($15 \pm 7\%$) than in control placentas ($2 \pm 1\%$) (Figure 4.1). Fetal weight in mid gestation was not significantly affected by restricted implantation (Table 4.1).

In control placentas, placentome weights ranged from 0.12 to 30g (Figure 4.1). However, most placentomes (89%) weighed between 0.5 to 14.9g, although the modal weight range category was the 5 to 9.9g range (Figure 4.1). Only two of the nine control placentas contained everted placentomes, thus placentome weight distribution was also analysed separately in control placentas with and without eversion (Figure 4.2). Control placentas which exhibited eversion had fewer placentomes in the 10 to 19g weight ranges and more placentomes in the 5 to 9.9g weight range when compared to either that of all control placentas or placentas with no eversion (Figure 4.2).

Restricted implantation increased the weight of the largest placentome to 50g instead of 30g in controls (Figure 4.1). In restricted placentas, the modal weight range category shifted from 5 to 9.9g to 0.5 to 4.9g, although this was not statistically significant. Following restricted implantation, all placentas contained everted placentomes. The proportion of placentomes which were everted was increased in restricted placentas, ($49 \pm 20\%$ compared to $11 \pm 10\%$ in controls; $p=0.05$) (Table 4.1; Figure 4.3).

Microscopic Parameters of Placental Structure at mid gestation

Following restricted implantation, "average" size placentomes were composed of similar proportions of fetal (51%) and maternal (43%) tissue to those in controls at mid gestation

(Table 4.2). The volume density of trophoctoderm increased following restricted implantation (+51%, $p=0.0001$) (Table 4.3 and Figure 4.4), as did volume densities of maternal crypts (+15%, $p=0.04$) and feto-maternal syncytium lining the maternal crypts (+35%; $p=0.006$) (Table 4.3 and Figure 4.4). Placentome size did not significantly influence the volume densities of any components. In contrast, placentome morphology was associated with significant changes in structure, such that everted placentomes had more trophoctoderm (+61% in controls compared to +7.5% in the restricted) ($p=0.03$) than non-everted placentomes (Table 4.4 and Figure 4.5).

Following restriction, surface density of trophoctoderm was increased (+33%; $p=0.0007$) (Table 4.5 and Figure 4.6), but the arithmetic mean barrier thicknesses of both trophoctoderm and feto-maternal syncytium were similar to those of control placentomes at mid gestation (Table 4.5 and Figure 4.7).

Following restriction, the total volumes of fetal villi (-32%) and connective tissue within fetal villi (-45%) were substantially reduced (Table 4.6). However, the total volumes of trophoctoderm and feto-maternal syncytium and the total surface area of these epithelia were similar in control and restricted placentas (Table 4.6).

Placental Structure and Fetal Growth

When control or restricted ewes were analysed separately, fetal weight did not correlate significantly with either placental weight or any other structural parameter of the placenta at mid gestation. However, when the control and restricted ewes were analysed together, fetal weight correlated with placentome number ($r=0.54$, $p=0.05$).

In control ewes, placental weight correlated positively with the volumes of fetal villi ($r=0.85$, $p=0.006$), connective tissue core of fetal villi ($r=0.83$, $p=0.006$) and surface area ($r=0.70$, $p=0.04$) (Figure 4.8) as well as other components (Table 4.7). In restricted ewes, placental weight correlated positively with the volumes of fetal villi ($r=0.99$, $p=0.009$), trophoctoderm ($r=0.99$, $p=0.005$) and feto-maternal syncytium ($r=0.99$, $p=0.01$) (Figure 4.8 and Table 4.7). When control and restricted ewes were analysed together, placental weight correlated with the

volumes of all morphometrically determined parameters except that of trophoctoderm (Table 4.7). Notably, the volume of fetal villi is the only component that consistently correlated with placental weight in both control and restricted placentas, whether analysed separately or together (Table 4.7).

Macroscopic Parameters of Placental Development in Late Gestation

Restricted implantation reduced the numbers of placentomes (-60%, $p=0.0001$) and placental weight (-45%, $p=0.0001$) in late gestation (Table 4.1). However, average placentome weight tended to be higher in restricted placentas (+36%, $p=0.07$) compared to control placentas in late gestation (Table 4.1).

Restricted implantation increased the proportion of large placentomes such that approximately 42% of all placentomes in restricted placentas were $\geq 10\text{g}$ compared to only 24% in control placentas (Figure 4.9). Restricted implantation reduced the number of placentomes in the 5 to 9.9g weight range ($p=0.0004$) and significantly increased the number of placentomes in the 15 to 19.9g ($p=0.005$) and the 20 to 24.9g ($p=0.04$) weight ranges (Figure 4.9).

Restricted implantation increased the proportion of everted placentomes present ($75\% \pm 9$ compared to $24\% \pm 14$ in controls) in late gestation ($p=0.0001$) (Table 4.1). In four of seven placentome weight range categories, restricted placentas had significantly more everted placentomes than control placentas ($p<0.05$) (Figure 4.10). In both control and restricted placentas, the proportion in each weight category which were everted appeared to decrease with increasing weight (Figure 4.10).

Placentome morphology at 120 days gestation was characterised before the adoption of "A to D" classification of placentome morphology (Vatnick *et al.*, 1991), hence no further analysis was possible.

Microscopic Parameters of Placental Structure in Late Gestation

Following restricted implantation, "average-size" placentomes were composed of similar proportions of fetal tissue (53%) and maternal tissue (37%) to those in control placentas in late gestation (Table 4.8).

In control placentas, there was a significant effect of placentome size on the volume densities of fetal villi and trophoctoderm, such that "large" placentomes had higher volume densities of fetal villi (+ 10%, $p = 0.01$) and trophoctoderm, (11%, $p = 0.008$) (Table 4.8). In contrast, following restriction "large" and "average-size" placentomes had similar volume densities of fetal villi and trophoctoderm, which were greater than those of similarly sized placentomes in control placentas ($p < 0.05$) (Table 4.8).

Because of the low numbers of "small" placentomes available for analysis from control placentas, the effect of restricted implantation upon placental structure was analysed with and without their inclusion (Table 4.8). Both analyses showed that restricted implantation reduced the volume densities of chorionic membrane and placental capsule, but increased that of fetal villi and trophoctoderm in late gestation (Table 4.8). Shrinkage was also greater following restricted implantation (Table 4.8). When "small" placentomes were included in the analysis, restricted implantation decreased the volume density of connective tissue within fetal villi (Table 4.8). When only "average size" and "large" placentomes were analysed, restricted implantation also reduced the volume densities of chorionic epithelium, haemophagous zone and of maternal tissue in the latter (Table 4.8).

Restricted implantation increased the surface densities of trophoctoderm (+22%, $p = 0.0001$) and feto-maternal syncytium in late gestation (+23%, $p = 0.0001$) regardless of placentome size, (Table 4.9 and Figure 4.11). In contrast, the arithmetic mean barrier thicknesses of both epithelial cell layers were not affected by both restricted implantation and placentome size (Table 4.9 and Figure 4.12).

In late gestation, placentome morphology influenced structure such that eversion increased the volume densities of fetal villi ($p = 0.0008$), and trophoctoderm ($p = 0.04$), and decreased those of

the haemophagous zone ($p=0.0003$), and the maternal tissue within the haemophagous zone ($p=0.03$) (Table 4.10). Eversion also increased the ratio of the volume of fetal villi to maternal crypts ($p=0.02$), and that of total fetal tissue (fetal villi plus chorionic epithelium, excluding the chorionic membrane) to total maternal tissue (maternal crypts + maternal tissue within the haemophagous zone + placentome capsule) ($p=0.007$) (Table 4.10). Placentome morphology did not influence either surface density or the arithmetic mean barrier thicknesses of trophoderm or feto-maternal syncytium (Table 4.11).

Restricted implantation reduced the volumes of all placental components in late gestation (Table 4.12). Within the parenchyma of the placentome, the volumes of the major functional components were substantially reduced:- fetal villi (-38%, $p=0.007$), trophoderm (-34%, $p=0.003$), connective tissue core of fetal villi (-53%, $p=0.06$), feto-maternal syncytium (-40%, $p=0.003$) as was the surface area of the exchange epithelia (-35%, $p=0.0001$) (Table 4.12).

Placental structure and fetal growth

Restricted implantation reduced fetal weight (-14%, $p=0.0004$) and increased the fetal to placental weight ratio (+59%, $p=0.0001$) in late gestation (Table 4.1).

In both control and restricted ewes there was a positive correlation between fetal weight and placental weight in late gestation ($r=0.77$, $p=0.08$, $N=6$ for control ewes compared to $r=0.78$, $p=0.004$, $N=11$ for restricted ewes) (Table 4.13 and Figure 4.13). Fetal weight also correlated positively with major structural parameters of the placenta including the volume of trophoderm ($r=0.82$, $p=0.05$ for control ewes vs $r=0.82$, $p=0.002$ for restricted ewes) and surface area ($r=0.81$, $p=0.05$ for control ewes vs $r=0.77$, $p=0.006$ for restricted ewes) (Table 4.13 and Figure 4.13). In addition, in the restricted ewes, fetal weight correlated with other placental parameters like placentome number ($r=0.65$, $p=0.03$), the volumes of fetal villi ($r=0.83$, $p=0.002$), of connective tissue core of fetal villi ($r=0.79$, $p=0.004$), and of feto-maternal syncytium ($r=0.71$, $p=0.01$) (Table 4.13 and Figure 4.13).

In both control and restricted ewes, placental weight correlated positively with the volumes of fetal villi ($r=0.86$, $p=0.03$ for control ewes vs $r=0.92$, $p=0.0001$ for restricted ewes) and

connective tissue core of fetal villi ($r=0.89$, $p=0.02$ for control ewes vs $r=0.91$, $p=0.0001$ for restricted ewes) and with surface area ($r=0.92$, $p=0.01$ for controls vs $r=0.82$, $p=0.002$ for restricted ewes) (Table 4.14). In addition, restricted implantation induced a significant correlation between placental weight and placentome numbers ($r=0.74$, $p=0.009$) and the volumes of trophoctoderm ($r=0.87$, $p=0.0004$) and feto-maternal syncytium ($r=0.93$, $p=0.0001$) (Table 4.14).

In control placentas, the volume density of fetal villi correlated positively with both the volume density of trophoctoderm ($r=0.85$, $p=0.0001$) and surface density ($r=0.73$, $p=0.003$), (Table 4.15). The volume density of trophoctoderm also correlated positively with surface density in control placentas ($r=0.70$, $p=0.005$) (Table 4.15). In restricted placentas, these positive correlations persisted but weakened (Table 4.15).

4.3.2. Effect of restriction of implantation on ontogeny of placental structural development

Macroscopic parameters in restricted placentas at 90 and 120 days gestation

There were no significant differences in placental weight, placentome number and mean placentome weight between the restricted placentas at 90 and 120 days gestation (Table 4.1). The proportion of everted placentomes (in terms of placentome numbers and the weight of the placenta composed of everted placentomes) was similar in the restricted placentas at the two gestational ages (Table 4.1). In both control and restricted sheep, there was a similar increase in fetal weight between 90 and 120 days (3.6-fold in restricted ewes compared to 3.7-fold in controls) (Table 4.1). However, the f:p ratio increased to a greater extent in restricted ewes ($\times 4.2$) compared to control ewes ($\times 3.3$) ($p<0.05$) (Table 4.1).

Within the restricted placentas at 90 and 120 days gestation, there were no significant differences in the proportions of placentomes present within all other weight ranges except the 30 to 35g weight range category. At 90 days gestation, restricted placentas had a significantly higher proportion of placentomes within this weight range ($3.9 \pm 0.9\%$ compared to $0.8 \pm 0.5\%$ at 120 days) ($p=0.005$) (Refer to Figures 4.1 and 4.9). Similarly, within the restricted placentas, there were no significant ontogenic differences in the distribution of everted

placentomes in all weight range categories except the 30 to 35g weight range. At 90 days gestation, $63 \pm 13\%$ of placentomes present within this weight range were everted compared to only $14 \pm 8\%$ at 120 days gestation ($p=0.003$) (Refer to Figures 4.3 and 4.10).

Microscopic parameters of placental structure

The ontogenic structural development within the restricted placentas was qualitatively similar to that in controls although generally less extensive. Thus following restriction of implantation, the volume density of chorionic epithelium ($+46\%$, $p=0.03$) and trophoctoderm ($+18\%$, $p=0.02$) increased while that of the connective tissue at the core of fetal villi (-39% , $p=0.0001$) and placentome capsule (-32% , $p=0.007$) decreased between 90 and 120 days gestation (Table 4.16 and Figure 4.14).

Between 90 and 120 days gestation, the surface density of trophoctoderm increased in the restricted placentas ($+47\%$, $p=0.0001$) but to a lesser extent than normal (Table 4.17 and Figure 4.15). In contrast, the barrier thicknesses of trophoctoderm (-28% , $p=0.003$) and fetomaternal syncytium (-43% , $p=0.0001$) in restricted placentas decreased to a greater than normal extent (Table 4.17 and Figure 4.16).

Comparison of total placental volumes of components, surface area and relative surface area

The relative surface area (the amount of surface area supplying a unit weight of fetus and placenta) decreased to a greater than normal extent (-51% , $p=0.0001$) with increasing gestation following restricted implantation (Table 4.18). In contrast, the total volumes of all other components and surface area for exchange in restricted placentas did not change with gestational age (Table 4.18).

4.4 DISCUSSION

Experimental restriction of implantation by pre-pregnancy excision of most endometrial caruncles, reduced the number of placentomes and placental weight in mid and late gestation. The latter occurred despite an increase in the range of placentome weights to much greater

weights and an increase in mean individual placentome weight at least in mid gestation, following restricted implantation.

A reduction in placental weight following restricted implantation despite increases in the numbers of large placentomes has been previously observed in late gestation (Robinson *et al.*, 1979, Alexander, 1964b). The present study has shown that the compensatory growth of individual placentomes in response to restricted implantation has occurred by 0.6 gestation and is already insufficient to maintain placental weight. Characterisation of placental development following restricted implantation to earlier stages of gestation may reveal when compensatory growth is initiated and hence the stimulus and mechanisms involved.

Restriction of implantation enhanced placentome eversion as previously described in late gestation. In addition, the present study has shown that increased placentome eversion was present by mid gestation. Either the stimulus that induces the eversion process is amplified, or occurs earlier in gestation in response to restricted implantation. While everted placentomes were present in all weight ranges normally and following restriction, fewer of the heavier placentomes appeared to exhibit the everted morphology. This suggests that placentomes that are able to grow substantially larger may not need to evert, or that the stimulus which promotes the growth of placentomes is separate from that which influences placentome eversion. One factor implicated in the latter process is progesterone (Robinson *et al.*, 1976; McNeil *et al.*, 1991).

The external appearance of an everted placentome suggests an extended haemophagous zone which covers the whole external surface of the placentome. However, the inspection of vertical sections through placentomes revealed that in non-everted placentomes, a substantial haemophagous zone is present but inverted and not visible externally. Thus morphometric analysis showed that in mid gestation, regardless of treatment, everted placentomes only have about half the size of haemophagous zone as the non-everted placentomes. In late gestation, this component is also significantly reduced to about 55% in controls and 58% following restriction in everted placentomes when compared to that of non-everted placentomes. The maternal tissue within the haemophagous zone which is the source of the extravasated

maternal blood is also reduced to half the size of that in non-everted placentomes. Although the volume density of chorionic epithelium, which is involved in the phagocytosis of the maternal erythrocytes present within the haemophagous zone to provide iron to the fetus (Steven *et al.*, 1981), is not significantly reduced, a reduced haemophagous zone may be connected to the hypoxic state of the IUGR fetus which is commonly associated with reduced placental size.

The everted morphology has previously been reported to raise fetal tissue:maternal tissue ratio in bovine placentomes (Andersen, 1926). The present study has for the first time demonstrated a similar phenomenon in ovine placentomes in late gestation but not in mid gestation. Non-everted placentomes in both control and restricted placentas at 120 days gestation were found to have a 1.12:1 ratio of fetal villi to maternal crypts suggesting a predominance of fetal over maternal tissue and in agreement with previous studies in which ratios of 1.36:1 (Mitchell and Stratford, 1987) and 1.3:1 (Caton *et al.*, 1984) were reported with no specification of placentome morphology. In the present study, the ratio of 1.12:1 in non-everted placentomes was significantly altered to 1.35:1 (fetal villi:maternal crypts) in everted placentomes, suggesting a relative increase of about 20% in fetal tissues. A similar finding was observed when the proportion of all fetal tissues combined (excluding the chorionic membrane) was compared with that of all maternal tissues combined. However, this study has not established the causes of placentome eversion. The 20% increase in the relative abundance of fetal compared to maternal tissue does not seem to be sufficient to account for the reversal in morphology, unless if accompanied by regional variations throughout the placentome in the relative abundances of fetal villi and maternal crypts and their components. Following restricted implantation the volume density of trophoctoderm increases, while that of the connective tissue core decreases. However, the increase in trophoctoderm volume density is greater in everted placentomes than in non-everted ones. In contrast, all placentomes regardless of morphology have similar volume densities of the connective tissue core. This suggests that placentome eversion may be related to these placentomes having a higher ratio of trophoctoderm to connective tissue. In general, at 120 days gestation, the everted morphology was consistently associated with a reduced volume of the haemophagous zone and the maternal tissue within it, but with an increased volume of

fetal villi. A study of the regional distribution of the volume densities of these components in everted compared to non-everted placentomes may help to elucidate the mechanism(s) of placentome eversion.

Progesterone promotes placentome eversion in early to mid pregnancy in sheep (Alexander and Williams, 1966; Rickets and Flint, 1980). The removal of endometrial caruncles from non-pregnant ewes increases the concentration of progesterone in jugular venous plasma of the ewes (mean concentration, 5.9 ng/ml, range, 4.3-9.7 ng/ml in seven cycles on days 8 to 13 of the estrus cycle), when compared with sham-operated control ewes (mean progesterone concentration, 2-3 ng/ml in six cycles) Robinson *et al.*, (1976). Also in sheep, hemi hysterectomy carried out on the 5th day of pregnancy, prior to blastocyst migration to the uterus, increased progesterone concentrations in maternal peripheral blood (Caton *et al.*, 1984). Therefore it is possible that in ewes with experimental restriction of implantation, early placental development occurs in an environment of greater than normal progesterone abundance.

The volumetric proportions of the placentome occupied by fetal villi were similar in control and restricted placentas in mid gestation but the cellular constituents of the fetal villi were different. In control placentas, the villi contained trophoctoderm and connective tissue in the ratio approximating to 1:1.4. As a result of restricted implantation this ratio reduced to 1:1.3 due to a significant increase in the volume proportion of the villi that was occupied by trophoctoderm. In contrast, the volume density at the core of the villi reduced by about 18% in restricted placentas although this did not attain statistical significance. At 120 days gestation, placentomes of restricted ewes were observed to contain about 17% more fetal villi and 27% more trophoctoderm than control placentomes while the volume density of fetomaternal syncytium did not change significantly. In addition, there was about 12% less connective tissue forming the core of the fetal villi in the placentomes from the restricted placentas, hence the villi were that much thinner in response to restricted implantation. Thus the changes of the constituent components of the villi suggest that in response to restricted implantation, fetal villi become thinner due to the loss of connective tissue. Hence the diffusion distance between fetal and maternal blood should be reduced following restricted

implantation. Thinning of villi has previously been demonstrated as an adaptive response in placental pathological conditions and in conditions of substrate insufficiency both in sheep and other species. Studies carried out in humans revealed reduced proportions of connective tissue in placentas of pregnancies complicated by pre-eclampsia and in those associated with SGA infants (Boyd and Scott, 1985). The decrease in connective tissue volume reduced parenchymal volume, despite an increase in the abundance of fetal capillaries (Boyd and Scott, 1985). Stereological analysis of the ovine placenta in pregnancy toxemia revealed a significant reduction of the villous volume density due to a combined decrease in that of the fetal mesenchymal cells in the core of the villi and of the trophoblast bordering the villi, at 120 days gestation (Mitchell and Stratford, 1987). In these placentas, maternal crypts predominated over fetal villi within the zona intima, as a result of combined increases in the volume densities of feto-maternal syncytium, connective tissue of the crypts and the vessels within them (Mitchell and Stratford, 1987). The fact that the volume of feto-maternal syncytium increased in response to pregnancy toxemia may suggest enhanced binucleate cell migration and fusion with the uterine epithelial cells. However, this study used too few numbers of ewes which may explain the absence of statistical significance in their results.

At mid gestation, the increase in trophoblast volume in the restricted placentas was paralleled by a relatively small but significant increase in the volume of feto-maternal syncytium (from 28% of maternal crypts in control placentomes to 30% in restricted placentas). The greater increase in the volume of trophoblast than that of feto-maternal syncytium may be compatible with the growth of the latter being in part determined by that of the former due to the migration of binucleate cells from the feto-maternal syncytium and their fusion with uterine epithelial cells to form trophoblast (Wooding, 1992).

Adaptational growth response to perturbation in other species also involves trophoblast cells but most of the information relates to more advanced stages of gestation than mid gestation. Histomorphometric analyses of human placentas, in maternal pre eclampsia, delivered by caesarean section at 40 weeks gestation, revealed a significant loss of villous connective tissue and the reduced size of the villi accompanied by a relative increase in syncytiotrophoblast and inter villous space volume (Teasedale, 1985). These factors could contribute towards the

improvement of the transplacental transfer of respiratory gases and nutrients (Teasedale, 1985). Other studies in human showed that the volumetric composition of the term placentas at high altitude had significantly greater volumes of trophoblast and stroma in their villi than placentas at low altitude (Jackson *et al.*, 1987). In light and electron microscopy studies of the effects of smoking on the human placentas, villous cytotrophoblast proliferation was evident (Van Der Veen and Fox, 1982, Jauniaux and Burton, 1990). This has been regarded as an adaptive response to enable syncytial repair since the syncytiotrophoblast undergoes necrosis in placentas of smokers (Jauniaux and Burton, 1990). Adaptive placental growth following the ligation of placental bridging vessels in rhesus monkeys also involves trophoblast cells (Novy *et al.*, 1981). Following ligation performed in mid-pregnancy between days 80 and 90 of gestation, the secondary placental disc atrophied by necrosis of all tissues except the syncytiotrophoblast. The primary disc hypertrophied and became heavier than the combined average weight of a primary plus a secondary disc. In the ligated placentas, there was a strong positive association between the DNA concentration (which continued to rise until term) and the amount of monkey chorionic somatomammotropin (mCS) (whose plasma concentration also rose steadily until term) which was not significantly altered by ligation. Since mCS secretion is primarily controlled by the mass of functioning trophoblast the results suggested the compensatory process had maintained trophoblastic elements.

In the present study, the alterations of the placentome components following restricted implantation were not significantly affected by placentome size. However, when placentomes were classified on the basis of their gross placentome morphology, in both control and restricted placentas the everted placentomes had greater proportions occupied by fetal villi and trophoctoderm than non-everted placentomes. The difference in the volume densities of trophoctoderm between placentomes of the two morphologies was much reduced in restricted placentas. In control placentas at mid and late gestation, everted placentomes had about 61 and 29% more trophoctoderm than non-everted placentomes respectively. In restricted placentas, everted placentomes had only 8% more trophoctoderm than non-everted placentomes at both gestational ages. This appears to suggest that the advantages of placentome eversion may diminish when placental size is restricted since the non-everted

placentomes also acquire a similar cellular structure to that of the everted placentomes in as far as the volume density of trophoctoderm is concerned.

Restricted implantation significantly increased the surface densities of the two epithelial cell layers at the feto-maternal interface (trophoctoderm and feto-maternal syncytium) but did not alter their arithmetic mean barrier thicknesses. This occurred in all placentomes regardless of size and gross morphology. However, unaltered arithmetic mean barrier thickness may not necessarily suggest the absence of an improvement in the total capacity for gaseous exchange, since Jackson *et al.*, (1985) found that for a constant arithmetic mean barrier thickness, highland placentas demonstrated great regional variation in thickness. This reduces harmonic mean thickness (not measured in the present study) and improves the gas conductance of the membrane. Harmonic mean thickness plays a principal role in the determination of placental diffusion for oxygen (Jackson *et al.*, 1985). Reduced proportion of fetal villi occupied by connective tissue in restricted placentas suggests a reduction in the barrier thickness separating maternal and fetal blood systems. Thus, it appears that the loss of connective tissue is the means by which the membrane diffusiveness is enhanced, concomitant with the increase in the surface area across the feto-maternal interface. Analysis of vascularisation of the fetal component should determine whether in sheep, the colonisation of the trophoctoderm by fetal capillaries that is normally observed after 100 days gestation (Björkman, 1965; Davies and Wimsatt, 1966; Mitchell, 1982) occurs earlier in restricted placentas.

The everted morphology was accompanied by increases in volume densities of trophoctoderm but not of feto-maternal syncytium and surface density of the epithelial cells. This may indicate that the stimulus which induces the overgrowth of fetal tissue over maternal tissue may be separate from that which induces the formation of feto-maternal syncytium resulting in further increases in surface density. Furthermore, the fact that within both control and restricted placentas the volume densities of fetal villi and trophoctoderm and also surface density positively correlated with each other but not with the volume density of feto-maternal syncytium may be an indication for the control of the volume density of this component being separate from that which controls the volume densities of fetal villi, trophoctoderm and surface density.

In absolute terms, by mid gestation, placental weight was significantly reduced by restricted implantation and so were the absolute volumes of most of the morphometrically determined components. However, the volumes of trophoctoderm, feto-maternal syncytium and also surface area were maintained at comparable levels with those of controls and hence fetal growth was not negatively affected. This shows that although the compensatory growth is unable to maintain total placental weight and the volumes of most components, it is nevertheless sufficient to maintain volumes of key functional components and hence placental function.

In late gestation, restricted placentas had significantly reduced absolute volumes of all morphometrically determined components. Thus, despite the increases in the volume densities of trophoctoderm and the surface density of the epithelial cells that were evident in individual placentomes, restricted placentas had significantly less total placental trophoctoderm and surface area when compared to controls. The ontogenic study of placentome components within restricted placentas between 90 and 120 days gestation revealed reduced increases in the volume densities of trophoctoderm and chorionic epithelium. The surface densities of the epithelial cells also exhibited an increase that was 13% less than that which occurred normally during the ontogeny of control placentas (chapter 3). Consequently, within the restricted placentas, placental surface area increased only by 26% compared to the 70% increase in the normal ontogeny between mid and late gestation. Thus at 120 days gestation placental surface area in restricted placentas ($6.51 \pm 0.5 \text{ m}^2$) was more comparable to that in control placentas at 90 days gestation ($5.9 \pm 0.4 \text{ m}^2$) than that at 120 days gestation ($10.07 \pm 0.8 \text{ m}^2$) causing a much greater decrease in the relative surface area (-51%) compared to that which occurred normally (-35%). This appears to suggest that within the restricted placentas, the extent of compensatory growth that occurs within the internal structure of the individual placentomes reduces as gestation progresses and becomes insufficient to maintain control volumes of the functional parameters by 120 days gestation. Thus following restricted implantation, a unit weight of feto-placental mass is supplied by a significantly reduced surface area in late gestation than occurs normally.

Morphometric studies of human placentas associated with IUGR have also demonstrated significant deficits in the volumes of functional components and surface area for diffusion (Aherne and Dunhill, 1966, Teasedale, 1984, Shen, 1992, Yu, 1992). In addition, the average placentas of women living at high altitude (3600m) contains smaller overall volume of villi, length, and a less extensive villous exchange area, implying that highland villi suffer a significant reduction in growth of functional components compared to their lowland counterparts (Jackson *et al.*, 1987a).

Restricted implantation did not reduce fetal weight by mid gestation. In addition, the reduction in fetal growth observed in late gestation in this study (-14%) was proportionately less than that of placental weight (-45%). In a previous study in which about 70% of uterine caruncles were removed prior to pregnancy, the number of placentomes that formed was about half that of normal twins and a third that of singletons (Mellor *et al.*, 1977). However, birthweights were only reduced to the level of normal twins (Mellor *et al.*, 1977). This demonstrates that the restricted placentas are able to support bigger fetuses in proportion and that these placentas are more functionally efficient than control placentas. In vivo measurements of placental substrate transfer in sheep suggest that the small placenta in restricted ewes has a reduced rate of substrate consumption, but an increased transfer capacity per gram of tissue for oxygen and glucose, compared to the normally grown placenta (Owens *et al.*, 1989). In addition, accelerated development in restricted placentas may have achieved an increased coefficient of substrate extraction, as occurs in late gestation in sheep (Owens *et al.*, 1987 a,b) and several other experimental animal species including goat, sow, guinea pig, rabbit, cat (Flexner and Gellhorn, 1942) rat (Rosso, 1974) and also in humans (Flexner *et al.*, 1948).

Comparison of data obtained for fetal growth at mid and late gestation suggests that in late gestation fetal growth is reduced as a consequence of the reduction in placental function in restricted placentas. In a previous study in sheep, placental ablation which was achieved by manual separation of placentomes into fetal and maternal tissues at 80 days gestation caused a 20% reduction in placental weight (Mellor *et al.*, 1977). This was responsible for inducing a significant reduction in fetal weight in late gestation (Mellor *et al.*, 1977). In rhesus monkeys,

Myers *et al.*, (1971) produced a 25% deficit in fetal growth at term by the ligation of placental bridging vessels after 110 days gestation. In contrast, results obtained by Novy *et al.*, (1981) who performed a similar type of vessel ligation in rhesus monkeys in mid-pregnancy found that the compensatory growth which was induced in the primary placental disc was sufficient to maintain placental function such that fetal weights at term were near normal. These results suggest that the placental compensatory growth becomes insufficient to maintain placental function after mid gestation although the cellularity of placentas in rhesus monkey continues to increase until term albeit accompanied by a reduction in mean cell size (Novy *et al.*, 1981). The explanation for the insufficiency of the compensatory growth in such placentas is not known although it is possible that it may be linked to placental cellular proliferation rate reducing with increasing gestation (Winick, 1971). In sheep however, the proliferative phase of placental growth stops around mid gestation (Kulhanek *et al.*, 1974, Vatnick and Bell, 1992). Therefore significant damage of placental structure occurring at or after mid gestation is likely to reduce placental functional capacity permanently.

At mid gestation, there was no significant correlation between fetal weight and placental weight or with the volumes of placental functional components in either control and restricted ewes. This suggested that even when placental size is restricted such as occurs following the removal of most implantation sites, placental parameters may not limit fetal growth at this stage in gestation. However, in late gestation, in control animals, fetal weight was found to correlate significantly with the placental volume of trophoctoderm and with surface area, but not with placental weight. The weight of restricted fetuses also correlated with placental weight, placentome numbers, the volumes of fetal villi and its connective tissue core, the volume of feto-maternal syncytium as well as that of trophoctoderm and also with surface area. These results suggest that normally, in late gestation, the major determinants of fetal growth are the volume of trophoctoderm and the amount of surface area across the feto-maternal interface. However, following restricted implantation, the restricted fetuses are at a greater risk than those of controls such that their growth critically depends on adequate functioning of all placental parameters. These results agree with those previously reported in sheep by Harding *et al.*, (1985) and (Caton *et al.*, 1984) in which carunclectomy prior to pregnancy as in the

present study, and hemi hysterectomy on day 5 of pregnancy, prior to blastocyst migration to the uterus, did not induce a significant correlation between fetal weight and placental weight prior to 125 days gestation. In contrast, Stegeman (1974) reported a positive correlation between fetal weight and total villous surface area by as early as 49 days of gestation. It is notable that Stegeman determined fetal age by fetal size which may have introduced undetermined errors in the results. However, Alexander (1964) also reported a weak but significant correlation ($r = 0.35$) between fetal weight and placental weight at 90 days gestation in sheep.

In late gestation, in both control and restricted ewes placental weight correlated positively with absolute volumes of the morphometrically determined parameters i.e. fetal villi, connective tissue core of fetal villi and with surface area. In addition, in the restricted ewes, placental weight also correlated with placentome numbers and the volumes of trophoblast and fetomaternal syncytium. The fact that surface area correlated strongly with both fetal weight and placental weight suggests that measuring placental weight is a quick and reliable way of measuring placental function.

4.5 SUMMARY

Experimental restriction of implantation induces compensatory growth within the placenta that is evident in mid gestation. Macroscopically, the compensatory growth involves enhanced individual placentome size and accelerated eversion of their morphology. Internally, there are increases in volume densities of fetal villi, and trophoblast to a greater extent and also fetomaternal syncytium to a lesser extent. Increases in surface densities are also evident. This seems to pinpoint the trophoblast as the major target for the stimulus that induces compensatory growth to improve efficiency of placentomes of a variety of sizes but especially those exhibiting the everted morphology.

Despite the compensatory growth, placental weight, and hence absolute volumes of several components are significantly reduced in mid gestation. In late gestation, most of the components are significantly reduced. Reduced volume of the connective tissue core of fetal

villi may have an overall effect of reducing the barrier thickness separating maternal and fetal bloods and enhance membrane diffusibility of substrates. In contrast, at the feto-maternal interface, volume and surface densities of trophoblast and feto-maternal syncytium are increased while the arithmetic mean barrier thicknesses of the epithelial tissues remain unaltered.

In mid gestation, the increases in volume and surface densities of the epithelial cells are sufficient to maintain total volumes of placental key functional components and total surface area to almost control levels. Therefore, overall placental function is promoted and hence fetal growth is maintained. In contrast, in late gestation, compensatory growth is insufficient to maintain all placental parameters and hence placental function is jeopardised. Thus the restricted placenta limits fetal growth and contributes significantly towards intrauterine growth retardation. However, the presence of high f:p ratios due to total placental weight and the volumes of its components being reduced to a greater extent than fetal weight suggests that the restricted placentas are more functionally efficient than those of controls.

In late gestation, placental weight correlates very highly with the morphometrically determined volumes of its functional components. Also the relationship between placental weight and fetal weight is similar to that of the morphometrically determined functional components and fetal weight in both control and restricted ewes. This suggests that at this stage in gestation, placental weight is a reliable index of function in normal pregnancy or that which is complicated by limited placental size.

TABLES AND FIGURES FOR CHAPTER 4

TABLE 4.1 Macroscopic parameters in well-fed control and experimentally restricted ewes bearing singletons at 90 and 120 days gestation

TREATMENT	NUMBER CARUNCLES REMOVED	NUMBER CARUNCLES LEFT	NUMBER PLACENTOMES FORMED	MEAN PLACENTOME WEIGHT (g)	TOTAL PLACENTAL WEIGHT (g)	% TOTAL PLACENTOME NO. EVERTED	% TOTAL PLACENTAL WEIGHT EVERTED	FETAL WEIGHT (g)	F:P RATIO
90 DAYS									
Control (N=9)	-	-	74 ± 6 ^a	7.5 ± 1.0 ^a	515 ± 42 ^a	11.3 ± 10 ^a	12.9 ± 12.5 ^a	716 ± 33 ^a	1.48 ± 0.16 ^a
Restricted (N=4)	75 ± 9 (N=3)	15 ± 0.3 (N=3)	33 ± 6 ^b	11.5 ± 1.4 ^b	340 ± 27 ^b	49 ± 20 ^b	54.4 ± 18.3 ^b	634 ± 46 ^a	1.87 ± 0.08 ^a
120 DAYS									
Control (N=6)	0	-	74 ± 2 ^a	7.4 ± 0.8 ^{ac}	545 ± 35 ^a	24 ± 14 ^{ab}	26 ± 15 ^{ab}	2654 ± 77 ^b	4.94 ± 0.26 ^b
Restricted (N=11)	67 ± 3	16 ± 1	30 ± 2 ^b	10.1 ± 0.7 ^{bc}	297 ± 22 ^b	75 ± 9 ^{bc}	76 ± 9 ^{bc}	2255 ± 97 ^c	7.88 ± 0.44 ^c

Values expressed as mean ± SE

Values with different letters are significantly different (p<0.05; LSMeans specific contrasts)

N= number of ewes

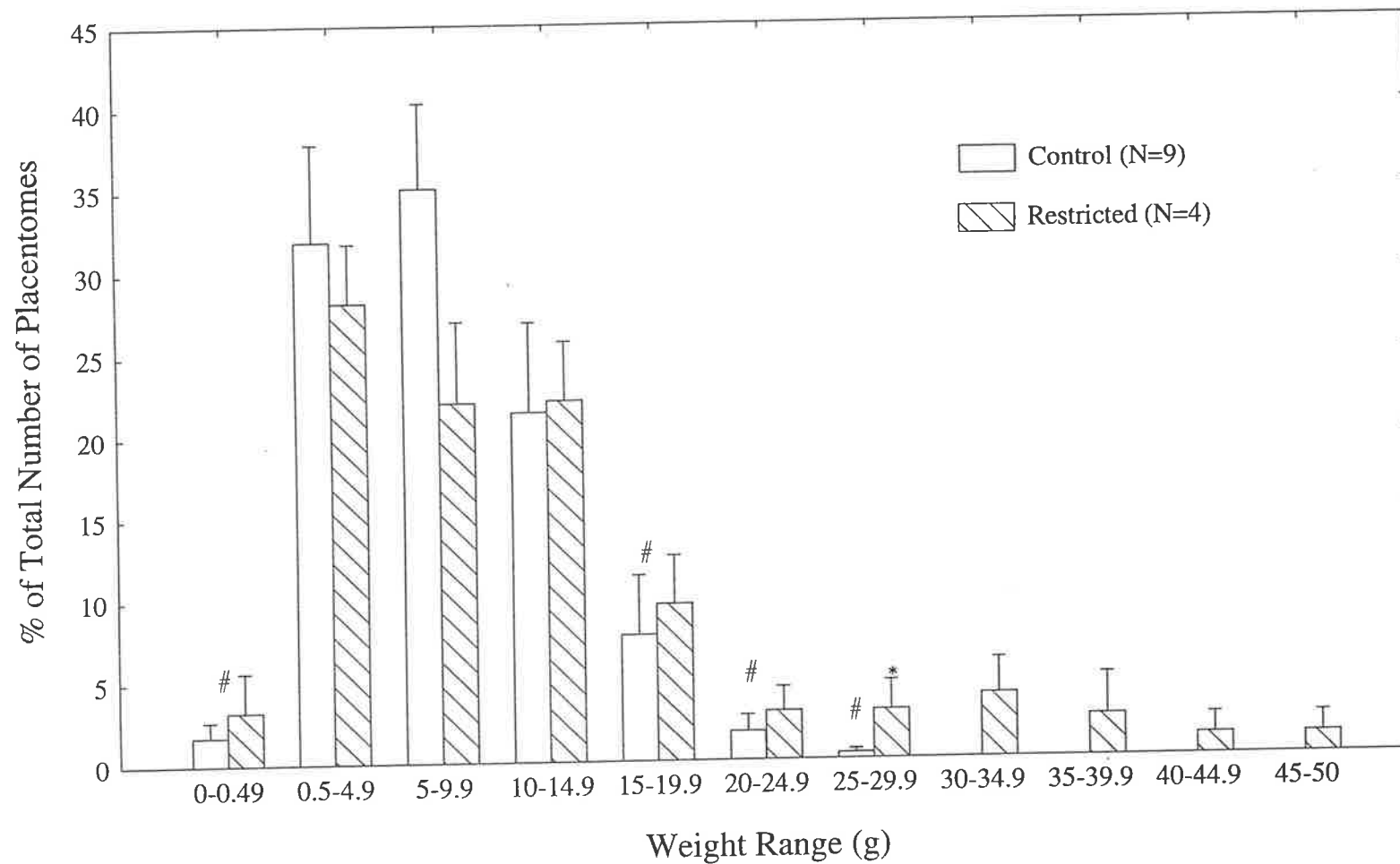


Figure 4.1 Placentome distribution within weight range categories in control (□) and restricted (▨) placentas at 90 days gestation (ANOVA; between factor analysis, testing effect of treatment; * $p < 0.05$; within factor analysis, testing differences between weight range categories and the model weight range category; 5-9.9g, # $p < 0.01$).

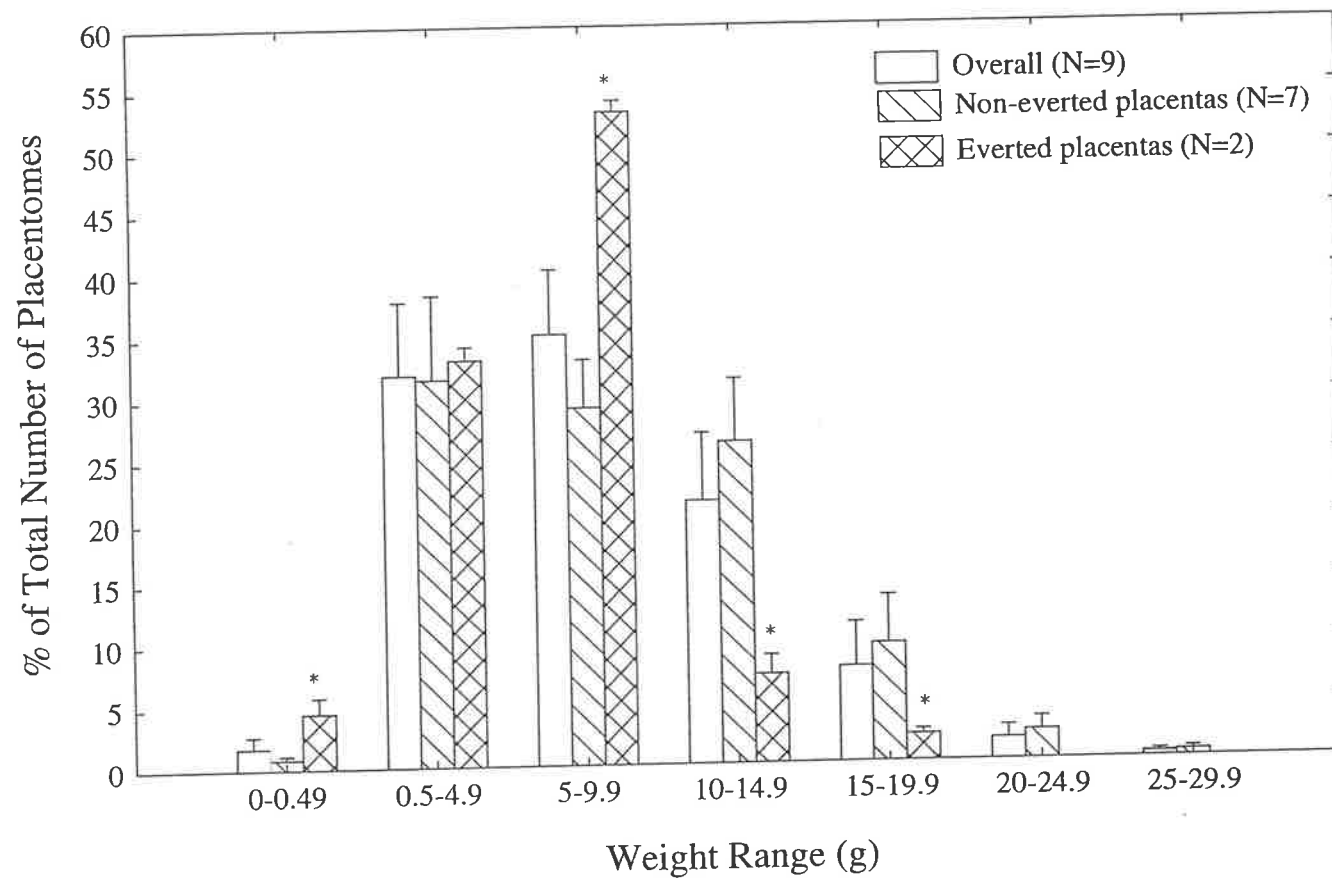


Figure 4.2 Placentome distribution within weight range categories in all control placentas combined (□), in control placentas without eversion (▨), and in control placentas with eversion (▩) at 90 days gestation (* $p < 0.05$; ANOVA; significant difference from overall distribution and from distribution in non-everted placentas).

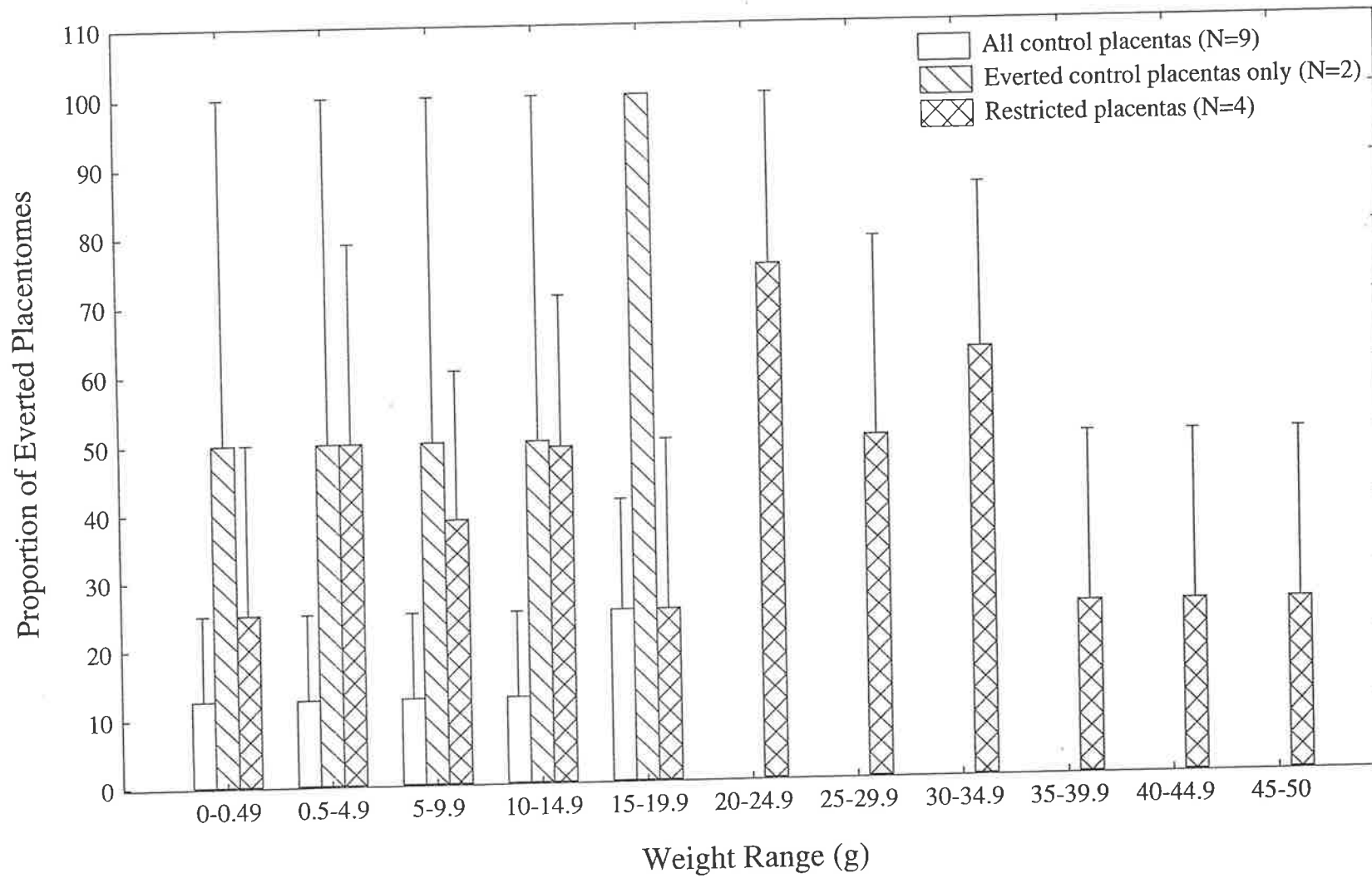


Figure 4.3 The proportion of everted placentomes within weight range categories in all control placentas combined (), only in control placentas with eversion (), and in restricted placentas () at 90 days gestation.

TABLE 4.2 Volume densities of placentome components in control and experimentally restricted placentas of singletons at 90 days gestation

TREATMENT	CONTROL (N=9)			RESTRICTED (N=4)		LSMeans specific comparisons (p=)
	"Small"	"Average-size"	"Large"	"Average-size"	"Large"	
<i>PLACENTOME SIZE</i>						
Weight (g)	2.9 ± 0.8 (n=2)	8.2 ± 0.5 (n=8)	16.3 ± 1.4 (n=7)	7.4 ± 1.2 (n=3)	28.1 ± 6.0 (n=5)	
<i>STRUCTURAL PARAMETERS</i>						
Chorionic membrane	0.107 ± 0.005	0.107 ± 0.011	0.115 ± 0.013	0.097 ± 0.012	0.090 ± 0.024	ns
Chorionic epithelium	0.024 ± 0.011	0.031 ± 0.004	0.022 ± 0.005	0.023 ± 0.003	0.022 ± 0.007	ns
Haemophagous zone (HMZ)	0.018 ± 0.01	0.069 ± 0.015	0.050 ± 0.017	0.024 ± 0.01	0.041 ± 0.017	ns
Maternal tissue in HMZ	0.007 ± 0.001	0.022 ± 0.004	0.021 ± 0.008	0.009 ± 0.001	0.012 ± 0.005	ns
Fetal villi	0.450 ± 0.027	0.363 ± 0.020	0.395 ± 0.032	0.387 ± 0.019	0.394 ± 0.019	ns
Trophectoderm	0.200 ± 0.046	0.162 ± 0.014	0.142 ± 0.025	0.224 ± 0.005	0.224 ± 0.013	0.0001
Connective tissue core	0.256 ± 0.074	0.199 ± 0.026	0.236 ± 0.034	0.163 ± 0.014	0.206 ± 0.015	ns
Maternal crypts	0.284 ± 0.022	0.278 ± 0.021	0.271 ± 0.023	0.351 ± 0.021	0.292 ± 0.034	0.04
Feto-maternal syncytium	0.081 ± 0.004	0.077 ± 0.006	0.072 ± 0.008	0.104 ± 0.01	0.090 ± 0.010	0.006
Shrinkage spaces	0.022 ± 0.003	0.042 ± 0.012	0.039 ± 0.010	0.037 ± 0.014	0.078 ± 0.027	ns
Placentome capsule	0.098 ± 0.010	0.082 ± 0.011	0.091 ± 0.008	0.076 ± 0.009	0.076 ± 0.011	ns
Fetal villi/ Maternal crypts	1.604 ± 0.219	1.359 ± 0.127	1.538 ± 0.200	1.116 ± 0.122	1.44 ± 0.208	ns
Total fetal tissue/Total maternal tissue	1.224 ± 0.084	1.079 ± 0.105	1.137 ± 0.134	0.949 ± 0.089	1.156 ± 0.154	ns

Values are mean ± SE; ANOVA; lsmeans specific comparisons excluding small placentomes; ns=no significant difference, N=number of ewes, n=number of placentomes morphometrically analysed; Total fetal tissue= chorionic epithelium + fetal villi (excluding chorionic membrane); Total maternal tissue= maternal crypts + maternal tissue in HMZ + placentome capsule

TABLE 4.3 Volume densities of placentome components in control and experimentally restricted placentas of singletons at 90 days gestation

TREATMENT	CONTROL (N=9)		RESTRICTED (N=4)		LSMeans specific comparisons
<i>PLACENTOME SIZE</i>	<i>"Average-size"</i>	<i>"Large"</i>	<i>"Average-size"</i>	<i>"Large"</i>	
Weight (g)*	8.2 ± 0.5 (n=8)	16.3 ± 1.4 (n=7)	7.4 ± 1.2 (n=3)	28.1 ± 6.0 (n=5)	(p=)
<i>COMPONENT</i>					
Chorionic membrane	0.111 ± 0.009		0.096 ± 0.012		ns
Chorionic epithelium	0.026 ± 0.004		0.024 ± 0.005		ns
Haemophagous zone (HMZ)	0.060 ± 0.008		0.042 ± 0.010		ns
Maternal tissue in HMZ	0.021 ± 0.005		0.012 ± 0.006		ns
Fetal villi	0.378 ± 0.012		0.386 ± 0.016		ns
Trophectoderm	0.152 ± 0.010		0.229 ± 0.013		0.0001
Connective tissue core	0.216 ± 0.013		0.178 ± 0.016		ns
Maternal crypts	0.275 ± 0.012		0.316 ± 0.016		0.04
Feto-maternal syncytium	0.074 ± 0.054		0.099 ± 0.007		0.006
Shrinkage spaces	0.040 ± 0.008		0.058 ± 0.010		ns
Placentome capsule	0.086 ± 0.006		0.069 ± 0.008		ns
Fetal villi/Maternal crypts	1.443 ± 0.082		1.288 ± 0.106		ns
Total fetal tissue/	1.106 ± 0.071		1.072 ± 0.092		ns
Total maternal tissue					

Values are expressed as least squares means ± SE; * Values are means ± SE;
ANOVA; LSMeans specific comparisons; excluding small placentomes; ns=no significant difference;
N=number of ewes; n=number of placentomes morphometrically analysed;
Total fetal tissue= chorionic epithelium + fetal villi (excluding chorionic membrane);
Total maternal tissue= maternal crypts + maternal tissue in HMZ + placentome capsule

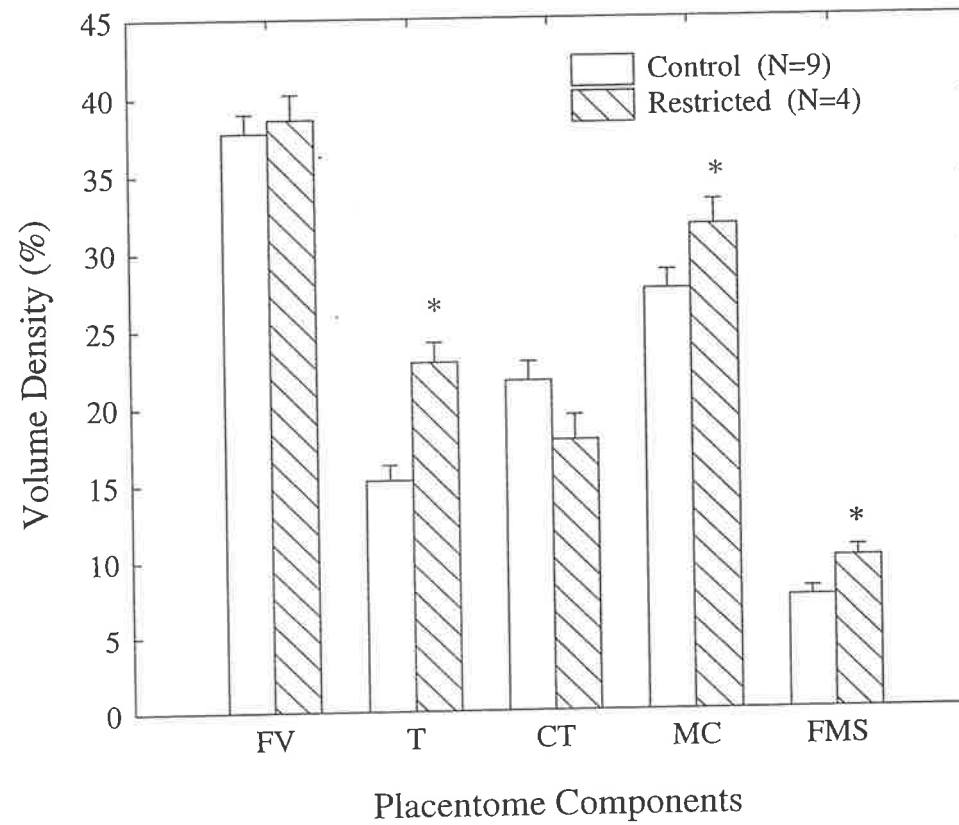


Figure 4.4 Volume densities of placentome components in control (□) and restricted (▨) placentas at 90 days gestation: Fetal villi (FV), Trophoctoderm (T), Connective tissue core of fetal villi (CT), Maternal crypts (MC) and Feto-maternal syncytium (FMS). ANOVA; L S Means Comparisons, * $p < 0.05$.

TABLE 4.4 Relationship between placentome morphology, volume density of components, and fetal to maternal tissue ratio in placentas of control and restricted ewes bearing singletons at 90 days gestation

TREATMENT	CONTROL (N=9)		RESTRICTED (N=4)		Overall Effect of Morphology (p=)
PLACENTOME MORPHOLOGY:	NON-EVERTED (n=16)	EVERTED (n=1)	NON-EVERTED (n=2)	EVERTED (n=6)	
Placentome weight (g)	11.5± 1.3	2.1	30± 20.4 (50.4; 9.56)	17.1± 4	
COMPONENT					
	0.111 ± 0.008	0.101	0.124 ± 0.021 (0.145; 0.104)	0.082 ± 0.018	ns
Chorionic epithelium (CEP)	0.026 ± 0.003	0.035	0.034 ± 0.006 (0.040; 0.028)	0.019 ± 0.004	ns
Haemophagous zone HMZ)	0.057 ± 0.011	0.028	0.056 ± 0.041 (0.097; 0.015)	0.028 ± 0.009	ns
Maternal tissue in HMZ (MT)	0.020 ± 0.004	0.008	0.018 ± 0.009 (0.028; 0.009)	0.008 ± 0.002	ns
Fetal villi (FV)	0.384 ± 0.018	0.423	0.400 ± 0.024 (0.376; 0.423)	0.389 ± 0.017	ns
*Trophectoderm	0.153 ± 0.013	0.247	0.212 ± 0.022 (0.190; 0.233)	0.228 ± 0.008	0.03
Connective tissue core	0.223 ± 0.020	0.182	0.188 ± 0.001 (0.187; 0.190)	0.191 ± 0.017	ns
*Maternal crypts (MC)	0.274 ± 0.014	0.305	0.244 ± 0.070 (0.173; 0.314)	0.338 ± 0.017	ns
*Feto-maternal syncytium	0.075 ± 0.004	0.085	0.087 ± 0.021 (0.066; 0.107)	0.098 ± 0.008	ns
Placentome capsule (PC)	0.087 ± 0.006	0.088	0.064 ± 0.022 (0.041; 0.086)	0.080 ± 0.007	ns
Fetal villi/Maternal crypts	1.467 ± 0.109	1.385	1.759 ± 0.409 (2.168; 1.350)	1.175 ± 0.101	ns
Total fetal tissue/ Total maternal tissue	1.119 ± 0.077	1.140	1.409 ± 0.304 (1.714; 1.105)	0.968 ± 0.064	ns
Shrinkage spaces	0.039 ± 0.007	0.025	0.069 ± 0.046 (0.115; 0.023)	0.060 ± 0.005	ns

Values are expressed as mean ±SE; ANOVA; LSMEANS testing effect of treatment and placentome morphology and their interaction using "small", "average-size", and "large" placentomes; Treatment had no significant effects and there was no significant interaction between treatment and placentome morphology;

Component names with (*) were significantly less in non-everted placentomes of control placentas when compared with everted placentomes

of restricted placentas (p<0.01; lsmeans; specific comparisons);

Component volume densities in "**bold**" were significantly different between non-everted and everted placentomes within each treatment (p< 0.05; lsmeans specific comparisons);

N= Number of ewes; n= Number of placentomes morphometrically analysed; ns= no significant difference

Total fetal tissue = CEP + FV (excluding CM); Total maternal tissue = MT + MC + PC

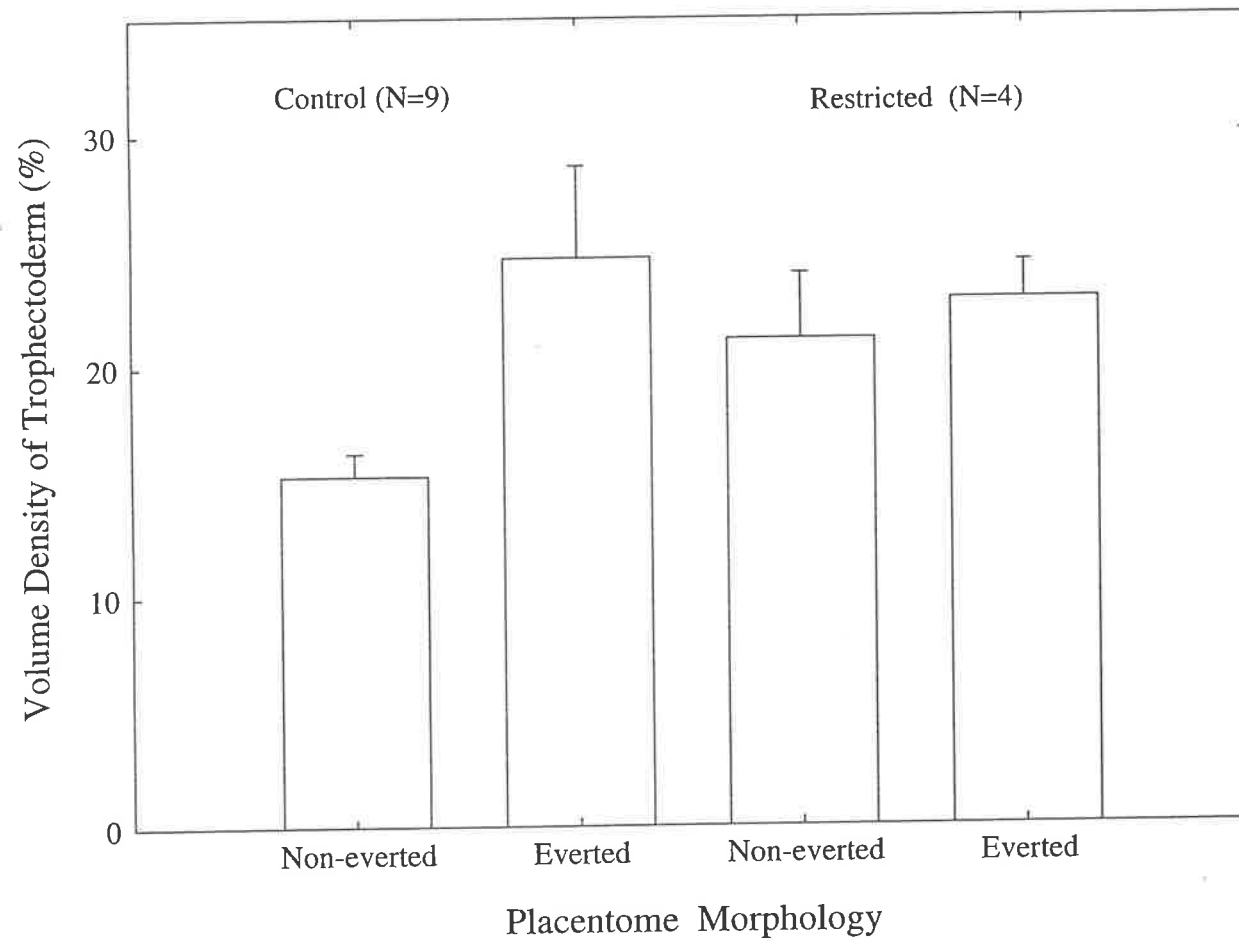


Figure 4.5 Comparison of trophoctoderm volume density in non-everted and everted placentomes in placentas of control and experimentally restricted singletons at 90 days gestation.
(Overall effect of placentome morphology, $p = 0.03$; ANOVA; L S Means Comparisons)

TABLE 4.5 Surface density and arithmetic mean barrier thickness of epithelial cell layers in placentomes from control and experimentally restricted ewes at 90 days gestation

TREATMENT	CONTROL (N=9)		RESTRICTED (N=4)		LSMeans specific comparisons
<i>PLACENTOME SIZE</i>	<i>"Average-size"</i>	<i>"Large"</i>	<i>"Average-size"</i>	<i>"Large"</i>	
Weight (g)*	8.2 ± 0.5 (n=8)	16.3 ± 1.4 (n=7)	7.4 ± 1.2 (n=3)	31.4 ± 6.6 (n=4)	(p=)
<i>PARAMETER</i>					
Surface density of trophoctoderm (cm ⁻¹)	116 ± 7		154 ± 8		0.0007
Barrier thickness of trophoctoderm (µm)	16.3 ± 0.9		18.0 ± 1.2		ns
Barrier thickness of feto-maternal syncytium (µm)	8.5 ± 0.5		7.7 ± 0.6		ns

Values are expressed as least squares means ± SE

* Values are means ± SE

ANOVA; LSMeans specific comparisons; excluding small placentomes

ns= no significant difference; N= Number of ewes; n= number of placentomes analysed morphometrically

No significant effect of placentome size and morphology and their interaction with treatment on surface area and arithmetic mean barrier thickness

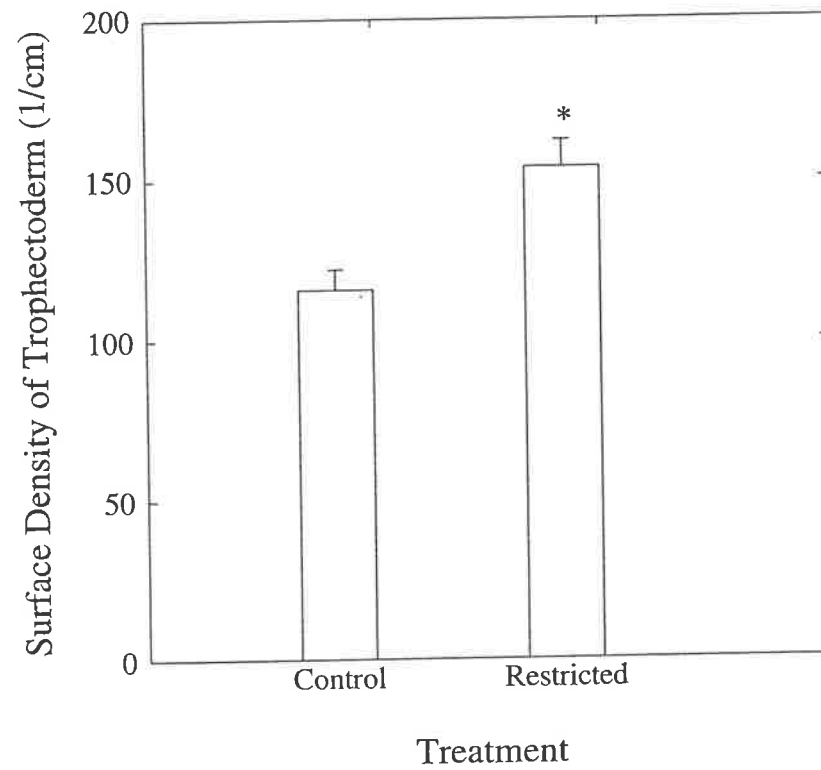


Figure 4.6 Surface density of trophectoderm in control (N=9) and experimentally restricted (N=4) ewes at 90 days gestation. (ANOVA; L S Means Comparison; * $p = 0.0007$).

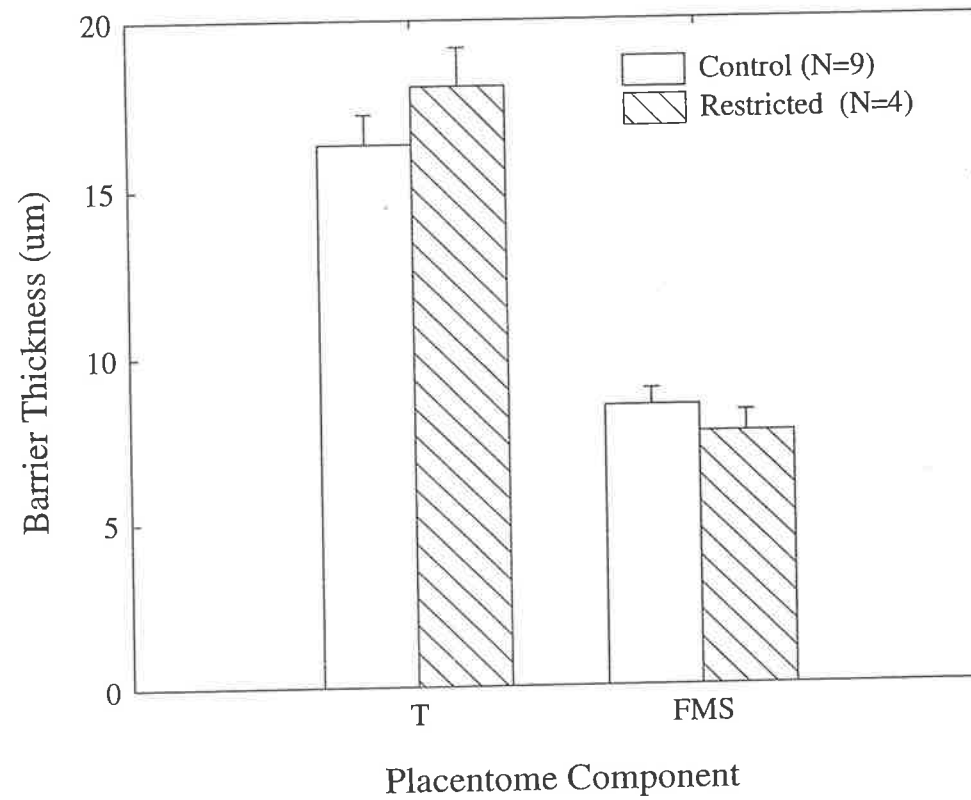


Figure 4.7 Arithmetic mean barrier thicknesses of the epithelial cell layers at the feto-maternal interface in placentas of control (□) and experimentally restricted (▨) ewes at 90 days gestation: Trophoctoderm (T) and Feto-maternal syncytium (FMS). (ANOVA; L S Means Comparison).

TABLE 4.6 Total placental volumes of components and surface area in control and restricted placentas at 90 days gestation

TREATMENT	CONTROL (N=9)	RESTRICTED (N=4)	<i>LSMeans specific comparisons</i>
Placental Weight (g)	515 ± 42	340 ± 27	0.0001
Chorionic Membrane	57.62 ± 8.67	29.00 ± 5.23	0.008
Chorionic Epithelium	13.09 ± 2.21	7.18 ± 1.30	ns
Haemophagous Zone	28.29 ± 7.31	9.90 ± 2.23	0.02
Maternal Tissue in Haemophagous Zone	10.48 ± 2.83	3.20 ± 0.36	0.02
Fetal Villi	200.58 ± 20.97	136.36 ± 15.73	0.01
Trophectoderm	83.51 ± 4.31	78.12 ± 6.78	ns
Connective Tissue Core	116.94 ± 20.71	64.12 ± 5.22	0.02
Maternal Crypts	141.65 ± 12.3	107.95 ± 7.89	ns
Feto-maternal Syncytium	38.21 ± 3.01	33.96 ± 4.52	ns
Shrinkage Spaces	17.73 ± 4.08	22.10 ± 10.92	ns
Placentome Capsule	45.43 ± 5.09	25.94 ± 3.79	0.002
Surface Area (m ²)	5.93 ± 0.44	5.18 ± 0.83	ns
Relative Surface Area (cm ⁻¹)	47.79 ± 2.32	52.28 ± 6.13	ns

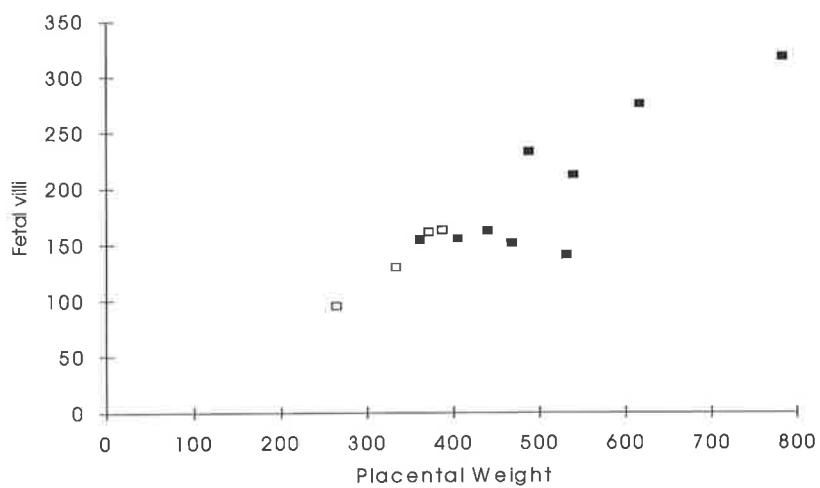
Values are expressed as Mean ± SE; ANOVA; LS Means Specific Comparisons excluding small placentomes;
Component placental volume = volume density of component x placental weight;
N = Number of ewes studied

TABLE 4.7 Correlation analysis between placental weight and morphometrically determined placental parameters in control and restricted ewes bearing singletons at 90 days gestation

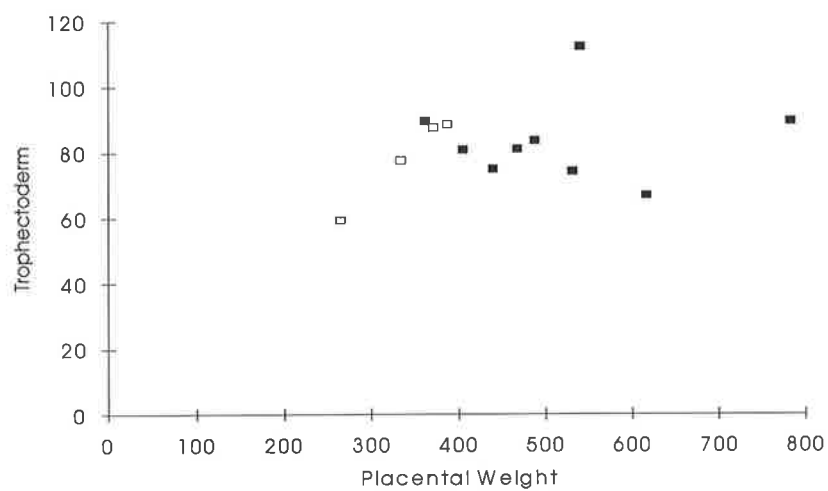
TREATMENT	CONTROL (N=9)	RESTRICTED (N=4)	CONTROL + RESTRICTED (N=13)
<i>PLACENTAL COMPONENT</i>			
Placentome Number	ns	ns	0.50 (p=0.08)
Chorionic Membrane	0.81 (p= 0.008)	ns	0.86 (p=0.0001)
Chorionic Epithelium	ns	ns	0.70 (p=0.008)
Haemophagous Zone	ns	ns	0.58 (p=0.004)
Maternal Tissue in the Haemophagous Zone	0.68 (p= 0.04)	ns	0.74 (p=0.004)
Fetal villi	0.85 (p= 0.004)	0.99 (p= 0.009)	0.89 (p=0.0001)
Trophectoderm	ns	0.99 (p= 0.005)	ns
Connective Tissue Core	0.83 (p= 0.006)	ns	0.85 (p=0.0002)
Maternal Crypts	0.65 (p= 0.06)	ns	0.73 (p=0.005)
Feto-maternal Syncytium	ns	0.99 (p= 0.01)	0.54 (p=0.05)
Placentome Capsule	0.81 (p= 0.008)	ns	0.88 (p=0.0001)
Surface Area	0.70 (p= 0.04)	ns	0.66 (p=0.02)

Values are correlation coefficients (outside brackets) and their probabilities (inside brackets). Correlation analysis using PROC CORR

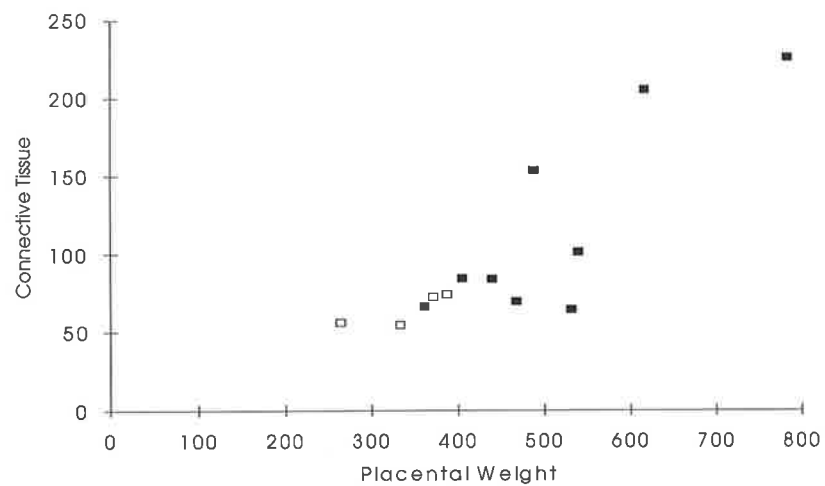
(i)



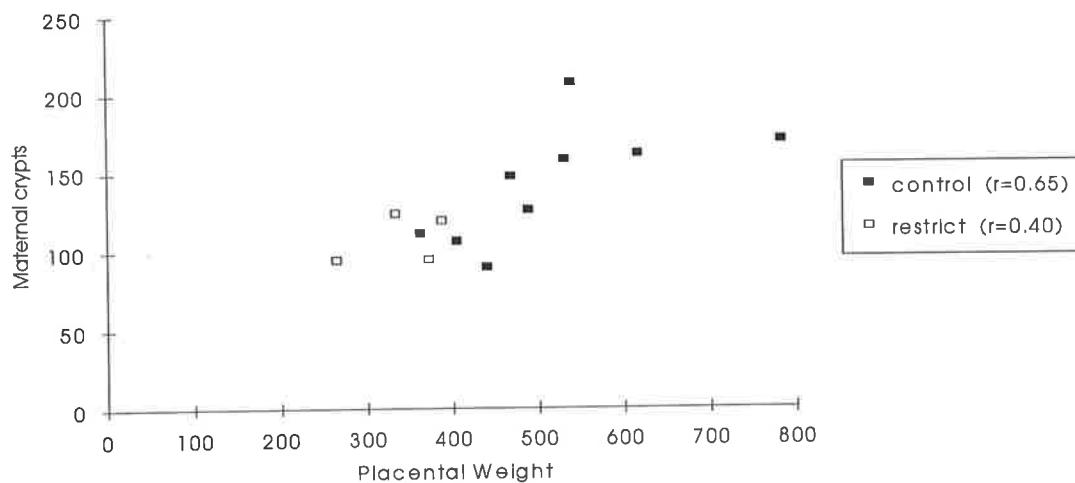
(ii)



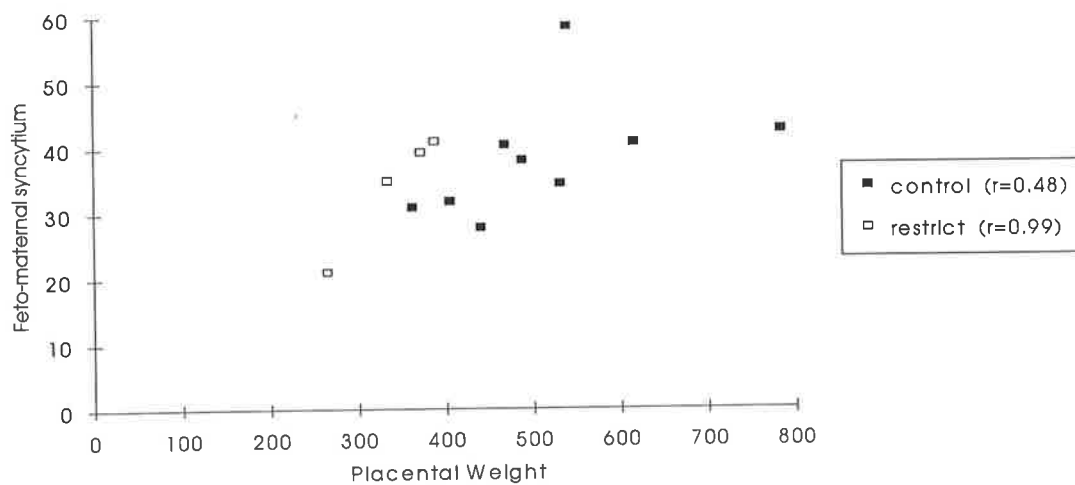
(iii)



(iv)



(v)



(vi)

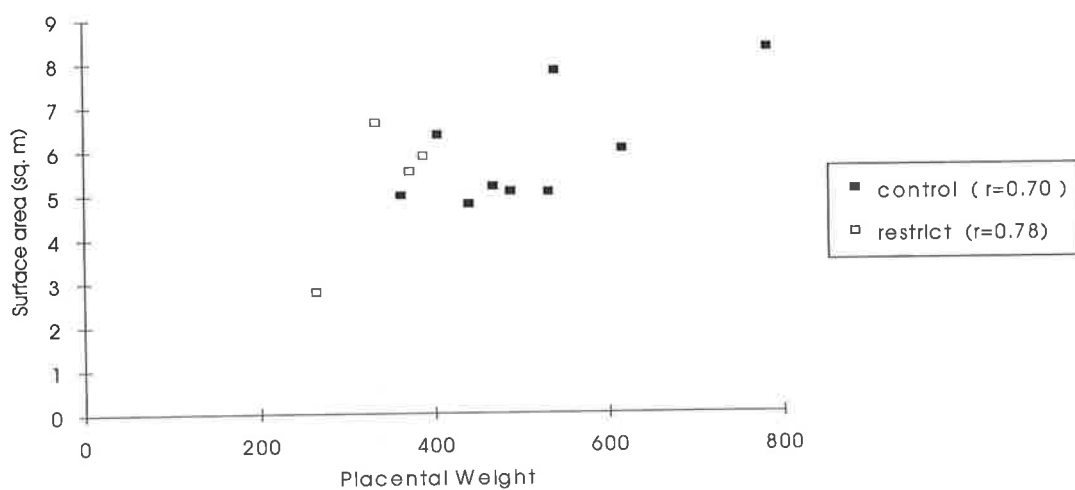


Figure 4.8: Relationships between placental morphometrically determined parameters and placental weight in control (N=9) and restricted ewes (N=4) bearing singletons at 90 days gestation. r = correlation coefficient.

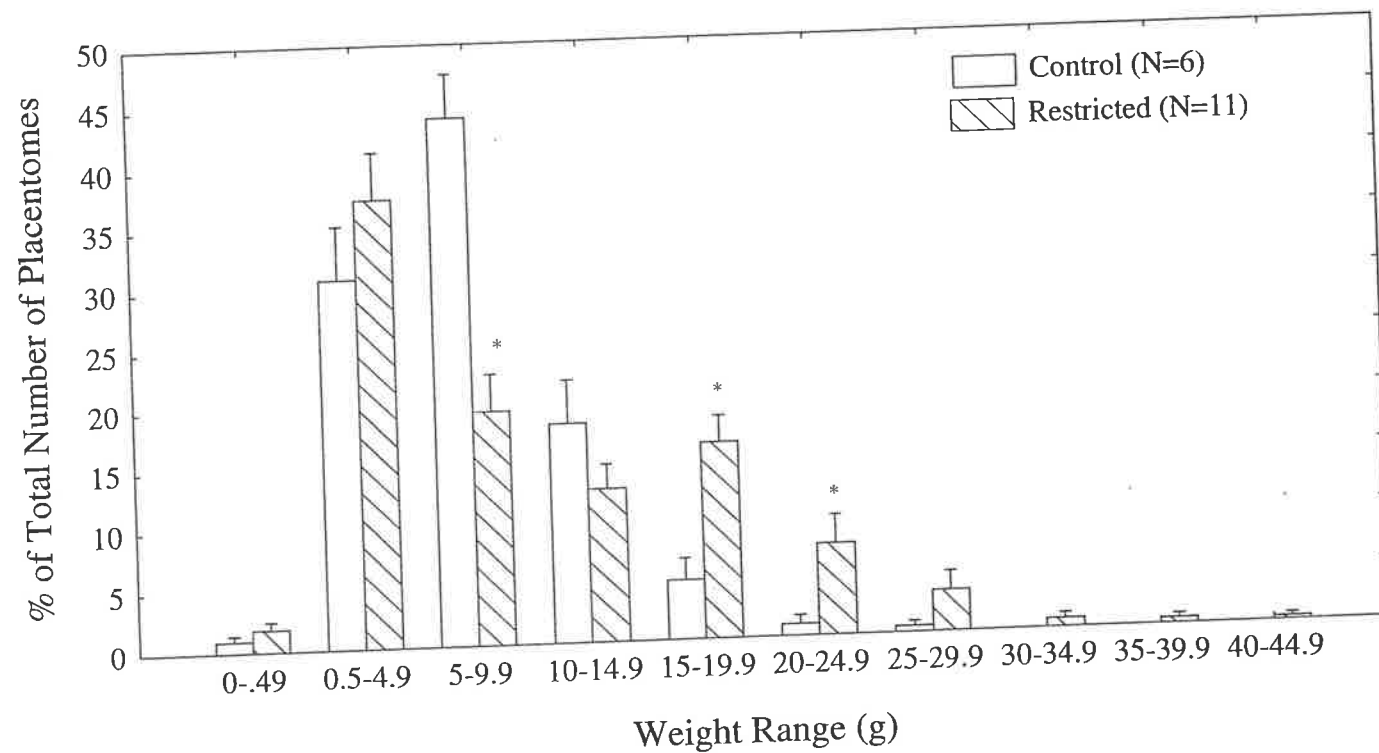


Figure 4.9 The distribution of placentomes within different weight range categories in Border-Leicester X Merino control (□) and restricted (▨) ewes at 120 days gestation (ANOVA; L S Means Comparisons; * $p < 0.05$).

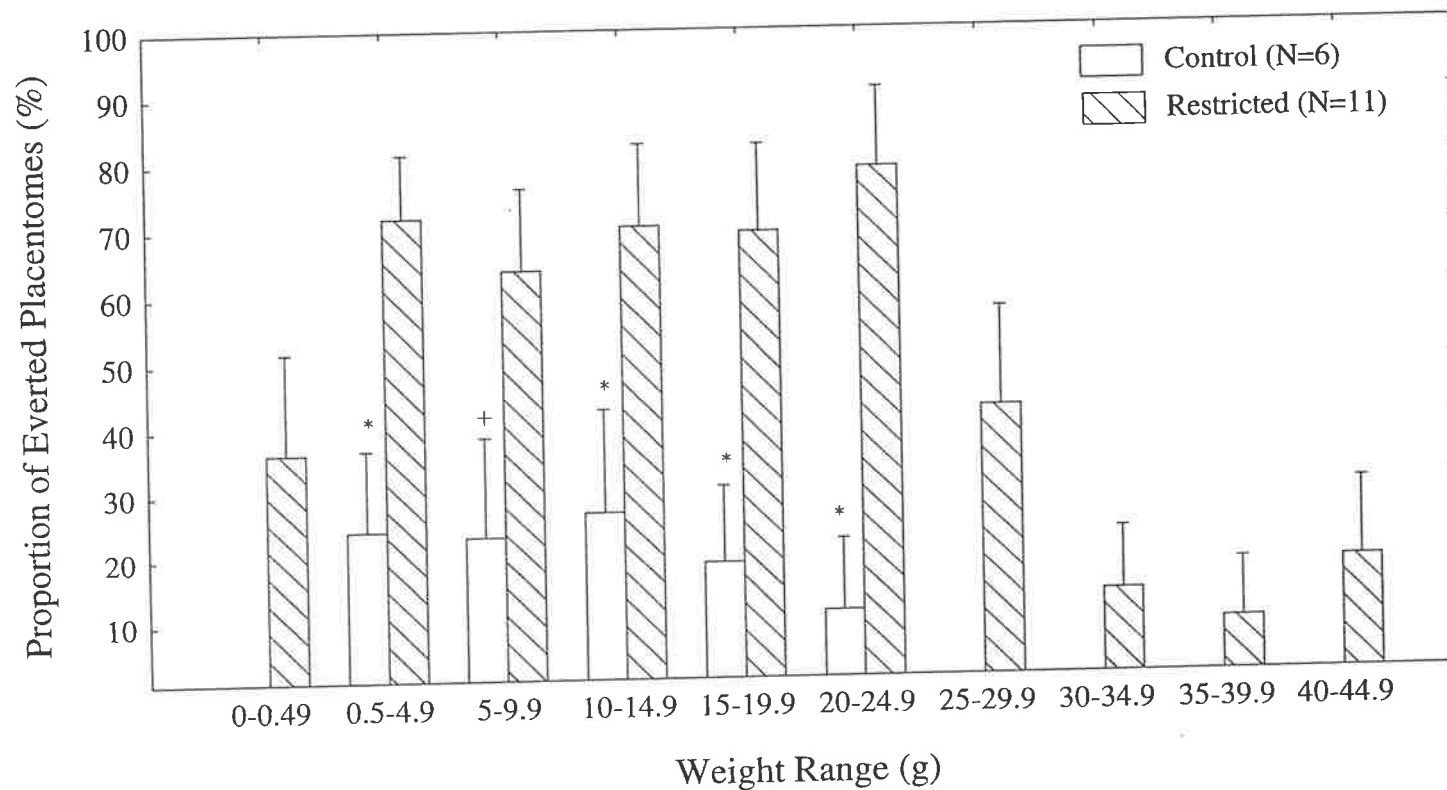


Figure 4.10 The proportion of everted placentomes within each weight range category in Border-Leicester X Merino control (□) and restricted (▨) ewes at 120 days gestation (ANOVA; L S Means Comparisons; * $p < 0.05$, + $p < 0.1$).

TABLE 4.8 Effect of restricted implantation on volume densities of individual placentome components at 120 days gestation

TREATMENT	CONTROL (N=6)			RESTRICTED (N=11)			(ANOVA)	
PLACENTOME SIZE	"Small" (n=2)	"Average-size" (n=6)	"Large" (n=6)	"Small" (n=6)	"Average-size" (n=8)	"Large" (n=11)		
Weight (g)	4.6 ± 0.6	7.4 ± 0.4	14.8 ± 0.4	3.3 ± 0.5	7.6 ± 0.4	21.4 ± 1.4	A1	A2
COMPONENT								(p)
Chorionic membrane	0.098 ± 0.017	0.137 ± 0.016	0.131 ± 0.019	0.095 ± 0.015	0.087 ± 0.011	0.100 ± 0.01	0.01	0.009
Chorionic epithelium	0.033 ± 0.002	0.053 ± 0.007	0.043 ± 0.006	0.053 ± 0.014	0.038 ± 0.003	0.033 ± 0.004	ns	0.02
Haemophagous zone (HMZ)	0.058 ± 0.029	0.066 ± 0.009	0.051 ± 0.013	0.065 ± 0.013	0.046 ± 0.006	0.038 ± 0.007	ns	0.06
Maternal tissue in HMZ	0.015 ± 0.006	0.020 ± 0.004	0.011 ± 0.002	0.018 ± 0.007	0.010 ± 0.001	0.008 ± 0.002	ns	0.01
Fetal villi	0.329 ± 0.038	0.332 ± 0.022	0.365 ± 0.037	0.391 ± 0.026	0.395 ± 0.015	0.397 ± 0.014	0.007	0.02
Trophectoderm	0.231 ± 0.018	0.206 ± 0.019	0.229 ± 0.025	0.318 ± 0.028	0.282 ± 0.017	0.270 ± 0.011	0.0007	0.003
Connective tissue	0.089 ± 0.016	0.125 ± 0.009	0.127 ± 0.013	0.077 ± 0.009	0.119 ± 0.011	0.104 ± 0.005	0.04	ns
Maternal crypts	0.353 ± 0.01	0.295 ± 0.018	0.284 ± 0.017	0.294 ± 0.021	0.323 ± 0.013	0.313 ± 0.01	ns	ns
Feto-maternal syncytium	0.122 ± 0.024	0.086 ± 0.007	0.084 ± 0.007	0.093 ± 0.008	0.099 ± 0.006	0.096 ± 0.005	ns	ns
Shrinkage spaces	0.008 ± 0.004	0.032 ± 0.009	0.029 ± 0.007	0.031 ± 0.007	0.052 ± 0.004	0.068 ± 0.007	0.0003	0.0001
Placentome capsule	0.102 ± 0.007	0.063 ± 0.013	0.083 ± 0.017	0.057 ± 0.015	0.054 ± 0.002	0.046 ± 0.006	0.004	0.005

Values are mean ± SE; ANOVA; lsmeans comparisons, testing effect of treatment; **A1**; analysis including small placentomes; **A2**; analysis excluding small placentomes;

N= Number of ewes, n= Number of placentomes morphometrically analysed;

ANOVA; Specific comparisons of least squares means of parameters in similar size placentomes between groups for example : "small" (control) vs "small" (restricted) etc.

Components in "**BOLDFACE ITALICS**" were significantly different between comparable size placentomes in control and restricted placentas; $p \leq 0.05$

ANOVA; Specific comparisons of least squares means testing the effect of size (within factor) on volume densities of placentome components.

Components in "**BOLDFACE**" were significantly different between average size and large placentomes in control placentas; $p \leq 0.01$

TABLE 4.9 Surface Densities and Arithmetic Mean Barrier Thicknesses in Control and Restricted Placentas at 120 Days Gestation.

TREATMENT:	CONTROL (N=6)		RESTRICTED (N=11)		
PLACENTOME Size	"Average-size"	"Large"	"Average-size"	"Large"	ANOVA
Weight (g) *	7.4 ± 0.2 (n=6)	14.8 ± 0.4 (n=6)	7.6 ± 0.4 (n=8)	21.4 ± 1.4 (n=11)	(p=)
PARAMETER					
Surface density of trophoderm (cm ⁻¹)	185 ± 7		226 ± 5		0.0001
Surface density of feto-maternal syncytium (cm ⁻¹)	175 ± 6		216 ± 5		0.0001
Arithmetic mean barrier thickness of trophoderm (µm)	13 ± 0.6		13 ± 0.5		ns
Arithmetic mean barrier thickness of feto-maternal syncytium (µm)	5 ± 0.2		4.4 ± 0.2		ns

Values are expressed as least squares means ± SE;

* Values are mean ± SE;

ANOVA; LSMeans comparisons, testing effect of treatment (between factor), placentome size (within factor), (excluding small placentomes) and their interaction; only treatment had significant effects;

N= Number of ewes, n= Number of placentomes, ns=no significant difference

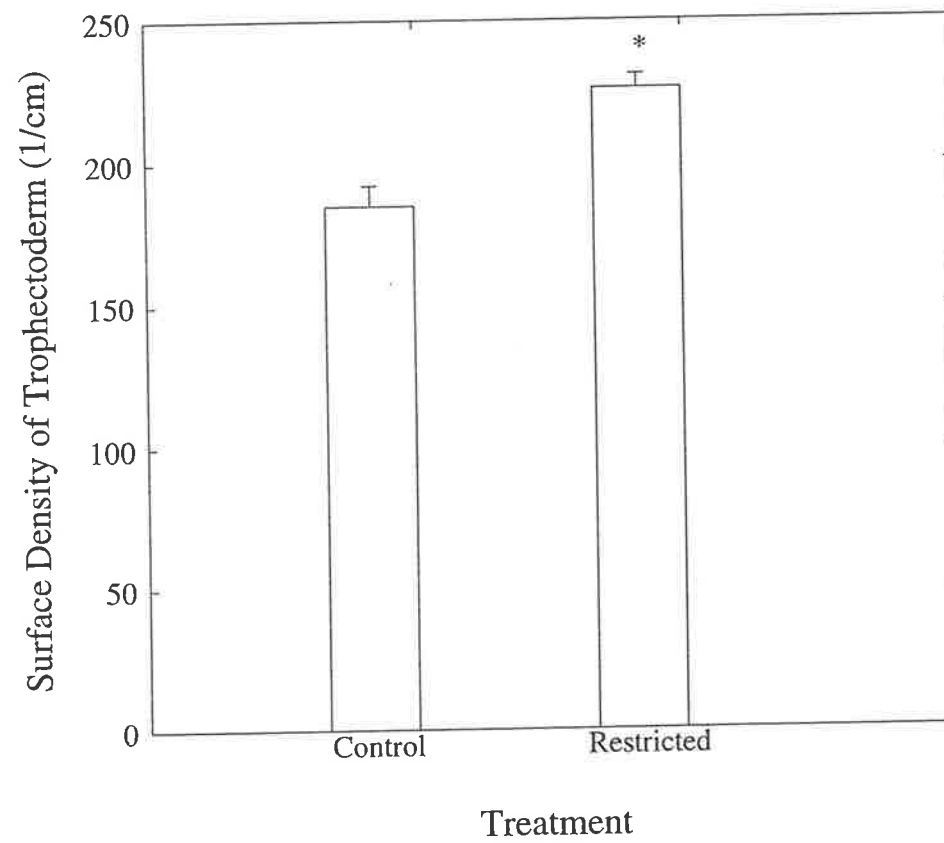


Figure 4.11 Surface density of trophectoderm in control (N=6) and restricted (N=11)

Border-Leicester X Merino ewes at 120 days gestation.

(ANOVA; L S Means Comparisons; * $p = 0.0001$)

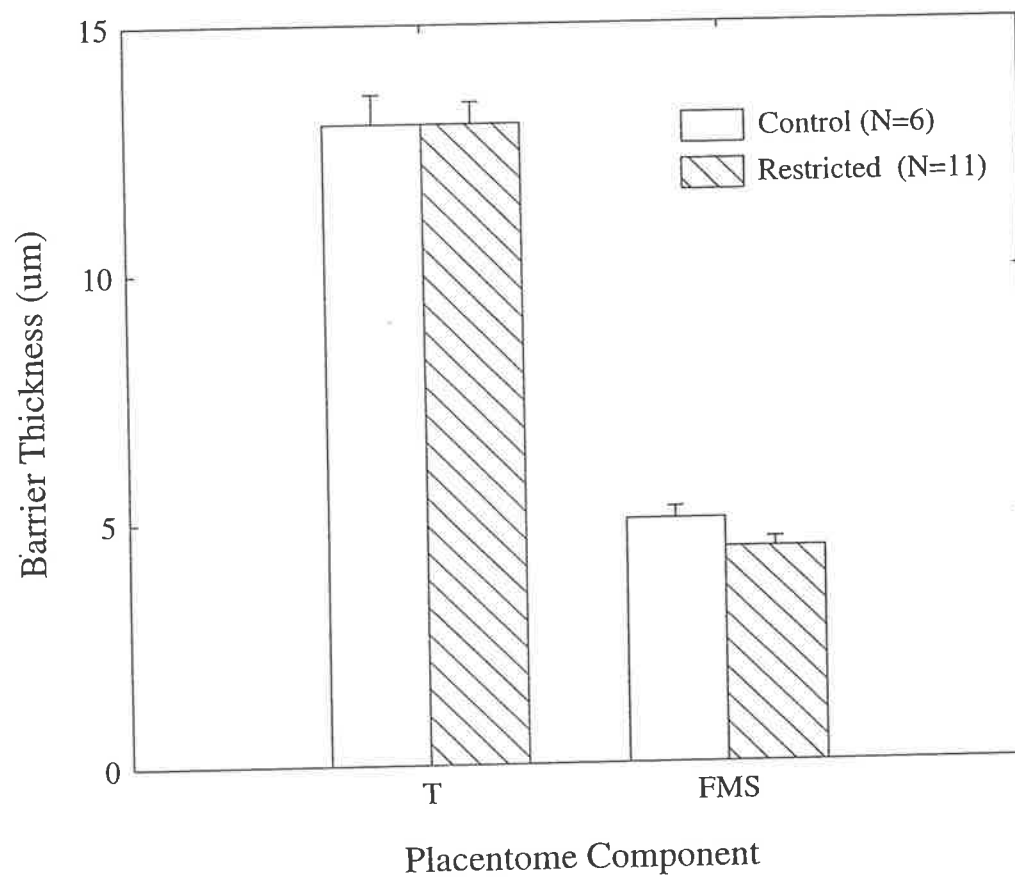


Figure 4.12 Arithmetic mean barrier thicknesses of the two epithelial cell layers at the fetomaternal interface; trophoctoderm (T) and fetomaternal syncytium (FMS) in control (□) and restricted (▨) Border-Leicester X Merino ewes at 120 days gestation (ANOVA; L S Means Comparisons).

TABLE 4.10 Relationship between placentome morphology, volume density of components, and fetal to maternal tissue ratio in control and restricted ewes bearing singletons at 120 days gestation

TREATMENT	CONTROL (N=6)		RESTRICTED (N=11)		ANOVA SUMMARY	
PLACENTOME MORPHOLOGY:	Non-everted (n=10)	Everted (n=4)	Non-everted (n=6)	Everted (n=22)	(LSMeans	Comparisons)
Placentome Weight (g)	10.1 ± 1.4	10.4 ± 2.2	10.5 ± 3.9	13.8 ± 1.7		
PARAMETER					Treatment (p=)	Morphology (p=)
* Chorionic membrane (CM)	0.138 ± 0.014	0.105 ± 0.012	0.098 ± 0.018	0.096 ± 0.007	ns	ns
Chorionic epithelium (CEP)	0.047 ± 0.004	0.043 ± 0.012	0.043 ± 0.003	0.038 ± 0.005	ns	ns
* Haemophagous zone (HMZ)	0.067 ± 0.008	0.037 ± 0.010	0.067 ± 0.010	0.039 ± 0.005	ns	0.0003
* Maternal tissue in HMZ (MT)	0.018 ± 0.003	0.009 ± 0.003	0.015 ± 0.005	0.009 ± 0.002	ns	0.03
* Fetal villi (FV)	0.323 ± 0.016	0.402 ± 0.043	0.354 ± 0.013	0.409 ± 0.01	ns	0.0008
* Trophectoderm	0.203 ± 0.015	0.261 ± 0.016	0.264 ± 0.017	0.286 ± 0.011	0.02	0.04
Connective tissue	0.120 ± 0.009	0.121 ± 0.016	0.100 ± 0.015	0.102 ± 0.005	ns	ns
Maternal crypts (MC)	0.295 ± 0.016	0.309 ± 0.015	0.318 ± 0.016	0.307 ± 0.008	ns	ns
Feto-maternal syncytium	0.088 ± 0.008	0.096 ± 0.007	0.097 ± 0.007	0.094 ± 0.004	ns	ns
* Placentome capsule (PC)	0.081 ± 0.013	0.067 ± 0.010	0.066 ± 0.012	0.045 ± 0.004	ns	ns
* Fetal villi/Maternal crypts	1.124 ± 0.078	1.329 ± 0.208	1.123 ± 0.052	1.355 ± 0.045	ns	0.02
* (FV + CEP)/ (MC + MT + PC)	0.962 ± 0.071	1.186 ± 0.160	1.007 ± 0.051	1.247 ± 0.046	ns	0.007
* Shrinkage spaces	0.024 ± 0.006	0.036 ± 0.011	0.045 ± 0.005	0.060 ± 0.005	0.01	ns

Values are mean ±SE

ANOVA SUMMARY; LSMeans comparisons, testing effect of treatment, placentome morphology and their interaction using "small", "average", and "large" placentomes; Treatment and morphology had significant effects. There was no significant effect of interaction between Treatment and Morphology on all components.

Values in "**BOLDFACE**" were significantly different between non-everted and everted placentomes within each treatment (p<0.05; LSMeans specific comparisons); Components names marked "*" were significantly different (p≤ 0.01; LSMeans specific comparisons) between non-everted placentomes of control placentas and everted

placentomes of restricted placentas; N= Number of ewes; n= Number of placentomes morphometrically analysed; ns= no significant difference

Values are mean $\pm$ SE; ANOVA SUMMARY; LSMeans comparisons testing effect of treatment; placentome morphology and their interaction using all "small", "average", and "large" placentomes; Treatment had significant effects. There was no significant effect of morphology and its interaction with treatment on surface densities and barrier thicknesses.

Components marked "*" were significantly different ($p < 0.01$) between non-everted placentomes of control placentas and everted placentomes of restricted placentas

N= Number of ewes; n= Number of placentomes morphometrically analysed; ns = no significant difference

TABLE 4.11 Relationship Between Placentome Morphology, Surface Density, and Arithmetic Mean Barrier Thicknesses in Control and Restricted Placentas at 120 Days Gestation.

TREATMENT PLACENTOME MORPHOLOGY	CONTROL (N=6)		RESTRICTED (N=11)		(LS Means comparisons)
	NON-EVERTED (n=10)	EVERTED (n=4)	NON-EVERTED (n=6)	EVERTED (n=22)	
Placentome Weight (g)	10.1 ± 1.4	10.4 ± 2.2	10.5 ± 3.9	13.8 ± 1.7	<i>Effect of Treatment</i>
		Surface Density (cm ⁻¹)			
* Fetal villi in zonae intima and intermediate only	167 ± 6	179 ± 6	199 ± 10	212 ± 8	0.009
* Fetal villi (including that in haemophagous zone on Chorionic epithelium)	181 ± 5	191 ± 4	210 ± 9	224 ± 7	0.006
* Maternal crypts in zonae intima and intermediate only	170 ± 6	180 ± 5	200 ± 10	212 ± 7	0.007
* Maternal crypts (including that in haemophagous zone on Chorionic epithelium)	181 ± 5	185 ± 7	213 ± 8	224 ± 6	0.006
		Barrier Thickness (µm)			
Trophectoderm in zonae intima and intermediate only	12.1 ± 0.7	14.5 ± 0.6	13.3 ± 0.8	13.8 ± 0.7	ns
Trophectoderm (including that in haemophagous zone on Chorionic epithelium)	13.7 ± 0.7	16.4 ± 0.4	13.5 ± 0.3	13.8 ± 0.6	ns
Feto-maternal syncytium in zonae intima and intermediate only	5.3 ± 0.6	5.3 ± 0.3	4.9 ± 0.4	4.5 ± 0.2	ns
Feto-maternal syncytium (including that in haemophagous zone on Chorionic epithelium)	5.5 ± 0.4	5.6 ± 0.4	5.0 ± 0.6	4.7 ± 0.2	ns

TABLE 4.12 Total Volumes of Placental Components, Surface Area, and Relative Surface Area in Control and Restricted Placentas at 120 Days Gestation

TREATMENT	CONTROL (N=6)	RESTRICTED (N=11)	ANOVA SUMMARY
Placental Weight (g)	545 ± 35	297 ± 22	<i>Effect of Treatment</i>
Placentome Numbers	74 ± 2	30 ± 2	<i>(LSMeans comparisons)</i>
COMPONENT			<i>(p=)</i>
Chorionic membrane	70.31 ± 6.70	28.80 ± 2.91	0.0001
Chorionic epithelium	24.95 ± 2.03	11.18 ± 1.89	0.0001
Haemophagous zone (HMZ)	31.01 ± 5.04	12.36 ± 2.10	0.01
Maternal tissue in HMZ	8.08 ± 1.34	3.04 ± 0.76	0.07
Fetal villi	193.23 ± 24.77	119.27 ± 9.71	0.007
Trophectoderm	122.25 ± 16.05	81.22 ± 7.06	0.003
Connective tissue	69.32 ± 9.57	32.58 ± 3.49	0.06
Maternal crypts	159.55 ± 14.55	91.40 ± 8.20	0.0002
Feto-maternal syncytium	47.09 ± 4.83	28.32 ± 3.02	0.001
Shrinkage spaces	17.08 ± 5.14	18.13 ± 2.14	0.9
Placentome capsule	39.02 ± 6.30	14.68 ± 1.89	0.0004
Surface area (SA) m ²	10.07 ± 0.69	6.51 ± 0.50	0.0001
Relative surface area (RELSA) cm ⁻¹	31.27 ± 1.54	25.36 ± 1.14	0.08

Values are mean ± SE

SA= (Surface density x Placental weight) m² RELSA =Surface Area/ (Fetal weight + Placental weight)

Component placental volume = volume density x placental weight

N = Number of ewes studied

TABLE 4.13 Correlation analysis between placental structure and fetal weight at 120 days gestation

			PLACENTAL PARAMETERS						
TREATMENT			PLACENTAL Wt(g)	PLACENTOME NUMBER	FETAL VILLI	TROPHECTODERM	CONNECTIVE TISSUE CORE OF FETAL VILLI	FETO- MATERNAL SYNCYTIIUM	SURFACE AREA (m ²)
CONTROL (N=6)	Fetal Wt(g)	<i>r</i>	0.77	-	-	0.82	-	-	0.81
		<i>p</i>	0.08	-	-	0.05	-	-	0.05
RESTRICTED (N=11)	Fetal Wt(g)	<i>r</i>	0.78	0.65	0.83	0.82	0.79	0.71	0.77
		<i>p</i>	0.004	0.03	0.002	0.002	0.004	0.01	0.006

r= correlation coefficient

p=coefficient probability

Fetal villi, trophoctoderm, connective tissue core of fetal villi and feto-maternal syncytium expressed as placental volume = volume density of component x placental weight

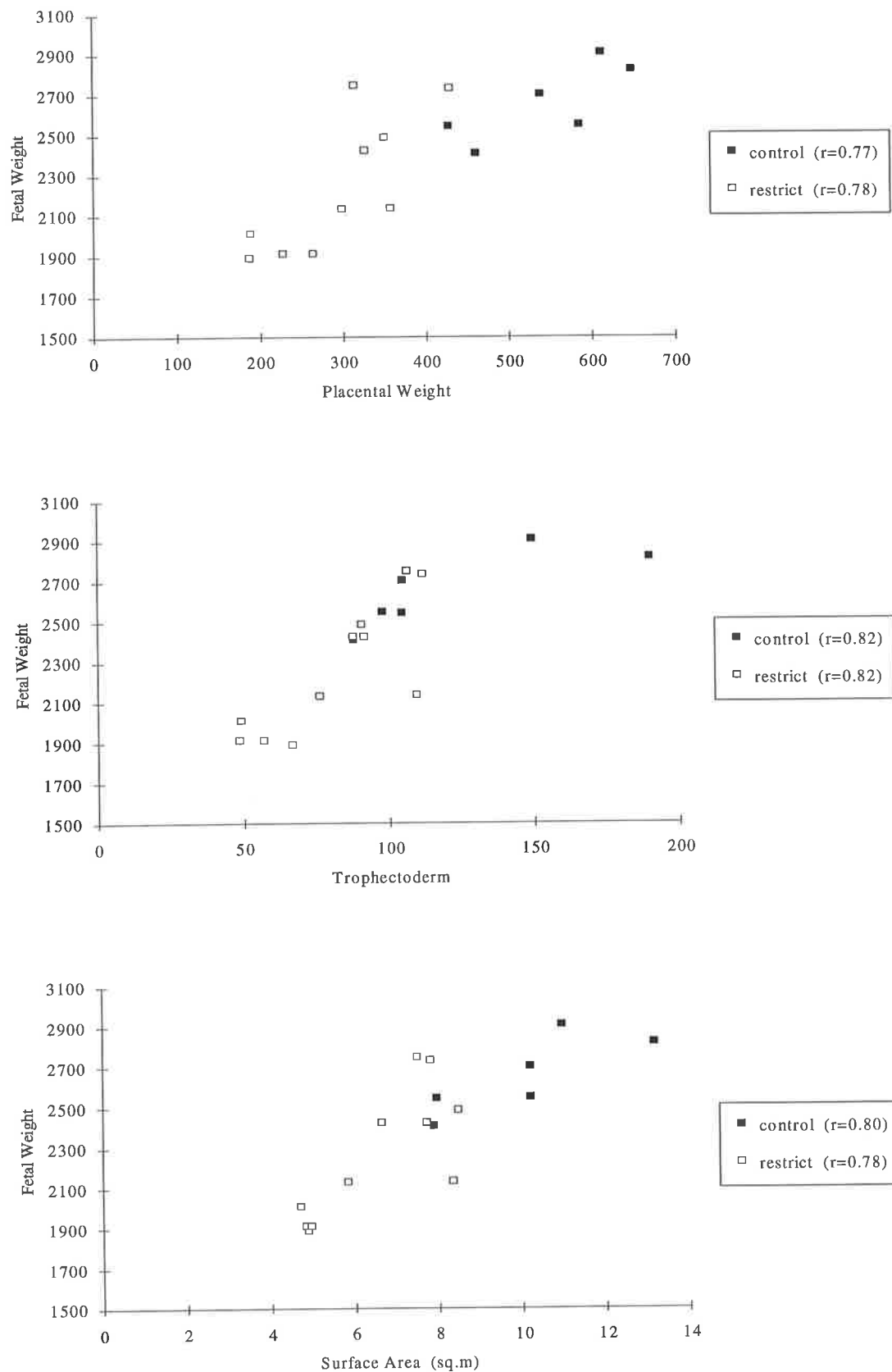


Figure 4.13: Relationship of fetal weight and placental parameters (viz placental weight, volume of trophoctoderm and surface area) in control (N=6) and restricted (N=11) ewes at 120 days of gestation.

TABLE 4.14 Correlation analysis between placental weight and its functional parameters at 120 days gestation

		CONTROL (N=6)	RESTRICTED (N=11)
<i>PLACENTAL PARAMETER</i>			
Placentome numbers	<i>r</i>	-	0.74
	<i>p</i>	-	0.009
Fetal villi	<i>r</i>	0.86	0.92
	<i>p</i>	0.03	0.0001
Trophectoderm	<i>r</i>	-	0.87
	<i>p</i>	-	0.0004
Connective tissue core of fetal villi	<i>r</i>	0.89	0.91
	<i>p</i>	0.02	0.0001
Feto-maternal syncytium	<i>r</i>	-	0.93
	<i>p</i>	-	0.0001
Surface area (m ²)	<i>r</i>	0.92	0.82
	<i>p</i>	0.01	0.002

r = correlation coefficient; *p* = coefficient probability N= number of ewes studied

Fetal villi, trophectoderm, connective tissue core of fetal villi and feto-maternal syncytium expressed as placental volumes

Component placental volume = volume density × placental weight

TABLE 4.15 Correlation Analysis of Various Morphometrically Determined Placentome Parameters at 120 days gestation

CONTROL (n=14)				
		Trophectoderm	Surface density	Feto-maternal syncytium
Fetal villi	r	0.85	0.73	0.21
	p	0.0001	0.003	ns
Trophectoderm	r		0.70	0.12
	p		0.005	ns
RESTRICTED (n=28)				
		Trophectoderm	Surface density	Feto-maternal syncytium
Fetal villi	r	0.51	0.63	0.20
	p	0.005	0.003	ns
Trophectoderm	r		0.43	0.32
	p		0.02	0.09

r= correlation coefficient *p*= coefficient probability

n = number of placentomes morphometrically analysed

ns= no significant correlation

surface density (cm⁻¹); Fetal villi, trophoctoderm and feto-maternal syncytium expressed as volume densities of the placentome

TABLE 4.16 Volume densities in placentomes of control and restricted placentas at 90 and 120 days gestation

GESTATIONAL AGE		90 DAYS				120 DAYS			
TREATMENT		CONTROL (N=9)		RESTRICTED (N=4)		CONTROL (N=6)		RESTRICTED (N=11)	
		"Average-size"	"Large"	"Average-size"	"Large"	"Average-size"	"Large"	"Average-size"	"Large"
<i>PLACENTOME SIZE</i>									
Weight (g)*		8.2 ± 0.5	16.3 ± 1.4	7.4 ± 1.2	28.1 ± 6.0	7.4 ± 0.4	14.8 ± 0.4	7.6 ± 0.4	21.4 ± 1.4
		(n=8)	(n=7)	(n=3)	(n=5)	(n=6)	(n=6)	(n=8)	(n=11)
<i>COMPONENT</i>									
Chorionic membrane		0.111 ± 0.009		0.096 ± 0.012		0.133 ± 0.010 \$		0.097 ± 0.007	
Chorionic epithelium		0.026 ± 0.004		0.024 ± 0.005		0.047 ± 0.004 \$		0.035 ± 0.003	
Haemophagous zone (HMZ)		0.060 ± 0.008		0.042 ± 0.010		0.056 ± 0.007		0.040 ± 0.005	
Maternal tissue in HMZ		0.021 ± 0.005 @		0.012 ± 0.006		0.015 ± 0.002		0.009 ± 0.001	
Fetal villi (FV)		0.378 ± 0.012		0.386 ± 0.016		0.350 ± 0.015 \$		0.399 ± 0.012	
Trophectoderm		0.152 ± 0.010 @		0.229 ± 0.013		0.220 ± 0.013 \$		0.271 ± 0.010	
Connective tissue core		0.216 ± 0.013		0.178 ± 0.016		0.124 ± 0.007		0.109 ± 0.005	
Maternal crypts (MC)		0.275 ± 0.012		0.316 ± 0.016		0.294 ± 0.001		0.313 ± 0.008	
Feto-maternal syncytium		0.074 ± 0.054 @		0.099 ± 0.007		0.086 ± 0.005		0.095 ± 0.004	
Shrinkage spaces		0.040 ± 0.008		0.058 ± 0.010		0.029 ± 0.006 \$		0.064 ± 0.004	
Placentome capsule		0.086 ± 0.006		0.069 ± 0.008		0.075 ± 0.007 \$		0.047 ± 0.005	
Fetal villi/Maternal crypts		1.443 ± 0.082		1.288 ± 0.106		1.212 ± 0.066		1.289 ± 0.051	
Total fetal tissue/Total maternal tissue		1.106 ± 0.071		1.072 ± 0.092		1.055 ± 0.057		1.191 ± 0.044	

Values are expressed as least squares means ± SE; * Values are means ± SE; ANOVA; lsmeans comparisons; excluding small placentomes
 ns=no significant difference; N=number of ewes; n=number of placentomes; Total fetal tissue= chorionic epithelium + fetal villi (excluding chorionic membrane)
 Total maternal tissue= maternal crypts + maternal tissue in HMZ + placentome capsule
 Values presented in "**BOLDFACE**" were significantly different between 90 and 120 days gestation within the restricted ewes (p<0.05);
 Values marked "@" were significantly different between control and restricted ewes at 90 days gestation (p≤0.05)
 Values marked "\$" were significantly different between control and restricted ewes at 120 days gestation (p≤0.05)

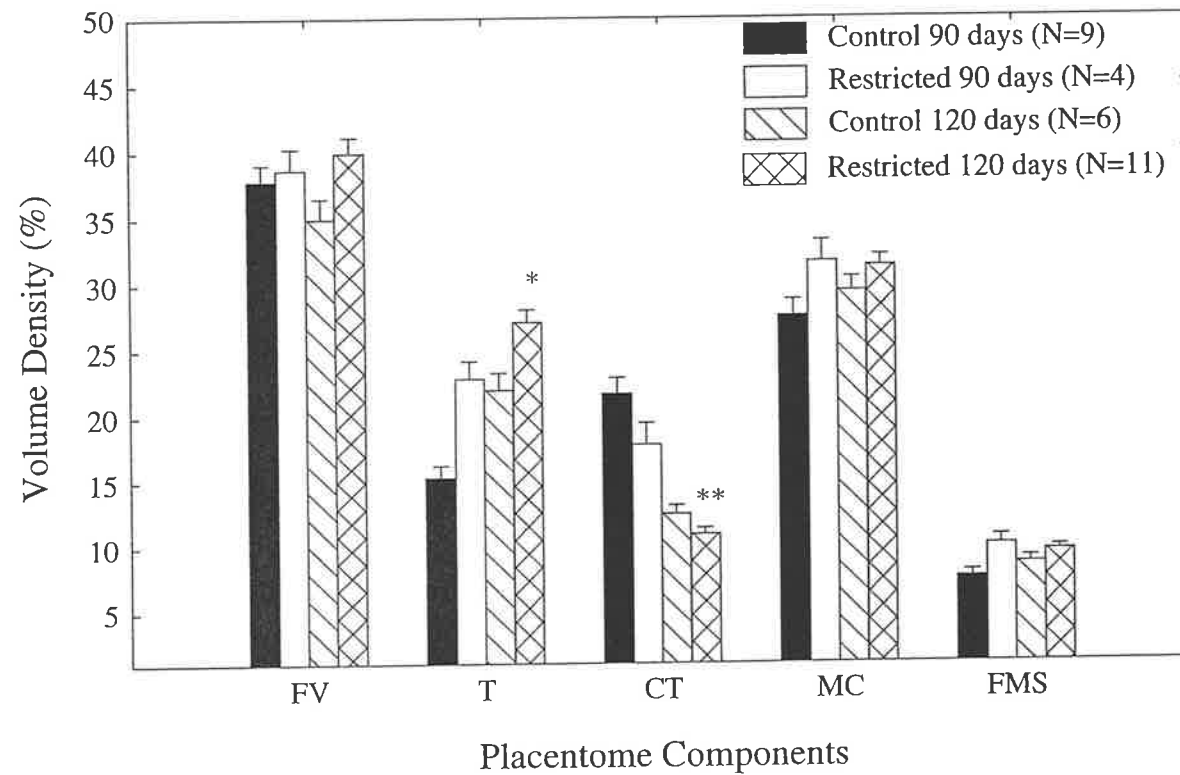


Figure 4.14 Volume densities of placentome components in control and restricted placentas at 90 and 120 days gestation: Fetal villi (FV), Trophoctoderm (T), Connective tissue core of fetal villi (CT), Maternal crypts (MC) and Feto-maternal syncytium (FMS).

* $p=0.02$; ** $p=0.0001$ ANOVA; LSMeans specific contrasts between restricted 90 days () and restricted 120 days ().

TABLE 4.17 Surface density and arithmetic mean barrier thicknesses of the epithelial cell layers in placentomes of control and experimentally restricted ewes at 90 and 120 days gestation

GESTATIONAL AGE		90 DAYS				120 DAYS			
TREATMENT		CONTROL (N=9)		RESTRICTED (N=4)		CONTROL (N=6)		RESTRICTED (N=11)	
<i>PLACENTOME SIZE</i>		<i>"Average-size"</i>	<i>"Large"</i>	<i>"Average-size"</i>	<i>"Large"</i>	<i>"Average-size"</i>	<i>"Large"</i>	<i>"Average-size"</i>	<i>"Large"</i>
Weight (g)*		8.2 ± 0.5 (n=8)	16.3 ± 1.4 (n=7)	7.4 ± 1.2 (n=3)	31.4 ± 6.6 (n=4)	7.4 ± 0.2 (n=6)	14.8 ± 0.4 (n=6)	7.6 ± 0.4 (n=8)	21.4 ± 1.4 (n=11)
<i>PARAMETER</i>									
@ Surface density of trophoctoderm (cm ⁻¹)		116 ± 7		154 ± 8		185 ± 7		226 ± 5	
Barrier thickness of trophoctoderm (µm)		16.3 ± 0.9		18.0 ± 1.15		13 ± 0.6		13 ± 0.5	
Barrier thickness of feto-maternal syncytium (µm)		8.5 ± 0.5		7.7 ± 0.6		5 ± 0.2		4.4 ± 0.2	

Values are expressed as least squares means ± SE; * Values are means ± SE; ANOVA; LSMeans specific comparisons; excluding small placentomes

ns= no significant difference; N= Number of ewes studied; n= number of placentomes morphometrically analysed

No significant effect of placentome size and morphology and their interaction with treatment on surface area and arithmetic mean barrier thickness

Values presented in "**BOLDFACE**" were significantly different between 90 and 120 days gestation within the restricted ewes (p<0.05);

Component name marked "@" was significantly different between control and restricted ewes at both gestational ages (p<0.05).

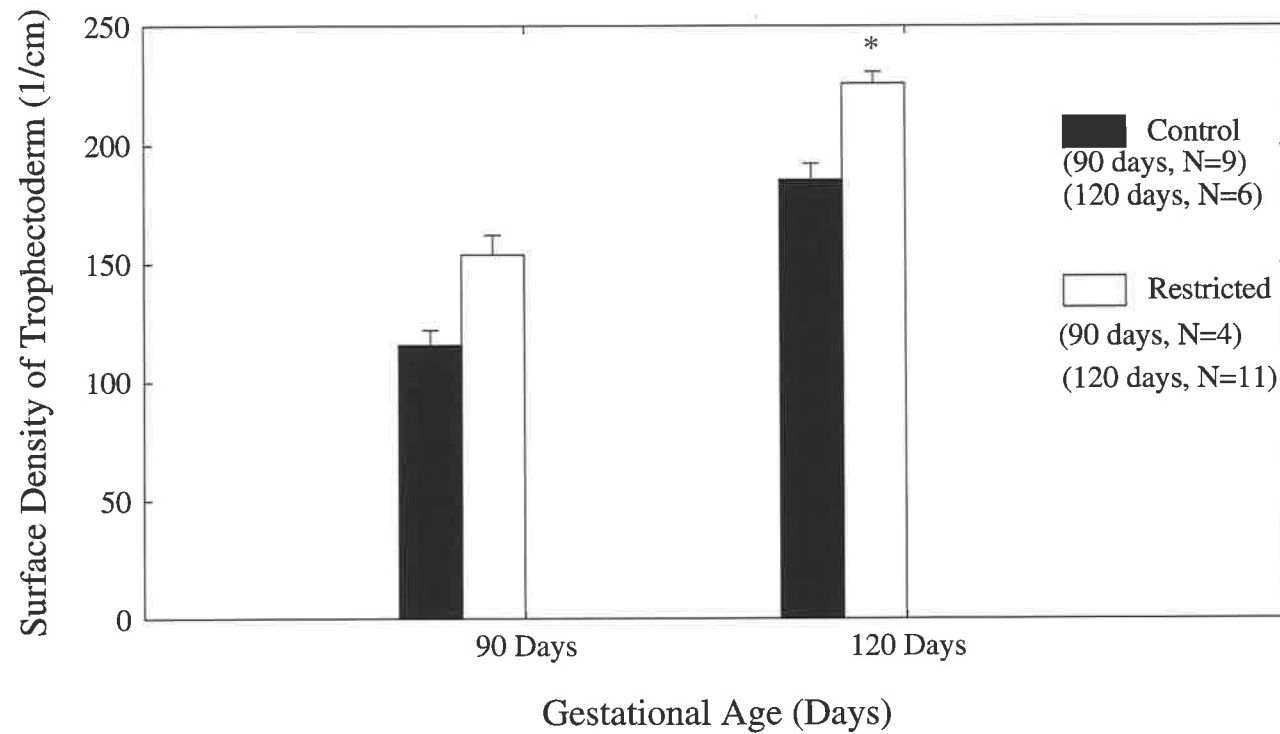


Figure 4.15 Surface density of trophoctoderm in control and experimentally restricted ewes at 90 and 120 days gestation.
(ANOVA; L S Means specific contrasts between restricted, 90 days and restricted, 120 days)

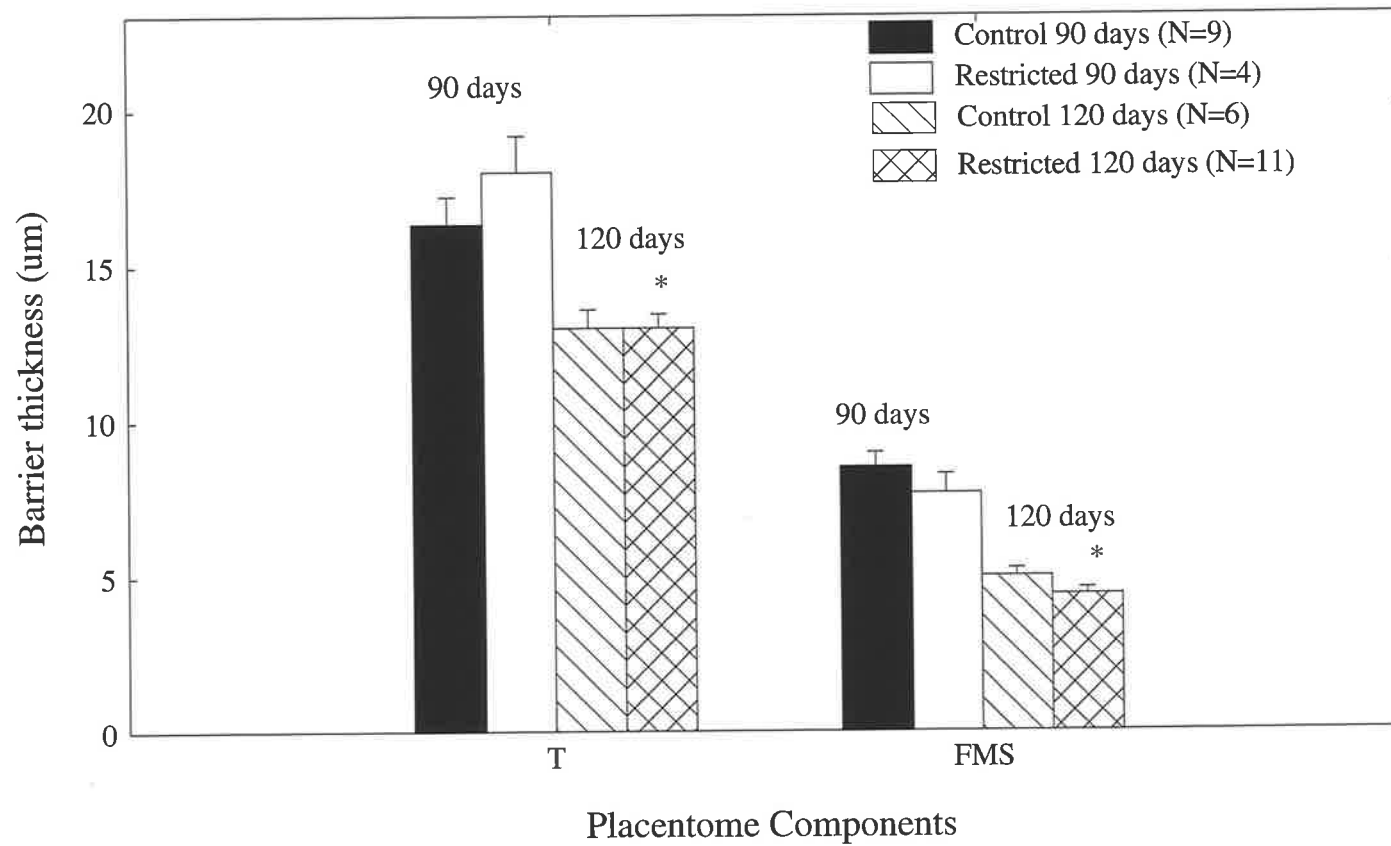


Figure 4.16 Arithmetic mean barrier thicknesses of the epithelial cell layers at the fetomaternal interface; trophoderm (T) and fetomaternal syncytium (FMS) in placentomes of control and experimentally restricted ewes at 90 and 120 days gestation. (* $p < 0.01$; ANOVA; L S Means specific contrasts between restricted, 90 days and restricted, 120 days).

Table 4.18 Total placental volumes and surface areas in control and restricted placentas of singletons at 90 and 120 days gestation

GESTATIONAL AGE	90 DAYS		120 DAYS	
TREATMENT	CONTROL (N=9)	RESTRICTED (N=4)	CONTROL (N=6)	RESTRICTED (N=11)
Placental Weight (g)	515 ± 42 @	340 ± 27	545 ± 35 \$	297 ± 22
Placentome Number	74 ± 6 @	33 ± 3	74 ± 2 \$	30 ± 2
Chorionic Membrane	57.62 ± 8.67 @	29.00 ± 5.23	70.31 ± 6.70 \$	28.80 ± 2.91
Chorionic Epithelium	13.09 ± 2.21	7.18 ± 1.30	24.95 ± 2.03 \$	11.18 ± 1.89
Haemophagous Zone	28.29 ± 7.31 @	9.90 ± 2.23	31.01 ± 5.04 \$	12.36 ± 2.10
Maternal Tissue in Haemophagous Zone	10.48 ± 2.83 @	3.20 ± 0.36	8.08 ± 1.34	3.04 ± 0.76
Fetal Villi	200.58 ± 20.97 @	136.36 ± 15.73	193.23 ± 24.77 \$	119.27 ± 9.71
Trophectoderm	83.51 ± 4.31	78.12 ± 6.78	122.25 ± 16.05 \$	81.22 ± 7.06
Connective Tissue Core	116.94 ± 20.71 @	64.12 ± 5.22	69.32 ± 9.57	32.58 ± 3.49
Maternal Crypts	141.65 ± 12.3	107.95 ± 7.89	159.55 ± 14.55 \$	91.40 ± 8.20
Feto-maternal Syncytium	38.21 ± 3.01	33.96 ± 4.52	47.09 ± 4.83 \$	28.32 ± 3.02
Shrinkage Spaces	17.73 ± 4.08	22.10 ± 10.92	17.08 ± 5.14	18.13 ± 2.14
Placentome Capsule	45.43 ± 5.09 @	25.94 ± 3.79	39.02 ± 6.30 \$	14.68 ± 1.89
Surface Area (m ²)	5.93 ± 0.44	5.18 ± 0.83	10.07 ± 0.69 \$	6.51 ± 0.50
Relative Surface Area (cm ⁻¹)	47.79 ± 2.32	52.28 ± 6.13	31.27 ± 1.54	25.36 ± 1.14

Values are expressed as Mean ± SE; ANOVA; LS Means Specific Comparisons; excluding small placentomes

Values presented in "**BOLDFACE**" were significantly different between 90 and 120 days gestation within the restricted ewes (p<0.05);

Values marked "@" were significantly different between control and restricted ewes at 90 days gestation; (p<0.05)

Values marked "\$" were significantly different between control and restricted ewes at 120 days gestation; (p<0.05)

N = Number of ewes studied; Component placental volume = volume density × placental weight

CHAPTER 5

EFFECT OF NATURAL RESTRICTION OF IMPLANTATION ON OVINE PLACENTAL STRUCTURAL DEVELOPMENT AT MID GESTATION

5.1 INTRODUCTION

Restricted implantation due to a reduction in the number of available implantation sites or caruncles occurs naturally in sheep when more than one conceptus is present because of competition which reduces the number of implantation sites available for each elongating blastocyst. In a singleton pregnancy, trophoblastic implantation recruits about 70% of the available caruncles for placental development (Alexander, 1964a; Stegeman, 1974). In a twin pregnancy, more or about 80% of the caruncles are recruited, but are shared by two developing placentas (Alexander, 1964a; Stegeman, 1974). Thus on average, the placenta of a twin is composed of about half the number of placentomes in a singleton placenta (Alexander, 1974; Robinson *et al.*, 1979; Vatnick *et al.*, 1991).

Placentomes of twin conceptuses are on average 30% heavier than those of singletons, but the combined weight of the placentas of twins is not twice that of singletons (Alexander, 1964a, b; 1974; Vatnick *et al.*, 1991). This suggests that the increased growth of individual placentomes following natural restriction of implantation is insufficient to maintain placental weight in twins

(Alexander, 1974). Following manual separation of placentomes into fetal and maternal tissue components, placentomes of twins had 45% more fetal tissue than those of singletons (Davies *et al.*, 1981), indicating that fetal tissue is largely responsible for the compensatory increase in individual placentome size observed.

Although the weight of the placenta of an individual twin is significantly smaller than that for a singleton, their total placental DNA content is similar suggesting that placentas of twins contain smaller cells than those of singletons (Vatnick *et al.*, 1991). This suggests that natural compensatory placental growth involves hyperplasia, which may largely occur during the first half of pregnancy and probably be completed by mid gestation (Kulhanek *et al.*, 1974; Vatnick and Bell, 1991; Vatnick *et al.*, 1991). However, changes in the heterogeneous

relative abundance of cell populations of the placenta might also contribute to this apparent hyperplasia.

The impact of compensatory placental growth in multiple pregnancy on the cellular composition of the placenta and the consequences for structural determinants of function have not been studied. In particular, it is not known whether natural and experimental restriction of implantation involve similar responses by the placenta, and whether there are limits to that compensation.

Therefore, the first aim of this study was to characterise placental structure in ewes carrying twins and triplets for comparison with those carrying singletons at mid gestation. A small study was included in this part in order to investigate the ontogeny of placental growth in twins compared to singletons between mid and late gestation.

The second aim was to determine if there are limits to the compensatory responses of placental structure to restricted implantation by increasing the extent of the restriction imposed. Hence, placental structure of ewes subjected to experimental restriction of implantation by carunclectomy and carrying twins or triplets was characterised.

5.2 MATERIALS AND METHODS

Animals, sample collection, morphometric methods and assessment of placental function.

This study was carried out in well-fed Border Leicester × Merino cross-bred ewes which carried singletons, twins or triplets. Some of the ewes had undergone carunclectomy prior to pregnancy to restrict implantation. All animals were killed at day 90 of gestation for the examination of uterine contents. A detailed description of the experimental design has been given in Chapter 2 and summarised in Table 2.3. However, in order to study the ontogenic growth of macroscopic parameters in twins compared to singletons, ewes were killed at either 90 or 120 days gestation. Fetal and placental parameters were recorded at post-mortem, and

placental samples were selected and processed for morphometric analysis as outlined in Chapter 2.

Volume densities of placentome components and surface densities of trophoctoderm were determined by morphometric analysis and used to calculate total placental volumes of all components, and placental exchange surface area and arithmetic mean barrier thicknesses of the epithelial cell layers at the feto-maternal interface. These parameters were in turn correlated

- a) with each other to study the association of different components within the placenta and
- b) with fetal weight to investigate placental function.

Statistical analysis and presentation of results

The effects of between factors, natural and experimental restriction of implantation and of within factors, placentome size and morphology and their interactions were assessed by analysis of variance. Specific comparisons between treatment groups were carried out by least squares means analysis.

Data for singletons without restricted implantation included in this chapter, for comparison with data for twins and triplets, are the same as those presented and discussed in Chapters 3 and 4 and referred to as control singletons at 90 days gestation in those chapters. Data for singletons with restricted implantation are the same as the restricted singletons at 90 days gestation in chapter 4.

5.3 RESULTS

5.3.1 Placental structure following natural restriction of implantation in ewes bearing twins and triplets

Macroscopic Parameters in twins and triplets without experimental restriction of implantation

Compared to placentas of singletons, placentas of twins and triplets had lower numbers of placentomes (-35% for twins and -47% for triplets) and placental weight (-32% for twins and

-46 % for triplets) ($p \leq 0.05$) (Table 5.1). Placentome numbers and placental weight were not significantly different in twins and triplets (Table 5.1). Singletons and triplets had comparable average fetal weights which were both lower (-21% for singletons and -20% for triplets) than the average fetal weight for twins (Table 5.1).

Mean placentome weights were similar in singletons, twins and triplets. The extent of eversion of placentomes, whether expressed as a proportion of placentome numbers everted or as a proportion of placental weight composed of everted placentomes, was similar in placentas of singletons, twins and triplets (Table 5.1).

Analysis of the weight distribution of placentomes revealed that in twins, $56 \pm 6\%$ of all placentomes were of average weight (5-9.9g) (Figure 5.1) compared to $36.2 \pm 4.8\%$ ($p = 0.02$) in singletons (Figure 5.1). In contrast, overall placentome weight distribution was similar in triplets and singletons (Figure 5.1)

Comparison of placentome distribution within weight range categories in placentas of twins and triplets showed that within placentome weights between 0 and 24.9g in which placentomes of both treatment groups were present, there were no significant differences in the placentome weight distribution (Figure 5.1). However, the largest placentomes in placentas of triplets weighed between 30 and 34.9g as opposed to only between 20 and 24.9g in placentas of twins (Figure 5.1). Overall, placentas of triplets did not contain a significantly increased number of large ($10g \geq$) placentomes when compared to a similar proportion of placentomes in placentas of twins (Figure 5.1)

In twins, everted placentomes were distributed equally in all weight range categories up to the 15-19.9g range, but without everted placentomes in the higher weight range categories (Figure 5.2). This distribution was similar to that observed in placentas of singletons (Figure 5.2). The distribution of everted placentomes in placentas of triplets was also similar to that in placentas of singletons and twins (Figure 5.2). In general, the proportion of everted placentomes tended to reduce with the increase in placentome weight.

on the ontogeny of placental weight. In addition, there was a significant interaction between the two treatment effects on the ontogeny of the proportion of large placentomes ($\geq 10\text{g}$) such that a significant increase in the proportion of large placentomes was evident in twins and not in singletons (Table 5.3). Both fetus number and gestational age had significant effects on the ontogeny of placentome eversion and f:p ratios (Table 5.2).

At both 90 and 120 days gestation, placentas of twins weighed 32% and 21% less respectively than those of singletons (Table 5.3). However, at 120 days gestation, individual placentome size (+35%; $p < 0.05$), the proportion of large placentomes (+2-fold) and the proportion of everted placentomes (+3-fold) were greater in twins than in singletons. Whereas in singletons, both average individual placentome weights and the proportions of everted placentomes were similar at the two gestational ages, in twins, there was an ontogenic increase in mean placentome weight (+36.5%; $p < 0.03$) between 90 and 120 days gestation. In addition, in twins, the proportion of large placentomes had increased 2.4-fold whereas that of everted placentomes had increased 4.3-fold by 120 days compared to that at 90 days. The increase in individual placentome weight caused a 22% increase in total placental weight in twins by 120 days compared to that at 90 days gestation whereas in singletons, placental weight only increased by 6% within the same period.

Microscopic Parameters.

In terms of the major tissue components (total fetal tissue and total maternal tissue), the volumetry of a placentome of average size from a placenta of a twin was 54.3% fetal tissue and 37.4% maternal tissue (Table 5.4). In triplets the average-size placentome was composed of about 60% fetal tissue and 37.7% maternal tissue (Table 5.4). Thus the volumetric composition of placentomes of comparable size in placentas of twins was comparable to that in singletons whereas in triplets, the volumetry demonstrated a relative increase of about 6% in total fetal tissues compared to that in singletons (Table 5.4).

There were no significant differences in volume densities of all individual cellular components in placentas of twins and triplets (Table 5.5). However, compared to singletons, placentomes of twins, had a higher volume density of trophoblast (+71%; $p = 0.0001$) and a

There were no significant differences in volume densities of all individual cellular components in placentas of twins and triplets (Table 5.5). However, compared to singletons, placentomes of twins, had a higher volume density of trophoctoderm (+71%; $p=0.0001$) and a lower volume density of the connective tissue core of the fetal villi (-36%; $p=0.0001$) (Table 5.5 and Figure 5.3). Also in placentomes of twins, the volume density of feto-maternal syncytium was higher (+46%; $p=0.0002$) while that of the placentome capsule was lower (-28%; $p=0.01$) than those of singletons (Table 5.5 and Figure 5.3).

Compared to singletons, placentomes of triplets had higher volume densities of chorionic epithelium (+ 43%; $p=0.002$) (Table 5.5), trophoctoderm (+ 40%; $p=0.0001$) and feto-maternal syncytium (+27%; $p=0.004$), but a lower volume density of the connective tissue core of the fetal villi (-47%; $p=0.0001$) (Table 5.5 and Figure 5.3)

Placentomes of twins and triplets had similar surface densities of trophoctoderm (Table 5.6 and Figure 5.4) which were 37% and 38% higher respectively than that in singletons ($p=0.0001$) (Table 5.6 and Figure 5.4). Placentomes of twins and triplets also had similar arithmetic mean barrier thicknesses of trophoctoderm and feto-maternal syncytium (Table 5.6 and Figure 5.5). which were 15% higher than that of singletons. However, due to a large standard error in twins, the value was not significantly different from that in singletons whereas that in triplets was significantly higher than those of singletons ($p=0.0008$). In contrast, in twins, the arithmetic mean barrier thickness of feto-maternal syncytium was significantly reduced (-20%; $p=0.03$) while that of triplets was only 9% lower than that of singletons (Table 5.6 and Figure 5.5)

There was no significant effect of placentome size on the cellular remodelling that occurred within the placentomes of multiple conceptuses. However, no comment can be made on the effect of placentome morphology on placentome components since only one everted placentome was morphometrically analysed.

In absolute terms, the volumes of all components and surface area were similar in placentas of twins and triplets (Table 5.7). Although in triplets most of the components, including those that are presumably directly related to function, were reduced in comparison to those in twins, this did not reach statistical significance. However, placentas of twins differed from those of singletons by having reduced volumes ($p < 0.05$) of maternal tissue in the haemophagous zone (-64%), fetal villi (-30%), the connective tissue core of the fetal villi (-58%), maternal crypts (-29%) and the placentome capsule (-54%) (Table 5.7). In contrast, the volumes of trophoctoderm, feto-maternal syncytium and also total placental surface area in twins were comparable to those in singletons (Table 5.7). Compared to singletons, placentas of triplets had lower volumes of most components ($p < 0.05$) including chorionic membrane (-44%), haemophagous zone (-48%), fetal villi (-50%), connective tissue core of fetal villi (-74%), maternal crypts (-41%), feto-maternal syncytium (-27%) and placentome capsule (-52%). The volume of trophoctoderm (-17%) as well as the total surface area for exchange (-24%) were also lower in triplets compared to singletons although this did not reach statistical significance (Table 5.7).

5.3.2 Experimental restriction of implantation in ewes bearing twins and triplets.

Macroscopic parameters.

Restricted twins.

Due to insufficient numbers of experimentally restricted twin-bearing ewes ($N=2$), results for effect of carunclectomy in twins may only be regarded as preliminary. Carunclectomy in twins, significantly reduced the number of placentomes formed (-72%) and total placental weight (-43%) ($p < 0.05$), but doubled the average weight of individual placentomes ($p < 0.05$) (Table 5.1). In addition, the proportion of everted placentomes increased three and a half-fold while the total weight of all everted placentomes as a proportion of total placental weight increased over four and a third-fold ($p < 0.05$) (Table 5.1).

Placentas of experimentally restricted twins also had less placentome numbers (about 50%) and placental weight than those of restricted singletons ($p < 0.05$), but similar mean placentome

weight and rate of eversion (Table 5.1). Fetal weights were also similar in these two treatment groups and were less than those of twins (Table 5.1).

Compared to placentas of twins, experimentally restricted placentas of twins contained a significantly greater proportion of "small" placentomes in 0-0.49g weight range category ($7.5 \pm 1.92\%$ versus $0.73 \pm 1.11\%$ in controls; $p=0.005$) (Figure 5.1). The proportion of placentomes within the 5-9.9g weight range was reduced ($22.5 \pm 10.13\%$ in restricted placentas versus $55.68 \pm 5.85\%$ in twins; $p=0.008$). Hence the model weight range slightly shifted to the left, such that the greatest proportion of placentomes were present within the 0.5 to 4.9g (Figure 5.1) following experimental restriction of implantation in twins. However the proportion of placentomes within the 0.5 to 4.9g range was not statistically significant from that in 5-9.9g weight range (Figure 5.1). In restricted twins, the range of placentome weights was increased such that 10% of placentomes weighed between 50 and 54.9g. In contrast, the largest placentomes in the placentas of twins weighed only 20 to 24.9g; making up only 1% of all placentomes (Figure 5.1). Overall, experimental restriction of implantation in twins did not significantly increase the proportion of large placentomes ($10g \geq$) (Figure 5.1).

In restricted twins, the distribution of everted placentomes appeared to increase in the higher weight range categories (Figure 5.2). This was in contrast to the observation made in restricted singletons (Figure 5.2). Also the proportion of the B morphology of eversion appeared to increase with restricted implantation in twins although this was not statistically significant due to a very high standard error (Table 5.8).

Restricted triplets

In triplets, further restriction of implantation by carunclectomy reduced placentome numbers (-54% ; $p=0.0001$) and placental weight (-39% ; $p=0.0003$) (Table 5.1). Mean placentome weight did not significantly increase in restricted placentas but the extent of eversion showed approximately a 5-fold increase ($p=0.0001$) both when expressed as a proportion of everted placentomes within the placenta and as the total weight of all everted placentomes expressed as a proportion of total placental weight (Table 5.1).

In triplets, experimental restriction of implantation increased the proportion of everted placentomes in all weight range categories except in the 0-0.49g weight range (Figure 5.2). The proportions of everted placentomes appeared to decrease at the higher placentome weight extreme (Figure 5.2). Restricted implantation increased the proportions of B, C, and D varieties of everted morphology, the increase for C, and D morphologies was significant ($p<0.05$) (Table 5.9).

Microscopic Parameters

Restricted Twins

In restricted placentas of twins, a placentome of average size was composed of 52% fetal tissue and 39.3% maternal tissue (Table 5.10). This volumetry showed a slight reduction in fetal tissues (2.7%) and a similar increase in maternal tissue (2%) when compared to that of comparable size placentomes in control twins.

In twins, experimental restriction of implantation caused the reduction of the volume density of chorionic membrane (-42%; $p=0.04$) whereas within the fetal villi, the volume density of trophoctoderm (+30%; $p=0.003$) increased.

Placentomes of restricted twins had a higher surface density of trophoctoderm (+33%; $p=0.002$) (Table 5.12 and Figure 5.7) but similar arithmetic mean barrier thicknesses of both trophoctoderm and feto-maternal syncytium (Table 5.12) to that in placentomes of control placentas.

In absolute terms, the volumes of all components in restricted placentas ($N=2$) were reduced when compared to those of control placentas ($N=6$) but the differences did not reach statistical significance (Table 5.7). However, placental function may have been impaired since fetal weight was lower (-40%; $p<0.05$) in the restricted ewes compared with that of ewes without experimental restriction of implantation (Table 5.1).

Restricted Triplets.

Within restricted placentas of triplets, placentomes of average size contained 52.6% fetal tissue and 40.8% maternal tissue thus showing a 7% reduction in fetal tissues and a 3% increase in maternal tissues in placentomes of restricted triplets compared to triplets (Table 5.13).

Following experimental restriction of implantation in triplets, placentomes had reduced volume densities of chorionic membrane (-30%; $p=0.01$) and placentome capsule (-35%; $p=0.01$) when compared with those in triplets (Table 5.14). The volume densities of fetal villi (+17%; $p=0.004$) and trophoctoderm (+19%; $p=0.004$) were significantly higher in placentomes of the restricted triplets compared to those of triplets (Table 5.14 and Figure 5.8). However, the surface density of trophoctoderm and arithmetic mean barrier thickness of both trophoctoderm and feto-maternal syncytium were not altered with further restriction of implantation by carunclectomy (Table 5.15).

There were no significant effects of placentome size on all these parameters. However, placentome morphology tended to be associated with reduced volume densities of haemophagous zone ($p=0.002$) in both triplets (-41%) and restricted triplets (-36%) (Table 5.16 and Figure 5.9). In addition, in triplets everted placentome morphology was associated with higher surface densities of trophoctoderm (+8% in triplets compared to +12% in restricted triplets; $p=0.002$) and not with higher volume densities of trophoctoderm as seen in chapters 3 and 4 (Table 5.17 and Figure 5.10). Placentome morphology was not associated with significant changes in arithmetic mean barrier thicknesses of the two epithelial cell layers across the feto-maternal interface (Table 5.17).

In absolute terms, the volumes of several components were reduced following experimental restriction of implantation in placentas of triplets ($p\leq 0.05$); chorionic epithelium (-50%), maternal crypts (-38%), placentome capsule (-62%) compared to placentas of triplets (Table 5.7). In addition surface area was not maintained (-36%) (Table 5.7), hence fetal growth was reduced (-27%; $p=0.003$) (Table 5.1).

Correlation Analysis of Placental Parameters with Fetal Weight.

Twins.

No significant correlations between fetal weight and placental parameters had emerged at this time in gestation in twins. Insufficient data for restricted ewes bearing twins made it impossible to detect if any correlations had emerged as a result of further restriction of implantation due to carunclectomy.

In twins, placental weight correlated significantly with the absolute volumes of chorionic membrane ($r=0.88$; $p=0.02$, $N=6$), fetal villi ($r=0.97$; $p=0.0009$, $N=6$), connective tissue core of fetal villi ($r=0.81$; $p=0.05$, $N=6$), maternal crypts ($r=0.84$; $p=0.04$, $N=6$) and also with surface area ($r=0.92$; $p=0.009$, $N=6$) (Table 5.18).

Triplets

In triplets, there was a positive correlation between fetal weight and absolute volumes of chorionic membrane ($r=0.91$; $p=0.01$, $N=6$), chorionic epithelium ($r=0.87$; $p=0.02$, $N=6$), and the maternal tissue within the haemophagous zone ($r=0.98$; $p=0.004$, $N=6$). In restricted triplets fetal weight correlated with the volumes of maternal crypts ($r=0.81$; $p=0.02$, $N=8$) and placentome capsule ($r=0.73$; $p=0.04$, $N=8$) (Table 5.19).

In both triplets and restricted triplets, there were significant positive correlations between placental weight and most morphometrically determined parameters. Correlation coefficients ranged from $r=0.78$ ($p=0.02$, $N=6$) for haemophagous zone in restricted triplets to $r=0.99$ for several components like fetal villi ($p=0.0002$) in triplets ($N=6$) (Table 5.19). In both triplets and restricted triplets, placental weight did not correlate with the volumes of placentome capsule and the maternal tissue within the haemophagous zone. In addition, placental weight in restricted triplets did not correlate with placentome number (Table 5.19).

5.3.3 Analysis of overall effects of natural restriction of implantation (twins and triplets versus singletons), experimental restriction of implantation (carunclectomy), and their interaction on placental parameters at 90 days gestation

Macroscopic Parameters

Overall, experimental restriction of implantation reduced placental weight ($p=0.0001$) and increased the proportion of everted placentomes within the placenta ($p=0.0001$) (Table 5.20). There was a significant increase in the proportions of C, and D varieties of everted morphology in restricted placentas (Tables 5.20 and 5.21). These effects were manifested equally in placentas of singletons, twins, and triplets except for the significant interaction between experimental and natural restriction of implantation on proportions of placentomes exhibiting the C morphology (Table 5.20).

There was a significant interaction between natural and experimental restriction of implantation on placentome numbers, such that the reduction of placentome numbers by experimental restriction of implantation was more pronounced in placentas of multiple conceptuses (Tables 5.20 and 5.21).

Experimental restriction of implantation was generally associated with increased placentome weight ($p=0.0007$). However, the resultant increase in the proportion of large placentomes ($10g\geq$) did not reach statistical significance ($p=0.06$) (Table 5.20).

Natural restriction of implantation also significantly influenced placental weight ($p=0.0001$) (Table 5.20) such that placental weight for each conceptus was reduced further with increased fetal number (Table 5.21). There were no significant effects of fetus number on mean placentome weight and also on the extent of eversion within the placenta although the latter tended to increase in placentas of twins and triplets when compared with that in singletons (Tables 5.20 and 5.21).

Microscopic Analysis.

i) Volume and Surface Densities and Arithmetic Mean Barrier Thicknesses.

Within the placentome, experimental restriction of implantation significantly reduced volume densities of chorionic membrane ($p=0.002$), chorionic epithelium ($p=0.04$), haemophagous zone ($p=0.03$) and placentome capsule ($p=0.02$). The volume densities of fetal villi ($p=0.006$) trophoctoderm ($p=0.0001$), maternal crypts ($p=0.007$) and feto-maternal syncytium ($p=0.0009$) were significantly higher in placentomes of restricted placentas when compared to those in placentomes of ewes without experimental restriction of implantation. These effects were manifested equally regardless of fetus number (Tables 5.22 and 5.23).

Generally, with increasing fetus number, there were significant increases in volume densities of chorionic epithelium ($p=0.003$), trophoctoderm ($p=0.0001$) and feto-maternal syncytium ($p=0.0001$) (Tables 5.22 and 5.23). The volume densities of the connective tissue core of the fetal villi ($p=0.0001$) and placentome capsule ($p=0.02$) were significantly reduced in multiple pregnancy (Tables 5.22 and 5.23). In contrast to the effects of experimentally restricted implantation, natural restriction of implantation was not associated with significant changes in volume densities of chorionic membrane, haemophagous zone, fetal villi and maternal crypts (Tables 5.22 and 5.23).

Overall, both natural and experimental restriction of implantation increased surface density of trophoctoderm ($p=0.0001$) (Tables 5.22 and 5.23). However, the arithmetic mean barrier thickness of trophoctoderm was increased ($p=0.0001$) in multiple pregnancy but was not altered in experimental restriction of implantation (Tables 5.22 and 5.23). The arithmetic mean barrier thickness of feto-maternal syncytium was not altered by both forms of restricted implantation (Tables 5.22 and 5.23).

ii) Absolute Volumes of Components and Surface Area.

In absolute terms, experimental restriction of implantation was generally associated with significant changes in volumes of all other placental components except those of the maternal tissue within the haemophagous zone, shrinkage spaces and surface area (Tables 5.24 and

5.25). On the other hand, natural restriction of implantation was associated with significant changes in volumes of components within the parenchyma of the placenta and also with surface area (Table 5.24). Except for the volume of trophoderm, which was similar in singletons and twins, all other components within the parenchyma of the placenta including surface area showed a tendency to decrease with increasing number of conceptuses (Table 5.25). In contrast to the overall effect of carunclectomy, increasing number of conceptuses did not alter volumes of chorionic membrane, chorionic epithelium, haemophagous zone and the maternal tissue within it (Tables 5.24 and 5.25).

5.4 DISCUSSION

Placentas of twins had significantly reduced numbers of placentomes when compared with placentas of control singletons as previously observed (Alexander, 1964a; Robinson *et al.*, 1979; Vatnick *et al.*, 1991). However, the present study has shown no macroscopic evidence of compensatory growth of individual placentomes of twins at 90 days of gestation such that average placentome weight and the extent of placentome eversion were similar to those of singletons. In twins, a high proportion of placentomes fall within the weight range of 5-9.9g ($55.68 \pm 5.85\%$; compared to only $36.17 \pm 4.77\%$ in placentas of singletons, $p=0.02$). Therefore the proportions of placentomes in all other weight range categories, including that of larger placentome weights, were reduced. Surprisingly, the largest placentome weight achieved in the placentas of twins was less (24.9g) than that in singleton placentas (29.9g). In addition, twins tended to have a higher proportion of "small" placentomes (0-4.9g) than singletons. These results reveal that placentomes of twins are not bigger than those of singletons in mid gestation.

Placentas of triplets had fewer placentomes than twins and lower placental weight than singletons. Mean placentome weights and proportions of everted placentomes in triplets were similar to those of singletons and twins. Thus further reduction in placental size due to the natural increase in number of conceptuses did not significantly enhance the adaptational growth response of the individual placentomes. In general, natural restriction of implantation did not alter mean placentome weight or the proportion of large placentomes ($10g \geq$). It is

therefore clear that in twins and triplets placental compensatory growth does not involve increases in placentome size and enhancement of placentome eversion by mid gestation.

Previous studies in late gestation in sheep indicate that the intact placentome in a placenta of a twin fetus is about 30% heavier than that of a singleton placenta (Alexander, 1964a). Also Davies *et al.*, (1981) reported a 45% increase in the weight of the fetal component of the placentome in twins compared to that of singletons. In contrast, Vatnick *et al.*, (1991) found no significant increase in mean weight of the intact placentomes of twins compared to singletons at 136 days gestation. In the present study, average weights of individual placentomes in twins and triplets were similar to those of singletons by mid gestation. This suggests that the adaptational increase in placentome size which has been reported previously in twins may occur after mid gestation; beyond the period when in singletons there is no significant increase in individual placentome size and hence placental size.

Comparisons of the ontogenic development of macroscopic parameters in singletons and twins between 90 and 120 days gestation revealed a significant increase in placentome size between the two gestational ages in twins as opposed to singletons. Consequently, in late gestation, placentomes of twins were larger (+35%) than those of singletons although placental weight of an individual twin was about -21% less than that of a singleton. Also, the proportion of everted placentomes had increased more than 4-fold over this time. These results are in agreement with those reported by previous workers (Alexander, 1964a; Davies *et al.*, 1981) but in addition, the present study demonstrates that increases in individual placentome weight and in proportions of everted placentomes in twins occur after 90 days gestation. In singletons, there is normally no significant increase in both parameters during this period of gestation.

Measurements of total placental DNA content indicate that placentas of singletons and twins contain similar amounts of DNA despite placental weight in the latter being significantly lower (Vatnick *et al.*, 1991). This suggests that there are similar total cell numbers in placentas of singletons and twins but that the latter contains smaller cells. Hence one component of the natural compensatory growth in multiple pregnancy involves hyperplastic

growth which occurs during the proliferative phase of placental growth and thus will probably be completed by mid gestation (Kulhanek *et al.*, 1974; Vatnick and Bell, 1991; Vatnick *et al.*, 1991).

In restricted placentas of twins, average placentome weight was doubled and the extent of eversion increased markedly when compared to placentas of twins. The proportion of placentomes within the 5 to 9.9g weight range was halved, and that of placentomes within the weight ranges 20 to 24.9g and 35 to 39.9g were significantly increased. However, the changes in placentome weight distribution were not adequate to significantly increase mean placentome weight as well as the proportion of large ($10g \geq$) placentomes when compared with those in placentas of triplets. In contrast, the extent of eversion increased several-fold following experimental restriction of implantation in triplets. These results show that following experimental restriction of implantation placentomes grow bigger and the incidence of placentome eversion increases in placentas of multiple conceptuses as in singletons. However, it appears that as numbers of conceptuses increase the ability of the placentomes to increase in size is reduced whereas their ability to evert is promoted. Thus these results confirm an observation made earlier (Chapter 4) that the stimulus for increased placentome size may be separate from that which is responsible for placentome eversion. However, in multiple pregnancy, the proportion of everted placentomes in higher weight range categories did not show an obvious decline as seen in placentas of singletons (Chapter 4).

From the macroscopic observations made in control and experimentally restricted singletons, twins and triplets, it appears that there is a temporal difference in the mechanism by which these two forms of restricted implantation induce adaptational growth of placentomes and their eversion. Following experimental restriction of implantation in singletons and twins, mean placentome size and the proportion of everted placentomes increase by mid gestation. In placentas of multiple conceptuses of normal ontogeny mean placentome size and eversion rate remain low and increase significantly after mid gestation. Thus in multiple pregnancy the stimulus for the growth of individual placentomes and that which enhances their eversion

occurs after mid gestation as opposed to earlier in gestation following experimental restriction of implantation.

The possibility of a high progesterone uterine environment around implantation time in experimentally restricted sheep (Robinson *et al.*, 1976) comparable to controls has already been discussed in the first part of this chapter. In ewes carrying twins and triplets, higher levels of progesterone would be expected due to increased numbers of corpora lutea (McNeil *et al.*, 1991). Thus it was surprising to find that placentas of twins and triplets had similar extents of eversion to those of singletons. This finding probably indicates that other factor(s) are also involved in the mechanism besides progesterone. In addition, the proportion of everted placentomes in restricted placentas of twins and triplets were similar to those of restricted singletons. Possibly, following carunclectomy, progesterone concentrations in singletons, twins and triplets are similar and exert comparable effects on placentome morphology. Alternatively, there may not be a linear relationship between the extent of placentome eversion and the levels of progesterone in mid gestation..

The proportions of fetal and maternal tissue were similar in placentomes of singletons and twins. However, in placentomes of twins, fetal villi contained greater volume densities of trophoctoderm but reduced volume densities of connective tissue; hence the villi were thinner than those of singletons. This should increase the relative abundance of trophoctoderm and also reduce the diffusion distance for substrate transfer. The volume density of feto-maternal syncytium was also greater in twins. Thus, compared to singletons, in the placentomes of twins, there may be more binucleate cells migrating to form feto-maternal syncytium per unit weight of tissue, probably due to the compensatory hyperplasia which occurs during the phase of rapid placental growth (Vatnick *et al.*, 1991). Increased volume densities of trophoctoderm and feto-maternal syncytium may be consistent with the greater cellularity in placentas of twins compared to singletons (Vatnick *et al.*, 1991). The present study has also shown that the surface density of trophoctoderm is greater whereas the arithmetic mean barrier thickness of feto-maternal syncytium is reduced in twins. The latter could partly be achieved by the formation of structures analogous to vasculosyncytial membranes, which are very thin areas of the syncytial plaques, observed previously in sheep (Mitchell, 1982). These may decrease

harmonic mean thickness by increasing membrane variability and thus enhance membrane diffusibility (Jackson *et al.*, 1988). Together the adaptational cellular changes occurring in placentomes of twins should substantially enhance placental transfer.

Surprisingly, the internal structure of placentomes of triplets did not demonstrate further structural remodelling beyond that observed in placentas of twins. Thus in triplets, volume densities of all placental components as well as surface densities and arithmetic mean barrier thicknesses of trophoctoderm and feto-maternal syncytium were similar to those of twins. Hence, the placentas of twins demonstrated maximum adaptational remodelling. It appears that the structural remodelling that was evident in placentas of twins may be sufficient to sustain the growth of a fetus with a smaller placental size as in triplets by mid gestation, such that placental function in triplets may not have to be further enhanced than that in placentas of twins. In both twins and triplets the adaptational process was sufficient to maintain absolute volumes of trophoctoderm, feto-maternal syncytium and total surface area comparable to those of singletons. In triplets, the placental volumes of these three parameters tended to be reduced but this did not reach statistical significance. Measurements of harmonic mean thickness and electron microscope morphometry of the trophoctoderm and feto-maternal syncytium brush border, for example, should demonstrate whether finer placental parameters of triplets are similar to or are better adapted to enhance transfer function than those in placentas of twins.

A qualitative comparison of the cellular remodelling occurring in placentomes of twins and that of experimentally restricted singletons suggested that the adaptational process occurred to a greater extent in the former. Natural restriction of implantation in twins induced a greater extent of structural remodelling especially in terms of reducing barrier thickness between fetal and maternal bloods compared to experimentally restricted placentas of singletons. Thus the stimuli for these processes may be amplified in twins. The relatively more advanced cellular remodelling in twins may reduce the need for increases in individual placentome weights at mid gestation. In contrast, limited cellular remodelling in experimentally restricted singletons may determine the need for more placentomes to grow bigger by mid gestation.

Following experimental restriction of implantation in both singletons and twins the volume densities of trophoctoderm and feto-maternal syncytium increased but to a lesser extent than the increase in surface density of trophoctoderm which was similar (33%) in both treatment groups. Carunclectomy did not induce a reduction of the arithmetic mean barrier thicknesses of the epithelial cell layers in both singletons and twins. Thus, significant increases in surface density of trophoctoderm and feto-maternal syncytium was a consistent feature following experimental restriction of implantation. This points to increased surface density as one of the key events in the mechanism of experimentally induced placental compensatory growth. However, the compensatory increases in volume and surface densities of the epithelial cell layers in restricted twins were insufficient to maintain absolute volumes of trophoctoderm (-26%), feto-maternal syncytium (-20%) and total placental surface area (-22%). This may have induced the 40% reduction in fetal weight observed in restricted twins. The absence of statistical significance of the reductions of the absolute volumes of the components may reflect the small sample (N=2) for restricted ewes bearing twins.

In restricted triplets the volume density of fetal villi increased (+17%) due to an increase in the volume density of the constituent trophoctoderm (+19%) while surface density and arithmetic mean barrier thicknesses of trophoctoderm and feto-maternal syncytium remained similar to those of triplets. Therefore, in comparison to singletons and twins, carunclectomy induced the least cellular remodelling response in triplets. The absolute volumes of trophoctoderm and feto-maternal syncytium were not significantly lower than those of placentas of triplets. However, due to the absence of an increase in surface density within the restricted placentas of triplets, placental surface area was very low ($2.9\text{m}^2 \pm 0.3$ compared to $4.5\text{m}^2 \pm 0.8$ in triplets) which may have impaired fetal growth as observed.

The average placental weights in both twins and triplets were significantly lower than that of singletons but fetal weight in twins was greater than while that of triplets was similar to the average fetal weight of singletons at mid gestation. This suggests that the placentas of multiple conceptuses are more functional and hence are able to support normal fetal growth and bigger fetuses in proportion. There was no significant correlation between fetal weight and placental weight in both twins and triplets. However, various placental sizes including

that of singletons, twins and triplets were compatible with normal fetal growth at 90 days gestation, suggesting that placental size may not be an appropriate index of function at this stage in gestation.

Experimental restriction of implantation induces a similar growth response in the placenta regardless of the number of conceptuses. The growth response involves increases in placentome size and eversion. In contrast, natural restriction of implantation is not associated with significant macroscopic placentome changes by mid gestation. Within the placentome, both forms of restricted implantation induce significant increases in volume and surface densities of trophoctoderm and feto-maternal syncytium. Following experimental restriction of implantation, these increases were usually paralleled by reductions in volumes of the chorionic membrane, chorionic epithelium, the haemophagous zone and the maternal tissue within this zone whereas in multiple pregnancy significant cellular remodelling was more limited to the parenchyma of the placentome.

5.5 SUMMARY

This work has confirmed that placentas of twin and triplet conceptuses differ from those of singletons by having reduced numbers of placentomes and placental weight. At 90 days gestation, average placentome weights and the extents of placentome eversion in placentas of multiple conceptuses are similar to those in placentas of singletons. However, the proportions of the everted morphologies C and D tend to increase in multiple pregnancy.

Within the internal structure of placentomes of twins, placental function is enhanced by increases in the volume densities of trophoctoderm and feto-maternal syncytium which achieve absolute volumes comparable to those in control singletons. In addition, reductions in the volume of the connective tissue core of the fetal villi and in the barrier thickness of feto-maternal syncytium should promote diffusiveness of the membrane. Due to increases in the surface density of the epithelial cell layers at the feto-maternal interface placentas of twins

have a surface area comparable to that of singletons. Thus overall placental function is enhanced and normal fetal growth is supported.

Placentas of triplets demonstrated no further enhancement of function beyond that observed in placentas of twins. Thus the adaptational cellular remodelling that is present in placentas of twins by mid gestation may be optimal at this gestational age, and may also be adequate to support normal fetal growth in conceptuses with even smaller placentas, as is the case with triplets.

The response to experimental restriction of implantation is similar in placentas of singletons, twins and triplets although placentome numbers tend to be much more reduced with increasing number of conceptuses. In twins, carunclectomy induces increases in volumes of trophoderm and feto-maternal syncytium but does not decrease their arithmetic mean barrier thicknesses. In triplets, except for increased volumes of fetal villi and trophoderm, there is no other evidence for enhanced placental function following experimental restriction of implantation. Therefore response to carunclectomy is limited in placentas of multiple conceptuses.

It appears that in sheep, placental surface area between 5 and 6m² is compatible with normal fetal growth in mid gestation. Thus failure of compensatory placental growth to achieve surface area of this magnitude impairs fetal growth. This is demonstrated in carunclectomised twins and triplets in which surface areas of $4.4 \pm 0.7\text{m}^2$ and $2.9 \pm 0.3\text{m}^2$ respectively were not compatible with normal fetal growth.

TABLES AND FIGURES FOR CHAPTER 5

TABLE 5.1 Comparison of macroscopic placental parameters, at 90 days gestation, of singletons, twins and triplets with and without experimental restriction of implantation

TREATMENT	NUMBER CARUNCLES REMOVED VERSUS NUMBER LEFT	NUMBER PLACENTOMES FORMED	MEAN PLACENTOME WEIGHT (g)	TOTAL PLACENTAL WEIGHT (g)	% TOTAL PLACENTOME NUMBER EVERTED	% TOTAL PLACENTAL WEIGHT EVERTED	FETAL WEIGHT (g)	F:P RATIO
Singletons (N=9)	-	74 ± 6 <i>a</i>	7.51 ± 1.03 <i>a</i>	515 ± 42 <i>a</i>	11 ± 10 <i>a</i>	12.93 ± 12.45 <i>a</i>	716 ± 33 <i>a</i>	1.48 ± 0.16 <i>a</i>
Restricted Singletons (N=4)	75 ± 9 versus 15 ± 0.3 (N=3)	33 ± 6 <i>b</i>	11.47 ± 1.4 <i>ab</i>	340 ± 27 <i>b</i>	49 ± 20 <i>b</i>	54.38 ± 18.29 <i>b</i>	634 ± 46 <i>ac</i>	1.87 ± 0.08 <i>ac</i>
Twins (N=6)	-	48 ± 3 <i>c</i>	7.44 ± 0.48 <i>a</i>	351 ± 25 <i>b</i>	18 ± 11 <i>a</i>	17 ± 11 <i>a</i>	908 ± 64 <i>b</i>	2.63 ± 0.22 <i>b</i>
Restricted Twins (N=2)	76/ 12 (N=1)	15 ± 5 <i>d</i>	15.16 ± 2.26 <i>b</i>	216 ± 42 <i>d</i>	38 ± 23 <i>b</i> (sheep 1: 60; sheep 2: 15)	48.99 ± 13.75 <i>b</i>	545 ± 131 <i>ac</i>	2.5 ± 0.12 <i>bc</i>
Triplets (N=6)	-	39 ± 7 <i>cb</i>	7.52 ± 1.1 <i>a</i>	278 ± 48 <i>bc</i>	11 ± 7 <i>a</i>	12.3 ± 6.68 <i>a</i>	728 ± 33 <i>a</i>	3.07 ± 0.58 <i>b</i>
Restricted Triplets (N=8)	68 ± 10 versus 14 ± 1 (N=3)	18 ± 3 <i>d</i>	10.7 ± 1.57 <i>ab</i>	169 ± 18 <i>d</i>	51 ± 10 <i>b</i>	66.6 ± 10.58 <i>b</i>	528 ± 39 <i>c</i>	3.32 ± 0.31 <i>d</i>

Values are expressed as mean ± SE

Values with different letters are significantly different; $p \leq 0.05$; ANOVA, LSMeans specific comparisons

N= number of fetuses studied

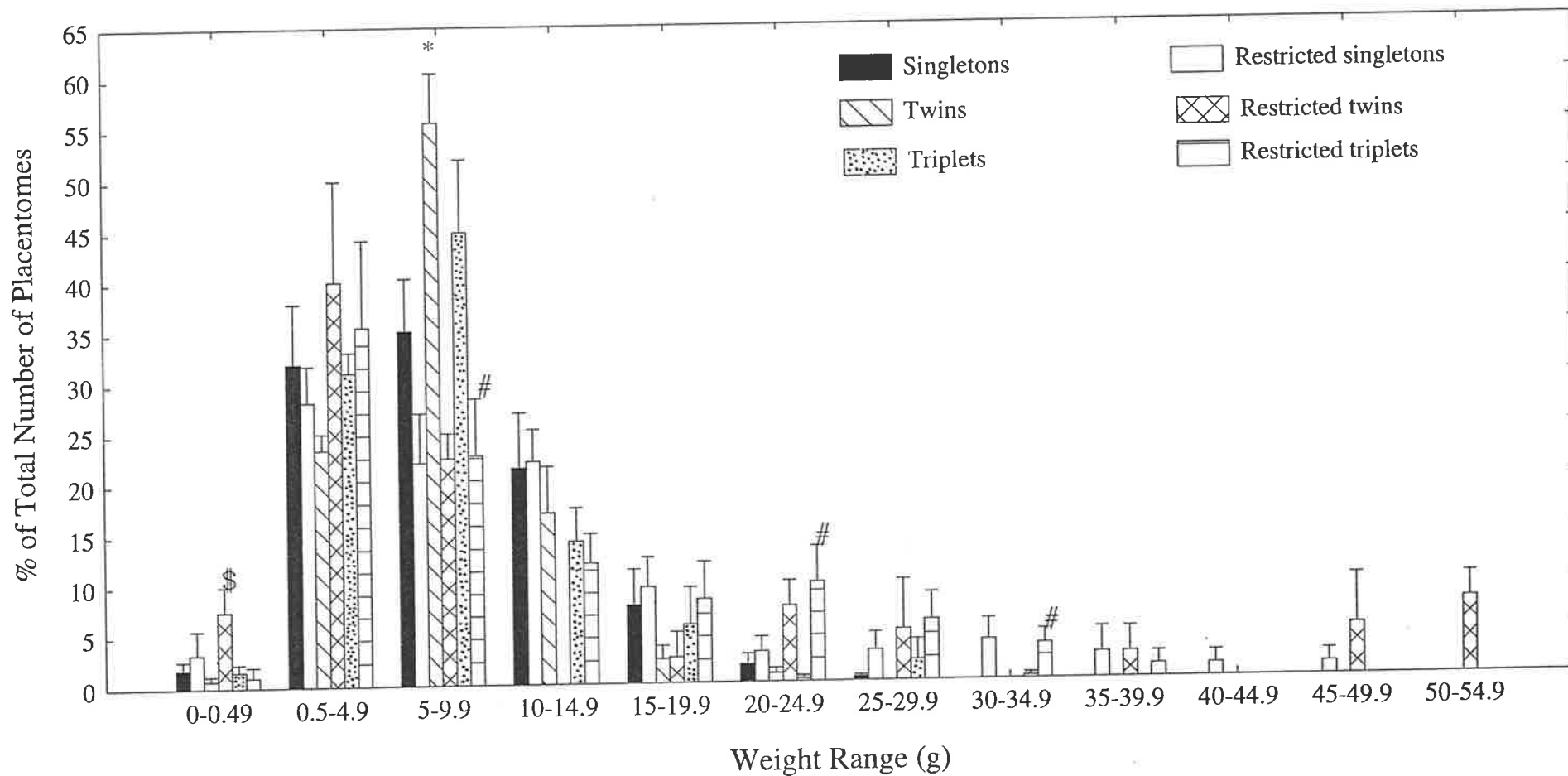


Figure 5.1 The distribution of placentomes within particular weight range categories in placentas of singletons, twins and triplets, with and without experimental restriction of implantation at 90 days gestation.

* $p = 0.02$; ANOVA; LSMeans specific contrasts, comparisons between twins and restricted twins; twins and singletons

\$ $p = 0.001$; comparisons between twins and restricted twins; # $p < 0.05$; comparison between triplets and restricted triplets

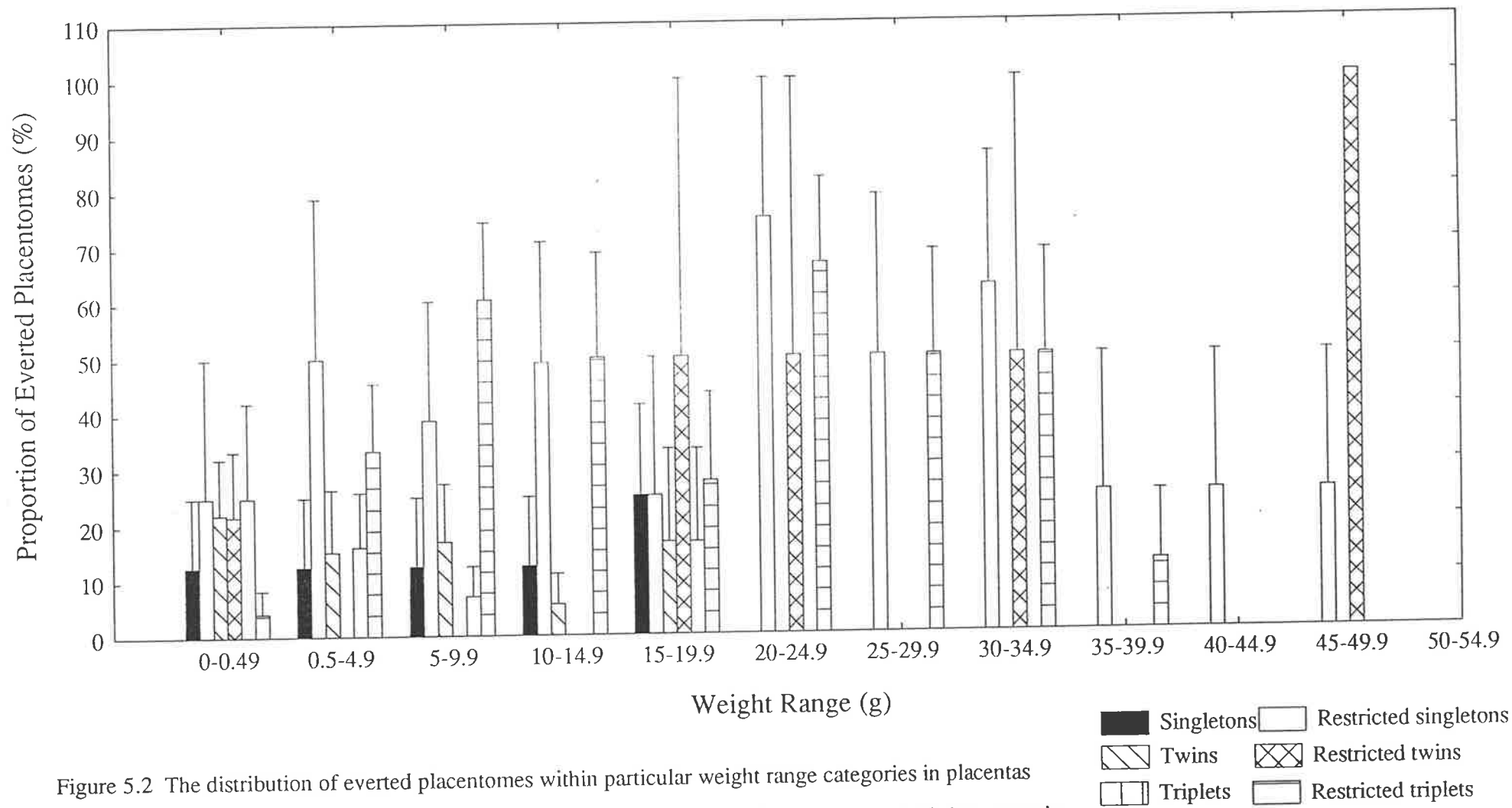


Figure 5.2 The distribution of everted placentomes within particular weight range categories in placentas of singletons, twins and triplets, with and without experimental restriction of implantation at 90 days gestation.

Table 5.2 ANOVA Summary: LSMeans comparisons; Overall effects of natural restriction of implantation (twins versus singletons), gestational age (90 and 120 days) and their interaction (combined effects of fetus number and gestational age), on the ontogeny of placental macroscopic parameters

PARAMETER	NATURAL RESTRICTION OF IMPLANTATION	GESTATIONAL AGE	INTERACTION
Placental weight (g)	0.0003	ns	ns
Placentome number	0.0001	ns	ns
Mean placentome weight (g)	ns	ns	ns
Proportion of large placentomes (%)	ns	ns	0.04
Proportion of everted placentomes (%)	0.02	0.007	ns
Fetal weight (g)	ns	0.0001	0.02
Fetal weight: Placental weight (F/ P Ratio)	0.0001	0.0001	ns

Values are probability values; ns = no significant effect

Table 5.3 Comparison of placental macroscopic parameters in singletons and twins between 90 and 120 days gestation

FETUS NUMBER	SINGLETONS		TWINS	
GESTATIONAL AGE (days)	90 (N=9)	120 (N=6)	90 (N=6)	120 (N=8)
Placental Parameter				
Placental weight (g)	515 ± 42	545 ± 35	351 ± 25	429 ± 16
Placentome Number	74 ± 6	74 ± 2	48 ± 3	44 ± 2
Mean placentome weight (g)	7.5 ± 1.0	7.5 ± 0.6	7.4 ± 0.5	10.0 ± 0.7 *
Proportion of large placentomes (10g≥) (%)	27.93 ± 9.25	24.35 ± 5.54	20.19 ± 5.96	49.31 ± 5.63 *
Proportion of everted placentomes (%)	11.49 ± 11.07	23.63 ± 13.8	17.53 ± 10.79	76.02 ± 11.27 **
Fetal weight (g)	716 ± 33	2654 ± 77 **	908 ± 64	2523 ± 73 **
Fetal weight/ Placental weight (F/P Ratio)	1.48 ± 0.16	4.94 ± 0.25 **	2.63 ± 0.22	5.92 ± 0.19 **

Values are expressed as mean ± SE

Values in "**bold**" are significantly different at the two gestational ages within singletons or twins (* p≤0.03, ** p≤0.002; LSMeans specific comparisons); N= Number of ewes studied

TABLE 5.4 Volume densities of components in placentomes of different sizes in ewes bearing singletons, twins and triplets at 90 days gestation

TREATMENT	SINGLETONS (N=9)			TWINS (N=6)			TRIPLETS (N=6)		
	"Small" Weight (g) (n=2)	"Average-size" (n=8)	"Large" (n=7)	"Small" (n=1)	"Average-size" (n=5)	"Large" (n=6)	"Small" (n=3)	"Average-size" (n=6)	"Large" (n=3)
<i>PlacentomeSize</i>	2.9 ± 0.8	8.2 ± 0.5	16.3 ± 1.4	3.8	7.1 ± 0.5	12.9 ± 1.0	3.2 ± 0.8	7.1 ± 0.6	17.6 ± 4.8
<i>COMPONENT</i>									
Chorionic membrane (CM)	0.107 ± 0.005	0.107 ± 0.011	0.115 ± 0.013	0.104	0.120 ± 0.019	0.112 ± 0.015	0.106 ± 0.007	0.146 ± 0.015	0.089 ± 0.012
Chorionic epithelium (CEP)	0.024 ± 0.011	0.031 ± 0.004	0.022 ± 0.005	0.027	0.040 ± 0.006	0.032 ± 0.004	0.05 ± 0.004	0.05 ± 0.005	0.037 ± 0.014
Haemophagous zone (HMZ)	0.018 ± 0.01	0.069 ± 0.015	0.050 ± 0.017	0.047	0.05 ± 0.005	0.041 ± 0.007	0.06 ± 0.015	0.044 ± 0.008	0.062 ± 0.013
Maternal tissue in HMZ	0.007 ± 0.001	0.022 ± 0.004	0.021 ± 0.008	0.017	0.012 ± 0.003	0.009 ± 0.003	0.039 ± 0.004	0.025 ± 0.005	0.014 ± 0.004
Fetal villi (FV)	0.450 ± 0.027	0.363 ± 0.020	0.395 ± 0.032	0.462	0.383 ± 0.005	0.404 ± 0.011	0.334 ± 0.019	0.363 ± 0.016	0.375 ± 0.026
Trophectoderm	0.200 ± 0.046	0.162 ± 0.014	0.159 ± 0.014	0.323	0.253 ± 0.022	0.266 ± 0.012	0.229 ± 0.019	0.261 ± 0.013	0.242 ± 0.015
Connective tissue core	0.256 ± 0.074	0.199 ± 0.026	0.236 ± 0.034	0.139	0.138 ± 0.018	0.139 ± 0.012	0.109 ± 0.003	0.101 ± 0.013	0.13 ± 0.012
Maternal crypts (MC)	0.284 ± 0.022	0.278 ± 0.021	0.271 ± 0.023	0.365	0.302 ± 0.015	0.306 ± 0.01	0.28 ± 0.01	0.275 ± 0.016	0.329 ± 0.02
Feto-maternal syncytium	0.081 ± 0.004	0.077 ± 0.006	0.072 ± 0.008	0.12	0.107 ± 0.014	0.109 ± 0.01	0.097 ± 0.002	0.097 ± 0.005	0.109 ± 0.01
Shrinkage spaces	0.022 ± 0.003	0.042 ± 0.012	0.039 ± 0.010	0.025	0.041 ± 0.012	0.047 ± 0.01	0.023 ± 0.01	0.016 ± 0.003	0.027 ± 0.004
Placentome capsule (PC)	0.098 ± 0.010	0.082 ± 0.011	0.091 ± 0.008	0.05	0.06 ± 0.010	0.063 ± 0.01	0.125 ± 0.039	0.077 ± 0.008	0.07 ± 0.011
Fetal villi/Maternal crypts	1.604 ± 0.219	1.359 ± 0.127	1.538 ± 0.200	1.266	1.281 ± 0.07	1.331 ± 0.061	1.168 ± 0.062	1.353 ± 0.129	1.142 ± 0.058
Total fetal tissue/Total maternal tissue	1.224 ± 0.084	1.079 ± 0.105	1.137 ± 0.134	1.130	0.964 ± 0.223	1.164 ± 0.061	0.900 ± 0.087	1.112 ± 0.079	1.017 ± 0.08

Values are expressed as mean ± SE; N= Number of ewes; n= Number of placentomes morphometrically analysed. Total fetal tissue= FV + CEP (excluding CM); Total maternal tissue = MC + PC. There were no significant differences between volume densities in placentomes of twins and triplets. Values presented in "**bold**" in twins and triplets were significantly different from those of singletons; $p \leq 0.01$ ANOVA; LSMeans specific comparisons, excluding small placentomes.

TABLE 5.5 Volume densities of placentome components in ewes bearing singletons versus those bearing twins and triplets at 90 days gestation

TREATMENT	SINGLETONS (N=9)		TWINS (N=6)		TRIPLETS (N=6)	
<i>PLACENTOME SIZE</i>	<i>"Average-size"</i>	<i>"Large"</i>	<i>"Average-size"</i>	<i>"Large"</i>	<i>"Average-size"</i>	<i>"Large"</i>
Weight (g)*	8.2 ± 0.5 (n=8)	16.3 ± 1.4 (n=7)	7.1 ± 0.5 (n=5)	12.9 ± 1.0 (n=6)	7.1 ± 0.6 (n=6)	17.6 ± 4.8 (n=3)
<i>COMPONENT</i>						
Chorionic membrane (CM)	0.111 ± 0.009		0.116 ± 0.010		0.127 ± 0.012	
Chorionic epithelium (CEP)	0.026 ± 0.004		0.035 ± 0.004		0.046 ± 0.005	
Haemophagous zone (HMZ)	0.060 ± 0.008		0.043 ± 0.009		0.050 ± 0.010	
Maternal tissue in HMZ	0.021 ± 0.005		0.010 ± 0.005		0.021 ± 0.006	
Fetal villi (FV)	0.378 ± 0.012		0.393 ± 0.015		0.367 ± 0.016	
Trophectoderm	0.152 ± 0.010		0.260 ± 0.012		0.255 ± 0.013	
Connective tissue core	0.216 ± 0.012		0.138 ± 0.015		0.111 ± 0.016	
Maternal crypts (MC)	0.275 ± 0.012		0.304 ± 0.014		0.293 ± 0.016	
Feto-maternal syncytium	0.074 ± 0.005		0.108 ± 0.006		0.101 ± 0.007	
Shrinkage spaces	0.040 ± 0.008		0.044 ± 0.009		0.019 ± 0.010	
Placentome capsule (PC)	0.086 ± 0.006		0.062 ± 0.007		0.073 ± 0.008	
Fetal villi/Maternal crypts	1.443 ± 0.082		1.308 ± 0.096		1.283 ± 0.106	
Total fetal tissue/Total maternal tissue	1.106 ± 0.071		1.073 ± 0.083		1.080 ± 0.092	

Values are expressed as least squares means ± SE; * Values are mean ± SE; N= Number of ewes; n= Number of placentomes morphometrically analysed.

Total fetal tissue= FV + CEP (excluding CM); Total maternal tissue = MC + PC

There were no significant differences between volume densities in placentomes of twins and triplets. Values presented in "**bold**" in twins and triplets were significantly different from those of singletons; $p < 0.01$; ANOVA; LSMeans specific comparisons (excluding small placentomes)

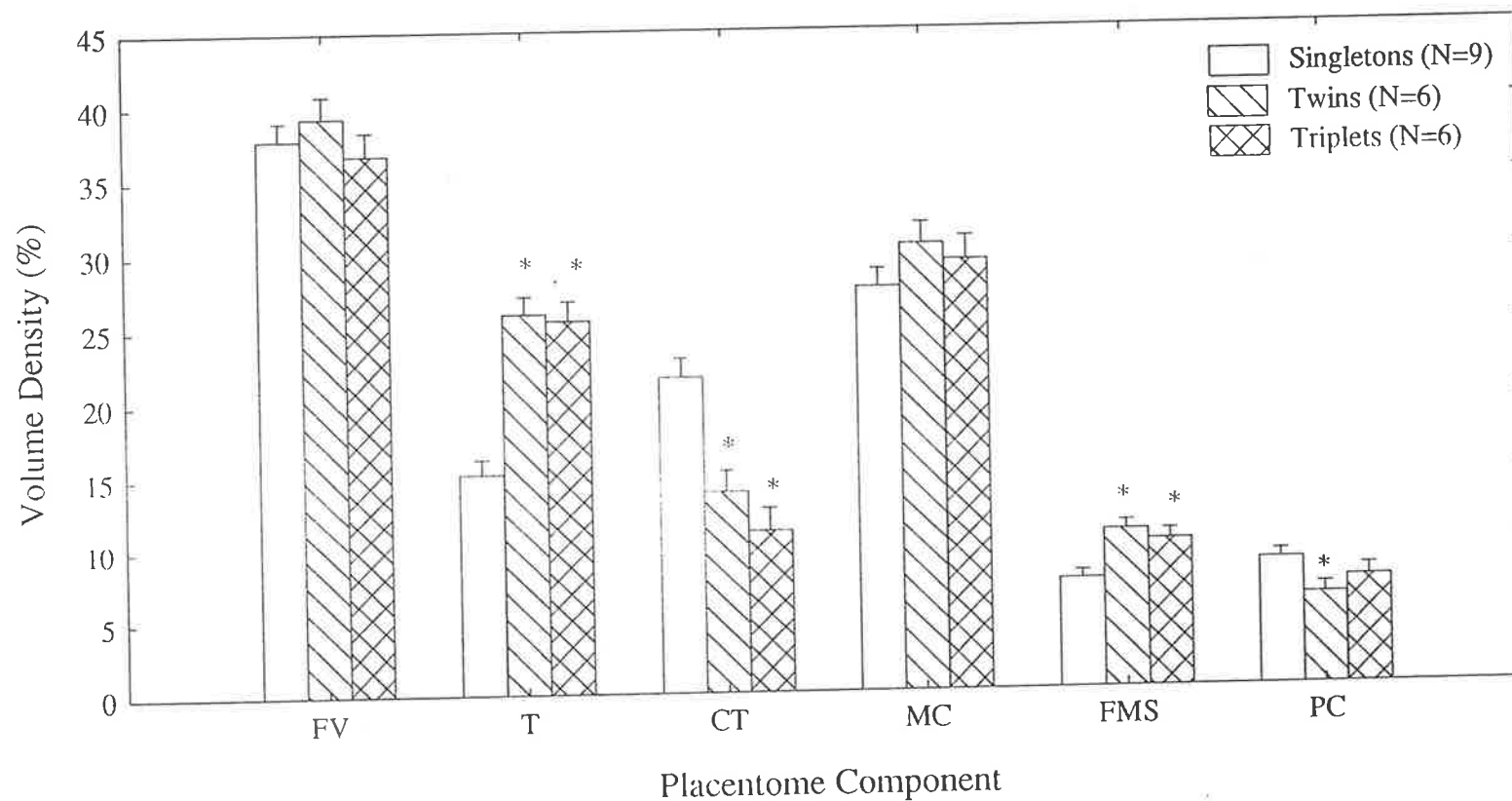


Figure 5.3 Volume densities of components in singletons (□), twins (▨) and triplets (▩) at 90 days gestation. Fetal villi (FV), Trophoctoderm (T), Connective tissue core of fetal villi (CT), Maternal crypts (MC), Feto-maternal syncytium (FMS) and Placentome capsule (PC). (* $p < 0.01$; ANOVA L S Means Comparisons. testing differences between components in placentas of multiple conceptuses and those of singletons). There were no significant differences between volume densities of components in twins and triplets.

TABLE 5.6 Surface density and arithmetic mean barrier thickness of epithelial cell layers in placentomes of singletons, twins and triplets at 90 days gestation

TREATMENT	SINGLETONS (N=9)		TWINS (N=6)		TRIPLETS (N=6)	
<i>PLACENTOME SIZE</i>	<i>"Average-size"</i>	<i>"Large"</i>	<i>"Average-size"</i>	<i>"Large"</i>	<i>"Average-size"</i>	<i>"Large"</i>
Weight (g) *	8.2 ± 0.5	16.3 ± 1.4	7.1 ± 0.5	13.5 ± 1.0	7.1 ± 0.6	17.6 ± 4.8
	(n=8)	(n=7)	(n=5)	(n=6)	(n=6)	(n=3)
<i>PARAMETER</i>						
Surface density of trophoctoderm (cm ⁻¹)	116 ± 6		159 ± 8		160 ± 8	
Barrier thickness of trophoctoderm (µm)	16.33 ± 0.89		18.77 ± 2		18.80 ± 1.15	
Barrier thickness of feto-maternal syncytium (µm)	8.5 ± 0.5		6.8 ± 0.6		7.67 ± 0.6	

Values are expressed as least squares means ± SE

* Values are mean ± SE

N= Number of ewes; n= Number of placentomes morphometrically analysed

There were no significant differences between the components in twins and triplets. Values presented in "**bold**" were significantly different in twins or triplets compared to singletons; p<0.05; ANOVA; LSMeans specific comparisons; excluding small placentomes

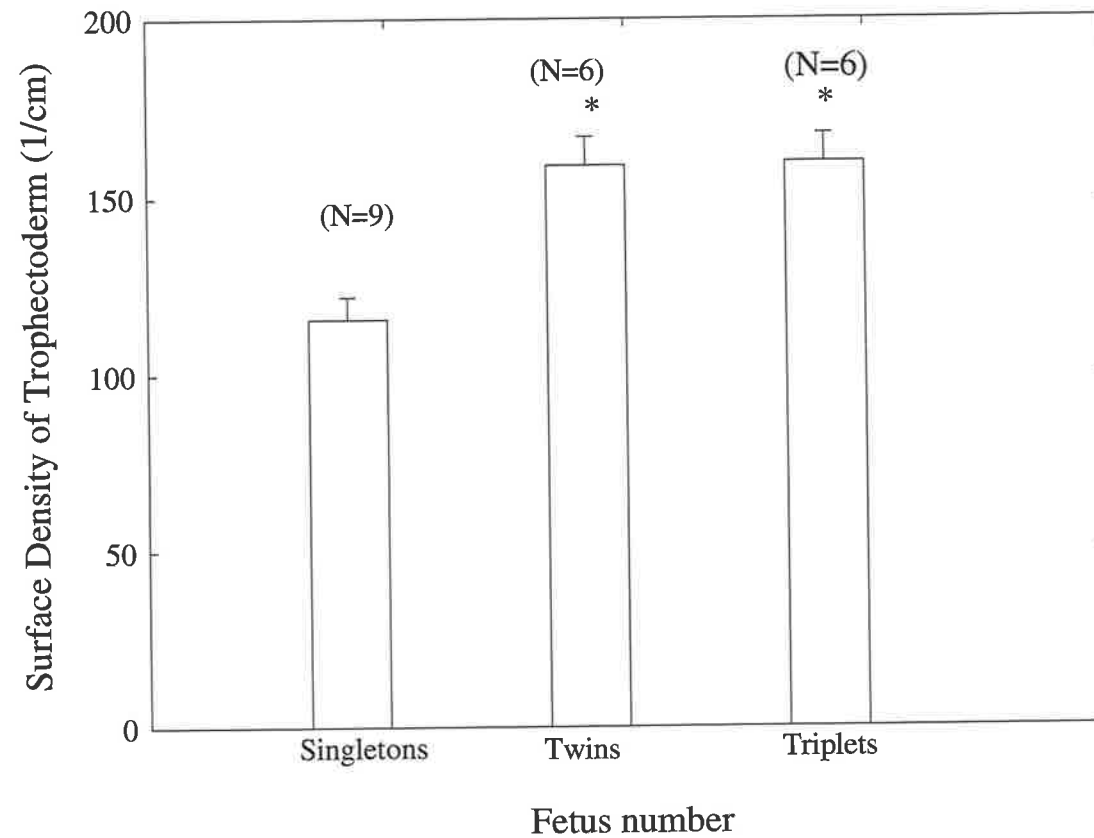


Figure 5.4 Surface density of trophoblast in singletons, twins and triplets at 90 days gestation.

* $p=0.0001$; ANOVA; L S Means Comparison; testing differences between the surface density in placentomes of the multiple conceptuses and that in placentomes of singletons.

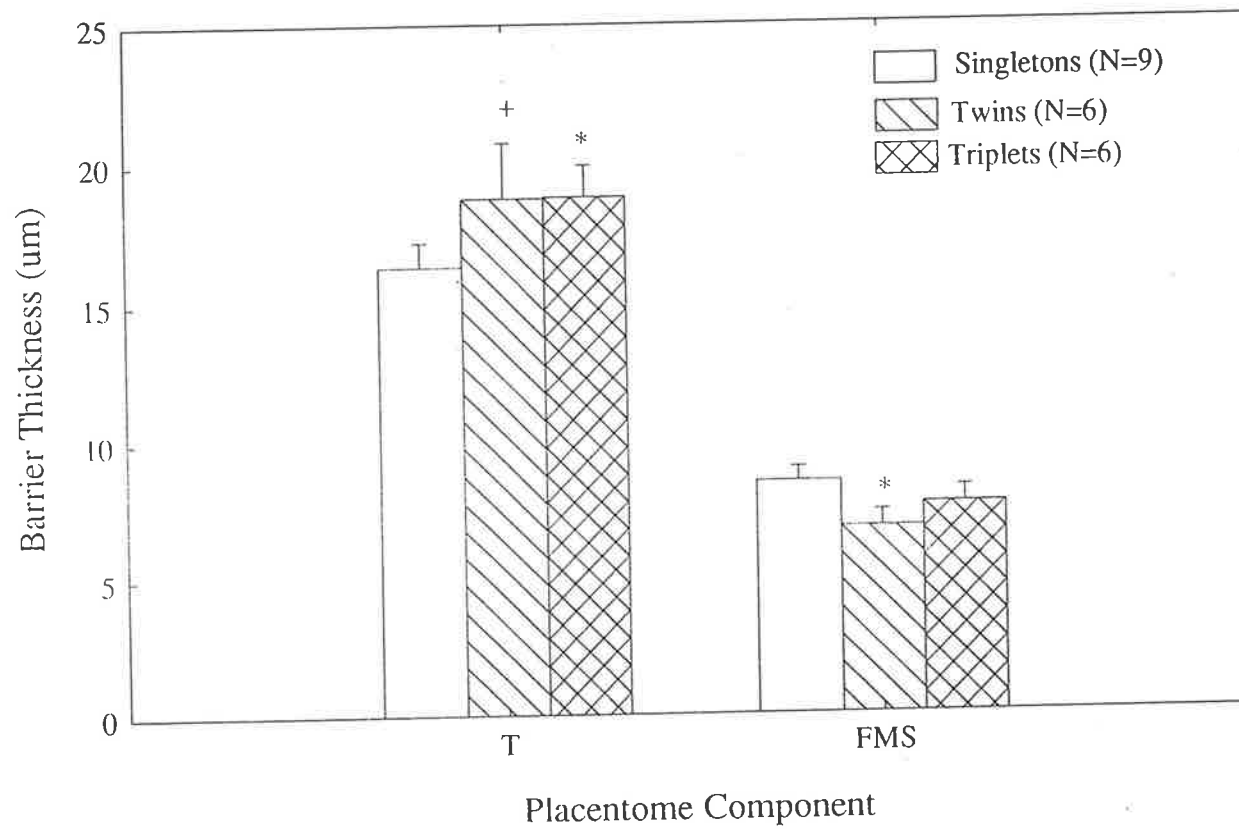


Figure 5.5 Arithmetic mean barrier thicknesses of Trophoctoderm (T) and Feto-maternal syncytium (FMS) at the feto-maternal interface in placentas of singletons (□), twins (▨) and triplets (▩) at 90 days gestation. (* $p < 0.05$; + $0.05 < p < 0.01$; ANOVA; LSMeans specific comparisons between values in placentomes of multiple conceptuses and singletons).

Values are mean $\pm$ SE; values with different letters are significantly different; $p \leq 0.05$; ANOVA, LSMeans specific comparisons

Component placental volume = volume density $\times$ placental weight, Surface area = surface density $\times$ placental weight;

Relative surface area = surface area / (fetal weight + placental weight)

TABLE 5.7 Total placental volumes of components, at 90 days gestation, in singletons, twins and triplets with and without experimental restriction of implantation

TREATMENT	RESTRICTED			RESTRICTED		RESTRICTED
	SINGLETONS (N=9)	SINGLETONS (N=4)	TWINS (N=6)	TWINS (N=2)	TRIPLETS (N=6)	TRIPLETS (N=8)
Placental weight (g)	515 ± 42 <i>a</i>	340 ± 27 <i>b</i>	351 ± 25 <i>b</i>	216 ± 42 <i>d</i>	278 ± 48 <i>bc</i>	169 ± 18 <i>d</i>
<i>COMPONENT</i>						
Chorionic membrane	57.62 ± 8.67 <i>a</i>	29.00 ± 5.23 <i>b</i>	41.19 ± 6.26 <i>ab</i>	17.65 ± 5.45 <i>b</i>	32.11 ± 4.52 <i>bc</i>	15.38 ± 2.61 <i>c</i>
Chorionic epithelium	13.09 ± 2.21 <i>a</i>	7.18 ± 1.30 <i>abc</i>	12.27 ± 1.48 <i>a</i>	5.41 ± 0.86 <i>abd</i>	13.05 ± 2.49 <i>a</i>	6.53 ± 1.46 <i>bcd</i>
Haemophagous zone (HMZ)	28.29 ± 7.31 <i>a</i>	9.90 ± 2.23 <i>b</i>	15.63 ± 0.79 <i>ab</i>	9.50 ± 4.31 <i>ab</i>	14.65 ± 3.16 <i>b</i>	6.25 ± 1.37 <i>b</i>
Maternal tissue in HMZ	10.48 ± 2.83 <i>a</i>	3.20 ± 0.36 <i>b</i>	3.73 ± 0.63 <i>b</i>	2.60 ± 0.39 <i>ab</i>	6.61 ± 1.7 <i>ab</i>	4.02 ± 1.19 <i>b</i>
Fetal villi	200.58 ± 20.97 <i>a</i>	136.36 ± 15.73 <i>b</i>	140.75 ± 10.65 <i>b</i>	89.31 ± 10.56 <i>bd</i>	99.58 ± 16.71 <i>bc</i>	71.76 ± 7.06 <i>cd</i>
Trophectoderm	83.51 ± 4.31 <i>a</i>	78.12 ± 6.78 <i>a</i>	92.71 ± 7.14 <i>a</i>	68.43 ± 9.11 <i>ac</i>	68.91 ± 12 <i>ab</i>	50.9 ± 4.54 <i>bc</i>
Connective tissue core	116.94 ± 20.71 <i>a</i>	64.12 ± 5.22 <i>bc</i>	49.14 ± 6.51 <i>bcd</i>	20.65 ± 1.75 <i>bcd</i>	30.66 ± 5.79 <i>bcd</i>	20.72 ± 2.4 <i>bd</i>
Maternal crypts	141.65 ± 12.3 <i>a</i>	107.95 ± 7.89 <i>ab</i>	108.06 ± 6.76 <i>b</i>	78.26 ± 20.06 <i>bc</i>	83.15 ± 17.45 <i>b</i>	51.26 ± 4.99 <i>c</i>
Feto-maternal syncytium	38.21 ± 3.01 <i>a</i>	33.96 ± 4.52 <i>ab</i>	38.09 ± 3.69 <i>ab</i>	30.51 ± 10.91 <i>abd</i>	27.79 ± 4.75 <i>bc</i>	18.45 ± 1.42 <i>cd</i>
Shrinkage spaces	17.73 ± 4.08 <i>ad</i>	22.10 ± 10.92 <i>ad</i>	14.98 ± 3.51 <i>abd</i>	3.61 ± 0.57 <i>ac</i>	5.72 ± 1.55 <i>bc</i>	6.93 ± 1.36 <i>bc</i>
Placentome capsule	45.43 ± 5.09 <i>a</i>	25.94 ± 3.79 <i>b</i>	20.93 ± 1.69 <i>b</i>	9.28 ± 1.47 <i>bc</i>	21.69 ± 4.11 <i>b</i>	8.22 ± 1.29 <i>c</i>
Surface area (m ²)	5.93 ± 0.44 <i>a</i>	5.18 ± 0.83 <i>a</i>	5.64 ± 0.61 <i>a</i>	4.38 ± 0.66 <i>ab</i>	4.50 ± 0.8 <i>a</i>	2.89 ± 0.25 <i>b</i>
Relative Surface area (cm ⁻¹)	47.79 ± 2.32 <i>abc</i>	52.28 ± 6.13 <i>abc</i>	44.63 ± 3.33 <i>abc</i>	58.59 ± 4.58 <i>ac</i>	43.11 ± 5.2 <i>ab</i>	41.53 ± 2.17 <i>ab</i>

Table 5.8 Proportions (%) of placentome everted morphologies B, C, and D at 90 days gestation in placentas of twins with and without experimental restriction of implantation

TREATMENT	EVERTED PLACENTOME MORPHOLOGY		
	B	C	D
TWINS (N=6)	16.53 $\pm$ 10.20	0.71 $\pm$ 0.45	-
RESTRICTED TWINS (N=2)	37.5 $\pm$ 22.5	-	-

Values are expressed as mean $\pm$ SE

Analysis carried out by ANOVA; LSMeans specific comparisons

N = Number of ewes or fetuses studied

Table 5.9 Proportions (%) of placentome everted morphologies B, C, and D at 90 days gestation in placentas of triplets with and without experimental restriction of implantation

TREATMENT	EVERTED PLACENTOME MORPHOLOGY		
	B	C	D
TRIPLETS (N= 6)	11.48 $\pm$ 5.85	0.41 $\pm$ 0.41	-
RESTRICTED TRIPLETS (N= 8)	29.15 $\pm$ 7.06	7.25 $\pm$ 2.21 *	12.59 $\pm$ 4.76 *

Values are expressed as mean $\pm$ SE

Analysis carried out by ANOVA; LSMeans specific comparisons (* $p \leq 0.05$)

N= number of fetuses studied

TABLE 5.10 Volume densities of components in placentomes of different sizes, at 90 days gestation, in twin-bearing ewes with and without experiemental restriction of implantation

TREATMENT	TWINS (N=6)			RESTRICTED TWINS (N=2)		LSMeans specific comparisons
PLACENTOME SIZE	"Small"	"Average-size"	"Large"	"Average-size"	"Large"	
Weight (g)	3.8 (n=1)	7.1 ± 0.5 (n=5)	12.9 ± 1.0 (n=6)	7.0 ± 0.7 (n=2)	22.7 ± 3.7 (n=2)	(p=)
COMPONENT						
Chorionic membrane	0.104	0.120 ± 0.019	0.112 ± 0.015	0.096 ± 0.021	0.064 ± 0.002	0.04
Chorionic epithelium	0.027	0.040 ± 0.006	0.032 ± 0.004	0.033 ± 0.0005	0.017 ± 0.002	ns
Haemophagous zone (HMZ)	0.047	0.05 ± 0.005	0.041 ± 0.007	0.066 ± 0.037	0.007 ± 0.003	ns
Maternal tissue in HMZ (MT)	0.017	0.012 ± 0.003	0.009 ± 0.003	0.016 ± 0.004	0.009 ± 0.005	ns
Fetal villi (FV)	0.462	0.383 ± 0.005	0.404 ± 0.011	0.387 ± 0.041	0.451 ± 0.024	ns
Trophectoderm	0.323	0.253 ± 0.022	0.266 ± 0.012	0.293 ± 0.028	0.348 ± 0.012	0.003
Connective tissue core	0.139	0.138 ± 0.018	0.139 ± 0.012	0.093 ± 0.01	0.102 ± 0.012	ns
Maternal crypts (MC)	0.365	0.302 ± 0.015	0.306 ± 0.01	0.326 ± 0.011	0.389 ± 0.037	0.06
Feto-maternal syncytium (FMS)	0.120	0.107 ± 0.014	0.109 ± 0.01	0.12 ± 0.019	0.154 ± 0.030	0.05
Shrinkage spaces	0.025	0.041 ± 0.012	0.047 ± 0.01	0.017 ± 0.004	0.017 ± 0.005	ns
Placentome capsule (PC)	0.050	0.06 ± 0.010	0.063 ± 0.01	0.051 ± 0.034	0.041 ± 0.003	ns
Fetal villi/Maternal crypts	1.266	1.281 ± 0.07	1.331 ± 0.061	1.196 ± 0.166	1.174 ± 0.172	ns
Total fetal tissue/Total maternal tissue	1.130	0.964 ± 0.223	1.164 ± 0.061	1.071 ± 0.05	1.077 ± 0.145	ns

Values are mean ± SE; ANOVA; Lsmeans specific comparisons; excluding small placentomes; N= Number of ewes; n= Number of placentomes morphometrically analysed; ns= No significant difference; Total fetal tissue = (CEP + FV; excluding CM); Total maternal tissue = (MT +MC + PC); All sampled placentomes were of normal morphology. There was no significant effect of placentome size on all components

TABLE 5.11 Volume densities of placentome components at 90 days gestation in twin-bearing ewes with and without experimental restriction of implantation

TREATMENT	TWINS (N=6)		RESTRICTED TWINS (N=2)		LSMeans specific comparisons
<i>PLACENTOMESIZE</i>	"Average-size"	"Large"	"Average-size"	"Large"	
Weight (g) *	7.1 ± 0.5 (n=5)	12.9 ± 1.0 (n=6)	7.0 ± 0.7 (n=2)	22.7 ± 3.7 (n=2)	(p=)
<i>COMPONENT</i>					
Chorionic membrane	0.116 ± 0.010		0.096 ± 0.012		0.04
Chorionic epithelium	0.035 ± 0.004		0.022 ± 0.008		ns
Haemophagous zone (HMZ)	0.043 ± 0.009		0.015 ± 0.017		ns
Maternal tissue in HMZ (MT)	0.010 ± 0.005		0.010 ± 0.010		ns
Fetal villi (FV)	0.393 ± 0.015		0.444 ± 0.028		ns
Trophectoderm	0.260 ± 0.012		0.339 ± 0.022		0.003
Connective tissue core	0.138 ± 0.015		0.103 ± 0.028		ns
Maternal crypts (MC)	0.304 ± 0.014		0.365 ± 0.028		0.06
Feto-maternal syncytium (FMS)	0.108 ± 0.006		0.136 ± 0.012		0.05
Shrinkage spaces	0.044 ± 0.009		0.015 ± 0.017		ns
Placentome capsule (PC)	0.062 ± 0.007		0.056 ± 0.013		ns
Fetal villi/Maternal crypts	1.308 ± 0.096		1.237 ± 0.184		ns
Total fetal tissue/Total maternal tissue	1.073 ± 0.083		1.091 ± 0.159		ns

Values are expressed as least squares means ± SE; * Values are mean ± SE;

ANOVA; Lsmeans specific comparisons; excluding small placentomes;

N= Number of ewes; n= Number of placentomes morphometrically analysed; ns= No significant difference;

Total fetal tissue = (CEP + FV; excluding CM); Total maternal tissue = (MT +MC + PC). There was no significant effect of placentome size on all components.

All sampled placentomes were of normal morphology.

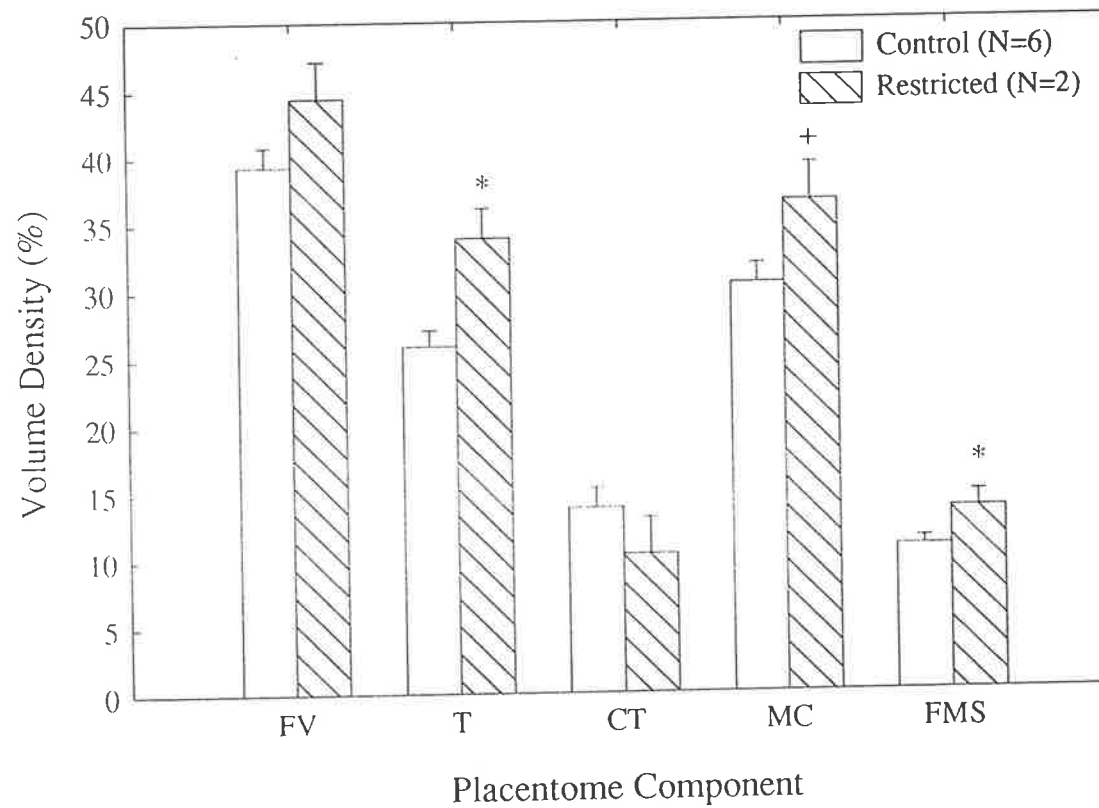


Figure 5.6 Volume densities of placentome components at 90 days gestation in twins with (hatched bar) and without (white bar) experimental restriction of implantation. Fetal villi (FV), Trophoctoderm (T), Connective tissue core of fetal villi (CT), Maternal crypts (MC) and Feto-maternal syncytium (FMS). (ANOVA; L S Means Comparisons; * $p < 0.05$; + $p = 0.06$).

TABLE 5.12 Surface density and arithmetic mean barrier thickness at 90 days gestation in placentomes of twin-bearing ewes with and without experimental restriction of implantation

TREATMENT	TWINS (N=6)		RESTRICTED TWINS (N=2)		<i>LSmeans specific comparisons</i>
<i>PLACENTOME SIZE</i>	<i>"Average-size"</i>	<i>"Large"</i>	<i>"Average-size"</i>	<i>"Large"</i>	
Weight (g) *	7.1 ± 0.5 (n=5)	12.9 ± 1.0 (n=6)	7.0 ± 0.7 (n=2)	22.7 ± 3.7 (n=2)	(p=)
<i>PARAMETER</i>					
Surface density of trophoctoderm (cm ⁻¹)	159 ± 8		212 ± 15		0.002
Barrier thickness of trophoctoderm (µm)	18.8 ± 2		17 ± 4		ns
Barrier thickness of feto-maternal syncytium (µm)	8.5 ± 0.5		6.4 ± 0.1		ns

Values are least squares means ± SE;

* Values are mean ± SE;

N= Number of ewes studied; n= Number of placentomes morphometrically analysed;

ANOVA; LSmeans specific comparisons; excluding small placentomes.

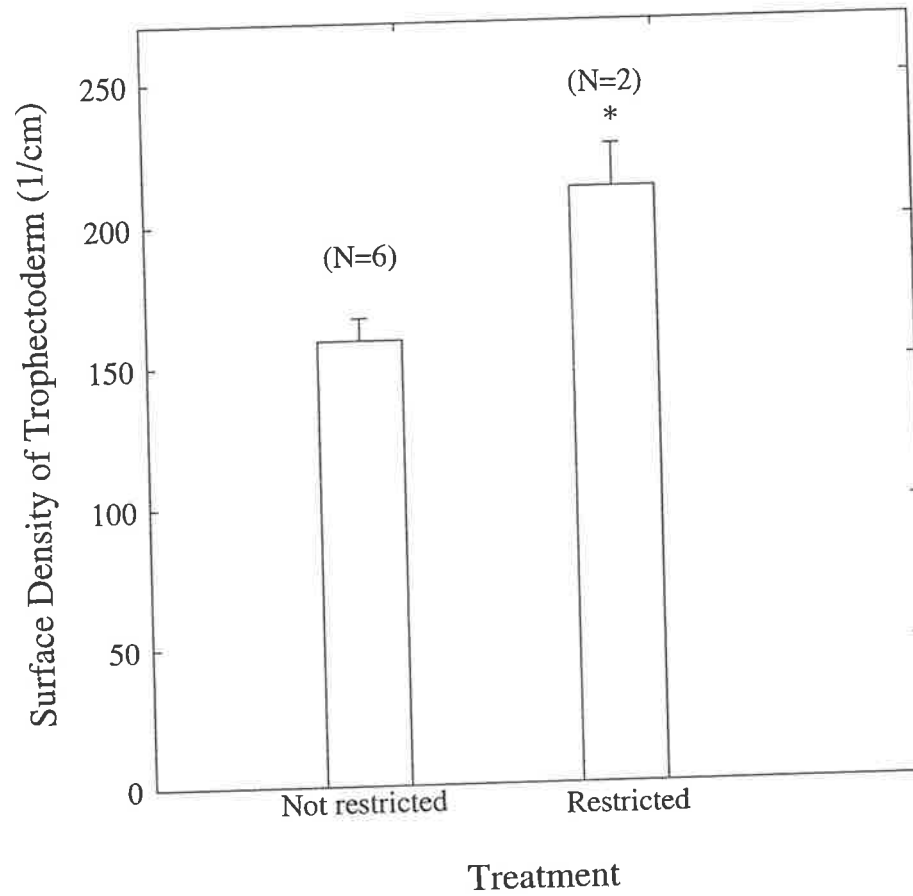


Figure 5.7 Surface density of trophoboderm at 90 days gestation in twins with and without experimental restriction of implantation. (ANOVA; L S Means Comparison; * $p = 0.002$).

TABLE 5.13 Volume densities of components in placentomes of different sizes, at 90 days gestation, in triplet-bearing ewes with and without experimental restriction of implantation

TREATMENT	TRIPLETS (N=6)			RESTRICTED TRIPLETS (N=8)			LSMeans specific comparisons
PLACENTOME SIZE	"Small"	"Average-size"	"Large"	"Small"	"Average-size"	"Large"	
Weight (g)	3.2 ± 0.8 (n=3)	7.1 ± 0.6 (n=6)	17.6 ± 4.8 (n=3)	2.9 ± 0.7 (n=2)	6.8 ± 0.8 (n=3)	18.7 ± 2.5 (n=11)	
COMPONENT							
Chorionic membrane (CM)	0.106 ± 0.007	0.146 ± 0.015	0.089 ± 0.012	0.082 ± 0.02	0.078 ± 0.016	0.093 ± 0.01	0.01
Chorionic epithelium (CEP)	0.05 ± 0.004	0.05 ± 0.005	0.037 ± 0.014	0.048 ± 0.012	0.042 ± 0.014	0.033 ± 0.005	ns
Haemophagous zone (HMZ)	0.06 ± 0.015	0.044 ± 0.008	0.062 ± 0.013	0.028 ± 0.01	0.046 ± 0.015	0.033 ± 0.004	ns
Maternal tissue in HMZ (MT)	0.039 ± 0.004	0.025 ± 0.005	0.014 ± 0.004	0.014 ± 0.001	0.038 ± 0.02	0.019 ± 0.009	ns
Fetal villi (FV)	0.334 ± 0.019	0.363 ± 0.016	0.375 ± 0.026	0.43 ± 0.023	0.406 ± 0.038	0.435 ± 0.012	0.004
Trophectoderm	0.229 ± 0.019	0.261 ± 0.013	0.242 ± 0.015	0.331 ± 0.047	0.284 ± 0.023	0.309 ± 0.01	0.004
Connective tissue core	0.109 ± 0.003	0.101 ± 0.013	0.13 ± 0.012	0.095 ± 0.021	0.124 ± 0.017	0.125 ± 0.007	ns
Maternal crypts (MC)	0.28 ± 0.01	0.275 ± 0.016	0.329 ± 0.02	0.291 ± 0.032	0.307 ± 0.025	0.312 ± 0.011	ns
Feto-maternal syncytium (FMS)	0.097 ± 0.002	0.097 ± 0.005	0.109 ± 0.01	0.111 ± 0.018	0.105 ± 0.008	0.115 ± 0.005	ns
Shrinkage spaces	0.023 ± 0.01	0.016 ± 0.003	0.027 ± 0.004	0.026 ± 0.005	0.031 ± 0.013	0.044 ± 0.009	ns
Placentome capsule (PC)	0.125 ± 0.039	0.077 ± 0.008	0.07 ± 0.011	0.085 ± 0.02	0.063 ± 0.011	0.043 ± 0.006	0.01
Fetal villi/Maternal crypts	1.168 ± 0.062	1.353 ± 0.129	1.142 ± 0.058	1.487 ± 0.089	1.34 ± 0.161	1.416 ± 0.073	ns
Total fetal tissue/Total maternal tissue	0.900 ± 0.087	1.112 ± 0.079	1.017 ± 0.08	1.226 ± 0.009	1.107 ± 0.102	1.275 ± 0.072	ns

Values are Mean ± SE; ; N= Number of ewes; n= Number of placentomes morphometrically analysed

ANOVA; LSMeans specific comparisons; ns= No significant difference

TABLE 5.14 Volume densities of placentome components at 90 days gestation in triplet-bearing ewes with and without experimental restriction of implantation

TREATMENT	TRIPLETS (N=6)		RESTRICTED TRIPLETS (N=8)		LSmeans specific comparisons
<i>PLACENTOME SIZE</i>	<i>"Average-size"</i>	<i>"Large"</i>	<i>"Average-size"</i>	<i>"Large"</i>	
Weight (g) *	7.1 ± 0.6	17.6 ± 4.8	6.8 ± 0.8	18.7 ± 2.5	
	(n=6)	(n=3)	(n=3)	(n=11)	(p=)
<i>COMPONENT</i>					
Chorionic membrane (CM)	0.127 ± 0.012		0.090 ± 0.009		0.01
Chorionic epithelium (CEP)	0.046 ± 0.005		0.035 ± 0.004		ns
Haemophegous zone (HMZ)	0.050 ± 0.010		0.040 ± 0.008		ns
Maternal tissue in HMZ (MT)	0.021 ± 0.006		0.023 ± 0.005		ns
Fetal villi (FV)	0.367 ± 0.016		0.429 ± 0.013		0.004
Trophectoderm	0.255 ± 0.013		0.304 ± 0.010		0.004
Connective tissue core	0.111 ± 0.016		0.125 ± 0.013		ns
Maternal crypts (MC)	0.293 ± 0.016		0.311 ± 0.013		ns
Feto-maternal syncytium (FMS)	0.101 ± 0.007		0.113 ± 0.006		ns
Shrinkage spaces	0.019 ± 0.010		0.042 ± 0.008		ns
Placentome capsule (PC)	0.073 ± 0.008		0.048 ± 0.006		0.01
Fetal villi/Maternal crypts	1.283 ± 0.106		1.399 ± 0.085		ns
Total fetal tissue/Total maternal tissue	1.080 ± 0.092		1.239 ± 0.074		ns

Values are expressed as Least Squares Means ± SE; * Values are mean ± SE;

N= Number of ewes; n= Number of placentomes; Total fetal tissue = FV + CEP (excluding CM); Total maternal tissue = MC + PC

ANOVA; LSMeans specific comparisons; excluding small placentomes; ns= No significant difference

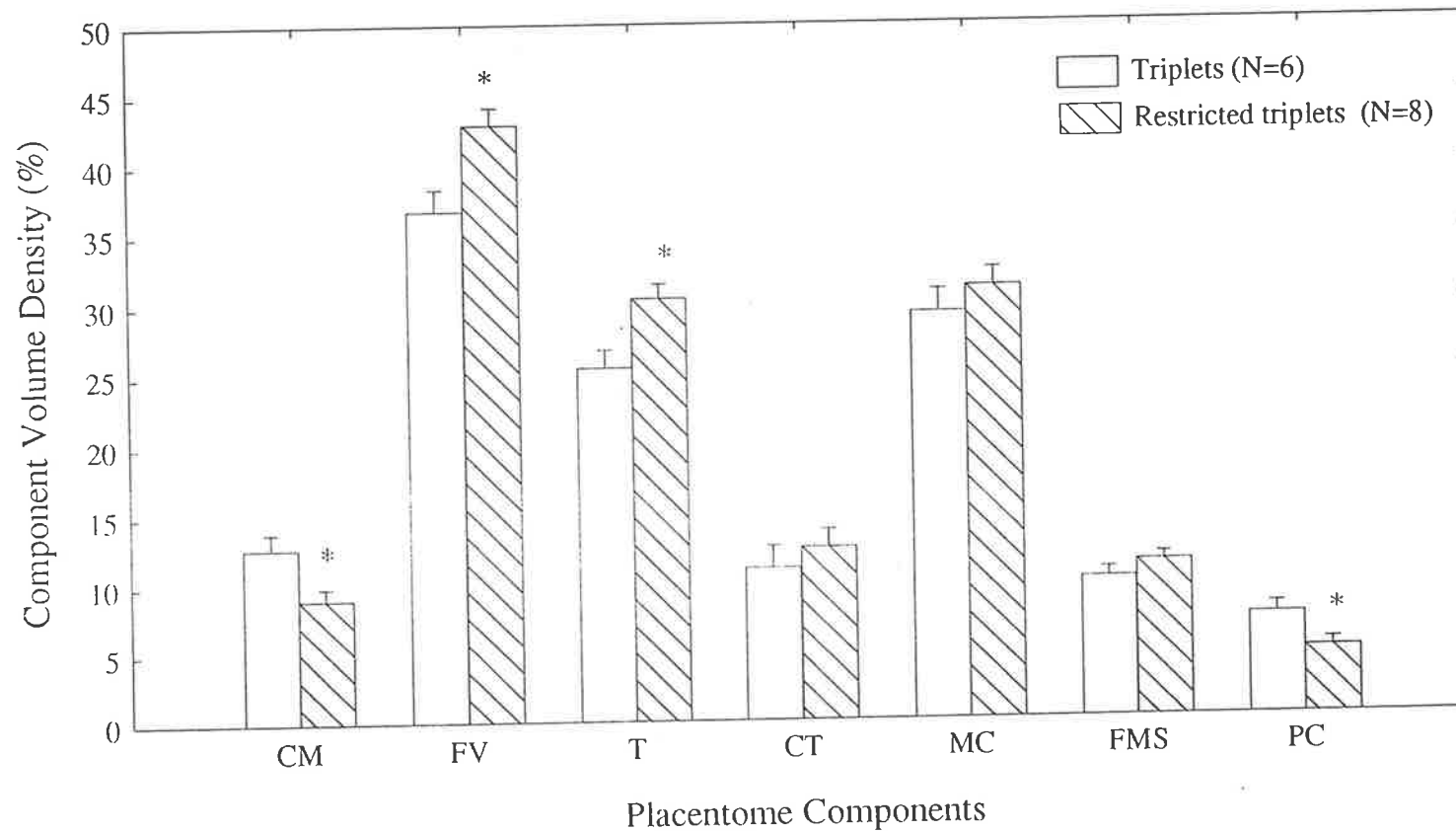


Figure 5.8 Volume densities of placentome components at 90 days gestation in triplets with (▨) and without (□) experimental restriction of implantation. Chorionic membrane (CM), Fetal villi (CM), Trophoctoderm (T), Connective tissue core of fetal villi (CT), Maternal crypts (MC), Feto-maternal syncytium (FMS) and Placentome capsule (PC). (ANOVA; L S Means Comparisons; * $p < 0.01$).

TABLE 5.15 Surface density and arithmetic mean barrier thicknesses of the epithelial cell layers, at 90 days gestation, in placentomes of triplet-bearing ewes with and without experimental restriction of implantation

TREATMENT	TRIPLETS (N=6)		RESTRICTED TRIPLETS (N=8)		LSMeans specific comparisons
PLACENTOME SIZE	"Average-size"	"Large"	"Average-size"	"Large"	
Weight (g)	7.1 ± 0.6	17.6 ± 4.8	6.8 ± 0.8	18.7 ± 2.5	
	(n=6)	(n=3)	(n=3)	n=11	(p)
PARAMETER					
Surface density of trophoctoderm (cm ⁻¹)	160 ± 8		177 ± 7		ns
Barrier thickness of trophoctoderm (µm)	18.80 ± 1.15		19.27 ± 0.92		ns
Barrier thickness of feto-maternal syncytium (µm)	7.67 ± 0.6		7.67 ± 0.49		ns

Values are Least Squares Means ± SE

ANOVA; Lsmeans specific comparisons; excluding small placentomes

N= Number of ewes; n= Number of placentomes

There was no significant effect of placentome size and morphology on surface density and barrier thickness

TABLE 5.16 Relationship between placentome morphology, volume density of components, and fetal to maternal tissue ratio at 90 days gestation, in placentas of triplet-bearing ewes with and without experimental restriction of implantation

TREATMENT	TRIPLETS (N=6)	RESTRICTED TRIPLETS (N=8)			ANOVA
<i>PLACENTOME MORPHOLOGY:</i>	<i>Non-everted</i>	<i>Everted</i>	<i>Non-everted</i>	<i>Everted</i>	<i>Effect of Morphology</i>
	(n=10)	(n=2)	(n=6)	(n=10)	
Placentome Weight (g)	9.3 ± 2.2	5.8 ± 3.6	11.5 ± 2.3	16.3 ± 3.5	(p=)
<i>COMPONENT</i>					
Chorionic membrane	0.121 ± 0.013	0.129 ± 0.02	0.103 ± 0.012	0.081 ± 0.007 *	ns
Chorionic epithelium (CEP)	0.046 ± 0.005	0.05 ± 0.002	0.032 ± 0.006	0.039 ± 0.006	ns
Haemophagous zone HMZ)	0.056 ± 0.007	0.033 ± 0.002	0.045 ± 0.006	0.029 ± 0.004 *	0.002
Maternal tissue in HMZ (MT)	0.025 ± 0.005	0.028 ± 0.004	0.009 ± 0.001	0.029 ± 0.011	ns
Fetal villi (FV)	0.362 ± 0.013	0.345 ± 0.035	0.409 ± 0.012	0.441 ± 0.015 *	ns
Trophectoderm	0.247 ± 0.008	0.254 ± 0.047	0.289 ± 0.008	0.319 ± 0.013 *	ns
Connective tissue core	0.115 ± 0.008	0.088 ± 0.018	0.126 ± 0.010	0.119 ± 0.009	ns
Maternal crypts (MC)	0.293 ± 0.014	0.276 ± 0.015	0.316 ± 0.019	0.304 ± 0.009	ns
Feto-maternal syncytium	0.099 ± 0.004	0.104 ± 0.009	0.107 ± 0.007	0.116 ± 0.004 *	ns
Placentome capsule (PC)	0.077 ± 0.006	0.128 ± 0.073	0.060 ± 0.013	0.047 ± 0.005 *	ns
Fetal villi/Maternal crypts	1.263 ± 0.084	1.207 ± 0.099	1.324 ± 0.107	1.463 ± 0.064	ns
(FV + CEP) / (MC + MT + PC)	1.052 ± 0.053	0.947 ± 0.221	1.175 ± 0.107	1.275 ± 0.059	ns
Shrinkage spaces	0.022 ± 0.003	0.011 ± 0.007	0.033 ± 0.008	0.044 ± 0.010	ns

Values are mean ± SE; ANOVA LSMeans; testing effect of treatment and placentome morphology and their interaction using "small", "average", and "large" placentomes; Treatment had no significant effects and there was no significant interaction between treatment and placentome morphology. Values in "**bold**" were significantly different ($p \leq 0.05$) between non-everted and everted placentomes within a particular treatment. Values marked (*) were significantly different ($p \leq 0.05$) from those of non-everted placentomes of non-restricted triplets. N = Number of ewes; n = Number of placentomes morphometrically analysed; ns = no significant difference

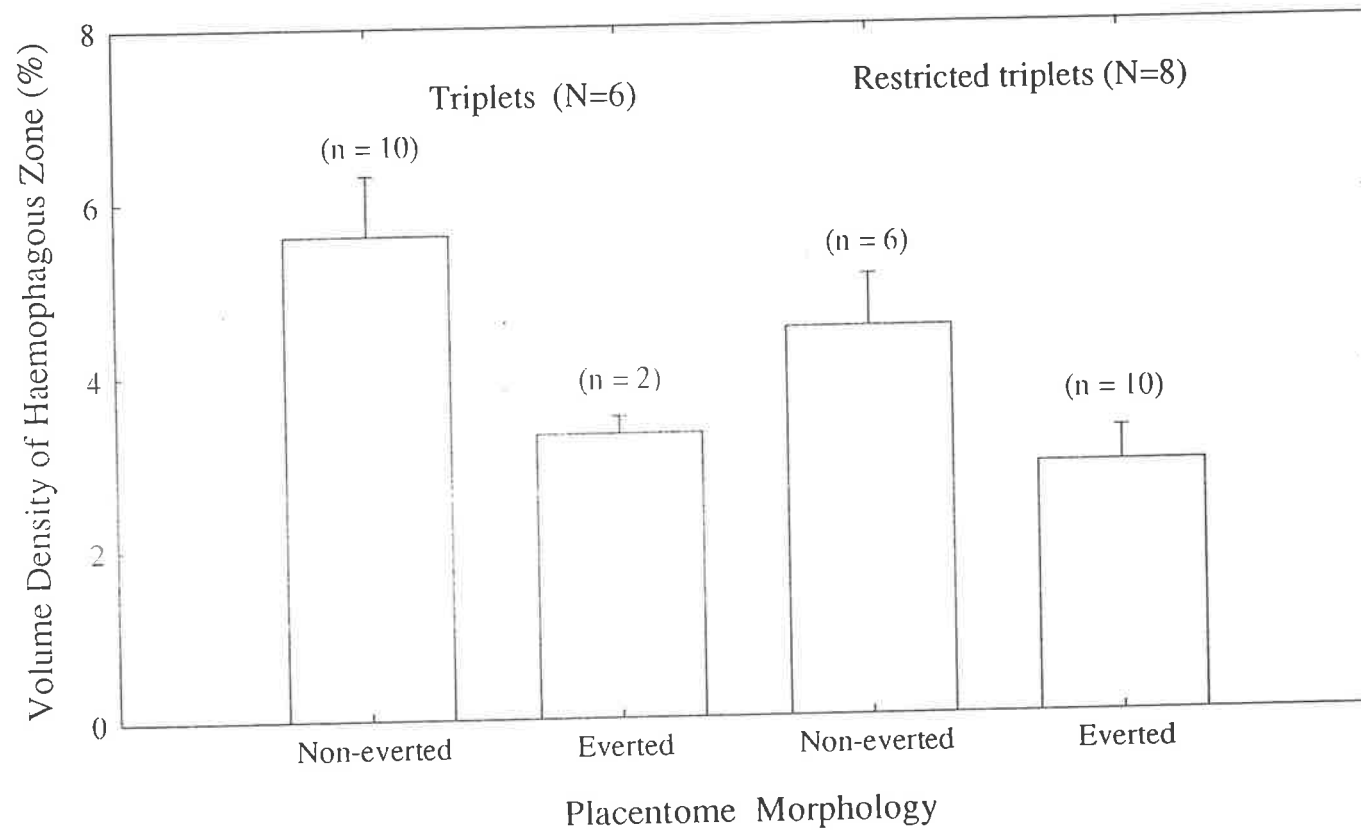


Figure 5.9 Gross placentome morphology and the volume density of the haemophagous zone at 90 days gestation in placentomes of triplets with and without experimental restriction of implantation

(Overall effect of placentome morphology; $p=0.002$; ANOVA; L S Means Comparisons)

N= Number of ewes studied; n = Number of placentomes morphometrically analysed

TABLE 5.17 Relationship between placentome morphology, surface density of trophoctoderm, and arithmetic mean barrier thickness of trophoctoderm and feto-maternal syncytium in placentomes of triplet-bearing ewes with and without experimental restriction of implantation

TREATMENT	TRIPLETS (N=6)		RESTRICTED TRIPLETS (N=8)		
<i>PLACENTOME MORPHOLOGY:</i>	<i>Non-everted</i> (n=10)	<i>Everted</i> (n=2)	<i>Non-everted</i> (n=6)	<i>Everted</i> (n=10)	
Placentome Weight (g)	9.3 ± 2.2	5.8 ± 3.7	11.5 ± 2.3	16.3 ± 3.5	
<i>PARAMETER</i>					@ Morphology (p=)
Surface density of trophoctoderm (cm ⁻¹)	158.60 ± 4.67	171.83 ± 3.94	163.21 ± 9.76	182.64 ± 6.13 *	0.002
Barrier thickness of trophoctoderm (µm)	18.54 ± 0.51	17.77 ± 3.26	20.02 ± 1.37	19.69 ± 0.64	ns
Barrier thickness of feto-maternal syncytium (µm)	7.7 ± 0.03	7.6 ± 0.05	7.1 ± 0.02	8.00 ± 0.06	ns

Values are expressed as mean ± SE; @ = ANOVA; LSMeans testing effect of morphology. The value marked (*) was significantly greater (p=0.04) than that for non-everted placentomes of non-restricted triplets (lsmeans, specific comparisons)

N = Number of ewes studied; n = number of placentomes morphometrically analysed

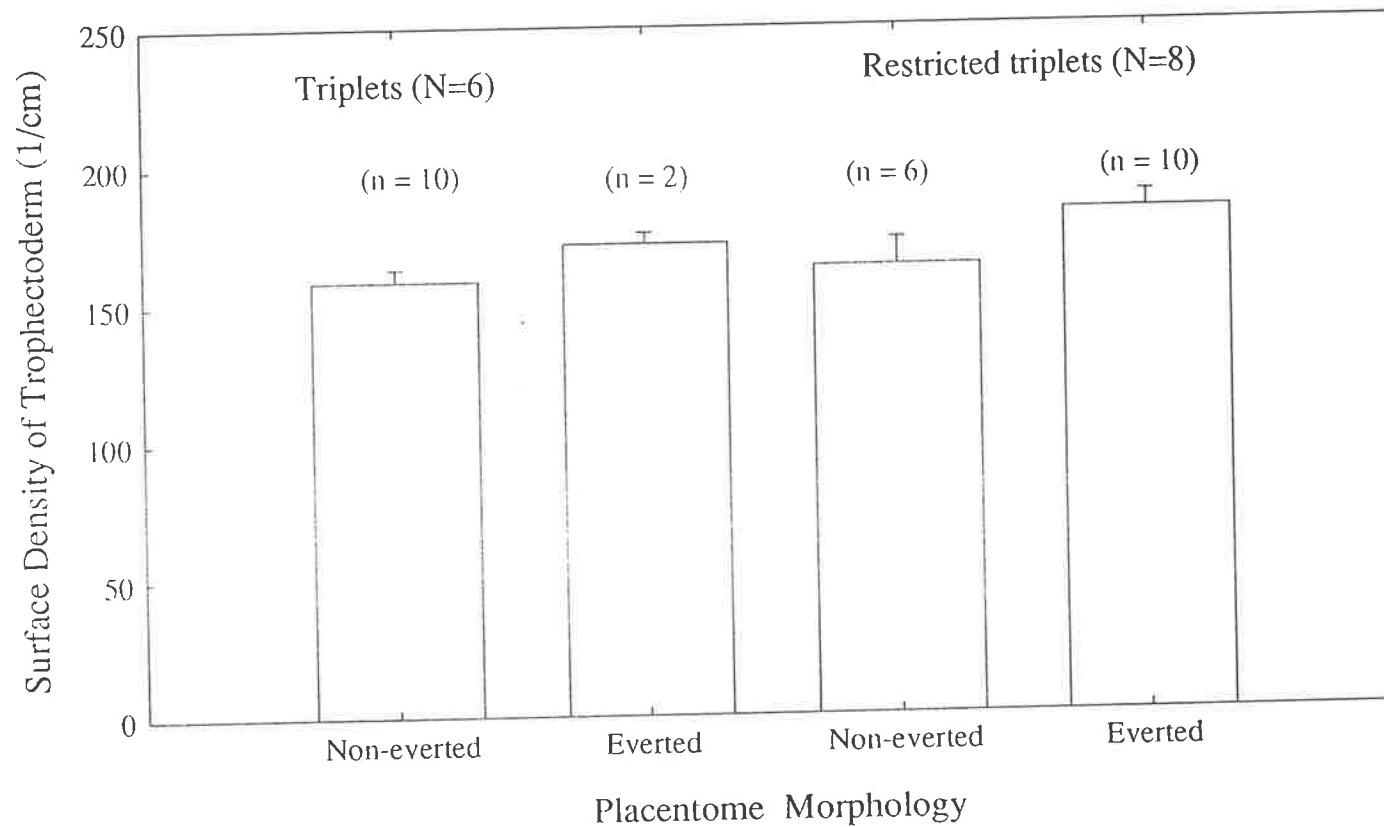


Figure 5.10 Gross placentome morphology and the surface density of trophoblast at 90 days gestation in triplets with and without experimental restriction of implantation.

(Overall effect of placentome morphology; $p=0.002$; ANOVA L S Means Comparisons).

N= Number of ewes; n = Number of placentomes morphometrically analysed

Table 5.18 Correlation analysis between placental weight and other placental parameters in twins at 90 days gestation

TREATMENT	TWINS (N= 6)
<i>PLACENTAL COMPONENT</i>	
Placentome Number	ns
Chorionic Membrane	0.88 (p= 0.02)
Chorionic Epithelium	0.79 (p= 0.06)
Haemophagous Zone (HMZ)	ns
Maternal Tissue in the HMZ	ns
Fetal villi	0.97 (p= 0.0009)
Trophectoderm	ns
Connective Tissue Core	0.81 (0.05)
Maternal Crypts	0.84 (p= 0.04)
Feto-maternal Syncytium	ns
Placentome Capsule	ns
Surface Area (m ²)	0.92 (p= 0.009)

Values are expressed as correlation coefficients (outside brackets) and their probabilities (inside brackets); ns = no significant correlation; N = number of ewes

Correlation analysis was done by ANOVA; PROC CORR

Placental cellular parameters expressed as placental volumes

Component placental volume = volume density × placental weight

Table 5.19 Correlation analyses between (a) placental weight and other placental parameters and (b) fetal weight and placental parameters at 90 days gestation in triplets with and without experimental restriction of implantation

TREATMENT	(a) PLACENTAL WEIGHT		(b) FETAL WEIGHT	
	TRIPLETS (N=6)	RESTRICTED TRIPLETS (N=8)	TRIPLETS (N=6)	RESTRICTED TRIPLETS (N=8)
<i>PLACENTAL COMPONENT</i>				
Placentome Number	0.82 (p=0.05)	ns	ns	ns
Chorionic Membrane	0.88 (p= 0.02)	0.84 (p= 0.01)	0.91 (p= 0.01)	ns
Chorionic Epithelium	0.87 (p= 0.03)	0.80 (p= 0.02)	0.87 (p= 0.02)	ns
Haemophagous Zone (HMZ)	0.85 (p= 0.03)	0.78 (p= 0.02)	ns	ns
Maternal Tissue in HMZ	0.79 (p=0.06)	ns	0.98 (p=0.004)	ns
Fetal villi	0.99 (p= 0.002)	0.95 (p= 0.0004)	ns	ns
Trophectoderm	0.95 (p= 0.003)	0.94 (p= 0.0004)	ns	ns
Connective Tissue Core	0.87 (p= 0.02)	0.95 (p= 0.0003)	ns	ns
Maternal Crypts	0.99 (p= 0.0003)	0.93 (p= 0.0007)	ns	0.81 (p= 0.02)
Feto-maternal Syncytium	0.99 (p= 0.0007)	0.94 (p=0.0004)	ns	ns
Placentome Capsule	ns	ns	ns	0.73 (p= 0.04)
Surface Area (m ²)	0.98 (0.0004)	0.96 (p= 0.0002)	ns	ns

Values are correlation coefficients (outside brackets) and their probabilities (inside brackets); ns = no significant correlation

Correlation analysis was done by ANOVA; PROC CORR

Placental cellular parameters expressed as placental volumes; placental volume = volume density $\times$ placental weight

N = Number of fetuses studied

TABLE 5.20 ANOVA Summary: Effect of natural and experimental restriction of implantation on uterine contents at 90 days gestation

COMPONENT	EXPERIMENTAL RESTRICTION	NATURAL RESTRICTION	INTERACTION
Placental weight (g)	0.0001	0.0001	ns
Placentome Numbers	0.0001	0.0001	0.01
Mean Placentome weight (g)	0.0007	ns	ns
Proportion of large placentomes (10g $\geq$) (%)	0.06	ns	ns
Proportion of Placenta (by weight) composed of everted placentomes (%)	0.0004	ns	ns
Everted Placentomes as a proportion of all placentomes in a placenta (%)	0.009	ns	ns
Morphology "B" Placentomes (%)	ns	ns	ns
Morphology "C" Placentomes (%)	0.004	0.03	0.04
Morphology "D" Placentomes (%)	0.01	ns	ns
Fetal weight (g)	0.0001	ns	ns
Fetal weight/Placental weight (F/P Ratio)	ns	0.0001	ns

Table 5.21 LSMeans comparisons of placental macroscopic parameters at 90 days gestation, in placentas of singletons, twins and triplets with and without experimental restriction of implantation

PARAMETER	EXPERIMENTAL RESTRICTION		NATURAL RESTRICTION		
	NOT RESTRICTED	RESTRICTED	SINGLETON	TWIN	TRIPLET
Fetal weight (g)	784 ± 25	569 ± 36	675 ± 34	726 ± 47	628 ± 31
Placental weight (g)	400 ± 14	235 ± 20	434 ± 18	278 ± 27	240 ± 18
Placentome number	56 ± 2	20 ± 3	55 ± 2	30 ± 4	29 ± 3
Mean placentome weight (g)	7.5 ± 0.8	12.5 ± 1.1	9.5 ± 1.0	11.3 ± 1.4	9.1 ± 0.9
Proportion of large placentomes (10g≥) (%)	23 ± 5	39 ± 7	37 ± 6	25 ± 9	31 ± 6
Fetal weight/Placental weight Ratio	2.2 ± 0.1	2.6 ± 0.2	1.7 ± 0.2	2.5 ± 0.2	3.1 ± 0.2
% Placental weight everted	7.2 ± 6.5	51 ± 9	18 ± 14	33 ± 8	37 ± 5
% Placentome number everted	7.1 ± 6.3	37 ± 9	7.5 ± 13.8	29 ± 7	30 ± 5
% B Placentomes	11.7 ± 4.6	27 ± 7	10.5 ± 8.0	27 ± 8.	20 ± 6
% C Placentomes	0.7 ± 1.4	8.8 ± 2.2	9.9 ± 2.4	0.4 ± 2.5	3.8 ± 1.7
% D Placentomes	1.2 ± 2.3	10.5 ± 3.7	11.4 ± 4.1	0.00 ± 4.3	6.3 ± 2.8

Values are expressed as Least Squares Means ± SE

TABLE 5.22 ANOVA Summary: Effect of natural and experimental restriction of implantation on volume density, surface density, arithmetic mean barrier thickness, and the ratio of fetal to maternal tissue at 90 days gestation

COMPONENT	EXPERIMENTAL RESTRICTION OF IMPLANTATION	NATURAL RESTRICTION OF IMPLANTATION
Chorionic membrane	0.002	ns
Chorionic epithelium	0.04	0.003
Haemophagous zone (HMZ)	0.03	ns
Maternal tissue in HMZ	ns	ns
Fetal villi	0.006	ns
Trophectoderm	0.0001	0.0001
Connective tissue core	ns	0.0001
Maternal crypts	0.007	ns
Feto-maternal syncytium	0.0009	0.0001
Placentome capsule	0.02	0.02
Shrinkage spaces	ns	ns
Fetal villi/Maternal crypts	ns	ns
Total Fetal Tissue/Total Maternal Tissue	ns	ns
Surface Density of Trophectoderm (cm ⁻¹)	0.0001	0.0001
Arithmetic mean Barrier thickness of Trophectoderm (μm)	ns	0.0001
Arithmetic Mean Barrier Thickness of Feto- Maternal Syncytium (μm)	ns	ns

Values are levels of significance for the effect of treatment factors on various placental parameters; ns = no significant effect
There were no significant effects of the interaction between natural and experimental restriction of implantation on all these parameters.

TABLE 5.23 Effect of natural and experimental restriction of implantation on volume densities of placental components by 90 days gestation

TREATMENT	EXPERIMENTAL	RESTRICTION	NATURAL	RESTRICTION	
COMPONENT	NOT RESTRICTED	RESTRICTED	SINGLETONS	TWINS	TRIPLETS
Chorionic membrane	0.118 ± 0.006	0.084 ± 0.008	0.103 ± 0.007	0.092 ± 0.011	0.108 ± 0.007
Chorionic epithelium	0.036 ± 0.002	0.027 ± 0.003	0.025 ± 0.003	0.029 ± 0.005	0.040 ± 0.003
Haemophagous zone (HMZ)	0.051 ± 0.005	0.031 ± 0.007	0.051 ± 0.006	0.029 ± 0.010	0.043 ± 0.006
Maternal tissue in HMZ	0.018 ± 0.003	0.015 ± 0.004	0.017 ± 0.004	0.010 ± 0.006	0.022 ± 0.004
Fetal villi	0.379 ± 0.008	0.420 ± 0.012	0.382 ± 0.010	0.418 ± 0.016	0.398 ± 0.010
Trophectoderm	0.222 ± 0.007	0.291 ± 0.009	0.191 ± 0.008	0.299 ± 0.013	0.279 ± 0.008
Connective tissue core	0.155 ± 0.008	0.135 ± 0.012	0.197 ± 0.010	0.120 ± 0.016	0.118 ± 0.010
Maternal crypts	0.291 ± 0.008	0.331 ± 0.011	0.296 ± 0.010	0.334 ± 0.016	0.302 ± 0.010
Feto-maternal syncytium	0.095 ± 0.004	0.116 ± 0.005	0.087 ± 0.004	0.122 ± 0.007	0.107 ± 0.004
Placentome capsule	0.074 ± 0.004	0.058 ± 0.005	0.078 ± 0.005	0.059 ± 0.007	0.060 ± 0.005
Shrinkage spaces	0.035 ± 0.005	0.038 ± 0.007	0.049 ± 0.006	0.030 ± 0.010	0.031 ± 0.006
Fetal villi/Maternal crypts	1.344 ± 0.055	1.308 ± 0.076	1.366 ± 0.067	1.272 ± 0.104	1.341 ± 0.068
Total Fetal Tissue/Total Maternal Tissue	1.087 ± 0.048	1.134 ± 0.066	1.089 ± 0.058	1.082 ± 0.090	1.160 ± 0.059
Surface Density of Trophectoderm (cm ⁻¹)	145 ± 4	181 ± 6	135 ± 5	185 ± 8	169 ± 5
Arithmetic mean Barrier thickness of Trophectoderm (µm)	17.97 ± 0.60	18.11 ± 0.83	17.18 ± 0.73	17.90 ± 1.12	19.03 ± 0.74
Arithmetic Mean Barrier Thickness of Feto-Maternal Syncytium (µm)	7.66 ± 0.32	7.25 ± 0.44	8.09 ± 0.39	6.61 ± 0.60	7.67 ± 0.40

Values are expressed as Least Squares Means ± SE

TABLE 5.24 ANOVA Summary: Effect of natural and experimental restriction of implantation on total placental volumes and surface areas at 90 days gestation, in placentas of singletons, twins, and triplets with and without experimental restriction of implantation

COMPONENT	NATURAL RESTRICTION	EXPERIMENTAL RESTRICTION
Chorionic membrane	ns	0.001
Chorionic epithelium	ns	0.002
Haemophagous zone (HMZ)	ns	0.03
Maternal tissue in HMZ	ns	ns
Fetal villi	0.0001	0.005
Trophectoderm	0.01	0.03
Connective tissue core	0.0002	0.03
Maternal crypts	0.0002	0.008
Feto-maternal syncytium	0.003	0.05
Placentome capsule	0.0001	0.0004
Shrinkage spaces	0.01	ns
Surface area (m ²)	0.05	ns
Relative surface area (cm ⁻¹)	0.008	0.03

There were no significant effects of the interaction between natural and experimental restriction of implantation on all these parameters

Placental cellular components expressed as placental volumes; placental volume = volume density $\times$ placental weight

Surface area = surface density $\times$ placental weight; Relative surface area = surface area / (fetal weight + placental weight)

TABLE 5.25 LSMeans of Absolute Volumes of Placental Components at 90 days gestation; Overall Effects of natural and experimental restriction of implantation

COMPONENT	EXPERIMENTAL	RESTRICTION	NATURAL	RESTRICTION	
	NOT RESTRICTED	RESTRICTED	SINGLETONS	TWINS	
Chorionic membrane	43.64 ± 3.68	20.67 ± 5.17	43.31 ± 4.98	29.42 ± 6.77	23.75 ± 4.48
Chorionic epithelium	12.80 ± 1.12	6.37 ± 1.58	10.13 ± 1.52	8.84 ± 2.06	9.79 ± 1.37
Haemophagous zone (HMZ)	19.52 ± 2.73	8.54 ± 3.82	19.09 ± 3.69	12.57 ± 5.01	10.45 ± 3.31
Maternal tissue in HMZ	6.94 ± 1.14	3.27 ± 1.59	6.84 ± 1.54	3.17 ± 2.09	5.32 ± 1.38
Fetal villi	146.97 ± 9.17	99.14 ± 12.87	168.47 ± 12.40	115.03 ± 16.85	85.67 ± 11.15
Trophectoderm	81.71 ± 3.93	65.82 ± 5.51	80.81 ± 5.31	80.57 ± 7.21	59.91 ± 4.77
Connective tissue core	65.58 ± 7.59	35.16 ± 10.64	90.53 ± 10.26	34.89 ± 13.94	25.69 ± 9.22
Maternal crypts	110.95 ± 6.44	79.16 ± 9.03	124.80 ± 8.71	93.16 ± 11.83	67.20 ± 7.82
Feto-maternal syncytium	34.69 ± 1.99	27.64 ± 2.80	36.08 ± 2.70	34.30 ± 3.66	23.12 ± 2.42
Placentome capsule	29.35 ± 2.15	14.48 ± 3.02	35.68 ± 2.91	15.11 ± 3.96	14.96 ± 2.62
Shrinkage spaces	12.81 ± 2.33	10.88 ± 3.26	19.91 ± 3.14	9.29 ± 4.27	6.32 ± 2.83
Surface area (m ²)	5.36 ± 0.31	4.15 ± 0.44	5.56 ± 0.42	5.01 ± 0.57	3.70 ± 0.38
Relative surface area (cm ⁻¹)	45.18 ± 1.97	50.80 ± 2.77	50.03 ± 2.67	51.61 ± 3.62	42.32 ± 2.40

Values are expressed as Least Squares Means ± SE

Volume of placental component = volume density × placental weight

Surface area = surface density × placental weight

Relative surface area = surface area / (fetal weight + placental weight)

CHAPTER 6

EFFECT OF MATERNAL UNDERNUTRITION ON THE PLACENTAL STRUCTURAL RESPONSE TO RESTRICTED IMPLANTATION BY MID GESTATION

6.1 INTRODUCTION

The nature of the stimulus to increased placentome size and eversion, increased trophoctoderm growth and other structural adaptations following natural or experimental restriction of implantation is unknown. While progesterone is implicated (Alexander and Williams, 1966; Robinson *et al.*, 1976; McNeil *et al.*, 1991), other factors may also be involved. In sheep, compensatory growth of the placenta increased placental weight in response to maternal undernutrition between 30 and 100 days of gestation, the period of establishment and maximum growth of the placenta (Faichney and White, 1987; McCrabb *et al.*, 1987, 1991). However, the majority of studies in sheep have found a reduction in placental growth subsequent to maternal undernutrition during mid gestation (McCrabb *et al.*, 1986; Everitt, 1964; Kelly, 1992). One explanation for these conflicting reports may be the presence of unrecognised variations in maternal condition prior to nutritional manipulation (McCrabb *et al.*, 1992). Undernutrition did not induce compensatory growth in placentas of either young ewes or mature ewes with a poor condition score at mating (Russel *et al.*, 1981), but did in mature ewes with good condition scores at mating. Thus maternal undernutrition in early/mid gestation can induce placental compensation providing maternal pre-pregnancy nutrition and thus condition score at mating are good. These studies raise the possibility that a specific nutritional deficit within the conceptus, secondary to inadequate or restricted early placental development induces some of the placental compensation observed following restricted implantation. Alternatively, the elevation of maternal progesterone levels in blood by undernutrition in early pregnancy (Cumming *et al.*, 1971; Oddy and Holst, 1991) may be responsible for the subsequent induction of placental compensation.

The placental response to alterations in maternal nutrition has been monitored largely by measuring placental weight achieved at certain periods in gestation and its relation to fetal weight (McCrabb *et al.*, 1986, 1987, 1990, 1992; Mellor and Murray, 1981, 1982; Mellor, 1983). In one study where fetal and maternal tissues were separated manually, ewes which were well-fed throughout pregnancy, had more fetal cotyledonary mass than ewes subjected to

undernutrition between 40 and 137 days (Davies *et al.*, 1981). This suggests some similarity to the placental response to experimental restriction of implantation in mid and late gestation (Chapter 4) and that in late gestation (Alexander, 1964b), which is characterised by overgrowth of fetal over maternal tissue (Alexander, 1964b). If maternal undernutrition and restricted implantation induce placental compensation by similar pathways then undernutrition in the first half of pregnancy should not affect or may even enhance the placental response to restricted implantation.

This chapter describes the investigation of the impact of maternal undernutrition prior to and during pregnancy on the placental response, by mid gestation, to experimental restriction of implantation. The aims were

- a) to determine whether inadequate maternal body reserves and undernutrition during pregnancy would enhance or inhibit placental cellular and structural response, to experimental restriction of implantation (Chapter 4) and
- b) to compare the placental response to undernutrition alone with that to restricted implantation at mid gestation, for evidence of a common placental response to a variety of perturbations.

6.2 MATERIALS AND METHODS

Treatment of animals, sample collection and processing

The study was carried out in Border-Leicester x Merino cross bred ewes. The manipulation of maternal nutrition has been described in Chapter 2 and summarised in Table 2.4. In brief, the animals were randomly divided into two groups. In one, the majority of visible endometrial caruncles were excised as described by Alexander (1964b). These operations were performed under general anaesthesia. One month after the surgical procedure, the animals were divided into four different groups. Two groups were underfed from 60 days prior to mating up to 90 days gestation. The other group were well-fed throughout the same period. All animals were killed at 90 days gestation and the uterine contents examined.

Post-mortem procedures for macroscopic examination of fetus and placenta, and placental tissue sampling for morphometric analysis were carried out as outlined in Chapter 2.

Statistical Analysis

The effect of experimental restriction of implantation, maternal undernutrition and their interaction on placental development was assessed by analysis of variance. Specific comparisons between treatments were carried out by least squares means and within treatments, by specific contrasts. Results are presented as means and standard errors.

6.3 RESULTS

6.3.1 Effect of experimental restriction of implantation, maternal undernutrition and their interaction on placental parameters; analysis of overall effects of treatment factors

Macroscopic placental growth and development

Analyses were carried out to test the overall effect of restricted implantation (regardless of the state of maternal nutrition) and that of maternal nutrition (regardless of the state of implantation) and their interaction on placental development. Experimental restriction of implantation reduced the number of placentomes formed (-43%; $p=0.02$) and placental weight (-35%; $p=0.004$) (Tables 6.1 and 6.2) but did not alter mean placentome weight or the proportion of large placentomes ($\geq 10\text{g}$). Eversion of placentomes increased 3.5 fold in restricted compared to control placentas ($p=0.02$), whether eversion was expressed as the proportion of the total number of placentomes or as a proportion of placental weight composed of everted placentomes. The proportion of everted placentomes of B morphology was increased five-fold ($p=0.02$) while that of those of the C morphology increased from 0.5% to 19% ($p=0.001$) following restricted implantation (Tables 6.1 and 6.2). In contrast, maternal undernutrition did not significantly alter any macroscopic parameters of placental growth and did not affect the response of macroscopic parameters of placental development to restricted implantation (Tables 6.1 and 6.2).

Cellular and structural placental growth and development

Restricted implantation induced remodelling of the internal structure of placentomes (Tables 6.3 and 6.4). There were significant increases in the volume densities of trophoctoderm (+24%; $p=0.008$) and feto-maternal syncytium (+28%; $p=0.0003$) in restricted placentas. The volume density of the placentome capsule was reduced by restriction of implantation (-21%; $p=0.03$) (Tables 6.3 and 6.4).

The surface density of trophoctoderm increased (+20%; $p=0.03$) but its arithmetic mean barrier thickness and that of feto-maternal syncytium did not change in response to restricted implantation (Tables 6.3 and 6.4).

Maternal undernutrition also increased the volume densities of trophoctoderm (+21%; $p=0.02$) and feto-maternal syncytium (+22%; $p=0.002$) as observed following restricted implantation (Tables 6.3 and 6.4). However, maternal undernutrition did not influence surface density, in contrast to the effect of restricted implantation (Tables 6.3 and 6.4).

Absolute Placental Volumes of Components and Placental Surface Area

Restricted implantation reduced the volumes of chorionic membrane (-46%; $p=0.01$), chorionic epithelium (-40%; $p=0.05$), fetal villi (-36%; $p=0.008$), and its connective tissue core (-48%; $p=0.02$), maternal crypts (-27%; $p=0.02$) and placentome capsule (-48%; $p=0.003$) (Table 6.5 and 6.6). The total volume of trophoctoderm tended to decrease in response to restricted implantation (-17%; $p=0.06$) (Table 6.6). In contrast, the volume of the feto-maternal syncytium and placental surface area were not altered by experimental restriction of implantation (Tables 6.5 and 6.6).

Maternal undernutrition alone did not affect the total placental volumes of all morphometrically determined placental components, placental surface area or arithmetic mean barrier thicknesses of trophoctoderm and feto-maternal syncytium (Tables 6.5 and 6.6). In addition, maternal undernutrition did not alter the absolute placental volumes of all components in experimentally restricted placentas (Tables 6.5 and 6.6).

Correlation analysis

Fetal weight did not correlate with placental weight or its morphometrically determined parameters in all treatment groups at mid gestation (data not shown).

6.3.2 Comparison of placental development in well-fed and underfed restricted ewes; specific comparisons of individual treatment groups

Macroscopic analysis of uterine contents

The total number of placentomes, total placental weight and fetal weight were similar in well-fed and underfed restricted ewes (Table 6.7). The overall distribution of placentomes in particular weight range categories was also similar in both treatment groups (Figure 6.1). However, underfeeding increased the proportion of the small placentomes in the 0-0.49g range by about 3.8-fold; from 3.3 to 12.3% ($p<0.01$).

Underfeeding tended to reduce the proportion of everted placentomes in all weight range categories (Figure 6.2). There was greater variability in the proportions of everted placentomes in well-fed restricted ewes therefore the differences in proportions of everted placentomes in weight range categories 0-9.9g between well-fed and underfed restricted ewes were not statistically significant. However, underfeeding significantly reduced the proportion of large everted placentomes ($p<0.01$) (Figure 6.2).

Due to insufficient data in the well-fed restricted group, the effect of undernutrition on the proportions of the different varieties of the everted placentome morphology cannot be evaluated.

Cellular and structural placental growth and development

Placentomes of underfed restricted ewes had higher volume densities of feto-maternal syncytium (+24%; $p=0.03$) than well-fed restricted ewes (Table 6.8 and Figure 6.3). There were no significant differences in volume densities of all other components (Table 6.8) nor in the surface density of trophoblast in placentas of well-fed and underfed restricted ewes

(Table 6.9). The arithmetic mean barrier thicknesses of trophoctoderm and feto-maternal syncytium were also similar in both treatment groups (Table 6.9). Notably, the volume density occupied by shrinkage spaces was significantly reduced (-60%) in placentomes of underfed restricted ewes compared to those of well-fed restricted ewes (Table 6.8).

In absolute terms placental volumes of all components and total surface area were similar in well-fed and underfed restricted ewes (Table 6.10).

6.3.3 Comparison of placental development in well-fed and underfed control ewes; specific comparisons of individual treatment groups

Macroscopic Analysis of Uterine Contents

There were no significant differences in most macroscopic parameters of the placenta and fetal weight between well-fed and underfed control ewes (Table 6.7). However, the number of placentomes formed was 15% lower in the underfed ewes. Overall, placentome weight distribution was similar in the well-fed and underfed ewes (Figure 6.1). A few large placentomes weighing between 40 and 50g were present in placentas of underfed ewes while the heaviest placentomes in the well-fed ewes weighed 30g. In the well-fed and underfed groups, the majority of the placentomes weighed between 0.5 to 9.9g (Figure 6.1). Although placentas of under-fed ewes had reduced numbers of everted placentomes in each of the weight range categories; the difference was not statistically significant (Figure 6.2).

Cellular and structural placental growth and development

The volumetry of the major tissue components (fetal tissue and maternal tissue) of a placentome of average size was not significantly altered by undernutrition (Table 6.11). In well-fed and underfed groups the placentome contained more fetal tissue (50% in well-fed compared to 55% in underfed ewes) than maternal tissue (~38% in both well-fed and underfed ewes) (Table 6.8). However, underfeeding significantly reduced the volume density of maternal tissue within the haemophagous zone (-57%; $p=0.01$) but increased that of trophoctoderm (+44%; $p=0.0009$) and feto-maternal syncytium (+22%; $p=0.02$) (Table 6.8 and Figure 6.4).

The surface density of trophoctoderm was not altered by underfeeding. In contrast, the arithmetic mean barrier thickness of trophoctoderm was significantly higher (+20%; $p=0.05$) while that of the feto-maternal syncytium was not altered by underfeeding (Table 6.9).

When placentomes were categorised according to their external morphologies and their components analysed, an overall effect of placentome morphology was detected on the volume density of maternal crypts ($p=0.05$) (Table 6.12). In addition, within the control treatment, everted placentomes had a significantly greater volume density of the placentome capsule compared to non-everted placentomes (+ 32%; $p=0.04$). Otherwise, all other components were not significantly different in non-everted and everted placentomes (Table 6.12). In addition, the surface density of trophoctoderm and its barrier thickness together with that of feto-maternal syncytium were not significantly altered with placentome morphology (Table 6.12).

Volumes of all placental components and placental surface area were similar in well-fed and underfed control ewes (Table 6.10).

Correlation analysis

There were no significant correlations between fetal weight and placental weight and placental functional components in both treatment groups (data not shown).

6.4 DISCUSSION

Placental responses to experimental restriction of implantation in well-fed ewes at both mid and in late gestation have been described in detail in Chapter 4. Briefly, the responses include increases in the average size of individual placentomes, the range in placentome weights and the proportion of everted placentomes present by mid gestation. Within the placentome, the volume densities of trophoctoderm and feto-maternal syncytium increase, while that of the connective tissue of the fetal villi decreases. The surface density of the epithelial cell layers also increases by mid gestation in response to experimental restriction of implantation. In

general, these responses to restricted implantation were observed in the current study and were only modified in part by maternal undernutrition.

Maternal undernutrition restrained the formation of very large placentomes following restricted implantation, reducing the upper limits of the range in placental weights from 50g to 35g. In addition, the proportion of placentomes in the lowest range category increased following a combination of experimental restriction of implantation and maternal undernutrition. Therefore, undernutrition appeared to limit the compensatory growth of placentomes which occurs in well-fed ewes with experimental restriction of implantation. This may indicate that compensatory increases in placentome size following restricted implantation require adequate maternal nutrition.

A combination of experimental restriction of implantation and maternal undernutrition tended to increase the rate of placentome eversion beyond that noted in well-fed ewes with experimental restriction of implantation. This suggests that low body reserves and further underfeeding throughout the first half of pregnancy do not inhibit and may enhance the rate of placentome eversion that occurs following experimental restriction of implantation (Chapter 4) and reported previously for well fed ewes (Alexander, 1964b; Robinson *et al.*, 1979). As previously described for well-fed ewes, (Chapter 3, 4), most everted placentomes of under-fed ewes subject to restricted implantation weighed between 0 and 9.9g and were less common in the higher weight range categories. Together with the current findings that maternal undernutrition limits compensatory increases in placentome size but does not limit their eversion, this further suggests that the mechanism that induces increased placentome size may be separate from that which enhances placentome eversion. Alternatively, for reasons not yet known, placentomes which grow larger may not need to evert.

The evidence for the possible involvement of progesterone in the mechanism for placentome eversion has already been discussed earlier in Chapters 3 and 4. There was a slightly enhanced rate of eversion in both underfed control ewes at mid gestation (similar to the eversion rate observed at 120 days in normal gestation) and underfed restricted ewes (slightly more than that which was observed in well-fed restricted ewes also at mid gestation). It is

possible that this enhanced rate of placentome eversion is a response to elevated levels in progesterone since undernutrition is known to raise the levels of the hormone in ewes (Cumming *et al.*, 1971; Oddy and Holst, 1991) and in gilts (Ashworth, 1991).

Maternal underfeeding alone reduced the volume density of the maternal tissue within the haemophagous zone. This zone is the source of the extravasated maternal blood which may act as a source of iron for the fetus (Amoroso, 1952; Steven *et al.*, 1981). Maternal undernutrition also substantially increased the volume density of trophoctoderm and to a lesser extent that of feto-maternal syncytium. Thus, the trophoctoderm is a major cellular target common to the compensatory responses to both experimental restriction of implantation and maternal undernutrition. Moreover, the lesser magnitude of the response of the feto-maternal syncytium, which is in part derived from trophoctoderm, is also consistently observed following both perturbations. This suggests that the increase in feto-maternal syncytium is secondary or a consequence of an increase in trophoctoderm abundance. This may suggest that the placenta reacts in a similar way to both forms of restriction but that the stimulus for increasing the size of these two components may be slightly weaker in the underfed control ewes when compared to that in well-fed ewes following experimental restriction of implantation.

In contrast to experimental restriction of implantation, maternal undernutrition did not increase the surface density of the two epithelial cell layers, trophoctoderm and feto-maternal syncytium.

A combination of maternal underfeeding and experimental restriction of implantation caused further increases in volume densities of trophoctoderm and feto-maternal syncytium which were more than those induced either by underfeeding or restricted implantation alone. This result suggested a stimulation by the two treatments to enhance the adaptive response by the epithelial cells on the feto-maternal interface. In addition, there was a significant reduction in the placentome capsule which may suggest a deeper invasion of the fetal villi into the maternal stroma.

The absolute volume of the connective tissue core of the fetal villi was reduced in underfed restricted ewes in comparison to that in well-fed and underfed control ewes. The reduction of the connective tissue core of the villi in the underfed restricted ewes would reduce the diameter of the fetal villi and increase the relative abundance of trophoctoderm beyond that which is achieved either by underfeeding or restricted implantation alone. Consequently, the barrier to diffusion between maternal and fetal blood systems may reduce substantially. A similar adaptive response has been observed in the ovine placenta in pregnancy toxemia (Mitchell and Stratford, 1987) and in the human placenta in pregnancies complicated by maternal pre-eclampsia (Teasedale, 1985) and in pregnancies at high altitude compared to those at sea level (Jackson *et al.*, 1987a, b). Thus the loss of connective tissue from the core of the villi is a common response to a variety of perturbations.

These results suggest that experimental restriction of implantation and maternal undernutrition before and during the first half of gestation may enhance the proliferation rate of trophoctoderm to increase cell numbers, cell size or both which maintain the volume of trophoctoderm. The increase in the volume of feto-maternal syncytium may suggest an increase in the rate of migration of binucleate cells (Wooding, 1992) beyond that which occurs normally in response to experimental restriction of implantation. However, underfeeding interferes with the mechanism by which epithelial cells are distributed or their shape alters to achieve an increase in surface density and reduce their arithmetic mean barrier thickness. Thus on average, restricted placentas of underfed ewes have a reduced surface area in comparison to that of placentas of control singletons, whereas in well-fed restricted ewes, the average surface area is comparable to that of control singletons. Similarly, although the average fetal weight in the underfed restricted ewes is only slightly less than that in well-fed restricted ewes, it is nevertheless significantly reduced in comparison to that of control singletons.

Restriction of maternal feed intake to 40% of *ad libitum* in guinea pigs reduces the weight of the peripheral labyrinth by 33% which in this species is equivalent to the exchange surface

area (Dwyer and Stickland, 1992). Thus, reduced surface for exchange at the feto-maternal interface may be a mechanism by which undernutrition reduces fetal growth (Dwyer and Stickland, 1992).

Infusion of IGF-I via the inferior vena cava in sheep for 10 days from 120 days gestation increased the proportion of larger placentomes weighing between 12 and 14g and the protein/DNA ratios in small placentomes ($5 < \%g$) but decreased the amount of tissue per unit amount of DNA in both average-size (5 to 9.9g) and large ($>10g$) placentomes (Lok *et al.*, 1993). The same experiments have also demonstrated reduced placentome eversion with IGF-I infusion (Owens *et al.*, 1994) which could explain the enhanced placentome eversion in the underfed ewes, since underfeeding would be expected to reduce both fetal and maternal plasma IGF-I levels (Oliver *et al.*, 1992). Gene disruption experiments also indicate a role for IGF-II and an unidentified receptor (XR) in placental growth in the mouse in which IGF-II gene deletion reduces placental growth (Baker *et al.*, 1993). Therefore the increase in the proportion of small placentomes and the reduction in that of large placentomes together with the enhanced placentome eversion, noted following underfeeding, may be responses to reduced concentrations of IGF-I in both fetal and maternal plasma. In guinea pig, maternal undernutrition is associated with reductions in the levels of both fetal and maternal IGF-I and fetal IGF-II and with reduced fetal and placental growth (Dwyer and Stickland, 1992).

The increased volumes of trophoctoderm and feto-maternal syncytium to a greater extent, would suggest the possible enhancement of the stimulus(i) for the mechanisms for these processes which may include the proliferation of uninucleate trophoctoderm and the transformation of some of the uninucleate cells into binucleate cells and their migration to form feto-maternal syncytium (Wooding, 1992).

6.5 SUMMARY

This study has shown that maternal undernutrition limits the increase in the size of individual placentomes following experimental restriction of implantation but does not alter and may enhance placentome eversion. Within the placentome, underfeeding enhances the increase in the volume densities of trophoctoderm and feto-maternal syncytium. This maintains the

absolute volume of trophoctoderm to control levels but almost doubles the volume of fetomaternal syncytium. The increases in these volume densities are not accompanied by increases in surface density. Thus underfeeding inhibits the promotion of growth of the surface area of the epithelial cell layers which is normally seen following restricted implantation. However, the reduction in the volume density of connective tissue from the villi may promote placental transport by reducing diffusion distance between maternal and fetal blood systems, whereas increased arithmetic mean barrier thickness of fetomaternal syncytium may reduce the efficiency of substrate diffusion. Overall placental function and hence fetal growth is reduced specifically in comparison to that of control singletons.

Comparison of the effects of maternal underfeeding prior to and throughout the first half of gestation and experimental restriction of implantation on placental growth revealed that the two forms of restriction have opposite effects on the growth of placentomes. Thus whereas experimental restriction of implantation enhances the growth of individual placentomes, maternal undernutrition limits the size of the largest placentomes and increases the proportion of the smallest placentomes. In contrast, both forms of restriction enhance placentome eversion and internally induce increases in the volume densities of the epithelial cell layers. However, undernutrition alone increases the arithmetic mean barrier thickness of trophoctoderm and inhibits the increase in surface density. Thus the mechanisms of placental compensatory growth induced by undernutrition alone and by experimental restriction of implantation in well-fed ewes may be driven by a common underlying stimulus which enhances placentome eversion and increases the volume densities of the epithelial cell layers. On the other hand, adequate nutrition may influence placental weight and surface area by determining the extent of the increases in the size of individual placentomes and the surface density of the epithelial cell layers.

TABLES AND FIGURES FOR CHAPTER 6

TABLE 6.1 Effect of experimental restriction of implantation, maternal undernutrition and their interaction on macroscopic placental growth and development at midgestation: Summary of ANOVA; analysis of overall treatment effects

COMPONENT	RESTRICTED IMPLANTATION (RI)	MATERNAL NUTRITION (MN)	RI*MN
Placental weight (g)	0.004	ns	ns
Placentome Numbers	0.02	ns	ns
Mean Placentome weight (g)	ns	ns	ns
% Proportion of large placentomes (≥ 10 g)	ns	ns	ns
Proportion of placenta composed of everted placentomes (by weight)	0.02	ns	ns
Proportion of placenta composed of everted placentomes (by number)	0.02	ns	ns
Placentome morphology % B	0.02	ns	ns
Placentome morphology % C	0.001	ns	ns
Placentome morphology % D	ns	ns	ns
Fetal weight (g)	0.04	ns	ns
Fetal weight/ Placental weight (F/P Ratio)	ns	ns	ns

Values represent levels of significance for the overall effects of restricted implantation (RI) (regardless of the level of maternal nutrition), maternal nutrition (MN) (regardless of state of implantation) and their interaction (RI * MN) on placental macroscopic parameters; ns = no significant effect.

TABLE 6.2 Effect of experimental restriction of implantation and maternal undernutrition on macroscopic placental growth and development at mid gestation: least squares means and standard errors; analysis of overall treatment effects

TREATMENT	RESTRICTED IMPLANTATION		MATERNAL NUTRITION	
	Control	Restricted	Well fed	Underfed
<i>PARAMETER</i>				
Placental weight (g)	497 ± 32	323 ± 43*	428 ± 36	392 ± 39
Placentome number	63 ± 7	36 ± 9*	53 ± 7	46 ± 8
Mean placentome weight (g)	8.3 ± 1.0	9.8 ± 1.3	9.5 ± 1.1	8.6 ± 1.2
(%) Proportion of large placentomes (≥10g)	30 ± 6	37 ± 8	37 ± 7	30 ± 8
% Placenta everted (by weight)	16 ± 9.10	57 ± 13*	33 ± 11	40 ± 12
% Placenta everted (by number)	15.1 ± 9	57 ± 13*	30 ± 11	43 ± 11
Placentome morphology % B	5 ± 4	25 ± 6.7*	10 ± 6	20 ± 5
Placentome morphology % C	0.5 ± 2	19 ± 4*	10 ± 4	9 ± 3
Placentome morphology % D	10 ± 6	14 ± 10	11 ± 9	13 ± 8
Fetal weight (g)	698 ± 36	566 ± 48*	675 ± 41	589 ± 44
Fetal weight/ Placental weight Ratio	1.50 ± 0.16	1.88 ± 0.22	1.68 ± 0.19	1.70 ± 0.20

Values are Least Squares Means ± SE for analysis of overall effects of restricted implantation (RI) (regardless of the level of maternal nutrition) and maternal nutrition (MN) (regardless of state of implantation) on placental macroscopic parameters.

Values marked * were significantly different from those of controls; $p < 0.05$; lsmeans comparisons)

TABLE 6.3 Effect of restricted implantation and maternal undernutrition on morphometrically determined parameters of placental growth and development at 90 days gestation: Summary of ANOVA

COMPONENT	RESTRICTION OF IMPLANTATION	MATERNAL NUTRITION	RI*MN
<i>VOLUME DENSITY:</i>			
Chorionic membrane	ns	ns	ns
Chorionic epithelium	ns	ns	ns
Haemophagous zone (HMZ)	ns	ns	ns
Maternal tissue in HMZ	ns	ns	0.05
Fetal villi	ns	ns	ns
Trophectoderm	0.008	0.02	ns
Connective tissue core	ns	ns	ns
Maternal crypts	ns	ns	ns
Feto-maternal syncytium	0.0003	0.002	ns
Placentome capsule	0.03	ns	ns
Shrinkage spaces	ns	0.03	ns
Fetal villi/ Maternal crypts	ns	ns	ns
Total Fetal Tissue/ Total Maternal Tissue	ns	ns	ns
Surface Density of Trophectoderm (cm ⁻¹)	0.03	ns	ns
Arithmetic Mean Barrier Thickness (μm):			
Trophectoderm	ns	ns	ns
Arithmetic Mean Barrier Thickness (μm):	ns	ns	ns
<i>Feto-maternal Syncytium</i>			

Values represent levels of significance for the overall effects of restricted implantation (RI) (regardless of the level of maternal nutrition), maternal nutrition (MN) (regardless of state of implantation) and their interaction (RI * MN) on placental morphometrically determined parameters; ns = no significant effect.

TABLE 6.4 Effect of restricted implantation and maternal undernutrition on microscopic morphometric parameters of placental growth and development at 90 days gestation: least squares means and standard errors

COMPONENT	TREATMENT		MATERNAL NUTRITION	
	CONTROL	RESTRICTED	WELLFED	UNDERFED
Volume density:				
Chorionic membrane	0.111 ± 0.006	0.103 ± 0.009	0.102 ± 0.007	0.112 ± 0.008
Chorionic epithelium	0.025 ± 0.002	0.024 ± 0.003	0.025 ± 0.002	0.025 ± 0.003
Haemophagous zone (HMZ)	0.047 ± 0.007	0.046 ± 0.011	0.047 ± 0.008	0.045 ± 0.01
Maternal tissue in HMZ	0.015 ± 0.002	0.012 ± 0.003	0.016 ± 0.002	0.012 ± 0.003
Fetal villi	0.397 ± 0.01	0.395 ± 0.015	0.385 ± 0.012	0.408 ± 0.014
Trophectoderm	0.186 ± 0.009	0.231 ± 0.013*	0.188 ± 0.01	0.228 ± 0.012*
Connective tissue core	0.207 ± 0.011	0.175 ± 0.016	0.203 ± 0.013	0.179 ± 0.015
Maternal crypts	0.283 ± 0.01	0.309 ± 0.015	0.295 ± 0.011	0.298 ± 0.014
Feto-maternal syncytium	0.083 ± 0.003	0.106 ± 0.005*	0.085 ± 0.004	0.104 ± 0.005*
Placentome capsule	0.085 ± 0.005	0.067 ± 0.007*	0.081 ± 0.005	0.071 ± 0.006
Shrinkage spaces	0.036 ± 0.006	0.044 ± 0.009	0.051 ± 0.007	0.028 ± 0.008*
Fetal villi/ Maternal crypts	1.45 ± 0.07	1.32 ± 0.10	1.38 ± 0.08	1.38 ± 0.10
Total fetal tissue/ Total maternal tissue	1.14 ± 0.05	1.11 ± 0.08	1.09 ± 0.06	1.15 ± 0.07
Surface density of trophoctoderm (cm ⁻¹)	120 ± 6	144 ± 9*	133 ± 7	131 ± 8
Arithmetic mean barrier thickness				
Trophectoderm (µm)	18 ± 0.8	18.8 ± 1.2	17.3 ± 0.9	19.5 ± 1.1
Feto-maternal syncytium (µm)	8.4 ± 0.4	8.8 ± 0.6	8.1 ± 0.5	9.0 ± 0.6

Values are Least Squares Means ± SE for analysis of overall effects of restricted implantation (RI) (regardless of the level of maternal nutrition) and maternal nutrition (MN) (regardless of state of implantation) on placental morphometrically determined parameters

Values marked * in restricted and underfed treatments were different from those of control and well-fed treatments respectively; p<0.03; ANOVA; lsmeans comparisons

TABLE 6.5 Effect of restricted implantation and maternal undernutrition and their interaction on volumes of placental components and placental surface area in midgestation: Summary of ANOVA

COMPONENT	RESTRICTION OF IMPLANTATION (RI)	MATERNAL NUTRITION (MN)	RI*MN
Chorionic membrane	0.01	ns	ns
Chorionic epithelium	0.05	ns	ns
Haemophagous zone (HMZ)	ns	ns	ns
Maternal tissue in HMZ	ns	ns	ns
Fetal villi	0.008	ns	ns
Trophectoderm	ns	ns	ns
Connective tissue core	0.02	ns	ns
Maternal crypts	0.02	ns	ns
Feto-maternal syncytium	ns	ns	ns
Placentome capsule	0.003	ns	ns
Shrinkage spaces	ns	ns	ns
Surface area (m ²)	ns	ns	ns
Relative surface area (cm ⁻¹)	ns	ns	ns

Values are levels of significance for the effects experimental restriction of implantation, maternal nutrition and their interaction on total placental volumes of components. Component placental volume = volume density $\times$ placental weight; surface area = surface density $\times$ placental weight. Relative surface area = surface area / (fetal weight + placental weight); ns = no significant effect.

TABLE 6.6 Effect of experimental restriction of implantation and maternal undernutrition on volumes of placental components and surface area in mid gestation; Least squares means; analysis of overall treatment effects

COMPONENT	RESTRICTION OF IMPLANTATION		MATERNAL NUTRITION	
	CONTROL (N=15)	RESTRICTED (N=8)	WELL FED (N=13)	UNDERFED (N=10)
Chorionic membrane	55.73 $\pm$ 5.69	30.22 $\pm$ 7.63*	43.31 $\pm$ 6.48	42.64 $\pm$ 6.96
Chorionic epithelium	12.13 $\pm$ 1.4	7.26 $\pm$ 1.9*	10.13 $\pm$ 1.59	9.27 $\pm$ 1.71
Haemophagous zone (HMZ)	22.11 $\pm$ 4.14	12.66 $\pm$ 5.56	19.09 $\pm$ 4.72	15.68 $\pm$ 5.07
Maternal tissue in HMZ	7.32 $\pm$ 1.53	4.24 $\pm$ 2.06	6.84 $\pm$ 1.75	4.71 $\pm$ 1.88
Fetal villi	199.12 $\pm$ 14.17	128.33 $\pm$ 19.01*	168.47 $\pm$ 16.15	158.98 $\pm$ 17.35
Trophectoderm	90.98 $\pm$ 4.74	75.35 $\pm$ 6.36	80.14 $\pm$ 5.40	85.51 $\pm$ 5.81
Connective tissue core	108.12 $\pm$ 12.20	55.83 $\pm$ 16.37*	90.53 $\pm$ 13.91	73.43 $\pm$ 14.94
Maternal crypts	140.90 $\pm$ 9.26	102.71 $\pm$ 12.42*	124.80 $\pm$ 10.55	118.80 $\pm$ 11.34
Feto-maternal syncytium	40.79 $\pm$ 4.42	42.67 $\pm$ 5.93	36.08 $\pm$ 5.04	47.38 $\pm$ 5.42
Placentome capsule	43.69 $\pm$ 3.68	22.66 $\pm$ 4.94*	35.68 $\pm$ 4.20	30.67 $\pm$ 4.51
Shrinkage spaces	15.70 $\pm$ 3.22	15.10 $\pm$ 4.32	19.91 $\pm$ 3.67	10.90 $\pm$ 3.94
Surface area (m ²)	5.84 $\pm$ 0.38	4.63 $\pm$ 0.51	5.56 $\pm$ 0.43	4.91 $\pm$ 0.47
Relative surface area (cm ⁻¹)	48.76 $\pm$ 2.32	50.96 $\pm$ 3.12	50.03 $\pm$ 2.65	49.69 $\pm$ 2.85

Values are least squares means $\pm$ SE;

Component placental volume = volume density $\times$ placental weight; Surface area = surface density $\times$ placental weight;

Relative surface area = surface area / (fetal weight + placental weight). Values marked * in the restricted treatment were different from those of controls; $p \leq 0.05$; ANOVA; LSMeans comparisons.

TABLE 6.7 Effects of experimental restriction of implantation and maternal undernutrition on macroscopic placental growth and development at mid gestation

TREATMENT	NUMBER CARUNCLES REMOVED	NUMBER CARUNCLES LEFT	NUMBER PLACENTOMES FORMED	MEAN PLACENTOME WEIGHT (G)	TOTAL PLACENTAL WEIGHT (G)	% TOTAL PLACENTOME NUMBER EVERTED	% TOTAL PLACENTAL WEIGHT EVERTED	FETAL WEIGHT (G)	F:P RATIO
Well-fed Control (N=9)	-	-	74 ± 6 ^a	7.5 ± 1.0 ^a	515 ± 42 ^a	11 ± 10 ^a	13 ± 12 ^a	716 ± 33 ^a	1.48 ± 0.16 ^a
Well-fed Restricted (N=4)	75 ± 9 ^a (N=3)	14.7 ± 0.3 ^a (N=3)	33 ± 6 ^b	11.5 ± 1.8 ^b	340 ± 27 ^{bc}	49 ± 20 ^{ab}	54 ± 18 ^{ac}	634 ± 46 ^{ab}	1.87 ± 0.08 ^a
Underfed Control (N=6)	-	-	63 ± 13 ^{ab}	9.1 ± 1.5 ^a	478 ± 58 ^{ab}	20 ± 16 ^{ab}	21 ± 16 ^{ab}	681 ± 57 ^a	1.53 ± 0.25 ^a
Underfed Restricted (N=4)	72 ± 5 ^a	15 ± 1 ^a	39 ± 9 ^b	8.1 ± 2.2 ^a	305 ± 55 ^c	65 ± 14 ^b	59 ± 19 ^{bc}	498 ± 111 ^b	1.88 ± 0.54 ^a

Values are expressed as mean ± SE;

Values with the same letter are not significantly different;

Significant difference; $p \leq 0.05$ (ANOVA, between factors: experimental restriction of implantation, maternal undernutrition,

LSMeans specific comparisons); N = number of ewes.

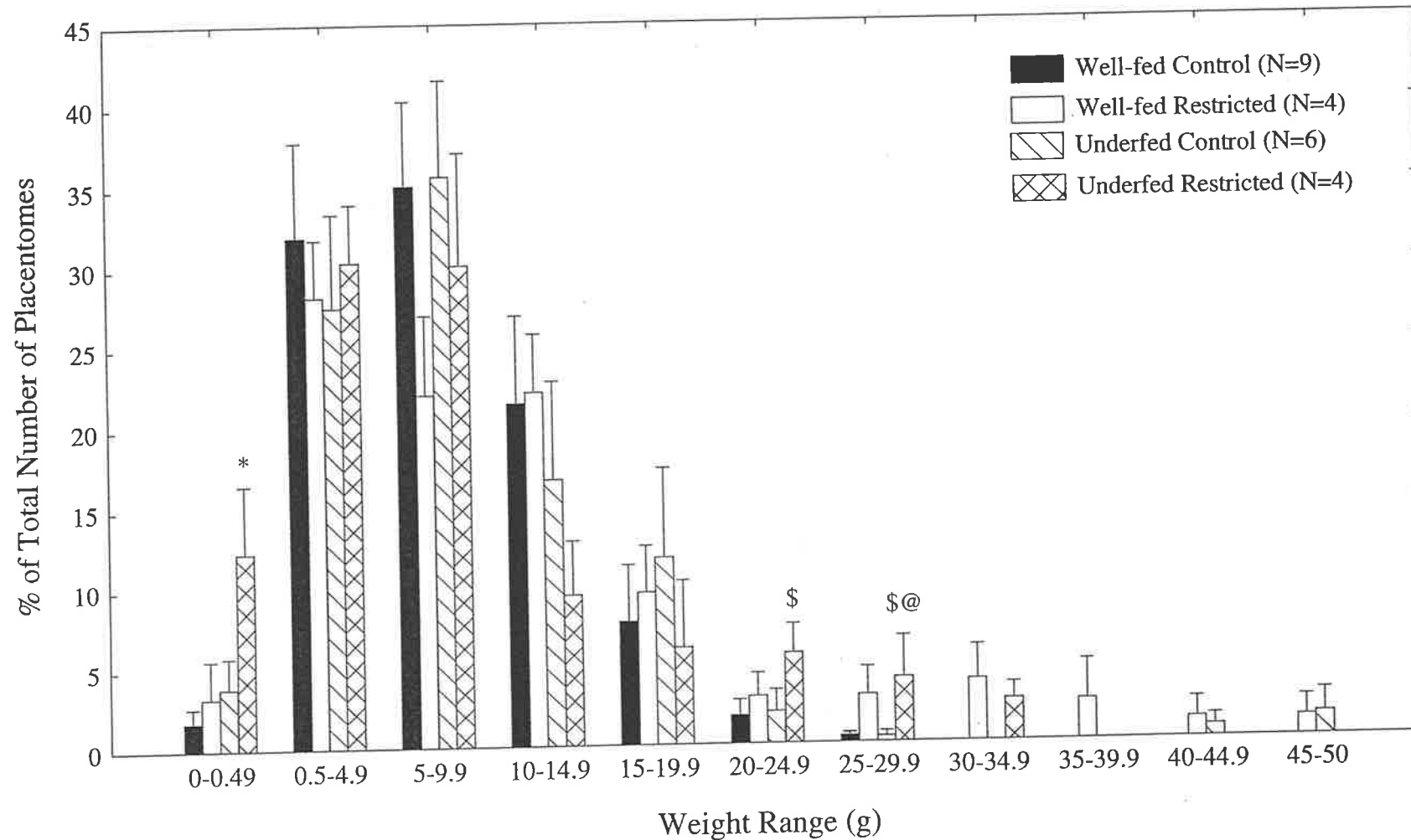


Figure 6.1. Placentome distribution within weight range categories in well-fed and underfed control and restricted ewes at 90 days gestation.

(* $p < 0.01$; proportion of placentomes in underfed restricted ewes significantly greater than all groups in the weight range category;
 \$ $p < 0.05$; proportion of placentomes in underfed restricted ewes greater than that in well-fed control ewes;
 @ $p < 0.05$; proportion of placentomes in underfed restricted ewes greater than that in underfed control ewes;
 (ANOVA; LSMeans specific contrasts).

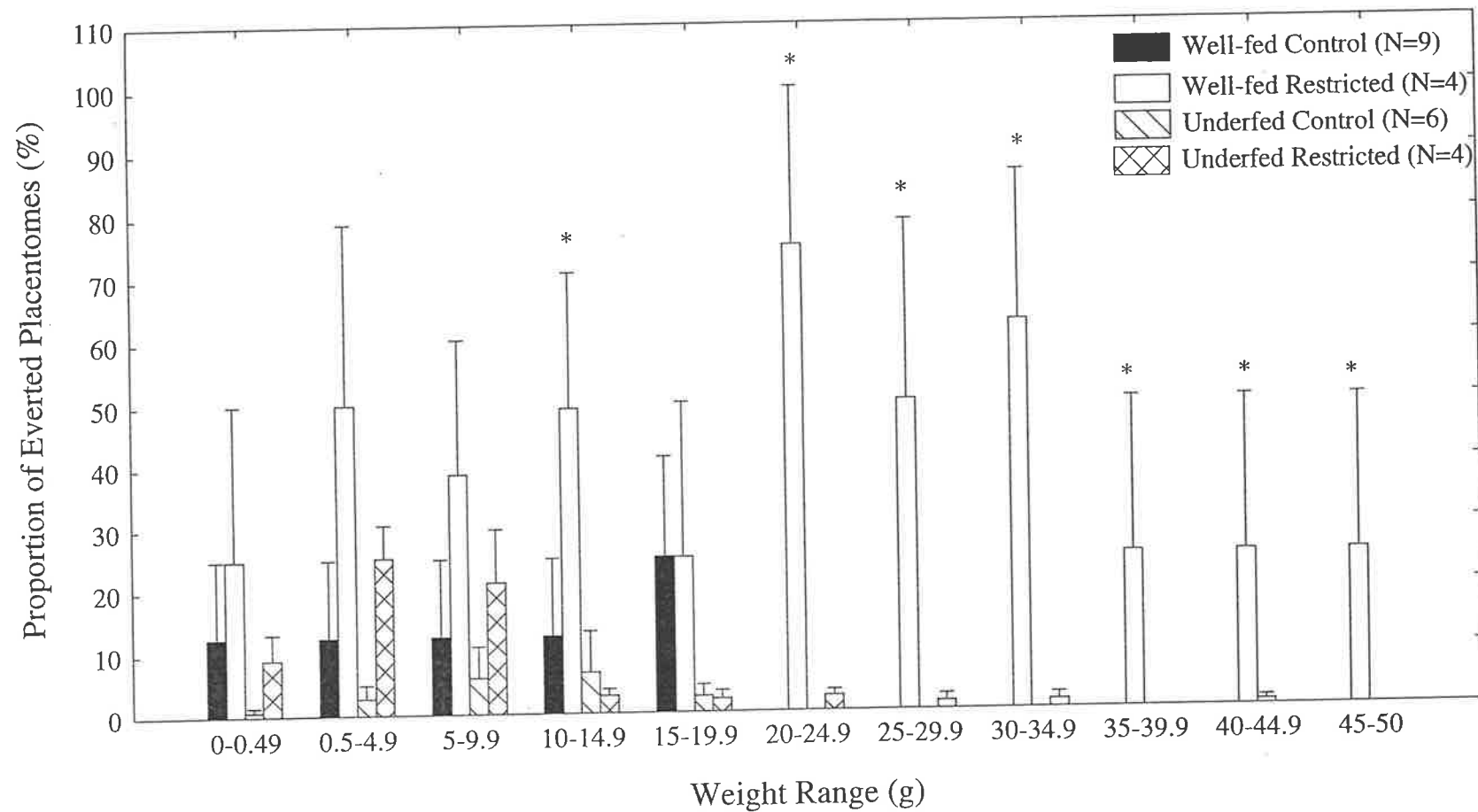


Figure 6.2 The distribution of everted placentomes within weight range categories in well-fed and Underfed control and restricted ewes at 90 days gestation (* $p < 0.05$; the proportion of placentomes in placentas of well-fed restricted ewes greater than those in all other groups; ANOVA; L S Means Specific Contrasts).

TABLE 6.8 Effect of experimental restriction of implantation and maternal undernutrition on volume densities of placentome components at 90 days gestation

TREATMENT	WELL-FED CONTROL (N=9)		WELL-FED RESTRICTED (N=4)		UNDER-FED CONTROL (N=6)		UNDER-FED RESTRICTED (N=4)	
PLACENTOME SIZE	"Average-size"	"Large"	"Average-size"	"Large"	"Average-size"	"Large"	"Average-size"	"Large"
Weight (g) *	8.2 ± 0.5 (n=8)	16.3 ± 1.4 (n=7)	7.4 ± 1.2 (n=3)	28.1 ± 6.1 (n=5)	7.3 ± 0.5 (n=6)	14.8 ± 1.4 (n=5)	6.2 ± 0.7 (n=3)	28.5 ± 2.7 (n=2)
<i>COMPONENT</i>								
Chorionic membrane	0.111 ± 0.008		0.093 ± 0.011		0.111 ± 0.009		0.113 ± 0.014	
Chorionic epithelium	0.026 ± 0.003		0.023 ± 0.004		0.024 ± 0.003		0.025 ± 0.005	
Haemophagous zone	0.060 ± 0.010		0.035 ± 0.013		0.033 ± 0.011		0.058 ± 0.017	
Maternal tissue in HMZ	0.021 ± 0.003		0.011 ± 0.004		0.009 ± 0.003		0.014 ± 0.005	
Fetal villi	0.378 ± 0.014		0.391 ± 0.019		0.417 ± 0.016		0.399 ± 0.024	
Trophectoderm	0.152 ± 0.012		0.224 ± 0.016		0.219 ± 0.014		0.237 ± 0.021	
Connective tissue core	0.216 ± 0.01		0.190 ± 0.020		0.199 ± 0.017		0.159 ± 0.026	
Maternal crypts	0.275 ± 0.013		0.314 ± 0.018		0.291 ± 0.015		0.305 ± 0.023	
Feto-maternal syncytium	0.074 ± 0.004		0.095 ± 0.006		0.091 ± 0.005		0.118 ± 0.008	
Shrinkage spaces	0.040 ± 0.008		0.063 ± 0.011		0.031 ± 0.009		0.025 ± 0.014	
Placentome capsule	0.086 ± 0.006		0.076 ± 0.008		0.085 ± 0.007		0.058 ± 0.011	
Fetal villi/Maternal crypts	1.443 ± 0.091		1.321 ± 0.125		1.450 ± 0.106		1.310 ± 0.158	
Total fetal tissue / Total maternal tissue	1.106 ± 0.069		1.078 ± 0.095		1.164 ± 0.081		1.136 ± 0.120	

Values are expressed as least squares means ± SE; * Values are mean ± SE; N= Number of ewes; n=Number of "average-size" and "large" placentomes analysed morphometrically. ANOVA; LSMeans specific comparisons (analysis excluding "small" placentomes);

Total fetal tissue = chorionic epithelium + fetal villi (excluding chorionic membrane);

Total maternal tissue = maternal crypts + maternal tissue within the haemophagous zone + placentome capsule. Values presented in "**bold**" for underfed control and underfed restricted treatments were significantly different from those of well-fed control and well-fed restricted treatments respectively; $p \leq 0.05$; ANOVA; lsmeans specific comparisons.

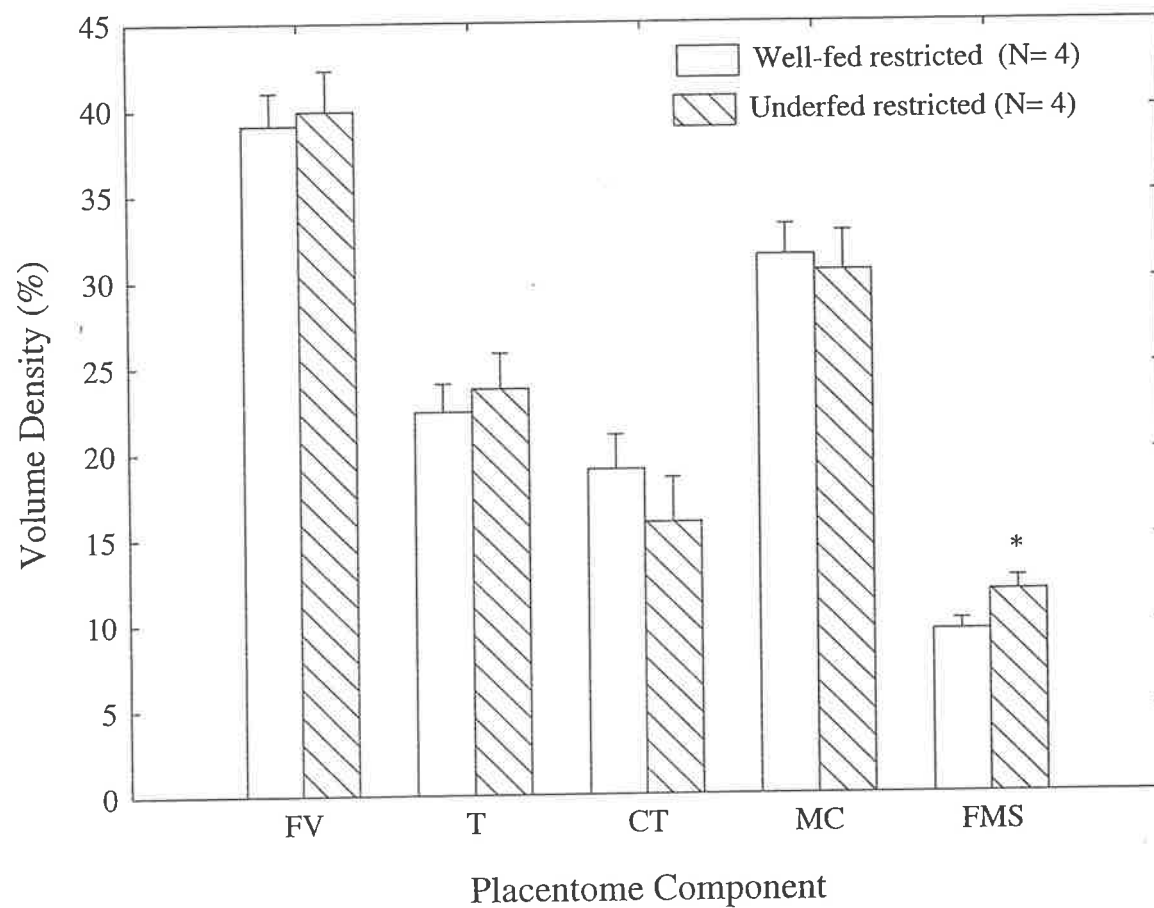


Figure 6.3 Comparison of volume densities of components in placentomes of well-fed restricted (□) and underfed restricted (▨) ewes at 90 days gestation: Fetal villi (FV), Trophoctoderm (T), Connective tissue core of fetal villi (CT), Maternal crypts (MC), Feto-maternal syncytium (FMS). (ANOVA; L S Means Comparisons; * $p = 0.03$).

TABLE 6.9 Effect of experimental restriction of implantation and maternal undernutrition on surface densities and arithmetic mean barrier thicknesses of the epithelial cell layers at 90 days gestation

TREATMENT	WELL-FED CONTROL (N=9)		WELL-FED RESTRICTED (N=4)		UNDERFED CONTROL (N=6)		UNDERFED RESTRICTED (N=4)	
<i>PLACENTOME SIZE</i>	<i>"Average-size"</i>	<i>"Large"</i>	<i>"Average-size"</i>	<i>"Large"</i>	<i>"Average-size"</i>	<i>"Large"</i>	<i>"Average-size"</i>	<i>"Large"</i>
Weight (g) *	8.2 ± 0.5 (n=8)	16.3 ± 1.4 (n=7)	7.4 ± 1.2 (n=3)	28.1 ± 6 (n=5)	7.3 ± 0.5 (n=6)	14.8 ± 1.4 (n=5)	6.2 ± 0.7 (n=3)	28.5 ± 2.7 (n=2)
<i>PARAMETER</i>								
Surface density of trophoctoderm (cm ⁻¹)	116 ± 8		150 ± 11		125 ± 9		138 ± 14	
Barrier thickness of trophoctoderm (µm)	16.33 ± 1.08		18.2 ± 1.5		19.66 ± 1.26		19.4 ± 1.9	
Feto-maternal syncytium (µm)	8.5 ± 0.54		7 ± 0.7		8.22 ± 0.63		9.8 ± 0.9	

Values are expressed as least squares means ± SE; * Values are mean ± SE

The value presented in "**bold**" was significantly different from that of well-fed control ewes; p = 0.05; ANOVA; LSMeans specific comparisons (analysis excluding small placentomes). N= Number of ewes; n=Number of average and large placentomes analysed morphometrically.

TABLE 6.10 Effect of experimental restriction and maternal undernutrition on placental volumes and surface areas; specific comparisons between treatment groups

TREATMENT	WELL-FED CONTROL (N=9)	UNDERFED CONTROL (N=6)	WELL-FED RESTRICTED (N=4)	UNDERFED RESTRICTED (N=4)
Placental Weight (g)	515 ± 42 a	478 ± 58 ab	340 ± 27 bc	305 ± 55 c
<i>COMPONENT</i>				
Chorionic membrane	57 ± 9 a	54 ± 9 ab	29 ± 5 bc	31 ± 6 ac
Chorionic epithelium	13 ± 2 a	11 ± 2 a	7 ± 1 a	7 ± 2 a
Haemophagous zone (HMZ)	28 ± 7 a	16 ± 5 a	10 ± 2 a	15 ± 4 a
Maternal tissue in HMZ	10 ± 3 a	4.15 ± 0.93 b	3.20 ± 0.36 b	5.27 ± 1.76 ab
Fetal villi	200.58 ± 20.97 a	197.67 ± 21.72 a	136.36 ± 15.73 ab	120.30 ± 22.62 b
Trophectoderm	83.51 ± 4.31 ab	98.44 ± 7.68 ab	78.12 ± 6.78 ab	72.58 ± 14.41 a
Connective tissue core	116.94 ± 20.71 a	99.31 ± 15.30 a	64.12 ± 5.22 ab	47.54 ± 14.41 b
Maternal Crypts	141.65 ± 12.3 a	140.14 ± 15.78 ab	107.95 ± 7.89 ac	97.46 ± 19.00 bc
Feto-maternal syncytium	38.21 ± 3.01 a	43.38 ± 4.46 a	33.96 ± 4.52 a	51.39 ± 17.94 a
Shrinkage spaces	17.73 ± 4.08 a	13.68 ± 2.24 a	22.10 ± 10.92 a	8.11 ± 2.18 a
Placentome capsule	45.43 ± 5.09 a	41.96 ± 6.84 ab	25.94 ± 3.79 bc	19.38 ± 4.80 c
Surface area (m ²)	5.93 ± 0.44 a	5.76 ± 0.53 ab	5.18 ± 0.83 ab	4.07 ± 0.88 b
Relative (cm ⁻¹)	47.79 ± 2.32 a	49.73 ± 2.14 a	52.28 ± 6.13 a	49.64 ± 6.50 a

Values are expressed as mean ± SE.

Component placental volume = volume density × placental weight; surface area = surface density × placental weight

Values with different letters are significantly different (p ≤ 0.05; ANOVA; LSMeans specific comparisons)

TABLE 6.11 Effect of maternal undernutrition on the volume densities of placentome components at 90 days gestation

TREATMENT	WELL-FED CONTROL (N=9)			UNDER-FED CONTROL (N=6)			ANOVA; LSMeans Comparisons
PLACENTOME SIZE	"Small"	"Average-size"	"Large"	"Small"	"Average-size"	"Large"	
Weight (g)	2.9 ± 0.8 (n=2)	8.2 ± 0.5 (n=8)	16.3 ± 1.4 (n=7)	3.2 (n=1)	7.3 ± 0.5 (n=6)	14.84 ± 1.37 (n=5)	(p=)
COMPONENT							
Chorionic membrane	0.107 ± 0.005	0.107 ± 0.011	0.115 ± 0.013	0.101	0.111 ± 0.006	0.109 ± 0.017	ns
Chorionic epithelium (CEP)	0.024 ± 0.011	0.031 ± 0.004	0.022 ± 0.005	0.009	0.025 ± 0.002	0.023 ± 0.004	ns
Haemophagous zone (HMZ)	0.018 ± 0.01	0.069 ± 0.015	0.050 ± 0.017	0.006	0.038 ± 0.007	0.028 ± 0.007	ns
Maternal tissue in HMZ (MT)	0.007 ± 0.001	0.022 ± 0.004	0.021 ± 0.008	0.001	0.010 ± 0.002	0.008 ± 0.002	0.01
Fetal villi (FV)	0.450 ± 0.027	0.363 ± 0.020	0.395 ± 0.032	0.412	0.41 ± 0.008	0.425 ± 0.016	ns
Trophectoderm	0.200 ± 0.046	0.162 ± 0.014	0.159 ± 0.014	0.165	0.228 ± 0.018	0.208 ± 0.023	0.0009
Connective tissue core	0.256 ± 0.074	0.199 ± 0.026	0.236 ± 0.034	0.245	0.183 ± 0.014	0.217 ± 0.006	ns
Maternal crypts (MC)	0.284 ± 0.022	0.278 ± 0.021	0.271 ± 0.023	0.341	0.284 ± 0.013	0.299 ± 0.015	ns
Feto-maternal syncytium	0.081 ± 0.004	0.077 ± 0.006	0.072 ± 0.008	0.105	0.089 ± 0.004	0.092 ± 0.006	0.02
Shrinkage spaces	0.022 ± 0.003	0.042 ± 0.012	0.039 ± 0.010	0.01	0.033 ± 0.005	0.028 ± 0.005	ns
Placentome capsule (PC)	0.098 ± 0.010	0.082 ± 0.011	0.091 ± 0.008	0.116	0.089 ± 0.009	0.079 ± 0.013	ns
Fetal villi/Maternal crypts	1.604 ± 0.219	1.359 ± 0.127	1.538 ± 0.200	1.208	1.466 ± 0.099	1.432 ± 0.070	ns
Total fetal tissue /Total maternal tissue	1.224 ± 0.084	1.079 ± 0.105	1.137 ± 0.134	0.920	1.156 ± 0.092	1.175 ± 0.080	ns

Values are expressed as mean ± SE; N= Number of ewes studied; n= Number of placentomes morphometrically analysed; ANOVA; LSMeans specific comparisons (excluding small placentomes)

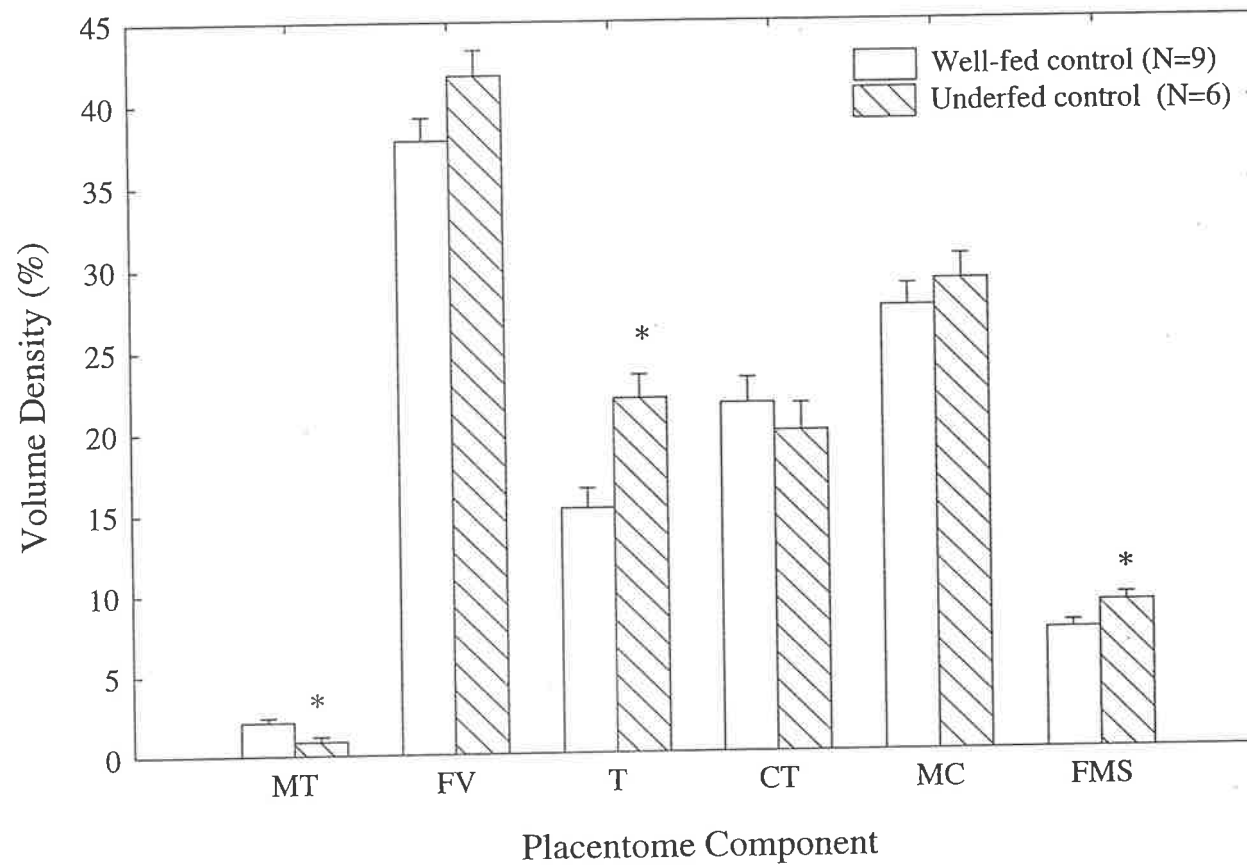


Figure 6.4 Comparison of volume densities of components in placentomes of well-fed (□) and underfed (▨) control ewes at 90 days gestation: Maternal tissue within the haemophagous zone (MT), Fetal villi (FV), Trophoctoderm (T), Connective tissue core of fetal villi (CT), Maternal crypts (MC), and Feto-maternal syncytium (FMS). (ANOVA; L S Means Comparisons; * $p \leq 0.02$).

Values are mean $\pm$ SE; ANOVA; LSMeans comparisons testing effect of treatment, placentome morphology and their interaction using "small", "average-size", and "large" placentomes. Restricted implantation had significant effects. There were no significant effects of interaction between treatment and placentome morphology; *The volume density of feto-maternal syncytium was significantly less in non-everted placentomes of control placentomes when compared with that of everted placentomes of restricted placentas ($p=0.002$); LSmeans specific comparisons); Component volume densities in "**BOLDFACE**" were significantly different between non-everted and everted placentomes within the control ewes ($p=0.04$); lsmeans specific comparisons); Total fetal tissue = FV + CEP (excluding CM); Total maternal tissue = MC + MT + PC; N= Number of ewes; n= Number of placentomes morphometrically analysed; ns= no significant difference

TABLE 6.12 Effect of experimental restriction of implantation on volume and surface densities and barrier thicknesses in non-everted and everted placentomes of underfed ewes at 90 days gestation

TREATMENT	CONTROL (N=6)		RESTRICTED IMPLANTATION (N=4)		ANOVA SUMMARY
<i>PLACENTOME MORPHOLOGY:</i>	<i>Non-everted</i>	<i>Everted</i>	<i>Non-everted</i>	<i>Everted</i>	<i>Effect of Morphology</i>
	(n=10)	(n=2)	(n= 3)	(n= 5)	(p=)
Placentome Weight (g)	10.3 ± 1.4	9.4 ± 6.2	13.3 ± 9	8.8 ± 4.3	
<i>COMPONENT:</i>					
Chorionic membrane (CM)	0.111 ± 0.009	0.106 ± 0.005	0.094 ± 0.020	0.113 ± 0.020	ns
Chorionic epithelium (CEP)	0.026 ± 0.002	0.010 ± 0.001	0.021 ± 0.007	0.027 ± 0.003	ns
Haemophagous zone (HMZ)	0.036 ± 0.005	0.005 ± 0.001	0.041 ± 0.012	0.066 ± 0.027	ns
Maternal tissue in HMZ (MT)	0.010 ± 0.001	0.002 ± 0.001	0.014 ± 0.004	0.019 ± 0.005	ns
Fetal villi (FV)	0.418 ± 0.009	0.408 ± 0.005	0.411 ± 0.025	0.382 ± 0.030	ns
Trophectoderm	0.223 ± 0.015	0.173 ± 0.008	0.225 ± 0.039	0.245 ± 0.011	ns
Connective tissue core	0.196 ± 0.010	0.233 ± 0.012	0.188 ± 0.045	0.135 ± 0.025	ns
Maternal crypts (MC)	0.286 ± 0.009	0.339 ± 0.003	0.327 ± 0.016	0.306 ± 0.010	0.05
*Feto-maternal syncytium	0.090 ± 0.004	0.103 ± 0.002	0.120 ± 0.012	0.120 ± 0.006	ns
Placentome capsule (PC)	0.081 ± 0.007	0.119 ± 0.003	0.057 ± 0.012	0.064 ± 0.010	ns
Shrinkage spaces	0.033 ± 0.003	0.011 ± 0.002	0.030 ± 0.009	0.024 ± 0.006	ns
Fetal villi/Maternal crypts	1.476 ± 0.061	1.204 ± 0.005	1.25 ± 0.025	1.25 ± 0.099	ns
Total fetal tissue/ Total maternal tissue	1.191 ± 0.060	0.911 ± 0.010	1.09 ± 0.019	1.066 ± 0.104	ns
Surface density of trophectoderm (cm ⁻¹)	125 ± 8	122 ± 5	125 ± 32	139 ± 8	ns
Barrier thickness of trophectoderm (µm)	20.11 ± 0.88	15.03 ± 0.12	20.43 ± 1.42	19.68 ± 0.71	ns
Barrier thickness of feto-maternal syncytium (µm)	8.20 ± 0.4	8.6 ± 0.4	11.50 ± 1.6	10.0 ± 0.5	ns

CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS

7.1 INTRODUCTION

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

7.2 ONTOGENY OF WEIGHT AND EVERSION

The present work has increased our understanding of placental structural development and its relationship to function. The study of the normal ontogeny of the ovine singleton placenta (Chapter 3) has confirmed that maximum placental size is attained in mid-gestation. In contrast to previous reports (Cloete, 1939; Alexander, 1964a; Stegeman, 1974) placental weight was observed to plateau until approximately 130 days gestation, after which it decreased by 26% by 140 days gestation. From mid-gestation to 120 days, average placentome weights remained the same while the proportion of everted placentomes remained low (between 10 and 25%). Thus during the first half of gestation, placental function is largely determined by the structure of the non-everted placentome. During this time, most everted placentomes tended to be of "small" and "average" sizes with relatively few large ones. However, by 130 days, 80% of the placentomes everted. Placentome eversion was complete by 140 days. The observation that 80% of all everted placentomes in late gestation were of the "B" morphology indicates that placental function at this time is largely determined by the structure that underlies this morphology. Notably, the completion of placentome eversion coincided with the 26% decline in placental weight. A systemic stimulus may be responsible for these rapid maturational changes in these two macroscopic placentome parameters, which may also be causally related. Placental maturation appears to involve reduction in connective tissue within fetal villi, reduction of the haemophagous zone and increased volume of fetal villi, due to significant increases in the volume of trophoblast. These characteristics were also generally associated with and may underlie the everted morphology of the placentome.

7.3 STEROIDS AND PLACENTOME EVERSION

The maturational changes in placentome size and morphology are coincident with the activation of the fetal hypothalamo-pituitary-adrenal axis (McMillan *et al.*, 1987; McIssac *et al.*, 1989). A major feature of this activation is a surge in circulating cortisol in the fetus, which may be associated with the developmental maturation of placental structure (Liggins *et al.*, 1976; Yang *et al.*, 1992; Ballard *et al.*, 1979). Conditions characterised by high maternal levels of progesterone in early pregnancy such as multiple ovulation, carunclectomy (Robinson *et al.*, 1976; McNeil *et al.*, 1991) and the administration of exogenous progesterone analogues (Alexander and Williams, 1966) are associated with increased placentome eversion. Exogenous glucocorticoids in mid pregnancy (Wintour *et al.*, 1994) and activation of the fetal HPA axis (Chapter 3) are also associated with increased eversion. Therefore it would appear that high levels of steroids/ glucocorticoids can enhance placentome eversion over most of gestation.

The effects of high levels of progesterone in the presence of low levels of glucocorticoids may be mediated via receptors for either progesterone or glucocorticoids. A similar mode of action would also apply for glucocorticoids. There is evidence for the cross reactivity and competition for receptors of high levels of progesterone and corticosteroids.

Following the activation of the HPA-axis, progesterone levels fall within the last week of pregnancy while the levels of oestrogen rise approximately two days prior to parturition (Thorburn *et al.*, 1972). Since the changes in these hormonal levels occur after the completion of placentome eversion (Chapter 3), it is unlikely that the mechanism by which glucocorticoids influence placentome eversion involves the effect which glucocorticoids have on progesterone and oestrogen levels.

7.4 MECHANISMS UNDERLYING WEIGHT CHANGE AND THE ONTOGENY OF THE REDUCTION IN BARRIER THICKNESS SEPARATING FETAL AND MATERNAL BLOOD SYSTEMS

One of the fundamental processes associated with the ontogenic maturation of the placenta in sheep is the reduction in the dimensions of the barrier separating fetal and maternal blood systems. In the ovine placenta this process involves a reduction in the connective tissue within the fetal villi and in the thickness of the epithelial cells forming the feto-maternal interface as illustrated in Table 7.1. Notably, the values determined in this study for the arithmetic mean barrier thicknesses of trophoctoderm and feto-maternal syncytium at 120 days gestation, were higher than those calculated from Mitchell's (1982) data. The calculation included surface area values which are themselves derived from surface densities. The latter may vary with the magnification at which tissues are examined (Baur, 1979). In this study, the $\times 400$ magnification was used compared to $\times 2200$ in Mitchell's study. Thus the differences in the values may partly reflect the magnification used since unlike estimates of volume densities which achieve stable values at magnifications ranging from $\times 100$ to $\times 10,000$, estimates of surface densities increase steadily and indefinitely as resolution increases with increasing magnification (pages 153-5, Weibel, 1979). Differences in the results may also reflect the small sample of animals ($N=2$) used in Mitchell's study compared to $N=9$ in the present study.

Another factor that contributes towards the reduction of the barrier to diffusion in the ovine placenta is the location of fetal blood vessels adjacent to the fetal trophoctoderm which appears to occur in late gestation (Björkman, 1965; Davies and Wimsatt, 1966; Mitchell, 1982) as in the human (Fox, 1967). Within the feto-maternal syncytium, a further reduction in barrier to diffusion may be achieved by the re-organisation of the contents of the syncytial plaques to achieve thinner areas that are more conducive to diffusion (Mitchell, 1982), that resemble the vasculosyncytial membranes observed in the human placenta (Fox, 1967). This would decrease the harmonic mean thickness and hence increase the overall diffusibility of the membrane (Jackson *et al.*, 1988).

The breakdown of the connective tissue within the fetal villi may in part involve the action of hyaluronidase which metabolises hyaluronic acid (West *et al.*, 1985). The partial breakdown of hyaluronic acid may destabilise the matrix of the connective tissue and induce its breakdown (West *et al.*, 1985). This process together with the angiogenic nature of hyaluronic acid may stimulate the invasion of capillaries, thus enhancing extensive angiogenesis and increased blood flow which is another feature of placental maturation (Rosenfeld *et al.*, 1974). In addition, uterine synthesis of proteins such as hyaluronic acid and hyaluronidase is optimal in a high progesterone environment (Ashworth *et al.*, 1991; Roberts and Bazer, 1982). Thus this process may also partly depend on adequate levels of progesterone.

The breakdown of the connective tissue within the fetal villi may be related to the reduction in the proportion of large placentomes as gestation proceeds. In late gestation there appears to be an increase in the proportion of the "average-size" placentomes (Chapter 3). This result suggested that large placentomes may undergo a reduction in size in late gestation; a process that may underlie the loss of placental weight that is observed in late gestation. In a recent study, IGF-I infusion into the fetus from day 120 elevated fetal plasma levels ~3-fold by 130 days. This treatment was associated with enhanced decrease of the volume density of the connective tissue core of the fetal villi as well as with a reduction in the mean arithmetic barrier thickness of fetal trophoctoderm (Owens *et al.*, 1994). In a similar study, increases in protein/DNA ratios in small placentomes ($\leq 5\text{g}$) and decreased amount of tissue per unit amount of DNA in both the average size (5 to 9.9g) and large placentomes ($\geq 10\text{g}$) were observed at 130 days gestation following chronic administration of IGF-1 (Lok *et al.*, 1993). In addition, reduced DNA synthesis in large placentomes ($\geq 10\text{g}$) was evident (Lok *et al.*, 1993). Thus there may be a possible role for IGF-1 in the process underlying the ontogenic reduction of placentome weight and size.

7.5 ONTOGENY OF SURFACE AREA

At 90 days gestation in sheep, total placental surface area is approximately 6m^2 and at 120 days, it is 11m^2 . This increase in surface area is compatible with a 3.7-fold increase in fetal growth during normal ontogeny in a singleton pregnancy as observed in this study (Chapter 3).

Previous studies in human (Baur, 1977; Aherne and Dunnill, 1966; Jackson *et al.*, 1993) and in rat, cat, cow and pig (Baur, 1977) have demonstrated that the structural principle of all placental types is to increase surface area for exchange although this proceeds at different rates in different species (Baur, 1977) as illustrated in (Table 7.2).

This study has also shown that the normal ontogenic maturation of the ovine placenta involves significant increases in surface density of trophoctoderm and feto-maternal syncytium which is responsible for the doubling of surface area for exchange between 90 and 120 days gestation. However, despite the increase in the absolute placental surface area, the amount of surface area supplying a gram of fetal and placental tissue decreased significantly between the two gestational ages. A similar developmental pattern in surface area has been previously observed in several species including cat, cow, horse, pig, rat and human (Baur, 1977; Rosso, 1974; Teasedale, 1980). Therefore, the reduction in the amount of surface area supplying a unit fetal and placental weight (relative surface area) in late gestation is a fundamental process of placental development. The value of relative surface area obtained for sheep in the present study closely resembles that obtained in the cow, the only other species with a cotyledonary placenta which has been morphometrically analysed. Together these values fall within the range observed for species with compact placentation and are approximately 7 times greater than those of species exhibiting diffuse placentation (Table 7.3) (Baur, 1977).

The placenta enhances its function by increasing transportation of substrates to counteract the reduced relative surface area in late gestation (Flexner and Gellhorn, 1942). This may be achieved by a combination of altering the membrane properties of the cells at the feto-maternal interface and increasing surface area as well as reducing the barrier thickness of the epithelial cell layers and the distance separating maternal and fetal blood systems (Flexner and Gellhorn, 1942). In sheep, placental glucose transfer is reported to increase approximately ten-fold during the second half of pregnancy (Molina *et al.*, 1991). The increase in the glucose transfer capacity may result from increased populations of the transfer proteins on the placental membrane (Morris *et al.*, 1985). The number of transport proteins may increase due to increased placental weight or surface area for exchange (Hay, 1994; in press). In the singleton placenta in sheep, placental weight does not increase during the second half of

gestation but surface area does (Chapter 3). Thus the increase in surface area could substantially account for the increased transfer capacity within the ovine placenta in late gestation.

The placenta also enhances its permeability with increasing gestation. For example, only 20% of the urea permeability that is observed at term in sheep, is available at mid-gestation (Kulhanek *et al.*, 1974). In goat, a species with placentation that closely resembles that of sheep, 25 to 30% of the total placental membrane permeability to sodium that was observed at 0.9 gestation was present at mid-gestation (Flexner and Gellhorn, 1942). Tremendous increases in the transfer rate of sodium per unit placental tissue have also been observed in the human (Flexner *et al.*, 1948) and in other species, such as the sow, guinea pig, rabbit, rat, cat (Flexner and Gellhorn, 1942). Also in rat, there is a marked increase in the rate of transfer of both glucose and α -amino-isobutyric acid per gram of placental tissue near term coincident with a fetal growth spurt (Rosso, 1974).

7.6 MECHANISMS OF PLACENTAL COMPENSATORY GROWTH

Enhancement of the normal ontogenic increase in surface density between 90 and 120 days was also a consistent feature of the compensatory growth process of the placenta following either natural or experimental restriction of implantation. At mid gestation, increases in surface density enabled the placentas of twins and triplets, and also those of the experimentally restricted singletons, to maintain placental and fetal weights at comparable levels to those of control singletons. In ewes carrying twins and triplets in which implantation was further restricted by carunclectomy, the compensatory increases in surface density were insufficient and the surface area achieved was significantly less than that of the control placentas. Similarly, at 120 days gestation, restricted placentas had significantly reduced surface area which was not compatible with normal rate of fetal growth. Thus it is clear from these results that there is a minimum surface area requirement that is compatible with normal fetal growth at various stages of development.

Clearly, experimental restriction of implantation induces a more limited response in placentas of multiple conceptuses at mid gestation and in placentas of restricted singletons in late

gestation in comparison to that which occurs in experimentally restricted placentas of singletons at mid gestation. The increase in surface density is just as great, but insufficient to completely compensate for the large reduction in total placental mass. Since no further increase in surface density could be induced in the normally grown placentas of twins and triplets compared to that which occurred in singletons, and also because further experimental restriction of implantation in the ewes carrying multiple conceptuses could not induce further compensatory growth in their placentas, there is a limit to which cellular dimensions may be modified to enhance placental structural characteristics related to function. *In vivo* measurements of the transfer function in sheep indicate that restriction of placental size is associated with increased efficiency of flow determined transport of antipyrine and facilitated transport of glucose per unit placental weight (Owens et al., 1987b). However, in absolute terms, the clearance of these substances decrease with the reduction in placental weight (Owens et al., 1987b). Thus the increased efficiency of substrate transfer per gram of placental tissue is insufficient to make up for the great reductions in placental mass.

It is clear that compensatory placental growth induced experimentally or naturally is manifested in a similar way. Thus, there is a general tendency towards increased individual placentome size, an enhanced rate of placentome eversion, and internally, a reduced barrier thickness between fetal and maternal blood systems and increased surface density to help maintain surface area. However, at mid gestation, the arithmetic mean barrier thickness of trophoderm was not altered following experimental restriction of implantation in singletons, but it decreased significantly following natural restriction of implantation.

A fundamental difference in the compensatory growth induced experimentally and naturally may concern the extent of compensatory hyperplasia that occurs during the phase of rapid placental growth within the first half of gestation. It has previously been shown that placentas of individual twins have DNA concentrations comparable to those of singleton placentas (Kulhanek *et al.*, 1974), although the twin placentas of the individual twins are smaller than those of singletons (Vatnick *et al.*, 1991).

7.7 RELATIONSHIP BETWEEN PLACENTAL WEIGHT AND HISTOLOGICAL STRUCTURE

At mid gestation, fetal weight did not correlate significantly with placental weight indicating either that the former may not be used as an index of placental function or that placental weight may not limit fetal growth at this stage in gestation. However, placental weight normally correlated significantly with the volumes of several of its morphometrically determined parameters including fetal villi and surface area. Following experimental restriction of implantation, at mid gestation, placental weight correlated with the volumes of trophoctoderm and feto-maternal syncytium in addition to that of fetal villi. Thus fetal villi consistently correlated with placental weight in control and restricted placentas. The lack of more significant correlations between placental weight and other placental parameters may reflect the small number of animals (N=4) used in the restricted treatment compared to N=9 for controls. However, the presence of strong positive correlations between placental weight and some of the key functional components such as surface area in control placentas suggests that at mid gestation placental weight may be a quick way of measuring normal placental function. Placental weight is particularly a good index of the volume of fetal villi. In late gestation, placental weight correlated positively with fetal weight, the relationship reaching statistical significance following restricted implantation. It should be noted that fewer control animals (N=6) were studied compared to N=11 for the restricted. Placental weight also consistently correlated significantly with the volume of fetal villi and that of the connective tissue core of the villi as well as with surface area. Thus placental weight is also a reliable index of fetal villi in late gestation. In addition, the fact that surface area correlated strongly with both placental and fetal weight in control and restricted ewes indicates that measuring placental weight is a quick and reliable way of measuring placental function in late gestation in both normally grown and restricted placentas.

At mid-gestation, a range of placental weights supported normal fetal growth. These include placentas of normally grown and experimentally restricted singletons as well as those of normally grown twins and triplets. However, it is clear that there is a limit beyond which placental weight may be reduced before its functional potential is jeopardised. Evidence of this is the experimentally restricted placentas of twins and triplets at 90 days and those of

restricted singletons at 120 days gestation which were not compatible with normal fetal growth.

7.8 MATERNAL NUTRITION AND PLACENTAL COMPENSATORY GROWTH

The results of Chapter 6 indicate that maternal low body weight at mating and sustained moderate undernutrition throughout the first half of pregnancy limited some macroscopic and microscopic responses to restricted implantation which usually occur by mid gestation in well fed ewes (Chapter 4). The results suggest that adequate nutrition levels are necessary for the increases in placentome size that occur during compensatory growth. Thus placentas of underfed restricted ewes contained a significantly higher proportion of small ($\leq 5g$) placentomes. Adequate levels of maternal nutrition may interact with those of progesterone to achieve required levels of protein synthesis which are necessary for cellular hypertrophy. Similar analyses later in gestation should establish the point in gestation when compensatory growth that is expressed by cellular components of placentomes is reduced by undernutrition. However, maternal undernutrition appeared to enhance placentome eversion. This could have resulted from increases in progesterone levels which have previously been reported to rise in undernourished ewes (Cumming *et al.*, 1971; Oddy and Holst, 1991) and gilts (Ashworth *et al.*, 1991). Internally, undernutrition inhibited the cellular response to increase surface density. This may suggest that the increase in surface area is also dependent on adequate levels of certain nutrients yet to be determined.

Overall, it is interesting that placental compensatory growth involves an accelerated process of the events that occur in normal ontogeny. This may suggest that the stimuli that are responsible for these events are exerted earlier or are amplified following either experimental perturbation or during normal multiple pregnancy.

7.9 LIMITATIONS OF THE WORK UNDERTAKEN

Limitations of the work presented in this thesis were largely related to sampling, at various levels of the sampling hierarchy. Firstly, the number of carunclectomised ewes used in different treatment groups were generally low. Thus the effect of carunclectomy was only analysed as a between factor, by comparing with controls and without categorising fetuses of

restricted ewes based on their size; as "normal" if their sizes are comparable to those of controls or "small" if their sizes are significantly less than those of controls (Robinson *et al.*, 1979; Harding *et al.*, 1985). This could have masked observations specific for different restricted fetal sizes. Only four ewes each were used in the studies of the effects of restricted implantation on placental development in mid-pregnancy (Chapter 4) and the effects of maternal undernutrition on the placental response to restricted implantation (Chapter 6). Furthermore, in the experiments involving twins, only two carunclectomised ewes were available to study the effects of further restriction of implantation in multiple pregnancy. Thus in this particular aspect of studies in twins, the results may only be regarded as preliminary.

In general, insufficient numbers of small placentomes ($\leq 5\text{g}$) were analysed. This is because the study was basically designed to analyse the structure of "average-size" and "large" placentomes which comprise at least 95% of all placentomes during the normal ontogeny of placental growth, both in mid and late gestation (Robinson *et al.*, 1979; and the present work). Following morphometric analysis of control placentas at 120 days gestation which suggested some structural differences between the "average-size" and "large" placentomes, it was considered necessary to investigate the structure of any available smaller than average placentomes. In the limited analyses which included the "small" placentomes, differences in volume densities of trophoblast and connective tissue core of the fetal villi and also barrier thicknesses of the epithelial tissues at the feto-maternal interface were evident, especially when these were compared with similar components in "large" placentomes. Thus the association between "small" placentome size and internal structure needs to be further characterised.

When placentomes were sampled at post mortem, their position in the uterus was not recorded (Chapter 3). Therefore, it has not been possible to relate directly the placentome morphometric characteristics with the position of the placentomes in the uterus, which in turn may be related to uterine blood flow patterns. Such information would enhance the understanding of the mechanisms involved in the growth of placentome size and the changes

of placentome morphology, which may largely be determined by the exposure of the placentomes to certain levels of substrates and hormones at particular stages in gestation.

One major drawback of this work may concern the quality of tissue fixation. The use of aldehyde fixatives and resin embedding should be considered in future to achieve better quality of tissue fixation and enable the cutting of thinner sections for light microscopy studies. This would have allowed alternative analyses, more especially those of barrier thickness. It should also enable the analysis of the vascularity of the tissues. In addition, electron microscopy morphometry of the surface area needs to be carried out since this will help analyse the contribution of the microvilli at the feto-maternal interface towards the increase in surface area. It should be borne in mind that the increase in surface area reported in this thesis excludes the possible contribution towards the absolute values due to likely variations in microvilli density or size.

Other limitations of this work are concerned with the fact that three other important parameters of the tissue were not analysed. These include (a) the vascularity of the fetal and maternal components (b) the abundance of binucleate cells and/or some measure of their function and (c) the harmonic mean barrier thicknesses of the trophoctoderm and feto-maternal syncytium, which measure the variability of the membrane thicknesses that is more directly related to the membrane diffusibility to substrates than arithmetic mean barrier thickness (Jackson *et al.*, 1988). Thus it has not been possible to relate the results of this work to the more direct measures of placental function.

7.10 CONCLUSIONS

The following conclusions were drawn from the work presented in this thesis:

1. The major stimulus for placentome eversion occurs in late gestation between 120 and 130 days of gestation and is associated with increased trophoctoderm.

2. Ontogenic structural maturation of the sheep placenta between 90 and 120 days involves increasing trophoderm volume, reducing connective tissue volume within fetal villi, reducing barrier thicknesses and doubling of the surface area of the apposed exchange epithelia.
3. Natural and experimental restriction of implantation induce similar compensatory changes in the placenta at mid-gestation. Macroscopically, the compensatory changes include increased placentome size and enhanced eversion. Microscopically, the volume and surface densities of the epithelial cell layers, trophoderm and feto-maternal syncytium, increase. In addition, the volume density of the connective core of fetal villi and the arithmetic mean barrier thickness of the epithelial cell layers decrease.
4. Experimental restriction of implantation induces changes which are sufficient to maintain placental volumes of key components, surface area, and hence fetal growth in mid, but not in late gestation.
5. Low maternal body reserves at mating and maternal undernutrition throughout the first half of gestation limit compensatory increases in placentome size and inhibit compensatory increases in surface density of the surface epithelia but do not alter other compensatory changes seen at mid gestation following restricted implantation.

7.11 CONCLUDING REMARK AND FUTURE DIRECTIONS

To the author's knowledge, this work is the first morphometric study relating placentome size and morphology in control ewes and also in ewes in which restricted implantation has been experimentally and/or naturally induced. Thus, many other parameters of the ovine placental structure still need to be characterised for a better understanding of the processes involved in the control of normal and compensatory placental growth in this species. Such parameters include those that may relate directly to placental function, such as the vascularisation of the maternal crypts and fetal villi and the harmonic mean thicknesses of the epithelial cell layers. In addition, factors that determine placentome size and morphology need to be determined. This may be carried out by the analysis of the uterine position of the placentomes in relation to blood flow patterns and concentrations of hormonal substances and metabolites within the

fetal and maternal blood. Maternal nutrition may also be varied accordingly to determine more precisely its effect on cellular proliferation and hypertrophy which should in turn determine the growth of placental size. Thus it is hoped that this work will provide a basis for further investigation of the mechanisms involved in the control of placental development normally and when placental size is restricted. The presence of similar characteristics of compensatory placental growth following experimental and natural restriction of implantation will improve our understanding of the processes involved in normal placental growth and development. Also, the presence of similar fundamental placental growth characteristics across a wide range of species with similar or different placental types may suggest that the knowledge acquired from morphometric studies in experimental animals may be applicable in both animal production and medical practice.

TABLES FOR CHAPTER 7

Table 7.1 Ontogeny of the reduction of the arithmetic mean barrier thickness of the epithelial cell layers in compact cotyledonary placentas of sheep.

Stage of gestation (fraction)	0.6	0.8	0.95	Term

Sheep (Border Leicester ×
Merino) (this study)

Component
(× 400)

trophectoderm	16.37 ± 0.6 (9)	14.15 ± 0.69 (6)	nd	nd
feto-maternal syncytium	8.4 ± 0.45 (9)	5.7 ± 0.5 (6)	nd	nd

Sheep (Corridale ewes)
(Mitchell 1982)*

(x 2200)

*trophectoderm	nd	5.9 (2)	5 (3)	nd
*feto-maternal syncytium	nd	3.7 (2)	3.45 (3)	nd

values for components marked () were calculated from surface area related to volume measurements in Table 6.V., page 250, Mitchell, 1982).*

Table 7.2 The ontogeny of surface area (sq. m) in several species of compact and diffuse placentas

Gestational stage (fraction)	0.6	0.8	Term
Compact placentas			
Sheep (Border-Leicester × Merino cross bred) (this study)	5.93 ± 0.44	10.07 ± 0.81	nd
European domestic cow	25	64	130
human	4		12
Diffuse placentas			
pig	0.25	0.4	0.6
horse	3.5	7	12

(adapted from Baur, 1977)

Values for cotyledonary placentas are presented in "**bold**"

nd not determined

Table 7.3 Relative surface areas in species with compact and diffuse placentas

Gestational stage (fraction)	0.6	0.8	Term
Compact placentas			
Sheep (Border-Leicester × Merino cross bred)	48	31	nd
European domestic cow	35	32	27.1
Rat			59.0
Guinea pig			33.3
Cat			65.6
German sheep dog			29.0
Sloth			44.3
Crab-eating monkey			65.5
Douc langur			53.8
Guereza			57.7
Leopard			93.5
Chimpanzee			30.2
Gorilla			42.0
Man			32.0
Seal			32.2
Saline			61.5
Giraffe			11.4(?)
Dwarf zebu			23.0
African elephant			26.6
Diffuse placentas			
European domestic pig			5.8
Dwarf hippopotamus			2.4
Dolphin			5.3
Llama			4.5
Pony			4.0
Sardinian dwarf donkey			7.6
Zebra			2.0
Somalian wild ass			3.9
Bactrian camel			2.5
Indian rhinoceros			2.5
Horse			4.4
Hippopotamus			6.7

Relative surface area is expressed as cm^2/cm^3 = surface area of the placenta divided by total volume (weight) of fetus plus placenta.

Values for cotyledonary placentas are presented in "**bold**"

nd = not determined

(adapted and modified from Baur, 1981).

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