THE DEVELOPMENT OF THE BOVINE PLACENTOME AND ASSOCIATED STRUCTURES DURING GESTATION

A thesis submitted in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

in

Veterinary Science

Massey University, Palmerston North,
New Zealand.

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2012

Dedication

This work is dedicated to my ***Lord Jesus Christ****,*

"Who is the beginning and the end"

In loving memories

My late dad, 'Samuel Oyin Oyedepo'

My late Kiwi dad 'William Bruce Teulon'

Thank you for the inspirations you have given to me.

Abstract

Placental development is a key influencer of fetal growth and development. However, limited information exists on many basic aspects of bovine placental development and the factors which affect it. To resolve this dearth of knowledge, studies of the bovine placenta were conducted to elucidate the relationship between its development and that of the fetus throughout gestation.

The first study investigated the effects by maternal nutrient restriction of two different groups of well fed heifers just prior to mating in two consecutive years to give an overall moderate weight gain during the first trimester averaging approximately 500g/day for controls compared to around 50g/day in the restricted groups Data obtainedby Day 90 of gestation, were subjected to principal component and factor analyses to evaluate the effect of nutrient restriction on placental growth. Placental growth was not directly affected by maternal nutrient restriction over the first 90 days of gestation, suggesting that significant underfeeding with weight loss is needed before placental development is affected. The study also showed that caruncle number is a major determinant of placental mass and, probably consequently, fetal mass.

The second study investigated changes in the relative contributions of fetal and maternal tissue to placentomes throughout pregnancy, using abattoir-collected material from Days 100 to 225 of gestation. Weights were measured and volumes were estimated using an air and water displacement method. Placentome number increased between Days 100 and 170 of gestation, but decreased thereafter. Mean placentome density did not show any biologically-significant variation over pregnancy. However, the contribution of maternal tissue (as determined through both weight and volume estimations) increased more rapidly with advancing gestation than did that of fetal tissue; with the consequence that maternal tissue weights were significantly greater than fetal weights by 200 days of pregnancy. The third study built upon these results by measuring placentome size *in-vivo* by trans-rectal ultrasound of dairy cows between Days 60 and 180 of pregnancy. To try to maximise the repeatability of measurements of placentome size, only those closest to the cervix were selected. The results of this study showed that there was a significant increase in placentome size between Days 60 and 180 of gestation, but that, a limits-of-agreement

analysis showed that placentome size was insufficiently closely associated with gestation age to be used as an accurate predictor of gestation age.

The final two studies examined the central zone of the feto-maternal interface of the placentome in greater detail; again using abattoir-collected material over similar period as in Study 2. In the first of these, (Study 4) stereology was used to estimate the relative volume densities, surface densities and total surface areas of the fetal and maternal components of bovine placentomes. The final study used lectin (*Dolichos buflorus* (DBA), *Glycine max* (SBA) and *Phaseolus vulgaris* leukoagglutinin (PHA-L)) histochemistry to attempt to characterize and quantify the distribution and origins of glycoproteins within the feto-maternal interface, with particular reference to the role of the binucleate cell. This was the first time that the lectin-binding properties of the bovine placetome had been objectively quantified throughout the second and third trimesters of pregnancy. Whilst the relative volume densities and surface densities of the tissues of the feto-maternal interface of the placentomes (i.e. binucleate cells, fetal trophoblast, fetal connective tissue, maternal connective tissue and maternal epithelium) did not change with gestational age, the total surface area of the feto-maternal interface increased throughout pregnancy. The presence of glycoprotein, as inferred from the patterns of lectin staining, was confined to fetal trophoblast and maternal epithelium and, in the case of DBA and, particularly, SBA, was especially prominent in binucleate cells. The latter staining probably reflects changes in the patterns of production of a key fetal regulator of maternal metabolism, placental lactogen, a glycoprotein whose origin from binucleate cells has previously been established.

Whilst it has long been established that placental size increases as pregnancy advances, this research has shown that the relationship between advancing gestational stage and placental mass is not a simple linear relationship, and even the combination of placentome size and number is not simply related to fetal size. This is, as the results of this thesis have shown, because of differential growth of the fetal and maternal components of the placentome, accompanied by progressive development of the critical interface between the mother and fetus at the central zone of apposition. It is believed that these studies have shown that in future a combination of ultrasonography, stereology and lectin histochemistry techniques could be used to quantify structural and cellular changes in the bovine placenta during gestation. This will be of value to underpin future investigations of situations in which

placental activity may be impeded to the detriment, not only of fetal growth, but also of the metabolic environment of the ensuing adult animal.

Acknowledgements

I give thanks to the Almighty God for giving me the strength, ability and wisdom to complete this work, without Him, I am nothing.

I would like to thank my chief supervisor, Dr Richard Laven, for his assistance and support from the beginning of this study right through to the end. Thank you for your brilliant ideas and for bringing out the best in me, especially my statistical skills. I am privileged to be co-supervised by Prof Tim Parkinson, I have learnt so much from you. I am very grateful for all your time, despite your busy schedule, your advice and learning from your wide scope of knowledge. Thanks to Dr Kevin Lawrence for your statistical advice and help during this study. I am honoured to have such a great team of supervisors.

I am thankful to Dr Matthew Perrot, Mike Hogan, Evelyn and Eugene for their help with histology. You are such a great team to have in the Institute. My sincere thanks to the Institute of Veterinary, Animal and Biomedical Science for regular postgraduate funding and providing me the opportunity to attend a conference in Australia. I would also like to thank the postgraduate office headed by Prof Kevin Stafford and the administrative section. Thanks to Debbie Hill, for all your assistance and willingness to help. You are one of the best administrators I have met. I can't forget Kristen Story, who is so supportive and full of smiles.

Very big thanks to Massey University for the Hardship Bursary and the Doctoral Alumni Bursary, which was of great help in paying my fees. I am also grateful to the prestigious Colin Holmes Inaugural Dairy Scholarship for the financial support and for allowing us to conduct part of the research at the Westpac research station supported by DairyNZ.

I would like to thank all my friends and office mates from IVABS, Chemistry, IFNHH, Aghort, Economics and Finance; Sharini, Lydia, Amy, Ali, Kruno, Gabriella, Maria, Sharifah, Sara, Asmad, Linda Laven, Lisanne, Santosh, Soffalina, Farihan, Mazidah, Noveline, Uttara, Beatrice, Jenny, Steve, Penny, Geraldine, Graham, Kate, Hope, Sue, Ganga, Edith, Yeshpwal, Deepa and many others. Thank you for always being there for me and for your help in putting the thesis together. Special thanks to my line manager, Mr

Andy Trow, for being so understanding and putting up with me for the last three years in Chemistry department.

My sincere thanks to my church members and friends from Emmanual Congregational church who stood by me, prayed and helped tremendously for the last few years. I will always remember my late Kiwi dad, who would have loved to see me finish this work. Thanks to my Kiwi mums and their families, Rosemary, Reine, and Nola. I would also like to thank my son's soccer team, Ruahine football club and especially Grant and Debra for taking care of KK while I was busy or out of town. Thanks to Bill Rowe for dropping and picking my kids up from school.

I am grateful to my family back in Nigeria, my mum, sisters, brother, uncle, cousins, nephews nieces and and my wonderful brother in-laws (Dapo Adeleke, Raphel Folorunsho and David Paul) for their constant calls, concern and prayers, I appreciate you all. Thanks to my friends, Dupe Orunmuyi, Taiye Olugbemi, Chris Daudu, Lekan Banwo, Joshua Ogunwole, Garce Jokthan and many others. My thanks go to Ahmadu Bello University for the study leave and fellowship granted to me. I also remember my 'Niaja kiwi' friends and family, the Adesanya's, Adelowo's, Folorunsho's, Ejiwale's, Awoshola's, Aruwa's, Beckleys and many others. My special thanks to my children, Samuel, Grace, Debra and KK for bearing with me through these years and taking care of themselves. You are the best kids anyone could ever ask for, thank God for you all. Lastly, but more important, I extend my thanks to my husband, Isaac for the support, statistical help and encouragement during the entire period of my study, I appreciate you.

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Chapter One
Introduction

In viviparous animals there needs to be a connection between the fetus and its dam that allows the physiological exchange of nutrients, gases, hormones and waste products. (Mossman 1987). This organ, the placenta, is most highly developed in eutherian (placental) mammals; there is a huge diversity of placental types and structures across this clade even though all eutherian placentae arise from the choriallantoic membrane, and develop from fusion or apposition of the chorion to the uterine epithelium with vascularisation spreading from the allantois to the chorion (Barclay *et al.* 1944).

Placental classification has classically been based on shape and structure. The bovine placenta is classified as cotyledonary (Grosser 1909) as it has multiple small areas of attachment which arise from pre-existing areas of non-glandular uterine epithelium (caruncles) overlain by fetal tissue (cotyledons). Villi form on the fetal tissue and become embedded into the crypts in the caruncle (Amoroso, 1952; Wooding and Burton, 2008). The structure of the bovine placentome was originally defined as syndesmochorial (Grosser 1909) as it was thought that fetal tissue was apposed to maternal endometrial connective tissue. However, Wooding (1992) showed that the chorion is directly apposed to the maternal epithelium (there being no loss or invasion of maternal tissue) although migration of specific binucleate cells from the fetal to the maternal epithelium does occur. So the bovine placenta is currently classified as synepitheliochorial (Grosser 1909; Wooding 1992).

Placental development is linked to and pivotal to fetal development. This means that the gross morphometry of the bovine placenta changes markedly as gestation progresses and if this placental development is markedly affected, then this can hinder fetal development. Studies in sheep have shown that stressors such as maternal nutrient restriction, carunculectomy, and high temperature can change placental size and shape and also affect fetal growth; the results of these studies suggest changes in placental size and shape, at least in sheep, can occur in an attempt to increase capacity of the placenta to transfer nutrients to the fetus (Fowden *et al.* 2006; Ward *et al.* 2006; Vonnahme *et al.* 2007). It is unclear whether similar changes in placental size and shape occur in response

to stressors in cattle because very few studies have been undertaken in that species. Such studies have been limited to the evaluation of nutritional stress and have only shown limited (Long *et al.* 2010) or, even, no effect (Hickson *et al.* 2009). These results suggest that the bovine placentome is refractory to nutritional stress, perhaps because the inherent capacity of the bovine placentome is such that even under significant stress sufficient capacity remains to transfer an adequate level of nutrients to the fetus. This is consistent with the finding in sheep that as part of the response to stressors, placentome shape changes to be more like that in cattle (Penninga and Longo 1998). Nevertheless, the limited number of studies means that such conclusions can only be tentative and further research is required to better establish the impact of stressors such as under-nutrition on placentome development in the cow.

One major disadvantage of evaluating placentome development in the cow compared to the sheep is the greater individual animal value which, particularly in dairy cattle, limits the feasibility of post-mortem measurement of placental development. Alternatives to post-mortem measurement of placentome size are thus required to properly elucidate the relationships of structure and function. Ultrasound measurement of placental morphometry has proved useful in sheep (Doize *et al*. 1997), but there is only limited evidence of the valued technique in cattle, despite its common use for pregnancy diagnosis in veterinary practice. Further research is required to assess whether ultrasound-based placentome measurement can be useful in both practice and research.

We also need to make better use of post-mortem material rather than simply using weight-based measurements. Migration of fetal binucleate cell across the maternal / fetal interface and their fusion with the maternal epithelium to form a transient trinucleate hybrid cell within the placentome is a key characteristic of the ruminant placenta (Amoroso 1952; Wooding 1982a). These binucleate cells are active factories which produce a wide range of different hormones and growth factors associated with fetal and placental growth such as bovine placental lactogen (bPL) (Duello *et al.* 1986) and pregnancy associated glycoproteins (Munson *et al.* 1989; Jones *et al.* 1994; Roberts *et al.* 1995). It is likely that the production of these and other glycoproteins varies markedly across gestation. However, although some studies have looked at lectin-binding of

glycoproteins in early and late pregnancy (Lehmann *et al.* 1992; Nakano *et al.* 2002; Klisch *et al.* 2008), no studies have yet been undertaken to quantify glycoprotein production across gestation in cattle. Quantification of glycoprotein production could provide valuable information on placental development and function but baseline data is required before such studies can be undertaken.

Another area which needs further investigation is the relationship between fetal and maternal placental development. The ratio of fetal to maternal tissue within the placentome can have a significant impact on placentome capacity and efficiency; alteration in the ratio may be one way in which the bovine placentome can adapt to stressors. However, published data on the normal ratio of fetal to maternal tissue is based principally of comparisons by weight (Reynolds *et al.* 1990; Laven and Peters 2001); although volume may reflect placental capacity better. An alternative to gross measurement of placenta mass is the application of stereology, which can be used to estimate the relative volume of all placentome components, even down to individual cell type, such as the fetal binucleate cell. This method could prove useful in such studies. However, as yet there has been only limited application of stereology to the bovine placentome. Both Farin *et al*. (2001) and Kannekens *et al*. (2006) used stereology but only at specific stages of gestation. Application of the technique across the whole of gestation could provide valuable baseline information for intervention studies.

So, despite the importance of cattle to the agricultural economy, there has been only limited study of placental development in cattle and there is a need to better characterise the normal situation in order to then identify how stressors such as nutrition impact on placental function. The present studies were undertaken to better characterise: (1) The association between placental parameters and nutritional stress; (2) how placental morphometry changes during gestation, particularly the relationship between fetal and maternal tissues and between weight and volume; (3) the value of *in vivo* measurement of placentomes by trans-rectal ultrasound as a research tool and as a tool for veterinary practitioners to estimate gestational age; and (4) the effect of stage of gestation on the glycoprotein make-up of the bovine placentome.

The objectives of this thesis are to:

1. Assess the relationships between placental measures in the first trimester of pregnancy and the impact of nutrition on these relationships.
2. Create a model to predict gestational age from measurements of placentome size using trans-rectal ultrasound and to assess the agreement between the model and actual gestational age.
3. Examine the effect of gestational age on the fetal and maternal components of the placentome, particularly their volume and density.
4. Use stereology to assess the effect of gestational age on the feto-maternal exchange area and on the relative volumes of fetal and maternal tissues, and to compare the latter results to those obtained by gross morphometry.
5. Characterise and quantify the changes in glycoproteins produced during gestation using lectin histochemistry combined with computer analysis of binding intensity.

Chapter Two

Literature review

Placental development is a key requirement for fetal growth during gestation such that impairment of placental growth could result in retarded fetal development as well as retention of the fetal membrane during parturition (Kelly 1992; Boos *et al.* 2003). The aim of this review is to review the development of the bovine placenta during pregnancy based on the changes in anatomy of both the gross and histological structures. This review will concentrate principally on the bovine placenta, with data from other ruminants such as sheep and goat included where there are no, or very limited, bovine data. The first section will focus on the gross morphology and morphometry of the bovine placenta with emphasis on the formation of the placentome. The next section will highlight the possible factors which could affect placental morphometry and review how such factors could affect placental growth during pregnancy. Histological changes within the feto-maternal unit of the normal and cloned placentomes using techniques which have been widely used in placental physiology to quantify binucleate cell populations and the production of glycoproteins in cattle (Kannekens *et al.* 2006) , yak (Liu *et al.* 2010) and human (Mayhew 2006a) during pregnancy will then be reviewed.

2.1. Formation of the bovine placenta

Placental formation starts after Day 18 to 19 of pregnancy in cattle, when the trophectoderm extends and the cells of the trophectoderm (trophoblast) establish themselves within the uterus (Mossman 1987). These cells play a key role in the growth of the placenta forming an intimate relationship with embryonic somatic cells or mesoderm to form the chorion, which thereafter becomes highly vascularized by fusing with the allantois to form the chorioallantois (Mossman 1987; Schlafer *et al.* 2000; Wooding and Burton 2008).

2.1.1. Gross morphology of the bovine placenta

The placenta is the specialised organ that develops during pregnancy in eutherian mammals to serve as the site of exchange of nutrients, waste products, hormones and enzymes between the mother and the fetus. The mammalian placentas have been classified based on morphological features such as placental arrangement (diffuse, cotyledonary, zonary and discoidal), feto-maternal interdigitation (villous, folded, lamellar, labyrinthine), layering of the interhaemal membranes (epitheliochorial, synepitheliolchorial, endotheliochorial, haemochorial) and placental separation at birth (deciduate, non- deciduate) (Amoroso 1952; Noakes *et al.* 2001; Schmidt 2005)

The bovine placenta is villous (Amoroso 1952; Mossman 1987) and cotyledonary, in which discrete fetal structures (cotyledons) interdigitate with discrete maternal structures (caruncles) to form the functional unit of the placenta (placentome) (Schlafer *et al.* 2000). The bovine placenta is synepitheliochorial, as the fetal chorion is apposed to maternal epithelium. Syncytium formation is only temporary in cattle, occurring early in gestation as a result of the migration and fusion of specialised trophoblast binucleate cells with the uterine epithelium (Wooding 1992). It is non-deciduate, as the maternal portion of the placenta is retained after parturition (Nickel and Seiferle 2004).

2.1.2. Placentome structure

The placentome is the primary area of the placenta through which the fetus absorbs oxygen and other nutrients and excretes carbon dioxide and other wastes. The placentome is made up of a fetal unit, the cotyledon, and a maternal section, the caruncle. The cotyledon is produced from the chorioallantoic membrane which develops villi that then extend into crypts in the maternal epithelium, resulting in a strong interlocking of the maternal and fetal tissues (Klisch *et al.* 2010) . This development occurs only where the chorioallantois is apposed to receptive areas of the endometrium (i.e. caruncles) (Atkinson *et al.* 1984). Caruncles are oval or round thickenings in the uterine mucosa which result from proliferation of sub-epithelial connective tissue. These caruncles are formed early in the fetal uterus and persist throughout the life of the cow (Schlafer *et al.*

2000; Schmidt 2005). They are present prior to pregnancy, but develop significantly in response to cotyledon development (Atkinson *et al*. 1984). The placentomes are formed at about 30 days of gestation as a result of the attachment of the chorioallantois to the uterine epithelium (Schlafer *et al.* 2000). At the site of the caruncle, localised tufts of chorionic villi develop at implantation and begin to interdigitate with the caruncular crypts, resulting in a placentome with a domed and ovoid mushroom-like structure (Laven and Peters 2001), about 10 to 12 cm long and about 2-3cm thick (Figure2.1).

During gestation, the structures of the villous trees of the placentomes are transformed to facilitate transplacental exchange of nutrients, oxygen and waste products through both passive and active exchange mechanisms (Leiser *et al.* 1997; Benirschke and Kaufmann 2000) . The placentome is thus the interface between the maternal endometrium and the fetal trophoblast. Normal placentome growth and development is essential for the establishment and maintenance of pregnancy and for normal fetal growth and development (Laven and Peters, 2001), hence, the placentome is a crucial component of the bovine placenta. The placentome is also a major site for placental steroidogenesis; its synthesis of progesterone not only supports the corpus luteum of pregnancy but also stimulates placentome growth itself (Hoffmann and Schuler 2002).

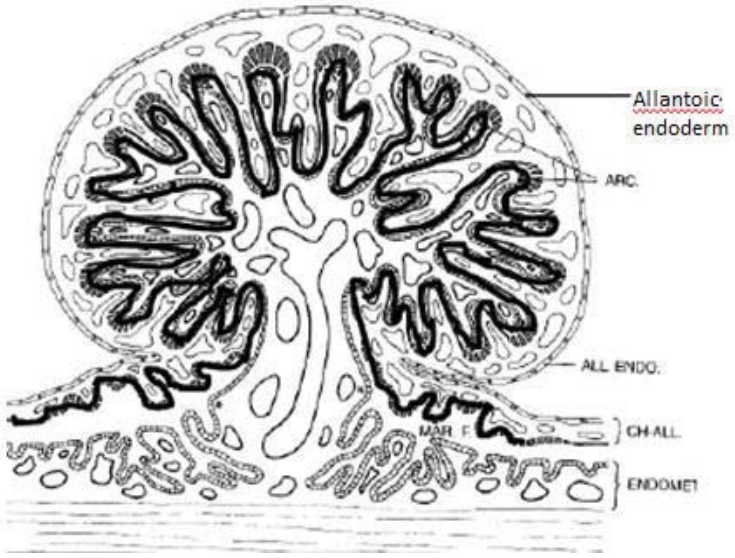


Figure 2.1: Diagrammatic representation of a normal bovine placentome (Adapted from Mossman, 1987) (ARC: arcade chorioallantois, ENDOMET: endometrium, CH ALL: chorioallantois

2.1.2.1. Placentome shape

Three shapes of placentomes have been reported in bovid ruminants (Figure 2.2): flat, concave and convex. The convex placentome is a mushroom-shaped structure with a distinctive endometrial stalk; the flat placentome (Laven and Peters, 2001) has a flatter, less convex shape and is non-pedunculated. For both of these placentomes fetal chorion covers the surface of the placentome. In contrast, in concave placentomes, maternal tissue completely surrounds the fetal tissue, and the shape is inverted (Figure 2.2).

The typical shape of the placentome depends on species. In sheep and goats, concave placentomes predominate (Penninga and Longo, 1998; Vatnick *et al*., 1991); in yak (*Bos mutus*) and African buffalo (*Syncerus caffer*), flat placentomes are the most common with a few convex placentomes (Schmidt *et al.* 2006; Liu *et al.* 2010); whilst in cattle (*Bos taurus*) convex placentomes predominate alongside a small number of flat ones (Laven and Peters, 2001).

Change in placentome shape in sheep occurs as a response to stressors such as under nutrition, cortisol administration, carunculectomy and high temperature (Vatnick *et al.*

1991; Steyn *et al.* 2001) but this has not, as yet, been reported for other ruminant species. Both Liu *et al*. (2010) and Laven and Peters (2001) studied change in placentome shape during gestation (in yak and cattle, respectively) and found no significant temporal changes, which might suggest that changes in placentome shape play little role in normal placental function in these species, although further research is required to prove this conclusively.

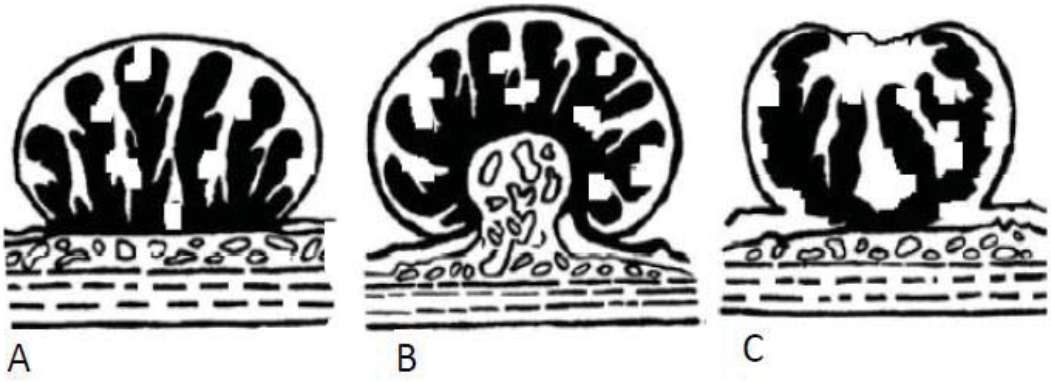


Figure 2.2: Schematic illustration of placentome shapes seen in bovid ruminants showing the maternal caruncular tissue as black and the fetal cotyledonary tissue as white. (A) Flat placentome with caruncular tissue attached to flat endometrium. (B) Normal convex bovine placentome with caruncular tissue attached to endometrial stalk. (C) Concave placentome- typical of ovine ruminants (Adapted and modified from Liu *et al.* (2010)

2.1.2.2. Placentome number

In cattle, the placentomes tend to be arranged in both pregnant and non-pregnant horns in rows of four lengthwise (Schlafer *et al*. 2000), although this pattern is often disrupted by the development of accessory and satellite placentation (Laven and Peters, 2001). The number of placentomes varies widely between individual cows. Mossman (1987) reported that the number of placentomes varied from around 50 to 175 per uterus, while Laven and Peters (2001) reported figures ranging from 40 to 120. The latter authors reported that the mean number of placentomes ranged from just over 50 per uterus in pregnancies of < 71 days to more than 70 in pregnancies of > 191 days of gestation. This apparent trend towards an increase in placentome number was almost significant at the

5% level (P=0.07). Data from other ruminants are inconclusive as to whether this is a real effect or not; Abdel Raouf and Badawi (1966), reported that in water buffalo (*Bubalus bubalis*), mean placentome number increased from early to late gestation but, in sheep and yak, there appears to be no significant effect of stage of gestation on placentome number (Redmer *et al*. 2009; Liu *et al*. 2010). Laven and Peters (2001) reported that placentome numbers were markedly higher in the pregnant horn than the non-pregnant one, with, on average, 28 more placentomes in the pregnant horn. There was no correlation between numbers of placentomes in the two horns, i.e. uteri with no or few placentomes in the non-pregnant horn did not have significantly more placentomes in the pregnant horn.

Abnormal placental development has been associated with changes in placentome number. Lee *et al*. (2004) reported that, in pregnancies resulting from the transfer of cattle embryos cloned by somatic cell nuclear transfer, the mean number of placentomes was significantly lower on Day 100 when compared with half siblings produced by artificial insemination (AI). This difference had disappeared by Day 150, probably because only those pregnancies with cloned embryos in which placentome numbers had been normal were represented in surviving pregnancies at Day 150. In addition, Miglino *et al*. (2007) suggested that the large numbers of small placentomes with diameter of <1 cm which were found in association with pregnancies resulting from somatic nuclear transfer could be a form of adaptation to allow such pregnancies to survive and continue to term. Furthermore, fetal membranes obtained from second trimester abortions of such pregnancies were associated with smaller number of placentomes and this may be one cause for pregnancy loss (Lee *et al*. 2004; Miglino *et al*. 2007). Nevertheless, even pregnancies with very low numbers of functional placentomes can survive to term. About 12 functional cotyledons ranging in size from 8 – 20 cm have been reported to provide sufficient capacity to support development to term and the birth of a live viable calf (Hill *et al.* 2001).

2.1.2.3. Placentome weight and size

Although placental development starts around Day 18 to 19 of pregnancy in cattle (Mossman 1987), placentomes do not become visible as discrete, oval, light coloured structures until around Day 30 of gestation. By Day 33, the placentomes can be seen as slight to discrete elevations on the uterine surface (King *et al.* 1979). Placentome length increases during gestation, with placentomes in the pregnant horn being significantly larger than those in the non-pregnant horn (Laven and Peters 2001). There is a significant linear association throughout most of pregnancy between gestation length and placentome length (Laven and Peters 2001). Also, larger individual placentomes have been found in twin pregnancies resulting in larger total mass than in singleton pregnancies (Wooding and Burton 2008).

However, placentas from pregnancies derived from somatic nuclear cloning were larger and were associated with higher maternal pregnancy serum protein (PSP60) concentrations between 4 to 6 months of gestation compared to control (artificial insemination) pregnancies in cattle. Specifically, nuclear cloning was associated with high concentrations of PSP60 between Days 150 and Day 180 of gestation and the presence of large cotyledons resulting from hydrallantois (Heyman *et al.* 2002). Placentome weight has been used to characterise placental size during pregnancy and in cattle, an exponential increase in weight during early to late gestation has been demonstrated (Reynolds *et al.* 1990; Laven and Peters 2001), although the total rate of increase is much less than that of the fetal weight (Ferrell *et al.* 1976; Ferrell and Ford 1980; Reynolds *et al.* 1990). The increase in fetal weight relative to placental weight is probably due to increases in placentome vascularity, thereby increasing feto-maternal exchange without affecting placental weight (Leiser *et al.* 1997). In contrast in the sheep placental weight does not substantially change after Day 90 of gestation (Barcroft and Barron 1946; Wallace 1948; Alexander 1964), despite the positive correlation between placental and fetal weights (Alexander 1964).

Feto-placental growth and placental vascular development have been investigated in sheep by varying maternal nutrient restriction during critical stages of placental development (Redmer *et al.* 2009). Those authors observed that the ratio of fetus to

placentome was the same for control and over-nourished sheep on Day 50, but differed on Days 90 and 130 of gestation, implying that maternal over-feeding does not influence placental mass during the first trimester of gestation (Redmer *et al.* 2009). However, whilst placentome weight can be a useful indicator for evaluating the pattern of placental growth during pregnancy, it is only a gross measure of the placenta and does not provide information about the relative weights of placentomal components (Reynolds and Redmer 1995). For instance, Reynolds *et al*.(1990) reported a 2-fold increase in maternal caruncle weight compared to fetal cotyledon weight between Days 100 and 250 of gestation. However, the DNA content of fetal cotyledon increased throughout gestation, whilst the DNA content within the caruncle remained constant, indicating a differential growth in cellular density within the placentome. The authors interpreted this to mean that the fetal cotyledon exhibited hyperplasia concurrently with increasing fetal growth towards the last trimester of pregnancy (Reynolds *et al.* 1990; Reynolds and Redmer 1995).

It is likely that the capacity of the bovine placenta exceeds that which is needed for fetal growth during the first trimester of pregnancy (Reynolds and Redmer 1995), indicating that placental weight may not accurately reflect placental function. Fetal growth and development could be affected by factors such as genes of parents, epigenetic state, maternal maturity and environmental factors including nutrition, stress and diseases and others (Redmer *et al.* 2004). These factors in turn influence placental growth and its functional capacity to transfer nutrients from the mother to the fetus in the ovine and bovine species (Bell and Ehrhardt 2002; Redmer *et al.* 2005). For example, in the normal placenta, transport of glucose to the fetus depends on the maternal to fetal trans-placental glucose concentration gradient (Bell *et al.* 1999) which is the active force responsible for glucose uptake and transfer to the fetus (Simmons *et al.* 1979).

2.1.2.4. Comparison between fetal, placentome, caruncle and cotyledon growth

Reynolds *et al.* (1990) reported that from mid- to late gestation placentomal tissues grow at a slower absolute rate than does fetal weight; while over the same time period the growth rate of the caruncle is greater than that of the cotyledon (Reynolds and Redmer 1995; Figure 2.3). Laven and Peters (2001) also reported that, from early to late gestation, the proportion of maternal tissue (by weight) increased relative to that of fetal tissue and that this effect was not dependent on placentome size.

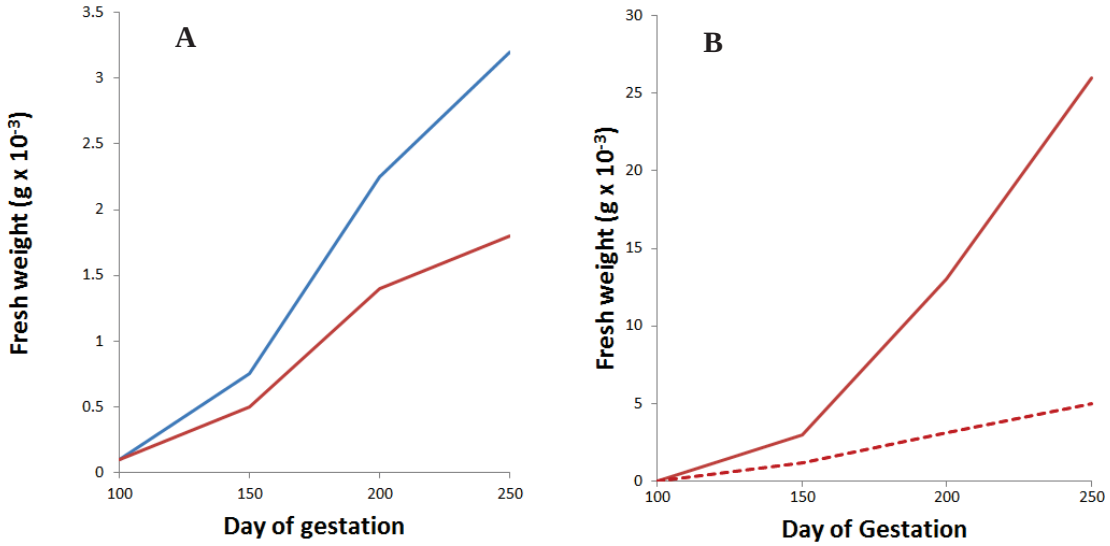


Figure 2.3: Change in (A) caruncular and cotyledonary weights and (B) fetal and placentomal weights by day of gestation in cows. In A, the blue line represents total caruncular weight and the red line total cotyledonary weight. In B, the solid line represents the fetal weight and the broken line total placentome weight. (Adapted from Reynolds et al., 1990)

2.2. Morphometry of the bovine placenta

2.2.1. Estimation of placentome growth *in vivo*

2.2.1.1. Ultrasonic measurements

Ultrasonography works on the basis of the pulse-echo principle, which involves sending a pulse to a known target and receiving the echo back from that target (Herring and Bjornton, 1985). Medical ultrasonography employs high frequency sound waves (20,000 Hz) to produce images of tissues and internal organs (Pierson *et al.* 1988). The probes, or transducers, in the ultrasound machine contain piezoelectric crystals that emit low intensity, high frequency sound waves that strike an interface between two tissues of different densities or acoustic impedances such as fluids, fat, muscle or bone. These sound waves create pressure disturbances that are projected and reflected back to the transducer (Williemse and Taverne 1989; Peters *et al.* 2004), thereby making the transducer act as a medium for receiving and sending echoes. The echoes from the tissues are then changed into electrical impulses that are processed to produce an image on a monitor showing a cross section of the tissue interface in various shades of grey, black and white dots (Reeves *et al*. 1984; (Ribadu and Nakao 1999) that correspond to tissue density and water content.

The type of images displayed on the monitor depend on the amplitude and frequency of the echo, the mode of the transducer, and the time taken for the echo to return to the transducer, which in turn relates to the depth and the size of tissue (Mannion 2006). Tissues such as gas and bone produce bright, hyperechoic images, as most of the sound waves are reflected, whilst anechoic black images are produced by tissues such as fluid in which most of the sound beams are absorbed or attenuated (Herring and Bjornton 1985).

There are three different modes of ultrasound used for medical imaging.

Amplitude (A)-mode. This is the simplest type of ultrasound utilising a transducer to emit and receive high frequency sound waves from tissue interfaces. The echoes produced from various depths are displayed as a one-dimensional image (Noakes *et al*., 2001).

Brightness (B)-mode uses a linear array of transducers simultaneously scanning a plane through the body that can be viewed as a two-dimensional image on screen. The higher the frequency of the transducer, the better the resolution of the image but the depth of the image is reduced (Rantanen and Ewing III 1981; Reeves *et al.* 1984). B mode ultrasound is commonly used to describe the bovine uterine lumen and for pregnancy diagnosis (Chaffaux *et al.* 1986) .

Motion (M)-mode: is a rapid sequence of B-mode scans where images follow each other in sequence on the screen, which helps visualisation and measurement of range of motion, as the organ boundaries that produce reflections move relative to the probe. M-mode is useful for accurate measurement of the dimensions of moving tissues such as the heart (Streeter and Step 2007).

Most ultrasound scanners used for reproductive research are B-mode trans-rectal real-time scanners (the latter refers to the ability of the scanner to image movement such as fetal motion or heartbeat as it happens (Griffin and Ginther 1992). Ultrasound transducers are available in different forms, such as the simple linear array, convex array, sector scanners, phased array and annular array. Of these, the simple and convex linear arrays are the ones that are most commonly used in large animal practice (Streeter and Step 2007).

The transducer is lubricated with a gel (such as carboxymethlcellulose) which increase contact with and/or remove the air between the rectal wall and the transducer (Griffin and Ginther 1992) . The ultrasound probe or transducer is then cupped in the operator's hand or placed in a fixed "extender" which is inserted into the cow's rectum. The scanning surface of the probe is placed in contact with the rectal wall to visualise structures within the pelvic region (Müller and Wittkowski 1986).

2.2.1.2. Accuracy of pregnancy diagnosis

Pregnancy diagnosis has two key roles in dairy cattle. Firstly, the identification of non-pregnant animals, which can then be managed appropriately (e.g. sent for re-breeding or

culled), and secondly, to identify the date of conception and, thus, the expected date of calving, so that drying off and calving management can be planned. Early pregnancy diagnosis aids the management of non-pregnant cattle and increases the accuracy of identifying the date of conception (Fricke and Lamb 2002).

Manual palpation of the uterus and its contents via the rectum has been commonly used to diagnose pregnancy in cattle, but the accuracy of this method, and earliest time of diagnosis, depends significantly on the expertise of the palpator (Williemse and Taverne, 1989). Trans-rectal ultrasonography has significant advantages as it requires less skill, is more accurate and faster (particularly in early gestation) and can be used to assess the number and sex of embryos, all without an adverse effect on the viability of the embryo or fetus (Kähn 1992; Romano and Magee 2001) . Ultrasonography can also be used to determine whether embryo or fetal death has occurred and crucially an accurate identification of ovarian structures in non-pregnant animals, which along with other ultrasound and clinical findings, allows more targeted treatments and management.

Pregnancy diagnosis in cattle using trans-rectal real-time ultrasonography (B-mode; 3.5 MHz transducer) was first described by Chaffaux *et al*. (1982) who examined the uterine lumen from Day 28 post insemination and identified the embryo within the amniotic vesicle from Day 35 of gestation. Soon after, other researchers reported detection of pregnancy in cattle as early as Day 9 post insemination (Boyd *et al.* 1988) and identification of the embryo within the uterine lumen between Days 12 and 14 after insemination. Additionally, enlargement of the vesicle was detected at about Day 19 , heartbeats from Days 22 to 28 (Boyd *et al.* 1988) and the amnion differentiated by Days 29 to 34. These workers used higher frequency 5.5 to 7.5 MHz transducers (Pierson and Ginther 1984; Boyd *et al.* 1988) . Ultrasound examination can also detect the vertebral column by Days 28-35, placentomes by Day 35, split hooves by Day 44, ribs by Day 52 and the umbilical cord by Day 60 (Curran *et al.* 1986; Kahn 1990; Fricke and Lamb 2002). Skeletal features such as the front and the hind limbs buds become visible between Days 29 to 31 (Fricke and Lamb 2002), and the skull can first be visualised between Days 70 and 84 (Kahn 1990).

Very early diagnosis of pregnancy in cattle is difficult because of the highly convoluted shape of the bovine uterine horn, which makes it difficult to get a clear view of the small amount of fluid present in early gestation. This fluid can also be confused with fluids that are produced 2-3 days after oestrus (Badtram *et al.* 1991). Hence, pregnancy diagnosis has low accuracy before Day 20 of gestation (Curran *et al.* 1986; Rajamahendran *et al.* 1994). Hughes and Davies (1989) also reported higher accuracy in early pregnancy diagnosis using a real-time trans-rectal ultrasound scanner with a 3.5 MHz transducer in heifers (on Days 22-28 of pregnancy) than in mature Friesian cows (on Days 30-35 of pregnancy). Romano *et al*. (2006) suggested that pregnancy diagnosis by trans-rectal ultrasound can be achieved three days earlier in heifers than in cows, with maximum sensitivity and negative predictive value on Days 26 and 29. Accurate pregnancy diagnosis using a 5.0 MHz transducer from 26- 28 days post ovulation has been reported, despite variations caused by different operators, image quality of the equipment and type of animal involved (Pierson and Ginther, 1984). This studies are summarised in Table 2.1

Table 2.1: Summary of accuracy or positive predictive value of ultrasound in early pregnancy diagnosis in cattle

Animal	n	Transducer MHz	Days post AI	*Accuracy or PPV (%)	Remarks	Reference
Cows	39	7.5	30	100	33 d 16	Boyd and Omran (1991)
Heifers	36	5	20-22	100	50 d 10 & 18	Kastelic *et al*. (1989)
Cows	148	5	21-25	PPV 68	NPV 64	Pieterse *et al*. (1990)
			26-23	PPV 90	NPV 97	
Heifers	321	5	21-27	PPV 89	NPV 89	Romano *et al*. (2006)
Cows	1079	5	24-30	PPV 88	NPV 96	
Cows, heifers	200	5	23-31	PPV 70	Cows: 65; heifers: 87	Badtram *et al*.(1991)
Cows, heifers	320	3	25-30	94	Embryo visible d 30	Hanzen and Delsaux (1987)
Cows	143	5	27-35	PPV 100	NPV 100 (after d 28)	Ivkov *et al*.(1992)
Cows	85	5	26-29	PPV 89	NPV 100	Willemse and Taverne (1989)
			30-33	PPV 90	NPV 94	
Cows	100	3.5/5.0	27-28	PPV 97	NPV 92%	Szenci (1992)
Cows	526	7.5	28-35	PPV 99	NPV 92%	Nation *et al*. (2003)
Cows	80	3.5	42	100	0% if >8years	Hughes and Davies (1989)
Cows, heifers	113	3.5	45	100		Chaffaux *et al*.1986
Cows	47	7.5	37-70	PPV 97.3	NPV 80%	Hunnam *et al*. 2009

PPV= Positive predictive value, NPV = Negative predictive value, AI = Artificial insemination, Heifer = Bred nulliparous female.
*Accuracy was defined as PPV/ PPV +NPV x 100

2.2.1.3. Accuracy of fetal age determination

2.2.1.3.1. Fetal aging by manual palpation

Estimation of gestational age is a major reason for pregnancy diagnosis in cattle. It can be used to predict calving date, allowing better management of calving cows, and it is also a crucial part of fertility management, as it provides information for key performance indicators such as conception rate and 6-week in-calf rate. Fetal aging has been determined from measurements of crown rump length and fetal fluid volumes from aborted fetuses (Winters *et al.* 1942; Maneely 1952; Richardson *et al.* 1990) before development of the technique of manual palpation via the rectum. Accurate fetal ageing by manual rectal palpation is limited to between Days 35 and 65 of gestation, after which accuracy reduces as gestation age increases, because of the of the distance of fetus from the rectum (Honey 1998). One of the problems of manual ageing is the overestimation and underestimation of fetal age during mid-pregnancy, which could result in approximately 49 days error in estimating fetal age (Macmillan and Moller 1977). Honey (1998) determined fetal age between Days 30 and 40 of gestation using the size of the amniotic vesicle and volumetric measurements of uterine fluids within the pregnant and non-pregnant horns, and found that the size of the amniotic vesicle increased with gestational age, although the amniotic fluid volume decreased.

Other studies have found a curvilinear relationship between gestation age and crown rump length which enabled creation of a model for prediction of gestational age from crown-rump length:

Fetal age = 65 + 2.3* crown-rump length (cm)

However, this relationship only allowed accurate prediction of gestational age between Days 60 to 210 (Richardson *et al.* 1990)

2.2.1.3.2. Fetal aging using trans-rectal ultrasound

Development of the conceptus has been characterised by trans-rectal ultrasound from Days 20 to 60 in terms of gross morphology, fetal heart beat, allantois, spinal cord, limb buds, amnion, optic vesicle, optic lens, split hooves, ribs and fetal movements (Curran *et al*., 1986). The efficiency and accuracy of fetal age determination by trans-rectal ultrasound varies with factors such as transducer frequency, gestation length, position and location of the fetus. Kähn (1989) examined the fetuses of 19 heifers between 2 months of gestation and full term

trans-rectally with a 3.5 MHz sector scanner and a 5.0 MHz linear array transducer. Digital electronic callipers were used to measure size of the fetal body organs and other body parts on the monitor. He reported increases in the size of the organs and body parts of the bovine fetus as gestational age increased, with crown-rump length showing the highest correlation to age of pregnancy. Using data from 300 beef cows, Wright *et al*. (1988) who used both linear and sector 3.5 MHz scanners also reported a high accuracy of predicting gestational age and calving dates from measuring fetal structures between Days 35 to 125. They showed a mean difference of 0.9 days between the actual and predicted calving dates and they concluded that ultrasonography was a useful technique for predicting calving date. White *et al*. (1985) evaluated head, trunk, nose, and uterine diameter as well as head and crown-rump length between Days 92 and 202 as predictors of gestational age and found crown rump-length to be the most accurate (± 4.5 days) and uterine diameter the least accurate (± 12.6 days) predictor (White *et al.* 1985). These studies are summarised in Table 2.2

Table 2.2: Association between fetal parameter measured and accuracy of determining gestational age

Animal	n	Fetal structures	Accuracy	Time interval (days)	Transducer MHz	Reference
Cows (beef)	300	Trunk diameter	0.8	35-125	3.5	Wright *et al.*, 1988*
		Head diameter	1.4			
		Uterine diameter	0.1			
		Nose diameter	-0.5			
Heifers	19	Crown-rump length	0.99	2-10 months	3.5/5.5	Kahn, 1989†
		Eye	0.97			
		Heart rate	0.70			
		Stomach	0.96			
		Cervical vertebrae	0.95			
		Head diameter	0.97			
Cows	174	Head diameter	0.95	92-202 days	3.5	White *et al.*, 1985†
		Head length	0.94			
		Trunk diameter	0.95			
		Nose diameter	0.94			
		Crown-rump length	0.91			
		Uterine diameter	0.93			

* Difference between actual and predicted calving dates, † Accuracy was estimated by correlation coefficients between growth of fetal organs and age

2.2.1.4 Placentome measurement

As described above, the ruminant placenta is cotyledonary in nature, with the fetal membranes attaching to the maternal tissue at specialised sites known as placentomes. These placentomes can be visualised by trans-rectal ultrasound as flattened, semi-circular elevations distributed over the surface of the uterine lumen. They can be detected by ultrasonography as early as Day 35 of gestation (Curran *et al.* 1986; Boyd and Omran 1991). Post-mortem studies have shown that placentomes grow in length from early to mid-gestation (Day 70-190) (Laven and Peters, 2001). This suggests that placentome measurement has the potential to be an accurate method of determining gestational age from early to mid-gestation. Doize *et al*. (1997) measured placentome size in sheep and goats using B-mode (5MHz) trans-rectal ultrasound from Days 30 to 90 of gestation. They correctly determined gestational age in 96% of does within a range of ±14 days, and 66% within a range of ±7 days (Doize *et al.* 1997). However in ewes they found that placentome size was too poorly associated with gestational age to be used as method of aging fetuses (R^2 = 0.16). A similar weak relationship (R^2 = 0.38) was also observed in Ossimi sheep (Ali and Hayder 2007). These data suggest that in goats, measuring placentome size using ultrasound can be a useful method of estimating gestational age from early to mid-gestation but this is not the case in sheep. However there may be differences between goat breeds; Karen *et al.* (2009) evaluated placentome size in Egyptian native goats and showed strong relationships in terms of correlation (R=0.90) and coefficient of determination (R^2=0.91). On the contrary, in red Sokoto goats only a weak correlation (R= 0.45) between placentome diameter and gestation age was reported (Nwaogu *et al.* 2010) .

In cattle, there is much more variation in placentome size, with the size of placentomes depending on the stage of gestation and location within the uterus. Effects of location include pregnant vs. non-pregnant horn and cervix vs. body vs. tip of horn with larger placentomes observed in the pregnant horn (Bjorkman 1954; Laven and Peters 2001). There may also be an interaction between these factors; Bjorkman (1954) observed a continuous growth of placentomes after implantation until full term (based on the measurement of placentomes closest to the fetus), although Laven and Peters (2001), in a study which measured placentomes randomly selected from throughout the uterus found placentome size did not increase significantly after Day 250 of gestation (Doize *et al.* 1997; Laven and Peters 2001).

This significant variation in size depending on site within the uterus along with other factors such the ultrasound technique used (transcutaneous (trans-abdominal) rather than trans-rectal) may explain why Hunnam *et al.*(2009b) failed to find a significant association between placentome size and gestational age. Further research is required to confirm this and to establish whether other factors such as breed or age of cow significantly affect the association between placentome size and gestational age in cattle.

2.3. Histology of the bovine placenta

2.3.1. Placentome development

Placentome formation occurs as a result of series of interactions between the extraembryonic chorioallantois and the maternal uterine caruncles (Steven 1975). This study found that uterine specimens from various stages of pregnancy in different ruminant species showed a common pattern of placentome formation before the development of species-specific features.

Placentomes from nulliparous heifers in the second month of gestation were examined using light and electron microscopy (King *et al*. 1979). Placentomes were visible as discrete, oval structures on Day 30 of gestation, whilst villi and crypts were formed by Day 33. Both the villi and the crypts subsequently lengthened and developed secondary branching. The major cell types found in the trophoblast were low columnar epithelial cells interspersed with darker-staining giant cells. Between Days 33 and 42, an extensive cytocavitary network is present in many of the uterine epithelial cells, but this subsequently regresses (King *et al.* 1979).

King *et al*. (1980) further investigated the development of the bovine placentome from Days 20 to 29 of gestation in pregnant heifers. Observation from both light and electron microscopy identified placentomes as discrete oval structures on the uterine luminal surface by Day 20 of gestation. By Day 20-21, the trophoblast was in close apposition to the caruncular epithelium, whilst mutual interdigitation of microvilli was present by Day 24 and this progressed to intimate attachment by Day 27. Maternal epithelium became uniformly cuboidal by Day 29 in contrast to the tall columnar to thin elongated cells present at the early stages (King *et al.* 1980).

2.3.2. Trophoblast cells of the feto-maternal interface

2.3.2.1. The binucleate cell

Development of the placentome begins with the migration of cells from the fetal trophectoderm into the maternal epithelium. This is followed by the fusion of the fetal cells with maternal cells forming a syncytium on the maternal side of the placenta. Trophoblast invasion is restricted inasmuch as there is no penetration of the trophoblast cells beyond the maternal epithelium (Pfarrer *et al.* 2001). The migrating fetal cells are known as binucleate cells (BNCs) and are characterised by two large nuclei and a highly granular cytoplasm. They are thought to be derived from uninucleate trophoblast cells by acytokinetic mitosis (Wooding 1992; Klisch *et al.* 1999). Binucleate cells are characteristic of every ruminant placenta and start to differentiate within the trophoblast (Wooding, 1992) from about 16 to 17 days after fertilization, i.e. just before implantation commences in cows (Bjorkman 1968). The chorionic epithelium is made up of 15 to 20% BNCs, with the remaining 80% consisting of uninucleate cells (Hradecky *et al.* 1988). These BNCs persist in the trophectoderm at a relatively constant proportion from implantation right up to one or two days before parturition, when there is a rapid decrease in numbers (Wooding *et al.* 1986). The syncytium formed in cattle is different from that seen in sheep and goats as, in the latter, it is maintained by BNCs migration throughout pregnancy (Wooding, 1992), whilst in the cow (and also in deer) the uterine caruncular syncytium is displaced by overgrowth of maternal unicellular cells which are different in structure from the columnar cells of the non-pregnant state (Wooding and Wathes 1980).The bovine placenta is thus classified as 'synepitheliochorial', because the syncytium formed by BNCs is transient (Wooding 1992) . It has therefore been suggested that the two main functions of the BNCs are, firstly, to form the feto-maternal syncytium that is essential for successful implantation and eventual growth of the placenta and, secondly, the production of protein and steroid hormones. The products of the BNCs include bovine placental lactogen (Duello *et al.* 1986) and prolactin related protein-I (PRP-I) (Milosavljevic *et al.* 1989), both of which belong to the growth hormone/prolactin family. These hormones synergise with progesterone and PGF_2 to stimulate cell growth (Grosser 1909; Wooding 1992).

In addition to the two functions outlined by Wooding (1992), it has been postulated (Laven and Peters, 2001) that the BNCs are also responsible for producing and maintaining the glycoprotein layer between the fetal and maternal epithelium. This suggestion was based on the linkage of two processes. Firstly, a chemical change in the glycoprotein layer appears to

be an important part of the process of placental release, inasmuch as such change does not occur in fetal membranes which are retained for prolonged periods postpartum (Bjorkman, 1954). Secondly, a reduction in the proportion of BNCs also appears to be a significant part of the process of placentome maturation and separation, as placental retention is associated with maintenance of the proportion of BNCs at normal pregnancy levels (Gross *et al.* 1986). Laven and Peters (2001) suggested that these two effects may be linked with the reduction in BNCs that occurs prior to parturition resulting in reduced production and a change in the glycoproteins which compose the feto-maternal interface.

2.3.3. Quantifying binucleate cell number and surface area of the placenta during gestation

BNCs are distributed in a random manner throughout the trophoblast. They are characterised by a dark-stained cytoplasm as a result of the presence of ribosomes and mature large Golgi bodies (Wango *et al.* 1990). Large membrane bound granules which occupy almost half of the volume of BNCs are produced by these Golgi bodies and can be detected in tissue sections using Periodic Acid Schiff (PAS) stain (Rodriguez *et al.* 2004). After the BNCs migrate and fuse with the maternal epithelium, they release their granules into the maternal tissue; these granules can be detected in both the maternal trinucleate cells and the syncytial plaques (Wooding and Wathes 1980). These processes of migration, fusion and granule release continue throughout gestation and are critical to the development of the conceptus (Wooding and Wathes 1980). Autoradiographic and immunocytochemical techniques, and visualisation by electron microscopy have shown that BNCs comprise about 20% of trophoblast cells, and of these, one fifth migrate through the feto-maternal junction during pregnancy (Wooding 1982b).

2.3.3.1. Stereology of the placenta

Stereology has been shown to be a useful method for describing and interpreting placental development and morphology at the cellular level in cattle (Kannekens *et al.* 2006; Ribeiro *et al.* 2008), yak (Kannekens *et al.* 2006; Ribeiro *et al.* 2008; Liu *et al.* 2010), sheep (Stegeman 1974) and humans (Mayhew 2006c). The 3-dimensional data derived from stereology, has been helpful in diagnostic studies and experimental manipulations such as cloning (Farin *et al.* 2001; Mayhew 2006a). Moreover, estimates of tissue volumes, surface

area thickness and feto-maternal ratio densities have been found to give reliable results for the human placenta, enabling quantification of the organ's ability to transfer oxygen and nutrients by passive diffusion (Mayhew 2006a),

Stereology works by using a basic randomised sampling technique, to obtain unbiased estimates of tissues distributions helping to overcome the heterogeneity of cells, tissues and organs which can vary according to the orientation of the tissue. Samples are, therefore, assumed to be representative of the entire organ (Mayhew 2006a; Howard and Reed 2010), giving 'true' values of the structures of interest. Basic terminologies and symbols have been developed to effectively use stereology to evaluate the morphometry of an object (Weibel 1969; Reid 1980; Howard and Reed 2010) (Table 2.3).

Stereology estimates volume density or volume fraction (V_V), surface area (S_V) i.e. surface area of the structures within a unit reference volume and numerical density (N_V) which is used to describe structures in 3D such as volume (V), surface area (S) and number (N) of the object of interest. (Reid 1980).With these symbols in place, basic stereology principles are based on the method used and the estimates needed to describe the object of interest.

Table 2.3: Definition of symbols used in stereology

Symbol	Definition	Dimension	Units
A	Test area	L^2	m^2
V	Volume of structure	L^3	m^3
S	Surface area of structure	L^2	m^2
N	Number of structures	-	-
P	Number of test points	-	-
I	Intersection of structure boundary with test line	-	-
L	Length of test line	L^1	M
P_A	Number of points per unit test area	$L^0/L^2 = L^{-2}$	m^{-2}
P_V	Number of points per unit test volume	$L^0/L^3 = L^{-3}$	m^{-3}
P_P	Point density or fraction. Number of points on structure per test point	-	-
I_L	Number of intersection per unit length of test line or intersection density	$L^0/L^1 = L^{-1}$	m^{-1}
V_V	Volume density or fraction. The total volume of a structure per unit test volume	$L^3/L^3 = L^0$	-
S_V	Surface area of structure per unit test volume	$L^2/L^3 = L^{-1}$	m^{-1}
N_A	Number of profiles of a structure per unit test area	$L^0/L^2 = L^{-2}$	m^{-2}
N_V	Number of structure per unit test volume. Numerical density	$L^0/L^3 = L^{-3}$	m^{-3}

Adapted from Howard and Reed (2010) and Reid (1980)

2.3.3.1.1. Stereological methods

Estimating reference volume

The total structural quantity in an organ or tissue can be estimated by using the Cavalieri method, which involves the use of a grid with known dimensions superimposed on the sample sections and point counting of the total number of points that fall on the sample section (Gundersen *et al.* 1988). Alternatively, it can be estimated by simple weighing and water immersion (Reid 1980; Howard and Reed 2010). The Cavalieri method was used to quantify placental morphology using placentomes obtained from cloned and normal Nelore bovines in their last trimester (Days 280-297) of gestation (Ribeiro *et al.* 2008). In that study each placentome was cut transversely and serially sectioned (to make a defined number of slices) after which the length of the placentome was divided by the number of slices to give the slab thickness. Thereafter, a test point system of known dimensions was placed on the slab to estimate the area from which an estimate of the reference volume could be obtained.

Estimating volume density/fraction

Volume density (fraction) is the proportion of the volume occupied by the object of interest (for example villi) within the total reference volume. This is estimated by point counting using a grid of known dimensions (Howard and Reed 2010).

Howard and Reed (2010) defined volume fraction [V_V(Y, ref)] as follows:

V_V(Y, ref) = Volume of phase Y in reference space/ Volume of reference space

For example, the volume fraction of BNCs is the volume of BNCs in the test sample / total volume of placentome. Volume fraction estimates ranges from 0-1, or can be expressed as percentages.

Reid, (1980) defined volume fraction as the ratio of cross sectional area occupied by the object of interest (A) to the total area of the section (A_T), i.e.: $V_V = A/A_T$

Liu *et al*. (2010) estimated volume density of the fetal villi, caruncular endometrium, fetal binucleate cells and fetal pynotic cells in Yak placentomes by using a 256 point grid system with a computer-assisted image analysis (Image–Pro Express). Ten fields of view representing a total of 1.090 mm^2 was studied in each placentome. Other authors estimated volume densities of fetal and maternal tissue components from placentomes obtained from somatic nuclear transfer recipient with hydrallantois (NTH) and artificial insemination (AI)

cows (Constant *et al.* 2006). The maternal and fetal tissue components were quantified using a 360 point grid superimposed on captured images. The volume densities of trophoblast, fetal connective tissues, maternal epithelium and connective tissues were calculated using the following formula: $V_V = P_a/P_T$, where P_a is the toatal number of points falling on the object of interest and P_T is the total number of points applied to the section

Total surface area and surface density

The surface area (S) of a tissue or structure of interest is often a valuable proxy measure of function; (Mayhew *et al.* 1984; Mayhew 2006b) i.e. increased surface area of the fetal/maternal interface in the placentome indicates increased capacity of the placentome to transfer nutrients or waste from the maternal to fetal tissue (or vice-versa). Total surface area can be estimated by multiplying surface density by the total volume of the reference space (Howard and Reed 2010). Surface density (S_V) is the surface area of the interface per unit volume of the reference area:

S_V(Y,ref) = Area of interface within the reference space (Y)/Volume of Y in reference space.

For the area of interface to be estimated accurately, a linear intercept method is required which uses linear test probes that cut across or intersect with the object of interest. This is achieved by using lines or (for vertical sections) cycloids of known length (Gundersen *et al.* 1988), projected at random on sections of tissues and then counting the number of times those lines intersect with the object of interest (Aherne and Dunnill 1966). The relationship between surface area per unit volume and number of intersections is shown in the following equation:

$S_V = 2*I_L$

Surface density is equal to twice the number of intersections falling between an interface, per unit length of test line (Smith and Guttman 1953).

Result of estimates derived using stereology

Kannekens *et al*. (2006) , demonstrated a feto-maternal exchange surface area of 18.5m^2 with a surface amplification factor of 108.1 in the bovine placentome within the second trimester of gestation compared to the fetomaternal exchange area of 27.9 m^2 reported in the yak placentome within a range of 121-150 days of gestation (Liu *et al.* 2010). This difference could be attributed to the number of placentas or species difference of animals used in these

studies. The total surface area estimated from stereology can be used to evaluate placenta function during gestation to meet the requirement for fetal growth as well as simplifying the estimates into standard units such as m^2 which makes it easy to understand (Kannekens *et al.* 2006; Liu *et al.* 2010).

Ribeiro *et al*. (2008), found no difference in all the placental parameters measured (which includes : number and volume of placentomes, villous volume and surface area, volume and number of star volume unit and placenta efficiency) between cloned and non-cloned bovine placentas between Days 280 and 297 of gestation. The authors concluded that placental deficiency in the cloned bovine embryos was not due to the villous component but could be due to changes in the trophoblast cells and the feto-maternal exchange area during gestation (Ribeiro *et al.* 2008).

A similar method was used to quantify the difference in fetal and maternal tissue volume in the bovine placentomes at 135 days of gestation(Kannekens *et al.* 2006). Their results showed a total volume of 470 mL in the fetal tissue and 430 mL in the maternal tissue, suggesting that the fetal tissue component is greater than the maternal component. However, it is important to realise that this conclusion was derived from placentomes using samples from a single cow.

2.3.4. Glycoprotein production during pregnancy

Different glycoproteins appear to play significant roles during pregnancy. Pregnancy-associated glycoproteins (PAGs) have been identified as potential moderators of interactions at the feto-maternal interface. These proteins appear to be made in BNCs, as they are stored in their secretory granules and released into the maternal tissue after the fusion of BNCs with maternal epithelial cells. Changes in PAG glycosylation pattern occur during pregnancy, indicating various carbohydrate-mediated functions at different stages of gestation (Klisch *et al.* 2008); a theory, which although intriguing, needs further evaluation. In particular, a better understanding is needed regarding how the glycoprotein layer develops during gestation and to identify the specific changes which occur as part of the process of placental maturation and release. In situ hybridization has been used to examine the expression patterns of ovine and bovine PAGs during pregnancy. Green *et al.* (2000) found that bovine PAGs 4, 5, and 9 were expressed strongly at Day 25 and earlier. Other investigators have shown correlations between bovine PAGs concentrations and stage of gestation and fetal number.

Patel *et al.*(1997) found significant increases in PAG concentration over gestation and higher concentrations in cows with twins than those with a singleton fetus.

2.3.5. Lectin

Lectins are proteins or glycoproteins which are capable of specific recognition of and reversible binding to carbohydrate moieties of complex glycoconjugates (carbohydrates attached to peptides or proteins) without altering their covalent structure. These molecules are of non-immune origin and bind to specific sugars. Thus, lectins can combine with insoluble carbohydrates moieties to promote cell adhesion. Both soluble and insoluble lectins have been found to bind to carbohydrates on suitable apposing cells, acting as bridges between these two cells. This behaviour of lectins plays an important role in cell-to-cell recognition (Ofek and Lis 1985). Lectins also play a key role during the initiation of infections, in the altered behaviour of cells during metastasis, and in the protection of neonates against environmental antigens. The specificity of lectins for certain sugars has allowed them to be used as probes to detect cell surface sugars, enzymes and immunoglobulins, and to identify tumorogenic cells (Singh *et al.* 1999).

The first lectin was discovered by Stillmark in 1888 from the seeds of the castor tree (*Ricinus communis).* He found that the extracts agglutinated red blood cells from various sources, although he did not realise that the plant lectin had binding sites for carbohydrate groups. More advances in lectin research have shown that the lectins are not restricted to plant cells but are ubiquitous, and have been found in fungi, bacteria, sea weed, sponges, molluscs, fish eggs, body fluids of invertebrates and lower vertebrates and from mammalian cell membranes (Sharon and Lis 1989) .

Gray *et al* (2005) postulated that the lectin galectin-15 had several biological roles in the conceptus, including endometrial interactions, uterine immune and inflammatory responses and placental morphogenesis and function. They found galectin-15 was present as multimers in the uterine lumen on Days 14 and 16 of pregnancy in sheep. In the endometrial epithelium and the trophectoderm of the conceptus, galectin-15 protein is present in intracellular sites and is associated with crystalline structures. Between Days 20 and 120 of pregnancy, galectin-15 mRNA is expressed by the luminal epithelium (superficial ductal glandular epithelium) of ewes. Thus galectin-15 is synthesized and secreted throughout gestation by the

endometrial luminal epithelium and/or superficial ductal glandular epithelium and is absorbed by the placenta to form crystals within the trophectoderm.

Woldesenbet *et al*. (2004) used immunofluorescence microscopy of caprine conceptus tissues collected during the apposition, adhesion and attachment phases of placentation to demonstrate the expression of lectin-like receptors for endometrial H-type L antigen.. They also found that carbohydrate-lectin interactions may facilitate attachment of uterine epithelial cells and trophectoderm during the early stages of placentation.

Lectins have been used to study various biological applications including improving the efficiency of associative nitrogen fixation in non-legume plants (especially rice and other cereal crops; (Mirelman 1986), detection of toxins in food and pharmaceuticals (Brown *et al.* 1991), bacterial typing and bone marrow transplantation (Lis and Sharon, 1986), prevention of bacterial infection and cancer metastasis (Beuth, 1995) and protection of newborns against environmental antigens (Davin *et al.* 1991). They have also been used in separation and purification technologies for carbohydrates, glycoconjugates, enzymes, immunoglobulins and cells (Lis and Sharon 1986).

2.3.6. Lectins and the Ruminant placentome

Lectins have been used to study the development of ruminant placentome because of their specific glycan-binding properties, which they exhibit due to: (1) Binding affinity of lectins for carbohydrate molecules and (2) protein-saccharide interactions. Lectin histochemistry and lectin western blotting was used to investigate the glycosylation of pregnancy associated glycoproteins (PAGs) and prolactin related protein-1 (PRP-1) (aspargine-linked lactosamine-type glycans terminating with N-acetyl –galactosamine) in bovine placental tissue. Klisch *et al.* (2006) showed that terminal N-acetyl-galactosamine (PAGs) (detected by *Dolichos biflorus* agglutinin, DBA) in placentomal BNC was greatly reduced prior to parturition compared to mid-pregnancy, whilst lactosamine–type N-glycans (detected by *Phaseolus vulgaris* leucoagglutinin, PHA-L) remained unaltered. Lectin western blots showed a reduction of terminal N-acetyl-galactosamine with DBA on PAGs at parturition. Klisch *et al.* (2006) concluded that the changes in PAGs during the last few weeks of gestation suggested an alteration in the functional properties of PAGs and that such changes could be used to investigate the functions of PAGs in placental development (Klisch *et al.* 2006) .

Nakano *et al.* (2002) used DBA to characterise bovine placental BNCs *in vitro* and a bovine trophoblastic cell line (BT-1) *in vivo*. They showed specific glycoconjugate binding of BNCs with DBA in primary culture *in vivo* but not in BNCs derived from BT-1 cell cultures (*in vitro*), suggesting that glycoproteins produced *in vivo* specifically bound to DBA and that this binding corresponded with the commencement of placentation (at about Day 30 of gestation) whilst such differentiation was not seen in uninucleate or binucleate cells in BT-1 cell culture.

Klisch *et al.* (2010) investigated the glycosylation pattern of the secretory granules within the BNCs of a variety of ruminant placentas, and showed that across the species there was a similar pattern of highly branched glycans that consisted of bisecting and terminal N-acetylgalactosamine (GlcNAc). This highly conserved glycosylation pattern of BNC granules suggests an early evolution of the ruminant BNC and that those cells play a functional role in the secretion of glycoproteins in the ruminant placenta.

Table 2.4: Summary table showing types of lectins, specificity, results and references

Type of Lectin	Specificity of glycans	Results	Reference
Galanthus nivalis (GNA)	Non reducing terminal α-D- mannose	Stained small subpopulations of granules in e BNCs of bovine and ovine species	Jones *et al*. (1994)
Pisum sativum (PSA)	α-D-mannose in non-bisected bi/tri-antennary	Similar result to GNA	Jones *et al*. (1994)
Phaseolus vulgaris (I-PHA) *Phaseolus vulgaris* Leucoagglutinin (PHA-L)	Tri/tetra-antennary, non-bisected complex N-linked sequences and lactosamine-type N-glycans	Many intensely stained BNC granules in bovine and ovine species (detected by PHA-L)	Jones *et al*. (1994) Klisch *et al*. (2006)
Triticum vulgaris (WGA)	Di-N-acetylchitobiosyl, N-acetyl lactosamine and some sialyl residues	Few intensively stained BNC granules in both species	Jones *et al*. (1994)
Tetragonlobus purpureus (LTA)	α -L-fucosyl terminals	Negative staining in both species	Jones *et al*. (1994)
Ulex europaeus-I (UEA-I)	L-Fucα1,2Galβ1,4GlcNAcβ 1-	Same as LTA	Jones *et al*. (1994)
Arachis hypogaea (AHA)	Galβ1,3GlcNAcα1- >Galβ1,4GlcNAcβ1-	Few intensively stained BNC granules in both species	Jones *et al*. (1994)
Erythrina cristagalli (ECA)	Galβ1,4GlcNAcβ1-	same as AHA	Jones *et al*. (1994)
Glycine max (SBA)	TerminalGalNAcα1->Galα1	Intensely stained (few-moderate) BNC granules in both species	Jones *et al*. (1994)
Limax flavus (LFA)	Certain sialyl terminals	Intense staining few bovine BNC granules while ovine BNC granules no staining.	Jones *et al*. (1994)
Maclura pomifera (MPA)	Galβ1,3GlcNAcα1->GalNAcα1-	Few ovine BNC were intensely stained as compared to the bovine species	Jones *et al*. (1994)

Wisteria floribunda (WFA)	GalNAcα1,6Galβ1- >GalNAcα1,3Galβ1-	Many granules intensely stained both species	Jones *et al*. (1994)
Dolichus biflorus (DBA)	GalNAcα1,3(L-Fucα1,2)Galβ1,3/4 GalNAcβ1- Terminal N-acetyl- galactosamine Placental lactogen	Intensely stained many to few BNC granules in both species. DBA bound strongly to the bovine BNC at mid-pregnancy specific staining was absent at term. DBA bound strongly to the cytoplasm of bovine BNC but not uninucleate cells.	Jones *et al*. (1994) Klisch *et al*. (2006) Nakano *et al*.(2002)
Maackia amurensis (MAA)	NeuNAcα2,3Galβ1-	Intensely stained many to few bovine BNC granules, and negative staining in ovine BNC	Jones *et al*. (1994)
Sambucus nigra (SNA)	NeuNAcα2,6Gal/GalNAc-	Intensely stained many to few granules in ovine BNC, negative staining for bovine BNC	Jones *et al*. (1994)

Several studies have reported that the granules in BNC are heavily glycosylated (Duello *et al.* 1986; Morgan *et al.* 1987; Wooding 1992) but, apart from lectin-binding studies on bovine endometrium (Munson *et al.* 1989) and bovine trophoblast of early gestation (Lehmann *et al.* 1992), there was limited information on the structure of the glycans that are contained in BNC. This lack of information prompted the study by Jones *et al.* (1994) who used high resolution light microscopy of the specific glycan-binding properties of 15 biotinylated lectins (in combination with perfusion–fixed, plastic embedded tissue) to investigate the glycosylation of BNCs in bovine and ovine placentas. Their results indicated a pronounced accumulation of saccharides in both species as well as the presence of different subtypes of N-glycans related to sialyl residues. The selectivity of certain lectins for ovine and bovine BNC granules was established (Table 2.4).

In water buffalo lectin-histochemistry with *Dolichos biflorus* agglutinin, *Vicia villisa* agglutinin and *Phaseolus vulgaris* leukoagglutinin was used in placentome between 2 and 10 months of gestation. Trinucleate feto-maternal hybrid cells were the typical outcome of the cell fusions and there was also a strong resemblance between water buffalo and cattle BNCs in terms of glycosylation pattern and characteristics of cell migration and fusions (Carvalho *et al.* 2006)

Another study, which used purified galectin-3 from midgestation ovine placenta, found that galectin-3 was generally associated with differentiation, morphogenesis and metastasis of the placenta. Results obtained by western blot analysis showed that the expression of galectin-3 was greatly decreased in the full-term placenta with respect to the middle of gestation. It was suggested that expression of galectin-3 is regulated throughout the period of development of the fetus (Iglesias *et al.* 1998).

2.4. Overall conclusion

The gross morphology of the bovine placentome changes as gestation progresses and this is crucial for normal fetal growth and placental development during gestation. The placentome is the functional unit of the placenta and is vital for the exchange of nutrients, gases and waste products between the fetus and the mother. Hence, without normal growth and development of the placentomes, normal fetal development will not occur.

Research has shown that changes in placentome size, shape, number, weight and feto-maternal tissue ratio during gestation depends on the species, stage of gestation, location of placentomes and the type of reproductive technology used for breeding.

Limited information exists on how the bovine placentome changes and copes with stressors such as nutrient restrictions during early gestation. In sheep, the shape of the placentomes change from concave to convex placentomes (i.e. to a shape that is similar to that of the normal bovine placentome) in order to cope with stress. This suggests that the bovine placenta has different ways of coping with increased fetal demands during pregnancy. Hence, the present study was investigated to evaluate changes in placentome weight, size, and density *in vitro* during gestation. Secondly, an *in vivo* study using ultrasonic technique was conducted to predict gestation age from placentome size during gestation.

This review has also shown that BNCs are important in the ruminant placenta growth and development. They fuse with the uterine epithelial cells to form trinucleate fetomaternal hybrid cells, produce and maintain the glycoprotein layer found between the feto maternal interfaces during pregnancy. Histology of the placentomes has been used to observe cellular changes within the placenta at different stages of gestation. Quantitative estimates of human and ruminant placenta obtained from stereology methods have been shown to be precise and reliable, as the randomised sampling technique removes systemic bias in the data collection. Stereology has been used to obtain 3D measurements such as volume, surface area, and densities of different organs and cells from 2D sections, thereby interpreting the anatomy of the cell or organ as well as for diagnostic purposes. Only one study has used stereology to demonstrate the volume and surface density of the feto-maternal contact area at a specific stage of pregnancy in cattle. Therefore this study will be one of the first to use stereology to estimate placentome volume and surface density from early to late pregnancy in cattle.

Furthermore, lectins which have the ability to recognise and bind to glycoconjugates, allows recognition and isolation of different types of cells, including BNC, in ruminant placentas. Lectins such as DBA, PHA-L, and SBA etc. have also been used to detect or isolate glycoprotein in BNC granules, because of their strong affinity to bind with surface

membranes and cytoplasm of BNC granules. However, there is only limited information on how those glycoproteins change over gestation.

Chapter Three

Factors Influencing Placental Morphometry in the First Trimester of Pregnancy in Angus Cows

3.1. Introduction:

The bovine placenta has discrete regions where there is co-development of both maternal (caruncular) and fetal (cotyledonary) tissue. It is through these regions, known as placentomes that the fetus obtains oxygen and nutrients and excretes waste products. The importance of placentome mass to the development of the fetus has been demonstrated by carunclectomy of sheep. Growth-retarded fetuses and reduced viability of lambs were observed when endometrial caruncles were removed prior to conception (Harding *et al.* 1985). Similarly, (Kelly 1992) found a high correlation between placental weight and birth weight and, consequently, the survivability and growth rate of both ovine and human neonates. Kelly (1992) examined placental growth in sheep by measuring the diameter of cotyledons from early to mid-gestation. He reported that nutrient restriction up to 90 days of gestation did not affect placental growth, but that there was a pronounced effect on placental growth when animals were nutrient-restricted later in gestation. Taken together, such data show that the size of the placenta directly affects its capacity to transfer nutrients which, can influence the growth rate of the fetus and, thus, birth weight (Fowden *et al.* 2006).

Zhu *et al.*(2007) evaluated the impact of nutrient restriction on placental development in cattle. In that experiment, 30 multiparous beef cows bred to a single sire were allocated to feed that either met nutrient requirements (NRC, 1996) for pregnant beef cattle (control: n=15) or were below NRC requirements (nutrient restricted: n=15) between Days 30 and 125 of gestation. By slaughter on Day 125, nutrient restricted cows had lost an average of 450g body weight / day whilst the control group had gained 250g /day. They reported that although fetal weight was lower in the restricted group, the difference was not significant ($p = 0.12$). However, the total weight of the placentomes (and both the caruncle and cotyledon components) were significantly reduced in the restricted group, which Zhu *et al.* (2007) concluded that placental efficiency had increased in cattle that had nutrient restriction in early gestation. Long *et al.*(2009) re-analysed the data from Zhu *et al.* (2007). Based on reduced fetal weight and asymmetric organ growth they grouped the fetuses of nutrient restricted cows into two groups; those that showed intrauterine growth restriction (n=4), or those which did not (n=6). This reanalysis showed that placentas from those

nutrient restricted cows with fetuses which had intrauterine growth restriction were smaller than those from nutrient-restricted cows with non-growth restricted fetuses and those from control pregnancies, and that there was no difference in placental weight between the latter two groups.

In contrast, other studies have shown no effect of maternal nutrient restrictions on placental development. Prior and Laster (1979) fed heifers one of three dietary energy levels to achieve weight gains of 0-100g/day (low), 500-600 g/day (medium) and 1,000-1100 g/day from pregnancy diagnosis (35-42 days) until slaughter on approximately day 90, 120, 150, 180, 210, 240 or 255. They reported high positive correlations between fetal weight, fetal fluid weight, cotyledon area, placental weight and uterine weight, but found no effect of energy restriction on fetal growth. Similarly, Hickson *et al*. (2009) reported that nutritional regimens which resulted in either moderate (500g/day) or low (50g/day) live weight gains in 15 month old Angus heifers during the first trimester of gestation had little or no effect on fetal or placental size at 90 days of gestation.

However, given that placental growth is dependent upon many factors other than nutrition, a reinvestigation of the data reported by Hickson *et al*. (2009) was undertaken to better determine the association between the factors that contribute to placental size. Data were subjected to principal component analysis (PCA), which helps to identify patterns in a dataset as well as the variability in that dataset, thus PCA summarizes the data without much loss of information (Jolliffe 2002). Hence this technique can be useful in visualising interrelationships between different variables, and to reveal and interpret groupings, similarities and differences (Herbert *et al.* 2000). Factor analysis (FA) was then used to further investigate these correlated variables and to identify the underlying factors that may account for the behaviour of the original variables within the data (Hair *et al.* 2006). The aim of this study was, therefore, to use PCA and FA to further interrogate the data produced by Hickson *et al.* (2009) in order to identify relevant underlying factors that might influence placental development within the first trimester of pregnancy in Angus heifers.

3.2. Materials and Methods

3.2.1. Animals and treatments

The data that were analysed were derived from two studies whose primary results have previously been reported (Hickson *et al.* 2009). Briefly, gravid uteri were collected on Day 90 or 91 of gestation from 18-month old Angus heifers, which had been fed to attain either moderate (451g/day in year 1 or 595 g/day in year 2; n=8 and 9, respectively) or low (118 g/day in year 1 or 7 g/day in year 2; n=9 and 15, respectively). Fasting live weights were measured on the day before slaughter.

3.2.2. Tissue Collection

The gravid uteri were collected and weighed intact and were then stored overnight at 4^{o}C. The total weight of the gravid uterus was recorded. The amniotic and allantoic fluid were then drained from the uterus, which was then opened to remove the placentomes and fetuses.

The following measurements were made:

- Fetal weight.
- Weight of the fetal membranes with the cotyledons, after manual separation of the fetal membranes (including the cotyledons) from the maternal caruncles.
- The weight of the uterus without fetus, fluid and fetal membranes/cotyledons
- Cotyledons were then severed from the fetal membrane, and were weighed and counted to give the total weight and number of cotyledons.
- Caruncles were then dissected from the uterus by cutting the caruncle from the stalk at the point of attachment to the uterus, after which they were counted and weighed.

3.2.3. Statistical Analysis

The data were subjected to analysis of variance with respect to treatment and year and simple correlations between the variables were examined using a Pearson correlation matrix.

Principal component analysis was undertaken to group the correlated variables and find linear combinations of those variables to produce new variables referred to as principal component scores or eigenvalues. Factor analysis was then carried out using varimax rotation of the

component scores to further reduce the number of variables. These variables were used to identify the underlying factors existing within the data for uterine parameters; and to determine whether differences between treatment groups could be detected in individual heifers' component scores. All analyses were undertaken using Proc Princomp for principal component analysis and Proc Factor for the factor analysis (SAS 9.1 SAS Institute Inc, Cary, USA), and graphics were generated using SAS Enterprise 4.1.

3.3. Results

3.3.1. Least square means of placental variables

The mean results for all the variables are summarised in Table 3.1. There were no significant differences between years or between low and moderate treatments for placental or fetal characteristics. For diet, the only significant variable was that mean maternal live-weight taken two days before slaughter was greater ($p < 0.001$) in both years for heifers on the moderate than the low treatment diet. All further analyses were undertaken using amalgamated data from both years.

Table 3.1: Means of Placental variables with Standard error (±SE) for low and moderate diet treatment

Variable measured (g, kg and mLs)	TREATMENT		
	LOW	MODERATE	P-value
Total weight of gravid uterus (g)	2160.1 ±64	2137.2±71.8	0.813^{NS}
Weight of uterus + caruncles only (g)	628.3± 16.9	604.2±19	0.35^{NS}
Weight fetal membrane + cotyledons (g)	256.1± 10.8	229.2±12	0.11^{NS}
Weight of fetus (g)	207.8±4.9	200.1±5.2	0.31^{NS}
Weight of cotyledons (g)	94.1±3.5	86.1±3.9	0.61^{NS}
Weight of caruncles (g)	87.0±3.4	87.1±3.8	0.97^{NS}
Maternal weight at slaughter (kg)	372.7±7.7	435.3±8.7	<0.0001
Total number of cotyledons (n)	90.4±5	91.9±5.6	0.84^{NS}
Total number of caruncles (n)	97.6±6.4	95.9±7.1	0.86^{NS}
Number of animals (n)	16	24	

NS = Not Significant

3.3.2. Correlation

The correlations between cotyledon or caruncle weight and the other measured variables are summarised in Table 3.2. Cotyledon and caruncle weight were positively correlated (P=0.005). Cotyledon weight was positively correlated with weight of the uterus (P<0.001 and P=0.02) and the total weight of the fetal membranes (P<0.001), although not with fetal or maternal weights. The correlations between caruncle weight and other parameters exhibited a similar pattern, except that the relationship with fetal membrane weight was not significant (P=0.1) whilst the relationship with fetal weight was significant (P=0.007).

3.3.3. Principal Component Analysis

Principal component analysis of pooled data from Years 1 and 2 showed that the first two principal components of the standardized data explained 92.5% of the total variation in the data, with 89.3% of the variation attributed to the first PC (PC1) and 3.2% to the second PC (PC2). The variation in PC1 was influenced by total weight of the gravid uterus (PC score: 0.47), uterus plus caruncles only (PC score: 0.43) and fetal cotyledon (PC score: 0.40). For PC2, the variation was influenced by the numbers of fetal cotyledons and maternal caruncles (PC score of 0.64 and 0.63, respectively).

Subsequent visualisation of PC1 and PC2 pattern of distribution of the variables via a scatter plot showed that heifers in both the low and moderate treatment groups had moderate PC1 scores (except for one outlier) and overlapped each other without any separation (Figure 3. 1); i.e. PCA did not discriminate between the two treatment groups, indicating that there was no influence of nutrition on the variables included in the analysis.

3.3.4. Factor Analysis

The results of the factor analysis and their loadings are shown in Table 3.3. The rotated factor pattern for Factor1 shows high positive loadings for total number of caruncles and cotyledons. Hence, Factor1 was labelled 'placentome number factor'. Factor 2 was characterised by the weight of the gravid uterus and of the uterus plus caruncle only, and was thus labelled as 'uterine mass factor'. Factor 3 reflects high and positive association with weight of fetal membranes with cotyledon and dissected cotyledon weight, and was named 'fetal tissue/ component factor'. Factor 4 showed positive loadings for the weight of caruncles and fetal weight and was therefore labelled 'fetal/caruncle weight factor'. There was no common variable shared by any of the factors.

Table 3.2: Correlation between cotyledon and caruncle weight and other variables

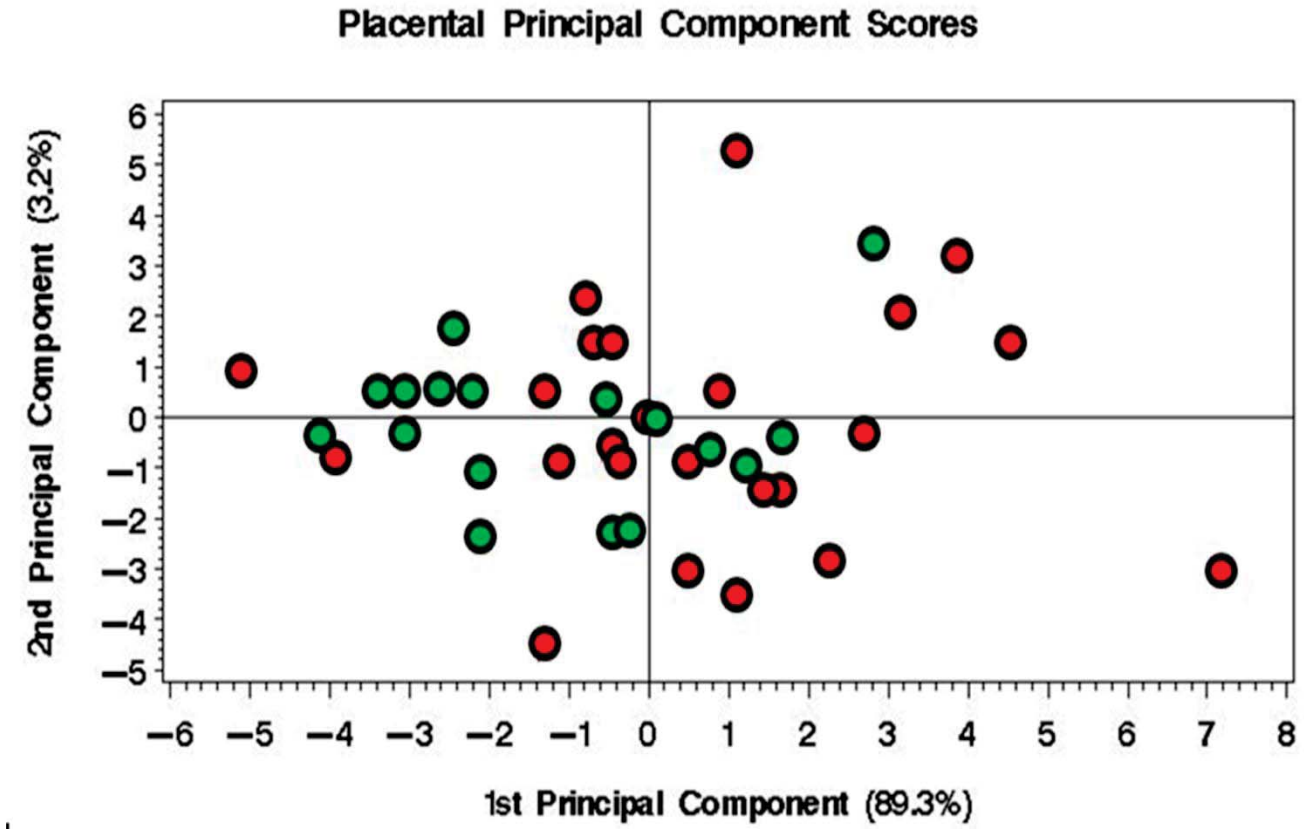
	Weight					**Cotyledon**		**Caruncle**	
Variable	Total weight of gravid uterus	Uterus + caruncles	Fetal membrane + cotyledons	Fetus	Maternal	Number	Weight	Number	Weight
Cotyledon weight	**0.50 (0.001)**	**0.35 (0.02)**	**0.61 (<.0001)**	0.25 (0.11)	-0.16 (0.31)	-0.07 (0.66)	1.00	0.05 (0.77)	**0.44 (0.005)**
Caruncle weight	**0.43 (0.005)**	**0.41 (0.007)**	0.26 (0.10)	**0.42 (0.007)**	0.08 (0.62)	-0.09 (0.56)	**0.44 (0.005)**	-0.08 (0.59)	1.00

P values are in bracket and bold when significant

Table 3.3: Result of Factor Analysis: Factors and Factor Loadings*

Variable	Factors			
	1	2	3	4
Total weight of gravid uterus	0.04	**0.85**	0.31	0.19
Weight of uterus + caruncle only	0.001	**0.92**	0.11	0.21
Weight fetal membrane + cotyledon	0.14	0.32	**0.83**	0.04
Weight of Fetus	-0.27	0.22	0.11	**0.71**
Weight of cotyledon	-0.06	0.09	**0.88**	0.26
Weight of caruncle	-0.004	0.16	0.17	**0.87**
Total number of cotyledon	**0.95**	-0.04	0.01	-0.09
Total number of caruncle	**0.93**	0.08	0.06	-0.14
% Total variance	27.81	26.36	24.28	21.51
% Cumulative Variance	27.81	54.17	78.45	99.96

*Factor loadings represent the correlation between the individual variable and the factor



Key: Low treatment **Moderate treatment**

Figure 3.1: A two dimensional Principal Components graph showing the distribution of the principal component scores for treatment groups (Low and moderate) within the 1st and 2nd principal components

3.4. Discussion

The results of the PCA confirm the report of Hickson *et al*. (2009) that there was no difference in placental variables in heifers on a moderate *versus* a low plane of nutrition during the first trimester of pregnancy, despite greater live weight in the animals on the higher plane of nutrition. This result is similar to that reported by Prior and Laster (1979), in which heifers were placed on low, medium and high diet levels from 35 days of gestation to slaughter. In that study and the study by Hickson *et al*. (2009) the heifers on the nutrient restricted group still gained weight during early gestation. In contrast, Zhu *et al.* (2007) did report a significant reduction in placentome mass associated with nutrient restriction, with cows on that diet losing an average of 250 g/day. It is therefore possible that in order to limit placental development nutrient restriction needs to be sufficient to result in weight loss. However even then fetal development may not be affected – with the initial analysis by Zhu *et al.* (2007) showing that fetal weight was not significantly affected ($p=0.12$) by the nutrient restriction despite the impact on placental development. A re-analysis of the data collected by Zhu *et al.* (2007) did show an effect of nutrient restriction on fetal growth (Long *et al.,* 2009). However this analysis was based on identifying two different groups based on fetal weight, and showed that both fetal weight and placental weight could be reduced by severe nutrient restriction, although this effect was not consistent in all cases being seen in only 4/10 animals. The reason for the difference between the two groups is not clear, but Long et al (2009) clearly shows that, in cattle, it is difficult to restrict placental development (and associated fetal growth) by restricting nutrition; this is consistent with the conclusions of this analysis and the findings of Prior and Laster (1979).

Although nutrition does not appear to be a major driver of placental capacity in cattle, the high correlations seen in this analysis and that of Prior and Laster (1979) between placental, fetal and uterine measurement suggests that placental development is dependent on a host of other factors, and that the dataset is extremely suited to PCA.

The first two principal components explained over 92% of the variation in the data. PC1 was moderately influenced by the weight of the contents of the gravid uterus, and the weight of the fetal placenta, whereas PC2 was strongly influenced by caruncle and cotyledon number. The factor analysis of these correlated components reduced the nine interrelated variables into four newly-defined uncorrelated variables each of which explained around 21 to 28% of the variation. The principal factor (28% of the variation) was placentome number (eigenvalue ≥ 0.93), showing that number of placentomes plays a significant role in placental capacity. This conclusion is consistent with the findings by Clarke *et al*. (1998) who found that maternal nutrient restriction in ewes from early to mid-gestation reduced cotyledon number thereby reducing placental size. However it seems in opposition to the finding reported by Laven and Peters (2001) that placentome number didn't affect total placentome weight. However, it is important to remember that the data in that study was from

normal cows where placental capacity tends to exceed requirements so placentome number is not a limiting factor, additionally if some cows with few placentomes respond to fetal demands by increasing placentome size whereas others do not that will obscure the relationship in normal cows between placentome number and placentome capacity.

The second factor is principally related to uterine mass (eigenvalue ≥0.85). Caruncle weight is included in this factor along with uterine weight but this combination is dominated by the weight of the uterus. Caruncle weight alone is not included in this factor. This finding is consistent with the findings of Ferrell (1991) who demonstrated the influence of maternal uterine environment on fetal growth and with Prior and Laster (1979) who concluded that uterine capacity is not influenced by fetal size, but that it increases to adjust to the fetal size. The third factor, is fetal membrane weight with cotyledon weight predominating. Interestingly, although cotyledon and caruncle weight are strongly correlated in the raw data, caruncle weight is the predominant part, along with fetal weight, of the fourth factor identified by the FA and is not part of the third factor. This suggests that fetal development is driven by caruncular development, consistent with the suggestion by Ferrell (1991) that fetal growth was facilitated by the growth and function of the caruncular tissues. This conclusion is not supported by data from normal animals (Laven and Peters 2001); however, as discussed earlier for placentome number it is likely that in normal animals the excess capacity of the bovine placenta obscures the relationship between the factors and that severe nutrient restriction (e.g. Long *et al.* 2009) is required to see this relationship. Cotyledon weight and caruncle weight are strongly correlated but the finding in this analysis that they are represented in different factors suggests that the development of the caruncle and the cotyledon are not as closely linked as that correlation would suggest.

Conclusions

This analysis has confirmed that moderate nutrient restriction in well fed heifers from approximately three weeks before pregnancy to 90 days of gestation does not affect placental development . Further analysis of the data has elucidated several links between placental and uterine factors which are not clear in simpler analyses because of the excess capacity possessed by the normal bovine placenta. Further research is required to better characterise the principal components and factors identified by this analysis

Chapter Four

The Effect of Gestational Age on the Density of the Bovine Placentome

4.1. Introduction

The development of the ruminant placentome during gestation has been characterised in terms of total mass and functional characteristics such as fetal and maternal blood flows (Wilson 2002; Vonnahme *et al.* 2003; Wallace *et al.* 2008), although , it has been difficult to explain the overall functional capacity of the placenta solely in such terms. However, if it were possible to determine the relative proportions of fetal and maternal tissues in bovine placentomes throughout gestation, it might allow a better understanding of the roles of the two compartments in providing support for the developing fetus. Four major studies have been previously undertaken on this subject; by Reynolds *et al*. (1990) and Laven and Peters (2001), who estimated the proportions of fetal and maternal tissue within the placentome based upon relative weights; by Laven and Peters (2006), who used image analysis data from placental histology; and by Kannekens *et al.* (2006), who used stereology which measures the relative volumes of each tissue to evaluate the feto-maternal relationship within the bovine placentome (though in only one cow). In order to compare their results with that of Reynolds *et al.* (1990) and Laven and Peters (2001), Kannekens *et al.* (2006) also estimated the density of the placentome by measuring the weight of three placentomes in air and water on Day 135 of gestation. They reported that placentome density was 1 g/mL and, using this figure, they estimated that the ratio of maternal: fetal tissue in the placentome was 0.92:1 (i.e. that there was less maternal than fetal tissue). By contrast, Reynolds *et al.* (1990) and Laven and Peters (2001) had reported that there was more maternal than fetal tissue (ratios of 1.5:1 and 1.1:1, respectively, at 150 days of gestation). Kannekens *et al.* (2006) suggested that these discrepancies were due to fetal tissue remaining within the caruncle when the placentome was manually separated. To explain the difference between the 0.92 figure reported by Kannekens *et al.* (2006) and the 1.1 figure calculated by Laven and Peters (2001) would require that 4% of the cotyledonary tissue would have remained within the caruncle; whilst to achieve the ratio reported by Reynolds *et al.* (1990) there would have had to be 13% of cotyledonary tissue remaining. However, both Reynolds *et al.* (1990) and Laven and Peters (2001) reported that there were only small amounts of fetal tissue left attached after manual separation. Furthermore, Reynolds *et al.* (1990) specifically rejected the notion that inadequate tissue separation was responsible for the maternal portion of the placentome being

larger by weight than the fetal portion. This suggests that the cause of the discrepancy reported by Kannekens *et al.* (2006) depends on factors other than merely insufficient separation of fetal and maternal tissue.

One possible explanation of these discrepancies is that Kannekens *et al.* (2006) estimated the ratio of maternal: fetal tissue in only one cow, so it is possible that individual cow-to-cow variation (which can be quite large; Laven and Peters 2006), may have affected their results. However, the estimate of 0.92:1 reported by Kannekens *et al.* (2006) was very similar to the expected ratio of maternal: fetal tissue by volume at the same stage (0.94:1) calculated by Laven and Peters (2006) using image analysis data from 47 uteri at varying stages of gestation. One other possible explanation is that the discrepancy occurred during the conversion of volumetric ratios to proportion by weight. Firstly, as with the ratio itself, it seems quite possible that there are significant individual cow variations in placentome density which could explain some of the variance. Additionally, Kannekens *et al.* (2006) assumed that maternal and fetal tissue had the same density, even though the two tissues are grossly and histologically different (Björkman 1954). There are no published data on either the individual variability of placentome density or on the comparison between the density of the caruncle and that of the cotyledon, so further research is required to establish whether the discrepancy between volumetric and weight measurements is due to incorrect assumptions in regard to placentome density.

Furthermore, even though Kannekens *et al.* (2006) established that stereology is a useful technique to study the feto-maternal relationships within the bovine placentome, knowledge of tissue density, particularly the relationship between density and gestational age, is needed to convert the volumetric data from stereology into tissue masses. However, no such studies have been published. It appears feasible that there are changes in tissue density with time, given that there are significant changes in placentome structure during gestation, particularly in regard to its vascularisation (Leiser *et al.* 1997). Support for this idea can be obtained by comparing weight-based data (Reynolds *et al.* 1990; Laven and Peters 2001) with volumetric data (Laven and Peters 2006). By volume, the ratio of maternal to fetal tissue decreased from 1:1 at 50 days of gestation to 0.8:1 at 250 days of gestation (Laven and Peters, 2006), whereas by weight, the ratio increased over gestation (1.1:1 on Day 70, increasing to 1.7:1 on Day 250 (Laven and Peters, 2001); or 0.8:1 on Day 100, increasing to 1.8:1 on Day 250 (Reynolds *et al.* 1990).

Thus, there remains a dearth of information about the relative contributions of the fetal and maternal components to the bovine placentome throughout gestation, as well as about whether or how these affect the overall density of the organ; and there is little consensus between the small number of earlier studies upon this subject. The aim of this study was therefore to establish how placentome density changes with gestation and to evaluate the relationship between the density of the caruncle and that of the cotyledon.

4.2. Materials and methods

4.2.1. Tissue collection and sampling

Uteri (n=24) from the second and third trimesters of pregnancy (Days 100 to 225 of gestation) were obtained from a local abattoir (Affco NZ Ltd., Feilding, New Zealand) and Massey University Veterinary Teaching Hospital. There were no details of breeds, body condition score or age of the cows selected for this study; however, records from the abattoir indicated that the majority of cows slaughtered were Friesian, Jerseys and Friesian x Jersey. The uteri were categorized into five stages of gestation by measuring the crown rump length of the fetus (Hammond 1927; Winters *et al.* 1942) to ensure that uteri were evenly collected across the range from 100 to 225 days:

Stage 1: Gestation age 100-125 days (n=5)
Stage 2: Gestation age range 126-150 days (n=5)
Stage 3: Gestation age range 151-175 days (n=5)
Stage 4: Gestation age range 176-200 days (n=5)
Stage 5: Gestation age range 201-225 days (=4)

Shortly after collection of the uteri, the fluid and the fetuses were carefully removed according to the procedure of Laven and Peters (2001) and taken to the laboratory within 45 minutes of slaughter for further measurements.

4.2.2. Recording

Each uterus was dissected to expose the pregnant and non-pregnant horns. Placentomes were then severed from the endometrium by cutting through the upper part of the caruncular stalk. Total placentome weight and number were measured and recorded. The total volume of the

placentomes was measured by water displacement. Placentomes were placed into a 5 litre container capacity, and the water displaced was then measured using firstly, a 1 litre capacity measuring cylinder graduated in 10 mL increments and, secondly using a 60 mL syringe.

For each uterus, 10 placentomes were selected by choosing every 8th placentome provided it was >15 mm diameter otherwise the next correct size was chosen and removed from both the pregnant and non-pregnant horn (Laven and Peters 2001) for further measurements. The selected placentomes were manually separated into cotyledon and caruncle by carefully peeling out the fetal cotyledon from the caruncle starting from the surface closest to the caruncular stalk. The remaining cotyledonary tissue within the caruncular crypts were then removed with forceps and added to the fetal tissues (Reynolds *et al.* 1990). The individual caruncles and cotyledons were weighed and their volumes were estimated by water displacement. Tissue densities were calculated from these data; and thereafter ratios between fetal and maternal weights, volumes and densities were also calculated.

4.2.3. Statistical analysis

Data were analysed by using the General Linear Model of SAS 9.2 (SAS 2011; SAS institute Inc. Cary, USA) to describe the change in total weight, volume and density with time (Gestation Stage). Changes in individual placentome components (10 placentomes >15 mm randomly selected from each uterus/cow) of weight, volume and density across gestation age were determined with repeated measures analysis of variance, in which individual placentomes were nested within cows. Relationships between gestation age and individual placentome, caruncle and cotyledon weights, volume and density were estimated by regression and correlation analysis. Thereafter, regression lines were fitted between placentome number, weight, volume, density and gestation age and prediction equations derived to predict placentome number, weight, volume and density from gestation age. The relationship between placentome number, total placentome weight and average placentome weight was further investigated by calculating the ratio of predicted numbers of placentomes and predicted total and mean placentome weight for each uterus sampled with the actual figures. The ratio of predicted to actual placentome numbers was then compared using linear regression to the ratio of predicted to actual mean or total placentome weight.

4.3. Results

The mean number of placentomes per uterus, the mean total placentome weight and volume and mean placentome density are shown for each stage in Table 4.1.

4.3.1. Placentome Number

The total number of placentomes per uterus varied significantly with gestational stage ($p=0.014$) (Figure 4.1).

Thus, the total placentome number increased between Day 100 and Day 170 and decreased thereafter (Figure 4.1). The relationship, as derived from GLM analysis was:

Total number of placentomes = $-0.013*(\text{gestational age})^2 + 4.467*\text{gestational age} - 285.448$ ($R^2=0.26$). Differentiating the equation indicates that the maximum placentome number was attained on Day 172 of gestation.

4.3.1. Total Placentome weight

Total placentome weight per uterus increased significantly ($p<0.0001$) from Days 100 to 225 of gestation (Table 4.2 and Figure 4.2). Placentome weight increased exponentially as gestation length increased (coefficient of determination (R^2) = 0.92; $p<0.001$; Table 4.2). There was no association between the total number of placentomes with total placentome weight (Correlation coefficient (R) = 0.26; p= 0.23; Table 4.3). However, there was a significant association between the ratio of predicted to actual placentome number and the ratio of predicted to actual mean placentome weight (Figure 4.3). Uteri which had more placentomes than predicted tended to have lesser placentomes than predicted whereas the uteri which had fewer tended to have greater placentomes ($R^2= 0.601$; $p <0.001$). The relationship between placentome number and total weight was less strong ($R^2= 0.17$; $p =0.04$) and also positive; i.e. uteri which had more placentomes than predicted tended to have greter total weights of placentomes than predicted

Table 4.1: Comparison between gestational stages in mean (SEM) gestational age, placentome number, total placentome weight and volume and mean density.

Gestation stage (n)	Gestational age (days)	Total number of placentomes	Total weight of placentomes (g)	Total volume of placentomes (mL)	Mean placentome density (g/mL)
1 (5)	117 (2.4)	63.6 (9.1)	842 (113.1)	779 (118.3)	1.09 (0.028)
2 (5)	139 (2.7)	75.0 (10.7)	1196 (71.7)	1134 (72.9)	1.06 (0.014)
3 (5)	156 (2.8)	90.0 (12.6)	1966 (336)	1940 (380.7)	1.03 (0.028)
4 (5)	189 (4.2)	94.6 (10.4)	3090 (177)	2943 (188.4)	1.05(0.013)
5 (4)	202 (1.2)	82.0 (19.5)	5050 (194)	4510 (198.9)	1.11 (0.014)
P- value		P<0.014	P<0.0001	P<0.0001	P=0.05

n = number of placentas collected per stage.

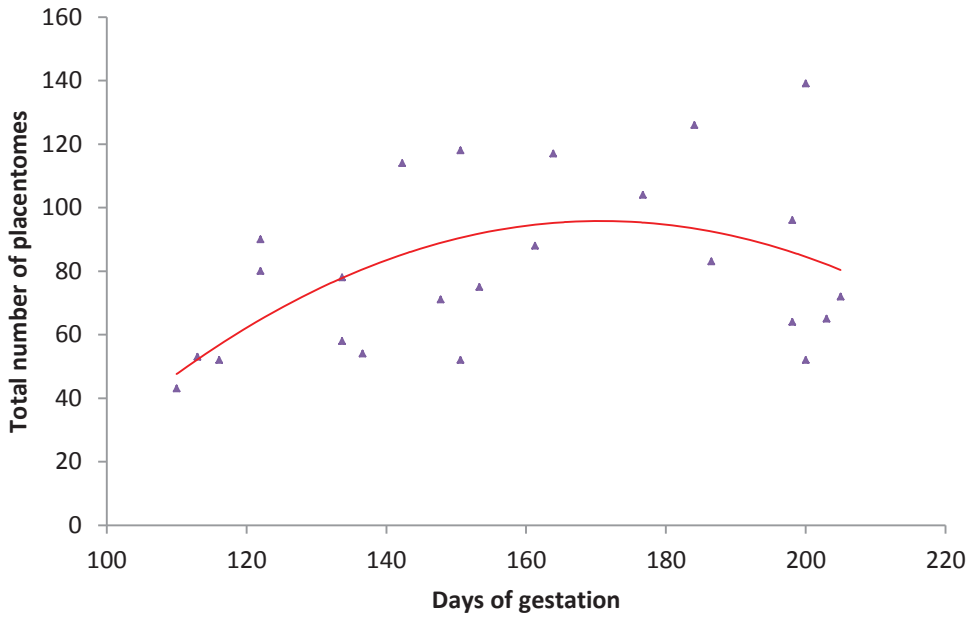


Figure 4.1: Change in total number of placentomes from Days 100 to 225 of gestation. Data from 24 uteri. The red line indicates the line of best fit.

Table 4.2: Relationship, as modelled using GLM, between gestational age (days) and weight (g), volume (mL) or density (g/mL) of placentomes and placentome components. Data from 24 uteri, and based on either all placentomes in the uterus (total) or ten randomly selected individual placentomes.

	Component	Weight		Volume		Density	
		Equation	R^2	Equation	R^2	Equation	R^2
Total	All Placentomes	$y = 74.344e^{0.020x}$	**0.92****	$y = 71.357e^{0.020x}$	**0.90****	$y = 3.713E\text{-}5x^2\text{-}0.012x + 1.956$	**0.33***
Individual placentome measurements	Intact Placentomes	$y = 3.5467e^{0.014x}$	**0.47****	$y = 3.308e^{0.014x}$	**0.47****	$y = -1.299E\text{-}5x^2+0.004x + 0.757$	0.06
	Maternal Caruncle	$y = 1.323e^{0.016x}$	**0.50****	$y = 1.213e^{0.016x}$	**0.50****	$y = -1.842E\text{-}5x^2+0.005x + 0.648$	0.05
	Fetal Cotyledon	$y = 2.536e^{0.011x}$	**0.35****	$y = 2.359e^{0.011x}$	**0.32****	$y = -1.035E\text{-}5x^2+0.003x + 0.833$	0.01
	Caruncle / cotyledon	$y = 1.1529x - 2.968$	**0.76****	$y = 1.523x - 2.864$	**0.77****	$y = 1.134e^{-0.094x}$	0.03

Significant (*P<0.05; **P<0.0001) associations (R^2) are in bold. Y = Component weight, volume or density; X = Gestational age as determined by crown rump length

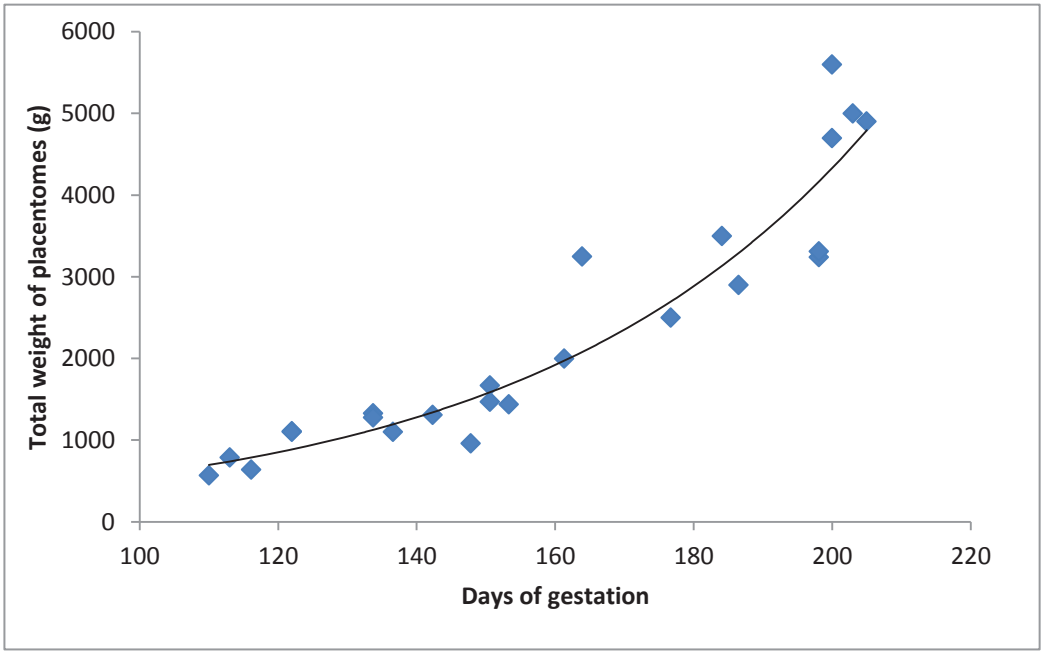


Figure 4.2: Relationship between the total weight of placentomes and gestational age. The black line shows the best line of fit; equation in Table 4.2.

Table 4.3: Correlation (p-value) between total number of placentomes per uterus with total placentome weight, volume and overall mean density of placentomes.

	Weight	Volume	Density (g/mL)
Placentome number	0.26 (0.23)	0.29 (0.17)	-0.31 (0.134)
Placentome weight (g)		0.99 (<0.001)	0.15 (0.49)
Placentome volume (mL)			0.07 (0.75)

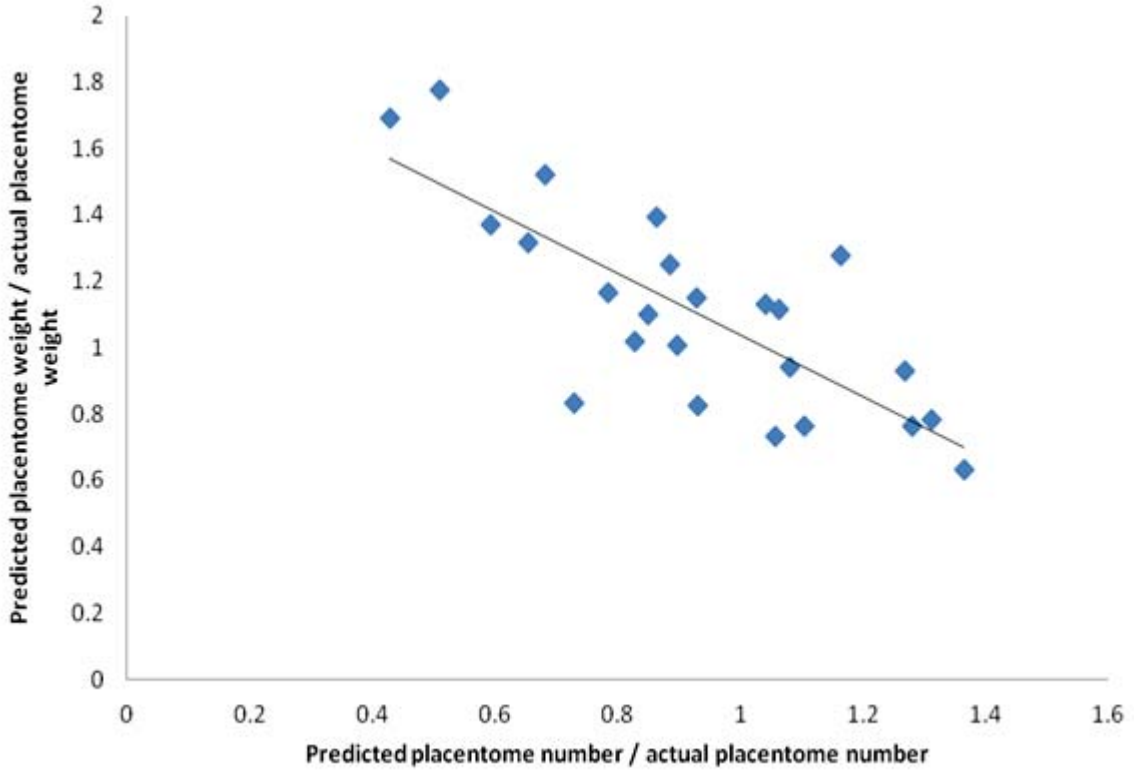


Figure 4.3: Relationship between ratio of predicted to actual placentome number and ratio predicted to actual mean placentome weight. The black line shows the best line of fit; predicted values were calculated using the equations in Table 4.2

4.3.3 Individual Placentomes

Figure 4.4 shows the effect of gestational age on the mean and range of placentome weight for the 10 randomly selected placentomes. As for total placentome weight, there was an exponential increase in mean individual placentome weight (R^2=0.47 and p=0.0001; Table 4.3). The mean weight of the individual placentomes varied from14.4 g on Day 110 to 65.9 g on Day 200. The mean weight of the individual placentomes increased following the same pattern as the mean placentome weight calculated from the data from all placentomes (Figure 4.5), but mean placentome weight of the individual placentomes was, on average, 8 g higher than mean placentome weight of all placentomes (i.e. total placentome weight / number of placentomes). This difference did not change with gestational age.

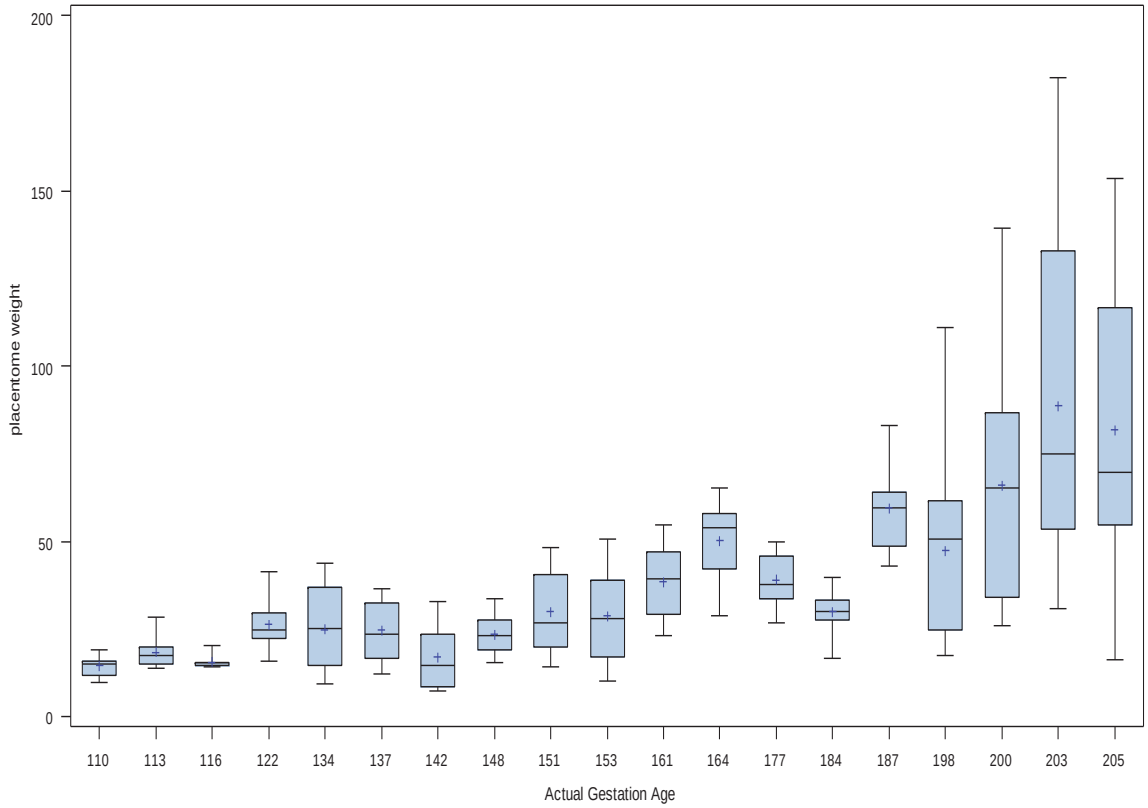


Figure 4.4: Box plots of weight (grammes) of ten randomly selected placentomes against gestational age (as estimated using fetal crown-rump length). Data from 24 uteri; two uteri were collected with estimated gestational ages of 122, 134, 151, 198 and 200 days. The black line (horizontal) in the box plot represents the mean for each uterus; the black vertical line above and below each box plot represents the range or spread of the data; the blue box represent the interquartile range or width of the data.

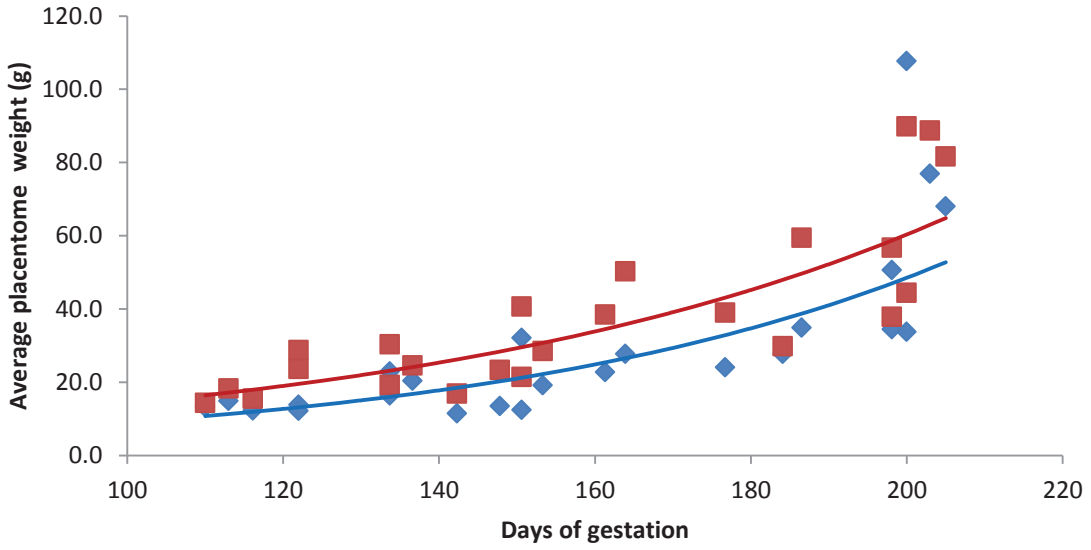


Figure 4.5: Comparison of mean placentome weight (as calculated from all placentomes; blue markers) and mean weight of ten selected (provided it was >15mm in size) individual placentomes.

4.3.2. Caruncle and cotyledon weight

Figure 4.6 shows the relationship between gestational age and the mean and range of caruncle weight for the 10 randomly selected placentomes. Caruncle weight increased exponentially with gestational age (R^2=0.50; $p<0.01$; Table 4.2). Mean caruncle weight increased from 6.9 g on Day 110 to 42.4 g by Day 200. Caruncle weight showed a strong correlation with placentome weight (R=0.98; $p<0.001$; Table 4.4).

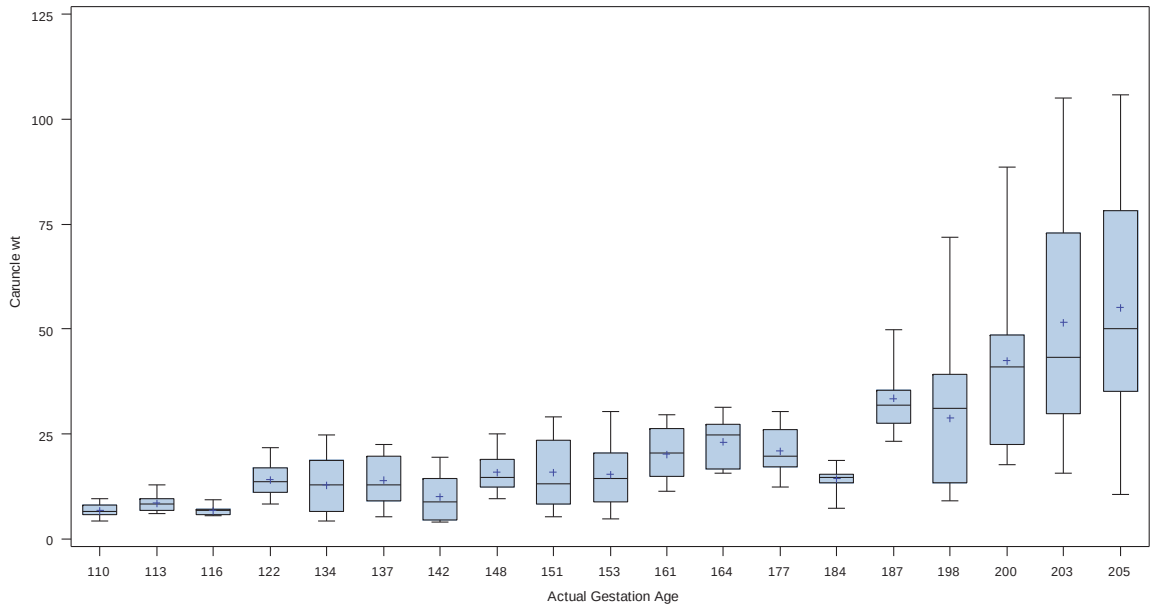


Figure 4.6: Box plots of weight (g) of caruncles dissected from ten randomly selected placentomes against gestational age (as estimated using fetal crown-rump length). Data from 24 uteri; two uteri were collected with estimated gestational ages of 122, 134, 151, 198 and 200 days. The black line (horizontal) in the box plot represents the mean for each uterus; the black vertical line above and below each box plot represents the range or spread of the data; the blue box represent the interquartile range or width of the data.

Table 4.4: Correlation of placentome and placentome component weight, volume and density. Data from 10 individual placentomes randomly selected (excluding placentomes <15mm in size) from the uteri of 24 cattle. All correlations were significant (P<0.001).

	Placentome volume	Caruncle weight	Caruncle volume	Cotyledon weight	Cotyledon volume
Placentome weight (g)	0.99	0.98	0.98	0.95	0.92
Placentome volume (mL)		0.98	0.98	0.95	0.93
Caruncle weight (g)			1.00	0.87	0.84
Caruncle volume (mL)				0.88	0.84
Cotyledon wt (g)					0.97

Figure 4.7 shows the relationship between gestational age on the mean and range of cotyledon weight for the 10 randomly selected placentomes. Cotyledon weight increased exponentially with gestational age (R^2=0.35; p<0.0001; Table 4.2) from a mean of 7.5 g on Day 110 to 23.5 g by Day 200. Cotyledon weight showed a strong correlation with both placentome and caruncle weight (R=0.95 and 0.87, respectively, p<0.001 for both). Fetal cotyledon weight increased at a slower rate (P<0.0001) than did caruncle weight (Figure 4.8). Caruncle and cotyledon weight were similar on Day 110 of gestation (6.9 g and 7.5 g, respectively) but, by Day 200, mean caruncle weight (42.4 g) was markedly greater than mean cotyledon weight (23.5 g).

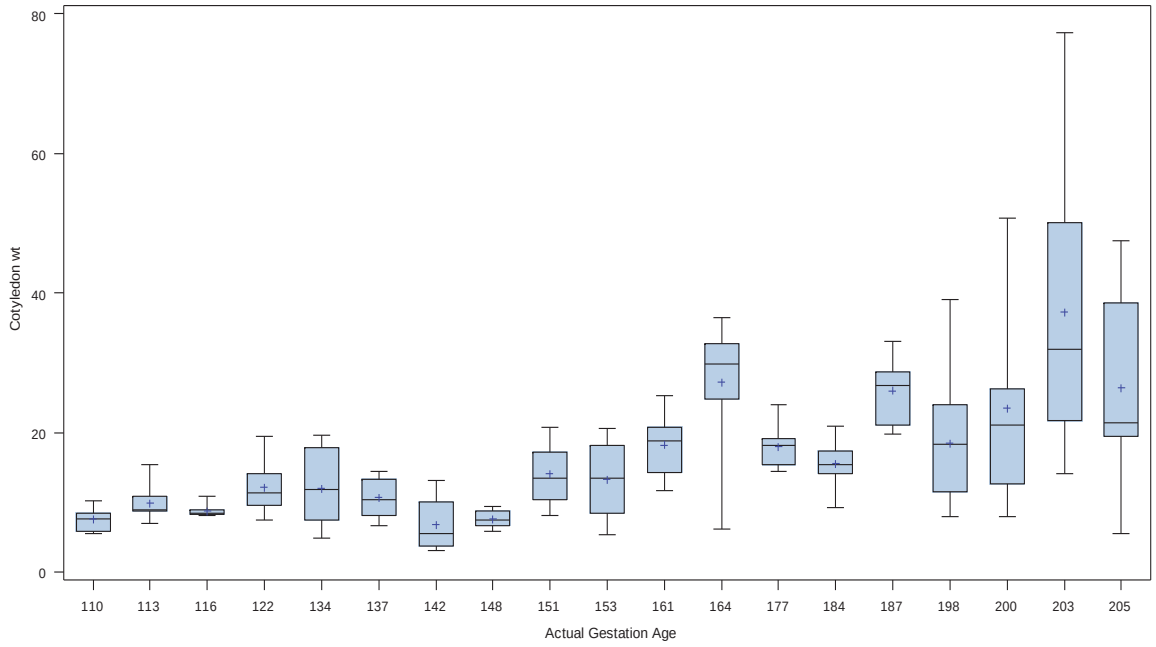


Figure 4.7: Box plots of weight of cotyledons dissected from ten randomly selected placentomes against gestational age (as estimated using fetal crown-rump length). Data from 24 uteri; two uteri were collected with estimated gestational ages of 122, 134, 151, 198 and 200 days. The black line (horizontal) in the box plot represents the mean for each uterus; the black vertical line above and below each box plot represents the range or spread of the data; the blue box represent the interquartile range or width of the data.

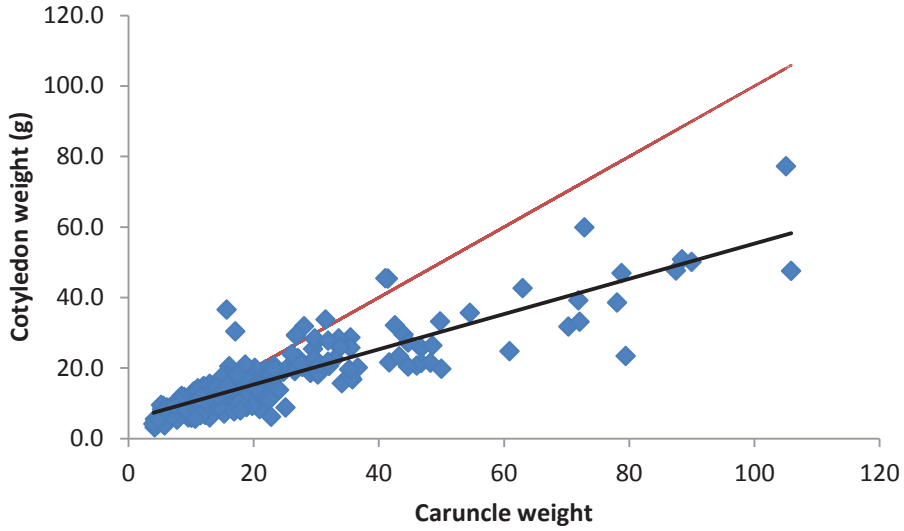


Figure 4.8: Relationship between individual cotyledon weight and caruncle weight between Days 100 to 225 of gestation. The black line indicates a line of best fit for the relationship between the fetal and maternal tissue while the red line is the line of identity, i.e. if cotyledon weight were equal to caruncle weight.

4.3.3. Total Placentome volume

Total placentome volume for each uterus increased significantly ($p<0.0001$) from Days 100 to 225 of gestation (Table 4.1 and Figure 4.9). Placentome volume increased exponentially as gestation length increased ($R^2=0.90$; $p<0.001$; Table 4.3). There was no correlation between the total number of placentomes and total placentome volume ($R=0.29$; $p=0.17$), but total placentome volume was strongly correlated to total placentome weight ($R=0.99$; $p< 0.001$; Table 4.3). Figure 4.10 shows the linear relationship between total placentome weight and volume.

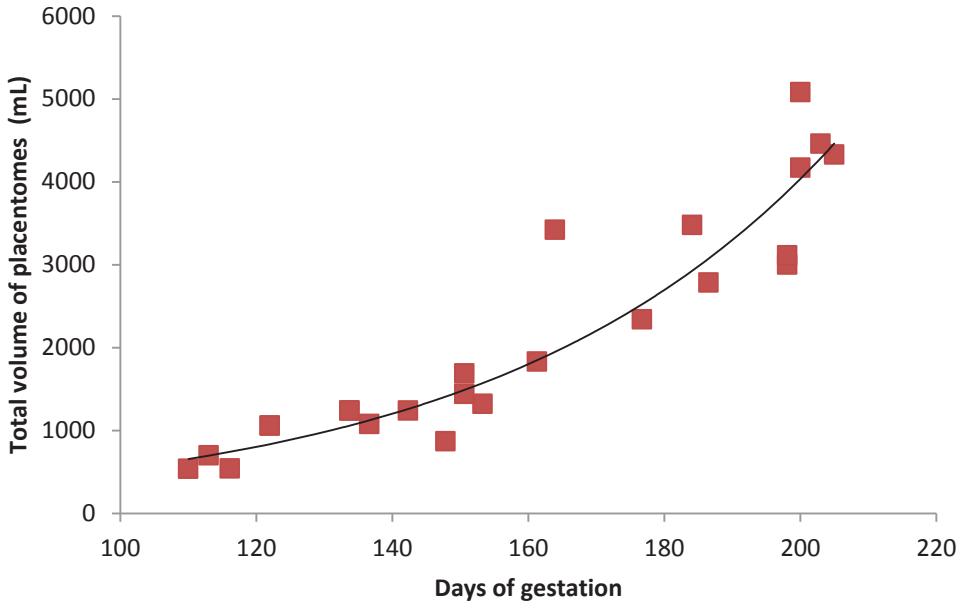


Figure 4.9: Relationship between the total volume of placentomes and gestational age. Data from 24 uteri. The black line shows the best line of fit; equation in Table 4.2.

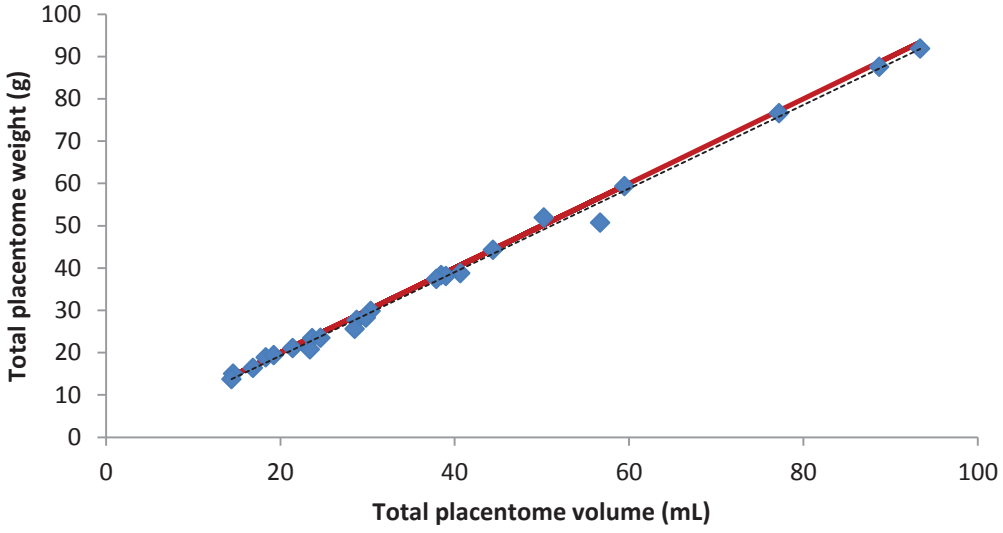


Figure 4.10: Relationship between total placentome weight and volume. The black dotted line is the line of best fit and the red solid line is the line of identity.

4.3.4. Individual placentome volume

Figure 4.11 shows the relationship between gestational age and the mean and range of placentome volume for the 10 randomly selected placentomes. As with total placentome volume, there was an exponential increase in individual placentome weight (R^2=0.47 and p<0.0001; Table 4.3) from a mean of 13.7 mL on Day 110, to a mean of 65.2 mL on Day 200 of gestation. The mean volume of the individual placentomes increased following the same pattern as the mean placentome volume calculated from the data from all placentomes, but mean placentome volume of the individual placentomes tended to be approximately 8 mL higher (Figure 4.11). This difference did not change with gestational age.

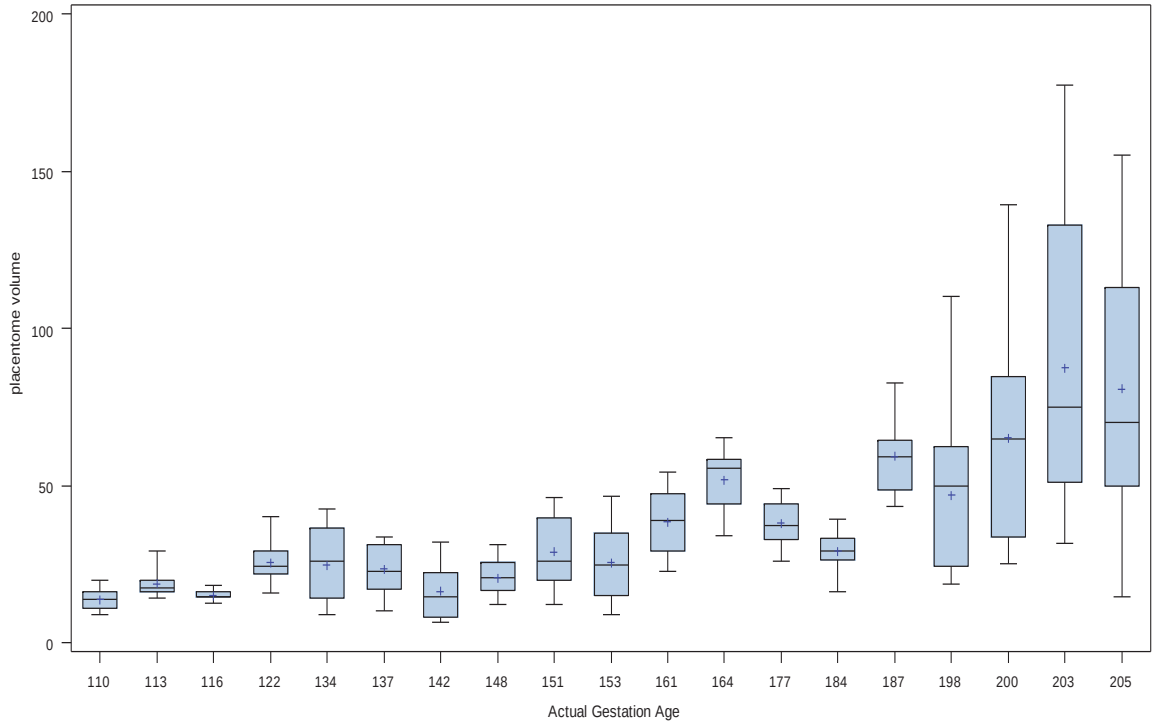


Figure 4.11: Box plots of volume(mLs) of ten randomly selected placentomes against gestational age (as estimated using fetal crown-rump length). Data from 24 uteri; two uteri were collected with estimated gestational ages of 122, 134, 151 198 and 200 days. The black line (horizontal) in the box plot represents the mean for each uterus; the black vertical line above and below each box plot represents the range or spread of the data; the blue box represent the interquartile range or width of the data.

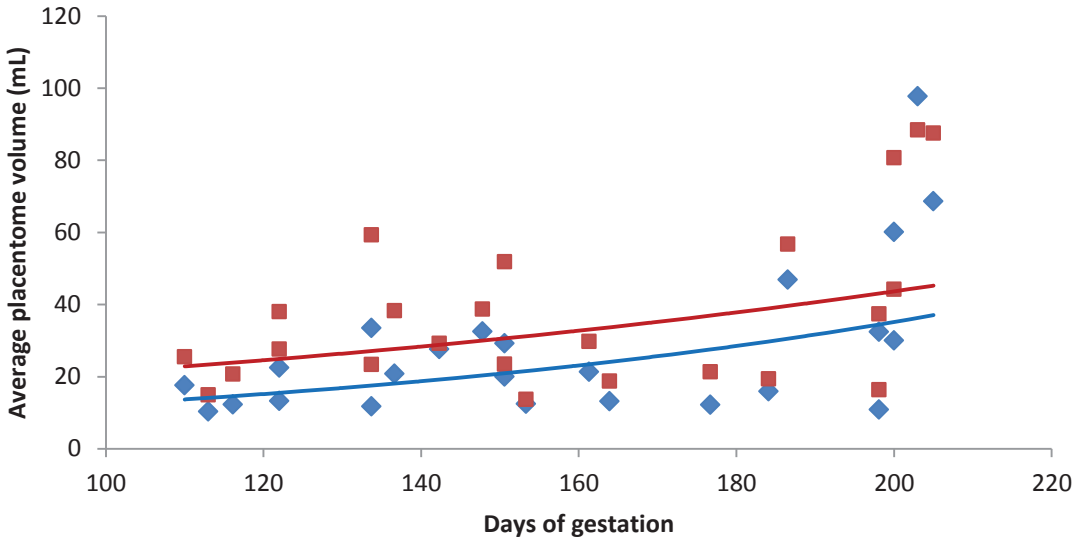


Figure 4.12: Comparison of mean placentome volume (as calculated from all placentomes; blue markers and solid line as line of best fit) and mean weight of ten selected (provided it was >15 mm in size, red markers and solid line as line of best fit) individual placentomes.

4.3.5. Caruncle and cotyledon volume

The relationship between gestational age and the mean and range of caruncle volume for the 10 randomly selected placentomes is shown in Figure 4.13. Caruncle volume increased exponentially with gestational age (R^2=0.50; p<0.0001; Table 4.3) from a mean of 6.6 mL on Day 110 to 42.1 mL by Day 200 of gestation. Caruncle volume was strongly correlated with placentome volume (R=0.98; p<0.001; Table 4.4).

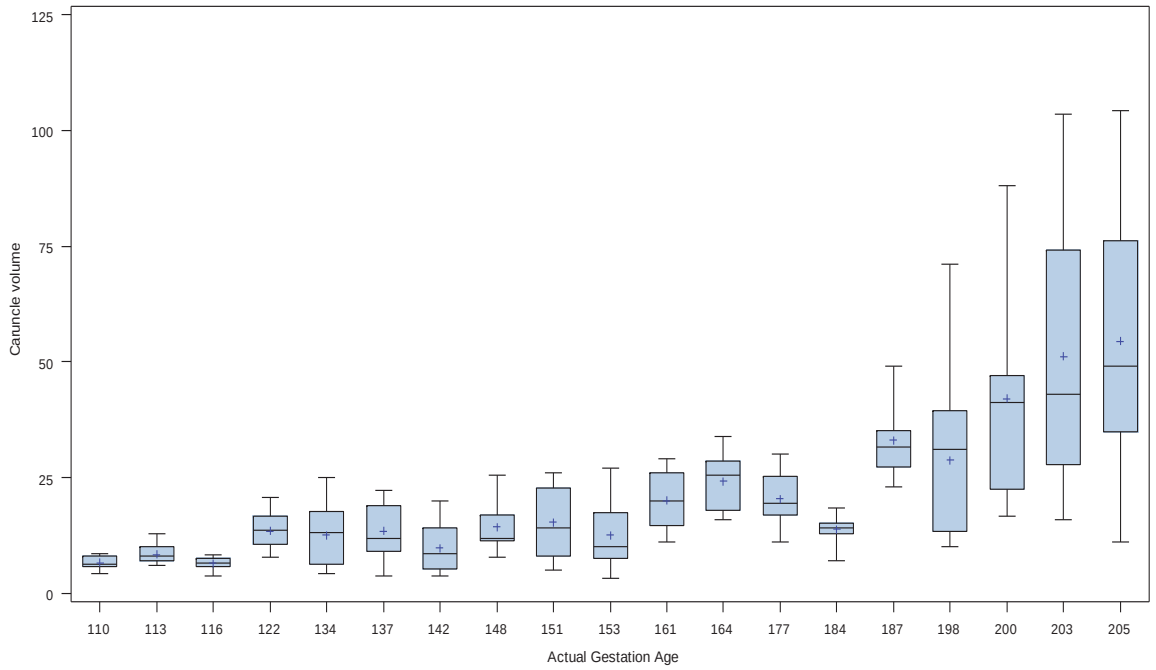


Figure 4.13: Box plots of volume (mLs) of caruncle dissected from ten randomly selected placentomes against gestational age (as estimated using fetal crown-rump length). Data from 24 uteri; two uteri were collected with estimated gestational ages of 122, 134, 151, 198 and 200 days. The black line (horizontal) in the box plot represents the mean for each uterus; the black vertical line above and below each box plot represents the range or spread of the data; the blue box represent the interquartile range or width of the data.

The relationship between gestational age and the mean and range of cotyledon volume for the 10 randomly selected placentomes is shown in Figure 4.14. Cotyledon volume increased exponentially with gestational age (R^2=0.32; p<0.0001; Table 4.3) from a mean of 7.0 mL on Day 110 to 23.1 mL by Day 200. Cotyledon volume showed a strong correlation with both placentome and caruncle volume (R=0.93 and 0.84, respectively, p<0.001 for both). Fetal cotyledon volume tended to increase at a slower rate than caruncle volume (Figure 4.15). Caruncle and cotyledon volume were similar on Day 110 of gestation (6.7 mL and 7.0 mL, respectively) but, by Day 200, mean caruncle volume (42.1 mL) was markedly greater than mean cotyledon volume (23.1 mL).

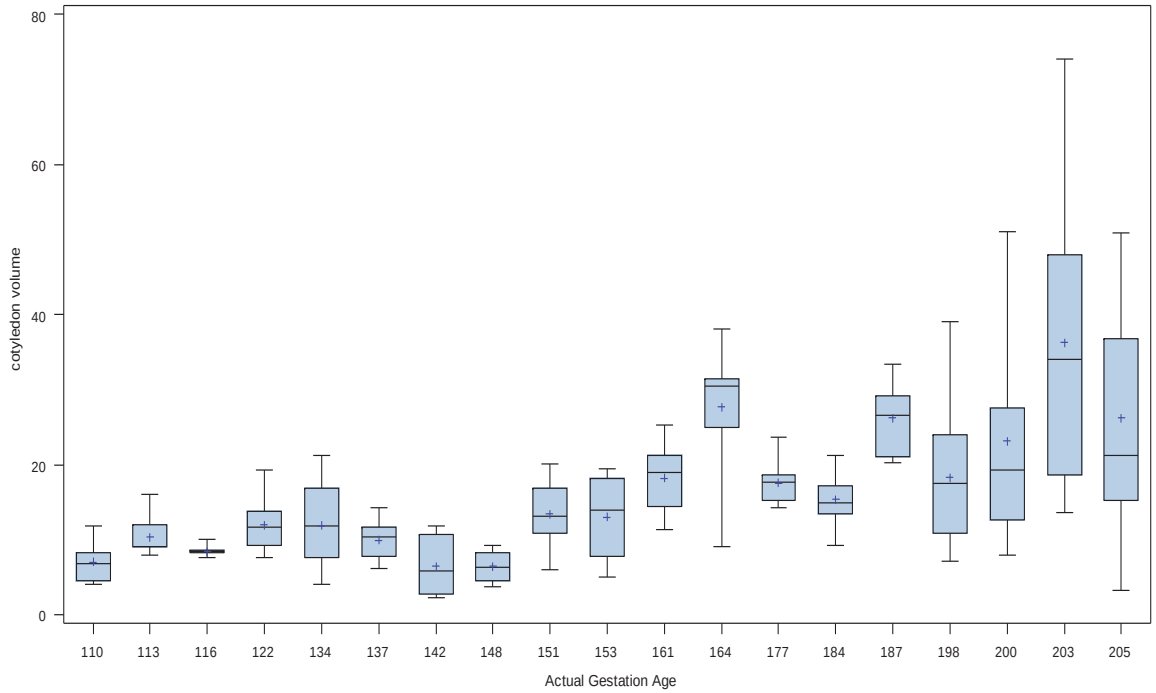


Figure 4.14: Box plots of volume (mLs) of cotyledon dissected from ten randomly selected placentomes against gestational age (as estimated using fetal crown-rump length). Data from 24 uteri; two uteri were collected with estimated gestational ages of 122, 134, 151, 198 and 200 days. The black line (horizontal) in the box plot represents the mean for each uteri; the black vertical line above and below each box plot represents the range or spread of the data; the blue box represent the interquartile range or width of the data.

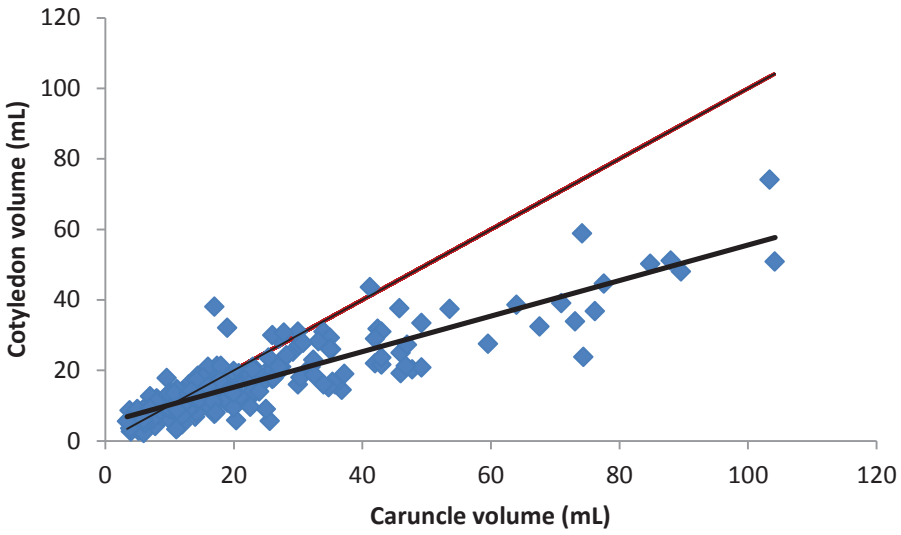


Figure 4.15: Relationship between individual cotyledon volume and caruncle volume from Days 100 to 225 of gestation. The black line indicates a line of best fit for the relationship between the fetal and maternal tissue while the red line is the line of identity, i.e. if cotyledon volume were equal to the caruncle volume.

4.3.6. Overall mean placentome density

Mean placentome density (total placentome weight/total placentome volume) decreased between Days 110 and 160, and then increased thereafter (Figure 4.16). This quadratic relationship between placentome density and gestational age was significant (R^2=0.33; p=0.015; Table 4.2). There was no association between overall placentome density and placentome weight, volume or number (p>0.135; Table 4.3).

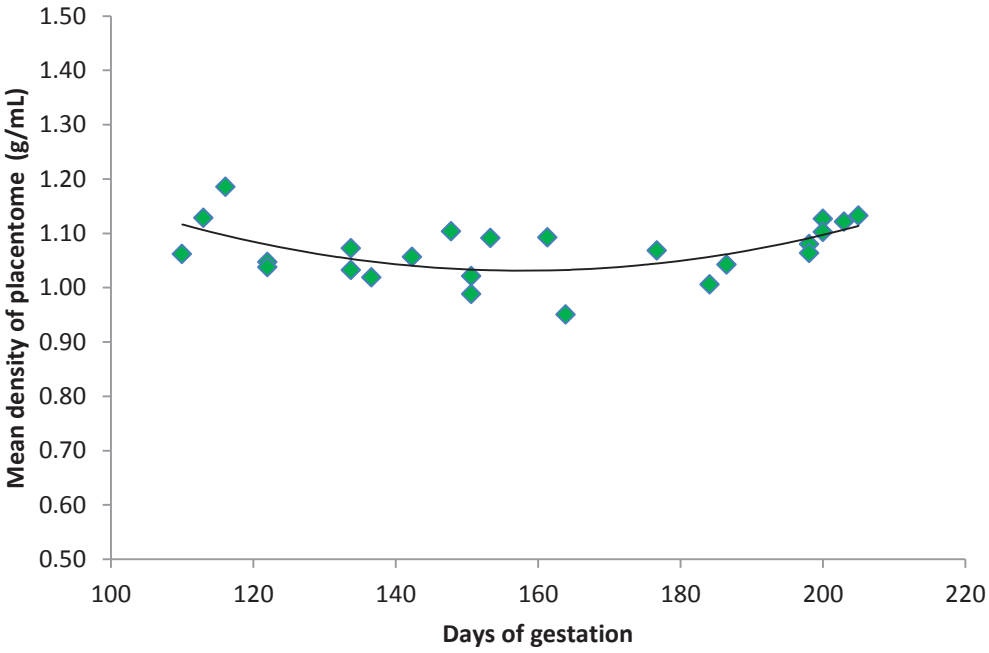


Figure 4.16: Relationship between the mean density of all placentomes (total placentome weight/total placentome volume) and gestational age. Data from 24 uteri. The black line shows the line of best fit; equation in Table 4.2. Differentiating the equation indicates that the minimum placentome density was attained on Day 162 of gestation when the density was 0.95 g/mL.

4.3.9 Individual placentome density

The relationship between gestational age and the mean and range of placentome density for the 10 randomly selected placentomes is shown in Figure 4.17. As for total placentome density, the model which identified the best fit between gestational age and individual placentome density was a quadratic one. However, in contrast to overall data, this was not a significant association (R^2=0.06; Table 4.3).

4.3.9.1 Caruncle and cotyledon density

The effects of gestational age on the mean and range of caruncle density for the 10 randomly selected placentome is shown in Figure 4.18. There was no significant association between caruncle density and gestational age (R^2=0.05; Table 4.3).

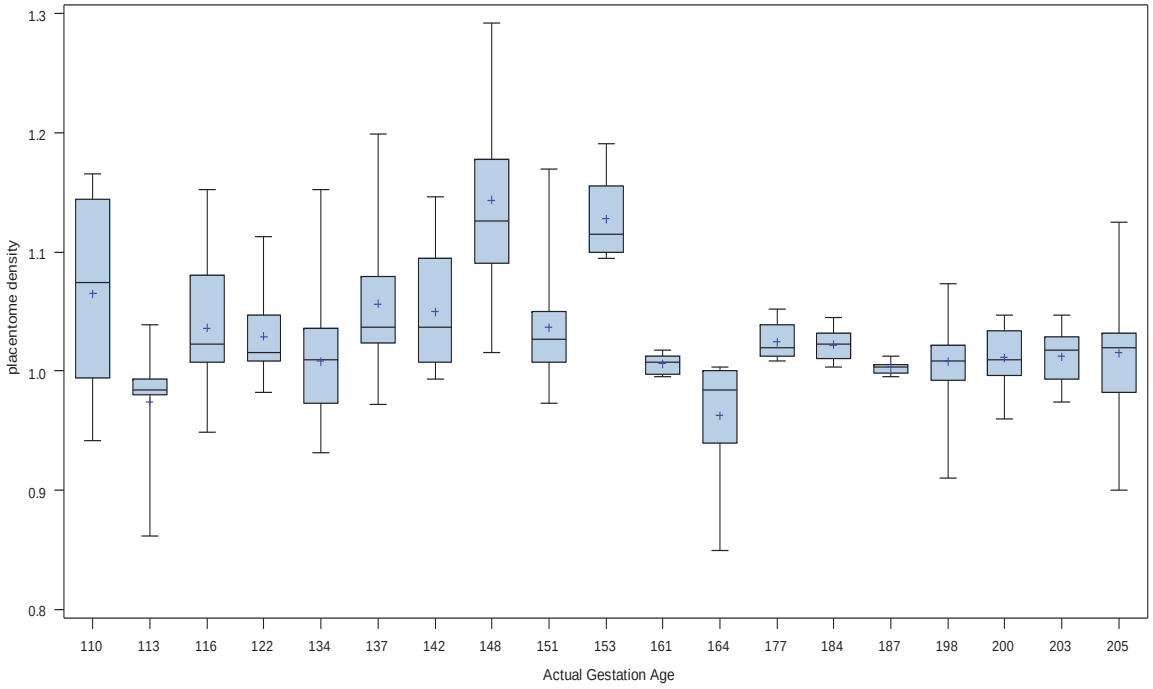


Figure 4.17: Box plots of density (g/mLs) of ten randomly selected placentomes against gestational age (as estimated using fetal crown-rump length). Data from 24 uteri; two uteri were collected with estimated gestational ages of 122, 134, 151, 198 and 200 days. The black line (horizontal) in the box plot represents the mean for each uterus; the black vertical line above and below each box plot represents the range or spread of the data; the blue box represent the interquartile range or width of the data.

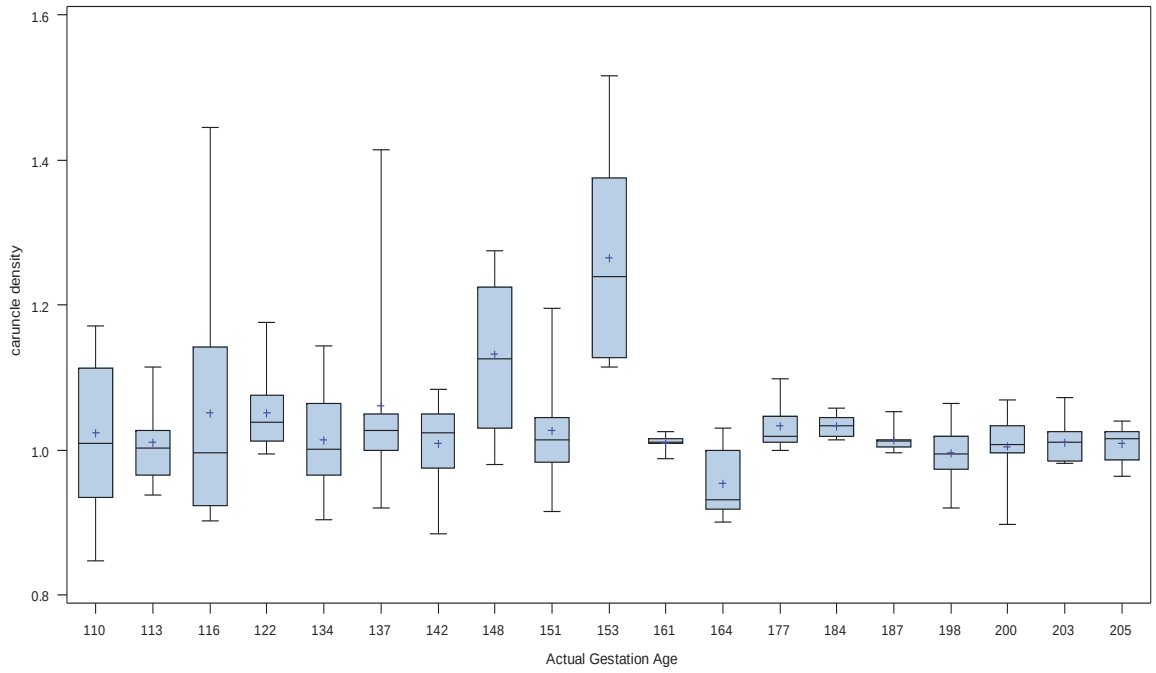


Figure 4.18: Box plots of density (g/mLs) of caruncles dissected from ten randomly selected placentomes against gestational age (as estimated using fetal crown-rump length). Data from 24 uteri; two uteri were collected with estimated gestational ages of 122, 134, 151, 198 and 200 days. The black line (horizontal) in the box plot represents the mean for each uterus; the black vertical line above and below each box plot represents the range or spread of the data; the blue box represent the interquartile range or width of the data.

The relationship between gestational age on the mean and range of cotyledon density for the 10 randomly selected placentomes is shown in Figure 4.19. As for caruncle density, there was no significant association between mean cotyledon density and gestational age (R^2=0.01; p=0.35; Table 4.3). When the individual data from each placentome was analysed there was a small but significant association between the caruncle and cotyledon density from the same placentome, even taking into account the effect of cow (R=0.05; p=0.01).

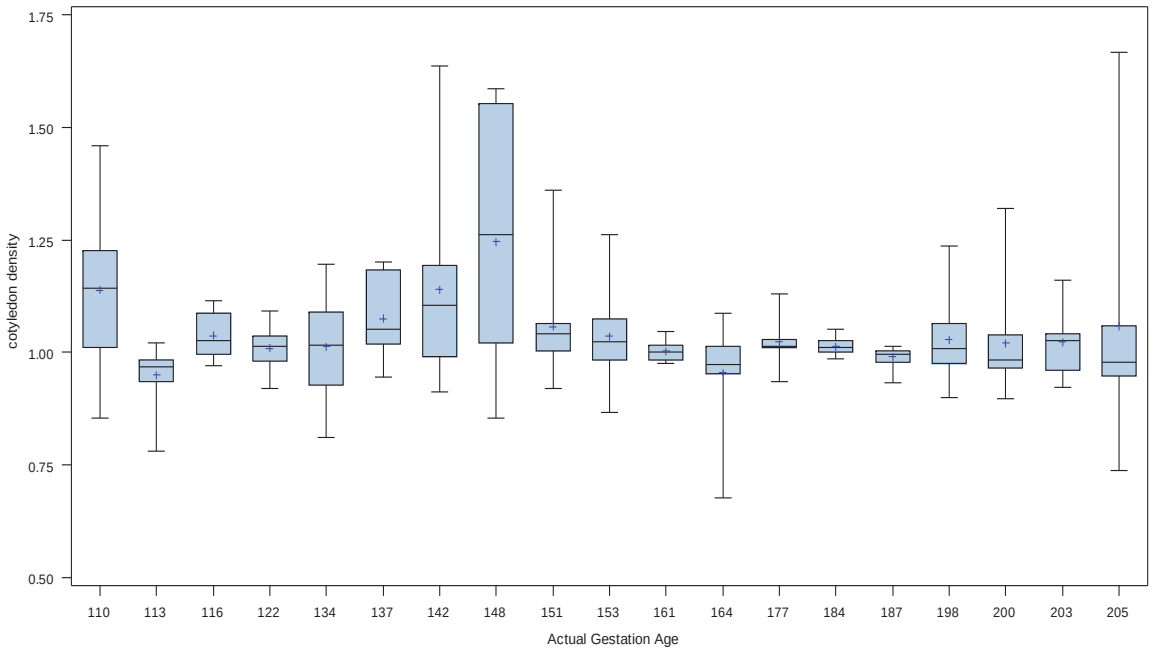


Figure 4.19: Box plots of density (mLs) of cotyledons dissected from ten randomly selected placentomes against gestational age (as estimated using fetal crown-rump length). Data from 24 uteri; two uteri were collected with estimated gestational ages of 122, 134, 151, 198 and 200 days. The black line (horizontal) in the box plot represents the mean for each uterus; the black vertical line above and below each box plot represents the range or spread of the data; the blue box represent the interquartile range or width of the data.

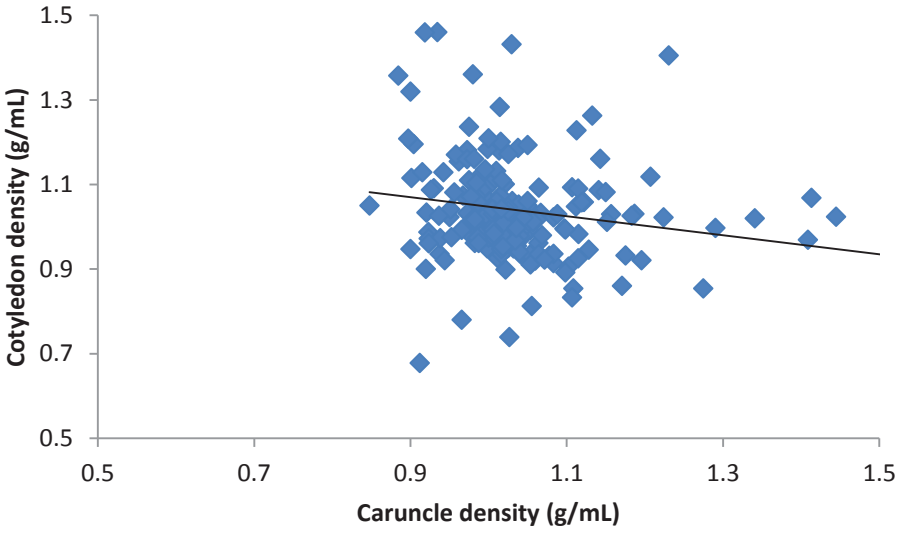


Figure 4.20: Relationship between individual cotyledon and caruncle density. The black line indicates the line of best fit.

4.4. Discussion

This study has provided new information on the relationship between volume and weight of the complete placentome and its maternal and fetal components, as well as providing additional baseline information on placental parameters such as placentome number. It has quantified, for the first time, that there is a small but significant change in overall placentome density within the second trimester of gestation, and this change is not dependent upon the relative contributions of maternal and fetal tissue. Moreover, it has quantified changes in placentome number over gestation, suggesting that, contrary to expectation, placentome number may not be fixed over the duration of pregnancy.

The total number of placentomes varied significantly with gestational age (Figure 4.1; P=0.014). Placentome number increased gradually from the first stage of gestation (Day 100-125; mean=64), reaching a maximum by Day 176-200 (mean = 95), and then decreasing to a mean of 82 placentomes by Day 225 of gestation The association between placentome number and gestation stage was relatively small (R^2=0.26); i.e. it explained only about a quarter of the total variation in placentome number. Previously Laven and Peters (2001) reported no significant association between placentome number and stage of gestation. However in that study, the effect of stage was almost significant (p=0.07) and the pattern of change seen (low numbers in early gestation, peaking in the second trimester, and then a small decrease over the third trimester) was similar. Further research is required to better establish how placentome numbers change during gestation and whether this has a significant impact on fetal development or placental capacity. Placentome weight increased exponentially as gestational age increased (R^2=0.92; P<0.0001; Figure 4.2). This was consistent with previous measures of placentome weight in cattle (Reynolds and Redmer 1995; Laven and Peters 2001), but differs from similar data from ewes carrying a single fetus, in which a significant decline in total cotyledon weight occured between Days 95 and 135 of pregnancy (Alexander 1964; Doize *et al.* 1997). On the other hand, ewes carrying twin pregnancies show a modest (albeit non-significant) increase in cotyledon weight over the same stage of pregnancy.

No evidence of an association between total placentome number and total placentome weight was found, consistent with the findings of Laven and Peters (2001). However additional analysis of this association, using the data from the present study, showed that uteri with more placentomes than predicted by the regression equations tended to have smaller

placentomes than predicted (and vice-versa). This suggests that there is a minimum weight of placentomes that each cow needs to achieve at each gestational stage and that this can be achieved either by multiple small placentomes or fewer larger placentomes. The drivers for this effect are unclear, but the estimated difference in average placentome weight is quite large (some uteri had placentomes which were 42% larger than predicted for their stage of gestation, others were 45% smaller). Some of this effect might be breed related; Dwyer *et al*.(2005) reported that Suffolk ewes have placentas with fewer larger cotyledons than Blackface ewes. No similar data are available for cattle. This increase in mean placentome size in uteri with fewer than predicted placentomes did reduce the impact of placentome number on total weight of placentomes but not completely as uteri with fewer than predicted placentomes still tended to have lower total placentome weights than predicted. Actual total placentome weight varied between 60 and 150% of predicted but most were between 80 and 120%. This great variability is again consistent with the conclusion by Laven and Peters (2001) that most placentas possess significantly more capacity than needed

Both caruncle weight and cotyledon weight increased exponentially with gestation age (R^2=0.50 and 0.35, respectively). At the individual placentome level, the relationship between caruncle weight and cotyledon weight was very strong. Cotyledon weight increased at a slower rate than caruncle weight; which was also found by Reynolds *et al*. (1990). Both that study and the present results therefore show that the proportion of placentome tissue which is caruncular in origin increases with increasing gestational age. Reynolds *et al.*. (1990) reported that by Day 200, the ratio of maternal tissue by weight was 1.65:1. In the present study the ratio of caruncle: cotyledon weight at that stage of gestation was 1.69: 1. If this increase was due to poor tissue separation it would imply that, assuming caruncle weight was actually equal to cotyledon weight, 21% of the tissue assigned as caruncle was actually cotyledon. This is exceedingly unlikely to be the situation, supporting the contention of Reynolds *et al*. (1990), that this phenomenon cannot be explained in such terms.

Placentome volume increased exponentially with gestational age (Figure 4.9). There was a strong relationship between total placentome weight and volume (R^2=0.99), such that the changes in placentome volume followed a very similar pattern to that seen for total placentome weight.

Both caruncle volume and cotyledon volume increased exponentially with gestational age (R^2=0.50 and 0.32, respectively; Figures 4.13 and 4.14). At the level of the individual

placentome, the relationship between caruncle volume and cotyledon volume was very strong. However, cotyledon volume increased more slowly than did that of the caruncle. The ratio of caruncle to cotyledon tissue by volume was greatest (at 1.67:1), in the samples from cows which had been pregnant for 200-225 days, the same stage at which the population of caruncular tissue by weight had been maximal. This finding is in contrast to that of Laven and Peters (2006), who reported, on the basis of image analysis data, that the ratio of maternal: fetal tissue by volume decreased with gestational age, so that by 200 days of gestation the ratio of maternal to fetal tissue was 0.85:1. This suggests that the two techniques – i.e. measuring relative volume by image analysis and by water displacement - are not measuring the same aspects of the fetal and maternal tissue contributions. This also seems likely to also be the case for the stereology data reported by Kannekens *et al.* (2006), in which, at Day 135 of pregnancy, the ratio of maternal to fetal tissue was 0.92:1. From the present study, the expected ratio of maternal: fetal tissue by gestational age at 135 days was approximately 1.5:1, much larger than that reported by Kannekens *et al.* (2006).

Thus, in contrast to the present findings based upon volumetric measurements, histological studies of the placentome (i.e. in which fetal and maternal tissue contributions have been estimated by stereology and image analysis), have indicated that fetal tissue grows faster than the maternal tissue during gestation (Kannekens *et al.* 2006; Laven and Peters 2006;). This apparent discrepancy can be resolved by realising that histology focuses on the central part of the placentome (fetal- maternal exchange area), but does not include the peripheral areas of the placentome, which volumetric measures do. Therefore, the two techniques are probably not directly comparable because they are measuring different portions of the placentomal tissue. Histological techniques give comprehensive details of the cellular growth during gestation between the fetal and maternal tissues, but does not evaluate what tissue is contributing the overall placentome in terms of weight or mass.

Overall mean density was 1.1 g/mL. Although placentome weight and volume were strongly related, there was a significant change in placentome density with gestational age (P=0.015; Figure 4.16). However, the range over which placentome density changed was small (decreased from 1.13 to 1.09) and appears unlikely to be of biological significance. This suggests that the hypothesis that changes in density of the bovine placentome during gestation might have a significant impact on the interpretation of stereology results was not supported.

Individual placentome density showed no significant effect of time, which is similar to those of the caruncle and cotyledon density. This supports the assumption that the change with time in total density was a statistical artefact, which may simply be due to the strong relationship between weight and volume. Individual placentomes within cows showed greater variation in density (Figure 4.17). Some of this effect may be measurement error as water displacement errors may have been relatively larger for the smaller placentomes than for the entire placenta, but the effect was still small, with the majority of placentomes having a density between 1.0 and 1.2 g/mL.

4.5. Conclusion

The consistent increase of the maternal tissue component to the bovine placentome found in this study indicates a continuum of development of placental function during gestation. Thus, anything that affects maternal caruncle size has the potential to also affect the functional capacity of the placenta and, hence, nutrient partitioning to the fetus. The mechanism responsible for the increase in maternal component of the placenta is not clear, but it is likely that the maternal components respond to fetal demands by increasing in size, thus increasing the number of crypts for the fetal villi for attachment as pregnancy advances. Volumetric measurement of the placentome showed a similar trend to weight; the change in density of the placentome was small and of limited range. Further research is required to investigate the growth mechanism of the fetal and maternal tissues within feto-maternal interface using stereology.

Chapter Five

Determination of Gestational Age in Cattle Using Trans-rectal Ultrasound Measurement of Placentomes.

5.1. Introduction

In ruminants, placentome development has been found to be positively correlated with gestational age (Laven and Peters 2001) and could therefore be useful in estimating the age of the fetus, particularly where breeding records are not available.

However although visualisation of the placentomes is a common part of trans-rectal ultrasound pregnancy diagnosis, and the relationship between placentome size and stage of pregnancy is often quoted in teaching material (e.g http://www.vetmed.lsu.edu/eiltslotus/theriogenology-5361/bovine_pregnanacy.htm), there are no published studies assessing the value of placentome measurement via trans-rectal ultrasound for determining gestational age in cattle. The only published peer-reviewed study of placentome measurement in cattle as a method for determining gestational age is that by Hunnam *et al*. (2009a) who measured placentome length and height over gestation period of 73 to 190 days by using transcutaneous ultrasonography over the right flank in 224 dairy cows. There were no significant relationship between gestation age and placentome length and height due to different sizes of placentomes closest to the fetus and lack of landmarks to standardise measurement of the placentomes (Hunnam *et al.* 2009a). Placentome size does vary significantly over the uterus, with this variability increasing over time (Chapter in this thesis(6); Laven and Peters 2001), with the variability greatest where placentome size is greatest, i.e. nearest the fetus and least where the placentomes are small, i.e. at the tips of the uterine horns. For monitoring gestational age, placentome variation for reasons other than gestational age needs to be limited. Hence this study measured placentomes closest to the cervix, where there is significant change with time but limited variation (Laven, unpublished observations; (Youngquist 1997). This has additional advantages in that cervical placentomes are easily accessible, particularly later in gestation when peri-fetal placentomes can become difficult to collect and in a study such as this, it is likely that repeat measurements are measuring a similar population of placentomes, which is less likely if the fetus or external anatomical land marks are used to identify the measurement site.

The aims of this study were to examine the time–course of placentome growth *in-vivo*, as visualised by trans-rectal ultrasound; and to determine whether there was a predictive relationship between placentome size and gestational age in cattle.

5.2. Material and Methods

5.2.1. Animals

The animals used for this study came from two farms, one in Manawatu (Farm A; n=28 cows; Friesian (n=12); Jersey (n=1); Crossbred n=15 breeds) and one in the Taranaki (Farm B; n=30 cows; Friesian (n=16); Jersey (n=14); Crossbred n=2 breeds). Both farms were spring-calving, pasture-based farms with limited additional supplement. The cattle selected for study from Farm A were a convenience sample of 28 cows selected on the basis of having conceived at the first inseminantion after an oestrus synchronisation programme of intravaginal progesterone plus GnRH-$PGF_{2\alpha}$-GnRH programme. The cattle from Farm B were also a convenience sample which was anticipated to be at the correct stage of pregnancy (approximately 60 days post conception). Cows from Farm A, were inseminated (Artificial inseminantion AI) between 22nd of October 2009 and 9-10th November 2009 and on 14th October 2009 for Farm B. Ultrasound scanning commenced on the 15th December 2009 and 8th January, 2010 for Farm A and Farm B respectively.

5.2.2. Ultrasonography examination

Animals were scanned trans-rectally, using a B-Mode real-time ultrasound scanner with a 7.5 MHz linear probe (Mindray DP6600, Mindray Szechuan, China) once every 10 days from Day 60 to Day 130 of gestation and once every 15 days from day 130 to Day 160 of gestation. On each occasion the placentomes for scanning were determined by locating the cervix using the ultrasound scanner and then moving the probe one probe length (about 6 cm) from the cervix. The pregnant and the non-pregnant horns were located to take measurements of placentomes

5.2.3. Measuring placentome size

Four to six placentomes were scanned on each occasion in both the pregnant and non-pregnant horn and were recorded as digital images for later analysis using image analysis. For each image, the probe was moved to minimise the difference between placentome length and width, (i.e. make the placentome image as circular as possible). The images were then transferred to a desktop computer for image analysis. Computer-assisted online image analysis software (ImageJ) was used to measure the placentomes as described below: (Figure 5.1)

a) Long axis: the length of the longest axis across the placentome
b) Area : this involves measurements around placentomes that were imaged and captured

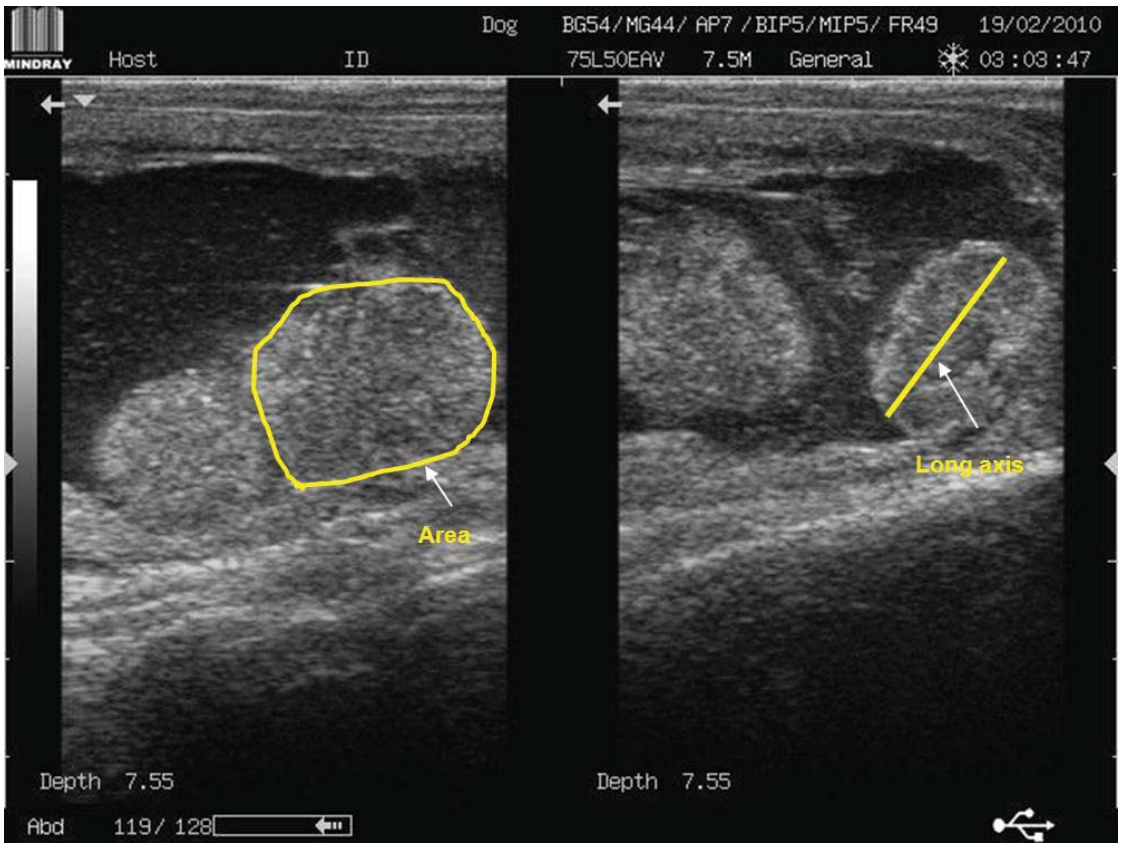


Figure 5.1: Images of placentomes showing measurements by using Image J software

5.2.4. Statistical analysis

The association between mean placentome length, area and gestation age were analysed using the correlation coefficient (R= 0.92). Due to the strong correlation between length and area, placentome length alone was used for further analysis.

The association between gestational age and placentome size was then analysed using mixed linear models (PROC MIXED, R statistics 2.14.2, 2012) with repeated measures (scan date). Initial data analysis showed that including cow as a random effect in the model did not appreciably increase the fit of the model, so further analysis of the relationship was undertaken using fixed effects alone. Placentome length (natural log transformed prior to use to normalise the residuals) was the response variable, and farm, breed (Jersey, or Friesian or crossbred), age (primiparous or multiparous), horn (non-pregnant or pregnant horn) and days pregnant were used as fixed effects.

The model used was:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_4 + \beta_5 x_5 + e$$

Where:

- y is log placentome length
- β_0 is the intercept,
- β_1 = the fixed effect of farm, x_1,
- β_2 = the fixed effect of breed, x_2,
- β_3 = the fixed effect of age, x_3,
- β_4 = the fixed effect of horn, x_4.
- B_5 = the fixed effect of days pregnant, x_5.
- e = is the residual error corresponding to observation

5.2.4.1. Limits of agreement analysis

The best model of placentome length, which included only days pregnant as a fixed effect, was then used to create a predicted gestational age, from the mean placentome length, for each cow at each time point. Limits-of-agreement analysis was then used to identify the agreement between the predicted and the actual gestational age. The first analysis calculated the limits-of-agreement using only the standard deviation of the difference between the predicted and actual gestational age ('basic' limits-of-agreement) (Bland and Altman 1999). As this analysis did not take into account the fact that the data included repeated pairs of measurements on the same subject or any association between method difference and gestational age, a second ('adjusted') limits-of-agreement was calculated as outlined by Bland and Altman (2007) for repeated measures where the true value of the measured quantity was changing, taking into account any association between method difference and gestational age.

This process was then repeated for gestational age predicted using log mean length calculated using data from both horns (adjusting the length of placentomes from the non-pregnant horn to account for the effect of horn on placentome size).

Finally, mean placentome length was calculated using data from all placentomes, ignoring the effect of horn, and a simple predictive model of placentome length, which included only days pregnant as a fixed effect, was then used to create a predicted gestational age for each cow at each time point. The limits of agreement analysis described above was then repeated using this model.

5.3. Results

5.3.1. Descriptive statistics

Tables 5.1 and 5.2 showed the mean and the standard deviation for placentome length and area for both pregnant and non-pregnant horn for breeds and on both farms.

Table 5.1: Mean ± Standard deviation for placentome length and area per breed for the pregnant and the non-pregnant uterine horns.

	Friesian	
Placentome dimensions	**Pregnant horn**	**Non-Pregnant**
Length (mm)	20.6±9.6	21.3±9.2
Area (mm^2)	249.1±218.5	256.3±200.7
	Jersey	
	Pregnant horn	**Non-Pregnant**
Length (mm)	18.4±8.9	19.2±8.1
Area (mm^2)	206.5±193.6	211.7±165.9
	Crossbred	
	Pregnant horn	**Non-Pregnant**
Length (mm)	21.8±14.3	21.3±9.5
Area (mm^2)	266.8±225.5	263.4±217.2

Table 5.2: Mean ± Standard deviation for placentome length and area per farm for the pregnant and the non-pregnant horns.

Placentome dimensions	**Farm A**	
	Pregnant horn	**Non-Pregnant horn**
Length (mm)	21.5±9.5	21.7±9.5
Area (mm^2)	272.6±225.7	271.0±214.1
	Farm B	
	Pregnant horn	**Non-Pregnant horn**
Length (mm)	19.0±9.2	20.0±8.5
Area (mm^2)	216.6±202.6	225.9±181.6

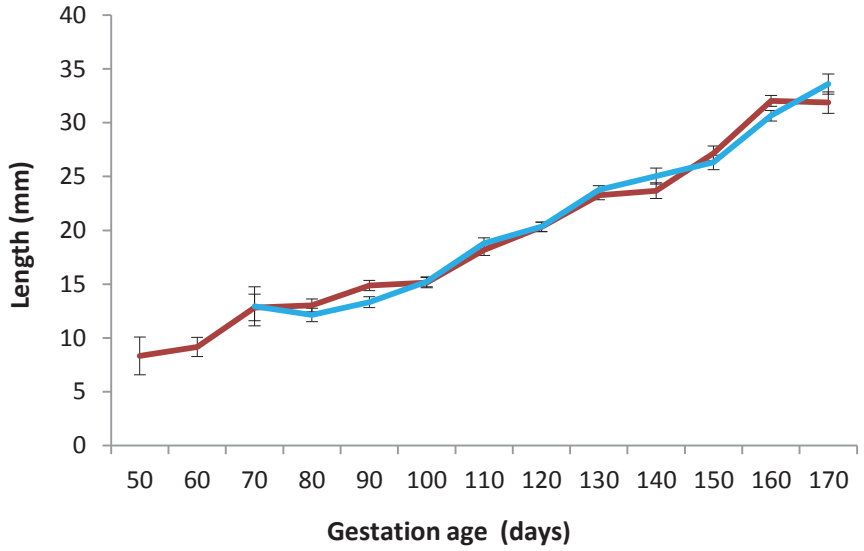


Figure 5.2: Placentome length (mm) with gestation age (days) for pregnant (red line) and non-pregnant horns in dairy cattle

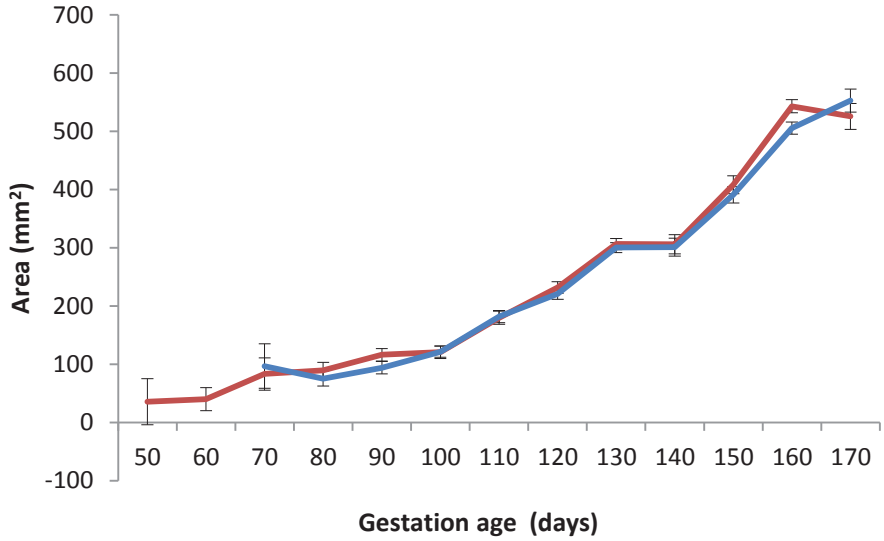


Figure 5.3: Placentome area (mm^2) with gestation age (days) for pregnant (red line) and non-pregnant horns in dairy cattle

5.3.2. Correlations between placentome dimensions and days pregnant

The correlations found between placentome length and area was very high (R= 0.92). The association between placentome length and area and days was also strong (R= 0.66 for both;

P<0.0001), with both placentome length and area increasing as gestation age advanced (Fig. 5.2 and 5.3). The association was stronger (R= 0.87, p<0.0001) when log placentome length was used.

5.3.3. Mixed model regression equations

Table 5.3 summarises the findings of the linear mixed model analysis. As suggested by the univariate analysis, there was a highly significant association between placentome length and gestational age. Log placentome length increased by 0.12 mm for every 10 days, i.e. placentome length increased by approximately 14% every 10 days. Placentome length was not significantly affected by breed, age and farm but placentomes in the non-pregnant horn were significantly smaller than placentomes in the pregnant horn (P = 0.048). This difference was approximately 0.15 mm in log placentome length equivalent to 15.8% in actual placentome length. Therefore for the initial limits of agreement analysis only data from placentomes identified as being in the pregnant horn were used and the equation used to predict days pregnant from placentome length was:

Predicted days pregnant = (log mean placentome length - 1.5322342) / 0.0118067

Table 5.3: Estimates of fixed effects of Days pregnant, breed, age, farm and horn (on length of placentome)

Effects	Estimate	Std. Error	DF	t-value	p-value
(Intercept)	1.700	0.0854	3466	19.917	0.000
Days pregnant	0.012	0.0003	3466	41.203	0.000
breed (Friesian) *	-0.029	0.0355	55	-0.828	0.411
breed (Jersey)*	-0.100	0.0573	55	-1.746	0.087
age	0.008	0.0104	55	0.768	0.446
farm	-0.003	0.0357	55	-0.089	0.929
horn (non-pregnant)[#]	-0.147	0.0744	3466	-1.981	0.048

*, crossbreds were used as reference breed; [#], pregnant horn was used as reference horn

5.3.4. Assessment of agreement

Figure 5.4 shows the relationship between predicted gestational age (using mean log placentome length from the pregnant horn only) and actual gestational age (from farm records). The data was split by farm for illustrative purposes.

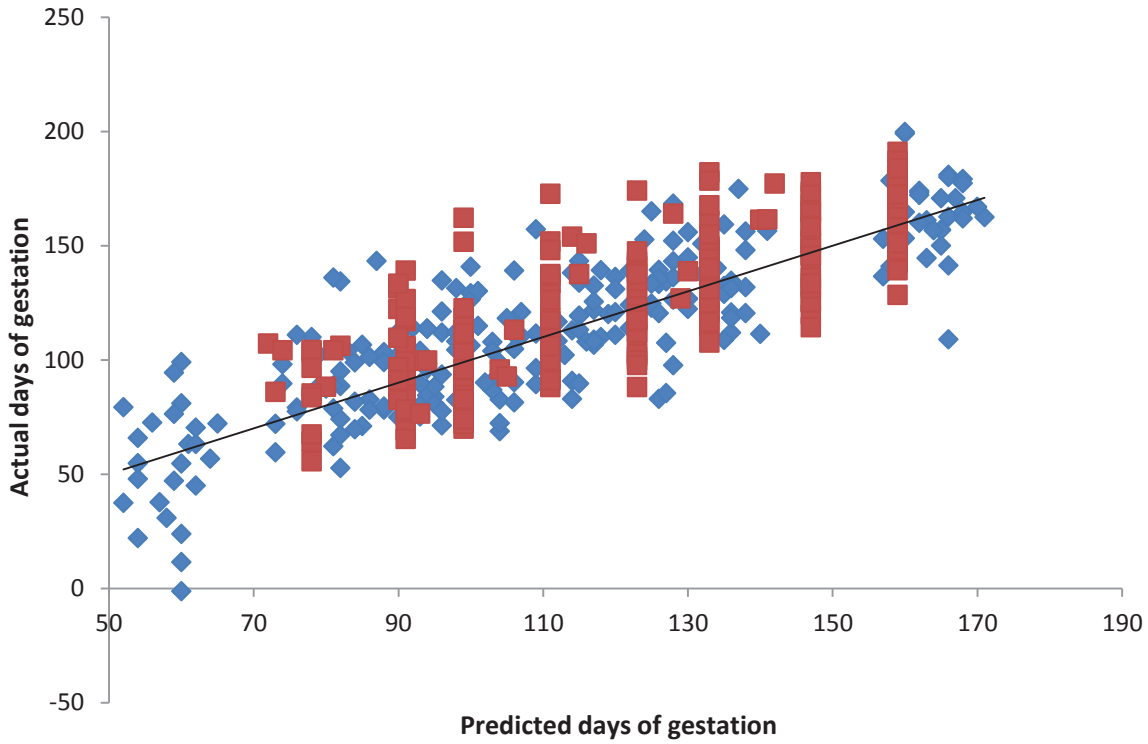


Figure 5.4: Relationship between actual gestational age and predicted gestational age (based on log placentome length from the pregnant horn only) for cows on farm A (■) and Farm B (◆). Solid black line is line of identity (actual = predicted). Predicted days pregnant = (mean log placentome length - 1.5322342) / 0.0118067

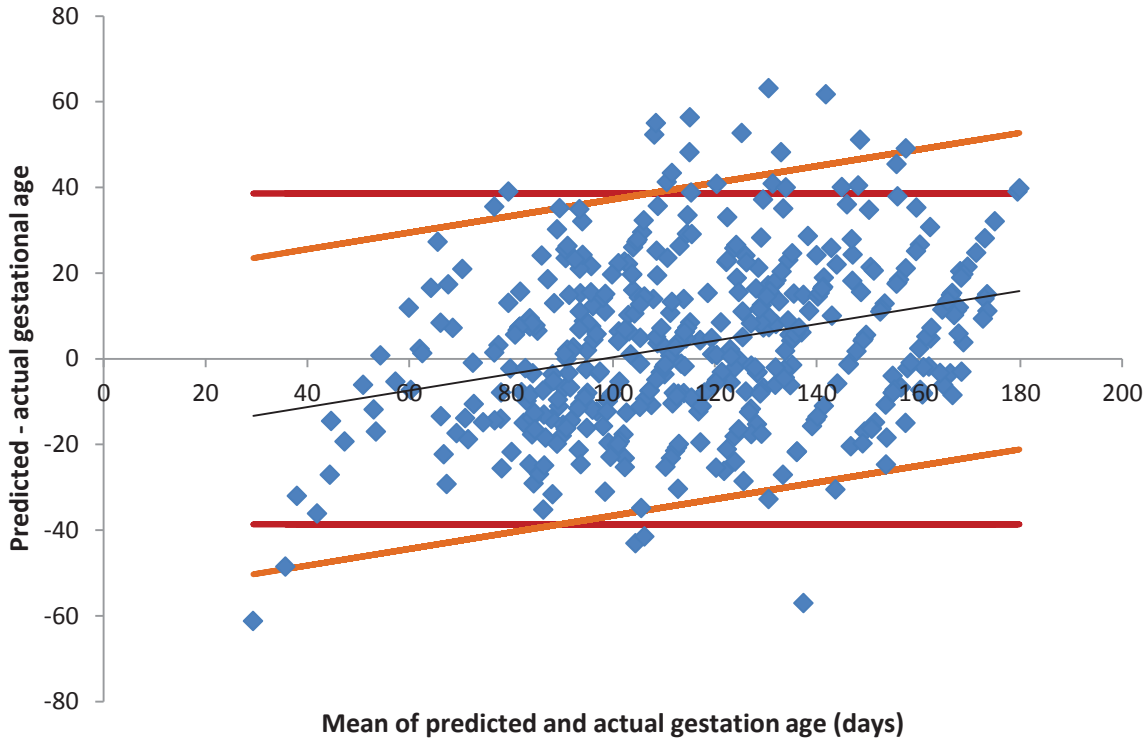


Figure 5.5: Scatterplot of difference between predicted (using log placentome length from the pregnant horn only) and actual gestational age and the mean of the two measures. Black line, line of best fit; red line, unadjusted limits of agreement; orange line, adjusted limits of agreement

Figure 5.5 illustrates the limits of agreement plot, showing the relationship between the difference between predicted gestational age (calculated using log mean length of placentomes in the pregnant horn only) and actual gestational age and the mean of those two ages. The estimated variance of the difference was 387.9. Hence the basic 95% limits of agreement were ± 38.6 days. Mean difference was 0.2 days, so gestational age, as estimated by using mean log placentome length (pregnant horn only), had a 95% chance of being between 38.8 days greater and 38.4 days less than actual age. These basic limits of agreement are illustrated by the red lines in Fig 5.5

Although the mean difference was almost 0, there was a significant but small association between expected difference and the mean of the two measures (R=0.28). Initially predicted age was smaller (negative) than actual age, but as gestation progressed the difference changed and became larger and positive; i.e. at gestational ages >100 days, predicted age was > actual age. The equation relating difference and days of gestation was:

Expected difference = (0.1938*mean gestational age) -19.008

This association was included in the calculation of the adjusted limits of agreement (accounting for the data coming from multiple observations per animal). Residuals did not vary with the mean of predicted and actual gestational age. Their variance was 354.4; hence the adjusted 95% limits of agreement were 36.9 days. These limits of agreement are illustrated by the orange lines in Fig 3.5. The effect of using the adjusted rather than the basic limits of agreement was moderate over the range of gestational ages evaluated in this study. At 60 days the adjusted 95% limits of agreement were 26 to -48, while at 170 days they were 52 to -22, compared to the 38.8 to -38.4 of the basic limits-of-agreement.

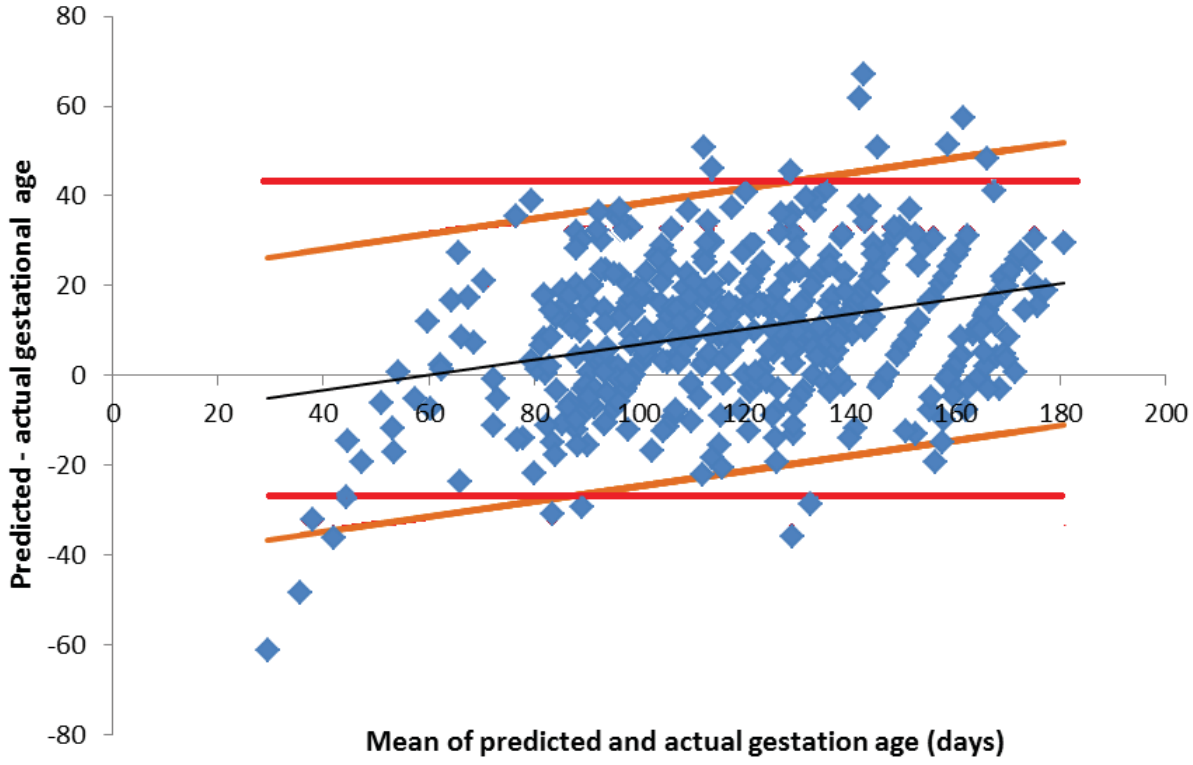


Figure 5.6: Scatterplot of difference between predicted (using log placentome length from both the pregnant and non-pregnant horns) and actual gestational age and the mean of the two measures. Black line, line of best fit; red line, unadjusted limits of agreement; orange line, adjusted limits of agreement

Figure 5.6 shows the second limits of agreement plot using data from both horns (to calculate mean log placentome length. Data from placentomes which had been identified as being in the non-pregnant horn were transformed using the conversion factor identified in Table 5.3,

i.e. their length was multiplied by 1.158 to account for the impact of horn on placentome size. The estimated variance of the difference was 283.5. Hence the basic 95% limits of agreement were ± 33 days. Mean difference was 10 days, so gestational age as estimated by using mean log placentome length (pregnant and non-pregnant horn) had a 95% chance of being between 43 days greater and 23 days less than actual age. These limits of agreement are illustrated by the red lines in Fig 5.6

In contrast to the previous analysis, the mean difference was markedly different from 0, but there was still a significant but small association between expected difference and the mean of the two measures (R=0.3). Initially predicted age was smaller (negative) than actual age, but as gestation progressed the difference increased and became larger and positive; i.e. at gestational ages >60 days, predicted age was > actual age. The equation relating difference and days of gestation was:

Expected difference = (0.1695*mean gestational age) -10.12

This association was included in the calculation of the adjusted limits of agreement (accounting for the data coming from multiple observations per animal). The residuals did not vary with the mean of predicted and actual gestational age. Their variance was 258.3; hence the 95% limits of agreement adjusted to account for the association between expected difference and gestational age were 31.5 days. These limits of agreement are illustrated by the orange lines in Fig 5.6. The effect of using the adjusted rather than the basic limits of agreement was moderate over the range of gestational ages evaluated in this study. At 60 days the adjusted 95% limits of agreement were 32 to -31.4, while at 170 days they were 50 to -13, compared to the 43 to -23 days of the basic limits-of-agreement.

Figure 5.7 shows the relationship between predicted gestational age (using mean placentome length calculated from all placentomes) and actual gestational age (from farm records), with the data split by farm for illustrative purposes. The equation used to create this graph was:

Estimated gestational age = (mean placentome length + 6.11) / 0.228).

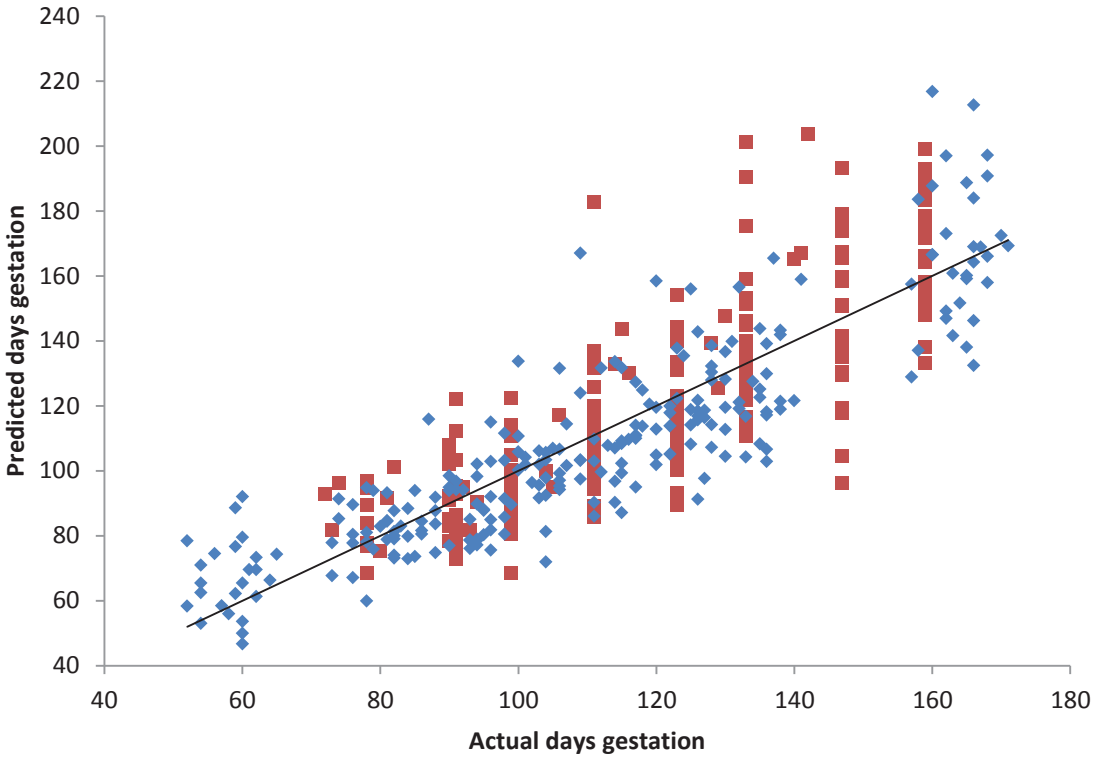


Figure 5.7: Relationship between actual gestational age and predicted gestational age (based on mean placentome length) for cows on farm A (■) and Farm B (◆). Solid line is line of identity (actual = predicted). Predicted days pregnant = (mean placentome length + 6.11) / 0.228

The limits of agreement analysis for this model is summarised in Figure 5.8. The estimated variance of the difference (Bland and Altman 2007) was 293.4. Hence the basic 95% limits of agreement were ± 33.7 days. Mean difference was 0 days, so estimated gestational age had a 95% chance of being between 33.7 days greater and 33.7 days less than actual age. These limits of agreement are illustrated by the red lines in Fig 5.8

As with the previous models there was a significant but small association between expected difference and the mean of the two ages. Initially predicted age was lower than actual age, but as gestation progressed the difference increased and became positive; i.e. at gestational ages >100 days, predicted age was > actual age. The equation relating difference and days of gestation was:

Expected difference = 0.17* gestational age - 19.1

This association was included in the calculation of the adjusted limits of agreement (accounting for the data coming from multiple observations per animal). The residuals did not vary with the mean of predicted and actual gestational age. Their variance was 269.5; hence the 95% limits of agreement adjusted to account for the association between expected difference and gestational age were ±32.2 days. These limits of agreement are illustrated by the orange lines in Fig 3.8. The effect of using the adjusted rather than the basic limits of agreement was moderate over the range of gestational ages evaluated in this study. At 60 days the adjusted 95% limits of agreement were 23.6 to -40.1, while at 170 days they were 41.8 to -22.5, compared to the ±34 of the unadjusted limits-of-agreement.

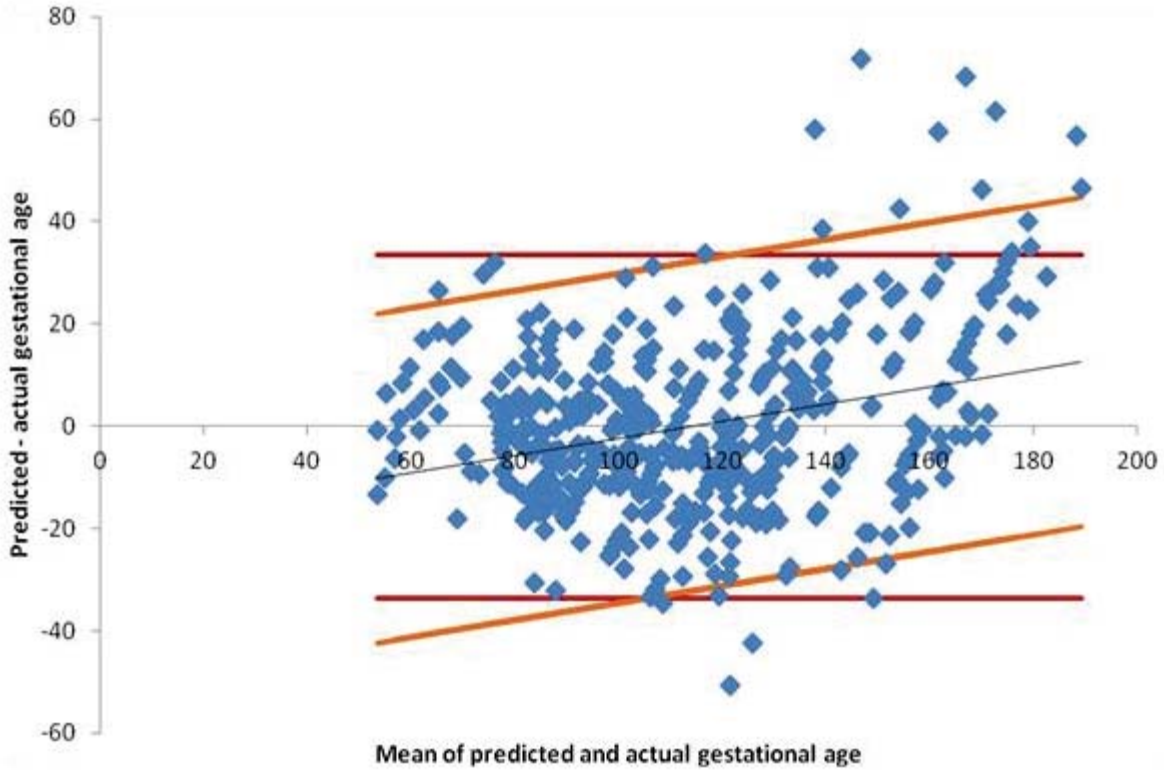


Figure 5.8: Scatterplot of difference between predicted (using placentome length) and actual gestational age and the mean of the two measures. Black line, line of best fit; red line, unadjusted limits of agreement; orange line, adjusted limits of agreement**.**

5.4. Discussion

Despite placentome size having been recommended as a measure of gestation length in the field (Zemjanis 1974; Matthews and Murton 2012) and ex vivo data showing that placentome size does increase over gestation (Laven and Peters 2001), this is the first study that has shown a significant association, *in vivo*, between placentome size and gestation length in cattle and characterised that relationship. Both mean placentome length and area, as measured using trans-rectal ultrasound, were significantly associated with gestation length between Day 60 and 180 of gestation. This is consistent with the report by Kohan-Ghadr *et al.* (2008) which showed a significant relationship between placentome length and gestational age between Day 80 and 180 (though not after in non-cloned embryos), but is in contrast to the report by Hunnam *et al.* (2009a), who found no significant relationship between placentome length or height and gestation length between Day 73 and 190 of gestation. The key difference between that study and the present one is that Hunnam *et al.* (2009a) measured placentome size trans-abdominally over a consistent site on the abdomen (the right caudodorsal flank fold) rather than trans-rectally at a consistent site within the uterus. This meant that not only was Hunnam *et al.* (2009a) unable to measure placentomes in a significant number of cows (over 35%), but the placentomes they measured would have been in the main body of the uterine horn but not at a consistent site in relation to the site of attachment of the fetus. This is important as placentome size varies significantly depending upon the proximity of the measured placentomes to the site of fetal attachment (Youngquist 1997; Laven and Peters 2001). In contrast, the placentomes selected in this study were all selected from the same place (near the cervix) to minimise the variation in placentome size unrelated to gestational age. This conclusion is supported by the results reported by (Kohan-Ghadr *et al.* (2008), who measured 12 placentomes across three specific sites within the uterus, close to the anterior cervical os and at the bifurcation of the pregnant horn (trans-rectal ultrasound), and the distal pregnant horn (trans-abdominal ultrasound) and showed that, in non-clone pregnancies, placentome size increased from day 80 to 180 (but not thereafter) , and also by the results reported by Bertolini *et al.* (2002), who measured placentome size using trans-rectal ultrasound at a consistent site (around the embryo and early fetus in the mid-region of the fetal horn) and were able to demonstrate differences in placentome size between *in vitro* and *in vivo* produced embryos (Bertolini *et al.* 2002). The advantage of measurement of cervical placentomes over mid-region placentomes is that, particularly in

later pregnancies identification and measurement of cervical placentomes is simpler and quicker.

The change with time in placentome length seen in this study was not affected by breed (Jersey vs. Friesian vs. crossbred, age of the cow (multiparous vs. primiparous) or farm. However site of placentome (pregnant vs. non-pregnant horn) was significant, with placentomes in the non-pregnant horn being 15% smaller than those in the pregnant horn. This difference was smaller than the mean difference reported by Laven and Peters (2001); that study compared placentomes from throughout the uterine horn not just cervical placentomes, again confirming that measuring cervical placentomes minimises the variation in placentome size unrelated to gestational age (Youngquist 1997) . The lack of impact of breed, age and farm, and the relatively small influence of horn meant that a simple equation could be used to predict gestational age from placentome length.

Over the period of this study (60 - 180 days of gestation) a significant linear relationship was found between log placentome length and gestational age. Although significant the correlation (R=0.87) was marginally lower than previously reported for fetal measurements, such as head diameter and crown rump length (R=0.91 to 0.99; (White *et al.* 1985; Kähn 1989). Such correlations are by far the most commonly reported relationship when the accuracy of gestational ageing is reported (e.g. Ali and Fahmy 2008; Riding *et al.* 2008; Hunnam *et al.* 2009). However, in order for a measure to be used as a predictor of gestational age a significant association is not sufficient, there needs to be significant agreement as well. Using correlation and/or regression does not identify bias (e.g. a consistent under prediction) or identify changes in the association as gestational age increases (e.g. a change from under to over prediction, or an increase in under prediction). Limits-of-agreement analysis (Bland and Altman 1999, 2007) identifies both of these and, in addition, provides information on the distribution of the difference between the predicted and the actual values. The results of these calculations can then be used to compare accuracy between techniques.

For this dataset using adjusted rather than basic limits of agreement had almost no impact on the size of the limits of agreement and only limited impact on their placing (see figures 5.5,5.6 and 5.8), so they have little advantage over the much simpler basic limits of agreement. The basic limits-of-agreement analysis showed that when mean log length of

placentomes in the pregnant horn was used to predict gestational age, 95% of predicted gestational ages were within 39 days of the actual gestational age, and that using data from placentomes in both the non-pregnant and pregnant horns (and accounting for the effect of horn on placentome size) decreased this to ±33 days (once the bias of 10 days was accounted for). Using means data from all measured placentomes rather than log data resulted in basic limits of agreement of ±34 days. Using log mean data from the pregnant horn only 42% (188/448) of predicted gestational ages were within 10 days of actual gestational age, this increased to 49% (218/448) when adjusted data from both horns was used and 48% (217/448) when using unadjusted data from both horns.

Published comparative data are limited, but what there are suggest that using placentome length to predict gestational age is significantly poorer than using other fetal data. White *et al.* (1985) estimated gestational age using trans-rectal ultrasound measurement of six measures in 32 beef cattle and reported residual standard deviations ranging from 4.5 (crown-rump length) to 12.6 days (uterine diameter). Using the predictive models reported by White *et al.* (1985) in 300 beef cattle, Wright *et al.* (1988) reported that the standard deviation of the difference between predicted and actual calving date was 9.0 days, when one of trunk, head, uterine or nose diameter was measured, equivalent to a limits of agreement of 17.6 days, just over half the smallest limits of agreement found in this study. Of the individual measures only uterine diameter had a limits of agreement similar to that reported here (31.5 days), both trunk and head diameter had markedly smaller limits-of-agreement (17.6 and 13.5, respectively) (Wright *et al.* 1988). In addition to a much reduced limits of agreement , Wright *et al.* (1988) also reported that a much higher proportion of predictions were within 10 days of the actual measure than in this study – 80.5% compared to 42 to 49% with this dataset. Interestingly the proportion calving within 10 days of predicted calving date reported by Wright *et al.* (1988) is exactly the same as that reported by Matthews and Morton (2012) based on manual rectal palpation of 10,487 dairy cows. It is likely that in the latter study the accuracy of ageing by manual palpation was increased to the same level as the trans-rectal ultrasound used by Wright *et al.* (1988) by the availability of data on insemination dates, as in cattle without insemination data only 60.7% calved within 10 days of predicted calving date. This is still better than the accuracy of this dataset, but does suggest that more accurate gestational ageing could be achieved if placentome measurement was combined with insemination data.

Nevertheless, although placentome measurement is inherently a less accurate measure of gestational age than measures such as crown-rump length and trunk or head diameter it does

have some advantages. Firstly, throughout gestation from approximately day 60 onwards, measuring placentomes is easier, simpler and more achievable than other measures. After day 60, four placentomes were measurable on all occasions in these cows; in contrast Kahn (1989) reported that accessibility ranged from 87% for head measurements to 56% for pelvic measurements. Placentome measurement may be of more value later in gestation when fetal measurements may not be possible in more than 1/3 of fetuses. This study suggests that this value may be enhanced by the lack of association between gestational age and variance of the difference between predicted and actual gestational age. In contrast, the variance for fetal measurements increases markedly as gestation progresses (White *et al.* 1985). However both Laven and Peters (2001) and Kohan-Ghadr *et al.* (2008) reported that late in gestation placentome growth ceases so further research is required to establish whether and when this hypothesis is of clinical value.

Conclusions

This study has shown that trans-rectal ultrasound measurement of the placentomes near to the cervix is an easily achievable simple technique, and that when placentomes are measured in this manner there is a significant association between placentome size and gestational age. However, the significance between cow variation in placentome size and placentome growth rate means that there is insufficient agreement between placentome size and gestational age for placentome size to be used as a predictor of gestational age when alternative fetal measures are available.

Chapter Six

The Use of Stereology Method to Estimate the Volume of Feto-Maternal Exchange Area of the Bovine Placentome during Gestation:

6.1. Introduction

In cattle (and other ruminants) the placentome is the specialized point of contact between the allantochorion and endometrium, where the vast majority of the feto-maternal exchange occurs (Liu *et al.* 2010).The placentome is made up of the caruncle (maternal tissue) and the cotyledon (fetal tissue). At the feto-maternal interface, where the exchange of nutrients and gases takes place, both the caruncle and the cotyledon consist of primary, secondary and tertiary branching villi. These develop throughout pregnancy and, indeed, their progressive development is essential for normal growth of the fetus. Failure of development of the placentome can be associated with abnormal development of the fetus, whilst failure of the final stages of maturation of the placentome is associated with retention of the fetal membranes (Boos *et al.* 2003)

Placentome development is complex with many factors involved but its success is essential for normal fetal growth during gestation. Better data on the normal process of development, particularly the relationship between the fetal and maternal components, would aid significantly in assessing how factors, such as nutrition, affect placentome development. Several methods have been used to assess the development of the bovine placentome. Some have required sophisticated equipment, for example Ferrell (1991) measured the RNA, DNA, and protein concentrations in the cotyledons and caruncle, and concluded that this changed in relation to cell size and cell number, while Pfarrer *et al.* (2001) followed the development of the vascular structure of the placentome using a scanning electron microscopic. Others have used easily available equipment; Ferrell (1991) measured the weight of fetal membranes, cotyledons and caruncles after separation at two time points near term – this was taken further by Laven and Peters (2001) who evaluated placentome, caruncle and cotyledon weight and size across most of gestation.

One study (Laven and Peters, 2006) measured tissue area using an image analyser. This gives relative volumes of tissue but is a long, slow technique limiting the amount of tissues which can be viewed and assessed. Stereology has significant speed advantages over simple image analysis. This method uses test grids to estimate surface area, volume density and absolute

volumes of the placenta. However, only two studies have used this method to examine the ruminant placenta: Kannekens *et al.*, (2006) evaluated the placenta from one cow at Day 135 of gestation, while Liu *et al.*, (2010) used stereology to evaluate thirty-one yak placentas from Day 60 to 211 of gestation. The latter study needs repeating in domestic cattle.

The aim of the present study was to use stereology to estimate the functional surface area and volume density of the feto-maternal unit within the bovine placentome from approximately Day 100 to 260.

6.2. Materials and Methods

6.2.1. Animals and tissue sampling

Uteri (n=25) from early to near term pregnant Friesian, Jersey and crossbred cows (100 to > 200 days of gestation) were obtained from a local abattoir (Affco NZ Ltd, Fielding , New Zealand) and Massey University Veterinary Teaching Hospital. These uteri were categorized into five stages of gestation by measuring the crown rump length of the fetus (Winters *et al.* 1942).

Stage 1: Gestation age 100-125 days (n=5),

Stage 2: Gestation age range 126- 150 days (n=5),

Stage 3: Gestation age range 151-175 days (n=5),

Stage 4: Gestation age range 176- 200 days (n=5),

Stage 5: Gestation age range 201- 260 days (n=5).

6.2.2. Tissue Preparation and Histological Techniques

All placentomes were were carefully dissected from the uteri and arranged according to their sizes. Every eighth placentome was selected provided it was > 15 mm diameter otherwise the next correct size was chosen to ensure unbiased sampling (Howard and Reed 2010). The selected placentomes were then fixed by perfusion fixation by using the syringe and needle to inject formalin into the tissue through the blood vessels and cut into half, followed by immersion in 10% formalin. Parallel cuts of 4 mm thick slices were made around the vertical axis of each placentome selected and one of these slices was randomly selected and cut into blocks, fixed in paraffin wax and embedded (Figure 6.1). Thereafter six 5 µm

thick tissues sections from each block were cut using a manual microtome, mounted and stained with Masson's trichrome (Bancroft *et al.* 2008).

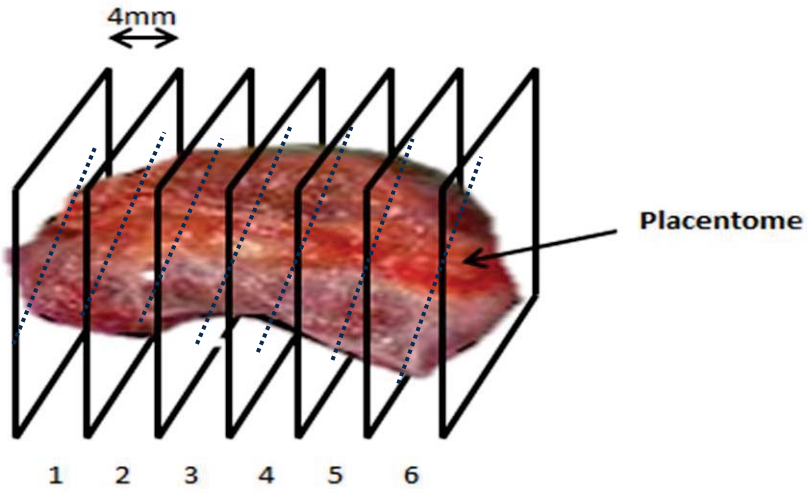


Figure 6.1: Illustrates a series of parallel cutting planes of 4mm thick slices of a placentome.

6.2.3. Stereology

One stained section was randomly selected per block per uterus and visualized using an Olympus light microscope (Olympus XC50, Japan) with an image magnification of 200X. A 9x9 uniformly spaced point grid was generated by using Image J computer-assisted software (http://rsb.info.nih.gov/ij/index.html) and overlaid on the sections. Three fields of view from the top, middle and lower part of the section were randomly selected and analysed (Laven and Peters 2006). Hence, 240 points were assessed per placentome making an overall total of 6000 per placenta (See Figures 6.2 and 6.3). To estimate the surface density and total surface area of the feto-maternal interface, a test grid comprising of eight uniformly spaced cycloids whose minor vertical axis were parallel to the vertical cutting direction of the sectioning plane was generated and overlaid on three fields of view on each placentome section using image J cycloid arc plugging (See Figure 6.4).

The following tissues of interest were distinguished, counted and volume densities were estimated in each section: binucleate trophoblast cells, fetal connective tissue, maternal epithelium, and maternal connective tissue.

Volume density

Volume density was defined as the 'volume proportion of one phase within a reference volume' (Howard and Reed 2010). The volume densities (Vv) of each tissue (binucleate trophoblast cells, fetal connective tissue, maternal epithelium, and maternal connective tissue) were estimated using the equation:

$Vv = P(i)/P(t)$,

where P(i) is the total number of points falling on the tissue of interest and P(t) is the total number of points falling within the placentomes.

Total Volume

Total placentome volume ($V_{(tot)}$) was obtained by measuring the volume of the total placentomes dissected from each uterus, by the water displacement method using a graduated measuring cylinder. The total volume of each of the tissues of interest (i.e. binucleate trophoblast cells, fetal connective tissue, maternal epithelium, and maternal connective tissue) in the whole placentome was estimated by multiplying total placentome volume by volume densities for each tissue of interest.

$V(i) = V_{(tot)} * Vv$

Surface density

Surface density is the area of an interface within a unit reference volume (Howard and Reed 2010). The number of intersections (I_i) crossing the feto-maternal junction was counted as described by Kannekens *et al.* (2006). The number of points falling within the field of interest (P_i) was then used to evaluate the length of the cycloid. The length of test line per point (l/p) was calculated by the computer-assisted software (Image J) estimated using the following equation (Howard and Reed 2010)

$$S_v = \frac{2\sum_{i=1}^{n} I_i}{l / p \bullet \sum_{i=1}^{n} P_i}$$

Total Surface Area

The total surface area of the fetomaternal interface (S_{tot} (FMI) in the placentome was estimated by multiplying the surface density (S_v) by the total volume of the placentome

$S_{tot} = S_v * V_{(tot)}$

Statistical analysis

Data were analysed using general linear models (SAS 9.2, SAS Institute) to obtain the least square means and standard error of means (SEM) for volume densities, surface densities, and total surface areas of bovine placentomes at different gestational ages. Analysis of variance was used to test for significant differences between different gestational stages. Tukey's range test was used to compare the means for individual stages where the ANOVA was significant.

To further analyse the effect of using stereology on the relationship between gestation and relative volume of maternal and fetal tissue a mixed model was used with volume of placentome tissue as the outcome variable, technique used (stereology or water displacement), type of tissue (fetal or maternal) and stage of gestation as fixed effects and cow as random effect. This was followed by a second mixed model with ratio of fetal to maternal tissue as outcome variable, technique used (stereology or water displacement) and stage of gestation as fixed effects and cow as random effect

6.3. Results

6.3.1. Relative volume densities (Vv)

Figure 6.2 shows a section of bovine placentome indicating both the maternal and fetal components. In Figure 6.3, a 9x9 grid has been placed on the sections showing examples of the points counted to estimate the relative volume densities of the placentome components. In Figure 6.4, eight cycloids have been superimposed upon the same section to estimate the

surface area of the feto-maternal interface. Data obtained from stereological analysis at different stages of gestation are shown in Table 6.1.

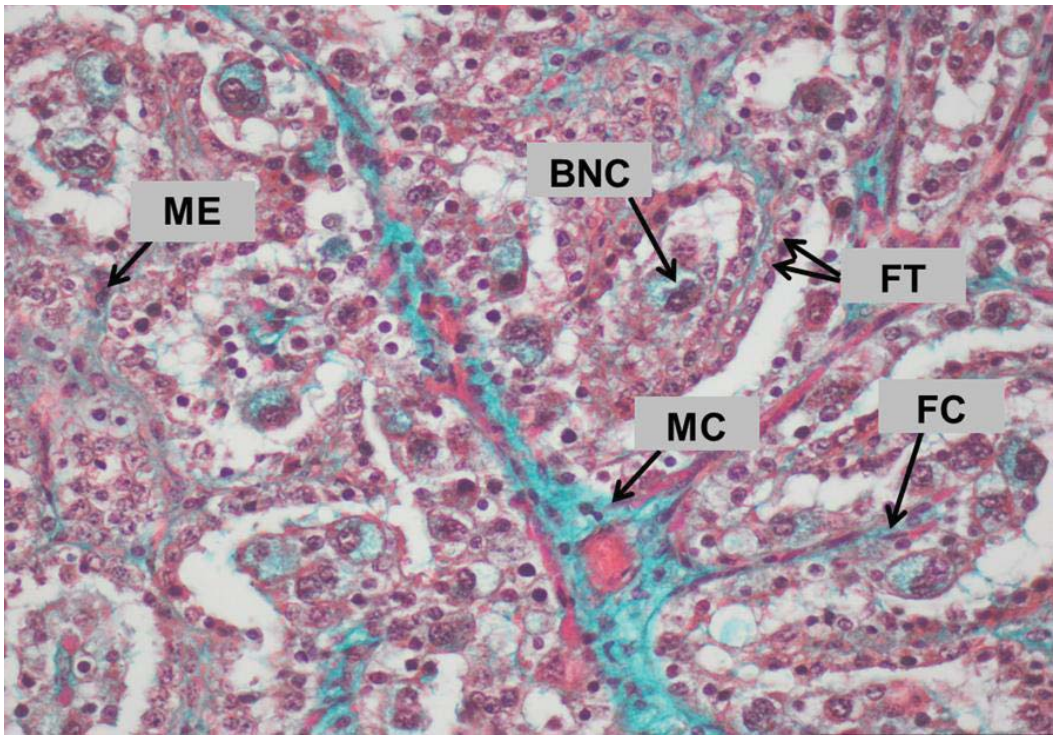


Figure 6.2: Bovine placentome at Gestation stage 2 (126 – 150 days) showing binucleate cells (BNC); fetal connective tissue (FC); fetal trophoblast (FT); maternal connective tissue (MC) and maternal epithelium (ME). Masson's Trichrome

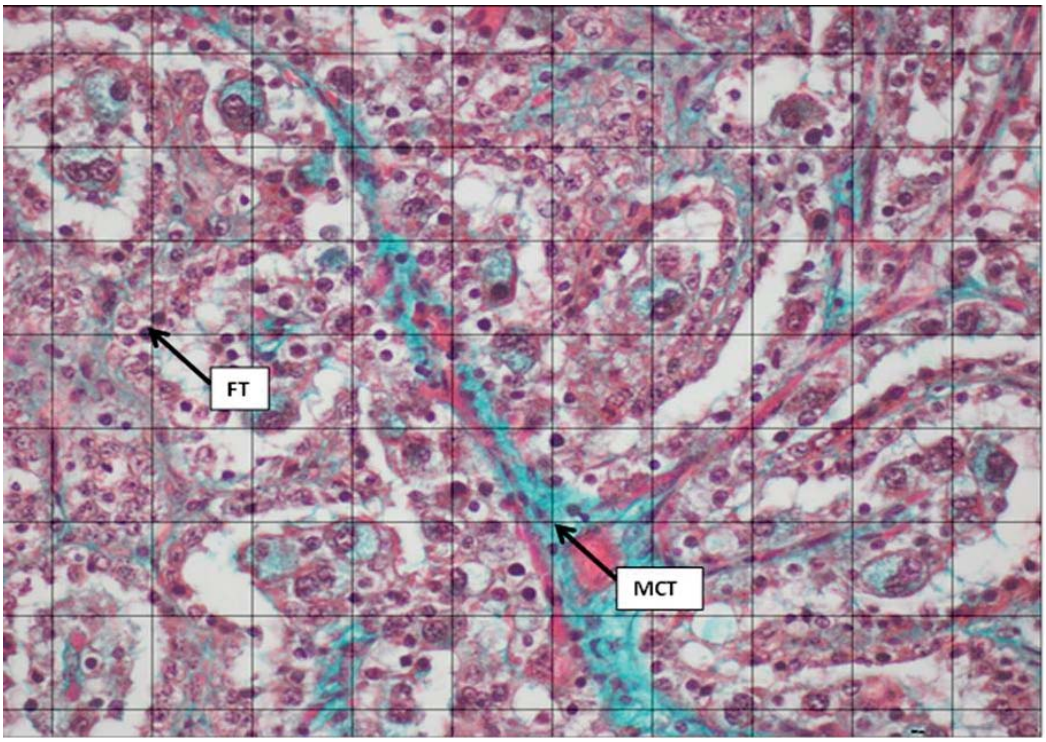


Figure 6.3: Bovine placentome at Gestation stage 2 (126-150 days) with a 9x9 point grid generated by image analysis (Image J software) overlaid on the image. Examples of points falling on a tissue of interest are represented by arrows (FT) fetal trophoblast; (MCT) Maternal connective tissue. Masson's Trichrome.

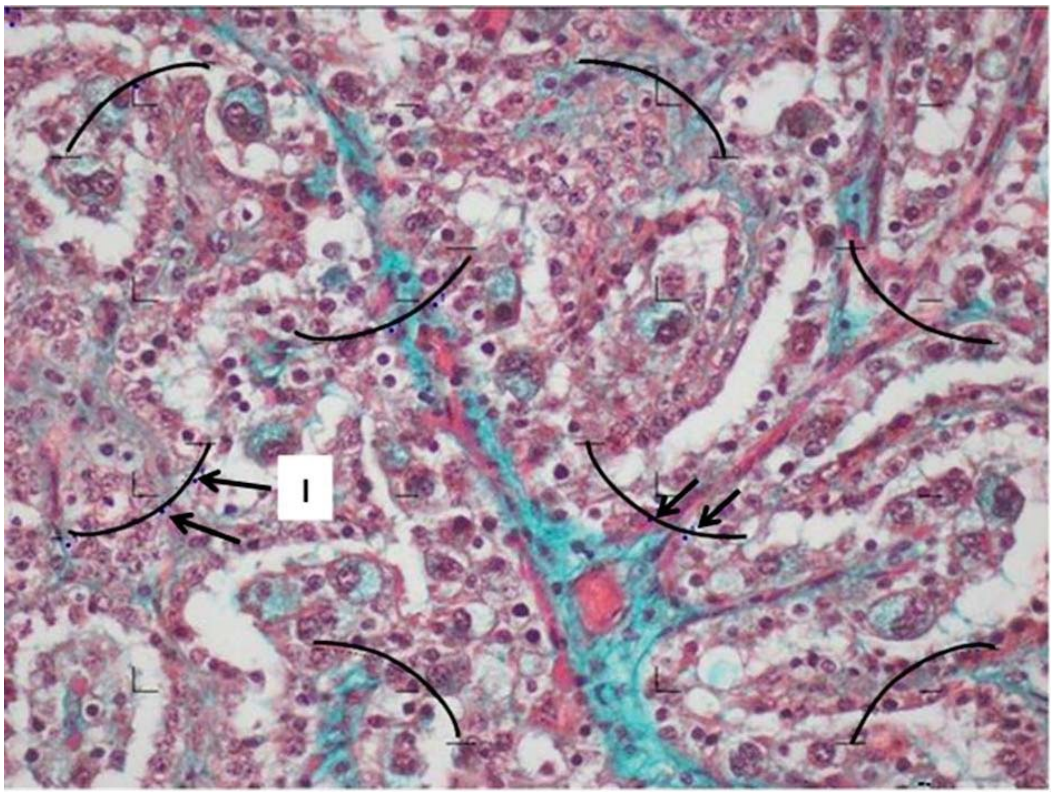


Figure 6.4: Bovine placentome at Gestation stage 2 (126-150 days) with cycloid grid generated from image analysis (Image J software) overlaid on the image. Examples of points

of intersection (I) crossing the feto-maternal interface are represented by arrows. Masson Trichrome.

Table 6.1: Volume densities (%) of placentome components and surface density of feto-maternal-interface (FMI) of bovine placentomes in each stage of gestation

	Volume Density %					
Gestation Stage	Binucleate cells	Fetal Trophoblast	Fetal Connective Tissue	Maternal Connective Tissue	Maternal Epithelium	Surface density (FMI) mm^{-1}
100-125	5.0 ± 1.82	42.4 ± 2.58	17.4 ±1.26	25.3 ±4.83	9.9 ±3.89	1.5±0.19
126-150	6.6 ±0.26	46.1 ± 6.53	12.0 ±1.77	26.67 ±4.06	8.6 ±1.55	1.9 ± 0.12
151-175	8.0 ± 0.92	43.6 ± 6.78	12.2±2.07	11.7 ±2.49	24.5 ±6.20	1.5 ± 0.10
176-200	6.5 ± 1.71	37.7 ± 2.91	16.3 ±1.53	24.6 ±6.04	14.8 ±4.90	1.6 ± 0.13
201-260	4.4 ± 1.34	40.9 ± 4.13	15.2 ±1.39	26.5 ±2.95	13.0 ±2.42	1.4 ± 0.15
P value	0.340	0.809	0.105	0.104	0.096	0.187

6.3.2. Binucleate cell (BNC)

The volume density of BNC did not change with gestational age (p =0.371). (Table 6.1; Fig 6.5)

6.3.3. Fetal Trophoblast

There was no effect of gestational age on fetal trophoblast cell (p= 0.809). (Table 6.1; Fig 6.6)

6.3.4. Fetal Connective Tissue

The relative volume density of fetal connective tissues did not change with gestation stage (p= 0.105) (Table 6.1; fig 6.7)

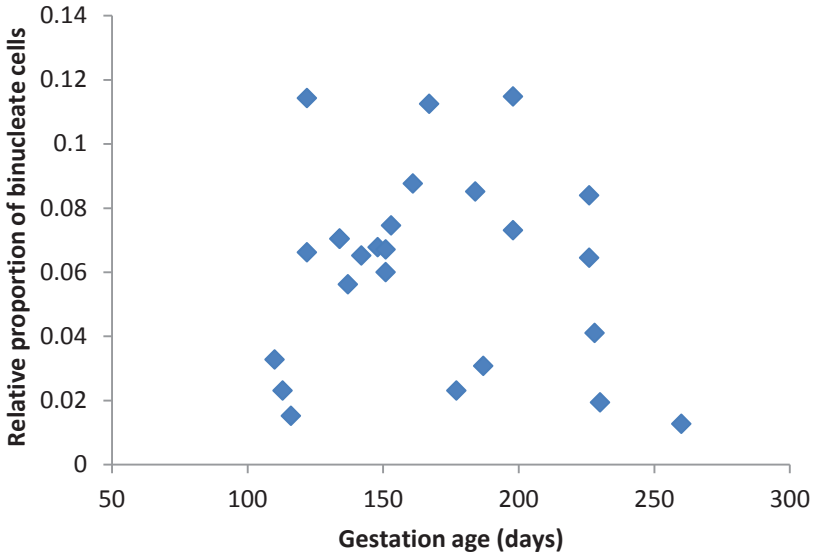


Figure 6.5: Change in relative volume density of binucleate cells of bovine placentomes sections from 100 to 260 days of gestation. Data from 25 uteri with a section randomly selected from each cow and 3 fields viewed per section

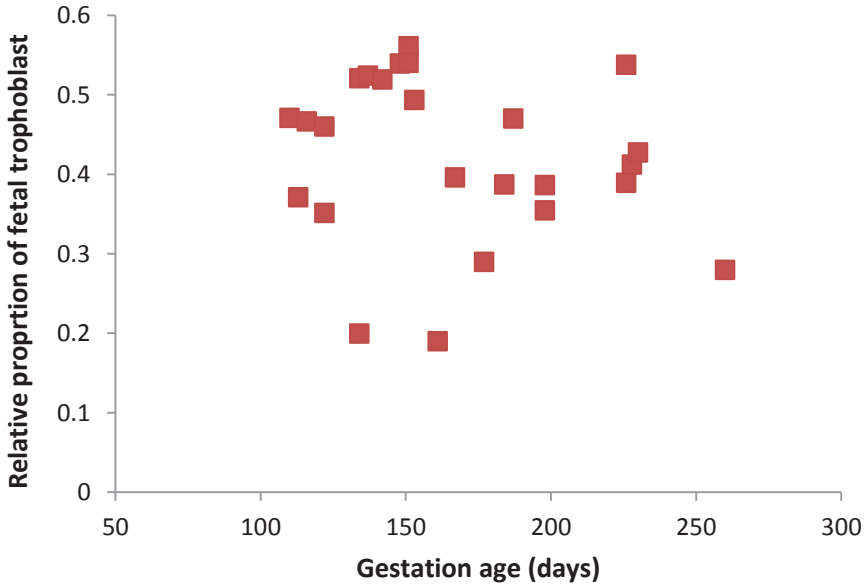


Figure 6.6: Change in relative volume density of fetal trophoblast cells of bovine placentomes sections from 100 to 260 days of gestation. Data from 25 uteri with a section randomly selected from each cow and 3 fields viewed per section

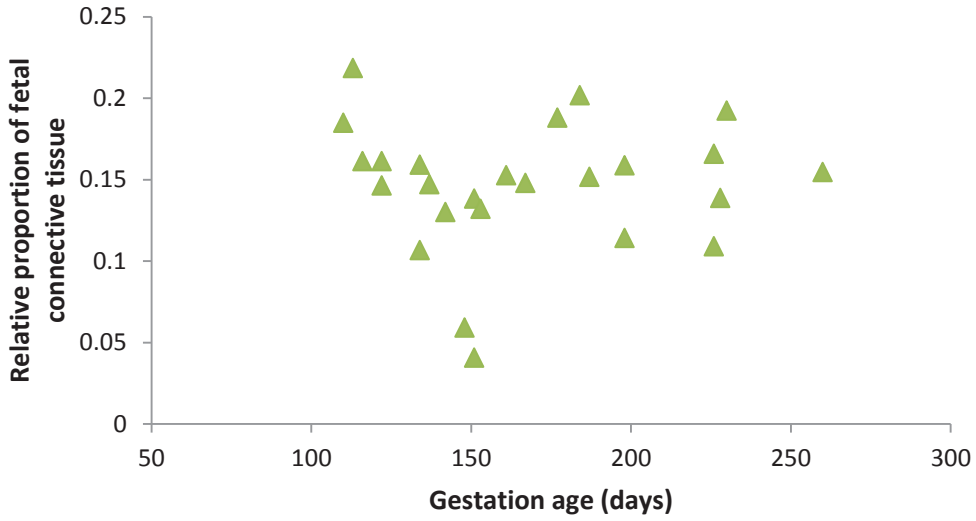


Figure 6.7: Change in relative volume density of fetal connective tissue of bovine placentomes sections from 100 to 260 days of gestation. Data from 25 uteri with a section randomly selected from each cow and 3 fields viewed per section

6.3.5. Maternal Connective Tissue

There was no relationship between maternal connective tissue and gestation age (p= 0.104). (Table 6.1; Fig 6.8).

6.3.6. Maternal Epithelium

Relative volume density of maternal epithelium was not affected by gestation age (p =0.096) (Table 6.1; Fig 6.9).

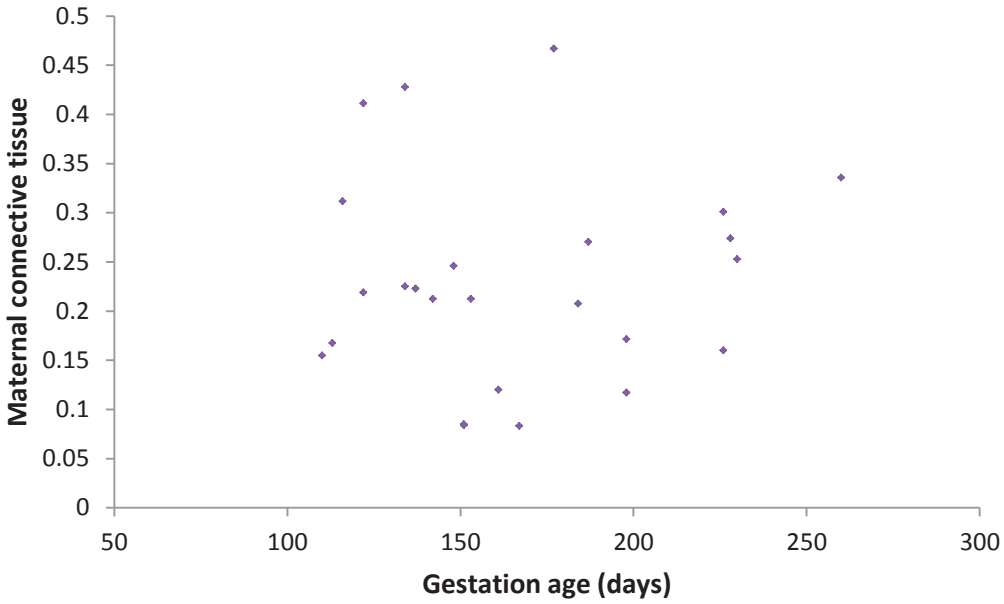


Figure 6.8: Change in relative volume density of maternal connective tissue of bovine placentomes sections from 100 to 260 days of gestation. Data from 25 uteri with a section randomly selected from each cow and 3 fields viewed per section

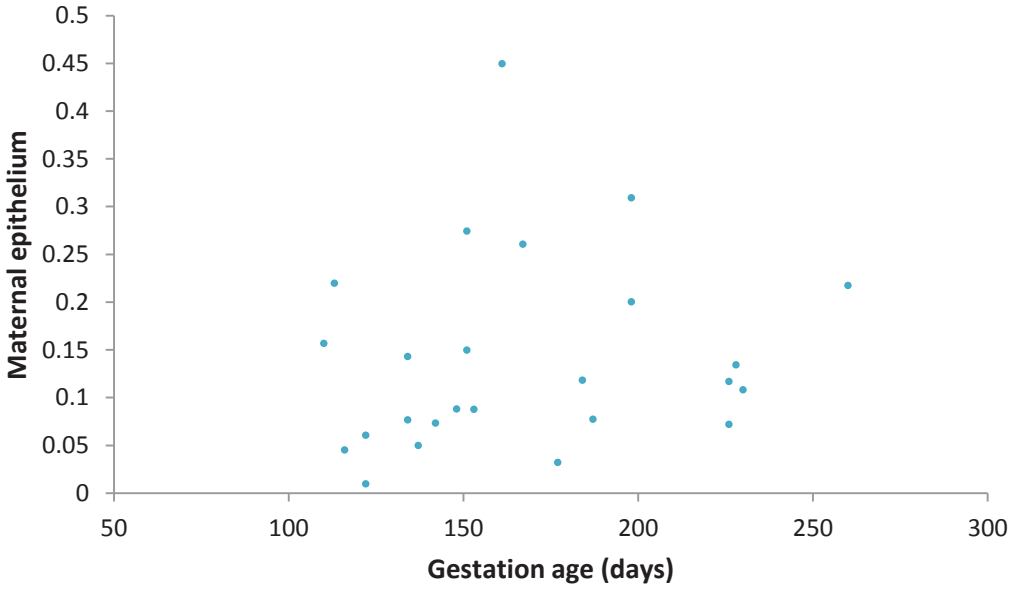


Figure 6.9: Change in relative volume density of maternal epithelium of placentomes sections from 100 to 260 days of gestation. Data from 25 uteri with a section randomly selected from each cow and 3 fields viewed per section

6.3.7. Maternal components (Maternal connective tissue + Maternal epithelium)

There was no marked change in proportion of the placentome which was maternal tissue over gestation (P = 0.656; Figure 6.10)

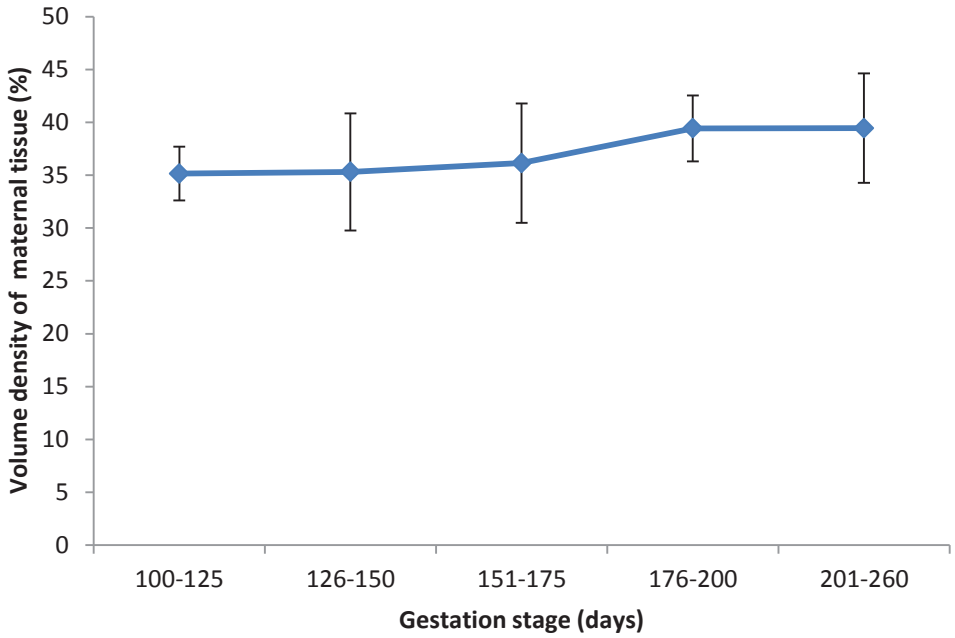


Figure 6.10: Change in relative volume density of maternal components of placentomes sections from 100 to 260 days of gestation. Data from 25 uteri with a section randomly selected from each cow and 3 fields viewed per section

6.3.8. Surface density

There was no significant effect of gestation stage on mean surface density (p= 0.187) (Table 6.1, Figure 6.11).

6.3.9. Total volumes and surface areas of placentome and components

Table 6.2 and Figure 6.12 show the total volumes of each of the tissues of interest calculated using the mean total volume of the placentome at each stage. All measures increased between stage 1 (100-125 days) and stage 5 (201 -260 days). There was a significant increase in placentome volume, binucleate cells and fetal trophoblast from Days 100 to 260 days of gestation (i.e stages 1-5) (P < 0.001 for all), however most of the increases between stages were not statistically significant at the 5% level. For all tissues, volume at stage 2 was not

significantly greater than stage 1, wich was consistent with the change in total placentome volume; volume at stage 3 was only greater than at Stage 1 for fetal and maternal epithelial tissue, not for connective tissue or BNC. By stage 4, all tissues were significantly larger by volume than at stage 1. There was a significant increase in all tissues between stage 4 and 5 (176 -260 days) except for maternal epithelium and BNC. The changes seen in the total surface area of the feto-maternal interface in stage 1 was significantly less than that at stage 3, which was significantly smaller than stage 4 which in turn was smaller than stage 5 (Table 6.2, Figure 6.13)

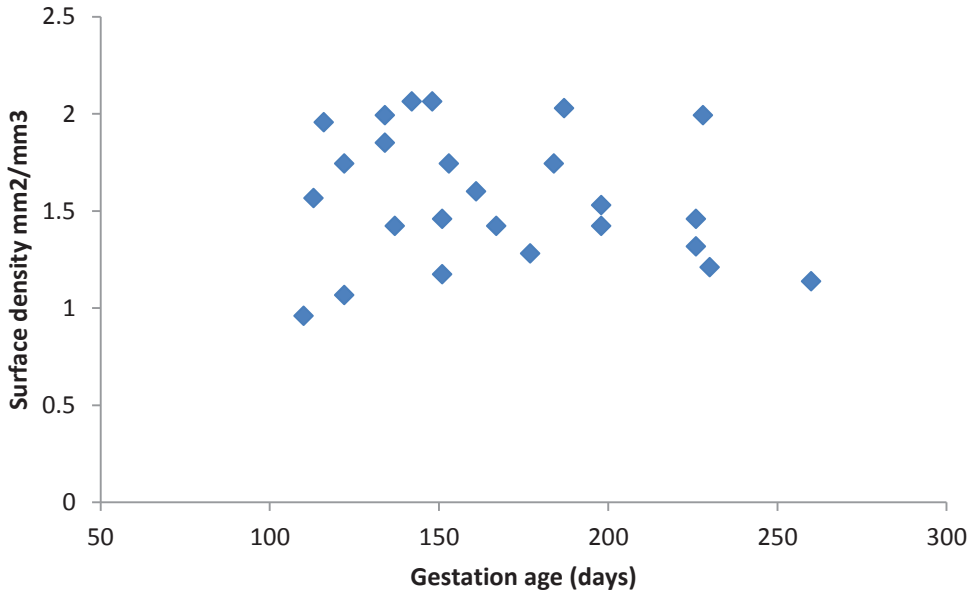


Figure 6.11: Change in surface density within the feto-maternal interface per unit of placentomes sections from 100 to 260 days of gestation. Data from 25 uteri with a section randomly selected from each cow and 3 fields viewed per section

Table 6.2: Total volume densities (mL) and Total surface area of feto-maternal interface (FMI) (means ± SE) of bovine placentomes in each stage of gestation

	Total Volume (mL)						
Stage of Gestation	Placentome volume	Binucleate cells	Fetal Trophoblast	Fetal Connective Tissue	Maternal Connective Tissue	Maternal Epithelium	Total surface area of FMI m^2
100-125	779± 118.3^{a}	47± 21.6^{a}	325± 46.6^{a}	133± 16.7^{a}	210± 62.4^{a}	68± 25.4^{a}	1.131 ± 0.21^{a}
126-150	1134± 72.9^{a}	75± 6.3^{ab}	515± 74.2^{ab}	140± 24.5^{a}	311± 57.3^{a}	99± 21.0^{a}	2.131 ± 0.19^{ab}
151-175	1940± 380.7^{b}	169 ± 55.5^{ab}	816 ± 166.5^{bc}	250± 73.8^{a}	211± 3.8^{a}	496 ± 154.5^{b}	2.841 ± 0.53^{b}
176-200	2940± 188.4^{c}	201± 57.1^{c}	1120± 120.9^{c}	479 ± 59.1^{b}	690± 123.3^{b}	454 ± 155.4^{b}	4.751± 0.54^{c}
201-260	4500± 154.3^{d}	200± 60.5^{c}	1861 ± 243.8^{d}	687± 72.1^{c}	1180± 112.2^{c}	579± 105.1^{b}	6.431± 0.75^{d}

Different superscripts within columns indicates means that are significantly different ($P<0.05$)

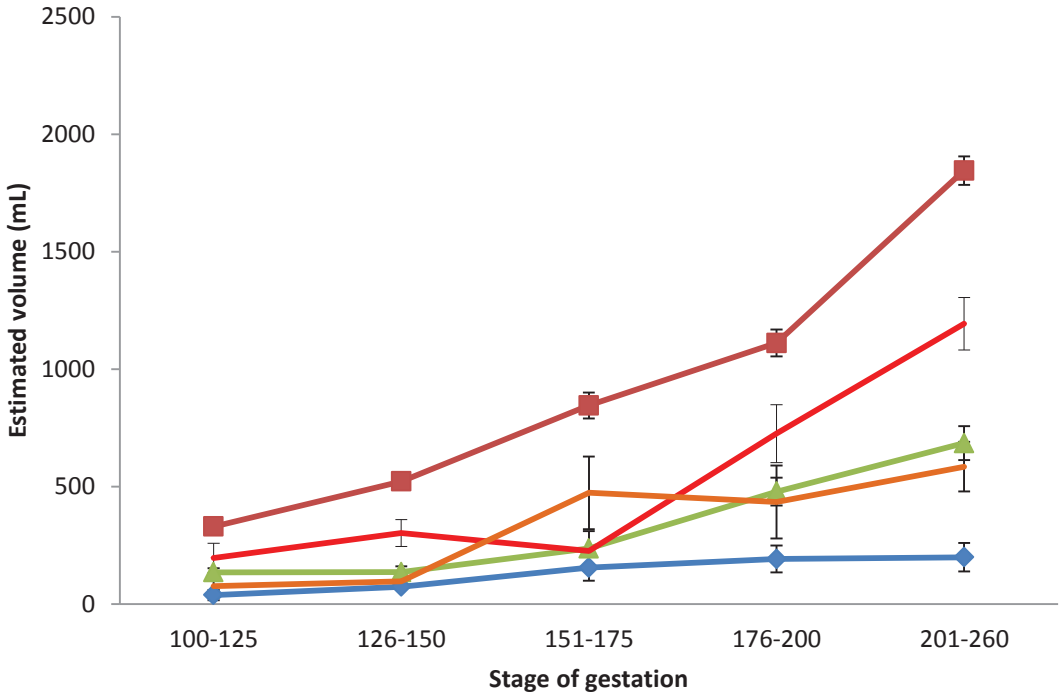


Figure 6.12: Change in total volume of placentome components; binucleate cells (◆), fetal trophoblast (■), fetal connective tissue (▲), maternal connective tissue (•) and maternal epithelium (•) from 100 to 260 days of gestation.

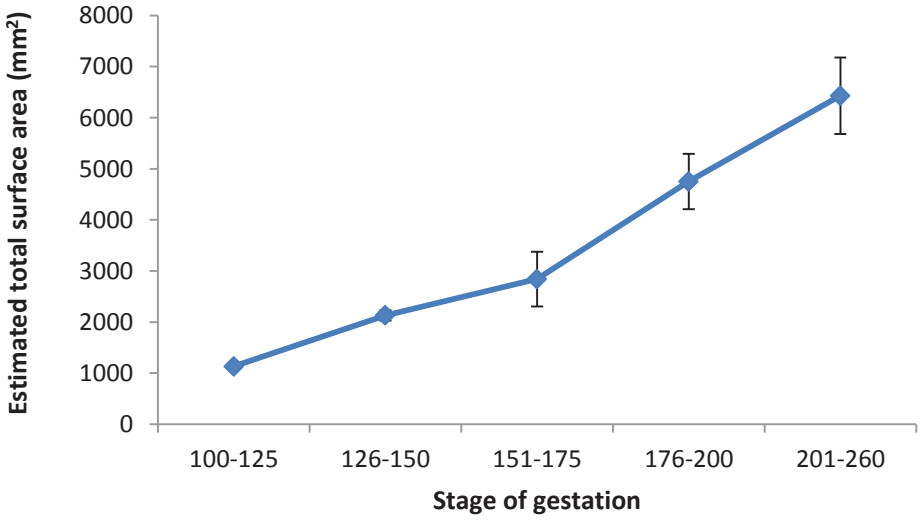


Figure 6.13: Change in estimated total surface area of the feto-maternal interface (FMI) of the bovine placentome from 100 to > 200 days of gestation. Data from 25 uteri with a section randomly selected from each cow and 3 fields viewed per section

6.3.10. Comparison between stereology and water displacement in estimates of the volume fetal and maternal tissue within the placentome

The total volume of both fetal and maternal components increased between Stage 1 and Stage 5 ($P < 0.0001$) (Table 6.3). There was a significant effect of stage on placentome volume and significant interaction between technique and tissue type and tissue type and stage ($P<0.001$ for all). All other main effects and interactions were not significant ($P>0.3$). This is illustrated in Fig 6.14.

For the second mixed model, with ratio of fetal to maternal tissue as the outcome variable, there was no significant effect of stage or interaction between stage and technique on tissue ratio ($P>0.6$) but there was a significant difference between estimates using stereology and those using water displacement technique ($P<0.001$). Overall mean ratio of fetal to maternal tissue was 0.844 using water displacement and 1.9 using stereology. Analysing the techniques separately, there was a significant association between gestational age and ratio of fetal to maternal tissue for water displacement ($P=0.006$) but not for stereology ($P=0.863$). This is illustrated in Figure 6.15.

Table 6.3: Effect of stage of gestation on mean volume of fetal and maternal placentome tissue estimated using either stereology or water displacement (Chapter 4 of the present study).

Stage of Gestation	Fetal tissue stereology (mL)	Maternal tissue Stereology (mL)	Fetal tissue volume (mL)	Maternal tissue volume (mL)
100-125	506 ± 79.9^{a}	274 ± 49.1^{a}	415 ± 62.98^{a}	364 ± 55.3^{a}
126-150	734 ± 73.3^{ab}	404 ± 77.7^{ab}	565 ± 36.3^{ab}	569 ± 36.6^{a}
151-175	1230 ± 266.2^{bc}	706 ± 167.7^{b}	837 ± 164.2^{b}	1100 ± 216.5^{b}
176-200	1800 ± 187.1^{c}	1140 ± 57.5^{c}	1260 ± 80.7^{c}	1680 ± 107.7^{c}
201-260	2930 ± 329.8^{d}	1580 ± 142.9^{d}	1630 ± 71.8^{d}	2880 ± 127.1^{d}
P value	<0.0001	<0.0001	<0.0001	<0.0001

Different superscripts within columns indicates means that are significantly different ($P<0.001$)

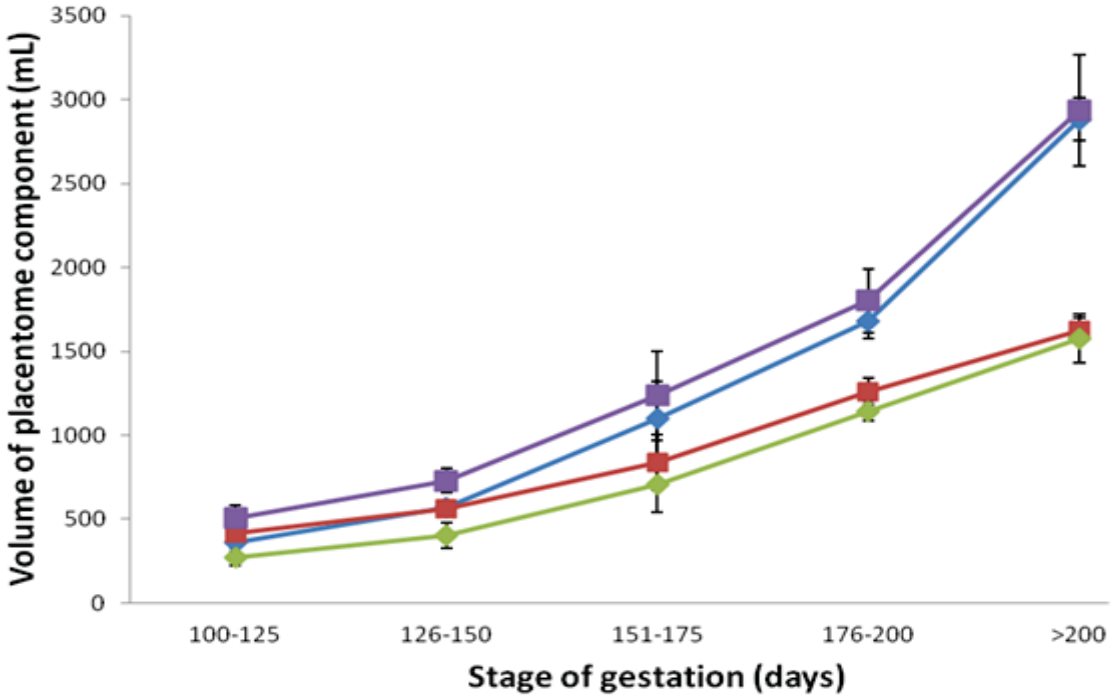


Figure 6.14: Change in the volume of fetal and maternal tissue in the placentome during gestation as estimated using stereology (fetal [■]; maternal [■]) and water displacement (fetal [◆]; maternal [◆]).

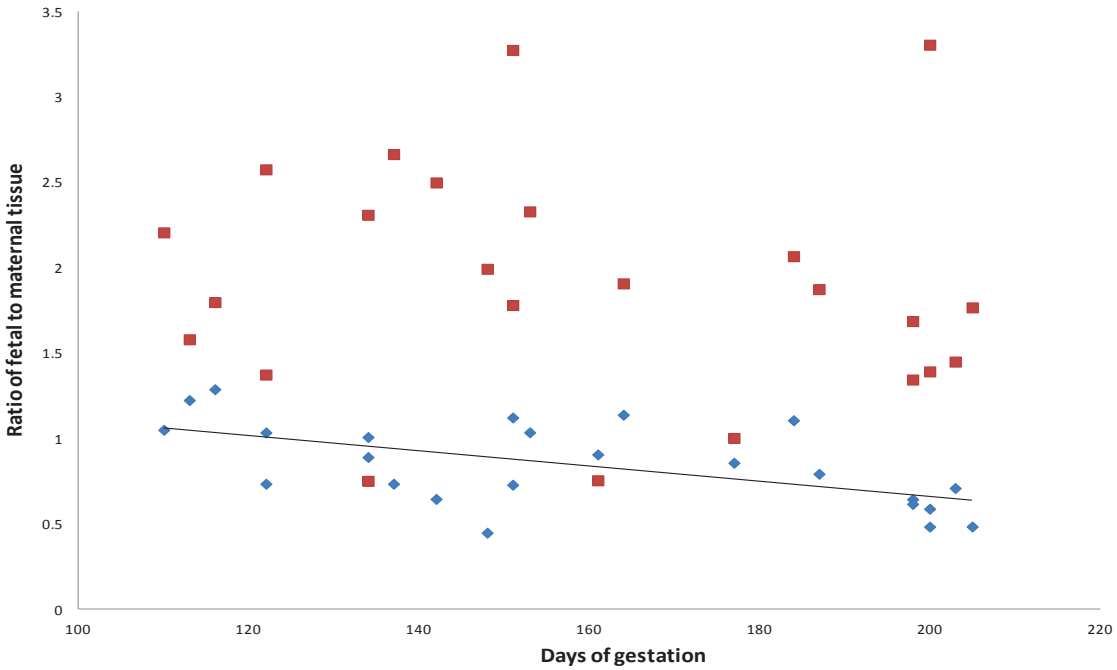


Figure 6.15: Effect of gestational age on ratio of fetal to maternal tissue (by volume) in placentome estimated using water displacement (◆) or stereology (◆). Solid line is line of best fit for water displacement (R^2= 0.333), there was no significant association for stereology (R^2=0.01).

6.4. Discussion

This study is the first study of cattle that has successfully used stereology to describe and quantify the changes in the fetal and maternal components of intact placentomes during gestation. The key findings of the study are that despite the marked change in volume of placentomes which occurs during gestation, there were no significant changes in the relative volume densities of any of the tissues measured (binucleate cells, fetal trophoblast, fetal connective tissue, maternal connective tissue and maternal epithelium) or in the surface density of the feto-maternal interface.

The first study to use stereology in the bovine placentome was Kannekens *et al.* (2006). This study was more of an evaluation of the technique as placentomes from only one cow at 135 days of gestation were used. The relative volume densities reported by Kannekens were 46% for fetal trophoblast, 6% for fetal connective tissue, 8% for maternal connective tissue and 40% for maternal epithelium (BNCs were not reported), whereas the equivalent figures for 126-150 days of gestation for this study were 46% , 12%, 26.7%, and 8.6% respectively. The figures for fetal trophoblast are similar in the two studies, but the proportion of fetal connective tissue recorded in this analysis is much higher than that reported by Kannekens *et al.* (2006). This difference is even more pronounced for maternal connective tissue, while the relative volume density of maternal tissue is significantly less. The reasons for these differences are unclear but are likely to be related to differences in classification of tissue. However this discrepancy is still present when fetal and maternal volume densities are compared. Kannekens *et al.* (2006) reported a relative volume density of maternal tissue of 48% compared to the 37% found in this study. The result reported by Kannekens *et al.* (2006) is consistent with that of Laven and Peters (2006) who reported that at around 135 days the proportion of maternal tissue (by volume) in the placentome was approximately 48%. The relative proportion of maternal tissue is also much lower than the proportion reported by Liu *et al.* (2010) in the yak at the same stage (57%). Some of this difference may be related to the measurement of BNC which would have been found in both maternal and fetal tissue and accounted for approximately 7% of relative volume density.

The results for surface density are more similar, (converting cm^{-1} to mm^{-1}) with the average surface density around $19/mm^{-1}$ compared to the $15.5/mm^{-1}$ reported by

Kannekens (2006). Both of these results are markedly lower than the 33.8/mm^{-1} reported by Liu *et al.*(2010).

Overall this study found no change in the relative volume densities of the measured tissues (and the sums of the fetal and maternal tissues) or in surface density of feto-maternal interface between 100 and 260 days of gestation. In contrast, Liu *et al.* (2010) reported that in the yak the relative volume density of BNC and caruncular endometrium decreased while that of fetal villi increased. Those authors reported no change in surface density over gestation – consistent with this study. These differences may reflect species differences in placentome development.

As total placentome volume changed significantly with gestational age, all tissue types increased similarly. The pattern of increase was similar for both total volume and tissue volume except that the difference between stages tended to be less distinct for the tissue types than for total placentome volume. However for all measures there was a significant difference between stage 4 (Day 176-200) and stage 5 (201-260), in contrast to the report by Liu *et al.* (2010) in yak where there was no change between Day 181-210 and >210. The reason for this difference was the marked increase in total placentome volume in cattle (2940 vs. 4500 mL, respectively) which did not occur in yak (2255 vs. 2206 mL, respectively)

The estimated total surface area of the feto-maternal interface increased similarly with gestational age. The estimated mean at Days 126-150 of gestation 2.131(± 0.19) m^2 is about 9 times smaller than the 18.5 m^2 reported by Kannekens *et al.* (2006). This difference is likely to be due to individual variation as the data reported by Kannekens *et al.*(2006) were from a single cow and their reported figure falls well within the distribution found in this study. The estimated surface area found in this analysis was smaller at all stages than the mean results reported by Liu *et al.*(2010); this was true even when the data from stage 5 (201-260 days) in this study were compared to the results reported by Liu *et al.*(2010) from 211+ days (64.3 vs. 65.13, respectively). This suggests that even though the total volume of placentomes in the yak is smaller, the surface area of the feto-maternal interface is greater. However it could be that the different method of estimating the volume of placentomes used by Liu *et al.*(2010) (Cavalieri principle) underestimated the placentome volume compared to water

displacement, but this does not explain the large difference in surface density between the two species which is the key driver of the increased total surface area of the feto-maternal interface. In contrast to a previous study using image analysis (Laven and Peters, 2006) which found that the ratio of fetal: maternal tissue by volume increased as gestation progressed, this study found no effect of gestational age on the relative proportions of fetal: maternal tissue by volume when volume was estimated by stereology. In contrast the ratio of fetal: maternal tissue by volume was estimated by water displacement of intact placentomes decreased as gestation progressed (consistent with the results reported by both Reynolds *et al.* 1990 and Laven and Peters 2001). Using stereology, Liu *et al.*(2010) reported results similar to Laven and Peters (2006), i.e. the proportion of fetal tissue by volume increased in yak placentomes as gestation progressed, except that the proportion of fetal tissue was much smaller in early gestation in the yak (36% compared to 50% in the cow) although proportions nearer term were similar (54 vs. 55%, respectively). The reason for the difference between this study and that reported by Laven and Peters (2006) is unclear.

Although there was no change with gestational age, the mean proportion of fetal tissue by volume estimated by stereology was always much greater than the proportion estimated by using water displacement of separated placentomes. As the density of placentome tissue is approximately 1 and consistent across gestation (see Chapter 4) this difference is consistent with the difference between the volume data from both Kannekens *et al.*(2006) and Laven and Peters (2006), who both used intact placentomes, and the weight data reported by Reynolds *et al.* (1990) and Laven and Peters (2001), who both manually separated placentomes into caruncles and cotyledons. Kannekens *et al.*(2006) concluded that the difference was due to insufficient separation of maternal and fetal, however both Reynolds *et al.*(1990) and Laven and Peters (2001) reported that there was only limited or no failure of separation, far below the level of separation required to produce the differences between stereology of intact placentomes and water displacement of separated placentomes seen in this analysis. The most likely reason for the difference is that the physical estimation of fetal and maternal proportion by volume using the water displacement method measures the whole placentome including both the tissue where exchange occurs and the non-exchange tissue such as the endometrial stalk and the non-villous areas of the cotyledon, whereas stereology focuses on the exchange area. The differences between the techniques are therefore due to the non-exchange

tissue having a higher proportion of maternal tissue than the exchange tissue. As stereology focuses on the exchange tissue it is likely that the results from stereology reflect placentome capacity better than the results from simple separation.

This study has confirmed that stereology is a useful technique to study placental development in the cow and has highlighted differences between cattle and yak in placentome development and relative tissue proportions. As stereology focuses on the interface between fetal and maternal villi, its results are focussed on placental capacity which cannot be measured by simpler methods such as placentome separation and water displacement as those measures include tissue which does not function as exchange tissue.

Chapter Seven

The Use of Lectins to Study the Development of the Bovine Placentome During Gestation

7.1. Introduction

Lectins or agglutinins are proteins of plant or animal origin that have the ability to bind to specific terminal sugars. Such sugars can be found throughout the cell, at sites which vary from tissue surfaces to internal structures. They can be present in the form of simple sugars, or as part of compounds that are linked by sugar moieties or in complex carbohydrates. This ability of lectins to bind to sugars means they can serve as markers to visualise glycoconjugates and, as different lectins bind to different conjugates, they can be used to examine the distribution of specific molecules in tissues or organs (Spicer and Schulte 1992). By linking biotinylated lectins to a label, such as a fluorescent substance which can be visualised under ultraviolet light, or an enzyme, such as horse radish peroxidase which produces a visible coloured product (Hoedt-Schmidt 1998), lectins can therefore be used as histochemical stains.

Glycoproteins have been recognised as a common component of cell membranes in the placenta (Zoli *et al.* 1991), so lectin histochemistry could be a useful method of identifying the patterns of glycoprotein distribution in the fetal and maternal tissues of which the placenta is composed. Because of the specificity of lectin binding to different sugars, lectin staining could also be used to help identify *in situ* which glycoproteins are constituents of the placenta.

Previous studies of lectin binding to ruminant placentas have focussed on characterising lectin binding properties of the binucleate cell of the fetal trophoblast. Lectins that have been used to examine binucleate cells include *Dolichos buflorus* (DBA), *Glycine max* (SBA) and *Phaseolus vulgaris* leukoagglutinin (PHA-L) (Nakano *et al.* 2002). Three studies of the bovine placenta have been published using different biotynylated lectins; during early pregnancy (Day 18 – 40 of gestation; Lehmann *et al*.1992), early to late pregnancy (Days 40-270; Munson *et al*.1989) and near term (Jones *et al.* 1994). The pattern of glycosylation and the changes observed during gestation suggests a functional role for glycoproteins in the development of the bovine placenta, particularly in terms of

the distribution and migration of binucleate cell (BNC) granules at different stages of pregnancy. These studies have been interpreted to suggest that glycoprotein in the trophoblast cells located in the feto-maternal unit could be responsible for cell-to-cell adhesion during placentation and for directing hormones to their receptors.

However, the previous studies of lectins binding to the bovine placenta have been relatively limited in scope, since either numbers of animals were small, or observations were limited to a narrow period of pregnancy; whilst the quantification of the intensities of lectin binding has only been made on a subjective basis.

The present study was therefore undertaken to characterise the changes in glycoprotein expression within the placentomes during gestation, expanding on the data produced by Jones *et al.* (1994) using three lectins (DBA, SBA, and PHA-L). Computer- assisted software developed was used to better quantify the intensities of the binding of lectins to the placentome tissue to augment the more qualitative descriptions of sites of binding.

7.2. Materials and Methods

7.2.1. Animals and tissue sampling

Uteri (n=25) from pregnant Friesian, Jersey and crossbred cows, that were between 100 and 260 days of gestation, were obtained from a local abattoir (Affco NZ Ltd, Fielding, New Zealand) and Massey University Veterinary Teaching Hospital. These uteri were categorized into five stages of gestation by measuring the crown rump length of the fetus :

Stage 1: Days 100-125 of gestation (n=5),

Stage 2: Days 126-150 of gestation (n=5),

Stage 3: Days 151-175 of gestation (n=5),

Stage 4: Days 176-200 of gestation (n=5),

Stage 5: Days 201-260 of gestation (n=5).

Initially, eight placentomes were randomly selected from each cow used in chapters 4 and 6 of this thesis. Two of these eight were randomly selected for lectin histochemistry. These placentomes were carefully dissected from the uteri and cut into slices of about 10 mm thick and immediately fixed in 4% formaldehyde. These slices of tissue were trimmed and embedded into paraffin wax before being cut into 5 µm thick sections. Sections were them mounted on super frost slides prior to lectin staining.

7.2.2. Lectin histochemistry

The protocol for lectin staining was as described by Vector Labs, Burlingame, CA, USA (http://www.ihcworld.com/_protocols/general_IHC/immunoenzyme_pod.htm).
Sections were deparaffinised in xylene twice for 5 mins and rehydrated in 100%, 90%, 70% and 40% ethanol for 2 mins each. Sections were then rinsed in water for 2 mins after which they were immersed in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) in a staining dish and heated in the water bath at 95˚C for 30 mins to expose the antigen for binding sites. Thereafter, the sections were washed with Tris-buffered saline (TBS) washing buffer (1 portion of 10x 0.5 M Tris base, 9% NaCl, 0.5% Tween 20, pH 7.6) before incubating the sections in 200 mL TBS buffer with 0.2 g trypsin at 37°C for 30 mins to digest the enzyme. Sections were rinsed three times in washing buffer and stained with one of three biotinylated lectins (*Dolichos buflorus* (DBA), *Glycine max* (SBA) or *Phaseolus vulgaris* leukoagglutinin (PHA-L); Vector Labs, Burlingame, CA, USA) and incubated with 2 µg/mL of lectin inTBS at room temperature (25°C) in a dehumidifying chamber for 1hour or at 4°C overnight. The sections were rinsed again with washing buffer (3x 5 mins) and finally incubated with fluorescent-conjugated streptavidin) (Alexa Fluor 546 Invitrogen, Grand Island, NY, USA) (2 µg/mL in TBS buffer) at room temperature, in the dark, for 1 hour to visualise the binding sites. After incubation, the sections were rinsed in washing buffer and covered with coverslips.

Three fields were randomly selected within the feto-maternal unit per cow per section. The sections were viewed immediately under an Olympus fluorescent microscope (Olympus BX51TRF, Japan) and photographed with an attached digital camera (Optronics version 070121-01B) and the images were captured and saved with MagnaFire 2.1C software (Olympus Centre valley, PA), 40x objective and 2 sets of

excitation filters. These images were the permanent record since the fluorescence fades out within few hours.

For negative controls, lectin was replaced with phosphate buffered saline (PBS) on the sections before incubation to ensure there was no non- specific binding. The positive or specificity control involves the pre-incubation of the biotynylated lectins (20 μg/mL) with inhibitory sugars such as 0.2 M N-acetylgalactosamine for DBA, SBA and PHA-L. Combinations of lectins and their inhibitory sugars were incubated at a temperature of 37°C for one hour before they were used for staining. These tests were carried out to ensure that lectins were binding via their specific binding sites.

Fluorescence-emitting cells within each section were qualitatively identified (i.e. the tissue type was described; Figure 7.1) prior to processing of the images using a computer-assisted software.

7.2.3. Processing

Fluorescence intensity measurement:

Computer-assisted image analysis software was used (custom developed software, with assistance from the School of Engineering, Massey University, Palmerston North, New Zealand) to measure the fluorescence intensity of the sections by transforming the coloured images into bright, medium and dark greyscale images. This was achieved by calculating the intensity value for each pixel from its red, green and blue colour components. Pixel intensity was scaled from 0 to 255 using colour splitting to differentiate the dark background, and categorised as minimal binding (0 to 35), medium binding (36 to 180) and maximum binding (181 to 255). An example of a computer transformed image is shown in Figures 7.2 which illustrates the analysis of an unprocessed (7.2a) and processed (7.2b) images of the placentomes showing minimal, medium and maximum binding intensity. The percentages of maximum binding (fluorescent intensity) on the transformed images were used to describe the pattern of lectin binding in each section (two sections per animal). Fluorescence intensity per pixel for each colour of the processed image were analysed and described to show the types of cells affected i.e. uninucleate or binucleate cells, maternal or fetal epithelium, and their relative staining intensity.

7.2.4. Statistical analysis

The data set used for analysis was generated by dividing the area of maximum binding by total area of image expressed as a percentage. The association between fluorescence intensity and gestational age was analysed using repeat measures (general linear model: SAS 9.2, SAS Institute, 2011) with fluorescence intensity as the dependent variable (two measures per cow) and stage of gestation and cow as a fixed effects

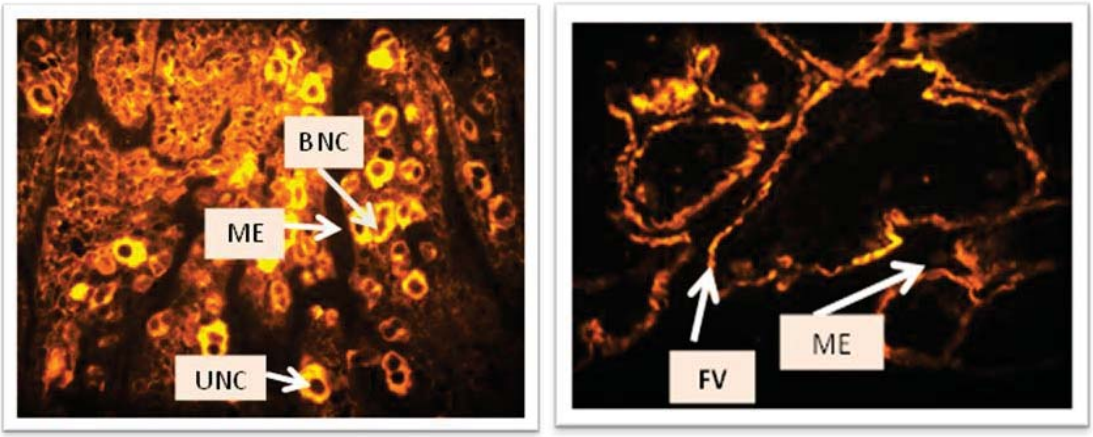


Figure 7.1: Lectin (DBA) staining of the feto-maternal unit showing the fetal and maternal components; binucleate cell (BNC), maternal epithelium (ME), uninucleate cell (UNC), fetal villi (FV). Magnification 400x.

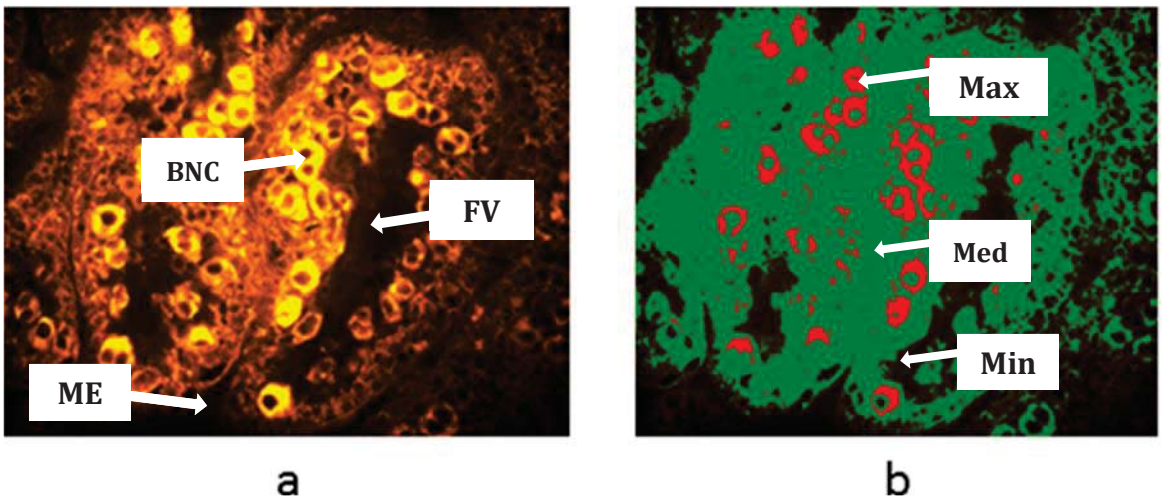


Figure 7.2: Unprocessed (a) and processed images (b) of DBA lectin binding to placental tissue visualised at magnification 400x. In Figure 7.2a, lectin binding is seen as area of yellow, orange and gold fluorescence. In Figure 7.2b, areas coloured black indicate minimal binding, green areas indicate medium binding, and red areas indicate maximum binding

7.3. Results

7.3.1. Validation of image analysis of binding intensity

Figure 7.3 showed that, across all three lectins and at all gestational stages, the programme clearly differentiated between minimal, medium and maximum binding

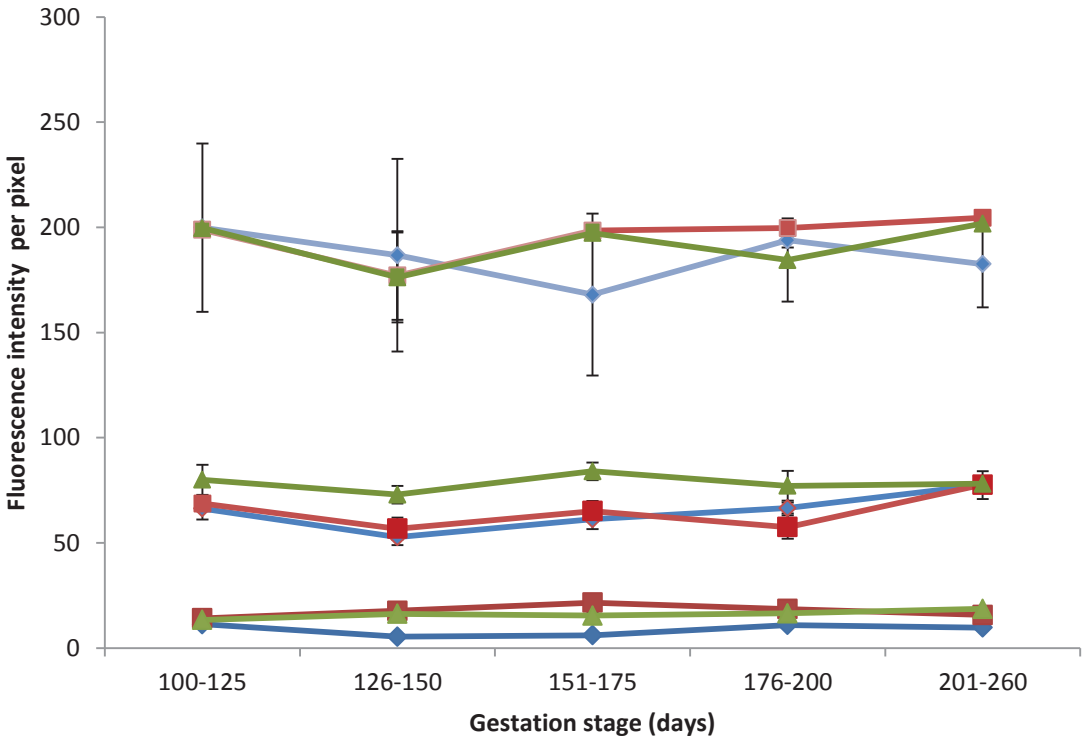


Figure 7.3: Mean fluorescence intensity per pixel for all lectin stains throughout all gestational stages, separated by image analysis into bright (intense binding; top group), medium (less intense binding; middle group) and dark (minimal binding) intensities. Red line, SBA binding; green line, PHA-L; blue line, DBA.

7.3.2. Patterns of lectin binding to placentome tissue

All three lectins bound to fetal trophoblast and maternal epithelium at varying intensities throughout gestation.

Phaseolus vulgaris leukoagglutinin (PHA-L) binding

Binding of PHA-L lectin to bovine placentome tissues was strong throughout gestation. Patterns of binding at different stages of gestation are illustrated in Figures 7.4 -7.9

Across all stages of gestation, PHA-L bound principally to the maternal and fetal epithelium. There was only limited binding to other fetal structures and no binding to maternal tissues other than the epithelium. There was no evidence of preferential binding to any specific cell type within the epithelia, i.e. binucleate cells were not specifically targeted by the lectin. There was no obvious pattern to the changes of binding (Figure 7.5) that occurred throughout gestation, although in sections where binding intensity was highest (Day 151-175 of gestation), there was strong binding to the rest of the fetal villi in addition to binding to the fetal epithelium. Although there marked differences between stages in the intensity of binding, the large between-cow variation meant that there was no significant association between stage and binding (P=0.11). However, the variance at each stage was heterogeneous (P=0.023).

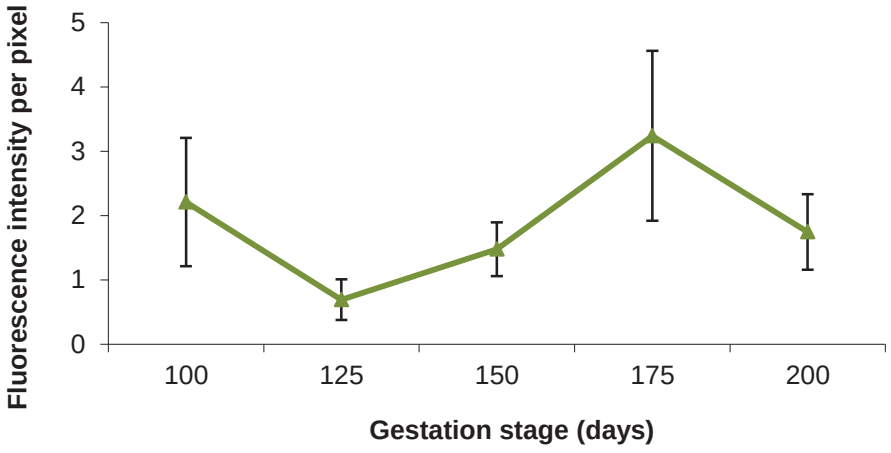


Figure 7.4: Mean (± SEM) fluorescence intensity for the binding of PHA-L to bovine placentomes between Days 100 and 260 of gestation.

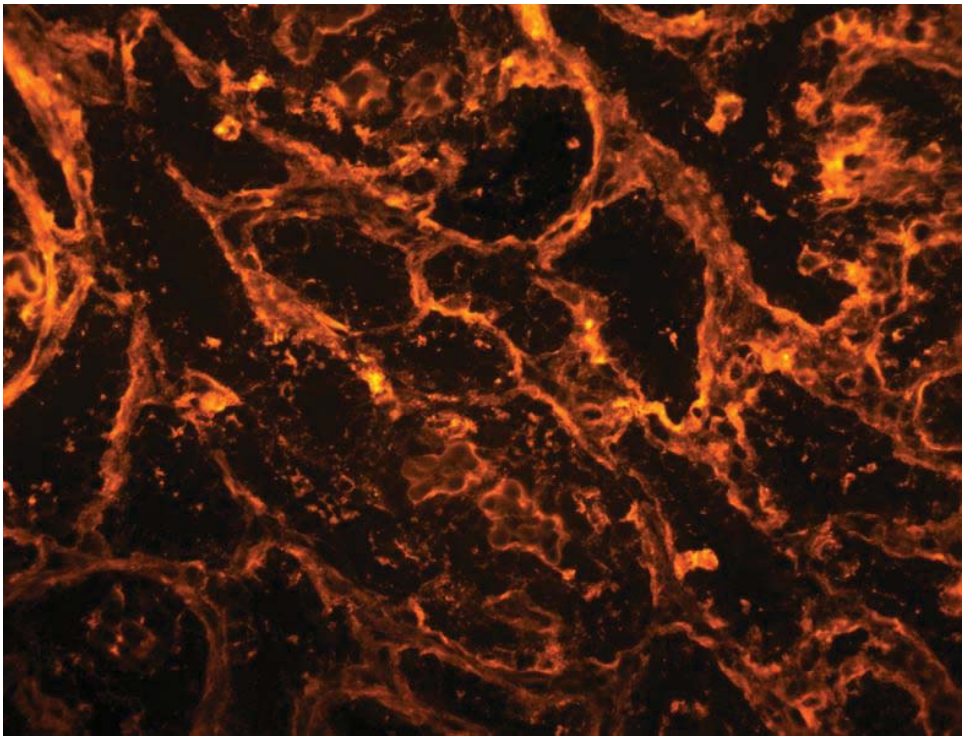


Figure 7.5: Binding of fluorescent-labelled biotinylated PHA-L to placentome tissue at Gestation Stage 1 (Days 100-125). Image captured at 400 x magnification

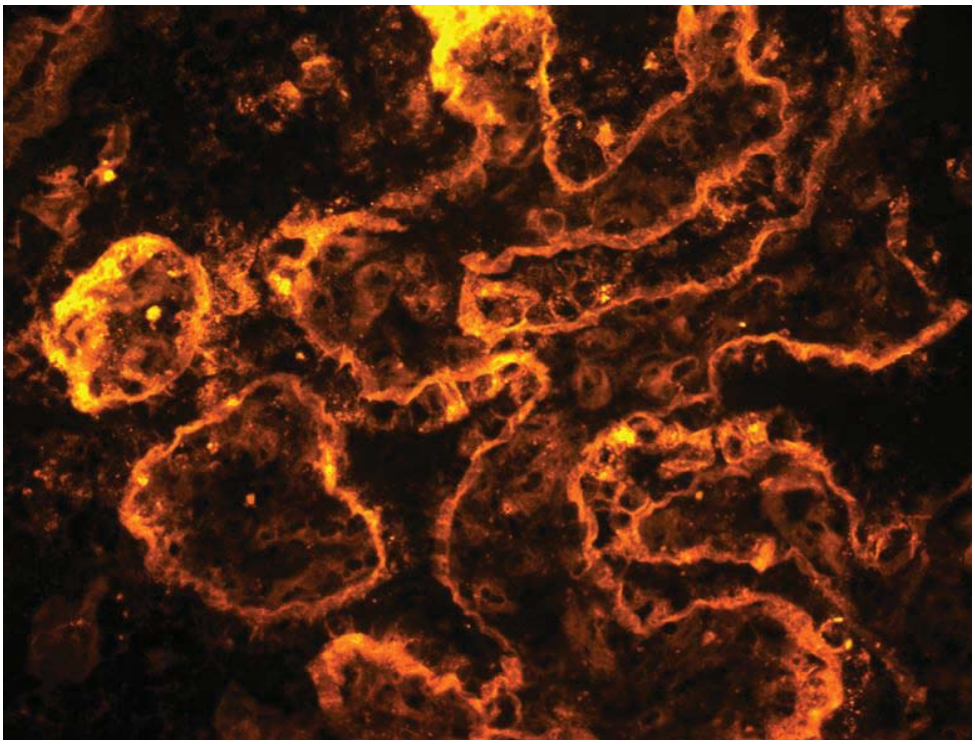


Figure 7.6: Binding of fluorescent-labelled biotinylated PHA-L to placentome tissue at Gestation Stage 2 (Days 126-150). Image captured at 400x magnification.

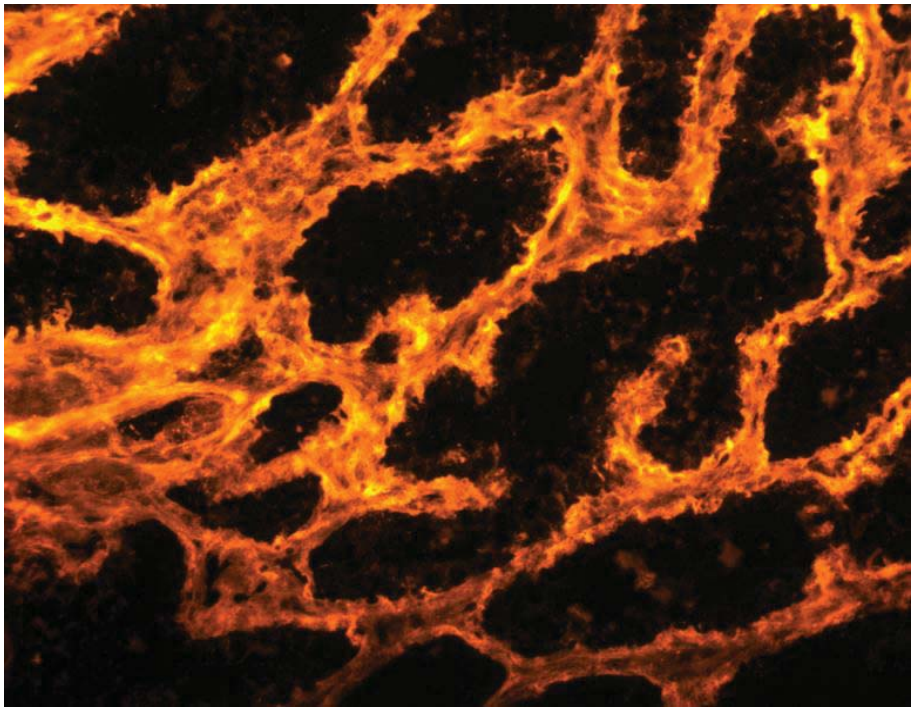


Figure 7.7: Binding of fluorescent-labelled biotinylated PHA-L to placentome tissue at Gestation Stage 3 (Days 151-175). Image captured at 400x magnification.

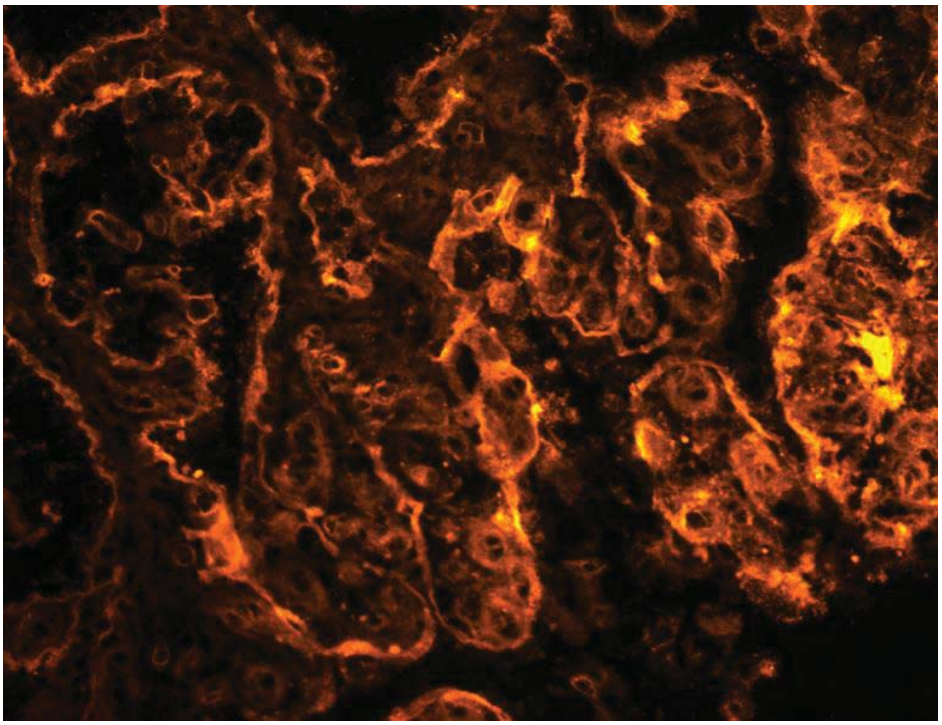


Figure 7.8: Binding of fluorescent-labelled biotinylated PHA-L to placentome tissue at Gestation Stage 4 (Days 176 -200). Image captured at 400x magnification.

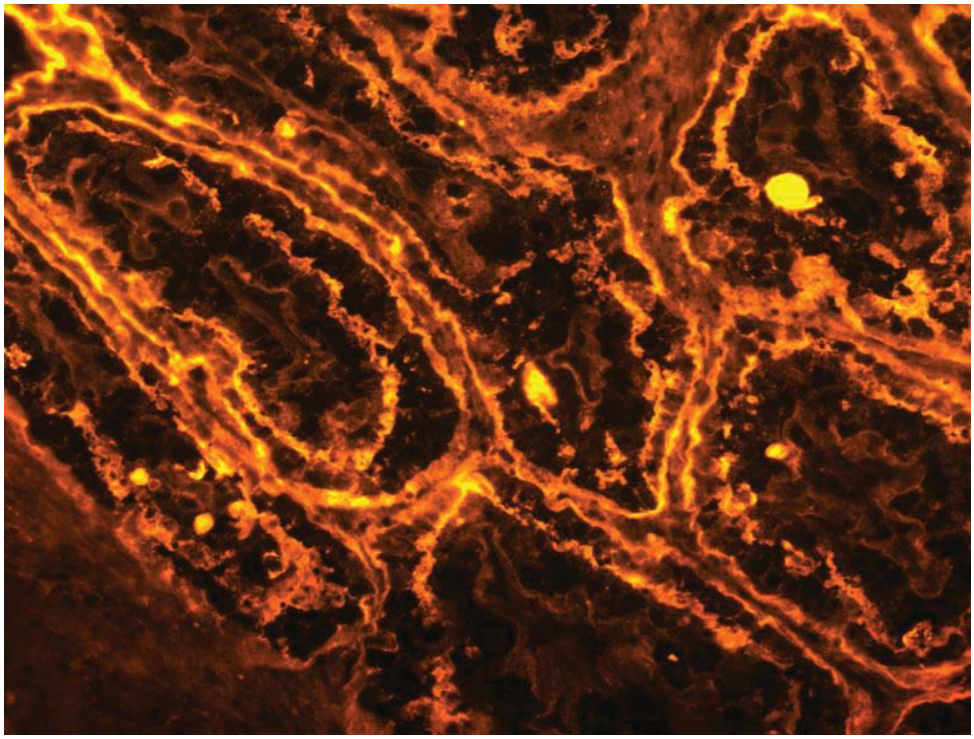


Figure 7.9: Binding of fluorescent-labelled biotinylated PHA-L to placentome tissue at Gestation Stage 5 (Days 201-260). Image captured at 400 x magnification.

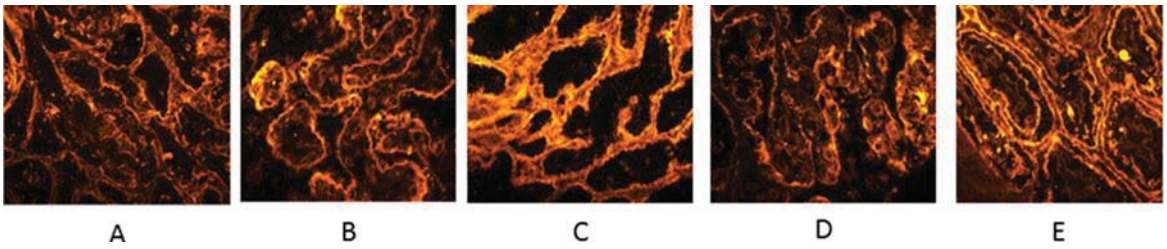


Figure 7.5-7.9: Binding of fluorescent-labelled biotinylated PHA-L to placentome tissue at gestation stages 1 – 5. (A) Days 100-125; (B) Days 126-150; (C) Days 151-175; (D) Days 176-200 and (E) Days 201-260). Image captured at 400x magnification.

Dolichos biflorus (DBA) binding

Binding of DBA lectin to bovine placentome tissues at different stages of gestation is illustrated in Figures 7.10-7.15.

Overall, DBA was mostly commonly bound to the maternal and fetal epithelium, although binding was also seen in a small number of binucleate/uninucleate cells. There was only limited binding to other fetal structures and no binding to maternal tissues other than the epithelium.

There was a significant effect of gestational stage on binding ($P<0.0001$ respectively). Binding was moderate to low initially (Days 100-125), but was lower on Days 125-150. Binding thereafter increased, and was maximal in the final gestation stage (Days 201-260). There was a gradual increase in binding pattern as gestational age increased but no visible change in the cells which exhibited DBA binding as fluorescence intensity increased.

Analysis of fluorescence intensity confirmed that the relationship was quadratic (DBA binding intensity = 0.9007- 0.7699*gestation stage + 0.1574 (gestation stage) ^{2} (R^2 = 0.34 and P=0.001) i.e. the binding decreased initially but then increased significantly after the third stage.

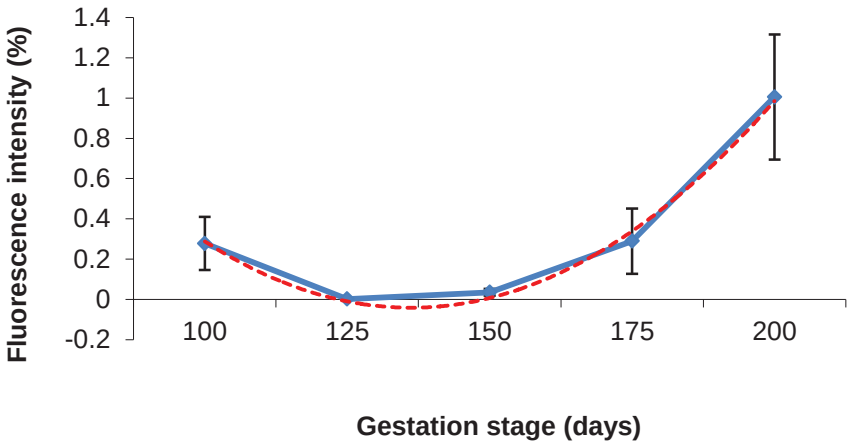


Figure 7.10 : Mean (± SEM) fluorescence intensity for the binding of DBA to bovine placentomes between Days 100 and 260 of gestation. The red dotted line shows the quadratic line superimposed on the data graph.

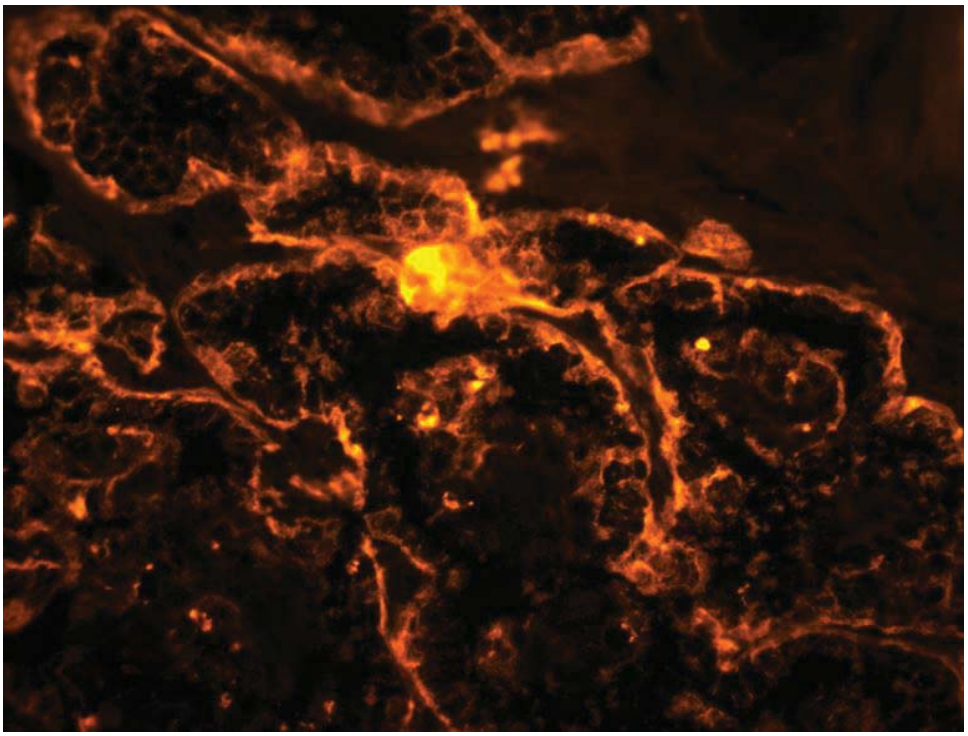


Figure 7.11: Binding of fluorescent-labelled biotinylated DBA to placentome tissue at Gestation Stage 1 (Days 100-125). Image captured at 400x magnification.

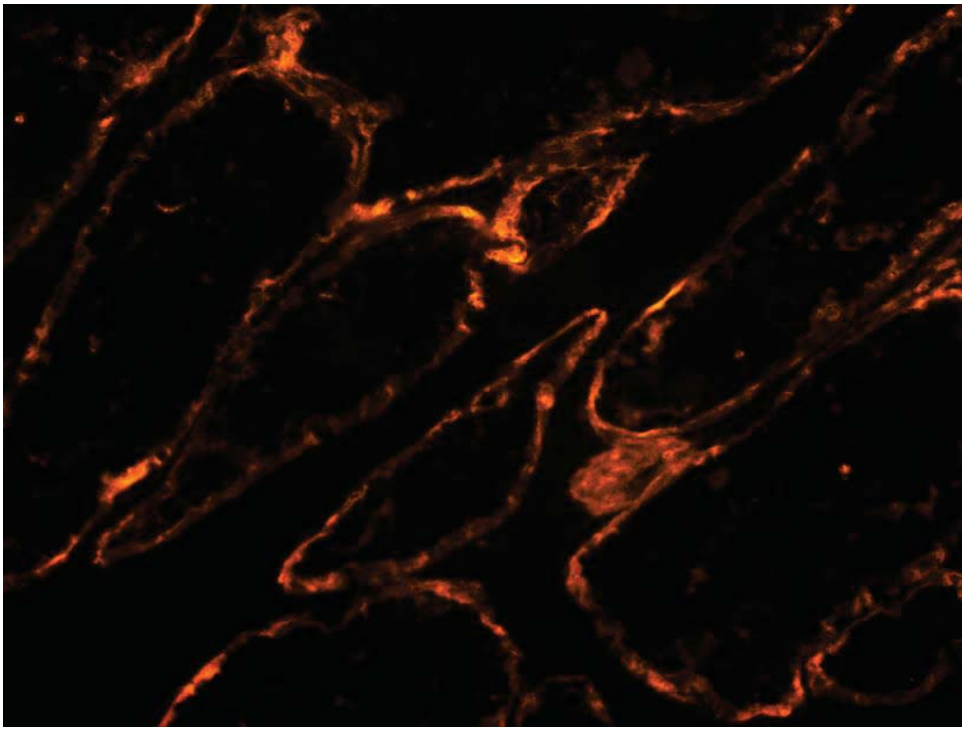


Figure 7.12: Binding of fluorescent-labelled biotinylated DBA to placentome tissue at Gestation Stage 2 (Days 126-150). Image captured at 400x magnification.

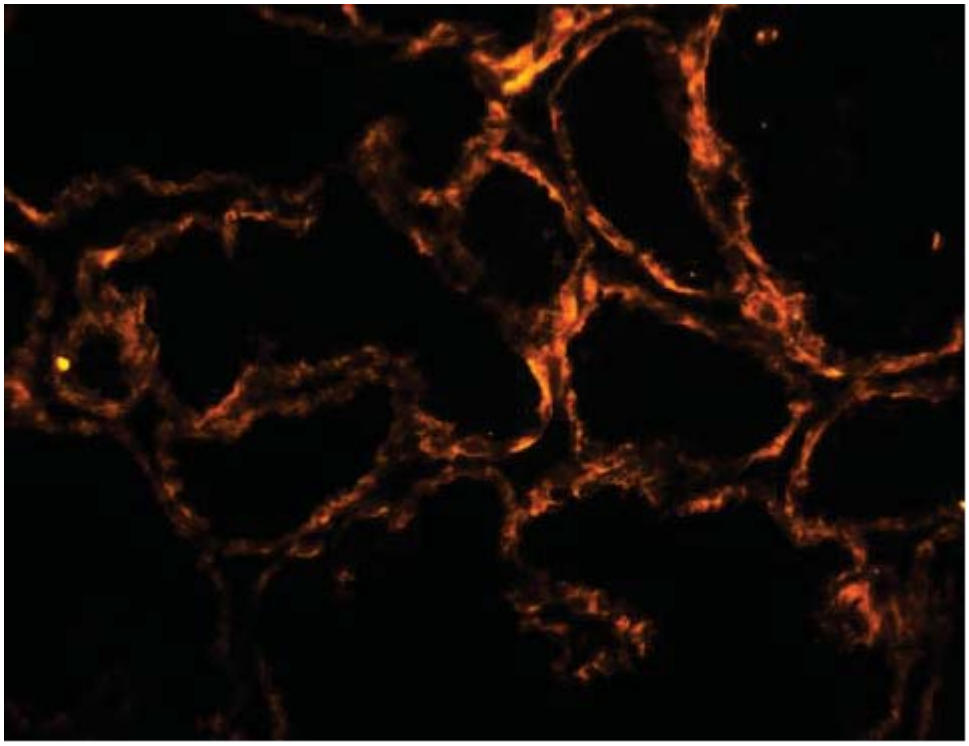


Figure 7.13: Binding of fluorescent-labelled biotinylated DBA to placentome tissue at Gestation Stage 3 (Days 151-175). Image captured at 400x magnification.

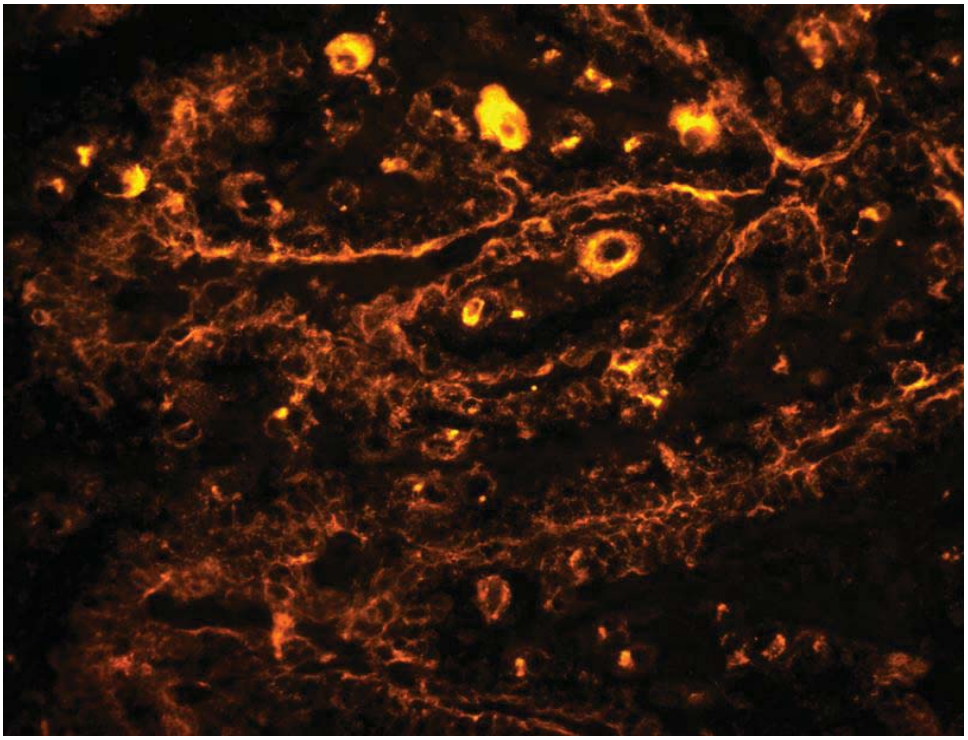


Figure 7.14: Binding of fluorescent-labelled biotinylated DBA to placentome tissue at Gestation Stage 4 (Days 176-200). Image captured at 400x magnification.

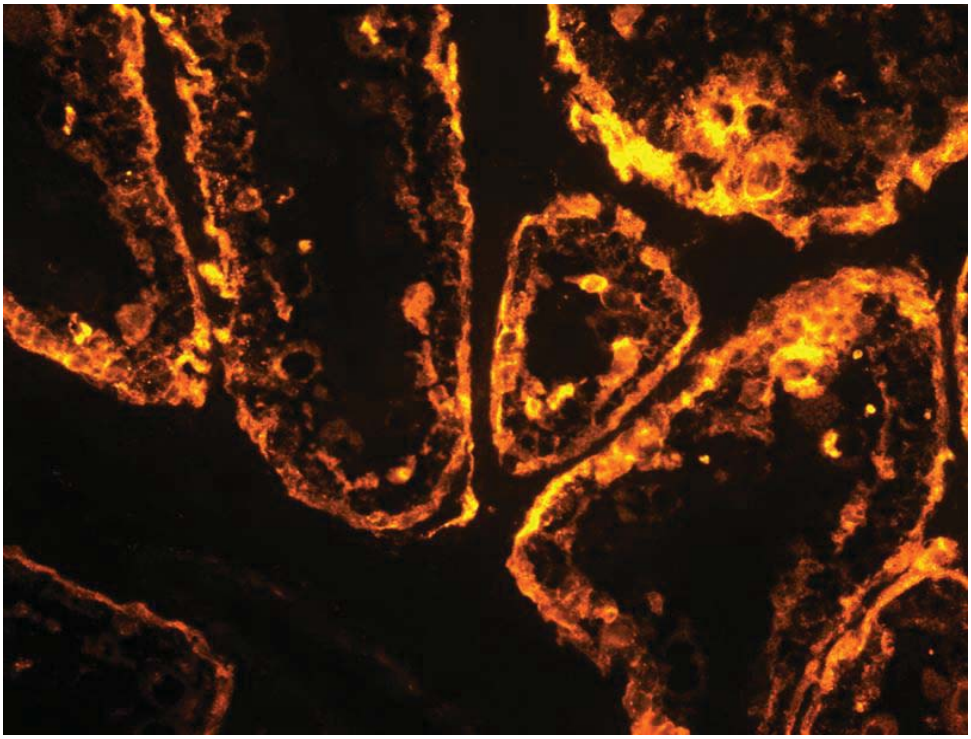


Figure 7.15: Binding of fluorescent-labelled biotinylated DBA to placentome tissue at Gestation Stage 5 (Days 201-260). Image captured at 400x magnification

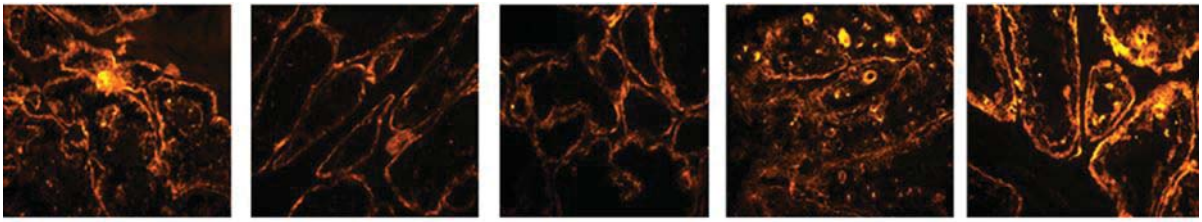


Figure 6.11 – 6.15: Binding of fluorescent-labelled biotinylated DBA to placentome tissue at gestation stages 1–5. (A) Days 100-125; (B) Days 126-150; (C) Days 151-175; (D) Days 176-200 and (E) Days 200-260). Image captured at 400x magnification.

***Glycine max* (SBA)**

Binding of PHA-L lectin to bovine placentome tissues at different stages of gestation is illustrated in Figures 7.16 -7.21

Overall, SBA binding followed a similar pattern (Figure 6.16) to that exhibited by DBA. SBA binding was mostly present in the maternal and fetal epithelium, although binding was also seen in some binucleate and regular epithelial cells (uninucleate cells). Binucleate cell binding was more prominent for SBA than DBA. There was only limited binding to other fetal structures and no binding to maternal tissues other than the epithelium, except at gestation Stage 5 when overall binding was at its peak.

There was a significant effect of gestational stage on binding ($P<0.0001$). However, between cow variation was not significant ($P=0.248$). Binding was moderate to low initially (Days 100-125), but was lower on Days 125-150. Binding thereafter increased, and was maximal in the final stage of gestation (Days 201-260). Analysis of fluorescence intensity confirmed that the relationship was quadratic (SBA binding intensity = 1.2059-1.0676*gestation stage + 0.2342 (gestation stage)^2 ($R^2 = 0.29$ and $P=0.008$), although the relationship was less strong than reported for DBA.

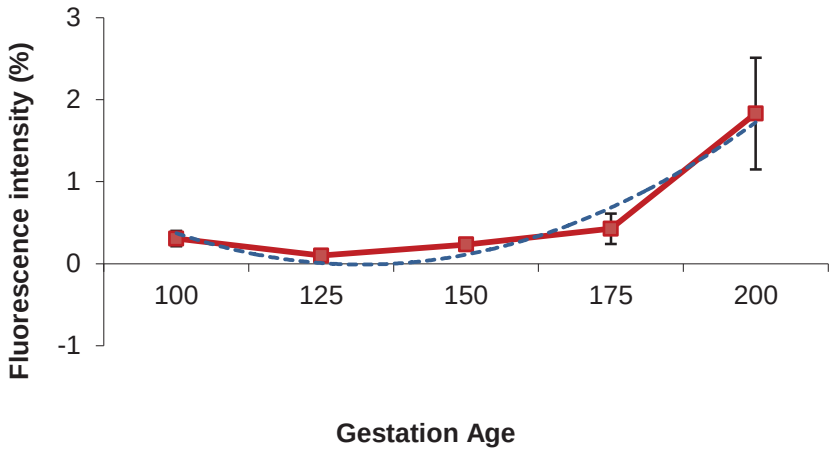


Figure 7.16: Mean (± SEM) fluorescence intensity for the binding of SBA to bovine placentomes between Days 100 and 260 of gestation. The blue dotted line shows the quadratic line superimposed on the data graph.

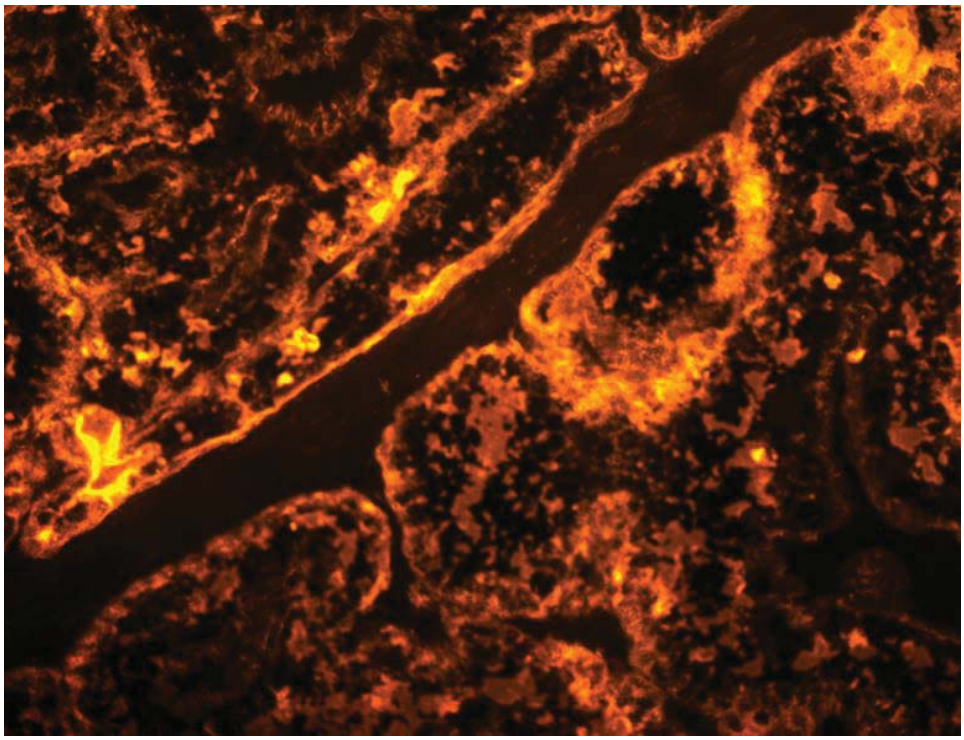


Figure 7.17: Binding of fluorescent-labelled biotinylated SBA to placentome tissue at Gestation Stage 1 (Days 100-125). Image captured at 400x magnification

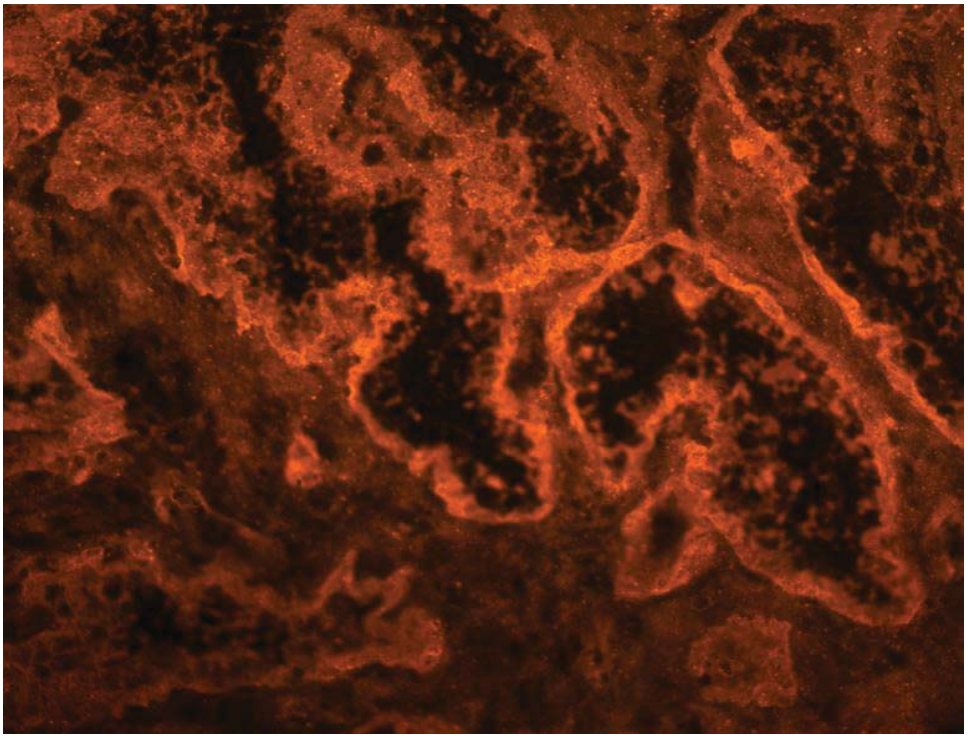


Figure 7.18: Binding of fluorescent-labelled biotinylated SBA to placentome tissue at Gestation Stage 2 (Days 126-150). Image captured at 400x magnification.

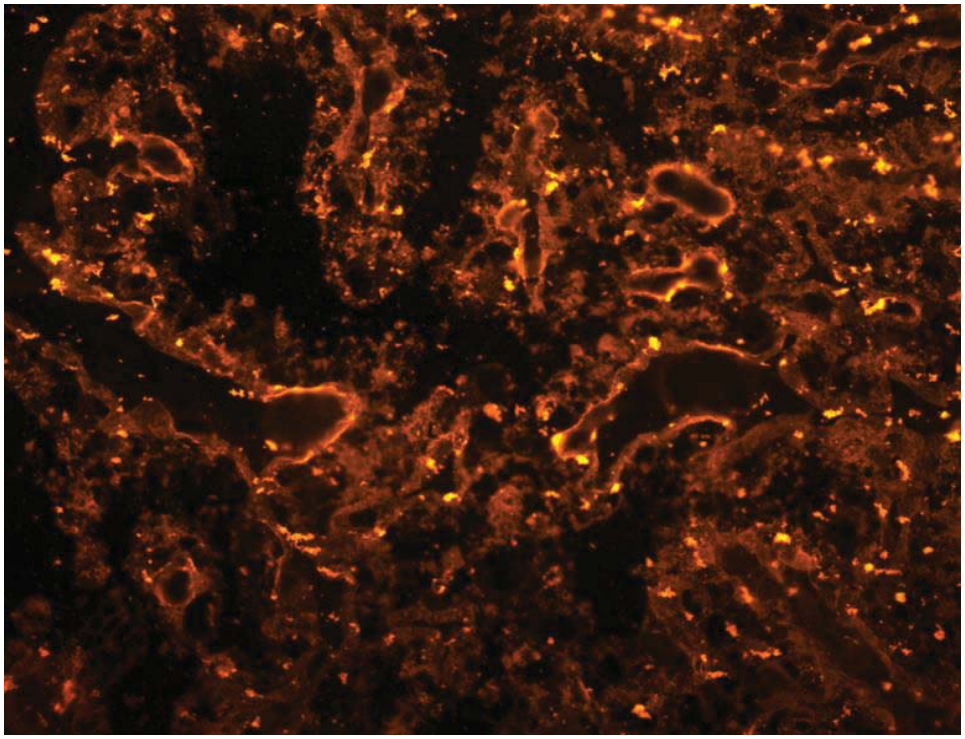


Figure 7.19: Binding of fluorescent-labelled biotinylated SBA to placentome tissue at Gestation Stage 3 (Days 151-175). Image captured at 400x magnification.

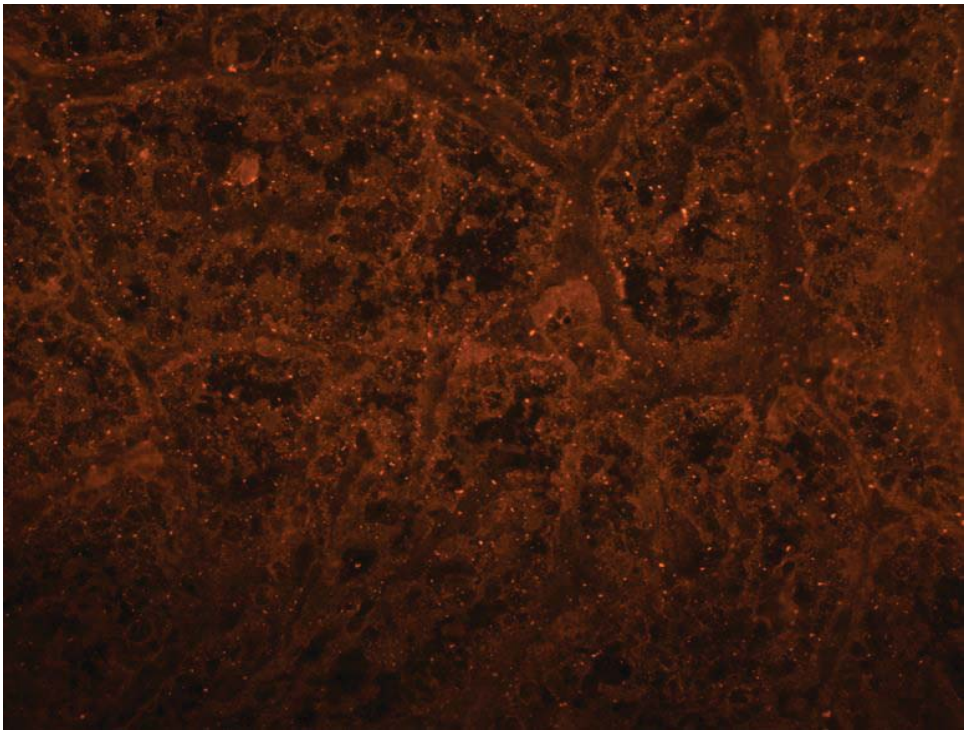


Figure 7.20: Binding of fluorescent-labelled biotinylated SBA to placentome tissue at Gestation Stage 4 (Days 176-200). Image captured at 400x magnification.

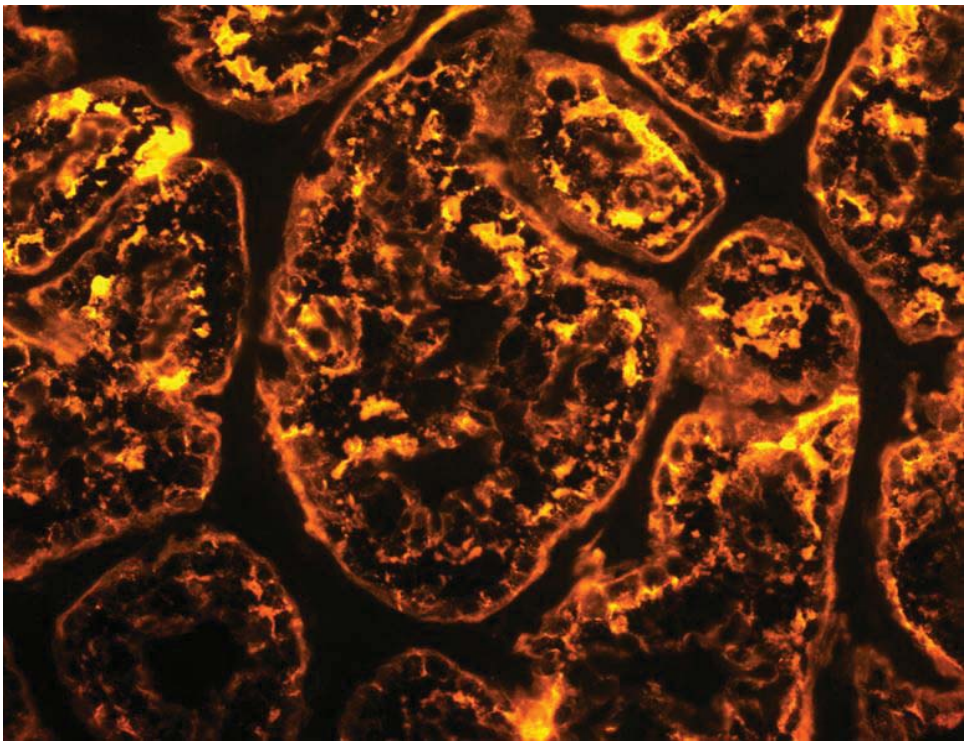


Figure 7.21: Binding of fluorescent-labelled biotinylated SBA to placentome tissue at Gestation Stage 5 (Days 201-260). Image captured at 400x magnification.

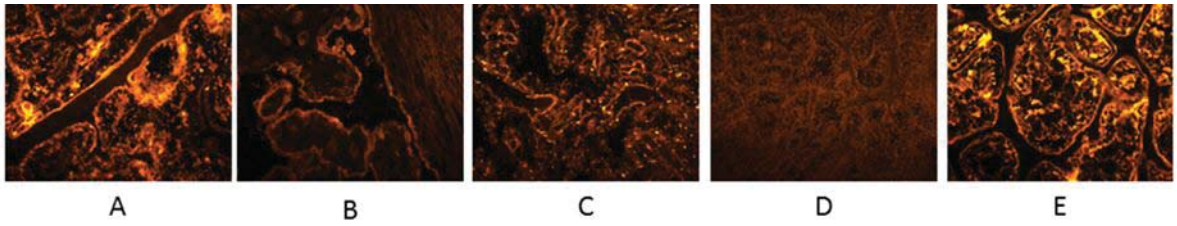


Figure 7.17– 7.21: Binding of fluorescent-labelled biotinylated SBA to placentome tissue at gestation stages 1–5. (A) Days 100-125; (B) Days 126-150; (C) Days 151-175; D) Days 176 200 and (E) Days 200-260). Image captured at 400x magnification.

7.4. Discussion

Glycoproteins play a significant role in the development of the bovine placenta, whether as the layer between the fetal and maternal tissue, which is thought to be important in the maintenance of adhesion between these two layers, or as pregnancy associated glycoproteins released into the maternal tissue after fusion between the maternal epithelium and the binucleate cells The presence of glycoproteins has been studied in many tissues via the use of lectin binding (Lis and Sharon 1986; Jones *et al.* 1994; Beuth *et al.* 1995); and, because individual lectins bind to specific carbohydrate groups, (Sharon and Lis 1989; Sharon 2007), lectin-binding studies not only reveal patterns of glycoprotein expression, but also reveal something of the chemical structure of the molecules that are present. Lectin binding studies have been used to characterise patterns of expression of glycoproteins involved in the placentation of non-ruminant species (Peel and Bulmer 1996), and, in ruminants, has particularly been used to characterize the role of binucleate cells in the production of glycoproteins. Despite this interest in the binucleate cell, relatively few studies have examined gestational changes in lectin binding in the broader spectrum of tissues of the ruminant placenta. Moreover, the studies that have been undertaken to date have been predominantly qualitative in nature, since binding has only been quantified in terms of subjective visual scoring. The present study is therefore the first in which binding intensities have not only been described on a qualitative basis in terms of binding to specific placental tissues, but in which they have also been fully quantified through the use of computer-assisted image analysis.

Previous authors have described the binding of a broad spectrum of lectins during early bovine pregnancies (Days 18-40 of gestation: Lehmann *et al.*, 1992), at a limited number of time points throughout gestation (Days 40-270: Munson *et al.*, 1989) or near term (Jones *et al.*, 1994) of bovine pregnancy; however, lectin binding in those studies

was described only in terms of subjective scoring of staining intensities. The present study has expanded the information provided by these earlier investigations, by objectively comparing binding intensities at different stages of gestation and thereby elucidating not only the different binding patterns of multiple lectins, but also quantifying differences of binding between individual animals.

In the present study, binding of PHA-L was greater than that of DBA and SBA at all stages of gestation except after Day 200. However, significant between-cow variation, which was much greater for PHA-L than for the other two lectins, meant that there was no significant change over gestation in binding. Whether the substantial level of between cow variation for PHA-L binding obscured small changes in its binding that were actually due to gestational stage is open to question. However, the present results were broadly consistent with a previous study of PHA-L binding to bovine binucleate cells (Jones *et al.* 1994), whilst a similar lack of significant difference through gestation was also reported by Klisch and Leiser (2003), who found no changes in PHA-L binding as gestation age increased.

Conversely, the pattern of binding by SBA and DBA showed a marked effect of gestational stage, in which intensities declined from Stage 1 (Days 100-125) to a nadir at mid gestation, and thereafter increased substantially to maximum values at Gestation Stage 5 (Days 200-260). DBA and SBA are specific for glycoproteins with terminal α-linked GalNac and α-/β-linked GalNac respectively. So this finding suggests that such glycoproteins increase in late gestation. Significant cow-to-cow variation was present for DBA, although not for SBA in binding intensity. Munson *et al.* (1989) reported a small decrease in binding intensity for DBA between Day 40 and 80 of gestation (from + to +/-), although the binding for both SBA decreased from +++ to ++ over the same time period of gestation. It is possible that, if the decrease in SBA and DBA binding seen in the present study between Gestational Stage 1 (Days 100-125) and mid-gestation were extrapolated backwards, higher binding might have been expected before Day 100 as well.

In near-term bovine placentomes, Jones *et al.* (1994) showed that PHA-L and DBA exhibited similar binding patterns to that of the present study (i.e. many BNC granules showed moderate to strong binding intensity but a few granules were intensely stained), whilst SBA was bound to only a moderate number of granules. This pattern is different from that seen at Stage 5 in this study (200+ days). Some of this difference may be due

to the fact that Jones *et al.*. (1994) used animals in the final days of gestation (Days 279 -285), such animals were not used in the present study (maximum of Day 260). If this is correct, it suggests that the pattern of DBA and SBA lectin binding, which in the present study had shown substantial changes towards the latter stages of pregnancy, might have continued to change during the last part of gestation. Another possibility is that the method of lectin histochemistry used in the current study (fluorescence-labelled lectin) could result in different patterns than those created when enzyme-labelled lectins are used (as they were by Jones *et al.* 1994). However, as the difference in the methods is simply in ways that bound lectins are visualised it is unlikely that major differences are due to the different techniques.

In contrast to the work of Lehmann *et al.* (1992) and Jones *et al.* (1994), PHA-L did not bind specifically to binucleate cells but, rather, there was strong binding throughout the fetal epithelium and villi and maternal epithelium. The reason for this difference is not clear. Further research is required to establish the cause of this and whether it is biologically significant.

Conclusions

This is the first study of lectin binding to use multiple cows at multiple stages from 110–260 days of gestation. It is also the first to use computer-aided quantification of binding rather than subjective assessment. Of the three lectins, PHA-L showed the strongest binding up to 200 days, although after that stage binding intensity was similar for all three lectins. There was marked between-cow variation in PHA-L which has not been reported before. Further research is required to establish the importance of this variability. This study has proven that computer-aided quantification of lectin binding can provide consistent and valuable results. More data from other lectins is required to better establish the changes in glycoproteins in bovine placentome tissue through gestation.

Chapter Eight
General Discussion

Placental development plays a crucial role in the development of the fetus in all eutherian mammals. Surprisingly, although the gross developmental changes that occur in the bovine placenta during gestation have long since been recognised and documented, knowledge about the changes that take place at the crucial interface between mother and fetus – the sites at which allantochorion and endometrium are most intimately apposed – is remarkably limited. The objectives of this thesis were therefore to extend knowledge of placental development in cattle and to better characterise factors which affect it.

The ruminant placentome and the associated synthepitheliochorial structure of the feto-maternal junction is unique to that group of mammals, so can only be studied in ruminants. However, even amongst ruminants there are significant differences in placentome structure between species and at different stages of gestation. For example, although the basic shape of the placentome in the sheep is generally similar to that in the cow the underlying arrangement of the maternal and fetal tissues is different. In the sheep, the caruncle is cup-shaped and holds the fetal tissue within its cup; in contrast the bovine caruncle is mushroom-shaped with the cotyledon forming a layer on top of it (Figure 2.2).

These apparently small differences in structure can have a significant impact on placental capacity and the responsiveness of the placenta to stressors. Previous studies have shown that a key response of the ovine placentome to stressors is a change in shape. Vatnick *et al.* (1991) reported that heat stress (40^{o}C) in mid-pregnancy resulted in a decrease in the proportion of placentomes which were the classical shape (A in Figure 2.2) and an increase in the proportion of flat and stalked placentomes (B and C in Fig 2.2). Similar changes in relative proportions of different placentome shapes have been reported by Steyn *et al.* (2001) when a moderate maternal nutrient restriction was imposed on ewes within the first trimester of pregnancy.

As both nutritional and heat stress are likely to reduce the supply of nutrients from the dam to the fetus, this suggests that the change in placentome shape is a mechanism for increasing placental efficiency without necessarily increasing placentome size. Hence, the preponderance of convex placentomes in the bovine placenta may indicate that

intrinsically there is a greater interface between the fetal and maternal tissue in the bovine compared to the ovine placentome. If this is the case, it has two consequences. Firstly, there may be significant unused capacity in the bovine placentome in normal pregnancies, which means that stressors have to be large and potentially of a severity to cause fetal loss by other means, before the capacity of the bovine placentome is exceeded. Secondly, because the bovine placentome is of convex morphology (i.e. it is intrinsically displays the shape of the stressed ovine placentome) it may be that it has no further capability for morphological changes in response to stresses. Hence, even in situations of gestational stress, bovine placentomes do not display a variety of morphologies. The only mechanism the cow therefore has to compensate for high demands is the development of accessory placentomes as in twins or in placental dysfunction such as hydrallantois in cloned pregnancies.

Studies of cloned animals have shown that significant alterations can occur when normal placentome development is impaired (Hill *et al.* 2000; Hashizume *et al.* 2002; Constant *et al.* 2006). Heavier and fewer placentomes have been reported from placentas on Day 70 of gestations that have resulted from the transfer of embryos produced from modified synthetic oviductal medium (mSOF) compared to those from *in vivo* embryos and embryos cultured in serum (Farin *et al.* 2006). However, these were catastrophic failures of placentation, inasmuch as there was failure of fetal survival to term; there is much less evidence that the small changes/stresses which induced changes in the ovine placenta would have effects in the bovine species.

There have been very few studies which have evaluated the impact of nutrition on placental development in cattle. The extent to which placental development is determined by the nutrition of the dam has been the subject of much debate, across a wide range of species (Clarke *et al.* 1998; Wu *et al.* 2004). This question was examined in this thesis by re-examining data derived from placentas from beef cows that were under moderate feed restriction during the first trimester of gestation. The conclusion that Hickson *et al.* (2009) drew from their analysis of the data was that nutrition was not a critical factor for placental development during early pregnancy. Further examination of these data, using principal component and factor analyses (Chapter 3) confirmed that there was no primary effect of nutritional regimen (moderate restriction *versus ad libitum* feeding) upon either placental development or fetal weight inasmuch as the

principal component analysis did not separate the treatment groups. However, there was a nutrition-independent effect of placental mass upon fetal development.

The work of Long *et al.* (2009) is of some significance in respect to the present study. They restricted feeding of mature pregnant cows during the first 125 days of gestation, and found that some (4/10) of these cows showed impaired placental and fetal development, whereas others (6/10) did not. In the affected cows, cotyledon weight and surface area were reduced, but in the non-affected animals cotyledon weight and surface area were not significantly different from controls. No effect of feeding on caruncular weight was found. A similar study (Zhu *et al.* 2007) that also used mature cows to examine maternal nutrient restriction on placental mass by Day 125 of gestation, showed reduced cotyledon and caruncle weights and placentome weight in nutrient-restricted cows compared to controls. However, placental efficiency was higher in nutrient-restricted cows thereby resulting in similar fetal weight for both controls and underfed cows. Hence, as in the work of Hickson *et al.*. (2009), who worked with 15-month old beef heifers, and as confirmed by the present re-analysis of their data, it appears that impairment of placental or fetal development cannot simply be attributed to a moderate level of nutrient restriction. Whether this situation persists through pregnancy was not resolved. However, Long *et al.* (2009) showed that restoring bodyweight after a period of feed restriction did not restore cotyledon weights to the values observed in control cattle; although this could be attributed to the slow absolute rate of growth of placentomal tissue compared to fetal growth in the last trimester of pregnancy (Reynolds *et al.* 1990) which, in turn, might infer that because placenta growth is disproportionately less than that of the fetus after mid-gestation any further increase in fetal demand during late gestation has to be met by increasing feed intake.

Taken together, the results of the factor analysis underlined the importance of the maternal caruncle in fetal development. This strongly supports previous research using carunculectomy to restrict fetal growth in sheep (Alexander 1964; Robinson *et al.* 1979; Harding *et al.* 1985), which showed significant retardation in fetal growth and increase in the size of the remaining placentomes. It is also consistent with the findings that mortality within the first trimester of pregnancy of cloned bovine and ovine embryos is associated with a reduced number of functional placentomes, an effect which is probably mediated by the inability of a placenta with too few functional placentomes to provide sufficient transport of nutrients between the mother and fetus (Cross 2005).

Clearly, therefore, factors which influence caruncle development will be expected to also affect fetal development. It is likely that vascularisation of the placentome is a key route by which the development of the caruncle affects the functional capacity of the placentome. Previous studies have shown the importance of placental vascularization in influencing the rate of exchange of nutrients and gases, together with blood flow from the placenta to the fetus (Lang *et al.* 2003; Reynolds *et al.* 2005). Luther *et al*. (2007) showed that the process was driven by vascularisation of the caruncle, which, in turn, implies that the latter is a major determinant of nutrient availability to the growing fetus. They also suggested that once vascularization is fully established in the first half of gestation, maternal nutrient restriction will not have a major effect on fetal growth. Further studies are needed to establish which factors can influence the functional capacity of the caruncle in bovine placentomes, particularly during the latter stages of gestation. Moreover, a better understanding of the baseline association between the fetal and maternal components of the placentome is needed to guide such studies: this was the aim of Chapter 4 in this thesis.

The weights and volume of the placentome and its components (i.e. caruncle and cotyledon) were measured to investigate the change in placentome density during gestation. There were no biologically significant changes in overall placentome density between Days 100 and 225 of gestation, despite the exponential increase in the weight and volume of the placentome and its components. No relationship between total placentome number and weight was found, which is consistent with the result of Laven and Peters (2001). These data suggest that there is a minimum weight of placentome that is required to sustain each gestational stage, but that this can be achieved either by multiple small placentomes or a few larger placentomes. Additionally, it is likely that in most cases the minimum weight to sustain fetal development is more than exceeded, which would mask any potential inverse relationship between placentome size and number. Whilst there are limited data from cattle to support this notion, there is some evidence from sheep for an inverse relationship between cotyledon size and number (Dwyer *et al.* 2005) .

Cotyledon weight increased more slowly than caruncle weight as gestation progressed; a finding that was similar to that of Reynolds *et al.* (1990). In the present study, there was more caruncular than cotyledonary tissue by Day 200 of gestation, resulting in a

caruncle: cotyledon weight ratio of 1.69:1. As the density of the placentome was effectively 1 g/mL these figures were the same for the caruncle:cotyledon ratio by volume. Given that the separation of the maternal and fetal components of the placentomes was manual, it is probable that some of the cotyledonary tissue would have remained in attached to caruncular tissue, but as both Reynolds *et al.* (1990) and Laven and Peters (2006) reported that the amount of attached tissue was negligible, it is likely that this ratio represents an accurate assessment of the relative weights and volumes of the maternal and fetal components of the placentome. By contrast, the volume results are remarkably different to those from Laven and Peters (2006), who found that the volume of fetal tissue increased more rapidly with gestational age than did that of maternal tissues, so that by Day 200 of gestation the ratio of maternal to fetal tissue (caruncle:cotyledon) was 0.85:1. It is important to note, however, that the estimate of Laven and Peters (2006) was based on estimation by volume of image analysis focussing on tissue within the villous section of the placentomes, whereas the present study, which used water displacement, measured all tissues including those outside of the area of villous exchange. Hence, it is likely that the two different techniques were not measuring the volume of identical parts of the placentome.

Such a conclusion could be equally applicable to results derived from stereology. For example, Kannekens *et al.* (2006) reported that, at 135 days of gestation, the ratio of maternal to fetal tissue was 0.92:1. Both stereology and the image analysis technique used by Laven and Peters (2001) rely on random sampling to overcome problems associated with tissue heterogeneity; however in both cases the selection of sections largely ignored the tissue surrounding the central zone of fetal and maternal apposition. Because this surrounding tissue is largely of maternal origin, methods based upon sampling only the area of apposition will therefore underestimate the amount of maternal tissue in the placentome as a whole. On the other hand, stereology focuses upon the area where fetal and maternal tissue are in apposition, which confers significant advantages in terms of measuring the relative functional capacity of the placentome; particularly as changes in the amount of tissue in the caruncular stalk probably has little impact on placentome capacity.

Placentome development during pregnancy was further investigated by using trans-rectal ultrasound to visualise the placentomes that are nearest to the cervix (Chapter 5). Again, it was remarkable that there was a dearth of systemic study in the literature of

the changes to the placentome, as visualised by trans-rectal ultrasonography, despite the plethora of material that has been written about the use of that imaging modality for pregnancy diagnosis and fetal aging (Chaffaux *et al.* 1986; Boyd *et al.* 1988; Kähn 1989; Kahn 1990). The present study used limits of agreement analysis (Bland and Altman 1999, 2007) to assess the congruence between the actual gestational age and that predicted from placentome size. The 95% limits-of-agreement varied depending on the measurement used, but were all >30 days; i.e. 95% of actual gestational ages were within ± >30 days of the predicted age. Further analysis showed that <50% of predicted gestational ages were within 10 days of the actual ages, irrespective of the data used. Previous studies have obtained closer difference of 9 days between actual and predicted gestational age based upon fetal nose diameter (using trans-rectal ultrasound) and of 10 days difference for manual rectal palpation combined with insemination data. Consequently, even though placentome size has previously been suggested as a reasonable measure of gestation age (Zemjanis 1974; Matthews and Murton 2012), over the range of gestational ages that were examined in this study, and even though the association between mean placentome size and gestational age was statistically significant, the degree of agreement was too poor for placentome size to provide a reliable method of estimating fetal age. Thus, measuring placentome size does not provide a useful alternative to measurements of the fetus, such as crown rump length, trunk or head diameter, as a means of aging pregnancies in the field.

On the other hand, the consistent measurement of placentomes around the cervix to minimize the variability in placentome size (Youngquist 1997; Laven and Peters 2001) and to increase the chance that the same placentomes were measured each time may provide a basis to explain the difference between this study and that of Hunnam *et al.* (2009a) who found no statistical relationship between gestational age (Days 73 to 190) and placentome size measured trans-abdominally over the right flank. Measuring the placentomes closest to the cervix is a simple and quick technique for pregnancy diagnosis and could be used as the method of choice for determining placentome size *in vivo*, although its value is likely to be limited to research investigations of the placentome itself.

The relative volume densities of the fetal (binucleate cells, trophoblast, connective tissue) and maternal (connective tissue and epithelium) tissue of intact bovine placentomes were estimated throughout gestation using stereology (Chapter 6). There

were no significant changes in the relative volume or surface densities between fetal and maternal tissue components between Days 100 and 260 of gestation. However, when the total volumes of placentomes at each gestation stage (as obtained from Chapter 4) were multiplied by the relative volume densities of placentome components, it was evident that the total volume of the fetal tissue increased at a faster rate with advancing gestational age than did the maternal tissue. Furthermore, the total surface area of the feto-maternal interface increased with gestation age, indicating that the feto-maternal contact area also increased with advancing gestational age. Together, these results show that the surface area becomes larger to meet the needs of the growing fetus as gestation advances. This result was consistent with the increase in total volume of fetal villi and binucleate cells that was described for the yak placentome between Days 60 and 210 of gestation (Liu *et al.* 2010) and for cattle on Day 135 of gestation (Kannekens *et al*. 2006). However, the present study was very much more extensive than these earlier investigations, inasmuch as it used 25 uteri from different cows collected from the abattoir over a wide range of gestational stages. As far as can be ascertained from the literature, this is the first time that the changes in functional surface area within the feto-maternal interface over a wide range of gestation age in cattle have been documented.

The key limitation to the present study was that the method of fixation of the tissue (immersion fixation with formalin) caused shrinkage of tissues (Dorph-Petersen *et al.* 2001), thereby creating gaps between the fetal and maternal tissue components. To prevent this, future studies should use formalin perfusion to preserve the architecture rather than immersion fixation. Nonetheless, the present results and those of previous authors suggest that stereology gives accurate quantitative estimates of the cellular, connective tissue and other components of fetal and maternal tissue which complement the absolute volume measurements described in the previous chapter of the thesis.

Binucleate cells are pivotal to the efficacy of ruminant pregnancies. They form in the trophoblast, after which they migrate and fuse with maternal uninucleate cells to form short-lived trinucleate cells, during which they deliver the granules containing placental lactogens and other pregnancy associated hormones to the maternal circulations (Wooding 1982b; Duello *et al.* 1986; Alvarez-Oxiley *et al.* 2008). The present study found that the proportionate contribution of binucleate cells to the placentome did not vary during gestation, but that there was an overall decrease in the total number of binucleate cells in the last trimester of gestation. The variation in binucleate cell number

during mid-gestation could be associated with the function of those cells in maintaining the glycoprotein layer between fetal and maternal epithelium; and, based upon the lectin-staining investigation of glycoproteins at the feto-maternal interface, such a role is compatible with the results of the present studies. The extent to which binucleate cell numbers affect the outcomes of pregnancy has not been established: placental lactogens derived from binucleate cells affect the dam during gestation (e.g. via hyperphagia, partitioning of nutrients towards adipose tissue deposition) and via post-natal effects upon lactation (Byatt *et al.* 1992; Anthony *et al.* 1995; Kann *et al.* 1999). Moreover, placental lactogens may have luteotrophic effects (Martal and Djiane 1977), with consequent progesterone-mediated effects upon embryo development (Flint *et al.* 1979; Spencer and Bazer 2002) . A reduction in binucleate cell migration in late pregnancy has been reported previously (Wooding and Wathes 1980; Wooding 1982a; Wooding and Burton 2008) probably due to changes in the migration of binucleate cells across the feto-maternal unit.

The result from the present study of normal bovine placentas collected from the abattoir between Days 100 to 260 of gestation show that there is a substantial individual variation between cows in the distribution of binucleate cells during pregnancy. Lee *et al.* (1985), who investigated the role of the binucleate cell in the outcome of individual ruminant (deer, sheep and cattle) pregnancies showed variation in the distribution of binucleate cells between species at different stages of gestation and a reduction in binucleate cell number by Day 283 of gestation in cattle. Abnormalities of placentas derived from cloned embryos have been associated with reduction in binucleate cell population by Day 222 of gestation (Bertolini *et al.* 2002; Miles *et al.* 2004), perhaps indicating that abnormal partitioning of nutrients and exchange of nutrients between the fetus and its mother underlie some of the fetal abnormalities that are associated with such pregnancies. In sheep, the fall in binucleate cell number in the last week of gestation is related to a reduction in placental lactogen concentrations in fetal and maternal circulation (Martal and Djiane 1977; Wooding 1982a), suggesting that the withdrawal of placental lactogens and pregnancy associated glycoprotein within the feto-maternal is involved in placentome maturation.

Glycoproteins are integral to the structure and function of the bovine placenta. The present study has successfully showed the expression patterns of different glycoproteins within the feto-maternal interface at different stages of gestation using qualitative and

quantitative methods. It is also the first study to develop a computer-assisted method to quantify the qualitative expression of glycoprotein showed by previous researchers, who showed the expression of glycoprotein during shorter periods of gestation. A small number of previous studies have used lectin binding to investigate the distribution of glycoproteins within the placenta (e.g. Munson *et al.* 1989; Jones *et al.* 1994); however, this is the first study to fully quantify lectin binding to the tissues of the bovine feto-maternal interface over a wide range of gestational stages. It is difficult to compare the present results with those reported previously, because those studies were either conducted over a limited period of gestation, or because small numbers of animals were used at each gestational stage. For example, Klisch and Leiser (2003) only had one placenta per stage while Munson *et al.* (1989) had a maximum of three. Methodological differences also make comparisons difficult, as previous studies were based upon subjective scoring, whereas the present study used computer-based image analysis to provide objective measurements of binding intensity.

The fluorescent-labelled lectins used in this study were *Phaseolus vulgaris agglutinins* (PHA-L), *Dolichus biflorus* (DBA) and *Glycine max* (SBA) lectins. The intensity of PHA-L binding was greater than that of either DBA and SBA until after Day 200 of gestation. However, as gestation advanced, the difference in binding between PHA-L and the other two lectins decreased, as there was no significant change in PHA-L binding intensity while both DBA and SBA binding intensity increased. Such changes have not previously been reported. Nonetheless, these changes in intensity are consistent with the findings of Jones *et al*. (1994) who reported that, in the near-term placenta, DBA binding was found on significantly more BNC granules than PHA-L, although SBA binding was less intense than either. Similarly, Klisch *et al.* (2006) reported that PHA-L binding remained high until the time of parturition. The present study found significant variation in PHA-L binding between individual cows; a result which has also not been reported previously. The small numbers of animals used in earlier studies would, however, probably have precluded the ability to reliably identify such between-animal variation.

Curiously, the present study found that PHA-L was not specifically bound to BNC, as has been reported by previous authors (Lehmann *et al.* 1992; Jones *et al.* 1994), but rather that there was strong binding throughout the fetal and maternal epithelium and

the fetal villi. PHA-L binds to a wide variety of glycoproteins, which includes tri/tetra-antennary, non-bisected complex N-Linked sequences, and lactosamine–type N-glycans (Klisch *et al.* 2006; 2008). Whilst it is possible to explain the high intensity of PHA-L staining observed in this study in terms of its widespread binding ability, it is less clear why the cellular patterns of binding differed from those reported in previous studies. It is unlikely to be attributable to methodological inadequacies, since negative control slides gave expected results; moreover binding was confined to fetal and maternal epithelia and was not distributed into tissues (e.g. connective tissue) that are poor candidates for PHA-L binding.

DBA and SBA increased polynomially with gestation age by binding strongly to a small number of binucleate and uninucleate cells as well as more generally to fetal and maternal epithelial cells. Specific binding of DBA has previously been reported (Nakano *et al*. 2002) as a characteristic of full differentiation of binucleate cells, so this intense staining is largely as expected. However, whilst DBA bound to fetal and maternal tissue, SBA only bound to fetal tissue. DBA and SBA are specific for glycoproteins with terminal α-linked GalNac and α-/β-linked GalNac respectively. Munson *et al.* (1989) showed stronger binding of DBA than SBA to intracellular binucleate cells in cows >80 days pregnant, which differs somewhat from the result from the present study. On the other hand, other studies of lectin binding in late pregnancy (Munson *et al.* 1989) also showed SBA binding to sub populations of BNCs to which DBA did not bind (Jones *et al.* 1994). The present study therefore suggests the presence of, and increase in, α-/β-linked GalNac types of glycoproteins as gestation age increases. Bovine binucleate cells granules contain a wide variety of glycoproteins, including variety of fetal bovine placental lactogen that has been characterised as a 200-amino acid glycoprotein hormone with higher molecular mass (31-33 kDA) than the standard (23 kDA) molecular mass of placental lactogen (Alvarez-Oxiley *et al.* 2008). Since PHA-L binds with lactosamine –type N-glycan, and DBA and SBA bind to other glycoproteins with smaller molecular mass, the result from the present study therefore are compatible with binding of PHA-L to bovine placental lactogens and terminal N-linked glycans by SBA and DBA during gestation. Thus, the present study has been able to add to knowledge on the distribution binucleate cells during bovine pregnancy, and has udentified which cellular components of the placentomal tissue produces specific glycoproteins during gestation. Previous studies that have quantified expression

of glycoprotein using different methods such as electrophoresis of the trophoblast, radioimmunoassay, lectin western blot and lectin elisa (Lee *et al.* 1985; Klisch *et al.* 2006; Alvarez-Oxiley *et al.* 2008) were unable to show what part of the placenta is responsible for the production of glycoprotein at the same time. The results of the present study will therefore provide valuable information for future examination of hormonal expression within the feto-maternal interface at different stages of gestation; as well as investigating abnormalities of placental maturation in events such as retention of the bovine fetal membranes.

Concluding statement

The main aim of this thesis was to investigate the changes in gross morphology and histology of the bovine placenta at different stages of gestation and also to examine factors that could be responsible for these changes. The aim was achieved by the following objectives:

The first objective was to evaluate the relationship between placental components with nutrient restriction in the first trimester of pregnancy. This question was answered by using principal component and factor analysis to identify the effect of nutrition on placental and fetal growth and placental factors that could influence placenta mass. The findings indicated that the placentas from beef heifers were not critically affected by moderate nutrient restriction in the first trimester of pregnancy, but the factor analysis identified the caruncle as a major determinant of placental and fetal mass. Previous studies of nutrient restriction in mature cows during the first trimester have shown that it affects the fetal cotyledon, suggesting that heifers respond to maternal nutrient restriction in a different way to that of mature cows.

The second study built upon these results by examining the changes in the volumes and densities of the fetal and maternal components throughout gestation. Placentome number increased after Day 100 and reached maximal values on Day 170, after which the number decreased. The overall mean placentome density was 1.1 g/mL as determined from the linear relationship between weight and volume of the placentomes. Although there was a statistically significant change in mean placentome density over gestation, it was small and no biological explanation could be proposed for it. Hence,

this result was not taken into consideration whilst interpreting volume densities in the stereological results.

The third study was undertaken to determine whether it is possible to predict gestational age from measuring placentome size *in vivo* using trans-rectal ultrasonography. The size of placentomes closest to the cervix in the pregnant and non-pregnant horn were measured throughout gestation, and regression equations were derived for predicted and actual gestation ages. The limits of agreement analysis showed that placentome size increased between Days 90 and 180, but that 95% of actual gestation ages were substantially over- or underestimated. This the first study to show a statistically significant relationship between gestation age and placentome size, based upon measurements of placentomes closest to the cervix . However, despite the statistical relationship, the results of the study showed that predicting gestational age from placentome size is unlikely to be sufficiently accurate to provide a useful method of aging pregnancies. Conversely, the consistent results for placentome size obtained by measuring those closest to the cervix, suggests that this may be a valuable technique for investigation of the placentome itself.

Thereafter, stereological techniques were used to assess changes within the feto-maternal exchange area of the bovine placenta at different stages of gestation and to compare with studies of gross morphometry in the earlier chapters. There was no change in relative volume and surface density during gestation, but the numbers of binucleate cells decreased by late gestation. Surface area increased as gestation age progressed, indicating that the feto-maternal interface of the bovine placentome expands with gestational age, thereby increasing the surface area for exchange of nutrients, oxygen and waste products between the mother and the fetus. The reduction in binucleate cell distribution within the interface may be related to the production of glycoprotein in the placenta.

Thus, the final study was conducted to characterise and quantify the changes in glycoprotein production during gestation. This was achieved by using histochemistry to examine binding patterns and intensities of lectins (PHA-L, DBA and SBA). Uniquely, this study fully quantified the binding intensities with specially-developed computer-assisted software. Results from the study indicate that the feto-maternal interface produces a wide variety of glycoproteins, which can probably be related to the

production of bovine placenta lactogens with different molecular weights, at different stages of gestation. This finding could be used to further investigate and quantify specific glycoprotein and their functions at different stages of gestation.

New data reported in this thesis have shown that bovine placentome development is not critically affected by nutrient restriction in the first trimester of pregnancy, with little or no change in density as gestation progressed. Placentome size increased between Days 60 and 180, but placentome size was considerably less accurate than using other fetal measurements. The relative volume densities of fetal and maternal tissue components was not affected by gestation stage but there was reduced binucleate cell population during late gestation, and total surface area increased with gestation age, suggesting a larger area for exchange of nutrients to meet fetal demand as gestation advanced. Lectin histochemistry successfully characterised and quantified glycoprotein produced within the feto-maternal interface during pregnancy. This study gives a wider knowledge of bovine placentome development and information for future investigation to better understand the factors that could influence the bovine placenta during pregnancy.

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