Effects of fetal sex and genetics on the bovine placenta – From baseline data to fetal programming and heterosis

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Thesis submitted for the degree of

Doctor of Philosophy in Veterinary Science

THE UNIVERSITY *of* ADELAIDE

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Adelaide, South Australia, Australia

July 2016

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Declaration

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CONSUELO AMOR. S. ESTRELLA

Abstract and Poster Presentations from this Research Project

1. Xiang, R. **Consuelo Amor S. Estrella**, C. Fitzsimmons, Z. Kruk, B. Burns, C.T. Roberts, S. Hiendleder. August 2013. **Sex-specific placental and fetal phenotypes in bovine at midgestation.** Abstract presented at the 44th Annual Conference of the Society for Reproductive Biology, Sydney, Australia.

2. **Estrella, Consuelo Amor S.,** R. Xiang, C. Fitzsimmons, Z. Kruk, B. Burns, C.T. Roberts, S. Hiendleder. November 2013. **Novel paternal genome effects on placental and fetal phenotype at midgestation.** Poster presented at the 8th World Congress of Developmental Origins of Health and Disease, Singapore.

3. **Estrella, Consuelo Amor S.,** K. Kind, R. Xiang, V. Clifton, C.T. Roberts, S.Hiendleder. August 2014. **Sexual dimorphism at midgestation: recapitulation of hallmarks of growth restriction in normal female fetuses.** Poster presented at the 45th Annual Conference of the Society for Reproductive Biology, Melbourne, Australia.

Acknowledgements

"Simplicity is the utmost perfection. Don't make it too complex, Cons."

– Leonardo da Vinci and always Stefan Hiendleder

"Someday Cons, you will be better than I am, otherwise I didn't do a good job."- SH

Thank you Stefan for the excellent training and supervision you provided. The laboratory and writing work done to the highest standards and "utmost perfection", the wisdom, and the eye for details – these are now part of me. You have moulded and polished me into what I am, and I am forever grateful for that. Thank you for your understanding despite my shortcomings and most of all, thank you for pushing me past my limits to reach my goals and achieve greater outcomes.

"Don't worry Cons, it will be over soon. Everything will be fine."-Karen Kind

"You learn more if things are not working." – KK

"Keep calm and redo your PCR." – Keep Calm series and KK

Thank you Karen for sharing your expertise and for teaching me the value of critical thinking; you have been more than a co-supervisor and mentor, you have always been there to listen, to encourage, and to help. You are instrumental to my survival in my journey as a PhD student. My gratitude to you is beyond words.

"I look forward to helping you with your PhD." – first email from Claire T. Roberts

"I know you will do your best." - CTR

"We have to work as a team. Older PhDs help younger ones. Later, you have to help others too, Cons." – CTR

Thank you Claire for your guidance and unwavering support; you inspired me to work hard independently and to always do my best. It was a great opportunity to learn from you and be under your care.

"*Run a good race, fight a good fight*." *– Dr. Benedicto A. Santiago, my father*

For your unconditional love and unceasing prayers, thank you Dad and Mom.

Thank you to the Australian Government for granting me the Australian Leadership Award.

Thank you to Dr. Dana Thomsen from SAVS for providing assistance on thesis editing.

To my friends, my fellow PhD students, past and present members of our lab group – thank you for the fun memories, for being part of my amazing four-year journey.

Dedication

I couldn't have gone this far without the love and support of my husband, Richard, and my two wonderful children, Richard James and Cedric Alva. They made it possible for two worlds to work together – PhD and family life. This thesis dissertation is the fruit of our sacrifice as a family.

Thesis Abstract

The placenta is a major determinant of fetal growth and central to fetal programming effects that impact postnatal performance and health. Most reports in human and animals on prenatal growth and programming are based on studies of term placentae and/or birth weight, but information at critical time points of development on (i) fundamental gross morphological and histomorphological characteristics and developmental changes of the placenta that could impact embryo/fetal growth, (ii) influence of sex-specific placental and umbilical cord phenotype on sex differences in fetal growth, (iii) contribution of placenta and umbilical cord in mediating effects of genetics and epigenetics on heterotic fetal growth, and (iv) differences in placental expression of insulin-like growth factor (IGF) system components between sexes and fetal genetic groups, are generally lacking. This information is highly relevant for both animal production and human health, and the present study used cattle, a major livestock species and valuable biomedical animal model, to address research questions and close gaps in knowledge.

Purebred and reciprocal cross *Bos taurus taurus* (Angus, A) and *Bos taurus indicus* (Brahman, B) concepti recovered at the late embryo (Day 48, n=60) and early accelerated fetal growth (Day 153, n=73) stages (term, 277-291) were used to examine effects of fetal sex and genetics on a broad range of conceptus traits. Data analyzed using general linear models included gross- and histomorphological parameters of the placenta, umbilical cord traits and fetal fluid volume as well as clinical-chemical parameters, circulating IGFs in cord blood, and tissue-specific transcript abundances of IGF system components.

The main findings include (i) the significant contribution of convex but not flat placentomes to embryo-fetal growth as revealed by the exclusive positive relationships between number of convex placentomes and embryo-fetal weights and the higher number and average weight of convex placentomes in placentae of $B_{paternal}\times A_{maternal}$ and A×A concepti,

which ultimately show the highest birth weights; (ii) fetal sex effects on placental and umbilical cord phenotype which mediate sex-specific fetal growth, where normal female fetuses display hallmarks of intrauterine growth restriction that provide a mechanism for sex-differences in susceptibility to non-communicable diseases; (iii) heterosis of B×A hybrids *in utero* that is characterized by polar overdominance of paternal B genome on umbilical cord phenotype and complements the superior maternal A genome effects on placenta, a clear indication of heterosis due to high capacity for nutrient extraction and high capacity for nutrient supply; and (iv) differences in circulating IGF2 and transcript abundance of *IGF2* in fetal brain and heart between sexes, as well as differences in expression of *IGF2*, *IGF2R*, *H19*, *AIRN* in placenta between fetal genetic groups demonstrate the important role of IGF2 for fetal growth at mid-gestation.

In conclusion, the results of the present study support the hypothesis that differences in placental and umbilical cord phenotypes between males and females and between purebreds and reciprocal cross hybrids determine variation in intrauterine growth at mid-gestation and contribute to programming of postnatal performance and health. Future studies to be explored include analyses of growth factors associated with development of the placental vasculature and umbilical cord, and involvement of additional imprinted gene clusters in prenatal growth and development.

Chapter 1 Introduction and Literature Review

1.1 Introduction

The placenta is an organ where fetal and maternal tissues are apposed for purposes of physiological exchange (Wooding and Burton, 2008b). Although the placenta is a transient organ, it performs a variety of functions with a common purpose of providing a favourable environment for fetal growth and development (Schneider, 1996). One of the critical functions of the placenta is to provide essential nutrients and oxygen to the fetus. Metabolites and gases from maternal circulation cross the placenta via fetal trophoblast cells in contact with maternal uterine tissue and enter fetal capillaries which culminate in umbilical cord vessels of the fetus. Thus, the placenta and umbilical cord vessels are vital components of the supply line of the fetus and the tight correlation observed between placental weight and birth weight (Greenwood et al., 2000, Salafia et al., 2008, Roland et al., 2012) demonstrates that the placenta is a primary determinant of fetal growth.

Impaired placental development or umbilical cord abnormality where the supply line of the fetus is affected, often leads to fetal growth restriction (Bruch et al., 1997, Chen et al., 2002, Platz and Newman, 2008, Morrison, 2008). Intrauterine growth restriction (IUGR) is associated with an increased risk of a range of neonatal problems, including increased mortality and morbidity (Rosenberg, 2008). In addition, epidemiological studies in human have shown that low birth weight is associated with increased standardized adult mortality ratios for coronary heart disease (Barker et al., 1993, Martyn et al., 1996) and incidence of type 2 diabetes (Forsén et al., 2000), indicating that adaptation to a suboptimal intrauterine environment may lead to programming of chronic diseases in adulthood (Barker, 1995, Godfrey and Barker, 2000). Fetal programming has been studied extensively in human and animal models and now forms the basis of the developmental origins of health and disease hypothesis (Vuguin, 2007, Gluckman et al., 2008, Longtine and Nelson, 2011, Hanson and Gluckman, 2015). Therefore, as a regulator of fetal growth, the placenta is also a major determinant of fetal programming (Lewis et al., 2011).

The impacts of fetal programming and growth restriction are highly relevant to domestic animal production. For example, IUGR is a major concern in livestock industries due to its postnatal consequences (Wu et al., 2006). Low birth weight in animals is associated with poor thermoregulation and high pre-weaning mortality with long-term effects on postnatal performance and carcass quality. This includes poor capacity to exhibit compensatory growth, low average daily gain and feedlot weight, older age at market, low hot carcass weight and small eye muscle area in beef cattle (Ferrell, 1989, Greenwood, 2006). Others report low efficiency of energy utilization in sheep and less lean and tender meat in pigs (Greenwood et al., 1998, Gondret et al., 2006). Similar to fetal growth restriction in human, IUGR in livestock is strongly associated with placental insufficiency (Wu et al., 2006, Barry et al., 2008).

Understanding the factors that impact on placental structure and function is therefore important, due to its key role in regulating fetal growth. For example, sexual dimorphism in placental characteristics, fetal biometric indices and birth weight have been reported in human and several large animal species, including sheep and cattle (Lubchenco et al., 1963, Alexander, 1964, Eley et al., 1978, Verburg et al., 2008, Eriksson et al., 2010, Almog et al., 2011, Melamed Nir et al., 2013). Sex-specific placental morphology and biometric indices and ratios, depicting sexual dimorphism in growth, have been associated with sex differences in long-term health outcomes in human (Forsén et al., 1999, Eriksson et al., 2010) and animals (Gilbert and Nijland, 2008). Newborns of different ethnic groups (Yeung et al., 2003) and animals with different genetic potential for growth, including hybrids, have different placental weights and birth weights (Anthony et al., 1986, Ferrell, 1991, Allen et al., 2002). These studies suggest that effects of sex and genetics on conceptus traits and birth weight are mediated by their effects on placental phenotype. However, while most reports in humans and animals are based on study of term placentae and birth weight, it is important to understand the influence of sex and genetics on placental and hence fetal growth, *in utero*.

Thus, examination of placental and fetal phenotypes at critical time points of development is required.

Studies on placental and fetal growth have implications for both human health and animal production and can be performed using a suitable animal model. In addition to mouse, livestock species including cattle, sheep and pig, are widely used as models for prenatal growth, fetal physiology and placental biology of human. However, differences in gestation period, placental morphology and gene expression, and fetal characteristics between human and animals should be considered when choosing a suitable model to answer specific research questions (**Table 1.1**). Cattle are valuable tools in biomedical research because they have a similar gestation period (Andersen and Plum, 1965) and carry a single conceptus with similar growth trajectory (Ott and Doyle, 1982, Ferrell, 1989) and maturity at birth as human (Martin, 2003, Dwyer, 2005, Xiang et al., 2013, Xiang et al., 2014). Although the cattle placenta is structurally different from human placenta, its similarity in growth pattern (Ferrell, 1989) and gene expression related to hormonal activity (Barreto et al., 2011) makes cattle placenta an appropriate model to understand pregnancy complications associated with placental dysfunction in human. Cattle have also been used for decades as models for *in vitro* fertilization and somatic cell nuclear transfer technologies (Constant et al., 2006, Bähr and Wolf, 2012) where fetal abnormalities such as large offspring syndrome observed in assisted reproduction technology (ART)-derived calves are highly similar to Beckwith-Wiedman syndrome seen in children conceived through ART (Constant et al., 2006, Chen et al., 2013, Polejaeva et al., 2016). In addition, developmental programming of progeny outcomes in beef cattle has important implications for the beef cattle industry (Sullivan et al., 2009, Funston and Summers, 2013, Hernandez-Medrano et al., 2015). Therefore, use of cattle in assessing effects of sex and genetics on placental and fetal characteristics may provide valuable insights for a better understanding of the impact of placental factors on fetal growth at critical time points of development.

Table 1.1 Comparison of gestation period and placental and offspring characteristics in human and animal models.

	Human	Cattle	Sheep	Pig	Mouse
Gestation period, weeks	40	40	21	16.3	3
Placental gross morphology (Wooding and Burton, 2008b)	Discoid	Cotyledonary	Cotyledonary	Diffuse	Discoid
Placental histomorphology (Wooding and Burton, 2008b)	Hemochorial	Synepitheliochorial	Synepitheliochorial	Epitheliochorial	Hemochorial
Placental growth (Ferrell, 1989, Wu et al., 2005)	Placental weight increase until near term	Placental weight increase until near term	Placental weight reaches maximum at mid-gestation	Placental weight increases until near term	Placental weight reaches a maximum later in gestation and declines near term
Placental interdigitation pattern (Telugu and Green, 2008, Gundling and Wildman, 2015)	Villous	Villous	Villous	Folded	Labyrinthine
Similarity in placental gene expression between human, cattle, and mouse (Barreto et al., 2011)	Shares expression of 93 genes with cattle and mouse	Shares expression of 167 genes with human, but 23 of those are specific in human and cattle, and not expressed in mouse or zebrafish	No data	No data	Shares expression of 157 genes with human, but 16 of those are specific in human and mouse, and not expressed in cattle or zebrafish
Number of offspring	One	One	One, two to four	Litter	Litter
Maturity of offspring at birth (Dwyer, 2005, Martin, 2003)	Intermediate between precocial and altricial	Precocial	Precocial	Intermediate between precocial and altricial	Altricial
Average birth weight, kg	3.2 (Battaglia and Lubchenco, 1967)	35 (Brown et al., 1993)	5 (Dwyer et al., 2005)	1.3 (Gondret et al., 2006)	0.0013 (Wagner and Christians, 2010)

In this review, fundamental information on development and characteristics of the bovine placenta with focus on beef cattle is presented, followed by discussion of the effects of factors such as fetal sex and genetics on placental development and characteristics. Evidences for sex-specific fetal adaptation leading to sex bias in adult diseases and the potential contributions of a sexually dimorphic placenta in mediating these effects are considered. Effects of maternal and paternal genetics on placental traits are also covered. For genes regulating fetal growth and development, literature reviewed here is limited to the insulin-like growth factor (IGF) system, which includes imprinted genes that play a pivotal role in regulating placental and fetal growth and development during the first half of pregnancy.

1.2 Literature Review

1.2.1 Development and structure of the bovine placenta

Prior to the establishment of placental structures, the bovine conceptus anchors itself halfway between the cervix and uterotubal junction and fills up the ipsilateral or gravid horn by elongating during days 13-17 post fertilization (Guillomot and Guay, 1982, Wooding and Burton, 2008a). This type of attachment is loose, but at day 18 of gestation the close apposition between the trophoblast and uterine epithelium renders the attachment more firm (Guillomot and Guay, 1982). Between days 19-20 adhesion occurs via microvillus interdigitation between the two tissues (Guillomot and Guay, 1982). The allantois emerges from the embryo proper at day 23 and initial vascularization is observed at day 26 (Curran et al., 1986, Assis Neto et al., 2010). The allantois extends rapidly and fuses with the chorion to form the chorioallantois at day 32 (Melton et al., 1951). The allantoic vessels connected to the umbilical cord are visible by day 33 (Assis Neto et al., 2010). Cotyledons begin to develop at the surface of the chorioallantois around this time, and interdigitation of the chorioallantois with the uterine caruncles progresses to placentome formation at day 37 (Assis Neto et al., 2010). This sequence of events happens at a later time in the contralateral or non-

gravid horn where development of placentomes begins at day 60 of gestation in this horn (Curran et al., 1986). The higher vascular perfusion of the endometrium in the ipsilateral horn compared to the contralateral horn during the periods of attachment (days 14-18), cotyledon development (days 25-30) and placentome formation (days 40-60), appear to support earlier placentome formation in the gravid horn (Ford et al., 1979, Silva and Ginther, 2010).

Establishment of the placental vasculature including umbilical cord vessels indicates the start of haemotrophic nutrition (Wooding and Burton, 2008a). As gestation progresses there is a corresponding increase in nutritional requirements of the fetus to sustain rapid growth. In human, cattle and sheep, placental growth is outpaced by fetal growth during the second half of pregnancy (Prior and Laster, 1979, Ott and Doyle, 1982, Ehrhardt and Bell, 1995). This is depicted by logarithmic increase in fetal weight compared with little or no significant increase in placental weight during the later stages of pregnancy. Although placental growth slows in the second half of pregnancy, this is compensated by extensive remodelling, particularly of the placenta's histomorphological architecture (Mayhew et al., 1994, Kaufmann et al., 2004, Reynolds et al., 2005a). This and the development of the placental microvasculature allows increased umbilical blood flow for more efficient transplacental exchange to support rapid fetal growth during this stage (Reynolds et al., 2005a).

1.2.1.1 Gross morphological characteristics of the bovine placenta

The cotyledonary placenta of cattle is organized into 75-120 placentomes (Roberts, 1971). Others report that total placentomes varied from 30-130 (Laven and Peters, 2001) and 20-150 (Wooding and Burton, 2008c). The number of placentomes initially increases with placental growth, reaches its highest number at mid-gestation and remains constant thereafter (Laven and Peters, 2001). In buffaloes and yaks, the majority of the placentomes are found in the gravid horn (Abdel-Raouf and Badawi, 1966, Liu et al., 2010), and in cattle the number of placentomes in the gravid horn is approximately twice the number of placentomes in the non-

gravid horn (Stickland and Purton, 1977, Laven and Peters, 2001). Although the cattle uterus contains a total of 89-155 total nodules or caruncles before pregnancy (Atkinson et al., 1984), the 65-88 placentomes present in the placenta near term at day 271 (Ferrell, 1991) indicate that not all caruncles participate in placentome development (Wooding and Burton, 2008c).

While the number of placentomes remains constant in the second half of pregnancy, placentome and total placental weight continue to increase, but at a slower rate relative to fetal weight (Prior and Laster, 1979, Laven and Peters, 2001). Thus, an increase in placental weight, rather than in placentome number, occurs to meet the increasing requirements of fetal growth. This is consistent with findings that weights of total placenta, cotyledon and fetal membrane are positively correlated with birth weight in cattle (Echternkamp, 1993), and the fact that total placenta weight accounts for 90% of variation in fetal weight in sheep (Greenwood et al., 2000). Low protein (Sullivan et al., 2009) or low to medium energy (Prior and Laster, 1979) diets fed to heifers beginning in the first trimester of gestation lead to higher cotyledonary weight which appears to be a compensatory mechanism to meet fetal demands for nutrients. Therefore, in cases of maternal dietary restriction, placental adaptations in weight may occur to facilitate transfer of available nutrients.

Placentomes vary in shape in ruminant species (Schmidt et al., 2006, Liu et al., 2010), and include flat and convex placentome types in cattle (Laven and Peters, 2001) and concave, flat and everted placentome types A to D in sheep (Vatnick et al., 1991, refer to **Table 1.2**). In cattle, it has been reported that flat placentomes are more common in the gravid than the non-gravid horn, but not all placentae examined and estimated to range from day 101-160 of gestation, contained at least one flat placentome (Laven and Peters, 2001). The flat placentome type was described to resemble placentome type C in sheep (Laven and Peters, 2001), while the "normal" placentome type appears to be convex and mushroom-shaped (Schmidt et al., 2006, refer to **Figure 1.1**). In sheep, types A and B predominate throughout gestation, though types C and D are often seen towards the end of gestation (Osgerby et al., 2004). Furthermore, early to mid-gestation nutrient restriction drives a change in placentome

shape from type A towards the type D without significantly decreasing total placental and fetal weights (Steyn et al., 2001) indicating that placental weight alone is not sufficient to explain changes in placental efficiency and functional capacity (Reynolds et al., 2005b). However, in cattle, comprehensive studies characterizing flat and convex placentome types and their roles in embryo-fetal growth have not yet been conducted.

Table 1.2 Placentome types in sheep.

	Type A	**Type B**	**Type C**	**Type D**
Classification (Vatnick, 1991; Ward et al., 2006)	Inverted, typical for sheep. Maternal tissue completely surrounding fetal tissue.	Fetal tissue has begun to grow over the maternal tissue.	Flat Maternal tissue on one surface and fetal tissue on the other.	Everted Resembles late gestation bovine placentome.
	 (black shading is fetal tissue)			
Placentome morphology at day 130 of gestation* (Vonnahme et al., 2008)				
Number	~ 58	-59%	-85%	-91%
Average weight	~ 8.48g	+25%	+78%	+141%
Average diameter	~ 2.50 cm	+12%	+45%	+64%

*Percentage relative to Type A, example, Type B is 59% lower in number than Type A

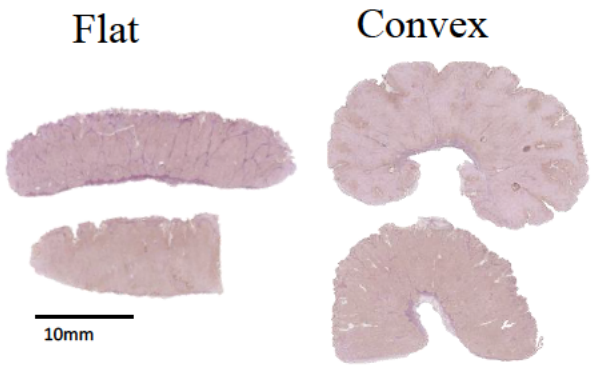

Figure 1.1 Bovine placentome types at mid-gestation (Estrella et al., Chapter 2). Histomicrograph images of flat (left) and convex (right) bovine placentome sections from Day 153 bovine resource of the present study. The bottom histomicrographs are only half of a placentome.

1.2.1.2 Histomorphological characteristics of the bovine placenta

Histological classification of the ruminant placenta is synepitheliochorial, wherein the uterine epithelium in contact with the chorion, remains intact throughout pregnancy (**Figure 1.2**, Wooding and Burton, 2008b). Histological features of the developing bovine placentome during the first and last trimesters are well described (King et al., 1979, King et al., 1980, Leiser et al., 1997, Leiser et al., 1998, Aires et al., 2014). Remodelling of the bovine placentome begins with attachment of the chorioallantois followed by interdigitation of fetal chorionic villi with the maternal caruncular crypts. The chorionic intermediate and secondary villi eventually branch out forming elaborate conical shaped villous trees (Leiser et al., 1998). A previous finding suggests that caruncular crypts and chorionic villi develop simultaneously (King et al., 1979), while a more recent report showed that placentome formation begins with proliferation of caruncular stromal cells leading to caruncular projections beginning at day 28 of gestation (Aires et al., 2014). Further studies may be needed to elucidate the functional significance of the caruncular stroma in placentome formation.

In human placentae, histomorphometric parameters describing placental growth and development include volume and volume density of different placental cell types (Mayhew, 2009). For example, a decrease in fetal mesenchyme volume indicates connective tissue attenuation and is part of the developmental process of bringing maternal and fetal circulations into close proximity (Rurak, 2001). Surface area of the villi and intervascular barriers are also morphometric indicators of transplacental exchange including nutrient transfer via passive diffusion (Mayhew, 2009). The contact zone between the mother and fetus is expressed as surface density (surface area per unit volume of the placenta), which is associated with increasing complexity of feto-maternal indentation patterns (Baur, 1977, Leiser et al., 1998). This and thinner barrier thickness contribute to increased placental efficiency as gestation progresses (Fowden et al., 2009). Although bovine placenta is cotyledonary and synepitheliochorial, compared to the discoid, hemochorial human placenta, similar histomorphometric measures are appropriate for analysis of the bovine placenta. Futhermore, histomorphometric measures have been used to examine placentae of other animal species including guinea pigs (Roberts et al., 2001), mouse (Coan et al., 2004), sheep (MacLaughlin et al., 2005), horse (Veronesi et al., 2010) and pig (Biensen et al., 1999).

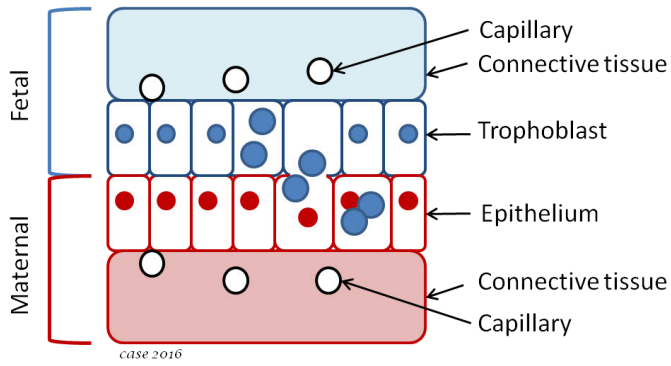

Figure 1.2 Diagrammatic representation of synepithelichorial placentation in bovine.

A synepitheliochorial placenta consists of six tissue layers separating fetal and maternal blood and has specialized trophoblast giant cells or binucleate cells (with two blue circles) capable of migrating and fusing with the uterine epithelium to form a syncytium (three circles).

Other unique characteristics of the synepitheliochorial ruminant placenta are the presence of binucleate cells and feto-maternal syncytium. Binucleate cells represent 20% of trophoblast cells throughout pregnancy in goat (Wango et al., 1990) and cattle (Wooding and Burton, 2008c), and 28-30% of trophoblast cells at 4-9 months of gestation in water buffalo (Carvalho et al., 2006). Although binucleate cells are common, mono- and trinucleate cells with similar features to binucleate cells are also observed, and the term 'trophoblast giant cell' is used to cover differences in nuclei number (Klisch et al., 1999b). Trophoblast giant cells migrate and fuse with the uterine epithelium to deliver pregnancy-associated glycoproteins and placental lactogen close to the maternal blood circulation (Wooding and Wathes, 1980). Further, trophoblast giant cells may be diploid or polyploid, suggesting that gene expression of biologically important compounds may be enhanced and higher quantities of these products are delivered to the uterine epithelium (Klisch et al., 1999a, Klisch et al., 2004). It was proposed that mononucleate cells, another type of trophoblast cell, which display typical features of a mononuclear cuboidal or columnar epithelial cell, may give rise to trophoblast giant cells (Klisch et al., 1999b). Mononucleate cells represent 75% of the trophoblast population and are found at the feto-maternal interface to facilitate nutrient exchange (Igwebuike, 2006).

While these studies described histological features of the bovine placentome, histomorphometric measurements of proportion and volume of bovine placental cell types and surface for transplacental exchange are limited to comparisons between placentae from pregnancies generated by artificial insemination versus those generated with *in vitro* fertilization embryos (Constant et al., 2006). Furthermore, others focused on differences between embryo culture systems (Miles et al., 2005, Miles et al., 2004) and study of angiogenesis (Vonnahme et al., 2007). Therefore, histomorphometric measurements of bovine placentomes from normal pregnancy sampled at different gestational ages are needed.

1.2.2 Placental insufficiency and hallmarks of intrauterine growth restriction

Intrauterine growth restriction (IUGR) and small for gestational age (SGA) are frequently used synonymously in human to describe low and/or restricted growth *in utero*, but these terms reflect different concepts (Hobbins, 2008). Small for gestational age is a statistical definition and denotes a fetus/neonate with anthropometric values below the tenth birth weight centile obtained from a reference population (Bertino et al., 2012). Thus a SGA neonate may be genetically small and normal and not affected by growth restricting processes that cause IUGR. On the other hand, IUGR refers to a functional condition and denotes fetuses unable to achieve their growth potential due to maternal, placental or fetal factors (Bertino et al., 2012). Placental insufficiency is associated with deterioration in the placenta's ability to deliver nutrient and oxygen to the fetus (Gagnon, 2003) and is a major cause of IUGR (Baschat et al., 2012). A reduction in placental size is associated with IUGR (Platz and Newman, 2008), while histomorphology of the placenta is also altered with significant reduction in diameter, density and branching of the placental villi, and number and area of fetal capillaries (Macara et al., 1996, Chen et al., 2002). Blood flow resistance is largely determined by the placental microvasculature and umbilical cord vessel tracts (Adamson, 1999), thus an umbilical cord that is thin with lower amount of Wharton's jelly (Di Naro et al., 2001) and with smaller cord vessels (Bruch et al., 1997) clearly indicates poor placental blood flow.

In addition to lower fetal weight in IUGR, there is asymmetric growth of fetal organs, where brain and heart growth are spared at the expense of the liver (Baschat et al., 2012). Asymmetric IUGR is a form of fetal adaptation to intrauterine undernutrition and indicative of preferential blood flow favouring the delivery of nutrients and oxygen to the brain and heart (Cetin et al., 2008). Animal models of IUGR where placental supply and umbilical blood flow are altered via surgical removal of endometrial caruncles prior to pregnancy or single umbilical artery ligation result in fetuses or neonates with similar phenotypes as seen in

human IUGR, i.e., high ratios of brain weight to liver weight, brain weight to body weight, and heart weight to body weight (Anthony et al., 2003, Barry et al., 2008, Morrison, 2008). Body composition is also altered where IUGR newborns have low ponderal index and limited fat stores (Petersen et al., 1988, Larciprete et al., 2005). These structural changes are associated with alterations in physiology and metabolism of the fetus. Impaired blood flow in IUGR correlates with high incidences of fetal hypoxia and acidosis because of decreased oxygen transport to the fetus and increased accumulation of carbon dioxide and/or lactate (Kingdom and Kaufmann, 1997, Marconi et al., 2006, Marconi and Battaglia, 2012). Clinical chemistry of cord blood from IUGR fetuses/newborns demonstrated lower concentrations of glucose, oxygen and cholesterol and higher carbon dioxide, lactate and haemoglobin concentrations and higher γ-glutamyl transferase activity in comparison to fetuses with normal heart rates and pulsatility indices or newborns with appropriate weight for gestational age (Pardi et al., 1993, Nieto-Diaz et al., 1996, Johnson et al., 2003). Differences in hormones regulating fetal growth are also evident. For example, high concentrations of circulating thyroid stimulating hormone and thyroxine and low levels of circulating insulin-like growth factor (IGF) 1, IGF2 and IGF binding proteins were reported for fetuses/newborns with IUGR (Thorpe-Beeston et al., 1991, Guidice et al., 1995). Expression of *IGF2* and *IGF1R* in placentae of IUGR newborns were upregulated (Abu-Amero et al., 1998) and in a negative relationship with birth weight (Iniguez et al., 2010), suggesting a compensatory increase in placental *IGF2* as a counter regulatory mechanism against deficiency in nutrient supply. Thus, a spectrum of markers, ranging from biometric indices and ratios to the clinical chemistry of cord blood and expression of IGFs in term placentae, is altered in IUGR fetuses and neonates.

1.2.3 Placenta mediates sex-specific growth

1.2.3.1 Sex-specific fetal adaptations

The Australian Bureau of Statistics projected that a male born in Australia from 2006 to 2008 would live 4.5 years less than an age-matched female. The lower life expectancy of males compared to females may be attributed to the higher mortality rate of males across all age groups, with ischaemic heart disease as the leading cause of death in adult males. Data from the USA shows that the fetal mortality rate of males is 6% higher than females (MacDorman and Gregory, 2013). Adverse perinatal outcomes including high incidence of premature births, true umbilical cord knots, and nuchal cord are also more common in males (Vatten and Skjærven, 2004, Aibar et al., 2012). Even in newborns with very low birth weights, morbidity and mortality rates are significantly higher in males than females (Stevenson et al., 2000).

While low birth weight is associated with cardiovascular disease in both sexes, biometric indices including birth length relative to placental weight in females and ponderal index relative to head circumference in males, have stronger associations with coronary heart disease than birth weight (Forsén et al., 1999). In men who were *in utero* during the November 1944-1945 Dutch Famine, a large placental surface area was associated with hypertension in later life (van Abeelen et al., 2011). In another study, hypertension in men is associated with a larger placental diameter whereas, in women, hypertension is associated with short diameter and smaller surface area of the placenta (Eriksson et al., 2010). This suggests that placental morphology and biometric indices and ratios are more sensitive indicators of sex-specific fetal adaptation than birth weight. It is likely that sex-specific structural, physiological and metabolic alterations in the fetus and placenta have long term outcomes that contribute to sex-bias in the prevalence of chronic diseases. Consistent with this are findings from animal models of placental insufficiency and maternal under-nutrition which resulted into sex-specific fetal programming of metabolic syndrome and hypertension

(Woods et al., 2005, Owens et al., 2007, Hernandez-Medrano et al., 2015). For example, male but not female progeny of mothers fed a moderately protein restricted diet during pregnancy became hypertensive in adulthood (Woods et al., 2001, Woods et al., 2005).

Several reports suggest female resilience in the presence of intrauterine insults. For example, growth-restricted female fetuses of untreated asthmatic mothers tend to adapt by slowing down their growth, contrary to male fetuses who maintain their growth trajectory despite the suboptimal intrauterine environment (Murphy et al., 2003). These different adaptation strategies prepare the female fetus to survive but render the male fetus vulnerable to further insults as gestation progresses (Clifton, 2010). Altered sex ratio favouring birth of more females shortly after the 1959 Great Leap Forward famine in China appears to be in agreement with the hypothesis that female offspring are 'nutritionally less expensive' to produce than males and thus would have better chances of surviving harsh environmental conditions (Myers, 1978, Song, 2012). Males born from mothers who smoke heavily during pregnancy have greater reduction in weight and fat as compared to females (Zarén et al., 2000). Head circumference at birth is also smaller in male, but not female, offspring of mothers who smoked heavily, suggesting males are more affected by smoking than females (Zarén et al., 2000). In a bovine model, biparietal diameter was lower in male fetuses whose mothers received a low, compared to high, protein peri-conception diet, whereas no differences were observed in female fetuses, again indicating that *in utero,* males are more sensitive to maternal dietary perturbations than females (Copping et al., 2014). Sex-specific fetal adaptations are most likely mediated by the placenta through observed sexual dimorphisms in placental structure, gene expression and function.

1.2.3.2 Sexual dimorphism in the placenta

Different placental characteristics are associated with cardiovascular disease in men and women (Eriksson et al., 2010) suggesting that sex-specific fetal adaptations to intrauterine insults are mediated by the placenta. Since the *Placenta fetalis* bears the same genetic make-

up as the fetus, it is expected to have the same autosomes and sex chromosomes as the fetus (Clifton, 2010). Therefore, placental morphology and function may underlie differences between placentae of males and females.

Placental weight and efficiency, as well as fetal weight, are increased in males compared to females (Thomson et al., 1969, Eley et al., 1978, Eriksson et al., 2010), indicating more nutrients are delivered by the placenta to support the male's greater propensity to grow. Male placentae have higher fetal capillary volume (Mayhew et al., 2008), larger umbilical cord vein diameter (Verburg et al., 2008) and longer umbilical cord (Georgiadis et al., 2014) with higher amount of Wharton's jelly than females (Filiz et al., 2011) suggesting sex differences in placental blood flow. Consistent with this are differences in blood flow resistance indices between male and female fetuses during the first trimester (Prefumo et al., 2003), last trimester (Verburg et al., 2008) and before induction of labour (Prior et al., 2013). Sex differences in placenta and umbilical cord morphology may contribute to the higher incidence of females being diagnosed or born with IUGR compared to males (Melamed et al., 2010, Borzsonyi et al., 2011, Aibar et al., 2012), however, this requires further study.

Sex-specific placental gene expression may contribute to sexually dimorphic fetal growth and adaptation. Sexual dimorphism in expression of several genes is evident in placentae of mice (Gabory et al., 2012) suggesting the presence of genes that are inherently regulated in a sex-specific manner. For example, as compared to males, genes highly expressed in female placentae include those with roles in immune regulation, indicating that female placentae have a greater immune response to varied stimuli (Sood et al., 2006). Consistent with this, more genes associated with growth, inflammation and immune pathways are altered in female placentae from pregnancies complicated with asthma relative to control and male placentae (Osei-Kumah et al., 2011). Interestingly, low and very high fat diets elicit up-regulation of genes associated with controlling uptake or excretion of compounds, ion and

fluid movement, in female placenta (Mao et al., 2010). This suggests that the female placenta's increased sensitivity to changes in maternal diet may provide protection against *in utero* perturbations. On the other hand, in mice, placentae of male, but not female, fetuses of mothers fed a high fat diet exhibited down-regulation of genes involved in connective tissue disorders and implicated in vascular development, suggesting that vascularity is affected more in placentae of males than females (Gabory et al., 2012). Furthermore, placentae of female fetuses from mice fed a high fat diet show global hypomethylation and greater changes in expression of imprinted genes from different clusters compared to placentae of the male fetuses (Gallou-Kabani et al., 2010). These studies demonstrate that male and female placentae have different transcriptomic and epigenomic responses to identical challenges *in utero*.

1.2.4 Fetal, maternal and paternal genetic effects on placental traits

The use of embryo transfer allows fetal genetic effects on placental characteristics and fetal weight to be examined separately from maternal effects. Embryos with *Bos taurus taurus* (Charolais) genetics transferred to cows with *Bos taurus indicus* (Brahman) genetics have heavier fetal weights than Brahman embryos transferred into Charolais cows demonstrating that Charolais fetuses have greater growth potential than Brahman fetuses regardless of maternal uterine environment (Ferrell, 1991). However, when compared with Charolais embryos in Charolais cows, embryos transferred into Brahman cows have lower fetal weights at day 272 of gestation (Ferrell, 1991). This study therefore indicates that fetal genome is the primary determinant of growth, but variation in the uterine environment can limit or support rapid fetal growth during the last trimester of gestation. Similar observations are seen in embryo transfer experiments in sheep (Sharma et al., 2012) and horse (Allen et al., 2002). Concurrent to differences in fetal weight, weight and amounts of RNA and DNA of the cotyledon are also affected by fetal genome, while maternal uterine environment did not

contribute to differences (Ferrell, 1991), indicating that the *Placenta fetalis* mediates the acquisitive drive of the fetus for nutrients.

Effects of maternal breed have been observed in placental membrane weight, average weight of placentomes (Bellows et al., 1993) and placentome number (Ferrell, 1991), suggesting maternal control of fetal growth mediated by the placenta varies between breeds. While effects of sire breed on fetal and birth weights are reported (Reynolds et al., 1980, Paschal et al., 1991), studies investigating sire breed effects on placental traits are limited to effects of *Bos t. taurus* (Angus) sires selected for high or low progeny birth weights on average placentome weight, placental fluid volume and ratio of total placentome weight to estimated fetal weight (Anthony et al., 1986). Information on parental and fetal genome effects on placental histomorphology and umbilical cord traits are also lacking.

1.2.5 Heterosis

Heterosis or hybrid vigor is a biological phenomenon where the hybrid F1 offspring exhibit increased level of growth or fitness relative to parental breeds. There are three conventional heterosis hypotheses based on standard genetic models, namely, dominance, overdominance and epistasis (Hochholdinger and Hoecker, 2007). Here, heterosis is thought to result from complementing action of superior dominant alleles inherited from both parents that suppress the effects of unfavourable alleles as in the case of the dominance hypothesis, and by the allelic or non-allelic gene interactions at multiple loci for overdominance and epistasis hypotheses, respectively (Hochholdinger and Hoecker, 2007). The terms “dominant” and “overdominant” are often used to describe hybrid performance or gene expression level, but this leads to confusion with the hypotheses mentioned above and thus these terms are avoided (Hochholdinger and Hoecker, 2007, Springer and Stupar, 2007). These conventional hypotheses are not sufficient to fully explain heterosis, as heterosis effects still cannot be predicted and frequently have to be determined in test-crosses (Lippman and Zamir, 2007, Springer and Stupar, 2007).

Modelling data have indicated that genomic imprinting, i.e., parent-of-origin specific monoallelic gene expression, can mimic overdominance (Chakraborty, 1989). In 1996, polar overdominance, a non-Mendelian model of inheritance, was proposed to explain the expression of generalized muscular hypertrophy in heterozygous lambs which inherited the callipyge mutation from their father (Cockett et al., 1996). Polar overdominance is present when the phenotype of one type of heterozygous progeny differs from the reciprocal cross depending on the parental origin of alleles and also differs from the two parental homozygotes (**Figure 1.3**). Changes in imprinted gene expression and trans-interaction between the products of reciprocally imprinted genes appear to be the underlying mechanisms of polar overdominance (Georges et al., 2004). Recent studies using intercrosses between large and small inbred strains of mouse, which focused on genomic imprinting (parent-of-origin) effects on phenotypic traits, revealed imprinting patterns of maternal and paternal expression and dominance imprinting. The latter mode of expression was further sub-classified as either bipolar or polar over- or underdominance (Wolf et al., 2008). In addition to sheep and mouse, similar patterns of polar overdominance were reported in pig (Kim et al., 2004) and human (Wermter et al., 2008).

Crossbreeding of *Bos taurus indicus* and *Bos taurus taurus* is widely used to exploit heterosis effects on reproductive and pre- and postweaning traits, including birth weight (Koger, 1980). These two types of cattle originate from different wild ancestors in terms of subspecies status and represent a maximum intraspecies divergence (Hiendleder et al., 2008). Birth weight is the endpoint of fetal growth and is a strong predictor of weaning weight and postnatal performance (Gregory et al., 1979, Thrift, 1997). Interestingly, *Bos t. indicus* (Brahman) sired progeny in *Bos t. taurus* (Angus) cows show polar overdominance in birth weight as these hybrids have significantly higher birth weights than either purebred or reciprocal cross which has birth weights similar to purebred Brahman calves (Reynolds et al., 1980, Brown et al., 1993). This polar overdominance pattern in birth weight persists after

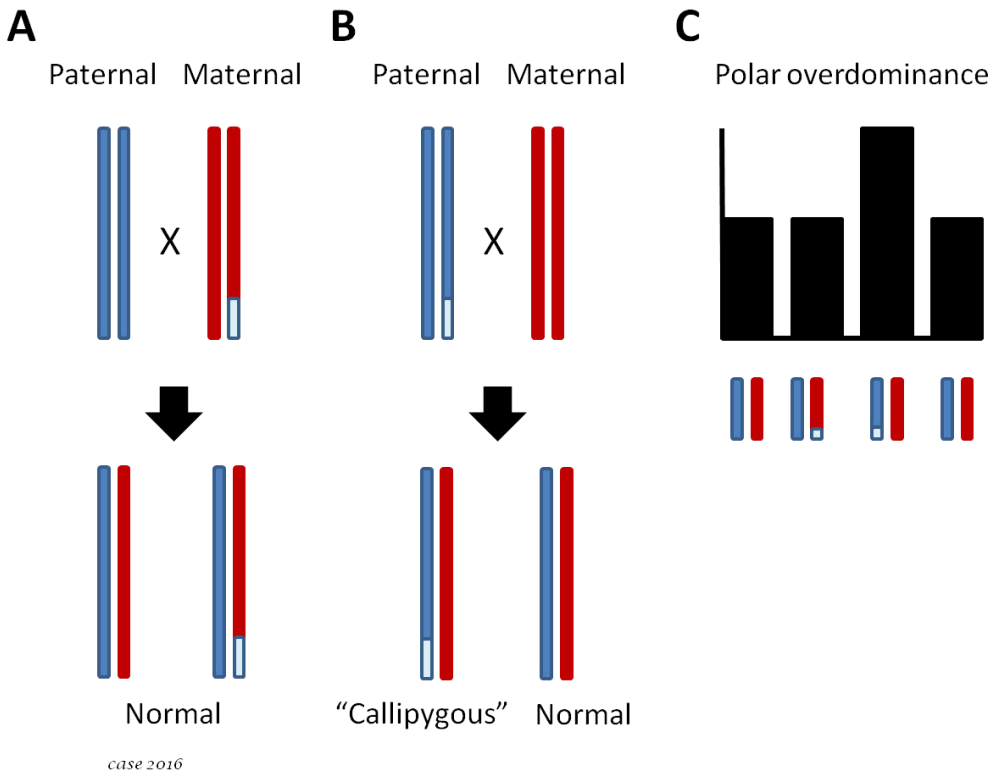

Figure 1.3 Diagrammatic representation of non-mendelian inheritance of the callipyge phenotype in sheep.

Callipygous animals are characterized by generalized muscular hypertrophy primarily around the hindquarters attributed to a dominant point mutation of the callipyge locus on ovine chromosome 18. (A) Non-callipygous ('normal') rams mated to heterozygous callipygous ewes produce non-callipygous ('normal') offspring. (B) Heterozygous callipygous rams mated to non-callipygous ewes produce 50% callipygous and 50% non-callipygous ('normal') offspring. (C) The callipygous heterozygote which inherited the mutation from the father displays polar overdominance compared to the other phenotypes of the homozygote and its reciprocal cross.

embryo transfer (Amen et al., 2007b, Dillon et al., 2015), and therefore excludes intrauterine effects of *Bos t. taurus* as cause of the observed fetal overgrowth. Others also reported sex differences in Brahman sired progeny with *Bos t. taurus* dams, where males are generally significantly heavier than females and heavier than both sexes of the reciprocal cross (Paschal et al., 1991, Amen et al., 2007a, Dillon et al., 2015). These studies suggest non-mendelian genetic factor and/or epigenetic factors of the conceptus as cause of this heterotic prenatal growth in one of the hybrids. However, so far, studies in cattle are limited to assessing heterosis in postnatal traits such as birth weight and additional studies are needed to determine the role of the placenta and umbilical cord in mediating heterotic fetal growth.

1.2.6 Insulin-like growth factor (IGF) system

1.2.6.1 Expression of IGFs in the placenta

The insulin-like growth factor (IGF) system plays a central role in regulating feto-placental growth and development (Roberts et al., 2008). IGF1 and IGF2 promote cell proliferation, differentiation, migration and survival primarily by binding with the IGF type 1 receptor (IGF1R) on target cell surfaces (Jones and Clemmons, 1995, Forbes and Westwood, 2008). IGFs may also bind to the insulin receptor (INSR) at a lower affinity than IGF1R, affecting both mitogenic and metabolic responses of the tissue. The IGF type 2 receptor (IGF2R) preferentially binds IGF2 for lysosomal degradation (Jones and Clemmons, 1995, Brown et al., 2009), although a direct signalling role of IGF2R in placenta has been reported (McKinnon et al., 2001).

In sheep, *IGF2* mRNA is highly expressed in the fetal mesoderm found at the tips of the invading placental villi and is moderately expressed in the caruncular stroma during early placental development (Reynolds et al., 1997). On the other hand, *IGF1* mRNA expression in the maternal caruncle is low and decreases as gestation progresses, while *IGF1* mRNA in the fetal placental tissue is undetectable (Reynolds et al., 1997). In sheep, *IGF1R* is moderately expressed in the caruncles prior to implantation, but is also undetectable in either maternal or

fetal portions of the placentome beginning day 30 of gestation when interdigitation between the caruncles and allantochorion occurs (Reynolds et al., 1997). Similarly, in cattle at day 16 of gestation, *IGF1* and *IGF1R* mRNA expression is low in the sub-epithelial stroma and caruncular stroma, respectively, while *IGF2* is again highly expressed in the caruncular stroma (Robinson et al., 2000). By day 25 of gestation, *IGF1* and *IGF1R* are expressed at low levels in placental tissue compared to the higher expression levels of *IGF2* and *IGF2R* (Moore et al., 2007). Beginning from the fetal stage at day 50 to mid-gestation, moderate to intense staining of IGF1 and IGF2 in fetal villi and maternal stromal and epithelial placental tissues is observed (Ravelich et al., 2004). In human placentae, examined from six weeks of gestation to term, *IGF2* is more abundantly expressed than *IGF1* in all gestational stages and is expressed in the chorionic mesoderm and cytotrophoblastic column of the placental villi, while *IGF1R* is expressed at low levels in all cell types (Han et al., 1996). Consistent with gene deletion studies in mice (Baker et al., 1993, Constancia et al., 2002), the higher *IGF2* expression indicates a more significant role for IGF2 in placental development than for IGF1. The low *IGF1R* expression in the sheep placentome suggests that IGF1R may not be mediating the actions of IGF2 in the placenta (Wathes et al., 1998). In human placenta, IGF2R is expressed in the maternal compartment facing the microvillous membrane surface of the syncytiotrophoblast (Fang et al., 1997). While IGF2R is commonly associated with IGF2 clearance, extravillous trophoblast cell invasiveness and migration in vitro is enhanced by IGF2 acting through IGF2R via inhibitory G-proteins and stimulation of the MAPK pathway (McKinnon et al., 2001). Thus, IGF2R can alternatively function as a signalling receptor (Harris et al., 2011).

The action of IGFs is modulated by six IGF binding proteins. These IGFBPs can sequester and bind IGFs and thus inhibit their action. The majority of IGFs in circulation are bound to the IGFBP3-acid labile subunit which functions as a reservoir for IGF release (Jones and Clemmons, 1995). IGFBP1, -2, and -4 are low molecular weight IGFBPs, where IGFBP1

and IGFBP2 more effectively inhibit IGF1 and IGF2, respectively, while IGFBP4 and IGFBP6 consistently inhibit the action of both IGFs (Jones and Clemmons, 1995, Duan and Xu, 2005). IGFBP5 can adhere to the extracellular matrix and may potentiate IGF1's effect on DNA synthesis (Jones and Clemmons, 1995). Expression of *IGFBP2,-3,* and *-4* is localized in the maternal caruncular stroma in sheep, which suggests that these binding proteins may be involved in controlling growth of the invading fetal villi during early gestation (Reynolds et al., 1997), while *IGFBP6* mRNA is detected in maternal placental villi (Osgerby et al., 2004). In cattle, IGFBP-1 is expressed in deep uterine glands but not in fetal villi during the first half of pregnancy, while IGFBP-2 is localized in deep stromal tissue of the maternal caruncle, and little or no staining is observed for IGFBP-3 in any placental tissue (Ravelich et al., 2004). In human placentae, all six IGFBPs are expressed in the maternal decidua, where *IGFBP1* is the most abundantly expressed binding protein (Han et al., 1996).

1.2.6.2 Imprinted genes of the IGF system

Each parent contributes equally to the genetic complement of the fetus, but a subset of placental genes is imprinted and expressed depending on whether they are paternally or maternally inherited (Reik and Walter, 2001). Since one of the alleles is transcriptionally silenced, imprinted genes are functionally haploid. Imprinting usually occurs through methylation of cytosines in areas rich in CpG dinucleotides, such as those found in transcription sites or promoter regions of many genes (Deaton and Bird, 2011).

Most imprinted genes are expressed in the placenta and are involved in regulating fetal growth (Reik and Walter, 2001). Based on the genetic conflict theory, paternally expressed genes function to increase resource allocation from the mother, thus promoting growth, whereas maternally expressed genes have the opposite role (Moore and Haig, 1991). A recent report showed the relationship between expression of key imprinted genes from human chorionic villi early in gestation and term placentae with biometric measurements and birth weight (Moore et al., 2015). For example, maternally expressed pleckstrin homology-like

domain family A member 2 (*PHLDA2*) and growth factor receptor-bound protein 10 (*GRB10*) from term placentae are negatively correlated with birth weight and head circumference, respectively. On the other hand, chorionic villi expression of paternally expressed *IGF2* and maternally expressed *IGF2R* are positively correlated to birth weight (Moore et al., 2015). These findings suggest *IGF2/IGF2R* are important in setting the growth potential of the fetus early in pregnancy and *PHLDA2* and *GRB10*, as maternally expressed genes, suppress growth in late pregnancy to avoid risk of a macrosomic newborn (Moore et al., 2015). It appears that while parental genomes in the conceptus have conflicting interests, they reach a point of cooperation because they share a common objective of perpetuating the offspring (Moore and Haig, 1991). Thus, the placental phenotype is the result of a carefully-regulated balance between expression of paternal and maternal genomes.

To date, there are 35 imprinted genes described in cattle (http://igc.otago.ac.nz) which include *IGF2R*, *IGF2*, and *H19*, that were first discovered using mouse models (Barlow et al., 1991, Bartolomei et al., 1991, DeChiara et al., 1991, Ferguson-Smith et al., 1991). The reciprocal functions of *IGF2* and *IGF2R* appear to recapitulate the conflict theory and disruption of their expression invariably results in small or overgrowth phenotypes. For example, gene deletion in mice has demonstrated that *igf2* deficiency results in smaller placentae and fetal growth retardation (DeChiara et al., 1990, Baker et al., 1993) while *igf2r* mutation in the maternal allele produces placental and fetal overgrowth (Lau et al., 1994, Ludwig et al., 1996). There are also studies showing alterations in *IGF2R* expression in placentae of large for gestational age newborns (Demetriou et al., 2014) and IUGR (Kumar et al., 2012). Furthermore, changes in expression of *IGF2* are associated with placental and fetal overgrowth in *inter*species hybrid dysgenesis in mouse (Zechner et al., 2002). This suggests that imprinted genes might also be involved in *intra*species mechanisms governing heterosis.

The maternally expressed *H19* downstream of the *IGF2* gene is involved in modulating *IGF2* expression in *cis* and *trans* (Wilkin et al., 2000, Gabory et al., 2009). Using

in vivo mouse models of tumorigenesis, *H19* has been implicated in tumour suppression (Yoshimizu et al., 2008). Furthermore, disruption of *H19* in mouse results in an overgrowth phenotype (Gabory et al., 2009). Differentiating cytotrophoblast of the human placenta expresses *H19 in vitro* (Rachmilewitz et al., 1992) and more recently, it was shown that a microRNA embedded in the first exon of *H19* targets *igf1r* thereby inhibiting placental growth during the second half of gestation (Keniry et al., 2012). The paternally expressed non-coding RNA anti-sense to *Igf2r*, *Airn*, was detected in bovine fetal liver samples but is only expressed at low levels in early concepti tissues (Farmer et al., 2013). *Airn* is involved in silencing *Igf2r* and organic cation transporters *OCT2/Slc22A2* and *OCT3/Slc22A3*, but its direct role in placental and fetal growth is still unclear (Latos et al., 2012, Monk, 2015).

1.3 Hypothesis and Research Aims

The placenta is central to fetal growth and development and a major determinant of fetal programming. While most reports in human and animals are based on studies of term placentae or birth weight, information at critical time points of development are generally lacking on (i) fundamental gross morphological and histomorphological characteristics of the placenta that contribute to increased efficiency and impact fetal growth, (ii) the influence of sex-specific placental and umbilical cord morphology on sexual dimorphism of fetal growth and its implications for postnatal health, (iii) contribution of placental and umbilical cord in mediating genetic/epigenetic effects on concepti traits leading to hybrid vigour in fetal growth, and (iv) differences in expression of the components of the insulin-like growth factor (IGF) system. These gaps in knowledge are highly relevant to both animal production and human health, and thus, cattle, a major livestock species and valuable biomedical model for human female reproduction and pregnancy, are best fit to be utilized for this research.

1.3.1 General Hypothesis

Differences in placental phenotype of male and female bovine concepti, and between purebreds and their reciprocal cross hybrids, unfold as the fetus enters accelerated growth at mid-gestation and determine variation in intrauterine growth which may programme postnatal phenotype.

1.3.2 Specific Hypotheses

1. In addition to placental weight, there are other gross morphological, histomorphological and molecular placental traits that contribute to increased placental efficiency and drive fetal growth in bovine.
2. Sex-specific placental and umbilical cord phenotypes affect nutrient availability and transfer differently between males and females, and contribute to sex-specific prenatal growth and adaptation which have long term implications for postnatal performance and health.
3. Heterosis in fetal growth and birth weight is predominantly controlled by the conceptus genome and mediated by the placenta with changes in abundances of imprinted transcripts of genes in the IGF system.

1.3.3 Research Aims

Purebred and reciprocal cross *Bos taurus taurus* (Angus, A) and *Bos taurus indicus* (Brahman, B) concepti recoved at the late embryo (Day 48) and onset of accelerated fetal growth (Day 153) stages (term 277-291 days) were used in addressing the following research aims:

1. To establish novel and baseline data on the gross morphological and histomorphological characteristics of the bovine placenta from late embryonic and early accelerated fetal growth stages to identify developmental changes and critical parameters for embyo-fetal growth;

2. To determine effects of fetal sex on placental-fetal phenotype, cord blood clinical-chemical parameters and tissue-specific transcript abundances of genes in the IGF system at the early accelerated fetal growth stage to unravel mechanism of sex-specific fetal growth and programming of sex-bias in postnatal disease;
3. To analyze effects of hybridization on gross morphological and histomorphological characteristics of the bovine placenta and on transcript abundances of key imprinted regulators of conceptus growth from the IGF system at the early accelerated fetal growth stage to map placental drivers of heterosis in birth weight and correlated postnatal performance.

1.4 Literature Cited

ABDEL-RAOUF, M. & BADAWI, H. M. 1966. Morphological study of uterine caruncles in Egyptian buffalo cows. *Zentralblatt für Veterinärmedizin Reihe A,* 13**,** 252-263.

ABU-AMERO, S. N., ALI, Z., BENNETT, P., VAUGHAN, J. I. & MOORE, G. E. 1998. Expression of the insulin-like growth factors and their receptors in term placentas: A comparison between normal and IUGR births. *Molecular Reproduction and Development,* 49**,** 229-235.

ADAMSON, S. L. 1999. Arterial pressure, vascular input impedance, and resistance as determinants of pulsatile blood flow in the umbilical artery. *European Journal of Obstetrics & Gynecology and Reproductive Biology,* 84**,** 119-125.

AIBAR, L., CARRILLO, M. P., MONTOYA, F., PUERTAS, A. & VALVERDE, M. 2012. Fetal sex and perinatal outcomes. *Journal of Perinatal Medicine,* 40**,** 271-276.

AIRES, M. B., DEGAKI, K. Y., DANTZER, V. & YAMADA, A. T. 2014. Bovine placentome development during early pregnancy. *In:* MENDEZ-VILLAS, A. (ed.) *Microscopy: Advances in Scientific Research and Education.* Spain: Formatex Research Center.

ALEXANDER, G. 1964. Studies on the placenta of the sheep (*Ovis aries L.*). *Journal of Reproduction and Fertility,* 7**,** 289-305.

ALLEN, W., WILSHER, S., TURNBULL, C., STEWART, F., OUSEY, J., ROSSDALE, P. & FOWDEN, A. 2002. Influence of maternal size on placental, fetal and postnatal growth in the horse. I. Development in utero. *Reproduction,* 123**,** 445-453.

ALMOG, B., SHEHATA, F., ALJABRI, S., LEVIN, I., SHALOM-PAZ, E. & SHRIM, A. 2011. Placenta weight percentile curves for singleton and twins deliveries. *Placenta,* 32**,** 58-62.

AMEN, T. S., HERRING, A. D., SANDERS, J. O. & GILL, C. A. 2007a. Evaluation of reciprocal differences in × backcross calves produced through embryo transfer: I. Birth and weaning traits. *Journal of Animal Science,* 85**,** 365-372.

AMEN, T. S., HERRING, A. D., SANDERS, J. O. & GILL, C. A. 2007b. Evaluation of reciprocal differences in Bos indicus × Bos taurus backcross calves produced through embryo transfer: II. Postweaning, carcass, and meat traits. *Journal of Animal Science,* 85**,** 373-379.

ANDERSEN, H. & PLUM, M. 1965. Gestation length and birth weight in cattle and buffaloes: a review. *Journal of Dairy Science,* 48**,** 1224-1235.

ANTHONY, R. V., BELLOWS, R. A., SHORT, R. E., STAIGMILLER, R. B., KALTENBACH, C. C. & DUNN, T. G. 1986. Fetal growth of beef calves. II. Effect of sire on prenatal development of the calf and related placental characteristics. *Journal of Animal Science,* 62**,** 1375-1387.

ANTHONY, R. V., SCHEAFFER, A. N., WRIGHT, C. D. & REGNAULT, T. R. H. 2003. Ruminant models of prenatal growth restriction. *Reproduction,* Supplement 61**,** 183-194.

ASSIS NETO, A. C., PEREIRA, F. T. V., SANTOS, T. C., AMBROSIO, C. E., LEISER, R. & MIGLINO, M. A. 2010. Morpho-physical recording of bovine conceptus (*Bos indicus*) and placenta from Days 20 to 70 of pregnancy. *Reproduction in Domestic Animals,* 45**,** 760-772.

ATKINSON, B. A., KING, G. J. & AMOROSO, E. C. 1984. Development of the caruncular and intercaruncular regions in the bovine endometrium. *Biology of Reproduction,* 30**,** 763-774.

BÄHR, A. & WOLF, E. 2012. Domestic animal models for biomedical research. *Reproduction in Domestic Animals,* 47**,** 59-71.

BAKER, J., LIU, J.-P., ROBERTSON, E. J. & EFSTRATIADIS, A. 1993. Role of insulin-like growth factors in embryonic and postnatal growth. *Cell,* 75**,** 73-82.

BARKER, D. J., OSMOND, C., SIMMONDS, S. J. & WIELD, G. A. 1993. The relation of small head circumference and thinness at birth to death from cardiovascular disease in adult life. *British Medical Journal,* 306**,** 422-426.

BARKER, D. J. P. 1995. Fetal origins of coronary heart disease. *British Medical Journal,* 311**,** 171-174.

BARLOW, D. P., STOGER, R., HERRMANN, B. G., SAITO, K. & SCHWEIFER, N. 1991. The mouse insulin-like growth factor type-2 receptor is imprinted and closely linked to the Tme locus. *Nature,* 349**,** 84-87.

BARRETO, R. S. N., BRESSAN, F. F., OLIVEIRA, L. J., PEREIRA, F. T. V., AMBROSIO, C. E., MEIRELLES, F. V. & MIGLINO, M. A. 2011. Gene expression in placentation of farm animals an overview of gene function during development. *Theriogenology,* 76**,** 589-597.

BARRY, J. S., ROZANCE, P. J. & ANTHONY, R. V. 2008. An animal model of placental insufficiency-induced intrauterine growth restriction. *Seminars in Perinatology,* 32**,** 225-230.

BARTOLOMEI, M. S., ZEMEL, S. & TILGHMAN, S. M. 1991. Parental imprinting of the mouse H19 gene. *Nature,* 351**,** 153-155.

BASCHAT, A. A., GALAN, H. L. & GABBE, S. G. 2012. Intrauterine growth restriciton. *In:* GABBE, S. G. (ed.) *Obstetrics: Normal and Problem Pregnancies.* USA: Elsevier Saunders.

BATTAGLIA, F. C. & LUBCHENCO, L. O. 1967. A practical classification of newborn infants by weight and gestational age. *The Journal of Pediatrics,* 71**,** 159-163.

BAUR, R. 1977. *Morphometry of the Placental Exchange Area,* New York, USA, Springer-Verlag.

BELLOWS, R. A., STAIGMILLER, R. B., ORME, L. E., SHORT, R. E. & KNAPP, B. W. 1993. Effects of sire and dam on late-pregnancy conceptus and hormone traits in beef cattle. *Journal of Animal Science,* 71**,** 714-23.

BERTINO, E., OCCHI, L. & FABRIS, C. 2012. Intrauterine growth restriction: neonatal aspects. *In:* BUONOCORE, G., BRACCI, R. & WEINDLING, M. (eds.) *Neonatology.* Italy: Springer Milan.

BIENSEN, N. J., WILSON, M. E. & FORD, S. P. 1999. The impacts of uterine environment and fetal genotype on conceptus size and placental vascularity during late gestation in pigs. *Journal of Animal Science,* 77**,** 954-959.

BORZSONYI, B., DEMENDI, C., NAGY, Z., TOTH, K., CSANAD, M., PAJOR, A., RIGO, J. J. & JOO, J. G. 2011. Gene expression patterns of insulin-like growth factor 1, insulin-like growth factor 2 and insulin-like growth factor binding protein 3 in human placenta from pregnancies with intrauterine growth restriction. *Journal of Perinatal Medicine,* 39**,** 701-707.

BROWN, J., JONES, E. Y. & FORBES, B. E. 2009. Keeping IGF-II under control: Lessons from the IGF-II–IGF2R crystal structure. *Trends in Biochemical Sciences,* 34**,** 612-619.

BROWN, M. A., THAREL, L. M., BROWN, A. H., JACKSON, W. G. & MIESNER, J. R. 1993. Genotype x environment interactions in preweaning traits of purebred and reciprocal cross Angus and Brahman calves on common bermudagrass and endophyte-infected tall fescue pastures. *Journal of Animal Science,* 71**,** 326-333.

BRUCH, J. F., SIBONY, O., BENALI, K., CHALLIER, J. C., BLOT, P. & NESSMANN, C. 1997. Computerized microscope morphometry of umbilical vessels from pregnancies with intrauterine growth retardation and abnormal umbilical artery doppler. *Human Pathology,* 28**,** 1139-1145.

CARVALHO, A. F., KLISCH, K., MIGLINO, M. A., PEREIRA, F. T. V. & BEVILACQUA, E. 2006. Binucleate trophoblast giant cells in the water buffalo (Bubalus bubalis) placenta. *Journal of Morphology,* 267**,** 50-56.

CETIN, I., BOITO, S. & RADAELLI, T. 2008. Evaluation of fetal growth and fetal well-being. *Seminars in Ultrasound, CT and MRI,* 29**,** 136-146.

CHAKRABORTY, R. 1989. Can molecular imprinting explain heterozygote deficiency and hybrid vigor? *Genetics,* 122**,** 713-717.

CHEN, C.-P., BAJORIA, R. & APLIN, J. D. 2002. Decreased vascularization and cell proliferation in placentas of intrauterine growth–restricted fetuses with abnormal umbilical artery flow velocity waveforms. *American Journal of Obstetrics and Gynecology,* 187**,** 764-769.

CHEN, Z., ROBBINS, K. M., WELLS, K. D. & RIVERA, R. M. 2013. Large offspring syndrome: A bovine model for the human loss-of-imprinting overgrowth syndrome Beckwith-Wiedemann. *Epigenetics,* 8**,** 591-601.

CLIFTON, V. L. 2010. Review: Sex and the human placenta: mediating differential strategies of fetal growth and survival. *Placenta,* 31**,** S33-S39.

COAN, P. M., FERGUSON-SMITH, A. C. & BURTON, G. J. 2004. Developmental Dynamics of the Definitive Mouse Placenta Assessed by Stereology. *Biology of Reproduction,* 70**,** 1806-1813.

COCKETT, N. E., JACKSON, S. P., SHAY, T. L., FARNIR, F., BERGHMANS, S., SNOWDER, G. D., NIELSEN, D. M. & GEORGES, M. 1996. Polar overdominance at the ovine callipyge locus. *Science,* 273**,** 236-238.

CONSTANCIA, M., HEMBERGER, M., HUGHES, J., DEAN, W., FERGUSON-SMITH, A. C., FUNDELE, R., STEWART, F., KELSEY, G., FOWDEN, A., SIBLEY, C. & REIK, W. 2002. Placental-specific IGF-II is a major modulator of placental and fetal growth. *Nature,* 417**,** 945-948.

CONSTANT, F., GUILLOMOT, M., HEYMAN, Y., VIGNON, X., LAIGRE, P., SERVELY, J. L., RENARD, J. P. & CHAVATTE-PALMER, P. 2006. Large offspring or large placenta syndrome? Morphometric analysis of late gestation bovine placentomes from somatic nuclear transfer pregnancies complicated by hydrallantois. *Biology of Reproduction,* 75**,** 122-130.

COPPING, K. J., HOARE, A., CALLAGHAN, M., MCMILLEN, I. C., RODGERS, R. J. & PERRY, V. E. A. 2014. Fetal programming in 2-year old calving heifers: peri-conception and first trimester protein restriction alters fetal growth in a gender-specific manner. *Animal Production Science,* 54**,** 1333-1337.

CURRAN, S., PIERSON, R. A. & GINTHER, O. J. 1986. Ultrasonographic appearance of the bovine conceptus from days 20 through 60. *Journal of the American Veterinary Medical Association,* 189**,** 1295-1302.

DEATON, A. M. & BIRD, A. 2011. CpG islands and the regulation of transcription. *Genes & Development,* 25**,** 1010-1022.

DECHIARA, T. M., EFSTRATIADIS, A. & ROBERTSEN, E. J. 1990. A growth-deficiency phenotype in heterozygous mice carrying an insulin-like growth factor II gene disrupted by targeting. *Nature,* 345**,** 78-80.

DECHIARA, T. M., ROBERTSON, E. J. & EFSTRATIADIS, A. 1991. Parental imprinting of the mouse insulin-like growth factor II gene. *Cell,* 64**,** 849-859.

DEMETRIOU, C., ABU-AMERO, S., THOMAS, A. C., ISHIDA, M., AGGARWAL, R., AL-OLABI, L., LEON, L. J., STAFFORD, J. L., SYNGELAKI, A., PEEBLES, D., NICOLAIDES, K. H., REGAN, L., STANIER, P. & MOORE, G. E. 2014. Paternally expressed, imprinted insulin-like growth factor-2 in chorionic villi correlates significantly with birth weight. *PLOS ONE,* 9**,** e85454.

DI NARO, E., GHEZZI, F., RAIO, L., FRANCHI, M., D'ADDARIO, V., LANZILLOTTI, G. & SCHNEIDER, H. 2001. Umbilical vein blood flow in fetuses with normal and lean umbilical cord. *Ultrasound in Obstetrics and Gynecology,* 17**,** 224-228.

DILLON, J. A., RILEY, D. G., HERRING, A. D., SANDERS, J. O. & THALLMAN, R. M. 2015. Genetic effects on birth weight in reciprocal Brahman–Simmental crossbred calves. *Journal of Animal Science,* 93.

DUAN, C. & XU, Q. 2005. Roles of insulin-like growth factor (IGF) binding proteins in regulating IGF actions. *General and Comparative Endocrinology,* 142**,** 44-52.

DWYER, C. M. 2005. Behavior: Maternal. *In:* POND, W. G. & BELL, A. W. (eds.) *Encyclopedia of Animal Science.* New York: Marcel Dekker.

ECHTERNKAMP, S. E. 1993. Relationship between placental development and calf birth weight in beef cattle. *Animal Reproduction Science,* 32**,** 1-13.

EHRHARDT, R. A. & BELL, A. W. 1995. Growth and metabolism of the ovine placenta during mid-gestation. *Placenta,* 16**,** 727-741.

ELEY, R. M., THATCHER, W. W., BAZER, F. W., WILCOX, C. J., BECKER, R. B., HEAD, H. H. & ADKINSON, R. W. 1978. Development of the conceptus in the bovine. *Journal of Dairy Science,* 61**,** 467-473.

ERIKSSON, J. G., KAJANTIE, E., OSMOND, C., THORNBURG, K. & BARKER, D. J. P. 2010. Boys live dangerously in the womb. *American Journal of Human Biology,* 22**,** 330-335.

FANG, J., FURESZ, T. C., LAURENT, R. S., SMITH, C. H. & FANT, M. E. 1997. Spatial polarization of insulin-like growth factor receptors on the human syncytiotrophoblast. *Pediatric Research,* 41**,** 258-265.

FARMER, W. T., FARIN, P. W., PIEDRAHITA, J. A., BISCHOFF, S. R. & FARIN, C. E. 2013. Expression of antisense of insulin-like growth factor-2 receptor RNA non-coding (AIRN) during early gestation in cattle. *Animal Reproduction Science,* 138**,** 64-73.

FERGUSON-SMITH, A. C., CATTANACH, B. M., BARTON, S. C., BEECHEY, C. V. & SURANI, M. A. 1991. Embryological and molecular investigations of parental imprinting on mouse chromosome 7. *Nature,* 351**,** 667-670.

FERRELL, C. L. 1989. Placental regulation of fetal growth. *In:* CAMPION, D. R., HAUSMAN, G. J. & MARTIN, R. J. (eds.) *Animal Growth Regulation.* USA: Springer US.

FERRELL, C. L. 1991. Maternal and fetal influences on uterine and conceptus development in the cow: I. Growth of tissues of the gravid uterus. *Journal of Animal Science,* 69**,** 1945-53.

FILIZ, A. A., RAHIME, B., KESKIN, H. L. & ESRA, A. K. 2011. Positive correlation between the quantity of Wharton's jelly in the umbilical cord and birth weight. *Taiwanese Journal of Obstetrics and Gynecology,* 50**,** 33-36.

FORBES, K. & WESTWOOD, M. 2008. The IGF axis and placental function. *Hormone Research in Paediatrics,* 69**,** 129-137.

FORD, S. P., CHENAULT, J. R. & ECHTERNKAMP, S. E. 1979. Uterine blood flow of cows during the oestrous cycle and early pregnancy: effect of the conceptus on the uterine blood supply. *Journal of Reproduction and Fertility,* 56**,** 53-62.

FORSÉN, T., ERIKSSON, J., TUOMILEHTO, J., REUNANEN, A., OSMOND, C. & BARKER, D. 2000. The fetal and childhood growth of persons who develop Type 2 diabetes. *Annals of Internal Medicine,* 133**,** 176-182.

FORSÉN, T., ERIKSSON, J. G., TUOMILEHTO, J., OSMOND, C. & BARKER, D. J. P. 1999. Growth in utero and during childhood among women who develop coronary heart disease: longitudinal study. *British Medical Journal,* 319**,** 1403-1407.

FOWDEN, A. L., SFERRUZZI-PERRI, A. N., COAN, P. M., CONSTANCIA, M. & BURTON, G. J. 2009. Placental efficiency and adaptation: endocrine regulation. *The Journal of Physiology,* 587**,** 3459-3472.

FUNSTON, R. N. & SUMMERS, A. F. 2013. Effect of Prenatal Programming on Heifer Development. *Veterinary Clinics of North America: Food Animal Practice,* 29**,** 517-536.

GABORY, A., RIPOCHE, M.-A., LE DIGARCHER, A., WATRIN, F., ZIYYAT, A., FORNÉ, T., JAMMES, H., AINSCOUGH, J. F. X., SURANI, M. A., JOURNOT, L. & DANDOLO, L. 2009. H19 acts as a trans regulator of the imprinted gene network controlling growth in mice. *Development,* 136**,** 3413-3421.

GABORY, A., FERRY, L., FAJARDY, I., JOUNEAU, L., GOTHIÉ, J.-D., VIGÉ, A., FLEUR, C., MAYEUR, S., GALLOU-KABANI, C., GROSS, M.-S., ATTIG, L., VAMBERGUE, A., LESAGE, J., REUSENS, B., VIEAU, D., REMACLE, C., JAIS, J.-P. & JUNIEN, C. 2012. Maternal Diets Trigger Sex-Specific Divergent Trajectories of Gene Expression and Epigenetic Systems in Mouse Placenta. *PLoS ONE,* 7**,** e47986.

GAGNON, R. 2003. Placental insufficiency and its consequences. *European Journal of Obstetrics & Gynecology and Reproductive Biology,* 110, Supplement**,** S99-S107.

GALLOU-KABANI, C., GABORY, A., TOST, J., KARIMI, M., MAYEUR, S., LESAGE, J., BOUDADI, E., GROSS, M.-S., TAURELLE, J., VIGÉ, A., BRETON, C., REUSENS, B., REMACLE, C., VIEAU, D., EKSTRÖM, T. J., JAIS, J.-P. & JUNIEN, C. 2010. Sex- and diet-specific changes of imprinted gene expression and DNA methylation in mouse placenta under a high-fat diet. *PLOS ONE,* 5**,** e14398.

GEORGES, M., CHARLIER, C., SMIT, M., DAVIS, E., SHAY, T., TORDOIR, X., TAKEDA, H., CAIMENT, F. & COCKETT, N. 2004. Toward molecular understanding of polar overdominance at the ovine callipyge locus. *Cold Spring Harbor Symposia on Quantitative Biology,* 69**,** 477-484.

GEORGIADIS, L., KESKI-NISULA, L., HARJU, M., RÄISÄNEN, S., GEORGIADIS, S., HANNILA, M. L. & HEINONEN, S. 2014. Umbilical cord length in singleton gestations: a Finnish population-based retrospective register study. *Placenta,* 35**,** 275-280.

GILBERT, J. S. & NIJLAND, M. J. 2008. Sex differences in the developmental origins of hypertension and cardiorenal disease. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology,* 295**,** R1941-R1952.

GLUCKMAN, P. D., HANSON, M. A., COOPER, C. & THORNBURG, K. L. 2008. Effect of in utero and early-life conditions on adult health and disease. *The New England Journal of Medicine,* 359**,** 61-73.

GODFREY, K. & BARKER, D. J. P. 2000. Fetal nutrition and adult disease. *The American Journal of Clinical Nutrition,* 71 (suppl)**,** 1344S-52S.

GONDRET, F., LEFAUCHEUR, L., JUIN, H., LOUVEAU, I. & LEBRET, B. 2006. Low birth weight is associated with enlarged muscle fiber area and impaired meat tenderness of the longissimus muscle in pigs12. *Journal of Animal Science,* 84.

GREENWOOD, P. L., HUNT, A. S., HERMANSON, J. W. & BELL, A. W. 1998. Effects of birth weight and postnatal nutrition on neonatal sheep: I. Body growth and composition, and some aspects of energetic efficiency. *Journal of Animal Science,* 76.

GREENWOOD, P. L., SLEPETIS, R. M. & BELL, A. W. 2000. Influences on fetal and placental weights during mid to late gestation in prolific ewes well nourished throughout pregnancy. *Reproduction, Fertility and Development,* 12**,** 149-156.

GREENWOOD, P. L., CAFE, L. M., HEARNSHAW, H., HENNESSY, D. W., THOMPSON, J. M. & MORRIS, S. G. 2006. Long-term consequences of birth weight and growth to weaning on carcass, yield and beef quality characteristics of Piedmontese- and Wagyu-sired cattle. *Australian Journal of Experimental Agriculture,* 46**,** 257-269.

GREGORY, K. E., SMITH, G. M., CUNDIFF, L. V., KOCH, R. M. & LASTER, D. B. 1979. Characterization of biological types of cattle-cycle III: I. Birth and weaning traits. *Journal of Animal Science,* 48**,** 271-279.

GUIDICE, L. C., DE ZEGHER, F., GARGOSKY, S. E., DSUPIN, B. A., DE LAS FUENTES, L., CRYSTAL, R. A., HINTZ, R. L. & ROSENFELD, R. G. 1995. Insulin-like growth factors and their binding proteins in the term and preterm human fetus and neonate with normal and extremes of intrauterine growth. *The Journal of Clinical Endocrinology & Metabolism,* 80**,** 1548-1555.

GUILLOMOT, M. & GUAY, P. 1982. Ultrastructural features of the cell surfaces of uterine and trophoblastic epithelia during embryo attachment in the cow. *The Anatomical Record,* 204**,** 315-322.

GUNDLING, W. E. & WILDMAN, D. E. 2015. A review of inter- and intraspecific variation in the eutherian placenta. *Philosophical Transactions of the Royal Society B: Biological Sciences,* 370.

HAN, V. K., BASSETT, N., WALTON, J. & CHALLIS, J. R. 1996. The expression of insulin-like growth factor (IGF) and IGF-binding protein (IGFBP) genes in the human placenta and membranes: evidence for IGF-IGFBP interactions at the feto-maternal interface. *The Journal of Clinical Endocrinology & Metabolism,* 81**,** 2680-2693.

HANSON, M. A. & GLUCKMAN, P. D. 2015. Developmental origins of health and disease – Global public health implications. *Best Practice & Research Clinical Obstetrics & Gynaecology,* 29**,** 24-31.

HARRIS, L. K., CROCKER, I. P., BAKER, P. N., APLIN, J. D. & WESTWOOD, M. 2011. IGF2 actions on trophoblast in human placenta are regulated by the insulin-like growth factor 2 receptor, which can function as both a signaling and clearance receptor. *Biology of Reproduction,* 84**,** 440-446.

HERNANDEZ-MEDRANO, J. H., COPPING, K. J., HOARE, A., WAPANAAR, W., GRIVELL, R. G., KUCHEL, T., MIGUEL-PACHECO, G., MCMILLEN, I. C., RODGERS, R. J. & PERRY, V. E. A. 2015. Gestational dietary protein is associated with sex specific decrease in blood flow, fetal heart growth and post-natal blood pressure of progeny. *PLOS ONE,* 10**,** e0125694.

HIENDLEDER, S., LEWALSKI, H. & JANKE, A. 2008. Complete mitochondrial genomes of *Bos taurus* and *Bos indicus* provide new insights into intra-species variation, taxonomy and domestication. *Cytogenetic and Genome Research,* 120**,** 150-156.

HOBBINS, J. C. 2008. Intrauterine growth restriction. *Obstetric Ultrasound: Artistry in Practice.* USA: Blackwell Publishing Inc.

HOCHHOLDINGER, F. & HOECKER, N. 2007. Towards the molecular basis of heterosis. *Trends in Plant Science,* 12**,** 427-432.

IGWEBUIKE, U. M. 2006. Trophoblast cells of ruminant placentas—a minireview. *Animal Reproduction Science,* 93**,** 185-198.

INIGUEZ, G., GONZALEZ, C. A., ARGANDONA, F., KAKARIEKA, E., JOHNSON, M. C. & CASSORLA, F. 2010. Expression and protein content of IGF-I and IGF-1 receptor in placentas from small, adequate, and large for gestational age newborns. *Hormone Research in Paediatrics,* 73**,** 320-327.

JOHNSON, J. K., GOPALAKRISHAN, K., JOSEPH, A. & LINDOW, S. W. 2003. Cord blood gamma glutamyl transferase activity in complicated pregnancies. *Journal of Obstetrics & Gynaecology,* 23 Supplement 1**,** S32-S74.

JONES, J. I. & CLEMMONS, D. R. 1995. Insulin-like growth factors and their binding proteins: biological actions. *Endocrine Reviews,* 16**,** 3-34.

KAUFMANN, P., MAYHEW, T. M. & CHARNOCK-JONES, D. S. 2004. Aspects of human fetoplacental vasculogenesis and angiogenesis. II. changes during normal pregnancy. *Placenta,* 25**,** 114-126.

KENIRY, A., OXLEY, D., MONNIER, P., KYBA, M., DANDOLO, L., SMITS, G. & REIK, W. 2012. The H19 lincRNA is a developmental reservoir of miR-675 that suppresses growth and Igf1r. *Nature Cell Biology,* 14**,** 659-665.

KIM, K.-S., KIM, J.-J., DEKKERS, J. M. & ROTHSCHILD, M. 2004. Polar overdominant inheritance of a DLK1 polymorphism is associated with growth and fatness in pigs. *Mammalian Genome,* 15**,** 552-559.

KING, G. J., ATKINSON, B. A. & ROBERTSON, H. A. 1979. Development of the bovine placentome during the second month of gestation. *Journal of Reproduction and Fertility,* 55**,** 173-180.

KING, G. J., ATKINSON, B. A. & ROBERTSON, H. A. 1980. Development of the bovine placentome from Days 20 to 29 of gestation. *Journal of Reproduction and Fertility,* 59**,** 95-100.

KINGDOM, J. C. P. & KAUFMANN, P. 1997. Oxygen and placental villous development: Origins of fetal hypoxia. *Placenta,* 18**,** 613-621.

KLISCH, K., HECHT, W., PFARRER, C., SCHULER, G., HOFFMANN, B. & LEISER, R. 1999a. DNA content and ploidy level of bovine placentomal trophoblast giant cells. *Placenta,* 20**,** 451-458.

KLISCH, K., PFARRER, C., SCHULER, G., HOFFMANN, B. & LEISER, R. 1999b. Tripolar acytokinetic mitosis and formation of feto-maternal syncytia in the bovine placentome: different modes of the generation of multinuclear cells. *Anatomy and Embryology,* 200**,** 229-237.

KLISCH, K., THOMSEN, P. D., DANTZER, V. & LEISER, R. 2004. Genome multiplication is a generalised phenomenon in placentomal and interplacentomal trophoblast giant cells in cattle. *Reproduction, Fertility and Development,* 16**,** 301-306.

KOGER, M. 1980. Efective crossbreeding systems utilizing Zebu cattle. *Journal of Animal Science,* 50.

KUMAR, N., LEVERENCE, J., BICK, D. & SAMPATH, V. 2012. Ontogeny of growth-regulating genes in the placenta. *Placenta,* 33**,** 94-99.

LARCIPRETE, G., VALENSISE, H., DI PIERRO, G., VASAPOLLO, B., CASALINO, B., ARDUINI, D., JARVIS, S. & CIRESE, E. 2005. Intrauterine growth restriction and fetal body composition. *Ultrasound in Obstetrics and Gynecology,* 26**,** 258-262.

LATOS, P. A., PAULER, F. M., KOERNER, M. V., ŞENERGIN, H. B., HUDSON, Q. J., STOCSITS, R. R., ALLHOFF, W., STRICKER, S. H., KLEMENT, R. M., WARCZOK, K. E., AUMAYR, K., PASIERBEK, P. & BARLOW, D. P. 2012. Airn

transcriptional overlap, but not its lncRNA products, induces imprinted Igf2r silencing. *Science,* 338**,** 1469-1472.

LAU, M. M., STEWART, C. E., LIU, Z., BHATT, H., ROTWEIN, P. & STEWART, C. L. 1994. Loss of the imprinted IGF2/cation-independent mannose 6-phosphate receptor results in fetal overgrowth and perinatal lethality. *Genes & Development,* 8**,** 2953-2963.

LAVEN, R. A. & PETERS, A. R. 2001. Gross morphometry of the bovine placentome during gestation. *Reproduction in Domestic Animals,* 36**,** 289-296.

LEISER, R., KREBS, C., KLISCH, K., EBERT, B., DANTZER, V., SCHULER, G. & HOFFMANN, B. 1997. Fetal villosity and microvasculature of the bovine placentome in the second half of gestation. *Journal of Anatomy,* 191 517-527.

LEISER, R., PFARRER, C., ABD-ELNAEIM, M. & DANTZER, V. 1998. Feto-maternal anchorage in epitheliochorial and endotheliochorial placental types studied by histology and microvascular corrosion casts. *Placenta,* 12 Supplement Trophoblast Research**,** 21-39.

LEWIS, R. M., CLEAL, J. K. & GODFREY, K. M. 2011. Chapter 78 - The placental role in fetal programming. *In:* RAMESH, C. G. (ed.) *Reproductive and Developmental Toxicology.* San Diego: Academic Press.

LIPPMAN, Z. B. & ZAMIR, D. 2007. Heterosis: revisiting the magic. *Trends in Genetics,* 23**,** 60-66.

LIU, B., CUI, Y., YANG, B., FAN, J., ZHAO, Z. & YU, S. 2010. Morphometric analysis of yak placentomes during gestation. *The Anatomical Record: Advances in Integrative Anatomy and Evolutionary Biology,* 293**,** 1873-1879.

LONGTINE, M. S. & NELSON, D. M. 2011. Placental dysfunction and fetal programming: The importance of placental size, shape, histopathology, and molecular composition. *Seminars in reproductive medicine,* 29**,** 187-196.

LUBCHENCO, L. O., HANSMAN, C., DRESSLER, M. & BOYD, E. 1963. Intrauterine growth as estimated from liveborn birth-weigth data at 24 to 42 weeks of gestation. *Pediatrics,* 32**,** 793-800.

LUDWIG, T., EGGENSCHWILER, J., FISHER, P., D'ERCOLE, A. J., DAVENPORT, M. L. & EFSTRATIADIS, A. 1996. Mouse mutants lacking the Type 2 IGF receptor (IGF2R) are rescued from perinatal lethality in Igf2 and Igf1r null backgrounds. *Developmental Biology,* 177**,** 517-535.

MACARA, L., KINGDOM, J. C. P., KAUFMANN, P., KOHNEN, G., HAIR, J., MORE, I. A. R., LYALL, F. & GREER, I. A. 1996. Structural analysis of placental terminal villi from growth-restricted pregnancies with abnormal umbilical artery doppler waveforms. *Placenta,* 17**,** 37-48.

MACDORMAN, M. F. & GREGORY, E. C. W. 2013. Fetal and perinatal mortality: United States, 2013. *National Vital Statistics Report,* 64**,** 1-23.

MACLAUGHLIN, S. M., WALKER, S. K., ROBERTS, C. T., KLEEMANN, D. O. & MCMILLEN, I. C. 2005. Periconceptional nutrition and the relationship between maternal body weight changes in the periconceptional period and feto-placental growth in the sheep. *The Journal of Physiology,* 565**,** 111-124.

MAO, J., ZHANG, X., SIELI, P. T., FALDUTO, M. T., TORRES, K. E. & ROSENFELD, C. S. 2010. Contrasting effects of different maternal diets on sexually dimorphic gene expression in the murine placenta. *Proceedings of the National Academy of Sciences,* 107**,** 5557-5562.

MARCONI, A. M. & BATTAGLIA, F. C. 2012. Intrauterine growth restriction: obstetric aspects. *In:* BUONOCORE, G., BRACCI, R. & WEINDLING, M. (eds.) *Neonatology.* Springer Milan.

MARCONI, A. M., PAOLINI, C. L., ZERBE, G. & BATTAGLIA, F. C. 2006. Lactacidemia in intrauterine growth restricted (IUGR) pregnancies: Relationship to clinical severity, oxygenation and placental weight. *Pediatric Research,* 59**,** 570-574.

MARTIN, R. D. 2003. Human reproduction: a comparative background for medical hypotheses. *Journal of Reproductive Immunology,* 59**,** 111-135.

MARTYN, C. N., BARKER, D. J. P. & OSMOND, C. 1996. Mothers' pelvic size, fetal growth, and death from stroke and coronary heart disease in men in the UK. *The Lancet,* 348**,** 1264-1268.

MAYHEW, T. M. 2009. A stereological perspective on placental morphology in normal and complicated pregnancies. *Journal of Anatomy,* 215**,** 77-90.

MAYHEW, T. M., JENKINS, H., TODD, B. & CLIFTON, V. L. 2008. Maternal asthma and placental morphometry: effects of severity, treatment and fetal sex. *Placenta,* 29**,** 366-373.

MAYHEW, T. M., WADROP, E. & SIMPSON, R. A. 1994. Proliferative versus hypertrophic growth in tissue subcompartments of human placental villi during gestation. *Journal of Anatomy,* 184**,** 535-543.

MCKINNON, T., CHAKRABORTY, C., GLEESON, L. M., CHIDIAC, P. & LALA, P. K. 2001. Stimulation of human extravillous trophoblast migration by IGF-II is mediated by IGF Type 2 receptor involving inhibitory G protein(s) and phosphorylation of MAPK. *The Journal of Clinical Endocrinology & Metabolism,* 86**,** 3665-3674.

MELAMED, N., YOGEV, Y. & GLEZERMAN, M. 2010. Fetal gender and pregnancy outcome. *The Journal of Maternal-Fetal & Neonatal Medicine,* 23**,** 338-344.

MELAMED NIR, MEIZNER ISRAEL, MASHIACH REUVEN, WIZNITZER ARNON, GLEZERMAN MAREK & YARIV, Y. 2013. Fetal sex and intrauterine growth patterns. *Journal of Ultrasound in Medicine,* 32**,** 35-43.

MELTON, A. A., BERRY, R. O. & BUTLER, O. D. 1951. The interval between the time of ovulation and attachment of the bovine embryo. *Journal of Animal Science,* 10**,** 993-1005.

MILES, J. R., FARIN, C. E., RODRIGUEZ, K. F., ALEXANDER, J. E. & FARIN, P. W. 2004. Angiogenesis and morphometry of bovine placentas in late gestation from embryos produced in vivo or in vitro. *Biology of Reproduction,* 71**,** 1919-1926.

MILES, J. R., FARIN, C. E., RODRIGUEZ, K. F., ALEXANDER, J. E. & FARIN, P. W. 2005. Effects of embryo culture on angiogenesis and morphometry of bovine placentas during early gestation. *Biology of Reproduction,* 73**,** 663-671.

MONK, D. 2015. Genomic imprinting in the human placenta. *American Journal of Obstetrics and Gynecology,* 213**,** S152-S162.

MOORE, T. & HAIG, D. 1991. Genomic imprinting in mammalian development: a parental tug-of-war. *Trends in Genetics,* 7**,** 45-49.

MOORE, K., KRAMER, J. M., RODRIGUEZ-SALLABERRY, C. J., YELICH, J. V. & DROST, M. 2007. Insulin-like growth factor (IGF) family genes are aberrantly expressed in bovine conceptuses produced in vitro or by nuclear transfer. *Theriogenology,* 68**,** 717-727.

MOORE, G. E., ISHIDA, M., DEMETRIOU, C., AL-OLABI, L., LEON, L. J., THOMAS, A. C., ABU-AMERO, S., FROST, J. M., STAFFORD, J. L., CHAOQUN, Y., DUNCAN, A. J., BAIGEL, R., BRIMIOULLE, M., IGLESIAS-PLATAS, I., APOSTOLIDOU, S., AGGARWAL, R., WHITTAKER, J. C., SYNGELAKI, A., NICOLAIDES, K. H., REGAN, L., MONK, D. & STAINER, P. 2015. The role and interaction of imprinted genes in human fetal growth. *Philosophical Transactions B,* 370**,** 20140074.

MORRISON, J. L. 2008. Sheep models of intrauterine growth restriction: fetal adaptations and consequences. *Clinical & Experimental Pharmacology & Physiology,* 35**,** 730-743.

MURPHY, V. E., GIBSON, P. G., GILES, W. B., ZAKAR, T., SMITH, R., BISITS, A. M., KESSELL, C. G. & CLIFTON, V. L. 2003. Maternal asthma is associated with reduced female fetal growth. *American Journal of Respiratory and Critical Care Medicine,* 168**,** 1317-1323.

MYERS, J. 1978. Sex ratio adjustment under food stress: maximization of quality or numbers of offspring? *The American Naturalist,* 112**,** 381-388.

NIETO-DIAZ, A., VILLAR, J., MATORRAS-WEINIG, R. & VALENZUELA-RUIZ, P. 1996. Intrauterine growth retardation at term: association between anthropometric and endocrine parameters. *Acta Obstetricia et Gynecologica Scandinavica,* 75**,** 127-131.

OSEI-KUMAH, A., SMITH, R., JURISICA, I., CANIGGIA, I. & CLIFTON, V. L. 2011. Sex-specific differences in placental global gene expression in pregnancies complicated by asthma. *Placenta,* 32**,** 570-578.

OSGERBY, J., WATHES, D., HOWARD, D. & GADD, T. 2004. The effect of maternal undernutrition on the placental growth trajectory and the uterine insulin-like growth factor axis in the pregnant ewe. *Journal of Endocrinology,* 182**,** 89-103.

OTT, W. J. & DOYLE, S. 1982. Normal ultrasonic fetal weight curve. *Obstetrics & Gynecology,* 59**,** 603-606.

OWENS, J. A., THAVANESWARAN, P., DE BLASIO, M. J., MCMILLEN, I. C., ROBINSON, J. S. & GATFORD, K. L. 2007. Sex-specific effects of placental restriction on components of the metabolic syndrome in young adult sheep. *American Journal of Physiology, Endocrinology, Metabolism,* 292**,** E1879-E1889.

PARDI, G., CETIN, I., MARCONI, A. M., LANFRANCHI, A., BOZZETTI, P., FARRAZZI, E., BUSCAGLIA, M. & BATTAGLIA, F. C. 1993. Diagnostic value of blood sampling in fetuses with growth retardation. *New England Journal of Medicine,* 328**,** 692-696.

PASCHAL, J. C., SANDERS, J. O. & KERR, J. L. 1991. Calving and weaning characteristics of Angus-, Gray Brahman-, Gir-, Indu-Brazil-, Nellore-, and Red Brahman-sired F1 calves. *Journal of Animal Science,* 69**,** 2395-2402.

PETERSEN, S., GOTFREDSEN, A. & KNUDSEN, F. U. 1988. Lean body mass in small for gestational age and appropriate for gestational age infants. *The Journal of Pediatrics,* 113**,** 886-889.

PLATZ, E. & NEWMAN, R. 2008. Diagnosis of IUGR: traditional biometry. *Seminars in Perinatology,* 32**,** 140-147.e

POLEJAEVA, I. A., RUTIGLIANO, H. M. & WELLS, K. D. 2016. Livestock in biomedical research: history, current status, and future prospective. *Reproduction, Fertility and Development,* 28**,** 112-124.

PREFUMO, F., VENTURINI, P. L. & DE BIASIO, P. 2003. Effect of fetal gender on first-trimester ductus venosus blood flow. *Ultrasound in Obstetrics and Gynecology,* 22**,** 268-270.

PRIOR, R. L. & LASTER, D. B. 1979. Development of the bovine fetus. *Journal of Animal Science,* 48**,** 1546-1553.

PRIOR, T., WILD, M., MULLINS, E., BENNETT, P. & KUMAR, S. 2013. Sex specific differences in fetal middle cerebral artery and umbilical venous doppler. *PLOS ONE,* 8**,** e56933.

RACHMILEWITZ, J., GILEADI, O., ELDAR-GEVA, T., SCHNEIDER, T., DE-GROOT, N. & HOCHBERG, A. 1992. Transcription of the H19 gene in differentiating cytotrophoblasts from human placenta. *Molecular Reproduction and Development,* 32**,** 196-202.

RAVELICH, S. R., BREIER, B. H., REDDY, S., KEELAN, J. A., WELLS, D. N., PETERSON, A. J. & LEE, R. S. F. 2004. Insulin-Like Growth Factor-I and Binding Proteins 1, 2, and 3 in Bovine Nuclear Transfer Pregnancies. *Biology of Reproduction,* 70**,** 430-438.

REIK, W. & WALTER, J. 2001. Genomic imprinting: parental influence on the genome. *Nat Rev Genet,* 2**,** 21 - 32.

REYNOLDS, L. P., BOROWICZ, P. P., VONNAHME, K. A., JOHNSON, M. L., GRAZUL-BILSKA, A. T., WALLACE, J. M., CATON, J. S. & REDMER, D. A. 2005a. Animal models of placental angiogenesis. *Placenta,* 26**,** 689-708.

REYNOLDS, L. P., BOROWICZ, P. P., VONNAHME, K. A., JOHNSON, M. L., GRAZUL-BILSKA, A. T., REDMER, D. A. & CATON, J. S. 2005b. Placental angiogenesis in sheep models of compromised pregnancy. *The Journal of Physiology,* 565**,** 43-58.

REYNOLDS, T. S., STEVENSON, K. M. & WATHES, D. C. 1997. Pregnancy-specific alterations in the expression of the insulin-like growth factor system during early placental development in the ewe. *Endocrinology,* 138**,** 886-897.

REYNOLDS, W. L., DEROUEN, T. M., MOIN, S. & KOONCE, K. L. 1980. Factors influencing gestation length, birth weight and calf survival of Angus, Zebu and Zebu cross beef cattle. *Journal of Animal Science,* 51**,** 860-867.

ROBERTS, C. T., OWENS, J. A. & SFERRUZZI-PERRI, A. N. 2008. Distinct actions of insulin-like growth factors (IGFs) on placental development and fetal growth: lessons from mice and guinea pigs. *Placenta,* 29**,** S42-S47.

ROBERTS, C. T., SOHLSTROM, A., KIND, K. L., EARL, R. A., KHONG, T. Y., ROBINSON, J. S., OWENS, P. C. & OWENS, J. A. 2001. Maternal food restriction reduces the exchange surface area and increases the barrier thickness of the placenta in the guinea-pig. *Placenta,* 22**,** 177-185.

ROBERTS, S. J. 1971. *Veterinary Obstetrics and Genital Diseases,* NY USA, Published by the Author.

ROBINSON, R., MANN, G., GADD, T., LAMMING, G. & WATHES, D. 2000. The expression of the IGF system in the bovine uterus throughout the oestrous cycle and early pregnancy. *Journal of Endocrinology,* 165**,** 231-243.

ROLAND, M. C. P., FRIIS, C. M., VOLDNER, N., GODANG, K., BOLLERSLEV, J., HAUGEN, G. & HENRIKSEN, T. 2012. Fetal growth versus birthweight: The role of placenta versus other determinants. *PLOS ONE,* 7**,** e39324.

ROSENBERG, A. 2008. The IUGR newborn. *Seminars in Perinatology,* 32**,** 219-224.

RURAK, D. 2001. Development and function of the placenta. *In:* HARDING, R. & BOCKING, A. (eds.) *Fetal Growth and Development.* UK: Cambridge University Press.

SALAFIA, C. M., ZHANG, J., CHARLES, A. K., BRESNAHAN, M., SHROUT, P., SUN, W. & MAAS, E. M. 2008. Placental characteristics and birthweight. *Paediatric and Perinatal Epidemiology,* 22**,** 229-239.

SCHMIDT, S., GERBER, D., SOLEY, J. T., AIRE, T. A. & BOOS, A. 2006. Histo-morphology of the uterus and early placenta of the African Buffalo (*Syncerus caffer*) and comparative placentome morphology of the African Buffalo and cattle (*Bos taurus*). *Placenta,* 27**,** 899-911.

SCHNEIDER, H. 1996. Ontogenic changes in the nutritive function of the placenta. *Placenta,* 17**,** 15-26.

SHARMA, R. K., BLAIR, H. T., JENKINSON, C. M. C., KENYON, P. R., COCKREM, J. F. & PARKINSON, T. J. 2012. Uterine environment as a regulator of birth weight and body dimensions of newborn lambs. *Journal of Animal Science,* 90**,** 1338-1348.

SILVA, L. A. & GINTHER, O. J. 2010. Local effect of the conceptus on uterine vascular perfusion during early pregnancy in heifers. *Reproduction,* 139**,** 453-463.

SONG, S. 2012. Does famine influence sex ratio at birth? Evidence from the 1959–1961 Great Leap Forward Famine in China. *Proceedings of the Royal Society of London B: Biological Sciences*.

SOOD, R., ZEHNDER, J. L., DRUZIN, M. L. & BROWN, P. O. 2006. Gene expression patterns in human placenta. *Proceedings of the National Academy of Sciences of the United States of America,* 103**,** 5478-5483.

SPRINGER, N. M. & STUPAR, R. M. 2007. Allelic variation and heterosis in maize: How do two halves make more than a whole? *Genome Research,* 17**,** 264-275.

STEVENSON, D. K., VERTER, J., FANAROFF, A. A., OH, W., EHRENKRANZ, R. A., SHANKARAN, S., DONOVAN, E. F., WRIGHT, L. L., LEMONS, J. A., TYSON, J. E., KORONES, S. B., BAUER, C. R., STOLL, B. J. & PAPILE, L.-A. 2000. Sex differences in outcomes of very low birthweight infants: the newborn male disadvantage. *Archives of Disease in Childhood - Fetal and Neonatal Edition,* 83**,** F182-F185.

STEYN, C., HAWKINS, P., SAITO, T., NOAKES, D. E., KINGDOM, J. C. P. & HANSON, M. A. 2001. Undernutrition during the first half of gestation increases the predominance of fetal tissue in late-gestation ovine placentomes. *European Journal of Obstetrics & Gynecology and Reproductive Biology,* 98**,** 165-170.

STICKLAND, N. C. & PURTON, M. D. 1977. A quantitative evaluation of placentome development in Zebu cattle (Bos indicus). *Anatomia, histologia, embryologia,* 6**,** 81-86.

SULLIVAN, T. M., MICKE, G. C., MAGALHAES, R. S., PHILLIPS, N. J. & PERRY, V. E. A. 2009. Dietary protein during gestation affects placental development in heifers. *Theriogenology,* 72**,** 427-438.

TELUGU, B. P. & GREEN, J. A. 2008. Comparative Placentation. *In:* SCHATTEN, H. & CONSTANTINESCU, G. M. (eds.) *Comparative Reproductive Biology.* Oxford, UK.: Blackwell Publishing Ltd.

THOMSON, A. M., BILLEWICZ, W. Z. & HYTTEN, F. E. 1969. The weight of the placenta in relation to birthweight. *British Journal of Obstetrics & Gynaecology,* 76**,** 865-872.

THORPE-BEESTON, J. G., NICOLAIDES, K. H., SNIJDERS, R. J., FELTON, C. V. & MCGREGOR, A. M. 1991. Thyroid function in small for gestational age fetuses. *Obstet Gynecol,* 77**,** 701.

THRIFT, F. A. 1997. Reproductive performance of cows mated to and preweaning performance of calves sired by Brahman vs alternative subtropically adapted breeds. *Journal of Animal Science,* 75**,** 2597-603.

VAN ABEELEN, A. F. M., DE ROOIJ, S. R., OSMOND, C., PAINTER, R. C., VEENENDAAL, M. V. E., BOSSUYT, P. M. M., ELIAS, S. G., GROBBEE, D. E., VAN DER SCHOUW, Y. T., BARKER, D. J. P. & ROSEBOOM, T. J. 2011. The sex-specific effects of famine on the association between placental size and later hypertension. *Placenta,* 32**,** 694-698.

VATNICK, I., SCHOKNECHT, P. A., DARRIGRAND, R. & BELL, A. W. 1991. Growth and metabolism of the placenta after unilateral fetectomy in twin pregnant ewes. *Journal of Developmental Physiology,* 15**,** 351-356.

VATTEN, L. J. & SKJÆRVEN, R. 2004. Offspring sex and pregnancy outcome by length of gestation. *Early Human Development,* 76**,** 47-54.

VERBURG, B. O., JADDOE, V. W. V., WLADIMIROFF, J. W., HOFMAN, A., WITTEMAN, J. C. M. & STEEGERS, E. A. P. 2008. Fetal hemodynamic adaptive changes related to intrauterine growth: the Generation R study. *Circulation,* 117**,** 649-659.

VERONESI, M. C., VILLANI, M., WILSHER, S., CONTRI, A. & CARLUCCIO, A. 2010. A comparative stereological study of the term placenta in the donkey, pony and Thoroughbred. *Theriogenology,* 74**,** 627-631.

VONNAHME, K. A., ZHU, M. J., BOROWICZ, P. P., GEARY, T. W., HESS, B. W., REYNOLDS, L. P., CATON, J. S., MEANS, W. J. & FORD, S. P. 2007. Effect of early gestational undernutrition on angiogenic factor expression and vascularity in the bovine placentome. *Journal of Animal Science,* 85**,** 2464-2472.

VONNAHME, K. A., ARNDT, W. J., JOHNSON, M. L., BOROWICZ, P. P. & REYNOLDS, L. P. 2008. Effect of morphology on placentome size, vascularity, and vasoreactivity in late pregnant sheep. *Biology of Reproduction,* 79**,** 976-982.

VUGUIN, P. M. 2007. Animal models for small for gestational age and fetal programing of adult disease. *Hormone Research in Paediatrics,* 68**,** 113-123.

WAGNER, P. K. & CHRISTIANS, J. K. 2010. Altered placental expression of PAPPA2 does not affect birth weight in mice. *Reproductive Biology and Endocrinology,* 8**,** 1-8.

WANGO, E. O., WOODING, F. B. P. & HEAP, R. B. 1990. The role of trophoblast binucleate cells in implantation in the goat: A quantatitive study. *Placenta,* 11**,** 381-394.

WARD, J. W., FORHEAD, A. J., WOODING, F. B. P. & FOWDEN, A. L. 2006. Functional significance and cortisol dependence of the gross morphology of ovine placentomes during late gestation. *Biology of Reproduction,* 74**,** 137-145.

WATHES, D. C., REYNOLDS, T. S., ROBINSON, R. S. & STEVENSON, K. R. 1998. Role of the insulin-like growth factor system in uterine function and placental development in ruminants. *Journal of Dairy Science,* 81**,** 1778-1789.

WERMTER, A. K., SCHERAG, A., D., M., REICHWALD, K., DURAND, E., NGUYEN, T. T., KOBERWITZ, K., LICHTNER, P., MEITINGER, T., SCHAFER, H., HINNEY, A., FROGUEL, P., HEBEBRAND, J. & BRONNER, G. 2008. Preferential reciprocal transfer of paternal/maternal DLK1 alleles to obese children: first evidence of polor overdominance in humans. *European Journal of Human Genetics,* 16**,** 1126-34.

WILKIN, F., PAQUETTE, J., LEDRU, E., MAMELIN, C., POLLAK, M. & DEAL, C. L. 2000. H19 sense and antisense transgenes modify insulin-like growth factor-II mRNA levels. *European Journal of Biochemistry,* 267**,** 4020-4027.

WOLF, J. B., CHEVERUD, J. M., ROSEMAN, C. & HAGER, R. 2008. Genome-wide analysis reveals a complex pattern of genomic imprinting in mice. *PLoS Genet,* 4**,** e1000091.

WOODING, F. B. & WATHES, D. C. 1980. Binucleate cell migration in the bovine placentome. *Journal of Reproduction and Fertility,* 59**,** 425.

WOODING, F. B. P. & BURTON, G. 2008a. Implantation, maternofetal exchange and vascular relationships. *In:* WOODING, F. B. P. & BURTON, G. (eds.) *Comparative Placentation.* Berlin, Hiedelberg: Springer-Verlag.

WOODING, F. B. P. & BURTON, G. 2008b. Placentation fundamentals. *In:* WOODING, F. B. P. & BURTON, G. (eds.) *Comparative Placentation.* Berlin, Hiedelberg: Springer-Verlag.

WOODING, F. B. P. & BURTON, G. 2008c. Synepitheliochorial placentation: ruminants (ewe and cow). *In:* WOODING, F. B. P. & BURTON, G. (eds.) *Comparative Placentation.* Berlin, Hiedelberg: Springer-Verlag.

WOODS, L. L., INGELFINGER, J. R., NYENGAARD, J. R. & RASCH, R. 2001. Maternal protein restriction suppresses the newborn renin-angiotensin system and programs adult hypertension in rats. *Pediatric Research,* 49**,** 460-467.

WOODS, L. L., INGELFINGER, J. R. & RASCH, R. 2005. Modest maternal protein restriction fails to program adult hypertension in female rats. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology,* 289**,** R1131-R1136.

WU, G., BAZER, F. W., HU, J., JOHNSON, G. A. & SPENCER, T. E. 2005. Polyamine Synthesis from Proline in the Developing Porcine Placenta. *Biology of Reproduction,* 72**,** 842-850.

WU, G., BAZER, F. W., WALLACE, J. M. & SPENCER, T. E. 2006. Board-invited review: Intrauterine growth retardation: Implications for the animal sciences. *Journal of Animal Science,* 84.

XIANG, R., GHANIPOOR-SAMAMI, M., JOHNS, W. H., EINDORF, T., RUTLEY, D. L., KRUK, Z. A., FITZSIMMONS, C. J., THOMSEN, D. A., ROBERTS, C. T., BURNS, B. M., ANDERSON, G. I., GREENWOOD, P. L. & HIENDLEDER, S. 2013. Maternal and paternal genomes differentially affect myofibre characteristics and muscle weights of bovine fetuses at midgestation. *PLOS ONE,* 8**,** e53402.

XIANG, R., LEE, A. M. C., EINDORF, T., JAVADMANESH, A., GHANIPOOR-SAMAMI, M., GUGGER, M., FITZSIMMONS, C. J., KRUK, Z. A., PITCHFORD, W. S., LEVITON, A. J., THOMSEN, D. A., BECKMAN, I., ANDERSON, G. I., BURNS, B. M., RUTLEY, D. L., XIAN, C. J. & HIENDLEDER, S. 2014. Widespread differential maternal and paternal genome effects on fetal bone phenotype at mid-gestation. *Journal of Bone and Mineral Research,* 29**,** 2392–2404

YEUNG, L. P. K., WONG, A. C. K., WANG, X., BIRMINGHAM, C. L., LEWICKA, S. & CHANOINE, J.-P. 2003. Different relationship between anthropometric markers and umbilical cord plasma leptin in Asian and Caucasian neonates. *Pediatric Research,* 53**,** 1019-1024.

YOSHIMIZU, T., MIROGLIO, A., RIPOCHE, M.-A., GABORY, A., VERNUCCI, M., RICCIO, A., COLNOT, S., GODARD, C., TERRIS, B., JAMMES, H. & DANDOLO, L. 2008. The H19 locus acts in vivo as a tumor suppressor. *Proceedings of the National Academy of Sciences of the United States of America,* 105**,** 12417-12422.

ZARÉN, B., LINDMARK, G. & BAKKETEIG, L. 2000. Maternal smoking affects fetal growth more in the male fetus. *Paediatric and Perinatal Epidemiology,* 14**,** 118-126.

ZECHNER, U., HEMBERGER, M., CONSTÂNCIA, M., ORTH, A., DRAGATSIS, I., LÜTTGES, A., HAMEISTER, H. & FUNDELE, R. 2002. Proliferation and growth factor expression in abnormally enlarged placentas of mouse interspecific hybrids. *Developmental Dynamics,* 224**,** 125-134.

Chapter 2

Remodelling of the bovine placenta: Comprehensive morphological and histomorphological characterization of the late embryonic and early accelerated fetal growth stages

Manuscript in preparation for submission to the journal **Placenta**, with the exception that figures are embedded in the text and citations are formatted in Harvard style to be consistent with the other chapters.

Statement of Authorship

Title of Paper	Remodelling of the bovine placenta: Comprehensive morphological and histomorphological characterization of the late embryonic and early accelerated fetal growth stages
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Manuscript format for submission to the journal Placenta, except that figures and tables are embedded on the text, and bibliography and citation follows the Harvard style of referencing to maintain consistency with the other chapters.

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Name of Principal Author (Candidate)	Consuelo Amor S. Estrella
Contribution to the Paper	Performed all immunohistochemical and histomorphometric analysis of bovine placentomes, measured umbilical cord vessel diameters, measured umbilical cord weight with the assistance of MM, characterized the Day 48 placenta with assistance from AD, performed all other statistical analyses, interpreted data based on reviewed literature, wrote manuscript and formatted accompanying Figures and Tables.
Overall percentage (%)	80%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
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i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

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Remodelling of the bovine placenta: comprehensive morphological and histomorphological characterization of the late embryonic and early accelerated fetal growth stages

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Abstract

Introduction: Placental function impacts growth and development with lifelong consequences for performance and health. We provide novel insights on placental development in bovine, an important agricultural species and biomedical model.

Methods: Concepti with defined genetics and sex were recovered from nulliparous dams managed under standardized conditions to study placental gross morphological and histomorphological parameters in relation to embryo-fetal weight at the late embryo (Day48) and early accelerated fetal growth (Day153) stages.

Results: Placentome number increased 3-fold between Day48 and Day153. Placental barrier thickness was thinner, volume and surface areas and densities of cell types were higher at Day153 than Day48. We confirmed two placentome types, flat and convex. At Day48, convex placentomes were higher in number and had a lower proportion of maternal connective tissue ($P<0.01$) than flat placentomes. However, this was reversed at Day153, where convex placentomes were lower in number and had greater volume of placental cell types ($P<0.01$- $P<0.001$) and greater surface area ($P<0.001$). Importantly, embryo ($r = 0.50$) and fetal ($r = 0.30$) weight correlated with number of convex but not flat placentomes.

Discussion: Extensive remodelling of the placenta increases capacity for nutrient exchange to support rapidly increasing embryo-fetal weight from Day48 to Day153. The cellular composition of convex placentomes, and exclusive relationships between convex placentome number and embryo-fetal weight, provide strong evidence for these placentomes as major drivers of prenatal growth. The difference in proportion of maternal connective tissue between placentome types at Day48 suggests that this tissue plays a role in determining placentome shape.

Keywords: cattle, placentomes, gross morphology, histomorphometry

2.1 Introduction

Prenatal growth and development strongly influence lifelong performance, health, and disease (Fowden et al., 2008, Gluckman et al., 2008, Barker et al., 2012) and are commensurate with placental delivery of nutrients and oxygen (Burton and Fowden, 2015). It is therefore important to understand developmental changes in placental structures that can impact intrauterine growth. The domestic cow (*Bos taurus taurus*, *Bos taurus indicus*) has a very similar gestation period to human and carries a single conceptus with similar growth trajectory and maturity at birth (Martin, 2003, Dwyer, 2005, Ferrell, 1989, Xiang et al., 2013, Xiang et al., 2014). This, and the fact that *in vitro* fertilization and somatic cell nuclear transfer cloning have been routinely practiced for decades in this species, makes them valuable models for biomedical research that are increasingly used beyond reproductive biology (Constant et al., 2006, Bähr and Wolf, 2012, Hernandez-Medrano et al., 2015). While investigation of angiogenesis and trophoblast migration has been conducted at different time points of pregnancy (Pfarrer et al., 2006a, Pfarrer et al., 2006b, Leiser et al., 1997), currently available data describing the basic gross morphology of the bovine placenta are limited and often based on specimens without clear information on breed, fetal sex, dam parity or gestational age (Laven and Peters, 2001, Assis Neto et al., 2010). Furthermore, previous histomorphological descriptions of the development of placentomes in normal pregnancy did not include morphometric analysis of placental cell types, barrier thickness or surface area (Björkman, 1969, King et al., 1979).

The cotyledonary placenta of cattle is organized into 75-120 placentomes (Roberts, 1971) and composed of fetal cotyledonary tissue interdigitating with caruncular crypts of the maternal endometrium (Mossman, 1987). The number of placentomes initially increases with placental growth and development but remains constant from mid-gestation (Laven and Peters, 2001). Placentomes vary in shape in ruminant species (Schmidt et al., 2006, Liu et al., 2010), and include flat and convex placentome types in cattle (Laven and Peters, 2001) and

concave, flat and everted types in sheep (Vatnick et al., 1991). In sheep, early to mid-gestation nutrient restriction drives the change in placentome shape from type A (concave) towards type D (everted), without significantly decreasing total placental and fetal weights (Steyn et al., 2001). Therefore, placental weight alone is not sufficient to explain changes in placental efficiency and functional capacity, and cannot provide a complete understanding of the effects of factors such as nutrition on placental development (Reynolds et al., 2005).

The cotyledonary placenta is further classified histologically as synepitheliochorial, based on the presence of the feto-maternal syncytium, and the number of cell layers between the maternal and fetal circulations (Wooding, 1992). In the bovine placenta, both maternal and fetal tissue layers include an epithelium, connective tissue and capillaries (Wooding and Burton, 2008). Trophoblast cells constitute the fetal epithelium covering the chorionic villi penetrating the caruncular crypts (King et al., 1979), while connective tissue forms the villous core and provides support to the fetal vasculature (Ockleford and Wakely, 1982). Maturation of chorionic villi involves the formation of long, elaborate and vascularized villous trees and shrinkage of the fetal mesenchyme (Rurak, 2001). These histomorphological modifications are essential to achieve a large placental surface area for exchange.

In the present study we used concepti from designed experiments with well-defined genetics and sex, recovered from nulliparous dams managed under standardized conditions at Day 48 and Day 153 of gestation, to obtain baseline data and study remodelling of the cattle placenta from early to mid-gestation. Comparison of late embryonic and early accelerated fetal growth stages, after adjustment for effects of fetal sex and genetics, revealed important fundamental information on gross and histomorphological features and developmental changes of the bovine placenta. These findings include (i) magnitude of increase in placental weight and placentome number, (ii) changes in numbers of flat and convex placentome types, (iii) increase in placental surface density and area, and (iv) decrease in placental connective tissue and barrier thickness from early to mid-gestation. Our data not only demonstrate

alterations to increase efficiency in supporting fetal growth but also indicate that the convex placentome, the number of which correlates with embryo/fetal weight, differs from the flat placentome in numbers present and in the proportion of maternal connective tissue at Day 48 and volume of placental cell types and surface area at Day 153. These data suggest that maternal connective tissue plays an important role in determining the shape of the placentome and that convex placentomes are significant drivers of embryo/fetal growth.

2.2 Methods

2.2.1 Animals

All animals and procedures used in this study were approved by The University of Adelaide Animal Ethics Committee (No.S-094-2005). We used two experimental animal cohorts to examine key parameters of the bovine placenta at two critical time points of conceptus development (term, 277 – 291 days; Andersen and Plum, 1965); the late embryo stage (Day 48, n=60; Assis Neto et al., 2010) and the early accelerated fetal growth stage (Day 153, n=72; Ferrell, 1989). Both resources were generated using *Bos taurus taurus* (Angus, A) and *Bos taurus indicus* (Brahman, B) genetics and contained purebred and reciprocal cross concepti to span the variation found in major domestic cattle breeds (Hiendleder et al., 2008). Nulliparous dams at 16-20 months of age were purchased from farms in South Australia and Queensland and transferred to Struan Agricultural Centre, Naracoorte, South Australia. Standard estrous cycle synchronization procedures were performed following an adjustment period of 3-4 weeks after animal purchase, as described (Anand-Ivell et al., 2011). Pregnancies were established using four (two Angus and two Brahman) and five (three Angus and two Brahman) bulls for Day 48 and Day 153 cohorts, respectively. Gestational age was counted as days post conception assuming conception within 24 h after artificial insemination. Animals in each cohort were fed and managed as one group. Dam weights at conception and prior to sacrifice were recorded. Rare twin

pregnancies were excluded from the experiment. Distribution of male and female animals in each genetic group of each experimental cohort is shown in **Table S2.1.**

2.2.2 Collection of samples and phenotype data

2.2.2.1 Late embryonic stage (Day 48)

Pregnant animals were sacrificed under standardized conditions in an abattoir. Entire uteri were recovered and, after opening the gravid horn by longitudinal incision, the seven largest visible placentomes were removed for weighing and two placentomes close to the embryo were removed for histology and fixed in ice cold 4% paraformaldehyde and 2.5% PVP-40 PBS for 24 h. Placentomes were then washed four times with 1% PBS and stored in 70% ethanol until embedding in paraffin. The embryo was removed by cutting the umbilical cord above the branching vessels. A photographic image of the entire embryo was taken before the umbilical cord was recovered for preservation in RNAlater (Qiagen, Chadstone, Australia). The *Placenta fetalis*, consisting of fetal membranes with cotyledons attached, was removed from the uterus and after weighing, *Placenta fetalis* and uterus were stored separately in 10% buffered formalin. Fetal fluids were collected and measured in a cylinder. We confirmed a 1:1 w/v relationship in collected fetal fluid sample and converted the milliliters measured to grams. Placental efficiency was calculated as embryo weight divided by *Placenta fetalis* weight as caruncles with signs of interdigitation (see below) were very small and few in numbers at this developmental stage. Caruncles in the gravid and non-gravid horns were counted and visually examined using a magnifying glass. Most caruncles in the gravid horn displayed a rough surface after detachment of the *Placenta fetalis*, indicating interdigitation between maternal caruncle and fetal cotyledon for placentome formation had occurred. In contrast, all visible caruncles in the non-gravid horn, and some caruncles distal to the embryo in the gravid horn, were smooth and showed no sign of interdigitation and therefore were not included in further analyses. Caruncles in the pregnant horn with clear signs of interdigitation, representing the maternal component of the

placentome, could further be assigned either of two shapes, convex if they were mound shape or flat if they had a level surface. Some caruncles in the pregnant horn (11.7%) had irregular shape and/or appeared to have an uneven surface. While we saw two major placentome shapes, handling (separation of the cotyledon with the caruncle) and storage of the uteri may have an effect on unevenness of the caruncular surface. Umbilical cord length was measured based on photographic images of embryos using AnalySIS Five software (Soft Imaging System Corp., Lakewood CO, USA), while cord weight was recorded after blotting off excess RNAlater (Qiagen, Chadstone, Australia). The sex of Day 48 embryos was determined by detection of the SRY gene by PCR as described previously (Anand-Ivell et al., 2011).

2.2.2.2 Early accelerated fetal growth stage (Day 153)

Entire uteri were recovered from pregnant animals at mid-gestation in an abattoir and opened as described above. The largest placentome was removed, weighed and placed in a Petri dish with the chorionic plate facing up. A 5 mm-thick cross section of this placentome was excised from the center of the placentome with dual mounted scalpel blades and fixed in ice cold 4% paraformaldehyde and 2.5% PVP-40 PBS for 24 h. The section was then washed four times with 1% PBS and stored in 70% ethanol until embedding in paraffin. After lifting the fetus from the uterus, the umbilical cord was cut immediately above the branching vessels, and fetal weight, cord weight and length were recorded. Fetal fluids were weighed and uteri with attached placentae vacuum-packed and frozen at –22 °C. After thawing, the *Placenta fetalis* was carefully detached from maternal caruncles and weighed. Caruncles associated with cotyledons in each horn were counted, cut off, weighed, inspected, and cross-sectioned to assign placentome shape as flat or convex. Placentome shape was classified as convex if the caruncle was mound shaped and at this developmental stage had the outer rim curled up. Flat placentomes, on the other hand, had an even and level surface. The *Placenta materna* weight was calculated as the sum of all individual caruncle weights. For comparisons with

the Day 48 embryo stage, placental efficiency was calculated by dividing fetal weight by that of the *Placenta fetalis*.

2.2.3 Immunohistochemistry and histomorphometric analysis of the placenta

Five micron thick longitudinal sections were cut from paraffin embedded samples and an indirect double label immunohistochemistry was performed as described previously (Roberts et al., 2001) with minor modifications. We used 10% porcine serum and 1% BSA in PBS to block non-specific binding and as a diluent for the anti-body. For Day 48 placentomes, a 1:1000 dilution of the mouse anti-human Vimentin clone Vim3B4 (DakoCytomation, Glostrup, Denmark) antibody and a 1:600 dilution of the mouse anti-human Cytokeratin AE1/AE3 (Millipore, Temecula LA, USA) antibody was used. Biotinylated goat anti-mouse antibody (DakoCytomation, Glostrup, Denmark) in 10% porcine serum at a 1:200 dilution was used as the secondary antibody. For Day 153 placentomes, the Vimentin antibody was used at 1:10 dilution and the Cytokeratin antibody was used at 1:400. Hematoxylin (Sigma, St. Louis MO, USA) and Eosin (Sigma, St. Louis MO, USA) were used as counterstains and negative controls without primary antibodies were included in each batch.

A high resolution image of the entire stained placental section was captured using the NanoZoomer C9600 slide scanner (Hamamatsu Photonics K.K., Hamamatsu City, Japan). Using NDP.view version 1.0.0 (Hamamatsu Photonics K.K., Hamamatsu City, Japan), ten fields at 200× magnification were selected using random systematic sampling; fields were 1 mm (Day 153) or 50 µm (Day 48) apart. Digital images of the fields were imported into Video Pro software (Leading Edge Pty., Adelaide, Australia) and using an L-36 Merz grid transparency overlaying the monitor screen, a total of 360 points were counted for each section. Volume densities of the placental trophoblast, fetal capillaries, fetal connective tissue, maternal epithelium, maternal capillaries, maternal connective tissue and maternal septae as ‘other’ tissues were computed as previously reported (MacLaughlin et al., 2005).

Trophoblast cells stained brown had one or two nuclei and were apposed to the maternal epithelium. Capillaries or blood vessels, located very close to trophoblast or maternal epithelial cells, were identified as having darkly stained circular or elongated opening (i.e., lumen of a blood vessel) with or without the presence of red blood cells. On the other hand, connective tissue appeared fibrous and not as darkly stained as a blood vessel or capillary opening. The volume in cm^3 of each placental component was determined based on the weight of analyzed placentomes after confirming a 1:1 w/v relationship in collected *Placenta materna* samples. Parameters for surface exchange, such as trophoblast and maternal epithelium surface density (cm^2/g), total surface area (cm^2), and barrier thickness (µm) were estimated using intercept counting. One field was counted five times and the coefficient of variation was less than 5%. Samples used for histomorphometric analyses comprised 41 (flat n=10, convex n=31) and 69 (flat n=27, convex n=42) placentomes from developmental stages Day 48 and Day 153, respectively, and had sections with clearly developed interdigitation betwen the fetal villi and maternal caruncle.

2.2.4 Statistical analyses

The data generated for Day 48 embryos and Day 153 fetuses were analyzed separately, because the experiments were conducted at different times, i.e., Day 48 and Day 153 concepti were generated in two consecutive years. We used the general linear model procedure of SAS (SAS Inst., Cary NC, USA) to calculate means and standard errors of means adjusted for fetal sex and genetics using the model:

$$y_{ij} = S_i + G_j + e_{ij}$$

where yij was the dependent variable, S (i = male, female) was the fetal sex effect and G (j = A×A, B×A, A×B, and B×B) was the fetal genetic effect.

To further analyze the effect of placentome type on histomorphometric characteristics, we used the model:

$$y_{ijk} = S_i + G_j + P_k + e_{ijk}$$

where yijk was the dependent variable, S (i = male, female) was fetal sex effect, G (j = A×A, B×A, A×B, and B×B) was the fetal genetic effect, and P (k = flat, convex) was the effect of the placentome type**. Table S2.2** shows the significance levels of the models and the variables with significant effect of placentome type. Least square means with standard errors of the means were compared using two-tailed *t*-test. Regression analyses were performed for embryo and fetal weight on placental characteristics, and Pearson product-moment correlation coefficients were computed for relationships between placental traits. The significance threshold was $P<0.05$ for all statistical tests performed.

2.3 Results

2.3.1 Gross morphological characteristics of the placenta

As expected from the rapid changes in embryo-fetal weight and the role of the placenta in fetal growth and development, we observed strong but nevertheless differentiated increases from Day 48 to Day 153 of gestation in umbilical cord length (12 fold) and weight (765 fold), *Placenta fetalis* weight (17 fold), placental efficiency (42 fold), and fetal fluid volume (33 fold, **Figure 2.1**). The *Placenta materna* weight was not determined at Day 48 as individual caruncles with interdigitation were very small. Placentome number ranged from 22 to 64 (mean 37.9 ± 0.71) at Day 48, and 35 to 212 (mean 100.5 ± 1.5) by Day 153. At Day 48, all placentomes were within the gravid horn, with no evident placentome formation in the non-gravid horn (**Figure 2.1**). At Day 153 of gestation, the majority (65%) of placentomes were located in the gravid horn. Of the placentomes in the gravid horn at Day 48, the majority (88.3%) were prominent and could be classified as either flat or convex, while the remaining 11.7% had an uneven surface and could not be classified or analyzed further. However, by

Day 153 all placentomes in the gravid and non-gravid horns, irrespective of size, were well developed and could be classified as flat or convex (see below).

Placentomes with flat or convex shape were the two distinct types found in the placenta of embryos and fetuses. Representative gravid uterine horns of Day 48 and Day 153 pregnancies, and histological sections of both placentome types, at both developmental stages, are shown in **Figure 2.2A, B**. At Day 48, uteri contained a higher proportion of convex placentomes (52%) than at Day 153 (20%, **Figure 2.2C-F**).

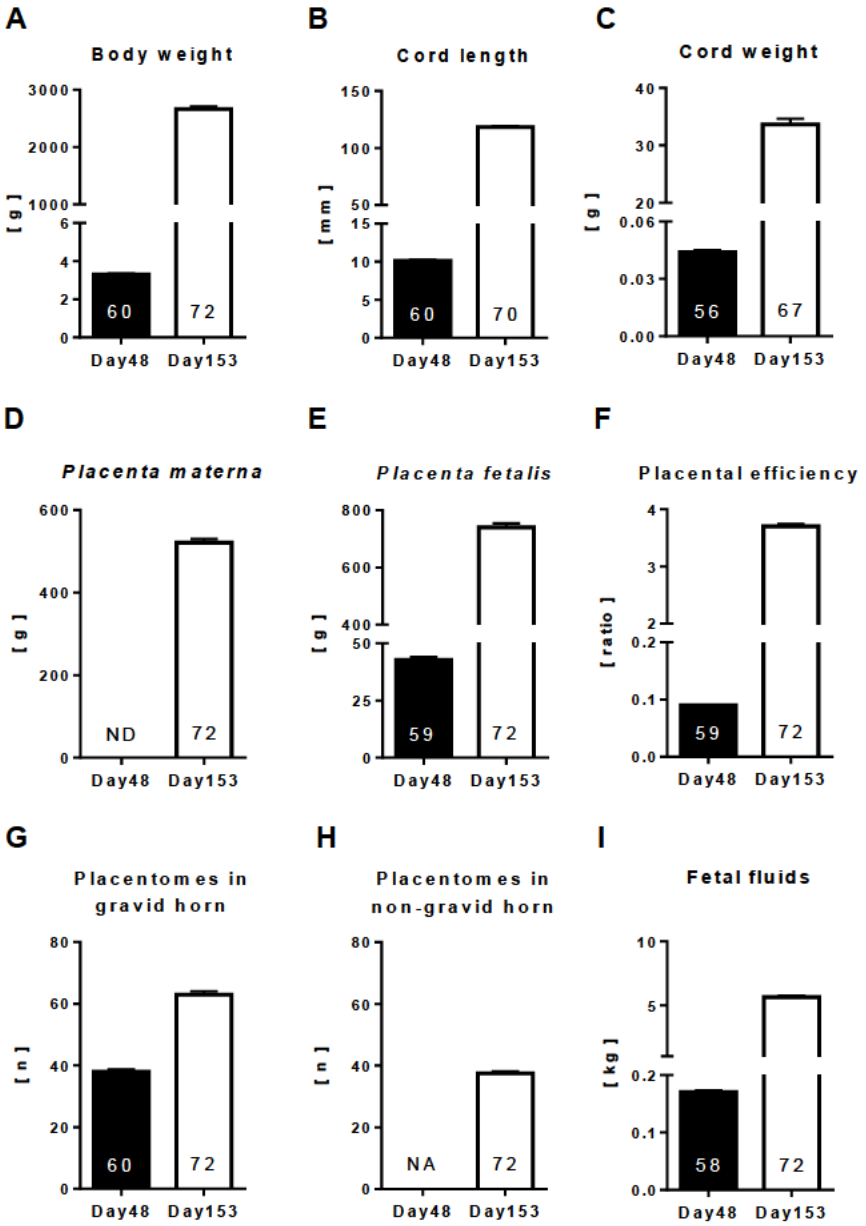

Figure 2.1 Key parameters of the bovine conceptus at Day 48 and Day 153 post conception.

(A) Embryo and fetal weight, (B,C) umbilical cord and (D-I) placental parameters. Bars show means ± SEM adjusted for effects of fetal sex and genetics. Number of individual sampled for each developmental stage is indicated inside bars. ND: not determined. NA: not applicable.

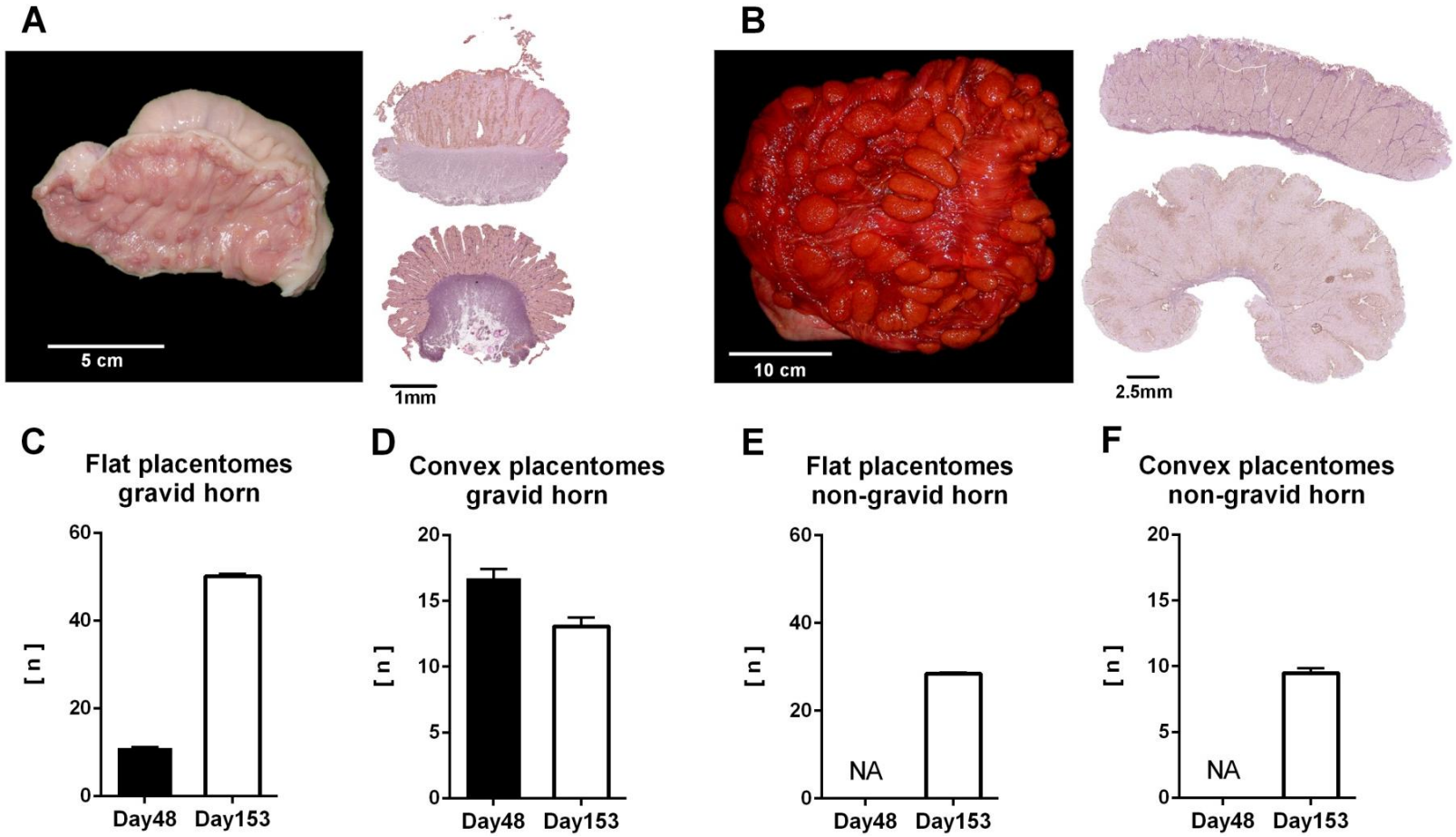

Figure 2.2 Gross morphological characteristics of the bovine *Placenta materna* at Day 48 (n=60) and Day 153 (n=72) of gestation.

(A, B) Representative uteri with the gravid horn opened and histomicrographs of flat (top) and convex (bottom) placentome types at both developmental stages. (C-F) Number of each placentome type in the gravid and non-gravid horn. Bars show means ± SEM adjusted for effects of fetal sex and genetics. NA: not applicable.

No correlations were detected between placental parameters at Day 48, but we identified significant relationships at Day 153. Here, placentome number in the gravid (r=0.60, $P<0.001$) and non-gravid horn (r=0.35, $P<0.01$), as well as total placentome number (r=0.55, $P<0.001$), correlated with *Placenta fetalis* weight. Correlations obtained for the gravid (r=0.52, $P<0.001$) and non-gravid (r=0.25, $P<0.05$) horns or for total placentome number (r=0.44, $P<0.001$) with *Placenta materna* weight were not as strong. As would be expected, *Placenta fetalis* and *Placenta materna* weights (r=0.60, $P<0.0001$) were also correlated. For reasons detailed above, placentome number in both gravid and non-gravid horns could only be analyzed at Day 153 and we found that these were correlated (r=0.44, $P<0.001$). Furthermore, the number of convex placentomes in the gravid and non-gravid horns was correlated (r=0.63, $P<0.001$) and we saw a similar relationship for flat placentomes (r=0.46, $P<0.001$).

2.3.2 Histomorphological characteristics of the placenta

The chorionic villi at Day 48 (**Figure 2.3A**) presented in a straight and long pattern with few emerging lateral branches as compared to the extensive branching of the chorionic villous trees at Day 153 (**Figure 2.3B**). In placentomes of both developmental stages, maternal epithelium and trophoblast cells were the predominant cell types in proportion and volume (**Figure 2.3E-H**). At Day 153, the volume density of the fetal connective tissue was lower (-50%) than at Day 48 (**Figure 2.3F**), while a marginal decrease in volume density of maternal connective tissue (-15%) was also observed (**Figure 2.3E)**. Maternal epithelium (+58%) and trophoblast (+54%) surface density, and maternal epithelium (302 fold) and trophoblast (292 fold) surface area were higher at Day 153 compared with Day 48 (**Figure 2.3I-J**), whereas maternal epithelium (-30%) and trophoblast (-39%) barrier thicknesses were thinner at Day 153 (**Figure 2.3K**).

Each placentome processed for histology was categorized as flat or convex. We analyzed data for each developmental stage in separate linear models with the effect of

placentome type fitted in addition to confounding effects of sex and genetics. We found that convex and flat placentomes were similar in weight at Day 48 (convex 0.19 ± 0.03 g, n= 31 *vs* flat 0.17 ± 0.03 g, n=10, $P>0.05$), but convex placentomes were significantly heavier than flat placentomes at Day 153 (convex 42.16 ± 1.6 g, n=42 *vs* flat 30.45 ± 1.9 g, n=27, $P<0.001$). The volume density of maternal connective tissue was 41% lower ($P<0.01$) in the convex placentome at Day 48 compared to flat placentomes, while volume densities of all other placental tissue types remained similar between the two placentome types (**Figure 2.4A**). At Day 153, volumes of maternal epithelium (+42%, $P<0.001$), maternal connective tissue (+39%, $P<0.01$), maternal capillaries (+32% $P<0.01$), trophoblast (+38% $P<0.001$) and fetal capillaries (+46% $P<0.01$) were higher in convex than flat placentomes, whereas no differences were observed between the placentome types at Day 48 (**Figure 2.4B**). Maternal (+39%, $P<0.001$) and trophoblast (+32%, $P<0.001$) surface areas were again higher in the convex than in the flat placentome at Day 153, while surface density and barrier thickness were similar between placentome types at both gestational stages of development **(Figure 2.4C**).

Figure 2.3

Representative histomicrographs of placentomes showing chorionic villous branching in (A) Day 48 and (B) Day 153 placentomes. (C-D) Placental types in Day 153 placentome; S – septa, a – maternal connective tissue, b – maternal capillary, c – maternal epithelium, d – fetal capillary, e – fetal connective tissue, f – trophoblast cell. Differences in (E,F) volume densities and (G,H) volume of tissues and (I-K) parameters indicating increased surface for nutrient exchange. Volume density for each stage totals to 1. Bars show means ± SEM adjusted for effects of fetal sex and genetics.

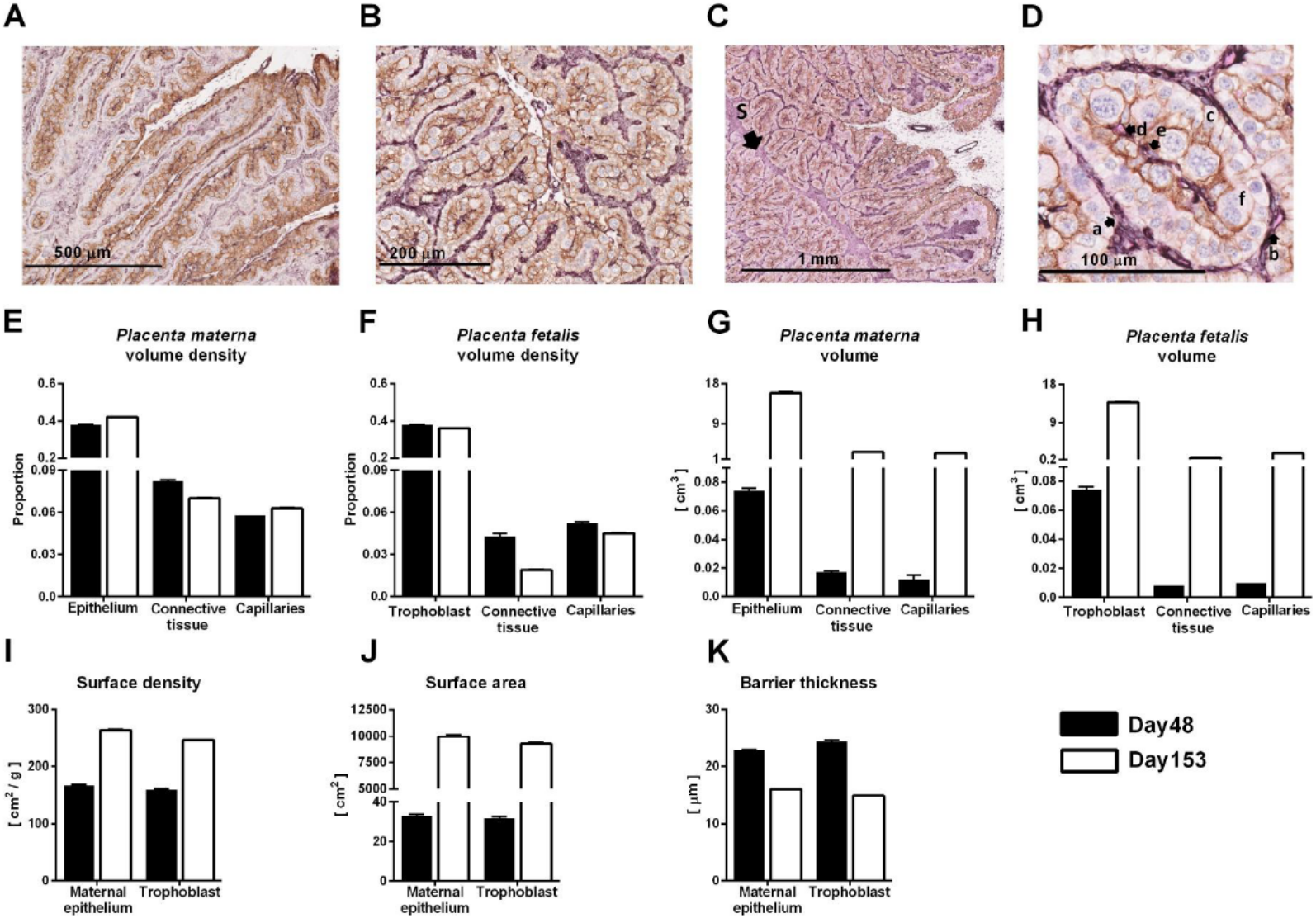

Figure 2.3 Histomorphological parameters of the bovine *Placenta materna* and *Placenta fetalis* at Day 48 (n=41) and Day 153 (n=69) of gestation.

2.3.3 Relationships between embryo / fetal weight and placental characteristics

Embryo-fetal body weight increased 807 fold from Day 48 to Day 153 of gestation (**Figure 2.1**) and weight at both developmental stages correlated with cord weight (embryo r=0.30 $P<0.05$; fetus r=0.55 $P<0.001$), *Placenta fetalis* weight (embryo r=0.42; fetus r=0.69, both $P<0.001$), number of placentomes in the gravid uterine horn (embryo r=0.52; fetus r=0.50, both $P<0.001$) and total number of convex placentomes (embryo r=0.50, $P<0.001$; fetus r=0.30 $P=0.01$, **Figure 2.5A**). *Placenta materna* weight was also correlated with fetal weight at Day 153 (r=0.70, $P<0.001$). At the histomorphological level, we found a positive relationship between embryo weight and fetal capillary volume density (r=0.32, $P<0.05$, **Figure 2.5B**), but none of the other gross morphological or histomorphological parameters tested were significantly correlated with embryo or fetal weight.

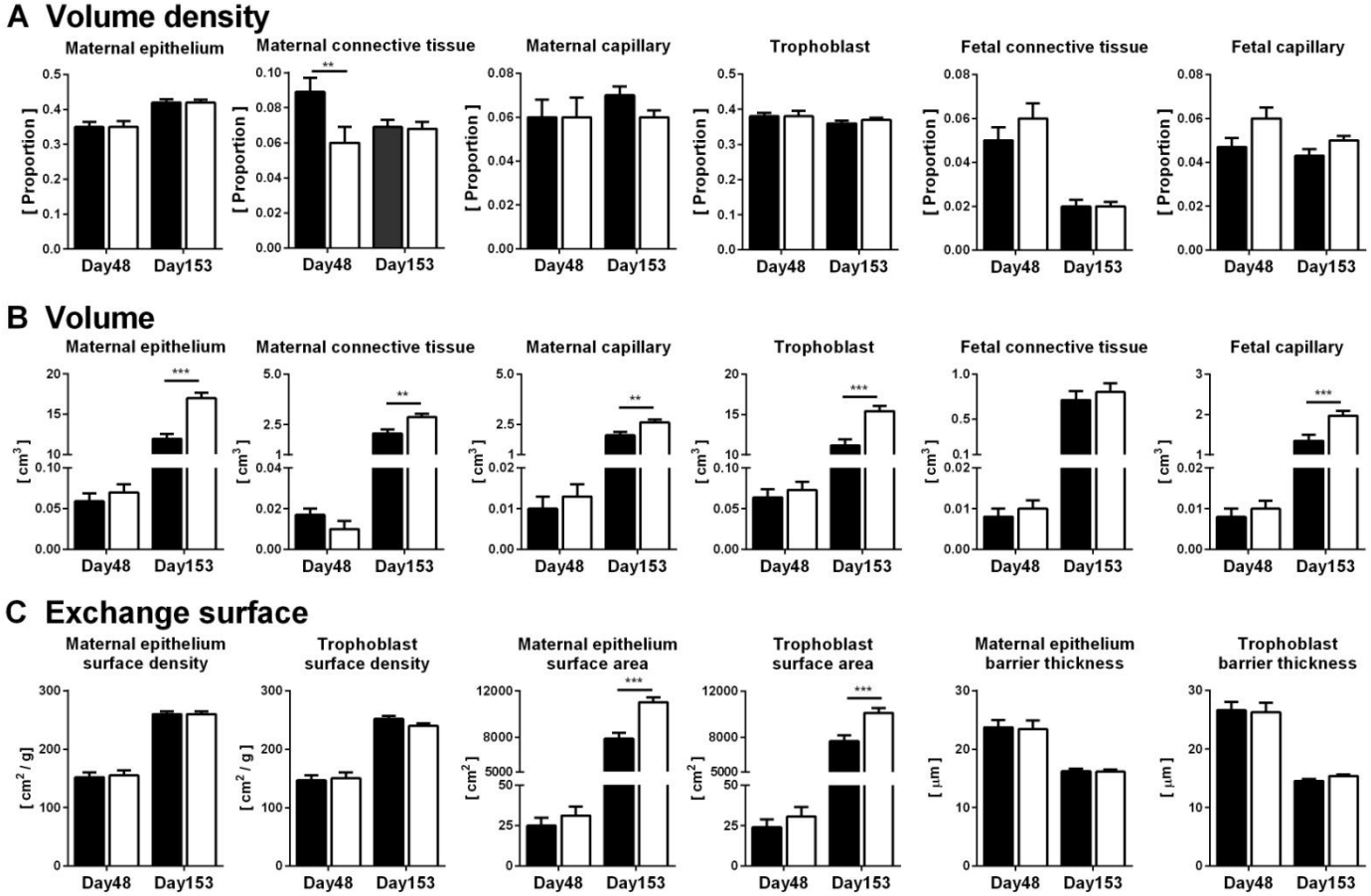

Figure 2.4 Histomorphological differences between flat (black bar) and convex (white bar) placentome types found in the bovine conceptus at Day 48 and Day 153 of gestation.

Volume densities for each placentome type at Day 48 and Day 153 totals to 1. Bars show least square means ± SEM adjusted for effects of fetal sex and genetics. Sample size of placentome type, Day 48 flat n=10, convex n = 31; Day 153 flat n=27, convex n=42. Statistical tests were performed using two-tailed t-test, where ***P<0.001, **P<0.01.

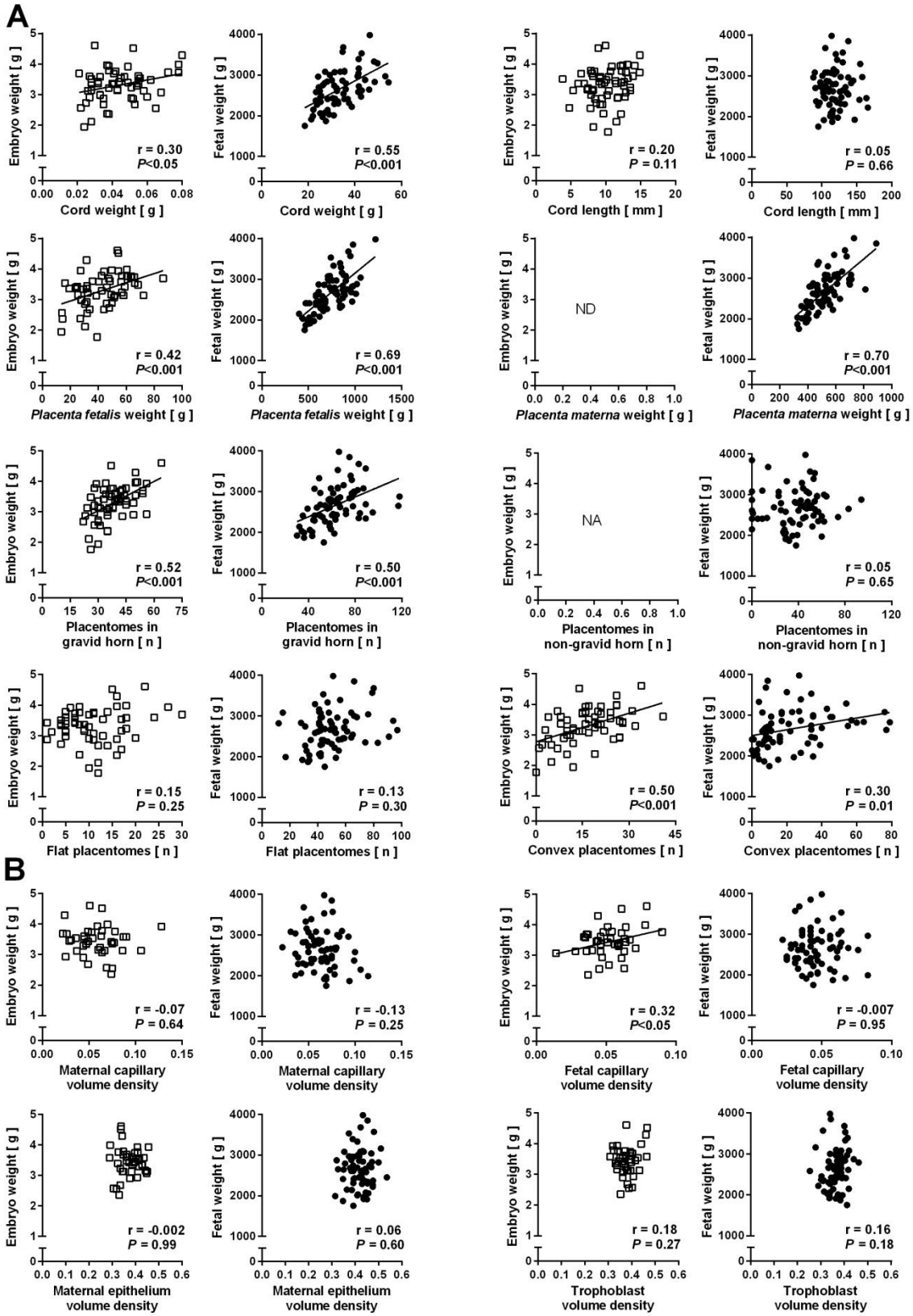

Figure 2.5 Relationships of embryo and fetal body weights with (A) gross morphological and (B) histomorphological characteristics of the placenta.

Regression lines and Pearson product moment correlation coefficients (r) with *P*-values for are indicated. $P<0.05$ are significant. ND: not determined, NA: not applicable.

2.4 Discussion

The present study utilized well-defined bovine embryo and fetal concepti to close significant gaps in knowledge about the bovine placenta and to provide baseline data for an important biomedical model that is also of global importance for animal production. Our data clearly demonstrate that the bovine placenta undergoes structural remodelling to facilitate greater feto-maternal exchange as gestation progresses from the late embryonic (Day 48) to the early accelerated fetal growth (Day 153) stage.

The *Placenta fetalis* at Day 48 was 12-fold heavier than the embryo, whereas, by Day 153, fetal weight was 4-fold and 5-fold heavier than the *Placenta fetalis* and *Placenta materna*, respectively. The critical role of nutrient supply and blood flow for early embryonic growth are also evident from significant positive relationships of embryo weight with placental and umbilical cord weights at Day 48 of gestation. To support rapidly increasing body weight the *Placenta fetalis* weight and placental efficiency increase by 17-fold and 42-fold, respectively, from Day 48 to Day 153. Although not all caruncles may be stimulated to form the *Placenta materna* (Telugu and Green, 2008), we found that the total number of placentomes formed had increased 3-fold between Day 48 and Day 153. The positive relationship between total placentome number and *Placenta fetalis* and *Placenta materna* weights for the Day 153 conceptus indicates that formation of fewer placentomes may constrain placental weight at mid-gestation. Others have reported no correlation between placentome number and total placentome weight using bovine uteri of different gestational ages derived from an abattoir (Laven and Peters, 2001). However, in our study, *Placenta fetalis* weight includes both cotyledon and fetal membrane and, rather than a comparison broadly across gestation, our efforts focused on Day 153, allowing an increased sample size, which may contribute to the contrasting results.

Similar to previous reports in cattle (Stickland and Purton, 1977, Laven and Peters, 2001) and other ruminant species (Abdel-Raouf and Badawi, 1966, Liu et al., 2010), our data

confirm that the number of placentomes is higher in the gravid than the non-gravid uterine horn at the embryo and fetal stages. Contact between the conceptus and endometrium during early gestation stimulates increased vascularity and uterine blood flow in the gravid horn compared to the non-gravid horn (Silva and Ginther, 2010). This, and anchorage of the conceptus to the endometrium supported by pressure build up from the fetal fluids (Leiser et al., 1998, Assis Neto et al., 2010), promote the early formation of placentomes in the gravid horn. We found clear evidence for interdigitation of embryonic with maternal tissue and thus placentome formation closer to the embryo in the gravid but not the non-gravid Day 48 uterine horn. This is consistent with reports that cotyledon formation starts from chorion adjacent to the embryonic zone to the chorionic tip but tightly interdigitated chorio-endometrial connection does not occur until Day 50 (Assis Neto et al., 2010) while placentome formation in the bovine non-gravid uterine horn was not evident until Day 60 of gestation (Curran et al., 1986). The distribution of placentomes near the embryo in the gravid horn at Day 48, and the positive correlation between placentome numbers in gravid and non-gravid horns at Day 153, indicate that placental development depends on and/or is initiated by the growth and function of the conceptus. Furthermore, we found a considerable variation in the number of placentomes ranging from 22–64 (mean 37.9 ± 0.71) at Day 48 and from 35–212 (mean 100.5 ± 1.5) at Day 153. Generally, placentome number described for cattle ranges from 75-120 (Roberts, 1971) and others have reported cotyledon numbers ranging from approximately 10-40 using *Bos t. indicus* specimens with estimated gestational ages of Day 40-50 (Assis Neto et al., 2010), and placentome number ranging from 42-160 using abattoir specimens with estimated gestational ages of Day 131-160 (Laven and Peters, 2001). This observed range in placentome numbers demonstrates that high variation in bovine placentome number in normal pregnancies and gestational age have to be taken into account when comparisons between experimental treatments, including embryo technologies such as *in vitro* fertilization or somatic cell nuclear transfer cloning, are made (Bertolini et al., 2002,

Hiendleder et al., 2004, Constant et al., 2006, Yang et al., 2007). Since dams used in the present study were managed under standardized conditions, it can be expected that the observed variation in placental parameters is due to random maternal effects, fetal sex and genetics.

We report for the first time the presence of convex and flat placentomes in the bovine placenta at Day 48 of gestation. We found more convex than flat placentomes in the gravid horn at Day 48, but there were more flat than convex placentomes in both uterine horns at Day 153. A previous study based on abattoir specimens reported that flat placentomes were more common in the gravid than the non-gravid horn. However, not all placentae examined that were estimated to range from Day 101-160 of gestation contained at least one flat placentome (Laven and Peters, 2001). In our defined resource, the high number and proportion of convex placentomes at Day 48 and the increase in number and proportion of flat placentomes at Day 153 suggests that flat placentomes develop later than the convex type placentomes. Placentome conversion has been reported in sheep (Vonnahme et al., 2006), however, whether conversion of convex to flat placentomes could contribute to the increasing numbers of flat placentomes observed at Day 153 in bovine is unknown. Interestingly, the significant difference between the volume density of flat and convex placentomes at Day 48 indicates that the maternal connective tissue of the caruncle may initially contribute in determining the shape of the placentome. It was recently shown that proliferation and/or hypertrophy of the caruncular connective tissue leads to the formation of caruncular projections at Day 28-33 of gestation which develop into trabecular networks where the fetal chorionic villi can insert and expand (Aires et al., 2014). It is possible that the conceptus stimulates the caruncular connective tissue to proliferate and develop projections and function as a mould for placentome formation.

The positive relationship of total number of convex placentomes with embryo and fetal weight in our data set suggests that in bovine this placentome type plays a more

significant role in nutrient transport to the fetus than flat placentomes. Convex placentomes sampled for histology were heavier than flat placentomes, and thus have greater volumes of placental cell types and surface area for transplacental exchange of nutrients, gases and wastes. The increase in number and proportion of flat placentomes as gestation progresses, and a negative correlation coefficient approaching significance (r=-0.23, P=0.051) for total number of flat and convex placentomes at Day 153 suggest that flat placentomes develop as the uterus grows and matures to further support fetal growth.

The present study generated histomorphometric data confirming that placental maturity is associated with profound tissue remodelling between Day 48 and Day 153 of gestation. Maternal epithelium and trophoblast cells had the highest volumes and volume densities amongst all placental cell types at both developmental stages, consistent with their key roles in placental transport. The decrease in fetal and maternal connective tissue volume density by Day 153 leads to closer proximity of fetal and maternal capillaries and facilitates greater substance transfer to the fetus. This is consistent with the reduction of placental barrier thickness by Day 153. Further, the long chorionic stem villi, which had few short branching intermediate villi at Day 48, developed into elaborate villous trees with extensive lateral branches and increased surface density and surface area by Day 153 favouring greater placental substance exchange. Of the placental cell types, only volume density of the fetal capillaries was found to be positively correlated with embryo weight. At Day 36 post insemination, bovine cotyledons become macroscopically visible and developing blood vasculature is evident (Assis Neto et al., 2010). The positive relationship between fetal capillary volume density and embryo weight suggests that by Day 48 the bovine embryo derives most of its nutrient supply through haemotrophic exchange of nutrients across the placenta.

In conclusion, our comprehensive gross and histomorphological parameters obtained from well-defined resources of Day 48 and Day 153 concepti demonstrated extensive

remodelling of the bovine placenta to facilitate rapid fetal growth and development. This includes decreased volume density of placental connective tissue, reduced barrier thickness and increased surface area for nutrient exchange by Day 153 of gestation. We describe for the first time, convex and flat placentome types in the bovine Day 48 placenta, which displayed different maternal connective tissue volume densities. At Day 153, convex placentomes were fewer, but had greater volume of placental cell types and surface area for placental exchange. These characteristics can explain the significant and exclusive positive relationship of embryo and fetal weights with the number of convex placentomes. This suggests a major role for convex placentomes in nutrient transfer to support fetal growth in the bovine. Further investigations are needed to determine potential effects of factors such as genetics and maternal nutrition on the variation in placentome shape.

Supplemental Tables

Table S 2.1, related to Methods. Numbers of male (M) and female (F) concepti with defined *Bos taurus taurus* (A) and *Bos taurus indicus* (B) genetics at the late embryonic (Day 48) and early accelerated fetal growth (Day 153) stages (term, 277- 291 days).
Sire genetics listed first.

Genetics	Sex	Day 48	Day 153
A × A	M	11	11
A × A	F	8	12
A × B	M	5	7
A × B	F	8	6
B × A	M	10	5
B × A	F	12	17
B × B	M	3	4
B × B	F	3	10

Table S 2.2, related to Methods. General linear models (Type III sums of squares) with R-squared and significance levels (*P*-values) of models and variables for placental parameters with significant effect of placentome type.

Parameters	R^2	*P*-value			
		Model	Genetics	Sex	Placentome type
Embryo stage (Day 48)					
Maternal connective tissue volume density	0.27	0.0460	0.0582	0.1106	0.0065
Fetal stage (Day 153)					
Volume					
Maternal epithelium	0.37	<0.0001	0.0138	0.6668	<0.0001
Maternal capillary	0.17	0.0327	0.4184	0.7525	0.0055
Maternal connective tissue	0.28	0.0008	0.0107	0.6730	0.0017
Trophoblast	0.36	<0.0001	0.1120	0.0604	<0.0001
Fetal capillary	0.28	0.0007	0.0832	0.6984	0.0004
Surface area					
Trophoblast	0.37	<0.0001	0.0118	0.5138	<0.0001
Maternal epithelium	0.44	<0.0001	0.0047	0.8031	<0.0001

2.5 Literature Cited

ABDEL-RAOUF, M. & BADAWI, H. M. 1966. Morphological study of uterine caruncles in Egyptian buffalo cows. *Zentralblatt für Veterinärmedizin Reihe A,* 13**,** 252-263.

AIRES, M. B., DEGAKI, K. Y., DANTZER, V. & YAMADA, A. T. 2014. Bovine placentome development during early pregnancy. *In:* MENDEZ-VILLAS, A. (ed.) *Microscopy: Advances in Scientific Research and Education.* Spain: Formatex Research Center.

ANAND-IVELL, R., HIENDLEDER, S., VIÑOLES, C., MARTIN, G. B., FITZSIMMONS, C., EURICH, A., HAFEN, B. & IVELL, R. 2011. INSL3 in the ruminant: a powerful indicator of gender- and genetic-specific feto-maternal dialogue. *PLOS ONE,* 6**,** e19821.

ANDERSEN, H. & PLUM, M. 1965. Gestation length and birth weight in cattle and buffaloes: a review. *Journal of Dairy Science,* 48**,** 1224-1235.

ASSIS NETO, A. C., PEREIRA, F. T. V., SANTOS, T. C., AMBROSIO, C. E., LEISER, R. & MIGLINO, M. A. 2010. Morpho-physical recording of bovine conceptus (*Bos indicus*) and placenta from Days 20 to 70 of pregnancy. *Reproduction in Domestic Animals,* 45**,** 760-772.

BÄHR, A. & WOLF, E. 2012. Domestic animal models for biomedical research. *Reproduction in Domestic Animals,* 47**,** 59-71.

BARKER, D. J. P., LAMPL, M., ROSEBOOM, T. & WINDER, N. 2012. Resource allocation in utero and health in later life. *Placenta,* 33 Supplement 2**,** e30-e34.

BERTOLINI, M., MASON, J. B., BEAM, S. W., CARNEIRO, G. F., SWEEN, M. L., KOMINEK, D. J., MOYER, A. L., FAMULA, T. R., SAINZ, R. D. & ANDERSON, G. B. 2002. Morphology and morphometry of in vivo- and in vitro-produced bovine concepti from early pregnancy to term and association with high birth weights. *Theriogenology,* 58**,** 973-994.

BJÖRKMAN, N. H. 1969. Light and electron microscopic studies on cellular alterations in the normal bovine placentome. *The Anatomical Record,* 163**,** 17-29.

BURTON, G. J. & FOWDEN, A. L. 2015. The placenta: a multifaceted, transient organ. *Philosophical Transactions B,* 370**,** 20140066.

CONSTANT, F., GUILLOMOT, M., HEYMAN, Y., VIGNON, X., LAIGRE, P., SERVELY, J. L., RENARD, J. P. & CHAVATTE-PALMER, P. 2006. Large offspring or large placenta syndrome? Morphometric analysis of late gestation bovine placentomes from somatic nuclear transfer pregnancies complicated by hydrallantois. *Biology of Reproduction,* 75**,** 122-130.

CURRAN, S., PIERSON, R. A. & GINTHER, O. J. 1986. Ultrasonographic appearance of the bovine conceptus from days 20 through 60. *Journal of the American Veterinary Medical Association,* 189**,** 1295-1302.

DWYER, C. M. 2005. Behavior: Maternal. *In:* POND, W. G. & BELL, A. W. (eds.) *Encyclopedia of Animal Science.* New York: Marcel Dekker.

FERRELL, C. L. 1989. Placental regulation of fetal growth. *In:* CAMPION, D. R., HAUSMAN, G. J. & MARTIN, R. J. (eds.) *Animal Growth Regulation.* USA: Springer US.

FOWDEN, A. L., FORHEAD, A. J., COAN, P. M. & BURTON, G. J. 2008. The placenta and intrauterine programming. *Journal of Neuroendocrinology,* 20**,** 439-450.

GLUCKMAN, P. D., HANSON, M. A., COOPER, C. & THORNBURG, K. L. 2008. Effect of in utero and early-life conditions on adult health and disease. *The New England Journal of Medicine,* 359**,** 61-73.

HERNANDEZ-MEDRANO, J. H., COPPING, K. J., HOARE, A., WAPANAAR, W., GRIVELL, R. G., KUCHEL, T., MIGUEL-PACHECO, G., MCMILLEN, I. C., RODGERS, R. J. & PERRY, V. E. A. 2015. Gestational dietary protein is associated with sex specific decrease in blood flow, fetal heart growth and post-natal blood pressure of progeny. *PLOS ONE,* 10**,** e0125694.

HIENDLEDER, S., BEBBERE, D., ZAKHARTCHENKO, V., REICHENBACK, H.-D., WENIGERKIND, H., LEDDA, S. & WOLF, E. 2004. Maternal-fetal transplacental leakage of mitochondrial DNA in bovine nuclear transfer pregnancies: potential implications for offspring and recipients. *Cloning and Stem Cells,* 6**,** 150-156.

HIENDLEDER, S., LEWALSKI, H. & JANKE, A. 2008. Complete mitochondrial genomes of *Bos taurus* and *Bos indicus* provide new insights into intra-species variation, taxonomy and domestication. *Cytogenetic and Genome Research,* 120**,** 150-156.

KING, G. J., ATKINSON, B. A. & ROBERTSON, H. A. 1979. Development of the bovine placentome during the second month of gestation. *Journal of Reproduction and Fertility,* 55**,** 173-180.

LAVEN, R. A. & PETERS, A. R. 2001. Gross morphometry of the bovine placentome during gestation. *Reproduction in Domestic Animals,* 36**,** 289-296.

LEISER, R., KREBS, C., KLISCH, K., EBERT, B., DANTZER, V., SCHULER, G. & HOFFMANN, B. 1997. Fetal villosity and microvasculature of the bovine placentome in the second half of gestation. *Journal of Anatomy,* 191 517-527.

LEISER, R., PFARRER, C., ABD-ELNAEIM, M. & DANTZER, V. 1998. Feto-maternal anchorage in epitheliochorial and endotheliochorial placental types studied by histology and microvascular corrosion casts. *Placenta,* 12 Supplement Trophoblast Research**,** 21-39.

LIU, B., CUI, Y., YANG, B., FAN, J., ZHAO, Z. & YU, S. 2010. Morphometric analysis of yak placentomes during gestation. *The Anatomical Record: Advances in Integrative Anatomy and Evolutionary Biology,* 293**,** 1873-1879.

MACLAUGHLIN, S. M., WALKER, S. K., ROBERTS, C. T., KLEEMANN, D. O. & MCMILLEN, I. C. 2005. Periconceptional nutrition and the relationship between maternal body weight changes in the periconceptional period and feto-placental growth in the sheep. *The Journal of Physiology,* 565**,** 111-124.

MARTIN, R. D. 2003. Human reproduction: a comparative background for medical hypotheses. *Journal of Reproductive Immunology,* 59**,** 111-135.

MOSSMAN, H. W. 1987. *Veterbrate Fetal Membranes,* USA, Rutgers University Press.

OCKLEFORD, C. D. & WAKELY, J. 1982. The skeleton of the placenta. *In:* HARRISON, R. J. & NAVARATNAM, V. (eds.) *Progress in Anatomy.* UK: Cambridge Unviersity Press.

PFARRER, C., WEISE, S., BERISHA, B., SCHAMS, D., LEISER, R., HOFFMANN, B. & SCHULER, G. 2006a. Fibroblast growth factor (FGF)-1, FGF2, FGF7 and FGF receptors are uniformly expressed in trophoblast giant cells during restricted trophoblast invasion in cows. *Placenta,* 27**,** 758-770.

PFARRER, C. D., RUZIWA, S. D., WINTHER, H., CALLESEN, H., LEISER, R., SCHAMS, D. & DANTZER, V. 2006b. Localization of vascular endothelial growth factor (VEGF) and its receptors VEGFR-1 and VEGFR-2 in bovine placentomes from implantation until term. *Placenta,* 27**,** 889-898.

REYNOLDS, L. P., BOROWICZ, P. P., VONNAHME, K. A., JOHNSON, M. L., GRAZUL-BILSKA, A. T., REDMER, D. A. & CATON, J. S. 2005. Placental angiogenesis in sheep models of compromised pregnancy. *The Journal of Physiology,* 565**,** 43-58.

ROBERTS, C. T., SOHLSTROM, A., KIND, K. L., EARL, R. A., KHONG, T. Y., ROBINSON, J. S., OWENS, P. C. & OWENS, J. A. 2001. Maternal food restriction reduces the exchange surface area and increases the barrier thickness of the placenta in the guinea-pig. *Placenta,* 22**,** 177-185.

ROBERTS, S. J. 1971. *Veterinary Obstetrics and Genital Diseases,* NY USA, Published by the Author.

RURAK, D. 2001. Development and function of the placenta. *In:* HARDING, R. & BOCKING, A. (eds.) *Fetal Growth and Development.* UK: Cambridge University Press.

SCHMIDT, S., GERBER, D., SOLEY, J. T., AIRE, T. A. & BOOS, A. 2006. Histo-morphology of the uterus and early placenta of the African Buffalo (*Syncerus caffer*) and comparative placentome morphology of the African Buffalo and cattle (*Bos taurus*). *Placenta,* 27**,** 899-911.

SILVA, L. A. & GINTHER, O. J. 2010. Local effect of the conceptus on uterine vascular perfusion during early pregnancy in heifers. *Reproduction,* 139**,** 453-463.

STEYN, C., HAWKINS, P., SAITO, T., NOAKES, D. E., KINGDOM, J. C. P. & HANSON, M. A. 2001. Undernutrition during the first half of gestation increases the predominance of fetal tissue in late-gestation ovine placentomes. *European Journal of Obstetrics & Gynecology and Reproductive Biology,* 98**,** 165-170.

STICKLAND, N. C. & PURTON, M. D. 1977. A quantitative evaluation of placentome development in Zebu cattle (*Bos indicus*). *Anatomia, histologia, embryologia,* 6**,** 81-86.

TELUGU, B. P. & GREEN, J. A. 2008. Comparative Placentation. *In:* SCHATTEN, H. & CONSTANTINESCU, G. M. (eds.) *Comparative Reproductive Biology.* Oxford, UK.: Blackwell Publishing Ltd.

VATNICK, I., SCHOKNECHT, P. A., DARRIGRAND, R. & BELL, A. W. 1991. Growth and metabolism of the placenta after unilateral fetectomy in twin pregnant ewes. *Journal of Developmental Physiology,* 15**,** 351-356.

VONNAHME, K. A., HESS, B. W., NIJLAND, M. J., NATHANIELSZ, P. W. & FORD, S. P. 2006. Placentomal differentiation may compensate for maternal nutrient restriction in ewes adapted to harsh range conditions. *Journal of Animal Science,* 84**,** 3451-3459.

WOODING, F. B. P. 1992. The synepitheliochorial placenta of ruminants: Binucleate cell fusions and hormone production. *Placenta,* 13**,** 101-113.

WOODING, F. B. P. & BURTON, G. 2008. Placentation fundamentals. *In:* WOODING, F. B. P. & BURTON, G. (eds.) *Comparative Placentation.* Berlin, Hiedelberg: Springer-Verlag.

XIANG, R., GHANIPOOR-SAMAMI, M., JOHNS, W. H., EINDORF, T., RUTLEY, D. L., KRUK, Z. A., FITZSIMMONS, C. J., THOMSEN, D. A., ROBERTS, C. T., BURNS, B. M., ANDERSON, G. I., GREENWOOD, P. L. & HIENDLEDER, S. 2013. Maternal and paternal genomes differentially affect myofibre characteristics and muscle weights of bovine fetuses at midgestation. *PLOS ONE,* 8**,** e53402.

XIANG, R., LEE, A. M. C., EINDORF, T., JAVADMANESH, A., GHANIPOOR-SAMAMI, M., GUGGER, M., FITZSIMMONS, C. J., KRUK, Z. A., PITCHFORD, W. S., LEVITON, A. J., THOMSEN, D. A., BECKMAN, I., ANDERSON, G. I., BURNS, B. M., RUTLEY, D. L., XIAN, C. J. & HIENDLEDER, S. 2014. Widespread differential maternal and paternal genome effects on fetal bone phenotype at mid-gestation. *Journal of Bone and Mineral Research,* 29**,** 2392–2404

YANG, X., SMITH, S. L., TIAN, X. C., LEWIN, H. A., RENARD, J.-P. & WAKAYAMA, T. 2007. Nuclear reprogramming of cloned embryos and its implications for therapeutic cloning. *Nature Genetics,* 39**,** 295-302.

Chapter 3 Intrauterine growth-restriction of the normal female conceptus can explain sex-bias in programming of postnatal disease

Manuscript in preparation for submission to **PloS Biology**, with the exception that figures and tables are embedded in the text and citations are formatted in Harvard style to be consistent with the other chapters.

Statement of Authorship

Title of Paper	Intrauterine growth-restriction of the normal female conceptus can explain sex-bias in programming of postnatal disease
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Manuscript format for submission to Plos Biology, except that figures and tables are embedded on the text, and bibliography and citation follows the Harvard style of referencing to maintain consistency with the other chapters.

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Overall percentage (%)	75%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

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Intrauterine growth-restriction of the normal female conceptus can explain sex-bias in programming of postnatal disease

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Abstract

The prevalence of major non-communicable diseases is sex-biased with mounting evidence for prenatal origins, but the mechanisms for sex-specific fetal programming are unknown. Here we demonstrate in a bovine model that at mid-gestation, normal females in comparison to males, manifest hallmarks of asymmetric intrauterine growth restriction (IUGR), including brain and heart sparing, lower ponderal index, higher γ-glutamyl transferase activity and lower cholesterol levels. Together with differences in placental histomorphology, and female-specific relationships of brain to body and brain to liver weight ratios with cord vessel diameters, our data indicate that redistribution of blood flow confers IUGR-like characteristics to the female conceptus. Furthermore, lower abundance of insulin-like growth factor 2 (IGF2) transcripts in brain and heart, and lower circulating IGF2 and IGF binding proteins implicate female-specific restriction of nutrient supply. These adaptations can explain the female's greater resilience to growth restricting intrauterine insults and sex-bias in programming of predisposition to disease.

3.1 Introduction

Epidemiological studies based on birth weight as a proxy for fetal nutrient supply have clearly demonstrated that diseases in adulthood such as type 2 diabetes and coronary heart disease can be programmed *in utero* (Barker, 1995, Rich-Edwards et al., 1997). The prevalence of these major non-communicable diseases is sex-biased with lower incidence of diabetes in females than in males under 60 years of age (Wild et al., 2004) and lower risk for females to die from cardiovascular disease before the age of 50 (Mikkola et al., 2013). While absolute differences in birth weight and length between females and males are small, large differences in body proportions namely, shortness in relation to placental size and in ponderal index in relation to head circumference at birth, suggest sex-specific prenatal growth and development (Forsén et al., 1999). Moreover, indicators for hypertension and coronary heart disease later in life, including ratios between placental parameters and fetal characteristics are sex-specific (Forsén et al., 1999, Eriksson et al., 2010). The critical role of the placenta in delivering substrates for growth and development manifests in tight correlations between placental and fetal weights with lower placental weight and efficiency in females than in males (Thomson et al., 1969, Almog et al., 2011). Females also have a shorter umbilical cord (Georgiadis et al., 2014), smaller cord vein diameter and higher cord artery pulsatility index (Verburg et al., 2008). Sexual dimorphism in the placenta and umbilical cord may therefore affect nutrient and oxygen supply through the feto-placental circulation and contribute to sex-specific differences in fetal growth that program long term health outcomes.

The terms small for gestational age (SGA) and intrauterine growth restriction or fetal growth restriction (IUGR, FGR), are frequently used synonymously to describe low and/or restricted growth *in utero* but reflect different concepts (Hobbins, 2008). SGA is a statistical definition and denotes a fetus/neonate with anthropometric values below the tenth birth weight centile obtained from a reference population and includes growth restricted as well as genetically small but otherwise normal fetuses and neonates (Bertino et al., 2012). IUGR, on

the other hand, refers to a functional condition and denotes fetuses unable to achieve their growth potential due to maternal, placental or fetal factors (Bertino et al., 2012). Biometric parameters and body proportions are used to further classify SGA/IUGR fetuses as symmetric or asymmetric (Rosso and Winick, 1974, Hobbins, 2008, Platz and Newman, 2008). Hallmarks of asymmetric growth restriction in humans and animal models include higher fetal brain to liver weight or brain to body weight ratio, i.e. "brain sparing" (Rosso and Winick, 1974, Campbell and Thoms, 1977, Boito et al., 2003, Platz and Newman, 2008) and increased fetal heart to body weight ratio, i.e. "heart sparing" (Morrison, 2008). Asymmetric growth is a result of reduced substrate supply and involves compensatory adaptive changes in placental, umbilical and fetal hemodynamics that include blood flow redistribution to brain and heart at the expense of other organs such as the liver (Hershkovitz et al., 2000, Cetin et al., 2008). This is reflected in the clinical chemistry of cord blood from IUGR newborns which has lower concentrations of glucose and cholesterol and higher γ-glutamyl transferase activity in comparison to appropriate weight for gestational age controls (Nieto-Diaz et al., 1996, Johnson et al., 2003). Reported changes in the insulin-like growth factor (IGF) system, which is a principal endocrine regulator of fetal growth (Fowden, 2003), include lower concentrations of cord blood IGF1, IGF2 and IGF binding proteins in IUGR (Guidice et al., 1995). Thus, a spectrum of clinical markers, ranging from biometric indices and ratios to the clinical chemistry of cord blood, is altered in IUGR fetuses and neonates.

Females have a lower birth weight and higher incidence of IUGR than males (Aibar et al., 2012, Melamed et al., 2010). The higher growth rate of males on the other hand is associated with greater vulnerability to growth restricting intrauterine insults such as maternal under-nutrition and smoking, with higher relative reduction of birth weight than in females (Zarén et al., 2000, Eriksson et al., 2010). We hypothesized that sexual dimorphism in growth and development of the normal conceptus, which has been linked to sex-specific differences in postnatal health, could involve the same underlying biological principles and

mechanisms that manifest in a more extreme and pathological form as clinical IUGR. To address this question, we generated a large set of normal concepti with a broad range of fetal weights using an outbred bovine model. This is the best fit animal model as dams carry a single fetus with gestation length, fetal growth curve and maturity at birth similar to human (Andersen and Plum, 1965, Ott and Doyle, 1982, Ferrell, 1989, Bolker, 2012, Xiang et al., 2014). Furthermore, sex-specific *in utero* programming of postnatal blood pressure was recently demonstrated in the bovine (Hernandez-Medrano et al., 2015). In the present study, comprehensive morphometric and histomorphological analyses combined with clinical-chemical, endocrine and gene expression data demonstrated for the first time that the smaller phenotype of female fetuses in normal pregnancy is an asymmetric IUGR phenotype consistent with female-specific redistribution of blood flow in response to constrained nutrient supply. These adaptations are established by mid-gestation, when the fetus enters the accelerated growth phase, and can explain greater resilience of females to insults later in pregnancy and lower risk to adverse perinatal and long term health outcomes.

3.2 Results

3.2.1 Hallmarks of growth restriction in normal female concepti

We took advantage of the diverse genetics available in the bovine model to generate, under standardized conditions, a resource of normal concepti recovered at week 21 (55% term) with fetal weights ranging from 1750-3980 g (Supplementary Table S3.1). Females weighed less (–15.0%), had lower skeletal muscle (–10.7%) and bone (–10.8%) mass and were shorter (–3.6%) and thinner (ponderal index, –9.6%) than males (Fig. 3.1). Absolute brain (–5.2%), liver (–14.9%), heart (–8.4%), kidney (–14.1%), and lung (–10.5%) weights were lower in females. However, relative to fetal weight, brain (+11.3%), heart (+8.5%), skeletal muscle (+4.6%) and bone (+5.3%) weights were higher in females than in males. Importantly, brain to liver weight ratio was also higher in females (+10.3%).

Placental (–9.3%) and umbilical cord (–14.7%) weights, placental efficiency (–7.1%), umbilical cord diameter (–6.1%), and umbilical vein (–6.5%) and artery (–7.8%) diameters, were all lower in females than in males, while umbilical cord length was similar between sexes (Fig. 3.2). In the female placenta, trophoblast volume (–15.2%), fetal connective tissue volume (–38.3%) and fetal connective tissue volume density (–32.0%) were also lower (Supplementary Table S3.2). All other placental tissue components and measurements of maternal and fetal exchange surface were similar in both sexes (Fig. 3.2, Supplementary Table S3.2).

In clinical-chemical analyses of cord blood serum, γ-glutamyl transferase activity was higher (+19.5%) and cholesterol serum concentration was lower (–8.5%) in females, while 17 other parameters were similar to males (Fig. 3.3A, B and Supplementary Table S3.3). Circulating plasma IGF2 (–9.6%) and total IGFBPs (–17.0%), but not IGF1, were lower in females (Fig. 3.3C). Furthermore, measurement of transcripts of 12 genes within the IGF system indicated lower abundance of *IGF2* transcript in female brain (–37.5%) and heart (–23.7%), but no differences in liver, skeletal muscle or placenta (Supplementary Fig. S3.1).

3.2.2 Hemodynamics implicated in female growth restriction

Sex-specific regression analyses of fetal body weight with placental weight and umbilical cord diameter revealed strong positive relationships in both males and females. However, significant positive relationships between fetal body weight and umbilical vein and artery diameters were only present in females. Furthermore, relationships of liver weight with umbilical vein and artery diameters were very similar and also female-specific (Fig. 3.4A, Supplementary Fig. S3.2). Regressions of brain to body weight ratio on placental weight and umbilical cord diameter indicated strong negative relationships in both sexes, but only females displayed significant negative relationships of brain to body weight ratio with umbilical vein and artery diameters. Highly similar female-specific relationships were obtained for brain to liver weight ratio (Fig. 3.4B, Supplementary Fig. S3.3).

Regression analyses of placental and liver weights on cord plasma IGF2 levels revealed significant negative relationships for female but not male concepti, while fetal weight was not affected by circulating IGF2. Moreover, brain to liver weight ratio displayed a female-specific positive relationship with circulating IGF2 (Fig. 3.5).

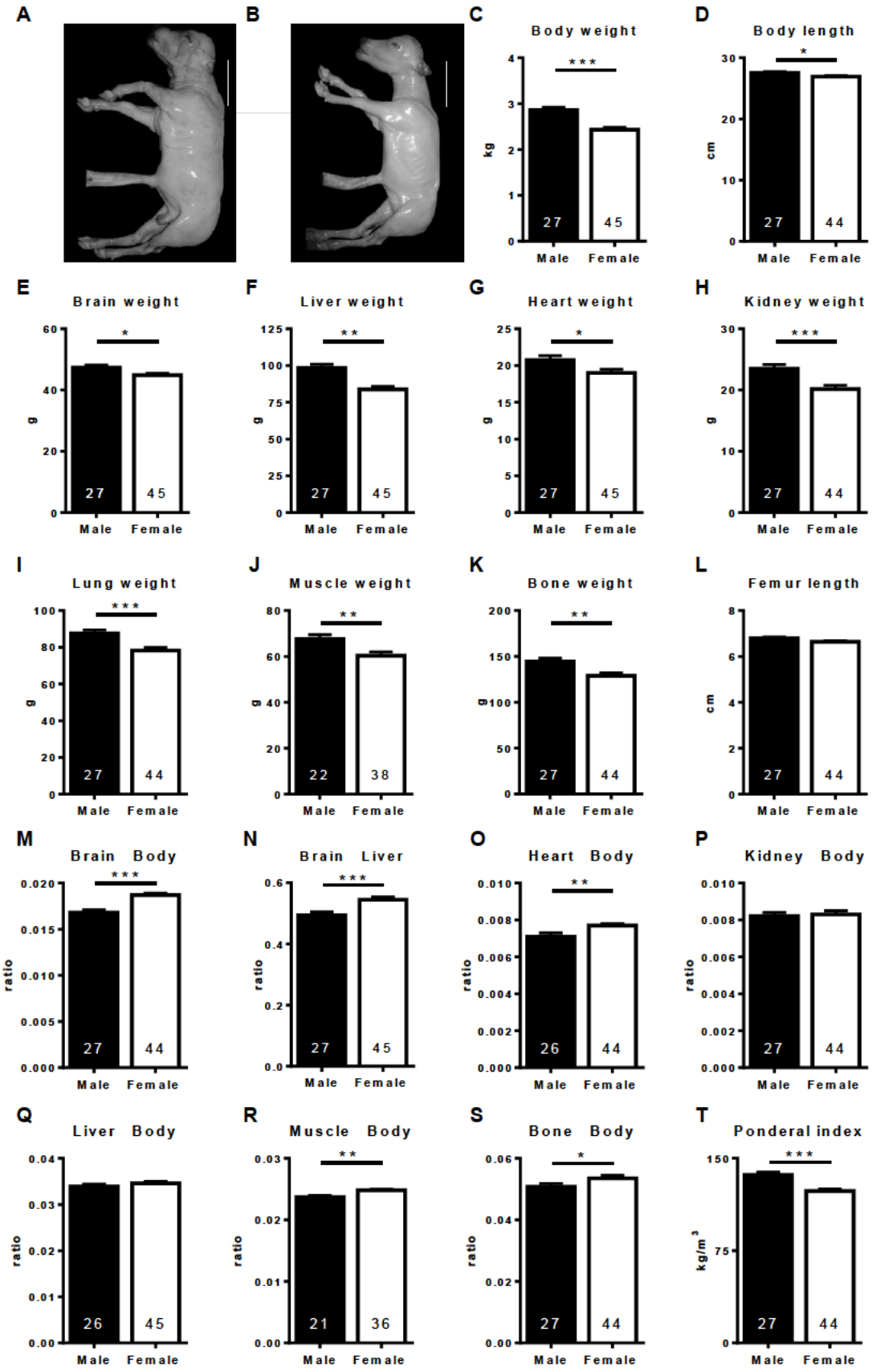

Figure 3.1 Female fetuses are growth restricted at mid-gestation.

Figure 3.1

Representative photographs of male (A) and female (B) fetuses recovered at Day 153 post conception. Scale bars: 10 cm. (C, E-I) Wet weights obtained after removal of fetuses from uteri. (D) Body length was measured along the spinal column including cervical, thoracic and lumbar vertebrae. (J,K) Muscle and bone mass were calculated based on cumulative weights of 8 representative dissected muscles and 12 major bones as described in methods. (M-T) Normal female fetuses show major indicators of IUGR. (M,N) Higher brain to liver weight and brain to fetal body weight ratios indicate 'brain sparing' and (O) higher heart to fetal body weight ratio, 'heart sparing', while (P,Q) kidney to fetal body weight and liver to fetal body weight ratios were not significantly different between sexes. (R,S) Higher relative muscle and bone mass and (T) lower ponderal index demonstrate that females are thinner than males. (C-T) Data are presented as least square means ± SEM for males and females with numbers of individuals indicated inside bars. (C-E, G-T) Two tailed *t*-test was used to calculate statistical difference between groups. **P*<0.05; ***P*<0.01; ****P*<0.001. (F) Wilcoxon two-sample test was used. Ws =1242, *z*=2.97, ***P*<0.01.

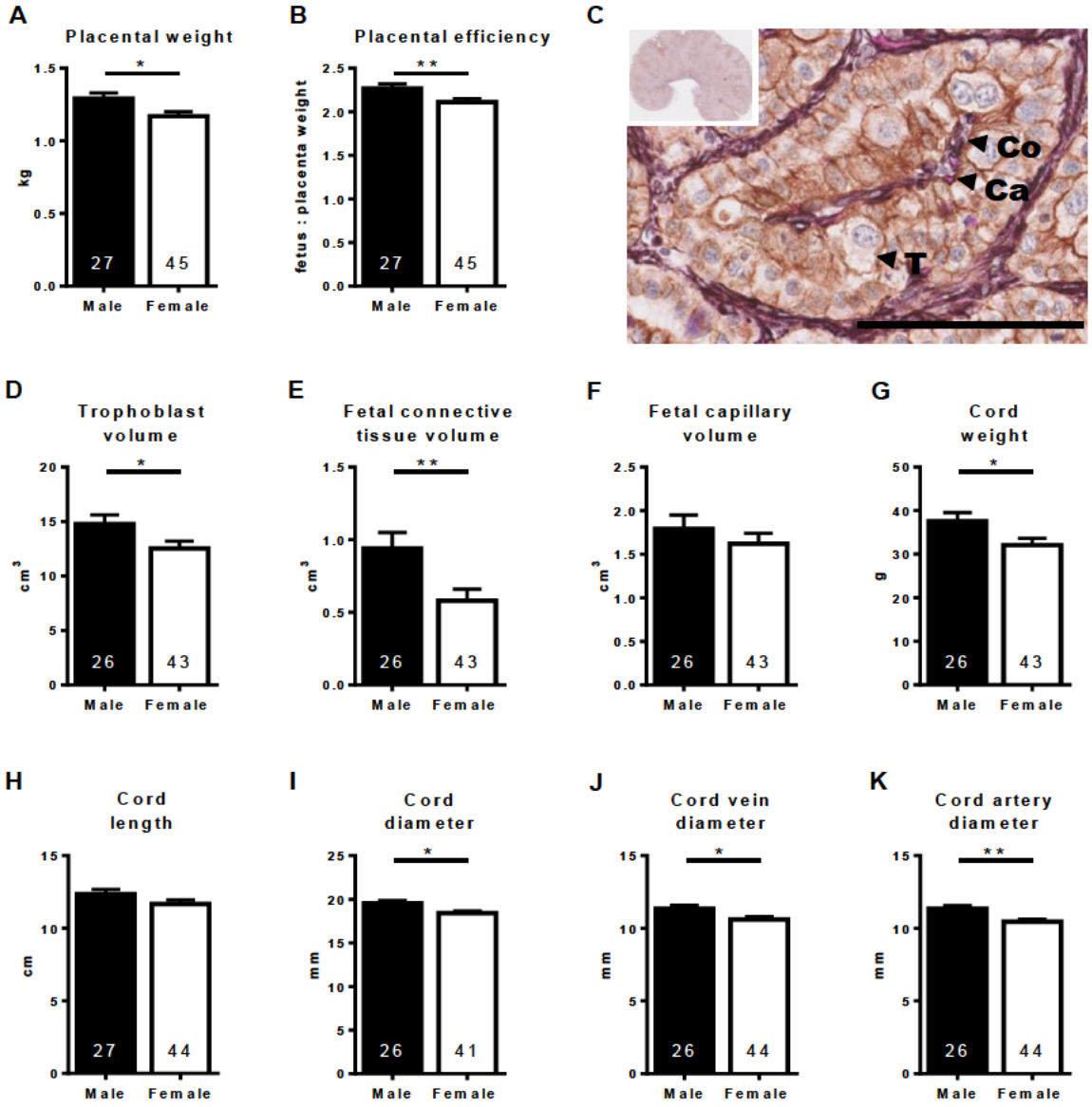

Figure 3.2 Placenta and umbilical cord phenotype evidence reduced nutrient supply and blood flow in female concepti.

Figure 3.2

(A) Placental weight and (B) placental efficiency are lower in females at mid-gestation (Day 153 post conception). (B) Placental efficiency calculated as fetal body weight : placental weight. (C) Representative photomicrograph shows histological detail of placenta (insert). Scale bar is 100 µM. Double label immunohistochemistry with Hematoxylin and Eosin as counterstains differentiates placental tissue components. T: Trophoblast, Co: fetal connective tissue, Ca:fetal capillary. (D-F) Placental tissue volumes calculated as volume density multiplied by the weight of the sample. (G-K) Umbilical cord characteristics. (A, B and D-K) Data are least square means ± SEM for males and females with numbers of individuals indicated inside bars. (A, B, D, E, G, H, J, K) Two tailed *t*-test was used to calculate statistical difference between groups. **P*<0.05; ***P*<0.01. (F, I) Wilcoxon two-sample test was used for fetal capillary volume, Ws =958, z=0.58, *P*>0.05 and for cord diameter, Ws =1043, z=2.04,**P*<0.05.

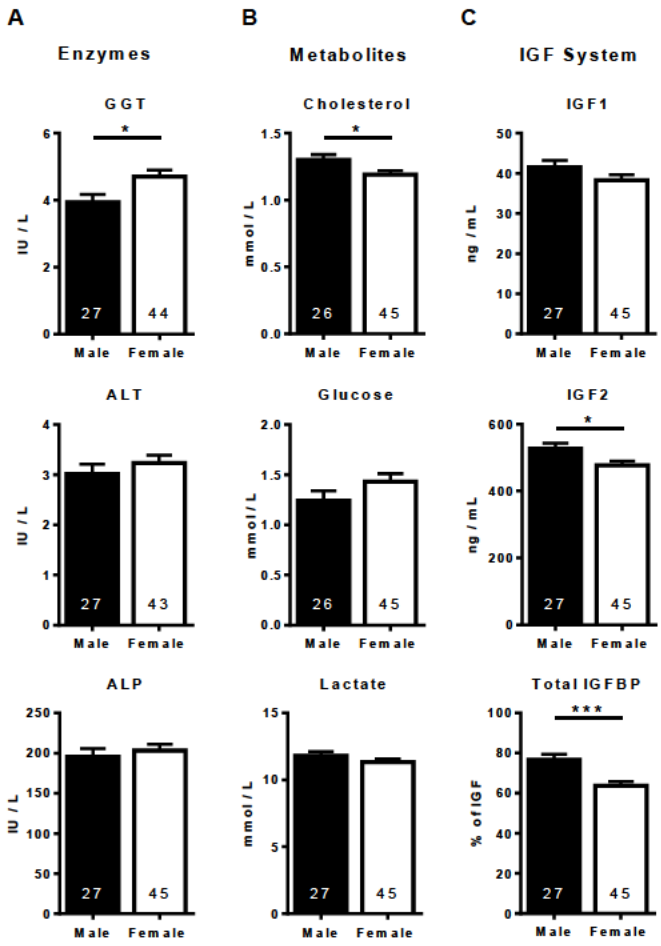

Figure 3.3 Cord blood indicators of growth restriction in female concepti implicate reduced nutrient supply and liver growth.

(A) As in neonates with intrauterine growth restriction (IUGR), γ-glutamyl transferase (GGT) is elevated in females at mid-gestation (day 153 post conception), indicating sex-specific differences in hepatocyte maturation and liver growth. Alanine transaminase (ALT) and Alkaline phosphatase (ALP) are similar in both sexes. (B) Metabolites previously associated with IUGR in neonates. As in IUGR, cholesterol is lower in females. Glucose and lactate remain unaffected at mid-gestation. (C) As in IUGR neonates, concentrations of circulating insulin-like growth factor (IGF) 2 and IGF binding proteins (IGFBP) are lower in females. Fetal IGF expression is positively regulated by nutrient supply and IGF2 signals fetal demand. (A-C) Data are least square means ± SEM for males and females with numbers of individuals indicated inside bars. With the exception of IGF1, two tailed *t*-test was used to calculate statistical difference between males and females. **P*<0.05; ****P*<0.001. (C) For plasma IGF1: Wilcoxon two-sample test, Ws =1029, z=0.5, *P*>0.05.

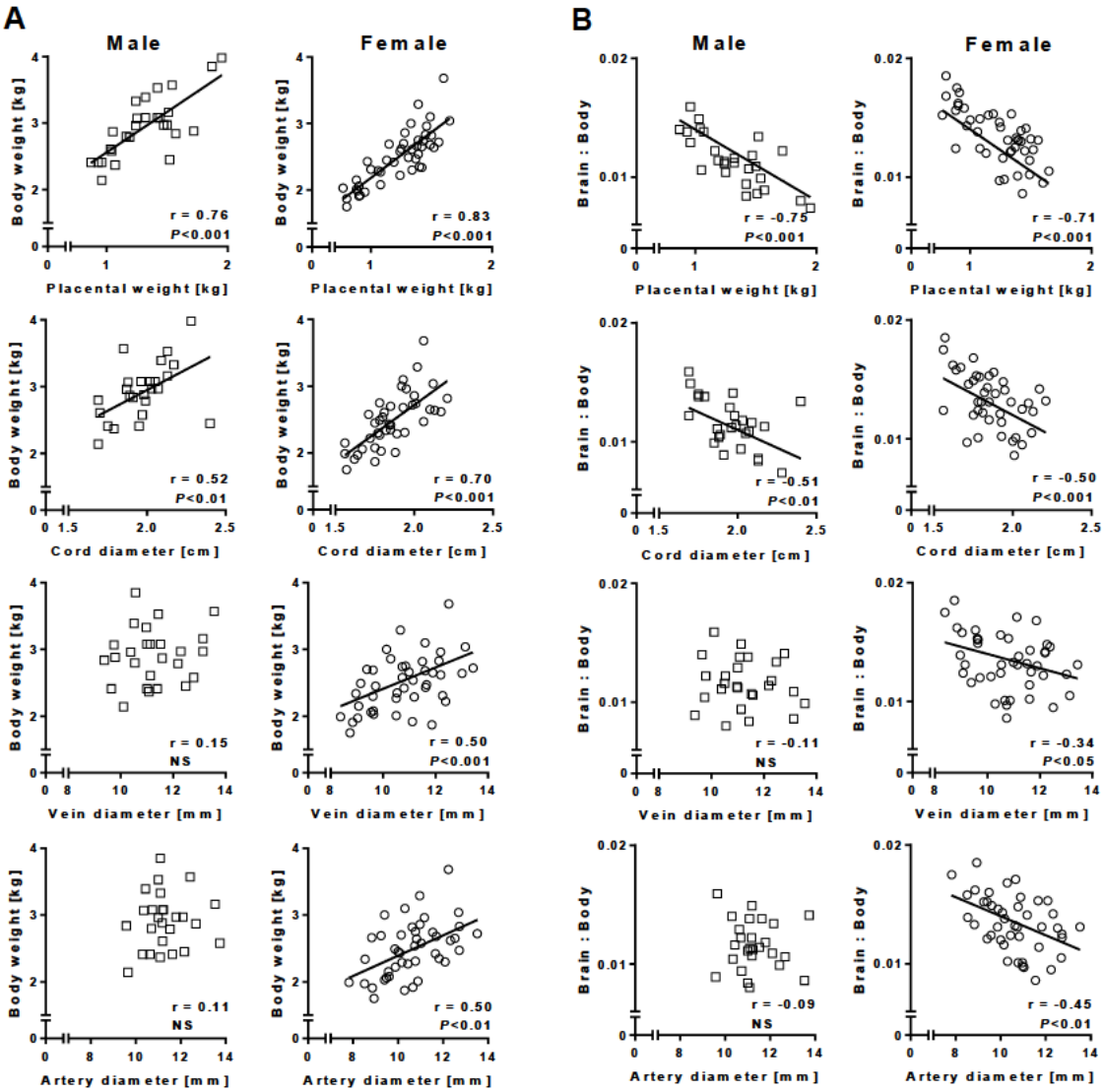

Figure 3.4 Phenotypic relationships of conceptus characteristics reflect female-specific hemodynamics.

(A) Sex-specific relationships of fetal body weight with placental weight and umbilical cord characteristics at mid-gestation (day 153). Female-specific positive regressions of body weight on cord vein and artery diameters demonstrate the growth limiting role of female cord vessels. (B) Relationships of brain to body weight ratio, an indicator of IUGR, with placental weight, umbilical cord diameter and cord vessel diameters. Female-specific negative regressions of brain to body weight ratio on umbilical vessel diameters suggest blood flow redistribution favouring the brain is enhanced with decreasing vessel diameters. Highly similar relationships were obtained for brain to liver weight ratio and are presented in Supplementary Figure S3. Regression lines for significant relationships and Pearson product moment correlation coefficients with *P*-values are indicated. NS: not significant, $P>0.05$.

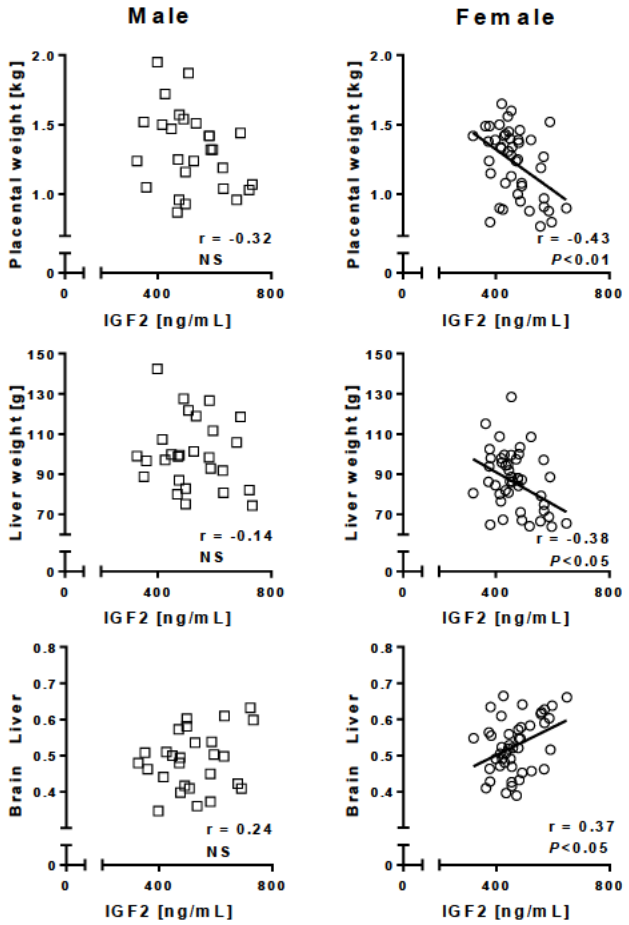

Figure 3.5 Phenotypic relationships provide evidence for involvement of the insulin-like growth factor axis in nutrient demand signalling of female fetuses.

Relationships of placental and fetal liver weights and brain to liver weight ratio with circulating fetal insulin-like growth factor 2 (IGF2) in male (left panel) and female (right panel) concepti at mid-gestation (day 153). Female-specific negative relationship of weights of organs crucial for fetal growth with IGF2, and the female-specific positive relationship of IGF2 with brain to liver weight ratio, a major indicator of IUGR, support a role of IGF2 as a fetal demand signal for nutrient supply. Regression lines for significant relationships and Pearson product moment correlation coefficients with *P*-values are indicated. NS: not significant, $P>0.05$.

3.3 Discussion

We have demonstrated that the female conceptus in normal pregnancy differs from the male in a striking array of features that are found in clinical IUGR (Table 3.1). These IUGR-like characteristics of females are expected to improve adaptive capacity and can explain the female's greater resilience to growth restricting intrauterine insults and sex-bias in fetal programming of predisposition to postnatal non-communicable disease.

At mid-gestation, upon entering accelerated fetal growth, females had a smaller placenta and lower body weight with lower organ weights than males and differed from males in major morphometric indicators of IUGR established in humans (Campbell and Thoms, 1977, Cetin et al., 2008, Platz and Newman, 2008) and animal models (Owens et al., 1994, Morrison, 2008). These include significantly higher brain to liver-, brain to body- and heart to body weight ratios of females. The higher relative lean body mass observed in IUGR newborns (Petersen et al., 1988) is also reflected in the female fetus of the present study, which is shorter and thinner and has a higher relative muscle and bone mass than males. In IUGR fetuses, these phenotypic asymmetries are a consequence of preferential blood flow redistribution to spare fetal brain and heart development in response to limited nutrient supply (Hershkovitz et al., 2000, Boito et al., 2003, Hobbins, 2008, Cetin et al., 2008).

Lower placental weight and efficiency in the female conceptus are consistent with previous data indicating sex-specific nutrient supply to the fetus (Thomson et al., 1969, Eriksson et al., 2010). However, our histomorphometric analyses of the placenta revealed additional differences between sexes, including lower trophoblast and fetal connective tissue volumes in females that are also found in the placenta of the IUGR neonate (Mayhew et al., 2003). Since trophoblast cells in apposition with the maternal epithelium facilitate oxygen and nutrient transfer (Wooding and Burton, 2008), and fetal placental connective tissue contains myofibroblasts whose contractile properties can compress or widen the intervillous space (Feller et al., 1985, Demir et al., 1997), these novel data suggest a mechanism where

Table 3.1 Biometric indicators of clinical intrauterine growth restriction (IUGR) and corresponding parameters in normal female concepti at mid-gestation as compared with males[A].

Clinical IUGR	Normal female concepti
Size and asymmetry (Platz and Newman, 2008)	
Smaller biparietal diameter, head circumference	Lower brain weight
Smaller abdominal circumference	Lower liver weight
Lower estimated fetal weight or birth weight	Lower fetal body weight
Elevated head to abdominal circumference ratio	Higher brain:liver weight ratio
Body composition (Cetin et al., 2008; Petersen et al., 2008)	
Thinness, low ponderal index	Thinner, lower ponderal index
Higher relative lean body mass	Higher muscle:fetal body weight ratio
	Higher bone:fetal body weight ratio
Reduced subcutaneous fat mass and lean mass	Lower muscle and bone mass, fat mass not determined
Placental characteristics (Platz and Newman, 2008; Mayhew et al., 2003)	
Smaller placenta	Lower placenta weight
Lower trophoblast volume	Lower trophoblast volume
Lower stromal volume	Lower fetal connective tissue volume/ volume density
Lower capillary volume	Capillary volume similar to males

Table 3.1 continued

Umbilical cord characteristics and blood flow	
(Bruch et al., 1997; Cetin et al., 2008; Raio et al., 2003)	
Increased blood flow resistance	Smaller umbilical cord vessel diameters
	Umbilical cord length similar to males
Lower umbilical venous volume flow, and/or smaller umbilical vein cross-sectional area	Smaller umbilical cord vein diameter
Thinner umbilical cord	Thinner umbilical cord
Centralization of fetal circulation or blood flow redistribution	Higher brain:liver weight ratio
	Higher brain:fetal body weight ratio
	Higher heart:fetal body weight ratio

[a] Mid-gestation is week 21.

differences in placental tissue composition contribute to lower nutrient transfer to the female fetus.

Substrates from trophoblast cells enter the fetal placental capillary networks that culminate in the umbilical cord vessels (Gude et al., 2004). In the present study, placental capillary volume and volume density did not differ between sexes, but umbilical cord vein and artery diameters of female concepti were smaller than those of males. In the course of uncomplicated pregnancy changes in umbilical vessel diameter correlate with changes in blood flow (Sutton et al., 1990) and in IUGR, lower umbilical cord vein cross-sectional area is associated with reduced venous flow (Boito et al., 2002). The effects of our observed differences in the female placental-umbilical vascular system on blood flow and nutrient supply are exacerbated by an umbilical cord that is similar in length to males, because blood flow resistance is proportionate to umbilical vessel length (Adamson, 1999, Sherwood, 2013). Furthermore, thicker cords contain higher volumes of Wharton's jelly, which enhances blood flow to the fetus (Di Naro et al., 2001) and lower cord diameter of females in the present study likely reflects less Wharton's jelly (Filiz et al., 2011). In any case, cord compression selectively increases resistance, especially in the venous outflow tract (Iwamoto et al., 1991, Adamson, 1999) and resistance to blood flow in smaller umbilical cord vessels of females is thus amplified by their thinner cord. Overall, our results indicate that an umbilical cord phenotype that is thin and long, with smaller vein and artery diameters, contributes to fetal growth restriction in females at mid-gestation.

The analyses of relationships between critical parameters of placental-fetal phenotype provided further evidence for sex-specific differences in blood supply to the fetus. In both sexes we observed strong positive relationships of fetal body- and liver weight with placental weight and umbilical cord diameter, but only females also displayed similar positive relationships of fetal body- and liver weight with both cord vessel diameters. This indicates that placental weight and umbilical cord diameter are general determinants of fetal growth,

while cord vessel diameters have critical growth-regulating effects in the female conceptus only. In IUGR brain to liver volume ratio is negatively correlated with umbilical venous volume flow relative to fetal weight, consistent with a restriction of fetal blood supply by smaller umbilical vein and artery diameters (Boito et al., 2003). Considering the IUGR-like phenotype of female concepti described in our study, it is therefore not surprising that we observed negative relationships of fetal brain to liver and fetal brain to body weight ratios with umbilical vein and artery diameters in females but not males. Taken together, our analyses of phenotypic relationships indicate that sex-specific phenotypes of both umbilical vein and artery contribute to lower blood flow in females, as described for asymmetric IUGR (Bruch et al., 1997, Raio et al., 2003).

High levels of cord blood γ-glutamyl transferase (GGT) activity at birth have been linked to hepatic immaturity (Garcia et al., 1987), placental insufficiency (Bartnicki et al., 1989) and IUGR (Johnson et al., 2003). This is consistent with higher GGT activity in cord blood of the female conceptus in the present study, which displayed lower placental capacity and a broad spectrum of other IUGR characteristics. Furthermore, we found that only in females were cord vessel diameters positive predictors of absolute liver weight, an indication for altered blood flow effects on liver growth and maturation. This is also consistent with the lower cholesterol levels we observed in the female conceptus; another characteristic of IUGR neonates (Pecks et al., 2012). Fetal *de novo* cholesterol synthesis occurs predominantly in the liver and demand for cholesterol is positively related to fetal growth rate (Haave and Innis, 2001). Thus, it appears that serum cholesterol is also affected by altered hemodynamics and lower perfusion and growth of the female fetal liver. Hypoglycemia is a common feature of IUGR neonates (Nieto-Diaz et al., 1996), but we found no evidence for lower cord blood glucose levels in the female conceptus at mid-gestation. This could be due to gestational age or experimental procedures, i.e., fasting dams prior to sacrifice (see methods).

The insulin-like growth factor (IGF) system is a major regulator of conceptus growth (Fowden, 2003) and fetal circulating IGF1 increases throughout gestation while IGF2 increases as gestation progresses and then declines in the third trimester (Reece et al., 1994, Carr et al., 1995). In the IUGR neonate cord serum levels of IGF1 and IGF2 are lower than in controls (Guidice et al., 1995) and, consistent with the described dominant role of IGF2 in early fetal growth and development (Gluckman, 1995), we found that cord serum IGF2, but not IGF1 levels, were lower in female concepti at mid-gestation. In addition, IGF2 gene expression in brain and heart tissue of females was lower than in males, while IGF1 gene expression was similar in all tissues studied. Our findings imply female-specific nutrient restriction, since fetal IGF expression is responsive to changes in placental nutritional supply (Owens et al., 1994, Gluckman, 1995). We found no relationship between fetal weight and fetal circulating IGF2 in either sex, indicating that growth promoting actions of IGF2 at mid-gestation are likely mediated through the placenta (Baker et al., 1993, Fowden, 2003). The female-specific negative relationships of placental and liver weights with circulating IGF2, and the female-specific positive relationship of brain to liver weight ratio with circulating IGF2, support the view that IGF2 may act as a fetal demand signal to the placenta, as previously demonstrated by gene deletion studies in mouse (Constancia et al., 2002, Constância et al., 2005). Growth-promoting effects of IGFs are regulated by binding proteins (IGFBPs) which transport IGFs in plasma, increase their half-life and regulate the availability of free IGFs (Rosenfeld et al., 1999). Lower IGFBP3 concentrations have been reported in IUGR neonates (Guidice et al., 1995) and we found lower cord serum total IGFBP levels in females at mid-gestation, potentially a compensatory mechanism to increase IGF availability in response to lower circulating IGF2. The mechanisms for differences in circulating IGFBPs are unclear; we found no sex-differences in expression of IGFBP1-6 genes in fetal brain, heart muscle, liver, skeletal muscle or placenta. However, lower circulating IGF2 levels observed in females were accompanied by similar differences in IGF2 gene expression in fetal heart

(see above), an organ that also displayed consistent trends (each $P<0.10$) for lower expression of IGFBP2, -3, and -6 genes in females. Thus, the present data indicate sex-specific expression of multiple components of the IGF axis.

We have shown that the normal female conceptus at mid-gestation is in a growth-restricting mode of development that resembles asymmetric IUGR. Female-specific relationships of fetal body weight and brain to liver weight ratios with umbilical cord artery and vein diameter indicate that the umbilico-placental vascular system is growth limiting for female fetuses. This, and reduction in size of critical umbilico-placental structures, creates a scenario where the female conceptus reorganizes distribution of venous blood flow to favor brain growth. Higher GGT and lower cholesterol levels in females as symptoms of altered liver perfusion lend further support to this phenotype, while lower circulating IGF2 indicates restricted nutrient supply or lower nutrient demand. Based on the extensive body of literature on prenatal programming of postnatal health and disease (Barker, 1995, Forsén et al., 1999, Eriksson et al., 2010, Hernandez-Medrano et al., 2015), we postulate that adaptation of females to lower nutrient supply by mid-gestation at the onset of accelerated growth has (i) long lasting physiological effects *per se* and (ii) confers resilience to the female fetus against insults later in pregnancy. This can explain better adjustment of females to changes in intrauterine environment (Clifton, 2010), higher risk for males of adverse perinatal outcomes (Vatten and Skjærven, 2004, Aibar et al., 2012), and ultimately, sex-bias in non-communicable disease later in life (Wild et al., 2004, Mikkola et al., 2013). Our study affirms the need for fetal sex-specific prenatal care to optimize health outcomes. Detailed molecular profiling of key tissues is now required to identify drivers of sex-specific fetal growth that will aid in identifying fetuses at risk and in developing intervention strategies.

3.4 Methods

3.4.1 Concepti

We used *Bos taurus taurus* (Angus, A) and *Bos taurus indicus* (Brahman, B) genetics to generate purebred and reciprocal cross concepti for comparison of placental and fetal parameters across a range of normal fetal weights (Supplementary Table S3.1). All animals and procedures used were approved by The University of Adelaide Animal Ethics Committee (No.S-094-2005). Nulliparous dams at 16-20 months of age were purchased from farms in South Australia and Queensland and transferred to Struan Agricultural Centre, Naracoorte, South Australia. Dams were managed and fed in one group. Pregnancies were established after standard estrous cycle synchronization procedures following an adjustment period of 3-4 weeks after animal purchase (Xiang et al., 2014). Conceptus sex was unknown until animals were sacrificed. A total of 45 female and 27 male concepti were recovered at day 153 ±1 post insemination (term 279-291 days, Andersen and Plum, 1965).

3.4.2 Collection of samples and phenotype data

Pregnant animals were fasted 24 h before sacrifice under standardized conditions in an abattoir. Entire uteri were removed and opened by longitudinal incision and the fetus removed after clamping and cutting the umbilical cord immediately above the placenta. Blood samples were collected in Lithium-Heparin-LH and Serum Z S-Monovettes® (Sarstedt) after cutting the cord above the clamp. Serum and plasma samples were obtained after centrifugation and stored frozen at –80 °C until further analysis. Fetal weight, cord weight and length and fetal organ weights (brain, heart, lung, liver and kidneys) were recorded and organ samples including skeletal muscle (*M. semitendinosus*) and placenta (cotyledon) stored in RNAlater (Ambion). A placentome close to the fetus was removed for histology and placed in a Petri dish with the chorionic plate facing up. A 5 mm-thick cross section through the center of this placental sample was excised and fixed in ice cold 4%

paraformaldehyde and 2.5% PVP-40 PBS for 24 h. The sample was then washed four times with 1% PBS and stored in 70% ethanol until embedding in paraffin. The entire umbilical cord was washed in 1% PBS and stored in 70% ethanol until further analysis of cord vessels. After collecting and weighing fetal fluids, eviscerated fetuses and uteri with attached placentae were vacuum-packed and stored frozen at –22°C until dissection for muscle, bone and placental parameters (see below). Ratios of organ to body weight were calculated by dividing organ weights by entire fetus weight. Brain to liver weight ratio was obtained in the same manner. Placental efficiency was calculated by dividing fetus weight by placental weight.

3.4.3 Fetal muscle, bone and ponderal index

Four representative muscles/muscle groups from front limb (*Musculus supraspinatus*), back (*M. longissimus dorsi*) and hind limb (*M. semimembranosus* and *M. quadriceps femoris*) were dissected and weighed (Xiang et al., 2013). Fetal muscle mass was calculated as the sum of the weights of these muscles. Twelve bones, *Os mandibulare*, *Os scapulare*, *Os humeri*, *Os radiale*, *Os ulnare*, *Ossa metacarpalia*, *Os costale VI*, *Os pelvis*, *Os femoris*, *Os tibiale*, *Ossa metatarsalia* and *Columna vertebralis* were removed and processed to obtain bone weights (Xiang et al., 2014). Fetal bone mass was calculated as the sum of the weights of the twelve bones. Femur length was measured between the most distal points of epiphyses and fetus length was measured as length of the spinal column. Ponderal index (PI) was calculated as fetus weight, kg/(total spine length, m)^3.

3.4.4 Umbilical cord and umbilical vessel diameters

Umbilical cord diameter was calculated from cord length and weight using the formula previously reported (Proctor et al., 2013). Cord artery and vein diameters were measured using a caliper after cross sectioning the cord at mid-length.

3.4.5 Immunohistochemisty and histomorphometric analysis of the placenta

Five µm thick longitudinal slices were cut from embedded samples and an indirect double label immunohistochemistry performed for three days as described previously (Roberts et al., 2001) with minor modifications. These included the use of 10% porcine serum and 1% BSA in 1% PBS to block non-specific binding, and 1:10 and 1:400 dilutions of the primary antibodies, mouse anti-human Vimentin clone Vim3B4 (DakoCytomation) and mouse anti-human cytokeratin AE1/AE3 (Millipore), respectively. Biotinylated goat anti-mouse antibody (DakoCytomation) in 10% pig serum at a 1:200 dilution was used as the secondary antibody. Hematoxylin (Sigma) and Eosin (Sigma) were used as counterstains and negative controls without primary antibodies were included in each batch.

A high resolution image of the whole stained placental section was captured using a NanoZoomer C9600 slide scanner (Hamamatsu Photonics K.K.). Ten fields at 200x magnification were selected and the first field's location chosen at random, followed by the other fields which were 1 mm apart, using the software NDP.view version 1.0.0 (Hamamatsu Photonics K.K.). Digital images of the fields were imported in Video Pro software (Leading Edge). With the aid of a L-36 Merz grid transparency overlaying the monitor screen, a total of 360 points were counted for each section. The volume densities of the different placental cell types including maternal epithelium, maternal capillaries, maternal connective tissue, trophoblast, fetal capillaries, fetal connective tissue and large maternal septa as 'other', were computed as previously reported (MacLaughlin et al., 2005). Trophoblast cells stained brown had one or two nuclei and were apposed to the maternal epithelium. Capillaries or blood vessels, located very close to trophoblast or maternal epithelial cells, were identified as having darkly stained circular or elongated opening (i.e., lumen of a blood vessel) with or without the presence of red blood cells. On the other hand, connective tissue appeared fibrous and not as darkly stained as a blood vessel or capillary opening. The volume in cm^3 of each placental component was determined based on the weight of analyzed placentomes after

confirming a 1:1 w/v relationship in collected *Placenta materna* samples. Other morphometric parameters such as trophoblast and maternal epithelium surface density (cm^2/g), total surface area (cm^2), and barrier thickness (µm) were estimated using intercept counting. A field was counted five times and the coefficient of variation was less than 5%.

3.4.6 Clinical-chemical parameters in cord serum

Cord serum samples were assayed for electrolytes (total Ca, Cl, Mg, Na, P, K), metabolites (albumin, cholesterol, creatinine, globulin, glucose, total protein, triglycerides, urea) and enzymes (ALP, ALT, GGT, GLDH) using the Beckman-Coulter AU Clinical Chemistry Analyser AU 480 (Beckman Coulter). Lactate and chloride were measured using a Radiometer 725 (Diamond Diagnostics).

3.4.7 Insulin-like growth factors (IGFs) and total IGF-binding protein (IGFBP) in cord plasma

Concentrations of cord plasma IGF1, IGF2 and total IGFBP binding were measured by RIA after separation of IGFs and IGFBPs by size-exclusion HPLC under acidic conditions as described previously for neonatal bovine plasma samples (Micke et al., 2010). Recovery of ^{125}I-IGF1 was 92.5 ± 0.5% for 9 HPLC runs of fetal plasma. Samples were assayed in triplicate in each assay. Plasma IGF1 concentrations were measured by analysis of neutralized HPLC fraction 3, in a RIA specific for IGF1 using a rabbit polyclonal antibody to human IGF1 (GroPep) (Francis et al., 1989). Total IGFBP binding protein (tIGFBP) concentrations were measured by analysis of neutralized fraction in the same assay. As IGFBP bind to and sequester ^{125}I-IGF1 in this assay, they can be measured due to their effect of reducing the amount of ^{125}I-IGF1 in the immunoprecipitated pellet, giving an apparent IGF concentration that reflects the total amount and binding affinity of IGFBP present in plasma (Sullivan et al., 2009). The inter-assay CV for HPLC separation and RIA of IGF1 was 5.4% (n = 5 assays) and the intra-assay CV for extraction and assay was 10.9% for a neonatal bovine plasma QC sample containing 43.9 ng/mL of IGF1. Plasma IGF2 concentrations were

measured by analysis of HPLC fraction 3 in a RIA specific for IGF2 (Carr et al., 1995). The inter-assay CV for HPLC separation and RIA of IGF2 was 2.1% (n = 3 assays) and the intra-assay covariance for extraction and assay was 13.7% for a neonatal bovine plasma QC sample containing 94.2 ng/mL of IGF2.

3.4.8 Expression of genes from the IGF system in placenta and fetal tissues

Relative expression levels of *IGF1*, *IGF2*, *IGF1R*, *IGF2R*, *INSRA*, *INSRB*, and *IGFBP1-6* were determined by real-time quantitative PCR (qPCR). RNA was extracted from brain (telencephalon), heart (apex), liver (*Lobus hepatis sinister*), skeletal muscle (*M. semitendinosus*) and placental (cotyledon) tissue using TRI Reagent (Ambion) in combination with ceramic beads (MoBio Laboratories) and a Precellys 24 tissue homogeniser (Bertin Technologies) following the manufacturer's instructions. RNA quantity and quality were assessed by NanoDrop ND-1000 spectrophotometer (Thermo Scientific) and Agilent RNA 6000 Nano Kit with Bioanalyzer 2100 (Agilent Technology). RNA integrity number averaged 8.4 for brain, 8.5 for heart, 8.1 for liver, 8.2 for skeletal muscle and 7.2 for placenta. After DNase treatment (RQ1-DNase, Promega), 500 ng RNA was reverse transcribed using Superscript III First-strand cDNA synthesis kit (Invitrogen) with random hexamer oligonucleotides following the manufacturer's instructions. Real-time qPCR reactions were performed in an Eppendorf Mastercycler ep realplex (Eppendorf Inc.) using 4 µL of 40-fold or 5.2 µL of 20-fold diluted cDNA, 0.8 µL of 5 pmol/µL primer mix and 6 µL of FastStart Universal SYBR Green Master (Roche Diagnostics) in a total volume of 12 µL. A standard curve comprised of a 2-fold serial dilution of pooled cDNA template for each fetal tissue over eight data points, as well as a non-template control, were included in each qPCR experiment. Fetal cDNA samples were measured in duplicate and cDNA standard curve data points in triplicate. Primer sequences and other details of amplicons from 12 target and five reference genes are shown in Supplemental Table S3.4. Target gene transcript abundance was

normalized to the geometric mean of the transcript abundances of the most stably expressed reference genes selected for each tissue using NormFinder (Andersen et al., 2004).

3.4.9 Statistical analyses

We used the general linear model procedure of SAS (SAS Inst.) and performed ANOVA to determine effects of fetal sex adjusted for fetal genetic effects on investigated parameters using the model:

$$y_{ij} = S_i + G_j + e_{ij}$$

where y_{ij} were bovine concepti characteristics, S ($_i$ = male, female) was fetal sex effect and G ($_j$ = A×A, B×A, A×B, and B×B) was fetal genetic effect. Tables S3.5 to S3.8 show general linear models and corresponding levels of significance of models and variables for parameters affected by fetal sex.

Least square means with standard errors of means for male and female fetuses were computed and compared using two-tailed *t*-test with a significance threshold of $P<0.05$. Data with residuals that failed to follow normal distribution and could not be normalized by logarithmic transformation were tested by Wilcoxon two-sample test with a significance threshold of two-sided $P< 0.05$ (Table S3.9).

To determine sex-specific relationships between gross morphological, histomorphological, endocrine and clinical-chemical parameters, the independent variable of interest was nested within fetal sex and sex-specific regression slopes were derived using the model:

$$y_i = S_i + X(S_i) + e_i$$

where y_i was the response variable, S ($_i$=male, female) the intercept estimate for fetal sex, and $X(S_i)$ corresponded to the independent variable nested within fetal sex. Sex-specific regression slopes and Pearson correlation coefficients were considered significant at $P<0.05$. Regressions and correlations were not adjusted for genetics in order to be able to show regression slopes and correlation coefficients with actual data points.

Acknowledgements

We are grateful for access to South Australian Research Development Institute research facilities and Struan Research Centre staff for animal management. We would also like to thank former members of the JS Davies Epigenetics and Genetics Group for their help in collection of samples and phenotype data. CASE is recipient of an Australia Awards Scholarship provided by the Australian Government and RX received a China Scholarship and an Adelaide University China Fee Scholarship provided by the China Scholarship Council and the University of Adelaide. AJ and MG-S received PhD Scholarships from the Iranian Ministry of Science, Research & Technology. ES receives a PhD Scholarship from the Libyan Ministry of Higher Education and Scientific Research. CR and VC are both supported by NHMRC Senior Research Fellowships (CTR GNT1020749, VCL APP1041918) and SH is a JS Davies Professorial Fellow. We thank the JS Davies Bequest for financial support.

Supplemental Tables

Table S 3.1, related to Results and Methods. Weight range and numbers of male (M) and female (F) fetuses with defined *Bos taurus taurus* (A) and *Bos taurus indicus* (B) genetics at mid-gestation (Day 153).

Sire genetics listed first. Least square means ± SEM are shown.

Fetal genetics	Sex	n	Weight (kg)
A × A	M	11	3.22 ± 0.10
A × A	F	12	2.79 ± 0.10
A × B	M	7	2.69 ± 0.12
A × B	F	6	2.26 ± 0.13
B × A	M	5	3.15 ± 0.14
B × A	F	17	2.64 ± 0.08
B × B	M	4	2.39 ± 0.16
B × B	F	10	2.04 ± 0.10

Table S 3.2, related to Figure 3.2. Histomorphometric placental parameters for male and female concepti at mid-gestation (Day 153).

Least square means ± SEM are shown.

	Male	**Female**	***P*-value[A]**
Volume density of placental tissues, V_d			
Maternal epithelium	0.413 ± 0.009	0.420 ± 0.007	0.5248
Maternal capillaries	0.062 ± 0.004	0.065 ± 0.003	0.5357
Maternal connective tissue	0.065 ± 0.004	0.071 ± 0.003	0.2700
Trophoblast	0.374 ± 0.007	0.358 ± 0.006	0.1096
Fetal capillaries	0.044 ± 0.003	0.046 ± 0.002	0.6283
Fetal connective tissue	0.025 ± 0.003	0.017 ± 0.002	0.0079
Others (maternal septa)	0.016 ± 0.003	0.021 ± 0.003	0.1548
Volume of placental tissues, cm^3			
Maternal epithelium	16.40 ± 1.02	14.74 ± 0.80	0.2026
Maternal capillaries	2.41 ± 0.18	2.28 ± 0.14	0.5713
Maternal connective tissue	2.62 ± 0.20	2.45 ±.0.16	0.4885
Trophoblast	14.77 ± 0.85	12.52 ± 0.67	0.0417
Fetal capillaries	1.79 ± 0.16	1.62 ± 0.12	0.5564
Fetal connective tissue	0.94 ± 0.11	0.58 ± 0.08	0.0075
Others (maternal septa)	0.64 ± 0.14	0.75 ± 0.11	0.5853

Table S 3.2 continued

Maternal and fetal exchange surface			
Trophoblast surface density, cm^2/g	244.27 ± 5.18	246.22 ± 4.06	0.7672
Maternal epithelium surface density, cm^2/g	255.97 ± 5.30	264.23 ± 4.14	0.2227
Trophoblast surface area, cm^2	9547.31 ± 540.86	8634.57 ± 424.09	0.1893
Maternal epithelium surface area, cm^2	10117.42 ± 597.48	9292.49 ± 468.49	0.2818
Trophoblast barrier thickness, µM	15.40 ± 0.38	14.67 ± 0.30	0.1405
Maternal epithelium barrier thickness, µM	16.34 ± 0.43	16.02 ± 0.34	0.5665

[A] Two-tailed *t*-test was used to identify significant differences at $P<0.05$ except for fetal capillary volume ($P>0.05$), volume densities of maternal septa ($P>0.05$) and fetal connective tissue ($P<0.01$), where Wilcoxon two-sample test was used.

Table S 3.3, related to Figure 3.3. Cord serum clinical-chemical parameters for male and female concepti at mid-gestation (Day 153).

Least square means ± SEM are shown.

	Male	Female	*P*-value[B]
Electrolytes, mmol/L			
Total calcium	3.21 ± 0.04	3.18 ± 0.03	0.5494
Chloride	101.24 ± 1.21	100.78 ±0 .95	0.6035
Magnesium	1.37 ± 0.03	1.37 ± 0.02	0.8901
Phosphorus	2.46 ± 0.06	2.40 ± 0.04	0.4021
Potassium	7.55 ± 0.18	7.19 ± 0.14	0.1343
Sodium	137.15 ± 1.49	135.96 ± 1.17	0.5608
Metabolites			
Albumin, g/L	17.21 ± 0.29	17.45 ± 0.22	0.5223
Cholesterol, mmol/L	1.30 ± 0.04	1.19 ± 0.03	0.0156
Creatinine, mmol/L	0.10 ± 0.00	0.10 ± 0.00	0.6244
Globulin, g/L	10.27 ± 0.16	10.29 ± 0.12	0.9482
Glucose, mmol/L	1.24 ± 0.10	1.43 ± 0.08	0.1341
Lactate, mmol/L	11.80 ± 0.31	11.37 ± 0.27	0.2314
Total protein, g/L	27.54 ± 0.46	27.96 ± 0.36	0.5364
Triglyceride, mmol/L	0.67 ± 0.02	0.63 ± 0.16	0.0870
Urea, mmol/L	5.14 ± 0.40	4.54 ± 0.32	0.2508

Table S 3.3 continued

Enzymes, IU/L[A]			
ALP	195.33 ± 10.14	203.04 ± 7.96	0.5534
ALT	3.01 ± 0.20	3.23 ± 0.16	0.3215
GGT	3.94 ± 0.23	4.71 ± 0.19	0.0120
GLDH	4.81 ± 1.36	7.35 ± 1.08	0.1967

[A] ALP: Alkaline phosphatase; ALT: Alanine transaminase; GGT: γ-glutamyl transferase; GLDH: Glutamate dehydrogenase.

[B] Two-tailed *t*-test was used to identify significant differences at $P<0.05$ except for chloride, sodium, creatinine and GLDH where Wilcoxon two-sample test was $P>0.05$.

Table S 3.4, related to Methods. PCR Primers, annealing temperatures and size of fragments used in real-time quantitative PCR measurement of transcript abundances for housekeeping and insulin-like growth factor (IGF) system target genes.

Gene		Primers[A]	Ta[B]	Size[C]	Accession no.[D]
VPS4A	F	GAA GAC AGA AGG CTA CTC GGG TG	60	106	NM_001046615.1
	R	ACA GAC CTT TTT GAA GTG TGT TGC T			
ACTB	F	CTC TTC CAG CCT TCC TTC CT	62	245	NM_173979.3
	R	CCA ATC CAC ACG GAG TAC TTG			
RPS9	F	TAG GCG CAG ACG GGC AAA CA	60	136	NM_001101152.2
	R	CCC ATA CTC GCC GAT CAG CTT CA			
GAPDH	F	GGG TCA TCA TCT CTG CAC CT	60	264	NW_003103940.1
	R	CAT AAG TCC CTC CAC GAT GC			
H3F3A	F	ACT GCT ACA AAA GCC GCT C	60	231	XM_003586223.1
	R	ACT TGC CTC CTG CAA AGC AC			
IGF1	F	GAT GCT CTC CAG TTC GTG TGC	58	140	NW_003103925.1
	R	TCC AGC CTC CTC AGA TCA CAG			
IGF1R	F	GAT CCC GTG TTC TTC TAC GTT C	58	100	XM_606794.3
	R	AAG CCT CCC ACT ATC AAC AGA A			
INSR-A	F	TCC TCA AGG AGC TGG AGG AGT	59	89	AJ488553
	R	TTT CCT CGA AGG CCT GGG GAT			
INSR-B	F	TCC TCA AGG AGC TGG AGG AGT	59	110	AJ320235
	R	TAG CGT CCT CGG CAA CAG G			
IGFBP1	F	ACC AGC CCA GAG AAT GTG TC	59	119	NW_003103902.1
	R	CTG ATG GCA TTC CAG AGG AT			
IGFBP2	F	CAC ATC CCC AAC TGT GAC AA	58	114	NW_001494682.3
	R	GAT CAG CTT CCC GGT GTT AG			

Table S 3.4 continued

IGFBP3	F	CTA CGA GTC TCA GAG CAC AG	58	103	NT_181996.1
	R	GTG GTT CAG CGT GTC TTC C			
IGFBP4	F	ATG TGC CTG ATG GAG AAA GG	57	106	NM_174557.3
	R	GCC ATC CTG TGA CTT CCT GT			
IGFBP5	F	CAA GCC AAG ATC GAA AGA GAC T	60	85	NM_001105327.1
	R	AAG ATC TTG GGC GAG TAG GTC T			
IGFBP6	F	GGA GAG AAT CCC AAG GAG AGT A	60	100	NM_001040495.1
	R	GAG TGG TAG AGG TCC CCG AGT			
IGF2	F	CTTCGCCTCGTGCTGCTATG	60	134	NM_174087.3
	R	GTCGGTTTATGCGGCTGGAT			
IGF2R	F	GATGGTAATGAGCAGGCTTACC	60	123	NM_174352.2
	R	ATCTCCTCCATCAGCCACTC			

[A] Forward (F) and reverse (R) primers are given. [B] Annealing temperature.
[C] Amplicon size in basepairs. [D] GenBank accession number of DNA sequence used for primer design.

Table S 3.5 General linear models (Type III sums of squares) with R-squared values and significance levels (*P*-values) of models and variables for fetal parameters with significant effect of sex.

Parameters	R-square	*P*-value		
		Model	Genetics	Sex
Body weight	0.61	<0.0001	<0.0001	0.0001
Body length	0.50	<0.0001	<0.0001	0.0224
Brain weight	0.15	0.0260	0.1709	0.0216
Bone weight	0.44	<0.0001	<0.0001	0.0014
Femur length	0.35	<0.0001	<0.0001	0.0510
Heart weight	0.57	<0.0001	<0.0001	0.0114
Kidney weight	0.52	<0.0001	<0.0001	0.0002
Lung weight	0.52	<0.0001	<0.0001	0.0005
Muscle weight	0.75	<0.0001	<0.0001	0.0022
Brain: Body	0.63	<0.0001	<0.0001	<0.0001
Brain:Liver	0.52	<0.0001	<0.0001	0.0007
Heart:Body	0.21	0.0034	0.0111	0.0045
Muscle:Body	0.61	<0.0001	<0.0001	0.0092
Bone:Body	0.21	0.0028	0.0025	0.0352
Ponderal Index	0.28	0.0020	0.1024	<0.0001

Table S 3.6 General linear models (Type III sums of squares) with R-squared values and significance levels (*P*-values) of models and variables for placental parameters with significant effect of sex.

Parameters	R^2	*P*-value		
		Model	Genetics	Sex
Placental gross morphology				
Total placenta weight	0.53	<0.0001	<0.0001	0.0164
Total placental efficiency	0.36	<0.0001	0.0002	0.0063
Cord weight	0.19	0.0098	0.0117	0.0228
Cord vein diameter	0.29	0.0002	0.0001	0.0102
Cord artery diameter	0.35	<0.0001	<0.0001	0.0014
Placental histomorphology				
Fetal connective tissue volume	0.14	0.0455	0.5993	0.0075
Trophoblast volume	0.17	0.0152	0.0294	0.0417

Table S 3.7 General linear models (Type III sums of squares) with R-squared values and significance levels (*P*-values) of models and variables for clinical-chemical and IGF parameters in fetal cord blood parameters with significant effect of sex.

Parameters	R^2	*P*-value		
		Model	Genetics	Sex
Clinical-chemical serum parameters				
Cholesterol	0.37	<0.0001	<0.0001	0.0156
GGT	0.38	<0.0001	<0.0001	0.0120
Fetal IGF plasma concentration				
Fetal IGF2	0.19	0.0071	0.0453	0.0234
Fetal IGFBP	0.35	<0.0001	<0.0001	0.0003

Table S 3.8 General linear models (Type III sums of squares) with R-squared values and significance levels (*P*-values) of models and variables for gene expression levels of IGF2 in selected fetal organs with significant effect of sex.

Parameters	R^2	*P*-value		
		Model	Genetics	Sex
IGF2 Brain	0.17	0.0246	0.0396	0.0343
IGF2 Heart	0.15	0.0321	0.3187	0.0062

Table S 3.9 Wilcoxon two-sample test results for parameters with significant effects of sex where residuals do not follow normal distribution after logarithmic transformation.

Parameters	Wilcoxon Two-sample Test Two sided Pr>/z/
Liver weight	0.0029, z = 2.97, Statistic = 1242
Cord diameter	0.0408, z = 2.05, Statistic = 1043
Fetal connective tissue volume density	0.0079, z = 2.65, Statistic = 1124

Supplemental Figures

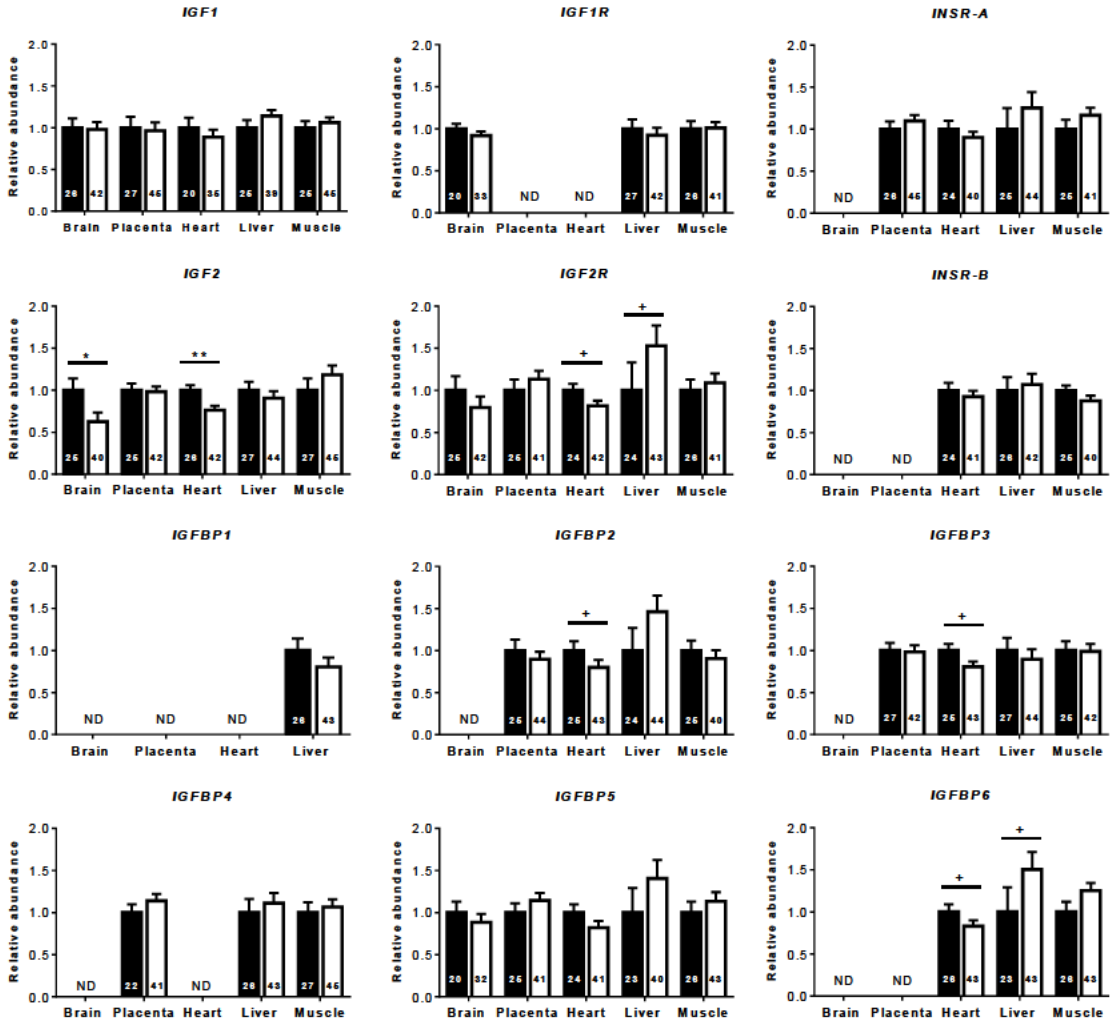

Figure S 3.1, related to Results. Transcript abundances for genes of the insulin-like growth factor (IGF) system in tissues of male and female concepti.

Transcript abundances measured for females (white bar) are compared within tissue relative to values obtained for males (black bar). Data are least square means ± SEM relative to males set as 1 with numbers of individuals indicated inside bars. PCR primers and annealing temperatures for target and housekeeper genes are presented in supplementary table S2. ND: Transcript not detected. Two tailed *t*-test was used to calculate statistical difference between groups. $^{+}P<0.10$; $^{*}P<0.05$; $^{**}P<0.01$. Wilcoxon two sample test was used for IGFBP2 in heart, $^{+}P<0.10$.

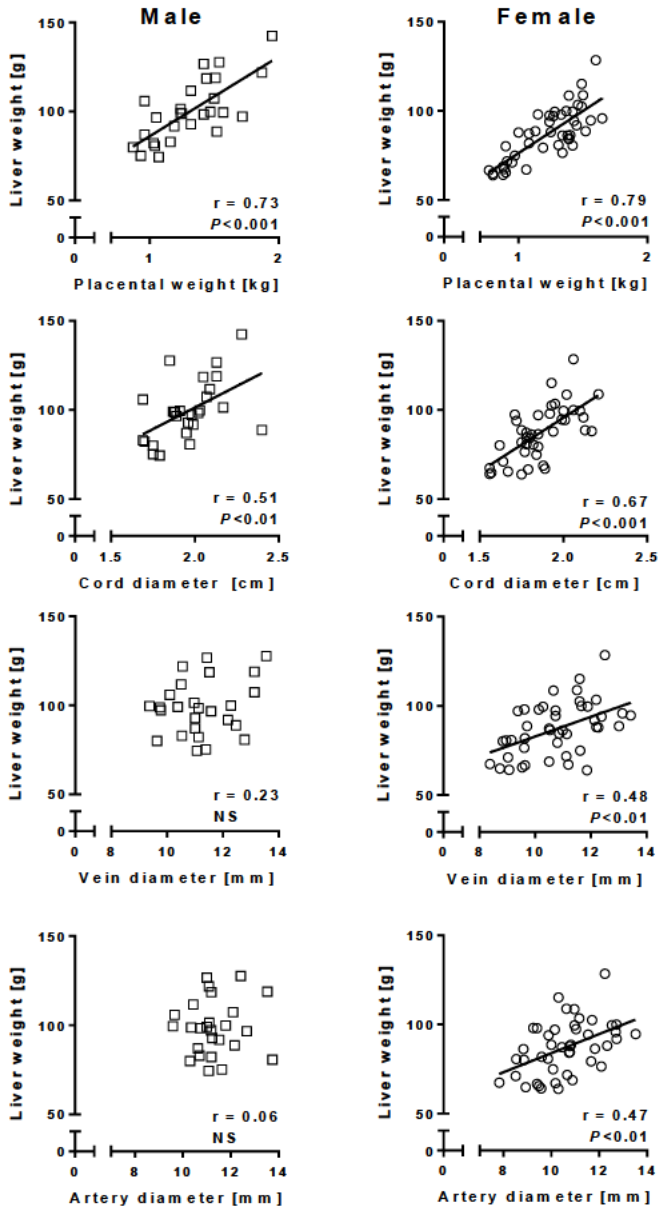

Figure S 3.2, related to Figure 3.4A. Relationships of liver weight with placental weight and umbilical cord characteristics in male and female concepti.

Vein: Umbilical cord vein. Artery: Umbilical cord artery. Regression lines for significant relationships, Pearson product moment correlation coefficients and *P*-values are indicated. NS: not significant, $P>0.05$.

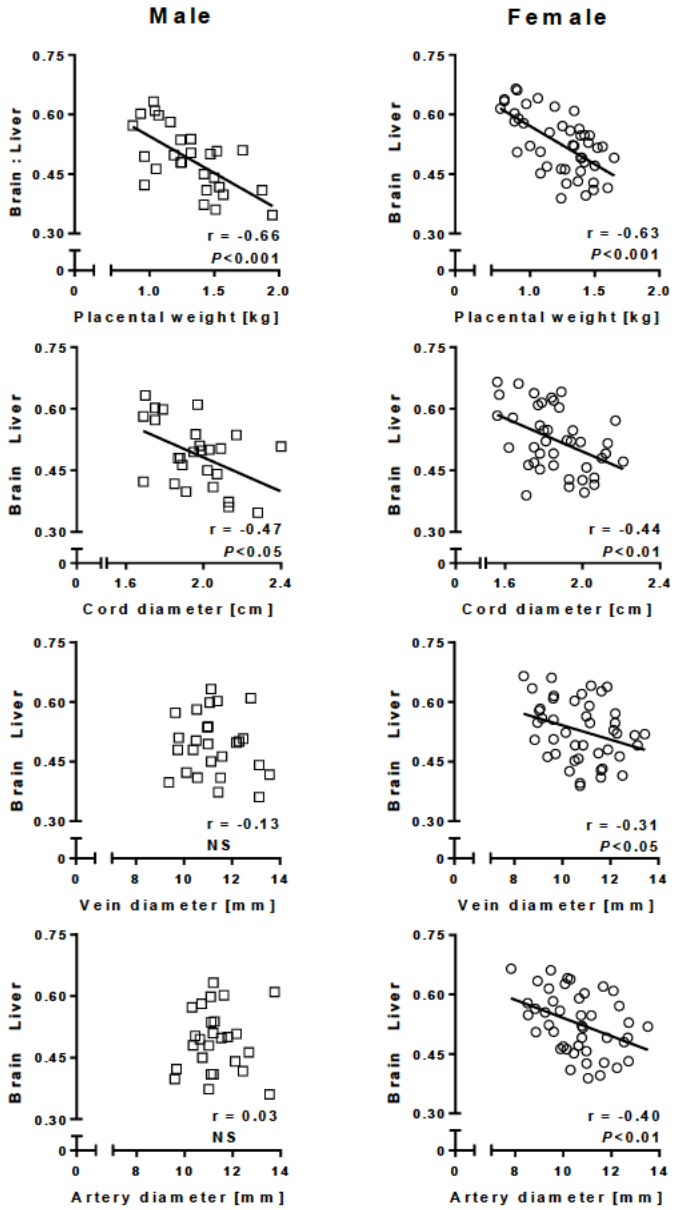

Figure S 3.3, related to Figure 3.4B. Relationships of brain to liver weight ratio with placental weight and umbilical cord characteristics in male and female concepti.

Vein: Umbilical cord vein. Artery: Umbilical cord artery. Regression lines for significant relationships, Pearson product moment correlation coefficients and *P*-values are indicated. NS: not significant, $P>0.05$.

3.5 Literature Cited

ADAMSON, S. L. 1999. Arterial pressure, vascular input impedance, and resistance as determinants of pulsatile blood flow in the umbilical artery. *European Journal of Obstetrics & Gynecology and Reproductive Biology,* 84**,** 119-125.

AIBAR, L., CARRILLO, M. P., MONTOYA, F., PUERTAS, A. & VALVERDE, M. 2012. Fetal sex and perinatal outcomes. *Journal of Perinatal Medicine,* 40**,** 271-276.

ALMOG, B., SHEHATA, F., ALJABRI, S., LEVIN, I., SHALOM-PAZ, E. & SHRIM, A. 2011. Placenta weight percentile curves for singleton and twins deliveries. *Placenta,* 32**,** 58-62.

ANDERSEN, C. L., JENSEN, J. L. & ØRNTOFT, T. F. 2004. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Research,* 64**,** 5245-5250.

ANDERSEN, H. & PLUM, M. 1965. Gestation length and birth weight in cattle and buffaloes: a review. *Journal of Dairy Science,* 48**,** 1224-1235.

BAKER, J., LIU, J.-P., ROBERTSON, E. J. & EFSTRATIADIS, A. 1993. Role of insulin-like growth factors in embryonic and postnatal growth. *Cell,* 75**,** 73-82.

BARKER, D. J. P. 1995. Fetal origins of coronary heart disease. *British Medical Journal,* 311**,** 171-174.

BARTNICKI, J., SLEDZIEWSKI, A., JÓŹWIK, M. & URBAN, J. 1989. Umbilical cord gamma-glutamyl transpeptidase and placental dysfunction. *International Journal of Gynecology & Obstetrics,* 29**,** 31-34.

BERTINO, E., OCCHI, L. & FABRIS, C. 2012. Intrauterine growth restriction: neonatal aspects. *In:* BUONOCORE, G., BRACCI, R. & WEINDLING, M. (eds.) *Neonatology.* Italy: Springer Milan.

BOITO, S., STRUIJK, P. C., URSEM, N. T. C., FEDELE, L. & WLADIMIROFF, J. W. 2003. Fetal brain/liver volume ratio and umbilical volume flow parameters relative to normal and abnormal human development. *Ultrasound in Obstetrics and Gynecology,* 21**,** 256-261.

BOITO, S., STRUIJK, P. C., URSEM, N. T. C., STIJNEN, T. & WLADIMIROFF, J. W. 2002. Umbilical venous volume flow in the normally developing and growth-restricted human fetus. *Ultrasound in Obstetrics and Gynecology,* 19**,** 344-349.

BOLKER, J. 2012. Model organisms: there's more to life than rats and flies. *Nature,* 491**,** 31-33.

BRUCH, J. F., SIBONY, O., BENALI, K., CHALLIER, J. C., BLOT, P. & NESSMANN, C. 1997. Computerized microscope morphometry of umbilical vessels from pregnancies with intrauterine growth retardation and abnormal umbilical artery doppler. *Human Pathology,* 28**,** 1139-1145.

CAMPBELL, S. & THOMS, A. 1977. Ultrasound measurement of the fetal head to abdomen circumference ratio in assessment of growth retardation. *British Journal of Obstetrics & Gynaecology,* 84**,** 165-174.

CARR, J., OWENS, J. A., GRANT, P., WALTON, P., OWENS, P. & WALLACE, J. 1995. Circulating insulin-like growth factors (IGFs), IGF-binding proteins (IGFBPs) and tissue mRNA levels of IGFBP-2 and IGFBP-4 in the ovine fetus. *Journal of Endocrinology,* 145**,** 545-557.

CETIN, I., BOITO, S. & RADAELLI, T. 2008. Evaluation of fetal growth and fetal well-being. *Seminars in Ultrasound, CT and MRI,* 29**,** 136-146.

CLIFTON, V. L. 2010. Review: Sex and the human placenta: mediating differential strategies of fetal growth and survival. *Placenta,* 31**,** S33-S39.

CONSTÂNCIA, M., ANGIOLINI, E., SANDOVICI, I., SMITH, P., SMITH, R., KELSEY, G., DEAN, W., FERGUSON-SMITH, A., SIBLEY, C. P., REIK, W. & FOWDEN, A. 2005. Adaptation of nutrient supply to fetal demand in the mouse involves interaction between the Igf2 gene and placental transporter systems. *Proceedings of the National Academy of Sciences of the United States of America,* 102**,** 19219-19224.

CONSTANCIA, M., HEMBERGER, M., HUGHES, J., DEAN, W., FERGUSON-SMITH, A. C., FUNDELE, R., STEWART, F., KELSEY, G., FOWDEN, A., SIBLEY, C. & REIK, W. 2002. Placental-specific IGF-II is a major modulator of placental and fetal growth. *Nature,* 417**,** 945-948.

DEMIR, R., KOSANKE, G., KERTSCHANSKA, S. & KAUFMANN, P. 1997. Classification of human placental stem villi:review of structural and functional aspects. *Microscopy Research and Technique,* 38**,** 29-41.

DI NARO, E., GHEZZI, F., RAIO, L., FRANCHI, M., D'ADDARIO, V., LANZILLOTTI, G. & SCHNEIDER, H. 2001. Umbilical vein blood flow in fetuses with normal and lean umbilical cord. *Ultrasound in Obstetrics and Gynecology,* 17**,** 224-228.

ERIKSSON, J. G., KAJANTIE, E., OSMOND, C., THORNBURG, K. & BARKER, D. J. P. 2010. Boys live dangerously in the womb. *American Journal of Human Biology,* 22**,** 330-335.

FELLER, A. C., SCHNEIDER, H., SCHMIDT, D. & PARWARESCH, M. R. 1985. Myofibroblast as a major cellular constituent of villous stroma in human placenta. *Placenta,* 6**,** 405-415.

FERRELL, C. L. 1989. Placental regulation of fetal growth. *In:* CAMPION, D. R., HAUSMAN, G. J. & MARTIN, R. J. (eds.) *Animal Growth Regulation.* USA: Springer US.

FILIZ, A. A., RAHIME, B., KESKIN, H. L. & ESRA, A. K. 2011. Positive correlation between the quantity of Wharton's jelly in the umbilical cord and birth weight. *Taiwanese Journal of Obstetrics and Gynecology,* 50**,** 33-36.

FORSÉN, T., ERIKSSON, J. G., TUOMILEHTO, J., OSMOND, C. & BARKER, D. J. P. 1999. Growth in utero and during childhood among women who develop coronary heart disease: longitudinal study. *British Medical Journal,* 319**,** 1403-1407.

FOWDEN, A. L. 2003. The insulin-like growth factors and feto-placental growth. *Placenta,* 24**,** 803-812.

FRANCIS, G., OWENS, P., MCNEIL, K., WALLACE, J. & BALLARD, F. 1989. Purification, amino acid sequences and assay cross-reactivities of porcine insulin-like like growth factor-I and -II. *Journal of Endocrinology,* 122**,** 681-687.

GARCIA, M. P., TUTOR, J. C., SANJOSE, M. E., PORTO, J. A., FRAGA, J. M., PAZ, J. M. & RODRIGUEZ-SEGADE, S. 1987. Cord serum gamma glutamyltransferase in newborns. *Clinical Biochemistry,* 20**,** 269-273.

GEORGIADIS, L., KESKI-NISULA, L., HARJU, M., RÄISÄNEN, S., GEORGIADIS, S., HANNILA, M. L. & HEINONEN, S. 2014. Umbilical cord length in singleton gestations: a Finnish population-based retrospective register study. *Placenta,* 35**,** 275-280.

GLUCKMAN, P. D. 1995. Insulin-like growth factors and their binding proteins. *In:* HANSON, M. A., SPENCER, J. A. D. & RODECK, C. H. (eds.) *Fetus and Neonate: Physiology and Clinical Applications.* UK: Cambridge University Press.

GUDE, N. M., ROBERTS, C. T., KALIONIS, B. & KING, R. G. 2004. Growth and function of the normal human placenta. *Thrombosis Research,* 114**,** 397-407.

GUIDICE, L. C., DE ZEGHER, F., GARGOSKY, S. E., DSUPIN, B. A., DE LAS FUENTES, L., CRYSTAL, R. A., HINTZ, R. L. & ROSENFELD, R. G. 1995. Insulin-like growth factors and their binding proteins in the term and preterm human fetus and neonate with normal and extremes of intrauterine growth. *The Journal of Clinical Endocrinology & Metabolism,* 80**,** 1548-1555.

HAAVE, N. C. & INNIS, S. M. 2001. Cholesterol synthesis and accretion within various tissues of the fetal and neonatal rat. *Metabolism: clinical and experimental,* 50**,** 12-18.

HERNANDEZ-MEDRANO, J. H., COPPING, K. J., HOARE, A., WAPANAAR, W., GRIVELL, R. G., KUCHEL, T., MIGUEL-PACHECO, G., MCMILLEN, I. C., RODGERS, R. J. & PERRY, V. E. A. 2015. Gestational dietary protein is associated with sex specific decrease in blood flow, fetal heart growth and post-natal blood pressure of progeny. *PLOS ONE,* 10**,** e0125694.

HERSHKOVITZ, R., KINGDOM, J. C. P., GEARY, M. & RODECK, C. H. 2000. Fetal cerebral blood flow redistribution in late gestation: identification of compromise in small fetuses with normal umbilical artery Doppler. *Ultrasound in Obstetrics and Gynecology,* 15**,** 209-212.

HOBBINS, J. C. 2008. Intrauterine growth restriction. *Obstetric Ultrasound: Artistry in Practice.* USA: Blackwell Publishing Inc.

IWAMOTO, H. S., STUCKY, E. & ROMAN, C. M. 1991. Effect of graded umbilical cord compression in fetal sheep at 0.6-0.7 gestation. *American Journal of Physiology - Heart and Circulatory Physiology,* 261**,** H1268-H1274.

JOHNSON, J. K., GOPALAKRISHAN, K., JOSEPH, A. & LINDOW, S. W. 2003. Cord blood gamma glutamyl transferase activity in complicated pregnancies. *Journal of Obstetrics & Gynaecology,* 23 Supplement 1**,** S32-S74.

MACLAUGHLIN, S. M., WALKER, S. K., ROBERTS, C. T., KLEEMANN, D. O. & MCMILLEN, I. C. 2005. Periconceptional nutrition and the relationship between maternal body weight changes in the periconceptional period and feto-placental growth in the sheep. *The Journal of Physiology,* 565**,** 111-124.

MAYHEW, T. M., OHADIKE, C., BAKER, P. N., CROCKER, I. P., MITCHELL, C. & ONG, S. S. 2003. Stereological investigation of placental morphology in pregnancies complicated by pre-eclampsia with and without intrauterine growth restriction. *Placenta,* 24**,** 219-226.

MELAMED, N., YOGEV, Y. & GLEZERMAN, M. 2010. Fetal gender and pregnancy outcome. *The Journal of Maternal-Fetal & Neonatal Medicine,* 23**,** 338-344.

MICKE, G., SULLIVAN, T., GATFORD, K., OWENS, J. A. & PERRY, V. 2010. Nutrient intake in the bovine during early and mid-gestation causes sex-specific changes in progeny plasma IGF-I, liveweight, height and carcass traits. *Animal Reproduction Science,* 121**,** 208-217.

MIKKOLA, T. S., GISSLER, M., MERIKUKKA, M., TUOMIKOSKI, P. & YLIKORKALA, O. 2013. Sex differences in age-related cardiovascular mortality. *PLOS ONE,* 8**,** e63347.

MORRISON, J. L. 2008. Sheep models of intrauterine growth restriction: fetal adaptations and consequences. *Clinical & Experimental Pharmacology & Physiology,* 35**,** 730-743.

NIETO-DIAZ, A., VILLAR, J., MATORRAS-WEINIG, R. & VALENZUELA-RUIZ, P. 1996. Intrauterine growth retardation at term: association between anthropometric and endocrine parameters. *Acta Obstetricia et Gynecologica Scandinavica,* 75**,** 127-131.

OTT, W. J. & DOYLE, S. 1982. Normal ultrasonic fetal weight curve. *Obstetrics & Gynecology,* 59**,** 603-606.

OWENS, J. A., KIND, K. L., CARBONE, F., ROBINSON, J. S. & OWENS, P. C. 1994. Circulating insulin-like growth factors-I and -II and substrates in fetal sheep following restriction of placental growth. *Journal of Endocrinology,* 140**,** 5-13.

PECKS, U., BRIEGER, M., SCHIESSL, B., BAUERSCHLAG, D. O., PIROTH, D., BRUNO, B., FITZNER, C., ORLIKOWSKY, T. & MAASS, N. 2012. Maternal and fetal cord blood lipids in intrauterine growth restriction. *Journal of Perinatal Medicine,* 40**,** 287-296.

PETERSEN, S., GOTFREDSEN, A. & KNUDSEN, F. U. 1988. Lean body mass in small for gestational age and appropriate for gestational age infants. *The Journal of Pediatrics,* 113**,** 886-889.

PLATZ, E. & NEWMAN, R. 2008. Diagnosis of IUGR: traditional biometry. *Seminars in Perinatology,* 32**,** 140-147.

PROCTOR, L. K., FITZGERALD, B., WHITTLE, W. L., MOKHTARI, N., LEE, E., MACHIN, G., KINGDOM, J. C. P. & KEATING, S. J. 2013. Umbilical cord diameter percentile curves and their correlation to birth weight and placental pathology. *Placenta,* 34**,** 62-66.

RAIO, L., GHEZZI, F., DI NARO, E., DUWE, D. G., CROMI, A. & SCHNEIDER, H. 2003. Umbilical cord morphologic characteristics and umbilical artery doppler parameters in intrauterine growth–restricted fetuses. *Journal of Ultrasound in Medicine,* 22**,** 1341-1347.

REECE, E. A., WIZNITZER, A., LE, E., HOMKO, C. J., BEHRMAN, H. & SPENCER, E. M. 1994. The relation between human fetal growth and fetal blood levels of insulin-like growth factors I and II, their binding proteins and receptors. *Obstetrics & Gynecology,* 84**,** 88-95.

RICH-EDWARDS, J. W., STAMPFER, M. J., MANSON, J. E., ROSNER, B., HANKINSON, S. E., COLDITZ, G. A., HENNEKENS, C. H. & WILLET, W. C. 1997. Birth weight and risk of cardiovascular disease in a cohort of women followed up since 1976. *BMJ,* 315**,** 396-400.

ROBERTS, C. T., SOHLSTROM, A., KIND, K. L., EARL, R. A., KHONG, T. Y., ROBINSON, J. S., OWENS, P. C. & OWENS, J. A. 2001. Maternal food restriction reduces the exchange surface area and increases the barrier thickness of the placenta in the guinea-pig. *Placenta,* 22**,** 177-185.

ROSENFELD, R. G., HWA, V., WILSON, L., LOPEZ-BERMEJO, A., BUCKWAY, C., BURREN, C., CHOI, W. K., DEVI, G., INGERMANN, A., GRAHAM, D., MINNITI, G., SPAGNOLI, A. & OH, Y. 1999. The insulin-like growth factor binding protein superfamily: new perspectives. *Pediatrics,* 104**,** 1018-1021.

ROSSO, P. & WINICK, M. 1974. Intrauterine growth retardation: a new systematic approach based on clinical and biochemical characteristics of this condition. *Journal of Perinatal Medicine* 2**,** 147.

SHERWOOD, L. 2013. *Human Physiology: From Cells to Systems,* USA, Brooks Cole, Cengage Learning.

SULLIVAN, T., MICKE, G., PERKINS, N., MARTIN, G., WALLACE, C., GATFORD, K., OWENS, J. & PERRY, V. 2009. Dietary protein during gestation affects maternal insulin-like growth factor, insulin-like growth factor binding protein, leptin concentrations, and fetal growth in heifers. *Journal of Animal Science,* 87**,** 3304-3316.

SUTTON, M. S. J., THEARD, M. A., BHATIA, S. J. S., PLAPPERT, T., SALTZMAN, D. H. & DOUBILET, P. 1990. Changes in placental blood flow in the normal human fetus with gestational age. *Pediatric Research,* 28**,** 383-387.

THOMSON, A. M., BILLEWICZ, W. Z. & HYTTEN, F. E. 1969. The weight of the placenta in relation to birthweight. *British Journal of Obstetrics & Gynaecology,* 76**,** 865-872.

VATTEN, L. J. & SKJÆRVEN, R. 2004. Offspring sex and pregnancy outcome by length of gestation. *Early Human Development,* 76**,** 47-54.

VERBURG, B. O., JADDOE, V. W. V., WLADIMIROFF, J. W., HOFMAN, A., WITTEMAN, J. C. M. & STEEGERS, E. A. P. 2008. Fetal hemodynamic adaptive changes related to intrauterine growth: the Generation R study. *Circulation,* 117**,** 649-659.

WILD, S., SICREE, R., ROGLIC, G., KING, H. & GREEN, A. 2004. Global prevalence of diabetes. *Diabetes Care,* 27**,** 1047-1053.

WOODING, F. B. P. & BURTON, G. 2008. Placentation fundamentals. *In:* WOODING, F. B. P. & BURTON, G. (eds.) *Comparative Placentation.* Berlin, Hiedelberg: Springer-Verlag.

XIANG, R., GHANIPOOR-SAMAMI, M., JOHNS, W. H., EINDORF, T., RUTLEY, D. L., KRUK, Z. A., FITZSIMMONS, C. J., THOMSEN, D. A., ROBERTS, C. T., BURNS, B. M., ANDERSON, G. I., GREENWOOD, P. L. & HIENDLEDER, S. 2013. Maternal and paternal genomes differentially affect myofibre characteristics and muscle weights of bovine fetuses at midgestation. *PLOS ONE,* 8**,** e53402.

XIANG, R., LEE, A. M. C., EINDORF, T., JAVADMANESH, A., GHANIPOOR-SAMAMI, M., GUGGER, M., FITZSIMMONS, C. J., KRUK, Z. A., PITCHFORD, W. S., LEVITON, A. J., THOMSEN, D. A., BECKMAN, I., ANDERSON, G. I., BURNS, B. M., RUTLEY, D. L., XIAN, C. J. & HIENDLEDER, S. 2014. Widespread differential maternal and paternal genome effects on fetal bone phenotype at mid-gestation. *Journal of Bone and Mineral Research,* 29**,** 2392–2404

ZARÉN, B., LINDMARK, G. & BAKKETEIG, L. 2000. Maternal smoking affects fetal growth more in the male fetus. *Paediatric and Perinatal Epidemiology,* 14**,** 118-126.

Chapter 4 Polar overdominance in combination with maternal genome effects drives mammalian heterosis *in utero*

Manuscript in preparation for submission to **PloS Biology**, with the exception that figures and tables are embedded in the text and citations are formatted in Harvard style to be consistent with the other chapters.

Statement of Authorship

Title of Paper	Polar over dominance in combination with maternal genome effects drives mammalian heterosis *in utero*
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Manuscript format for submission to PloS Biology, except that figures and tables are embedded on the text, and bibliography and citation follows the Harvard style of referencing to maintain consistency with the other chapters.

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Overall percentage (%)	80%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date: February 14, 2016

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Contribution to the Paper	Designed the experiment, interpreted data, worked with CASE to finalize manuscript
Signature	Date: 14/02/2016

Polar overdominance in combination with maternal genome effects drives mammalian heterosis *in utero*

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Abstract

Heterosis, defined as the superior performance of F_1 hybrids over their parents, has been used for centuries to increase yield in plants and animals. However, the biological basis of heterosis is poorly understood as it does not follow standard genetic models. Based on theoretical prediction that genomic imprinting may mimic overdominance and heterosis, we investigated if imprinting effects could explain heterosis. We used purebred and reciprocal cross *Bos taurus taurus* (Angus, A) and *Bos taurus indicus* (Brahman, B) cattle that display one of the strongest known heterotic phenotypes in mammals. We intercepted concepti at mid-gestation, when the fetus enters accelerated growth but does not yet display heterosis in weight, to map drivers of heterosis in placenta as the major organ regulating prenatal growth that predicts postnatal performance. Our analyses at the gross morphological, histomorphological and molecular levels revealed 9 maternal, 3 paternal and 9 polar over/underdominance patterns consistent with genomic imprinting effects but only 2 with additive genetic effects. Strikingly, placental polar overdominance patterns at midgestation mirrored polar overdominance in birthweight. We found that increased nutrient supply via maternal A genome effects on placental phenotype, combined with increased nutrient transfer capacity via polar overdominance effects of paternal B genome on umbilical cord phenotype, provide the basis for heterosis in birth weight of B×A hybrids. Polar overdominance in expression of imprinted *IGF2R* in *Placenta fetalis* of B×A hybrids, and correlation of transcript abundance with number of feto-maternal syncytia in placenta, are consistent with an active signalling role of IGF2R in placenta and a further indicator of superior placental performance as the driver of heterosis. In conclusion, we have shown that phenotypic expression patterns consistent with imprinting effects on placental and umbilical cord parameters and in agreement with the conflict of interest theory of genomic imprinting drive mammalian heterosis *in utero*.

4.1 Introduction

Heterosis is defined as the superior phenotype of first generation hybrids compared to their parents. This has been used for centuries globally to generate superior plant and animal phenotypes, but heterosis is also starting to be recognized in human health and disease (Campbell et al., 2007). However, the mechanistic basis of heterosis is still poorly understood and conventional heterosis hypotheses based on standard genetic models, i.e., dominance, heterozygosity *per se* with overdominance, and epistasis, have generally failed to fully explain heterosis. As a result, heterosis effects cannot be predicted and frequently have to be determined in test-crosses (Hochholdinger and Hoecker, 2007, Lippman and Zamir, 2007, Springer and Stupar, 2007). Work in plants has nevertheless demonstrated that significant heterosis effects can be caused by single genetic loci (Krieger et al., 2010).

Modelling data have long indicated that genomic imprinting, i.e., parent-of-origin specific monoallelic gene expression, can mimic overdominance (Chakraborty, 1989). More recently, epigenetic mechanisms, which regulate gene expression without changing the DNA sequence, have been correlated with heterosis in plants. This includes changes in DNA methylation, histone modifications and expression of non-coding RNAs involved in RNA interference (He et al., 2013, Groszmann et al., 2013). Potential epigenetic components in mammalian heterosis, on the other hand, have not yet been determined. Recent studies in crosses between inbred strains of mice to identify imprinting effects on phenotypic traits demonstrated phenotypic patterns representing maternal, paternal and dominance imprinting (Cheverud et al., 2008, Wolf et al., 2008b). Dominance imprinting was further sub-classified into either bipolar, or polar over- or underdominance (Cheverud et al., 2008, Wolf et al., 2008a). In polar over- and underdominance, one type of heterozygous progeny differs from the reciprocal cross depending on the parental origin of alleles and differs from the two parental homozygotes. Polar overdominance was first reported in sheep manifesting generalized muscle hypertrophy due to the Callipyge mutation (Cockett et al., 1996). The

non-Mendelian and paternal mode of inheritance of the Callipyge phenotype correlates with changes in imprinted gene expression and is caused by a mutation in a non-coding RNA and thought to arise from trans-interaction between transcripts of reciprocally imprinted genes (Georges et al., 2004, Gao et al., 2015). Polar overdominance has since been reported for a range of phenotypes in other mammals including human, mouse and pig (Kim et al., 2004, Cheverud et al., 2008, Wermter et al., 2008, Boysen et al., 2010).

Crosses between *Bos taurus indicus* and *Bos taurus taurus* display one of the most pronounced heterotic phenotypes known in mammals with significant heterosis in a broad range of phenotypic traits including pre- (+11%) and postnatal (+19%) growth (Brown et al., 1993a, Brown et al., 1993b). The two types of cattle originate from different wild ancestors with subspecies status and represent maximum *intra*species divergence (Hiendleder et al., 2008). Interestingly, the significantly higher birth weight of *B. t. indicus* sired hybrids is consistent with polar overdominance. This pattern persisted after embryo transfer (Amen et al., 2007, Dillon et al., 2015) suggesting that epigenetic factors of the conceptus and not maternal intrauterine effects are responsible for significantly higher prenatal growth of hybrids.

Birth weight is a proxy for prenatal growth and development and a strong predictor of postnatal performance in mammals (Lubchenco et al., 1963, Holland and Odde, 1992). Fetal programming of birth weight and postnatal phenotype is a well-established paradigm in human and animal models, with a central role of the placenta providing nutrients and oxygen for the conceptus (Morrison, 2008, Barker et al., 2012,). The conflict theory of evolution of imprinting is based on resource allocation to offspring, where paternally expressed genes are growth promoting and maternally expressed genes have an opposing growth limiting effect (Moore and Haig, 1991). It is therefore not surprising that imprinted genes play a key role in the placenta, where a carefully regulated balance between the expression of genes from the paternal and maternal genomes is required (McMinn et al., 2006).

The insulin-like growth factor (IGF) system regulates feto-placental growth and development (Roberts et al., 2008) and includes paternally expressed *IGF2* and maternally expressed *IGF2R* (Barlow et al., 1991, DeChiara et al., 1991), which recapitulate the conflict theory with growth promoting and growth inhibiting functions of ligand (IGF2) and receptor (IGF2R), respectively (Haig and Graham, 1991). Disruption of paternal *IGF2* expression results in a smaller placenta and fetal growth retardation (DeChiara et al., 1990, Baker et al., 1993) in mouse while disruption of maternal *IGF2R* expression results in placental and fetal overgrowth (Lau et al., 1994, Ludwig et al., 1996). Two long non-coding RNAs, the maternally expressed *H19* downstream of *IGF2* (Gabory et al., 2010) and the paternally expressed anti-sense to *IGF2R*, *AIRN* (Latos et al., 2012), are involved in *IGF2/IGR2R* imprinting. More recently, *H19*, a master regulator of an imprinted gene network that controls growth in mouse (Gabory et al., 2009), was shown to harbour microRNAs that limit placental growth (Keniry et al., 2012). Furthermore, *AIRN* is involved in the regulation of imprinted placental genes including organic cation transporters, *OCT2/Slc22a2* and *OCT3/Slc22a3* (, Sleutels et al., 2002, Monk, 2015).

The central role of the placenta as a target for genomic imprinting (Wang et al., 2013), and changes in expression of imprinted genes such as *IGF2* (Zechner et al., 2002) associated with placental and fetal overgrowth due to *inter*species hybrid dysgenesis in mouse, lead us to hypothesize that *intra*species mammalian heterosis involves changes in imprinted gene expression and is programmed via the placenta *in utero*. To address this, we intercepted purebred and reciprocal cross *B.t. indicus* and *B.t. taurus* concepti at mid-gestation as they enter accelerated growth before displaying heterosis in fetal weight to capture the drivers of heterosis in birth weight. We found polar overdominance in fetal fluid volume, umbilical cord weight, umbilical artery and vein diameters and placental *IGF2R*/*AIRN* expression in *B.t. indicus* sired *B.t. indicus* × *B.t. taurus* concepti which mirrored reported (Brown et al., 1993b) polar overdominance in birth weight. Together with positive correlations between

abundances of imprinted transcripts and placental characteristics our findings indicate for the first time that heterosis in birth weight is driven by non-Mendelian placental effects that change placental-umbilical phenotype and increase nutrient supply to the fetus.

4.2 Results

4.2.1 Phenotypic patterns of gross morphological, histological and molecular parameters of purebred and hybrid concepti

The four different combinations of *Bos t. taurus* (Angus, A) and *Bos t. indicus* (Brahman, B) maternal and paternal genomes revealed twenty-one genomic imprinting patterns (Wolf et al., 2008a), including maternal- and paternal expression, polar over- and underdominance, and bipolar dominance, but only two additive patterns (Chen, 2013), for parameters significantly affected by fetal genetics (Figure 4.1-4.5, Table 4.1).

Maternal or paternal genome effects are characterized by similar means for a hybrid and its corresponding maternal- or paternal purebred with significant differences between the different respective hybrid-purebred combinations. We found maternal genome effects for fetal body weight and the majority of gross and histomorphological placental parameters, including *Placenta materna*- and *Placenta fetalis*- weights, number of placentomes in the gravid horn, number and average weight of convex placentomes in both uterine horns, volume of trophoblast cell, surface area of maternal epithelium and trophoblast, and surface density of maternal epithelium (Figure 4.1-4.3). In all these parameters B×A (paternal genome listed first) and A×A concepti displayed higher values than A×B and B×B concepti. Paternal genome effects were present in umbilical cord length and number and proportion of syncytia with higher values for B×A and B×B than for A×B and A×A concepti (Figure 4.1, 4.4).

In polar over- and underdominance, the phenotypic value for one of the reciprocal hybrids is significantly higher or lower than those for the three other genome combinations. We observed polar overdominance in B×A concepti for fetal fluid volume, cord weight, cord artery and vein diameters and placental *IGF2R* and *AIRN* transcript abundances (Figure 4.1,

4.5). We also classified maternal connective tissue volume as polar overdominant in the B×A hybrid as comparisons with A×B and B×B concepti were significant (*P<0.05*) and comparison with A×A approached significance at *P<0.10* (Table 4.1). Maternal epithelium volume was included although the P-value (*P=0.13*) for the B×A and A×A comparison was higher. A clear pattern of polar underdominance was observed for one parameter, *Placenta fetalis* efficiency, in the B×A combination (Figure 4.1).

Additive expression patterns, where the reciprocal hybrids are not significantly different and similar to the parental mean, were found for *IGF2* and *H19* transcript abundances in the placenta (Figure 4.5). We found no significant fetal genetic effects on *IGF1-*, *INSRA-* and *IGFBP2-5* transcript abundances and placental expression of *IGF1R* and *IGFBP1* and *-6* in our bovine resource was below detection limit.

4.2.2 Relationships between imprinted gene expression and gross morphological and histomorphological phenotype

Fetal weight and *Placenta fetalis* weight were both negatively correlated with placental expression of *IGF2* and *H19*. In contrast, *Placenta materna* weight was not correlated with these transcripts. Furthermore, the total number of syncytia was positively correlated with *IGF2*, *IGF2R*, *H19*, and A*IRN* expression, while proportion of syncytia was positively correlated with *IGF2* and *H19* only (Figure 4.6).

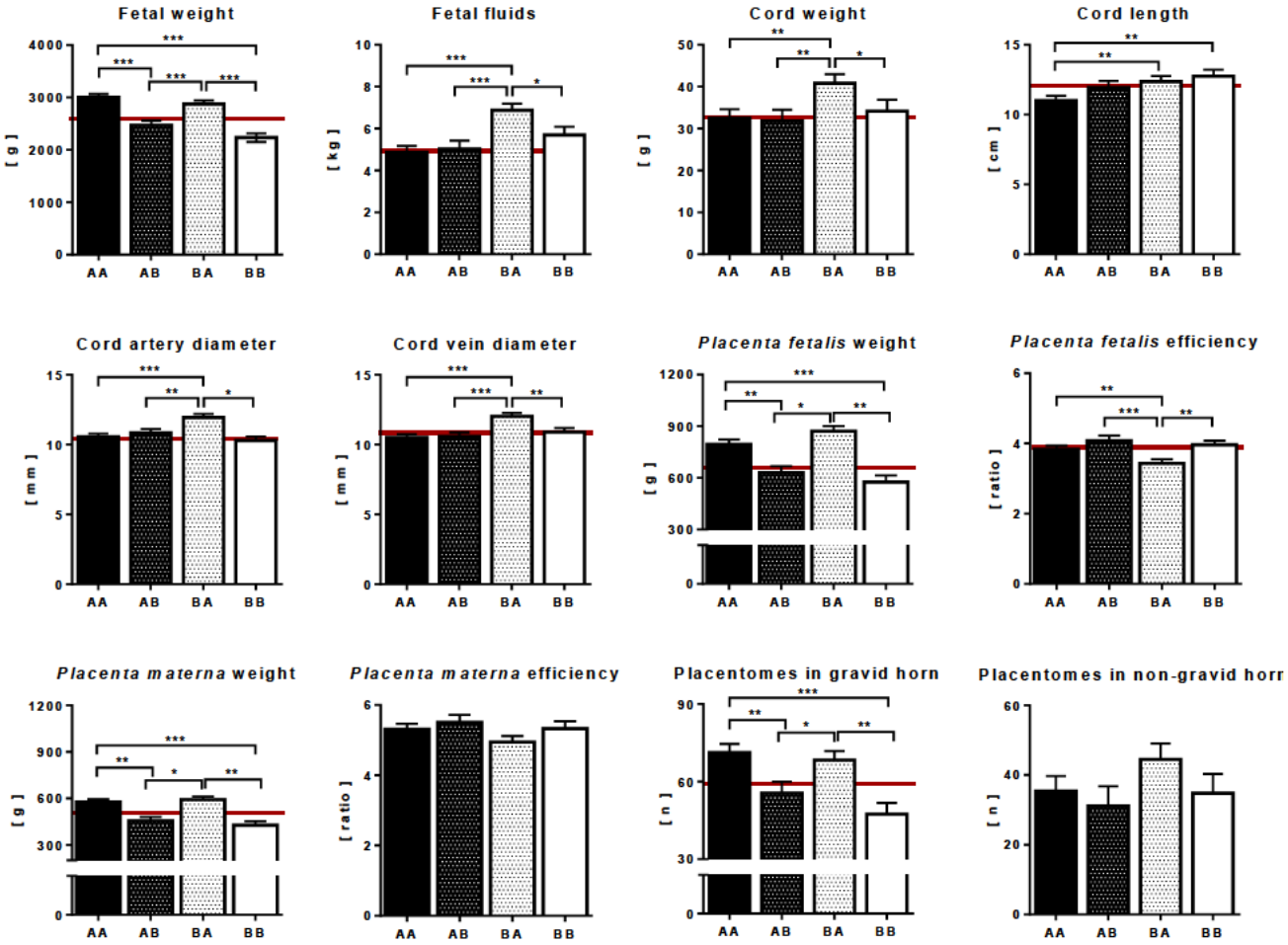


Figure 4.1 Fetal weight, placental weights, and placentome number in gravid horn display maternal expression pattern, while only cord length displays paternal expression pattern. The B×A hybrid exhibits polar overdominance on cord phenotype and fetal fluid volume. Purebred (A×A, B×B) and reciprocal cross (B×A, A×B, paternal genome listed first) concepti at Day 153 gestation. Data presented as least square means ± SEM. Two tailed t-test used to compare means where ***P<0.001, ** P<0.01, **P<0.05. Red line represents the mid-parent value, i.e., mean value of AA and BB.

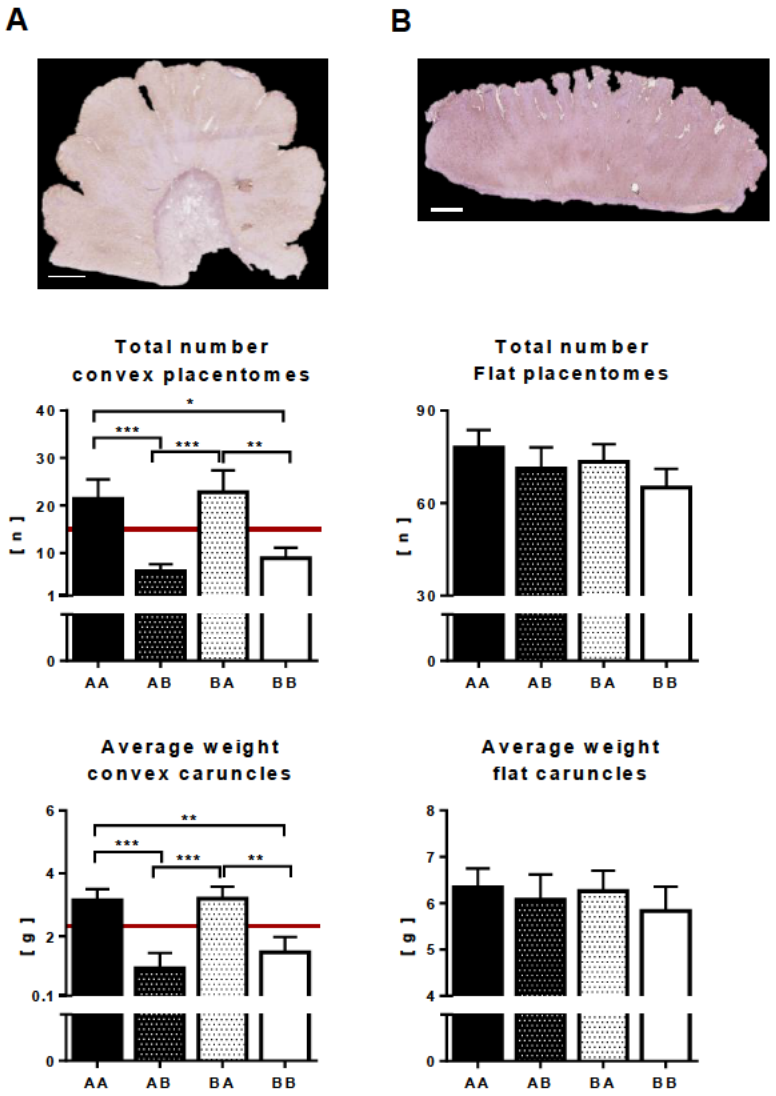


Figure 4.2 Convex and flat placentome types in bovine at mid-gestation, where total number and average weight of convex placentomes show maternal genome effects. Purebred (A×A, B×B) and reciprocal cross (B×A, A×B, paternal genome listed first) placentae. Data presented as least square means ± SEM. Two tailed t-test used to compare means where ***P<0.001, ** P<0.01, **P<0.05. Red line represents the mid-parent value, i.e., mean value of AA and BB. Representative photomicrographs of convex (A) and flat (B) bovine placentomes. Scale bar 2.5mm.

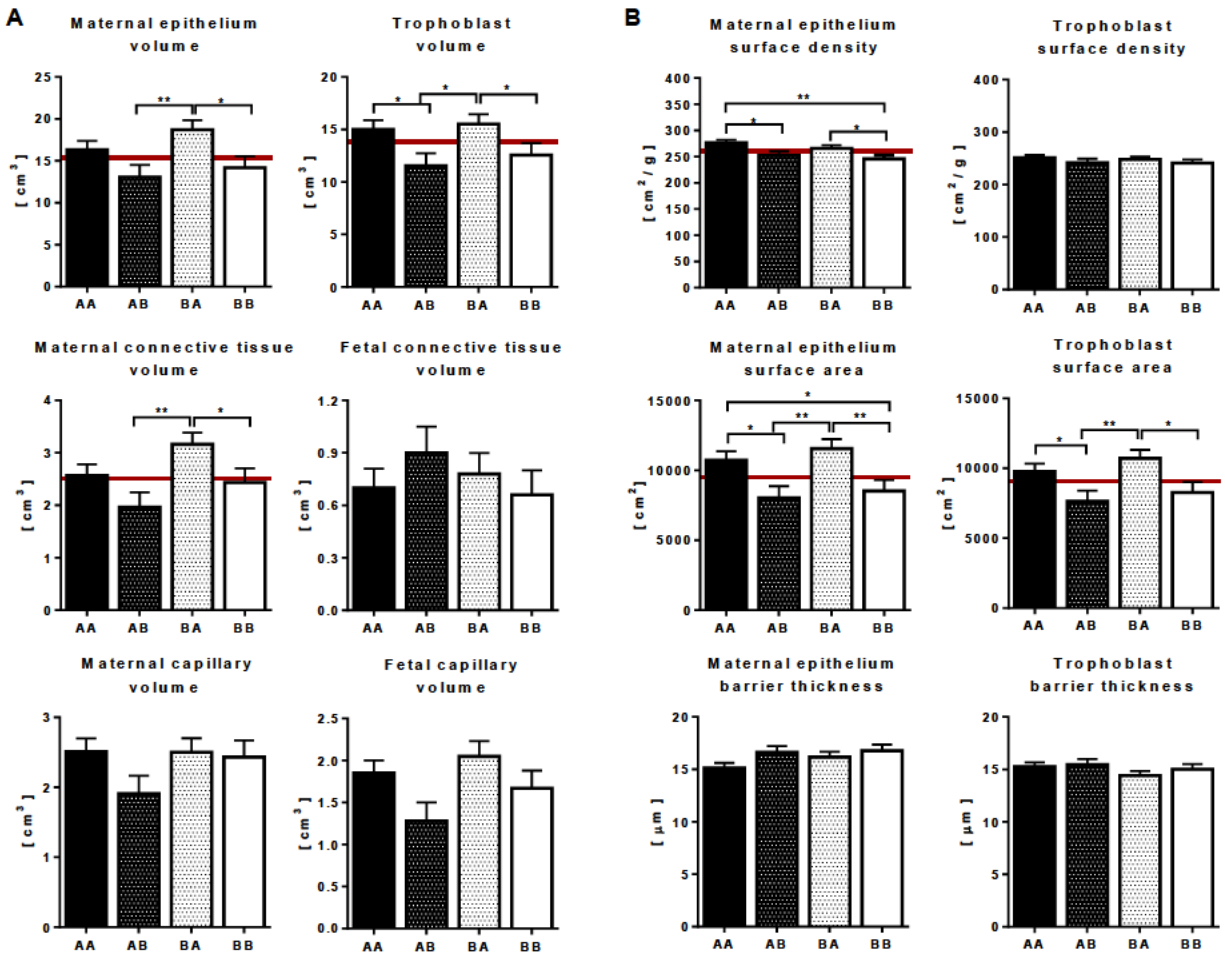


Figure 4.3 Volume of trophoblast, surface density of maternal epithelium and surface area of maternal epithelium and trophoblast show maternal expression pattern, while volumes of maternal epithelium and maternal connective tissue show polar overdominance of the B×A hybrid.

Purebred (A×A, B×B) and reciprocal cross (B×A, A×B, paternal genome listed first) placentae. Data for volume of placental cell types (panel A) and measurements reflecting placental exchange (panel B) are presented as least square means ± SEM. Two tailed t-test used to compare means where ***P<0.001, ** P<0.01, **P<0.05. Red line represents the mid-parent value, i.e., mean value of AA and BB.

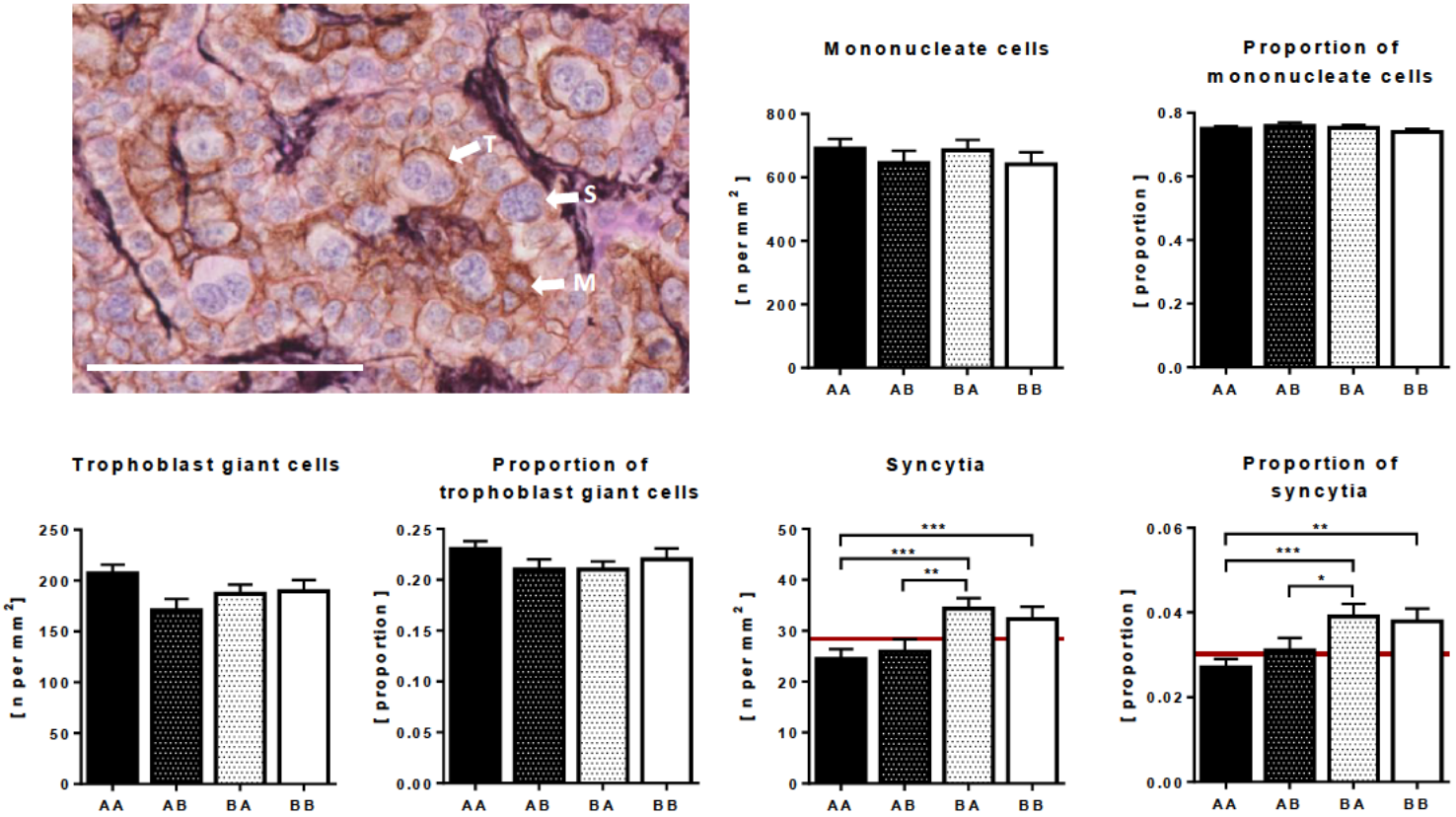


Figure 4.4 Number and proportion of different trophoblast cell types, where number and proportion of feto-maternal syncytia display paternal expression pattern.

Purebred (A×A, B×B) and reciprocal cross (B×A, A×B, paternal genome listed first) placentae. Representative photomicrograph showing different trophoblast cell types: mononucleate cell (M), trophoblast giant cell (T) and feto-maternal syncytium (S). Scale bar is 100 µM. Data presented as least square means ± SEM. Two tailed t-test used to compare means where ***P<0.001, ** P<0.01, **P<0.05. Red line represents the mid-parent value, i.e., mean value of AA and BB.

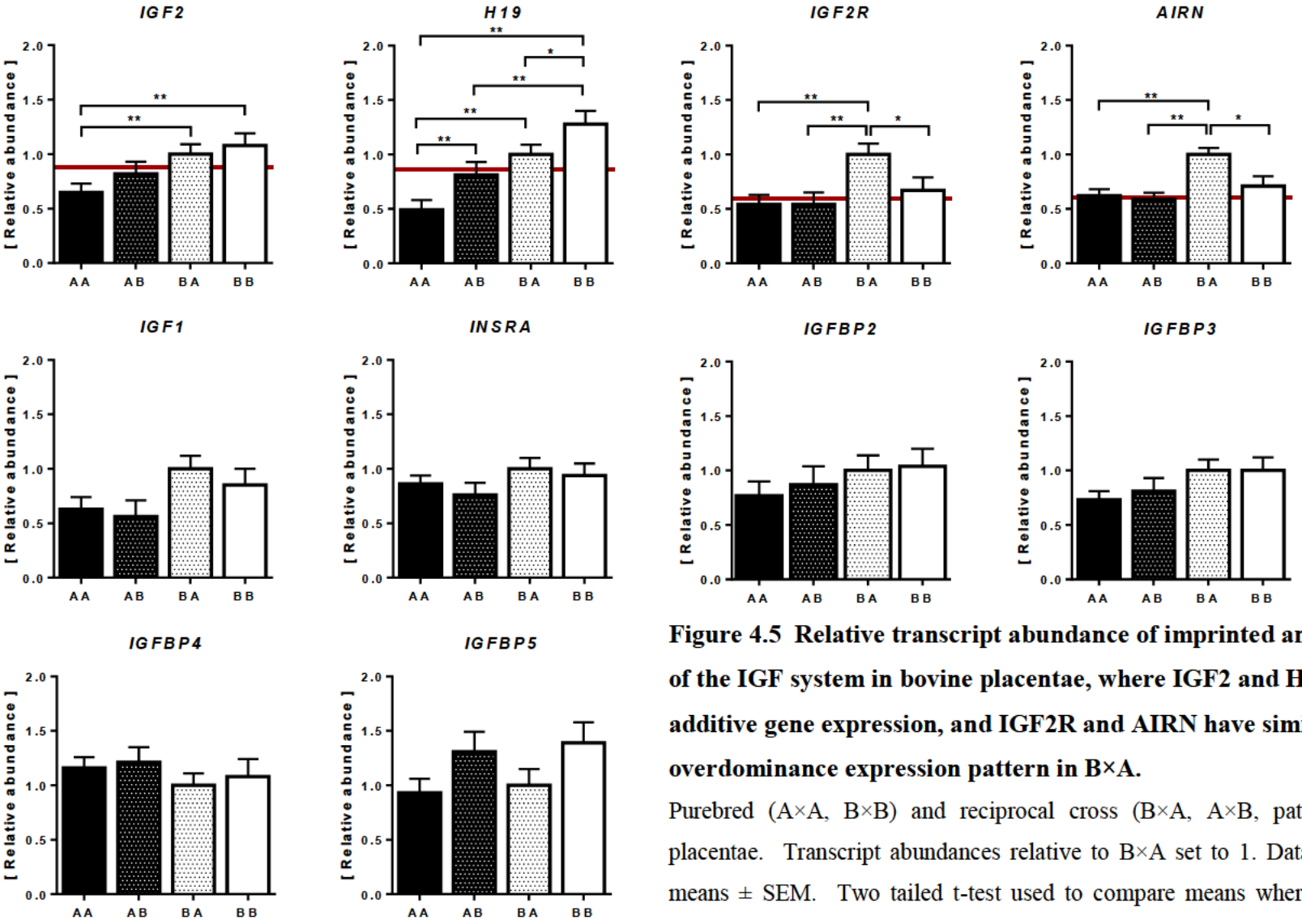
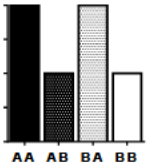
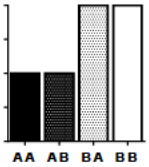
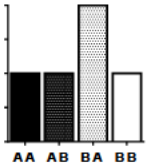
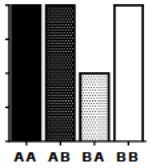
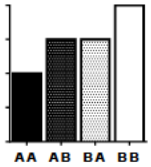


Figure 4.5 Relative transcript abundance of imprinted and non-imprinted genes of the IGF system in bovine placentae, where IGF2 and H19 have similar additive gene expression, and IGF2R and AIRN have similar polar overdominance expression pattern in B×A.

Purebred (A×A, B×B) and reciprocal cross (B×A, A×B, paternal genome listed first) placentae. Transcript abundances relative to B×A set to 1. Data presented as least square means ± SEM. Two tailed t-test used to compare means where ***P<0.001, ** P<0.01, **P<0.05. Red line represents the mid-parent value, i.e., mean value of AA and BB. *IGF1R*, *IGFBP1* and *IGFBP6* are below detection limit.

Table 4.1 Summary of phenotypic patterns of conceptus traits with significant effects of fetal genetics at mid-gestation according to the classification system of Wolf et al. (2008) and Chen (2013). A + in the respective column designates observed expression pattern.

Conceptus traits[1]	Phenotypic expression pattern				
	Maternal	Paternal	Polar Over-dominance	Polar Under-dominance	Additive
					
Gross morphology					
Fetus weight	+				
Fetal fluids			+		
Cord weight			+		
Cord length		(+)[2]			
Cord artery diameter			+		
Cord vein diameter			+		
P. fetalis weight	+				
P. fetalis efficiency				+	
P. materna weight	+				
Placentome number in gravid horn	+				
Total number of convex placentomes	+				
Average weight convex caruncles	+				
Histomorphology					
Maternal epithelium volume			(+)[2]		
Trophoblast epithelium volume	+				
Maternal connective tissue volume			(+)[2]		
Maternal epithelium surface density	(+)[2]				
Maternal epithelium surface area	+				
Trophoblast surface area	+				
Number of syncytia		+			
Proportion of syncytia		+			
Transcript abundances					
IGF2					+
H19					+
IGF2R			+		
AIRN			+		

[1]Only parameters with significant fetal genetic effects were included. [2] One comparison between two least square means $P>0.05$.

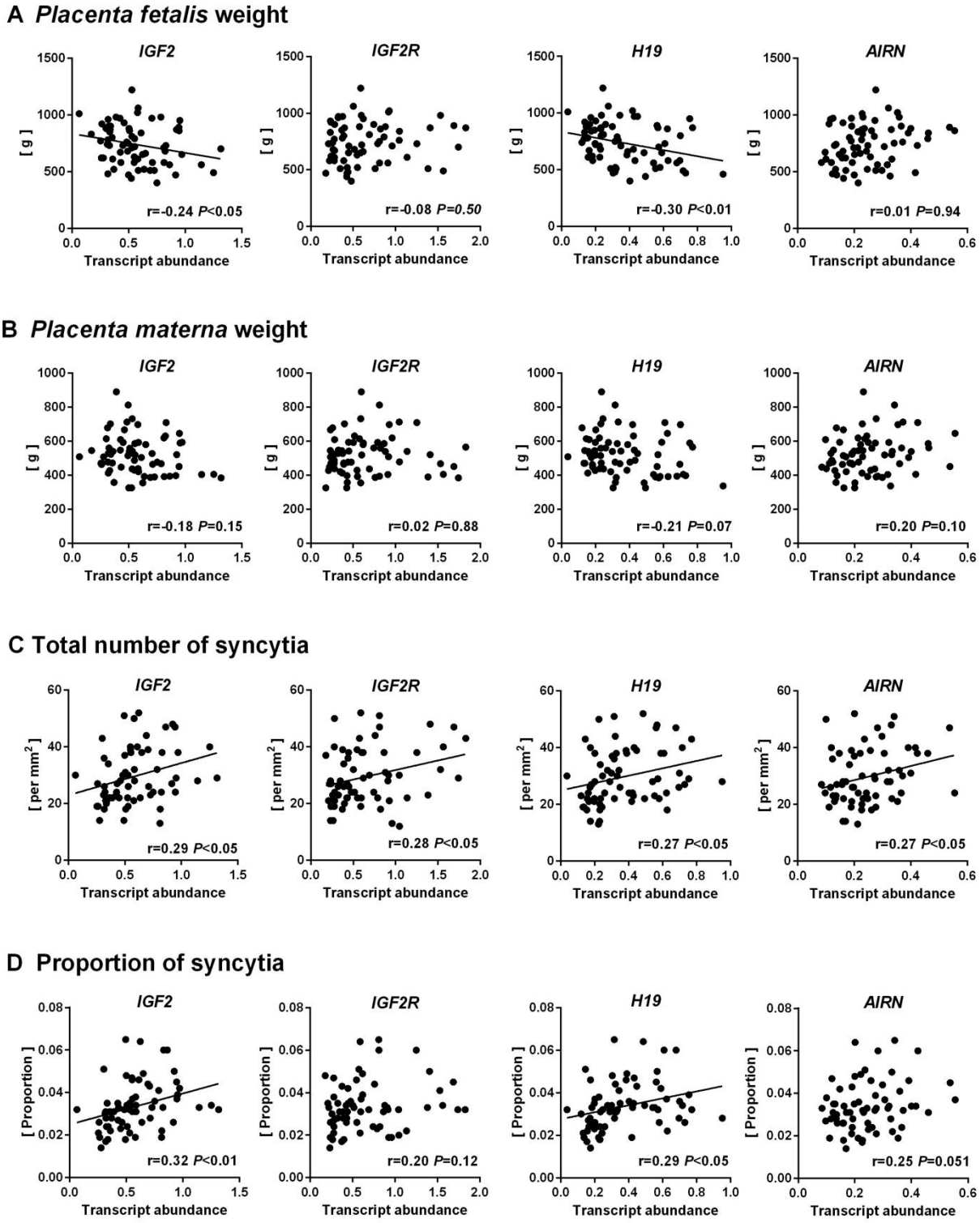


Figure 4.6 Relationship between imprinted genes of the IGF system and *Placenta fetalis* weight (A), *Placenta materna* weight (B), number (C) and proportion of syncytia (D).

Pearson product moment correlation coefficients and significant regression lines with *P*-values as indicated.

4.3 Discussion

We hypothesized that mammalian heterosis is programmed *in utero* and driven by heterotic changes in placental phenotype and imprinted gene expression. To address this, we established a model with purebred and reciprocal cross *Bos t. taurus* (Angus, A) and *B. t. indicus* (Brahman, B) concepti and identified polar overdominance effects in combination with maternal and paternal genome effects at the gross morphological, histomorphological and gene expression level as drivers of fetal growth and heterosis in birth weight of the B×A hybrid (Reynolds et al., 1980, Brown et al., 1993b).

Birth weight of *Bos t. indicus*-sired progeny from *Bos t. taurus* dams through natural mating and embryo transfer are consistently higher compared to purebreds and reciprocal cross hybrid, indicating that difference in birth weight is not primarily due to the maternally provided intrauterine environment, but instead may be regulated by epigenetic mechanisms. Based on patterns reported for mouse postnatally (Cheverud et al., 2008, Wolf et al., 2008a), we identified phenotypic expression patterns consistent with maternal genomic imprinting for the majority of gross morphological placental parameters. Here, the B×A hybrid conceptus, which, upon entering accelerated growth, does not yet display heterosis in fetal weight, clearly indicates maternal A genome effects which are superior to the A×B cross and B×B purebred. A higher placental weight, also reflected in an increased number of placentomes and increased number and weight of convex placentomes, as drivers of fetal growth (Estrella et al., Chapter 2), provides the basis for increased nutrient supply to the B×A fetus in accelerated growth. Polar overdominance effects on umbilical cord weight and cord blood vessel diameters in B×A concepti facilitate better access to this nutrient source as all three parameters are strong indicators for enhanced blood flow and increased nutrient transfer (Adamson, 1999, Di Naro et al., 2001, Barbieri et al., 2011). Polar overdominance in fetal fluid volume provides an additional nutritive and hormonal advantage to the B×A fetus for increased growth as these fluids are a rich reservoir of electrolytes, metabolites, amino acids

and growth factors (Baetz et al., 1975, Tabatabaei and Mamoei, 2012) that are absorbed by the fetus through swallowing, through the lungs or the intramembranous route (Beall et al., 2012).

The placental nutritive advantage of the B×A hybrid through positive maternal A genome effects (see above) was also evident in placental histomorphological indicators of nutrient exchange, including volume of trophoblast, surface area of maternal epithelium and trophoblast, and surface density of maternal epithelium. Furthermore, we observed polar overdominance in volume of maternal connective tissue and maternal epithelium of the B×A hybrid and speculate that this could reflect effects of the hybrid conceptus on *Placenta materna* growth (Estrella et al., Chap 2). Maternal connective tissue is stimulated to proliferate and form caruncular projections where the chorionic villi can interdigitate and develop (Aires et al., 2014), while the maternal epithelium, in apposition with the trophoblast, is an important structure for nutrient transport (Wooding and Burton, 2008).

The superior paternal B genome effects on number and proportion of feto-maternal syncytia with superior B genome effects in B×A and B×B concepti demonstrate the key role of paternal genome in trophoblast cell migration and syncytium formation. Trophoblast giant cells are the source of placental lactogen that is delivered close to the maternal circulation upon fusion of trophoblast binucleate cells with maternal epithelium to form a syncytium (Wooding and Beckers, 1987, Wooding, 1992). Placental lactogen binds to the growth hormone receptor (Byatt et al., 1990) and promotes physiological adjustment of the mother and enhanced uterine histiotrophic nutrient delivery (Soares, 2004). Thus, trophoblast migration leading to syncytium formation may be a means through which the conceptus can influence maternal physiology by signalling nutrient demand.

We found strong polar overdominance in expression of *IGF2R* in B×A placenta and detected no *IGF1R* transcript. The latter finding is consistent with results in sheep where *IGF1R* is not detectable in placentomes from day 30 of gestation onwards (Reynolds et al.,

1997). In human extravillous trophoblast, cell invasiveness and migration *in vitro* is enhanced by IGF2 acting through IGF2R via inhibitory G-proteins and stimulation of the MAPK pathway (McKinnon et al., 2001). In light of superior B genome effects on number and proportion of syncytia (see above), and the polar overdominance in *IGF2R* expression, we interpret our data such that IGF2R mediates IGF2 action on trophoblast migration leading to syncytium formation at similarly high levels in B×A and B×B genome combinations. However, relative to the B×B purebred excess IGF2R in the B×A hybrid is available to bind other ligands, including transforming growth factor-beta (Sue et al., 1995), which was reported to stimulate trophoblast proliferation in bovine (Munson et al., 1996). The present study also shows that the smaller placenta of the B×B conceptus is compensated for by high IGF2 expression and syncytium formation driven by the paternal genome. The negative correlation between *IGF2* and placental weight, and the positive correlation between *IGF2* and the number and proportion of feto-maternal syncytia, further support a role of IGF2 in placental development by predominantly promoting trophoblast invasion over that of proliferation (Dunk et al., 2009).

The expression patterns of *H19* and *AIRN* were highly similar to expression of *IGF2* and *IGF2R*, respectively, as could be expected for co-regulated reciprocally imprinted transcripts from the two loci. Invading trophoblast cells and differentiating cytotrophoblasts express *H19 in vitro* (Rachmilewitz et al., 1992, Mutter et al., 1993). The long non-coding RNA *H19* has anti-proliferative function as a tumour suppressor (Yoshimizu et al., 2008) and encodes miRNA-675, a negative regulator of placental growth that targets *igf1r* transcript (Keniry et al., 2012). This explains the negative relationship between *H19* transcript level and *Placenta fetalis* weight, although the comparatively low correlation coefficient (r =- 0.30) and positive relationships between the other investigated imprinted transcripts and placental parameters such as number of syncytia, point to a network of other regulatory factors including IGF2R (see above). The cis-acting long non-coding RNA *AIRN* plays an important

role in regulating imprinted expression of *IGF2R*, but has no direct role in fetal growth regulation in mouse (Wutz et al., 2001, Sleutels et al., 2002). *AIRN* was detected in bovine fetal liver early in gestation (Farmer et al., 2013), and our study confirms *AIRN* expression in bovine fetal placenta. The positive relationship between *AIRN* expression and number of syncytia suggests *AIRN* plays an important role in trophoblast migration, most likely by regulating imprinted expression of IGF2R. Changes in *AIRN* expression may also impact expression of neighbouring organic cation transporters OCT2/Slc22a2 and OCT3/Slc22a3 (Monk, 2015).

We observed polar underdominance in *Placenta fetalis* efficiency, i.e., gram fetal weight per gram placental weight, of the B×A hybrid conceptus, which is significantly lower than those of all other genome combinations. This initially surprising result for the hybrid combination with the highest birth weight can be explained by considering developmental stage, high fetal and placental weights that are already well above the parental mean, and observed polar overdominance effects in placental expression of *IGF2R* and *AIRN*, fetal fluid volume and cord phenotypes of the B×A genome combination. Thus, lower placental efficiency most likely merely indicates that at the onset of general accelerated fetal growth the drivers of superior hybrid growth, including high placental weight, are already in place before rapid fetal weight increase is manifest.

The effects of maternal and paternal genome on conceptus traits in the present study provides further evidence for the conflict hypothesis where the paternal genome seeks to maximize, and the maternal genome seeks to limit, resource extraction from the mother (Moore and Haig, 1991). The paternal genome, where B is superior to A, affects number and proportion of syncytia suggesting that fetal demand for nutrients is largely determined by the paternal genome. Consistent with the conflict hypothesis, we observed a majority of placental traits with maternal expression pattern, where maternal A is superior to B. Polar overdominance in umbilical cord phenotype and fetal fluid volume of the B×A conceptus is

driven by the B paternal genome and its interaction with the maternal A genome. This is a further indicator that the paternal genome affects nutrient transfer to the fetus via enhanced blood flow and nutrient access through the fetal fluids to support heterotic fetal growth.

In conclusion, we have shown that heterosis unfolds *in utero* and is driven by non-additive polar over- and underdominance, maternal- and paternal- genome effects in a number of parameters critical for fetal growth including imprinted transcripts of the IGF system. The superiority of the B×A hybrid in birth weight is clearly visible and mirrored in observed polar overdominance patterns caused by the novel combination of paternal *Bos t. indicus* and maternal *Bos t. taurus* genomes. This specific paternal and maternal genome combination is characterized by high capacity for nutrient extraction and high capacity for nutrient supply.

4.4 Methods

4.4.1 Animals

All animals and procedures used in this study were approved by The University of Adelaide Animal Ethics Committee (No.S-094-2005). We used *Bos t. taurus* (Angus, A) and *Bos t. indicus* (Brahman, B) (epi)genetics and recovered purebred (A×A and B×B) and reciprocal cross (A×B, B×A, paternal genome listed first) concepti at the onset of accelerated fetal growth (Day 153, term 277-291 days) (Andersen and Plum, 1965, Ferrell, 1989). Animals were purchased from farms in South Australia and Queensland, and were transferred to and managed as one group at Struan Agricultural Centre, Naracoorte, South Australia. Pregnancies were established in nulliparous dams at 16-20 months of age using standard estrous cycle synchronization procedures following an adjustment period of 3-4 weeks after purchase, as described (Anand-Ivell et al., 2011). Two Brahman and three Angus sires were used.

4.4.2 Collection of samples and phenotype data

Entire uteri were recovered from pregnant animals in an abattoir and opened by longitudinal incision. Rare twin pregnancies were excluded from the experiment. After cutting the largest placentome, a 5 mm-thick cross section was excised from the center with dual mounted scalpel blades and fixed in ice cold 4% paraformaldehyde and 2.5% PVP-40 PBS for 24 h. The section was then washed four times with 1% PBS and stored in 70% ethanol until embedding in paraffin. The fetus was removed from the uterus, and the umbilical cord was cut immediately above the branching cord vessels. Fetal weight, cord weight and length were recorded. Allantoic and amniotic fluids were weighed combined as fetal fluids. Uteri with attached placentae were then vacuum-packed and stored frozen at –22 °C. After thawing in the laboratory, the *Placenta fetalis* was detached from the maternal caruncles and weighed. Caruncles in each horn were counted, cut off, weighed and cross-sectioned to assign placentome shape as flat or convex. A placentome shape was classified as convex if the caruncle was mound shaped and had the outer rim curled up. Flat placentomes, on the other hand, had an even and level surface. The *Placenta materna* weight was calculated as the sum of individual caruncle weights. Placental efficiencies were calculated by dividing fetal weight with the *Placenta fetalis* and *Placenta materna* weights.

4.4.3 Placental histology

Five µm thick longitudinal slices were cut from paraffin embedded samples and an indirect double label immunohistochemistry was performed as described previously (Roberts et al., 2001) with minor modifications. We used 10% porcine serum and 1% BSA in 1% PBS to block non-specific binding and as a diluent for the anti-body. Vimentin clone Vim3B4 (DakoCytomation, Glostrup, Denmark) antibody was applied on each section at 1:10 dilution and the Cytokeratin AE1/AE3 (Millipore, Temecula LA, USA) antibody was used at 1:400. Biotinylated goat anti-mouse antibody (DakoCytomation, Glostrup, Denmark) in 10% porcine serum at a 1:200 dilution was used as the secondary antibody. Hematoxylin (Sigma, St. Louis

MO, USA) and Eosin (Sigma, St. Louis MO, USA) were used as counterstains and negative controls without primary antibodies were included in each batch.

A high resolution image of the whole stained placental section was captured using the NanoZoomer C9600 slide scanner (Hamamatsu Photonics K.K., Hamamatsu City, Japan). Using NDP.view version 1.0.0 (Hamamatsu Photonics K.K., Hamamatsu City, Japan), ten fields at 200× magnification were selected using a random systematic order; fields were 1 mm apart. Digital images of the fields were imported into Video Pro software (Leading Edge Pty., Adelaide, Australia) and using an L-36 Merz grid transparency overlaying the monitor screen, a total of 360 points were counted for each section. The volume densities of the placental trophoblast, fetal capillaries, fetal connective tissue, maternal epithelium, maternal capillaries, maternal connective tissue and maternal septae as 'other' tissues were computed as previously reported (MacLaughlin et al., 2005). Trophoblast cells stained brown had one or two nuclei and were apposed to the maternal epithelium. Capillaries or blood vessels, located very close to trophoblast or maternal epithelial cells, were identified as having darkly stained circular or elongated opening (i.e., lumen of a blood vessel) with or without the presence of red blood cells. On the other hand, connective tissue appeared fibrous and not as darkly stained as a blood vessel or capillary opening. The volume in cm^3 of each placental component was determined based on the weight of analyzed placentomes after confirming a 1:1 w/v relationship in collected *Placenta materna* samples. Parameters for surface exchange, such as trophoblast and maternal epithelium surface density (cm^2/g), total surface area (cm^2), and barrier thickness (µm) were estimated using intercept counting. A field was counted five times and the coefficient of variation was less than 5%.

Trophoblast cell types were further classified and counted as mononucleate, trophoblast giant cell or feto-maternal syncytium. Mononucleate cells were cuboidal or columnar in shape with a small cytoplasm and a nucleus. On the other hand, trophoblast giant cells were identified on the basis of having an enlarged cytoplasm with one or two nuclei.

The feto-maternal syncytium was multinuclear and present alongside the maternal epithelia. Proportion of each trophoblast cell type was computed by dividing with the total number of trophoblast cells. All ten fields per placentome sample were counted and analyzed. Data was adjusted to number or proportion of trophoblast cell type per given area of 1 mm^2.

4.4.4 Expression of genes from the IGF system in placenta

Relative expression levels of *IGF1*, *IGF2*, *IGF1R*, *IGF2R*, *INSRA*, *INSRB*, and *IGFBP1-5* were determined by real-time quantitative PCR (qPCR). RNA was extracted from placental (cotyledon) tissue using TRI Reagent (Ambion) in combination with ceramic beads (MoBio Laboratories) and a Precellys 24 tissue homogeniser (Bertin Technologies) following the manufacturer's instructions. RNA quantity and quality were assessed by NanoDrop ND-1000 spectrophotometer (Thermo Scientific) and Agilent RNA 6000 Nano Kit with Bioanalyzer 2100 (Agilent Technology). After DNase treatment (RQ1-DNase, Promega), 500 ng RNA was reverse transcribed using Superscript III First-strand cDNA synthesis kit (Invitrogen) with random hexamer oligonucleotides following the manufacturer's instructions. Real-time qPCR reactions were performed in an Eppendorf Mastercycler ep realplex (Eppendorf Inc.) using 4 µL of 40-fold or 5.2 µL of 20-fold diluted cDNA, 0.8 µL of 5 pmol/µL primer mix and 6 µL of FastStart Universal SYBR Green Master (Roche Diagnostics) in a total volume of 12 µL. A standard curve comprised of a 2-fold serial dilution of pooled cDNA template over eight data points, as well as a non-template control, were included in each qPCR experiment. Placental cDNA samples were measured in duplicate and cDNA standard curve data points in triplicate. Primer sequences and other details of amplicons from 11 target and two reference genes used in normalization of transcript abundances are shown in Supplementary Table S4.1**.** Target gene transcript abundance was normalized to the geometric mean of the transcript abundances of the most stably expressed reference genes selected using NormFinder (Andersen et al., 2004).

4.4.5 Statistical analyses

We used the general linear model procedure of SAS (SAS Inst., Cary NC, USA) to calculate means and standard errors of means for each fetal genetic group adjusted for the effects of fetal sex using the model:

$$y_{ij} = S_i + G_j + e_{ij}$$

where yij was the dependent variable, S (i = male, female) was the fetal sex effect and G (j = A×A, A×B, B×A, and B×B, sire listed first) was the fetal genetic effect. Table S4.2 shows a summary of significance levels of models and variables for parameters with significant effect of fetal genetics. Interactions between fetal genetics and sex were not significant for investigated parameters and therefore removed from the model. Least square means with standard errors of the means were compared using two-tailed t-test. Regression analyses and Pearson product-moment correlation coefficients were computed for relationships of imprinted gene expression with fetus weight and placental traits. The significance threshold was $P<0.05$ for all statistical tests performed.

Supplemental Tables

Table S 4.1, related to Methods. PCR Primers, annealing temperature (Ta) and size of fragments used in real-time quantitative PCR.

Gene	Primers		Ta	Size	Accession no.
VPS4A	F	GAA GAC AGA AGG CTA CTC GGG TG	60	106	NM_001046615.1
	R	ACA GAC CTT TTT GAA GTG TGT TGCT			
GAPDH	F	GGG TCA TCA TCT CTG CAC CT	60	264	NW_003103940.1
	R	CAT AAG TCC CTC CAC GAT GC			
IGF1	F	GAT GCT CTC CAG TTC GTG TGC	58	140	NW_003103925.1
	R	TCC AGC CTC CTC AGA TCA CAG			
IGF1R	F	GAT CCC GTG TTC TTC TAC GTT C	58	100	XM_606794.3
	R	AAG CCT CCC ACT ATC AAC AGA A			
INSR-A	F	TCC TCA AGG AGC TGG AGG AGT	59	89	AJ488553
	R	TTT CCT CGA AGG CCT GGG GAT			
IGFBP1	F	ACC AGC CCA GAG AAT GTG TC	59	119	NW_003103902.1
	R	CTG ATG GCA TTC CAG AGG AT			
IGFBP2	F	CAC ATC CCC AAC TGT GAC AA	58	114	NW_001494682.3
	R	GAT CAG CTT CCC GGT GTT AG			
IGFBP3	F	CTA CGA GTC TCA GAG CAC AG	58	103	NT_181996.1
	R	GTG GTT CAG CGT GTC TTC C			
IGFBP4	F	ATG TGC CTG ATG GAG AAA GG	57	106	NM_174557.3
	R	GCC ATC CTG TGA CTT CCT GT			
IGFBP5	F	CAA GCC AAG ATC GAA AGA GAC T	60	85	NM_001105327.1
	R	AAG ATC TTG GGC GAG TAG GTC T			
IGFBP6	F	GGA GAG AAT CCC AAG GAG AGT A	60	100	NM_001040495.1
	R	GAG TGG TAG AGG TCC CCG AGT			
IGF2	F	CTT CGC CTC GTG CTG CTA TG	60	134	NM_174087.3
	R	GTC GGT TTA TGC GGC TGG AT			

Table S 4.1 continued...

IGF2R	F	GAT GGT AAT GAG CAG GCT TAC C	60	123	NM_174352.2
	R	ATC TCC TCC ATC AGC CAC TC			
AIRN	F	AAT CTC TTG CGG AGT GTT CAT	57	136	DQ835615.1
	R	CTC TGT TGT ATC GTG TCT TTC G	57		
H19	F	TCA AGA TGA CAA GAG ATG GTG CTA	60	171	NR_003958.2
	R	GGT GTG GGT CGT CCG TTC	60		

Forward (F) and reverse (R) primers are given. GenBank accession number of DNA sequence used for primer design.

Table S 4.2 General linear models (Type III sums of squares) with R-squared values and significance levels (*P*-values) of models and variables for fetal and placental parameters with significant effect of fetal genetics.

Parameters	R^2	*P*-value		
		Model	Genetics	Sex
Fetal body weight	0.61	<0.0001	<0.0001	<0.0001
Placental gross morphology				
Placenta fetalis weight	0.44	<0.0001	<0.0001	0.1726
Placenta materna weight	0.47	<0.0001	<0.0001	0.0042
Total placenta weight	0.53	<0.0001	<0.0001	0.0164
Placenta fetalis weight	0.32	<0.0001	0.0008	0.0097
Total placental efficiency	0.36	<0.0001	0.0002	0.0063
Placentome number gravid horn	0.34	<0.0001	<0.0001	0.7898
Total number of convex placentomes	0.24	0.0008	0.0003	0.6545
Average weight of convex placentome	0.33	<0.0001	<0.0001	0.1880
Cord weight	0.19	0.0098	0.0117	0.0228
Cord diameter	0.24	0.0020	0.0075	0.0074
Cord vein diameter	0.29	0.0002	0.0001	0.0102
Cord artery diameter	0.35	<0.0001	<0.0001	0.0014
Placental histomorphology				
Maternal epithelium volume	0.16	0.0202	0.0123	0.2026
Maternal connective volume	0.16	0.0263	0.0121	0.4885
Trophoblast volume	0.17	0.0152	0.0294	0.0417
Maternal epithelium surface density	0.18	0.0094	0.0057	0.2227
Maternal epithelium surface area	0.21	0.0047	0.0025	0.2818
Trophoblast surface area	0.18	0.0120	0.0076	0.1893
Total number of syncytia	0.24	0.0013	0.0013	0.5710
Proportion of syncytia	0.26	0.0006	0.0009	0.3949

Table S 4.2 continued...

Gene expression of IGFs in placenta				
IGF2	0.18	0.0131	0.0062	0.8131
IGF2R	0.22	0.0045	0.0067	0.4891
H19	0.38	<0.0001	<0.0001	0.2591
AIRN	0.33	<0.0001	<0.0001	0.9582

4.5 Literature Cited

ADAMSON, S. L. 1999. Arterial pressure, vascular input impedance, and resistance as determinants of pulsatile blood flow in the umbilical artery. *European Journal of Obstetrics & Gynecology and Reproductive Biology,* 84**,** 119-125.

AIRES, M. B., DEGAKI, K. Y., DANTZER, V. & YAMADA, A. T. 2014. Bovine placentome development during early pregnancy. *In:* MENDEZ-VILLAS, A. (ed.) *Microscopy: Advances in Scientific Research and Education.* Spain: Formatex Research Center.

AMEN, T. S., HERRING, A. D., SANDERS, J. O. & GILL, C. A. 2007. Evaluation of reciprocal differences in Bos indicus × Bos taurus backcross calves produced through embryo transfer: II. Postweaning, carcass, and meat traits. *Journal of Animal Science,* 85**,** 373-379.

ANAND-IVELL, R., HIENDLEDER, S., VIÑOLES, C., MARTIN, G. B., FITZSIMMONS, C., EURICH, A., HAFEN, B. & IVELL, R. 2011. INSL3 in the ruminant: a powerful indicator of gender- and genetic-specific feto-maternal dialogue. *PLOS ONE,* 6**,** e19821.

ANDERSEN, C. L., JENSEN, J. L. & ØRNTOFT, T. F. 2004. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Research,* 64**,** 5245-5250.

ANDERSEN, H. & PLUM, M. 1965. Gestation length and birth weight in cattle and buffaloes: a review. *Journal of Dairy Science,* 48**,** 1224-1235.

BAETZ, A. L., HUBBERT, W. T. & GRAHAM, C. K. 1975. Developmental changes of free amino acids in bovine fetal fluids with gestational age and the interrelationships between the amino acid concentrations in the fluid compartments. *Journal of Reproduction and Fertility,* 44**,** 437-444.

BAKER, J., LIU, J.-P., ROBERTSON, E. J. & EFSTRATIADIS, A. 1993. Role of insulin-like growth factors in embryonic and postnatal growth. *Cell,* 75**,** 73-82.

BARBIERI, C., CECATTI, J. G., SURITA, F. G., COSTA, M. L., MARUSSI, E. F. & COSTA, J. V. 2011. Area of Wharton's jelly as an estimate of the thickness of the umbilical cord and its relationship with estimated fetal weight. *Reproductive Health,* 8**,** 32.

BARKER, D. J. P., LAMPL, M., ROSEBOOM, T. & WINDER, N. 2012. Resource allocation in utero and health in later life. *Placenta,* 33 Supplement 2**,** e30-e34.

BARLOW, D. P., STOGER, R., HERRMANN, B. G., SAITO, K. & SCHWEIFER, N. 1991. The mouse insulin-like growth factor type-2 receptor is imprinted and closely linked to the Tme locus. *Nature,* 349**,** 84-87.

BEALL, M. H., VAN DEN WIJNGAARD, J. P. H. M., VAN GEMERT, M. & ROSS, M. G. 2012. Chapter 1 - Water Flux and Amniotic Fluid Volume: Understanding Fetal Water Flow. *Nephrology and Fluid/Electrolyte Physiology: Neonatology Questions and Controversies (Second Edition).* Philadelphia: W.B. Saunders.

BOYSEN, T. J., TETENS, J. & THALLER, G. 2010. Detection of a quantitative trait locus for ham weight with polar overdominance near the ortholog of the callipyge locus in an experimental pig F2 population. *Journal of Animal Science,* 88.

BROWN, M. A., BROWN, A. H., JACKSON, W. G. & MIESNER, J. R. 1993a. Genotype x environment interactions in postweaning performance to yearling in Angus, Brahman, and reciprocal-cross calves. *Journal of Animal Science,* 71**,** 3273-9.

BROWN, M. A., THAREL, L. M., BROWN, A. H., JACKSON, W. G. & MIESNER, J. R. 1993b. Genotype x environment interactions in preweaning traits of purebred and reciprocal cross Angus and Brahman calves on common bermudagrass and endophyte-infected tall fescue pastures. *Journal of Animal Science,* 71**,** 326-333.

BYATT, J. C., WELPLY, J. K., LEIMGRUBER, R. M. & COLLIER, R. J. 1990. Characterization of glycosylated bovine placental lactogen and the effect of enzymatic deglycosylation on receptor binding and biological activity. *Endocrinology,* 127**,** 1041-1049.

CAMPBELL, H., CAROTHERS, A. D., RUDAN, I., HAYWARD, C., BILOGLAV, Z., BARAC, L., PERICIC, M., JANICIJEVIC, B., SMOLEJ-NARANCIC, N., POLASEK, O., KOLCIC, I., WEBER, J. L., HASTIE, N. D., RUDAN, P. & WRIGHT, A. F. 2007. Effects of genome-wide heterozygosity on a range of biomedically relevant human quantitative traits. *Human Molecular Genetics,* 16**,** 233-241.

CHAKRABORTY, R. 1989. Can molecular imprinting explain heterozygote deficiency and hybrid vigor? *Genetics,* 122**,** 713-717.

CHEN, Z. J. 2013. Genomic and epigenetic insights into the molecular bases of heterosis. *Nature Reviews Genetics,* 14**,** 471-482.

CHEVERUD, J., HAGER, R., ROSEMAN, C., FAWCETT, G., WANG, B. & WOLF, J. 2008. Genomic imprinting effects on adult body composition in mice. *Proceedings of the National Academy of Sciences of the United States of America,* 105**,** 4253 - 4258.

COCKETT, N. E., JACKSON, S. P., SHAY, T. L., FARNIR, F., BERGHMANS, S., SNOWDER, G. D., NIELSEN, D. M. & GEORGES, M. 1996. Polar overdominance at the ovine callipyge locus. *Science,* 273**,** 236-238.

DECHIARA, T. M., EFSTRATIADIS, A. & ROBERTSEN, E. J. 1990. A growth-deficiency phenotype in heterozygous mice carrying an insulin-like growth factor II gene disrupted by targeting. *Nature,* 345**,** 78-80.

DECHIARA, T. M., ROBERTSON, E. J. & EFSTRATIADIS, A. 1991. Parental imprinting of the mouse insulin-like growth factor II gene. *Cell,* 64**,** 849-859.

DI NARO, E., GHEZZI, F., RAIO, L., FRANCHI, M., D'ADDARIO, V., LANZILLOTTI, G. & SCHNEIDER, H. 2001. Umbilical vein blood flow in fetuses with normal and lean umbilical cord. *Ultrasound in Obstetrics and Gynecology,* 17**,** 224-228.

DILLON, J. A., RILEY, D. G., HERRING, A. D., SANDERS, J. O. & THALLMAN, R. M. 2015. Genetic effects on birth weight in reciprocal Brahman–Simmental crossbred calves. *Journal of Animal Science,* 93.

DUNK, C., HUPPERTZ, B. & KINGDOM, J. 2009. Development of the placenta and its circulation. *In:* RODECK, C. (ed.) *Fetal Medicine: Basic Science and Clinical Perspective.* Second ed.: Elsevier Limited.

FARMER, W. T., FARIN, P. W., PIEDRAHITA, J. A., BISCHOFF, S. R. & FARIN, C. E. 2013. Expression of antisense of insulin-like growth factor-2 receptor RNA non-coding (AIRN) during early gestation in cattle. *Animal Reproduction Science,* 138**,** 64-73.

FERRELL, C. L. 1989. Placental regulation of fetal growth. *In:* CAMPION, D. R., HAUSMAN, G. J. & MARTIN, R. J. (eds.) *Animal Growth Regulation.* USA: Springer US.

GABORY, A., JAMMES, H. & DANDOLO, L. 2010. The H19 locus: Role of an imprinted non-coding RNA in growth and development. *BioEssays: news and reviews in molecular, cellular and developmental biology,* 32**,** 473-480.

GABORY, A., RIPOCHE, M.-A., LE DIGARCHER, A., WATRIN, F., ZIYYAT, A., FORNÉ, T., JAMMES, H., AINSCOUGH, J. F. X., SURANI, M. A., JOURNOT, L. & DANDOLO, L. 2009. H19 acts as a trans regulator of the imprinted gene network controlling growth in mice. *Development,* 136**,** 3413-3421.

GAO, Y.-Q., CHEN, X., WANG, P., LU, L., ZHAO, W., CHEN, C., CHEN, C.-P., TAO, T., SUN, J., ZHENG, Y.-Y., DU, J., LI, C.-J., GAN, Z.-J., GAO, X., CHEN, H.-Q. & ZHU, M.-S. 2015. Regulation of DLK1 by the maternally expressed miR-379/mir_544 cluster may underlie callipyge polar overdominance inheritance. *Proceedings of the National Academy of Sciences of the United States of America,* 112**,** 13627-13632.

GEORGES, M., CHARLIER, C., SMIT, M., DAVIS, E., SHAY, T., TORDOIR, X., TAKEDA, H., CAIMENT, F. & COCKETT, N. 2004. Toward molecular understanding of polar overdominance at the ovine callipyge locus. *Cold Spring Harbor Symposia on Quantitative Biology,* 69**,** 477-484.

GROSZMANN, M., GREAVES, I. K., FUJIMOTO, R., PEACOCK, W. J. & DENNIS, E. S. 2013. The role of epigenetics in hybrid vigour. *Trends in Genetics,* 29**,** 684-690.

HAIG, D. & GRAHAM, C. 1991. Genomic imprinting and the strange case of the insulin-like growth factor II receptor. *Cell,* 64**,** 1045 - 1046.

HE, G., HE, H. & DENG, X. W. 2013. Epigenetic variations in plant hybrids and their potential roles in heterosis. *Journal of Genetics and Genomics,* 40**,** 205-210.

HIENDLEDER, S., LEWALSKI, H. & JANKE, A. 2008. Complete mitochondrial genomes of *Bos taurus* and *Bos indicus* provide new insights into intra-species variation, taxonomy and domestication. *Cytogenetic and Genome Research,* 120**,** 150-156.

HOCHHOLDINGER, F. & HOECKER, N. 2007. Towards the molecular basis of heterosis. *Trends in Plant Science,* 12**,** 427-432.

HOLLAND, M. D. & ODDE, K. G. 1992. Factors affecting calf birth weight: A review. *Theriogenology,* 38**,** 769-798.

KENIRY, A., OXLEY, D., MONNIER, P., KYBA, M., DANDOLO, L., SMITS, G. & REIK, W. 2012. The H19 lincRNA is a developmental reservoir of miR-675 that suppresses growth and Igf1r. *Nature Cell Biology,* 14**,** 659-665.

KIM, K.-S., KIM, J.-J., DEKKERS, J. M. & ROTHSCHILD, M. 2004. Polar overdominant inheritance of a DLK1 polymorphism is associated with growth and fatness in pigs. *Mammalian Genome,* 15**,** 552-559.

KRIEGER, U., LIPPMAN, Z. B. & ZAMIR, D. 2010. The flowering gene SINGLE FLOWER TRUSS drives heterosis for yield in tomato. *Nature Genetics,* 42**,** 459-463.

LATOS, P. A., PAULER, F. M., KOERNER, M. V., ŞENERGIN, H. B., HUDSON, Q. J., STOCSITS, R. R., ALLHOFF, W., STRICKER, S. H., KLEMENT, R. M., WARCZOK, K. E., AUMAYR, K., PASIERBEK, P. & BARLOW, D. P. 2012. Airn

transcriptional overlap, but not its lncRNA products, induces imprinted Igf2r silencing. *Science,* 338**,** 1469-1472.

LAU, M. M., STEWART, C. E., LIU, Z., BHATT, H., ROTWEIN, P. & STEWART, C. L. 1994. Loss of the imprinted IGF2/cation-independent mannose 6-phosphate receptor results in fetal overgrowth and perinatal lethality. *Genes & Development,* 8**,** 2953-2963.

LIPPMAN, Z. B. & ZAMIR, D. 2007. Heterosis: revisiting the magic. *Trends in Genetics,* 23**,** 60-66.

LUBCHENCO, L. O., HANSMAN, C., DRESSLER, M. & BOYD, E. 1963. Intrauterine growth as estimated from liveborn birth-weigth data at 24 to 42 weeks of gestation. *Pediatrics,* 32**,** 793-800.

LUDWIG, T., EGGENSCHWILER, J., FISHER, P., D'ERCOLE, A. J., DAVENPORT, M. L. & EFSTRATIADIS, A. 1996. Mouse mutants lacking the Type 2 IGF receptor (IGF2R) are rescued from perinatal lethality in Igf2 and Igf1r null backgrounds. *Developmental Biology,* 177**,** 517-535.

MACLAUGHLIN, S. M., WALKER, S. K., ROBERTS, C. T., KLEEMANN, D. O. & MCMILLEN, I. C. 2005. Periconceptional nutrition and the relationship between maternal body weight changes in the periconceptional period and feto-placental growth in the sheep. *The Journal of Physiology,* 565**,** 111-124.

MCKINNON, T., CHAKRABORTY, C., GLEESON, L. M., CHIDIAC, P. & LALA, P. K. 2001. Stimulation of human extravillous trophoblast migration by IGF-II is mediated by IGF Type 2 receptor involving inhibitory G protein(s) and phosphorylation of MAPK. *The Journal of Clinical Endocrinology & Metabolism,* 86**,** 3665-3674.

MCMINN, J., WEI, M., SCHUPF, N., CUSMAI, J., JOHNSON, E. B., SMITH, A. C., WEKSBERG, R., THAKER, H. M. & TYCKO, B. 2006. Unbalanced placental expression of imprinted genes in human intrauterine growth restriction. *Placenta,* 27**,** 540-549.

MONK, D. 2015. Genomic imprinting in the human placenta. *American Journal of Obstetrics and Gynecology,* 213**,** S152-S162.

MOORE, T. & HAIG, D. 1991. Genomic imprinting in mammalian development: a parental tug-of-war. *Trends in Genetics,* 7**,** 45-49.

MORRISON, J. L. 2008. Sheep models of intrauterine growth restriction: fetal adaptations and consequences. *Clinical & Experimental Pharmacology & Physiology,* 35**,** 730-743.

MUNSON, L., WILHITE, A., BOLTZ, V. F. & WILKINSON, J. E. 1996. Transforming growth factor beta in bovine placentas. *Biology of Reproduction,* 55**,** 748-755.

MUTTER, G. L., STEWART, C. L., CHAPONOT, M. L. & POMPONIO, R. J. 1993. Oppositely imprinted genes H19 and insulin-like growth factor 2 are coexpressed in human androgenetic trophoblast. *American Journal of Human Genetics,* 53**,** 1096-1102.

RACHMILEWITZ, J., GILEADI, O., ELDAR-GEVA, T., SCHNEIDER, T., DE-GROOT, N. & HOCHBERG, A. 1992. Transcription of the H19 gene in differentiating cytotrophoblasts from human placenta. *Molecular Reproduction and Development,* 32**,** 196-202.

REYNOLDS, T. S., STEVENSON, K. M. & WATHES, D. C. 1997. Pregnancy-specific alterations in the expression of the insulin-like growth factor system during early placental development in the ewe. *Endocrinology,* 138**,** 886-897.

REYNOLDS, W. L., DEROUEN, T. M., MOIN, S. & KOONCE, K. L. 1980. Factors influencing gestation length, birth weight and calf survival of Angus, Zebu and Zebu cross beef cattle. *Journal of Animal Science,* 51**,** 860-867.

ROBERTS, C. T., OWENS, J. A. & SFERRUZZI-PERRI, A. N. 2008. Distinct actions of insulin-like growth factors (IGFs) on placental development and fetal growth: lessons from mice and guinea pigs. *Placenta,* 29**,** S42-S47.

ROBERTS, C. T., SOHLSTROM, A., KIND, K. L., EARL, R. A., KHONG, T. Y., ROBINSON, J. S., OWENS, P. C. & OWENS, J. A. 2001. Maternal food restriction reduces the exchange surface area and increases the barrier thickness of the placenta in the guinea-pig. *Placenta,* 22**,** 177-185.

SLEUTELS, F., ZWART, R. & BARLOW, D. P. 2002. The non-coding Air RNA is required for silencing autosomal imprinted genes. *Nature,* 415**,** 810-813.

SOARES, M. J. 2004. The prolactin and growth hormone families: Pregnancy-specific hormones/cytokines at the maternal-fetal interface. *Reproductive Biology and Endocrinology,* 2**,** 1-15.

SPRINGER, N. M. & STUPAR, R. M. 2007. Allelic variation and heterosis in maize: How do two halves make more than a whole? *Genome Research,* 17**,** 264-275.

SUE, S. R., CHARI, R. S., KONG, F. M., MILLS, J. J., FINE, R. L., JIRTLE, R. L. & MEYERS, W. C. 1995. Transforming growth factor-beta receptors and mannose 6-phosphate/insulin-like growth factor-II receptor expression in human hepatocellular carcinoma. *Annals of Surgery,* 222**,** 171-178.

TABATABAEI, S. & MAMOEI, M. 2012. Changes in the biochemical composition of fetal fluids and maternal blood serum during different days of gestation in cattle. *Comparative Clinical Pathology,* 21**,** 1005-1012.

WANG, X., MILLER, D. C., HARMAN, R., ANTCZAK, D. F. & CLARK, A. G. 2013. Paternally expressed genes predominate in the placenta. *Proceedings of the National Academy of Sciences,* 110**,** 10705-10710.

WERMTER, A. K., SCHERAG, A., D., M., REICHWALD, K., DURAND, E., NGUYEN, T. T., KOBERWITZ, K., LICHTNER, P., MEITINGER, T., SCHAFER, H., HINNEY, A., FROGUEL, P., HEBEBRAND, J. & BRONNER, G. 2008. Preferential reciprocal transfer of paternal/maternal DLK1 alleles to obese children: first evidence of polor overdominance in humans. *European Journal of Human Genetics,* 16**,** 1126-34.

WOLF, J. B., CHEVERUD, J. M., ROSEMAN, C. & HAGER, R. 2008a. Genome-wide analysis reveals a complex pattern of genomic imprinting in mice. *PLoS Genet,* 4**,** e1000091.

WOLF, J. B., HAGER, R. & CHEVERUD, J. M. 2008b. Genomic imprinting effects on complex traits: A phenotype-based perspective. *Epigenetics,* 3**,** 295-299.

WOODING, F. B. P. 1992. The synepitheliochorial placenta of ruminants: Binucleate cell fusions and hormone production. *Placenta,* 13**,** 101-113.

WOODING, F. B. P. & BECKERS, J. F. 1987. Trinucleate cells and the ultrastructural localisation of bovine placental lactogen. *Cell and Tissue Research,* 247**,** 667-673.

WOODING, F. B. P. & BURTON, G. 2008. Implantation, maternofetal exchange and vascular relationships. *In:* WOODING, F. B. P. & BURTON, G. (eds.) *Comparative Placentation.* Berlin, Hiedelberg: Springer-Verlag.

WUTZ, A., THEUSSL, H. C., DAUSMAN, J., JAENISCH, R., BARLOW, D. P. & WAGNER, E. F. 2001. Non-imprinted Igf2r expression decreases growth and rescues the Tme mutation in mice. *Development,* 128**,** 1881-1887.

YOSHIMIZU, T., MIROGLIO, A., RIPOCHE, M.-A., GABORY, A., VERNUCCI, M., RICCIO, A., COLNOT, S., GODARD, C., TERRIS, B., JAMMES, H. & DANDOLO, L. 2008. The H19 locus acts in vivo as a tumor suppressor. *Proceedings of the National Academy of Sciences of the United States of America,* 105**,** 12417-12422.

ZECHNER, U., HEMBERGER, M., CONSTÂNCIA, M., ORTH, A., DRAGATSIS, I., LÜTTGES, A., HAMEISTER, H. & FUNDELE, R. 2002. Proliferation and growth factor expression in abnormally enlarged placentas of mouse interspecific hybrids. *Developmental Dynamics,* 224**,** 125-134.

Chapter 5 General Discussion

The placenta is central to fetal growth and birth weight and a major determinant of programming postnatal performance and lifelong health. We used a bovine model with *Bos taurus taurus* (Angus, A) and *Bos taurus indicus* (Brahman, B) genetics and intercepted purebred and reciprocal cross concepti at mid-gestation (Day 153) before the fetus enters accelerated growth to investigate effects of fetal sex and genetics on concepti traits, with special focus on gross morphology and histomorphology of the placenta and gene expression of the insulin-like growth factor (IGF) system. In addition, we used another experimental cohort consisting of purebred and reciprocal cross embryos recovered at the late embryonic stage (Day 48), where the transition from embryo to fetus occurs, to assess developmental changes in gross morphology and histomorphology of the placenta and their association with fetal growth.

5.1 Developmental changes in gross morphology and histomorphology of the bovine placenta associated with embryo-fetal growth

The present study comprehensively characterized bovine placentae recovered at Day 48 and Day 153 of gestation and identified placental characteristics that were positively related to embryo and fetal weights. Interestingly, we observed that the two placentome types, i.e., convex and flat, differ in number and in several histomorphometric parameters. At Day 48 of gestation, higher numbers of convex placentomes were present, compared to flat placentomes, and convex placentomes had lower volume density of maternal connective tissue. By Day 153, convex placentomes are fewer in number, but have a higher volume of different placental cell types and greater surface area for transplacental exchange than flat placentomes. These characteristics may therefore explain the significant and exclusive positive relationship of embryo and fetal weights with the number of convex placentomes. Furthermore, we found that number and average weight of convex placentomes in the four fetal maternal and paternal genome combinations displayed a maternal expression pattern, where concepti with maternal A genome (B×A and A×A) had higher values than fetuses with

maternal B genome (A×B, B×B), while number of flat placentomes did not differ between groups (Chapter 4). Not surprisingly, B×A and A×A concepti had heavier fetal weights at mid-gestation compared to the other two genome combinations. Therefore, our findings suggest a major role for convex placentomes in nutrient transfer to support fetal growth in the bovine.

At Day 48 of gestation, the majority (88.3%) of placentomes that had developed according to signs of interdigitation of fetal cotyledon and caruncle were characterized as convex or flat. The remaining smaller number of developed placentomes (11.7%) was irregular or had an uneven surface and may have been adversely affected by handling while the *Placenta fetalis* was detached or when uteri were stored (**See Appendix 1**). Histological sections confirmed only two types of placentomes, convex and flat, where convex placentomes had lower maternal connective tissue volume density at Day 48 of gestation. It is possible that maternal connective tissue at Day 48 participates in determining placentome shape by functioning as a mould. A more recent study showed that shortly after implantation the maternal stroma forms caruncular projections where the chorionic villi can interdigitate and develop (Aires et al., 2014). Consistent with our findings, a previous report had also identified two types of placentomes in the cattle placenta at a fetal crown rump length of 9 cm (approximately Day 60 of gestation). These were termed 'pedunculated' and 'non-pedunculated', the latter type also being typical for buffalo placentomes (Schmidt et al., 2006). The pedunculated placentome forms a mushroom-like structure with a caruncular stalk and corresponds to the convex placentome type observed in the present study. On the other hand, the non-pedunculated placentome is dome shaped without a caruncular stalk and corresponds to the flat placentome type observed by us. At Day 153, convex placentomes were lower in number but had greater volumes of different placental cell types and surface areas than flat placentomes. Since we used different animal cohorts to study concepti at different developmental stages, it was not possible to directly monitor the growth of these two

placentome types. Thus, it would be difficult to draw conclusions as to whether convex placentomes can transform into flat placentomes or are stretched as the uterus grows, which could potentially result in lower numbers of convex placentomes at Day 153. Further studies monitoring the ontogenesis of the two placentome types are required to answer specific questions on potential placentome type transformation in bovine.

Chapter 4 reports significant effects of fetal genetics on the number and proportion of feto-maternal syncytia, which displayed a paternal expression pattern. Data on number and proportion of different trophoblast cell types, i.e., mononucleate cells, trophoblast giant cells (or binucleate cells) and feto-maternal syncytia, were not included in Chapter 2, as complete data on these cell types were not available for Day 48 concepti. However, the percentage of mononucleate cells and binucleate cells was reported to remain stable throughout gestation at about 75% and 20%, respectively in cattle (Igwebuike, 2006, Wooding and Burton, 2008). Furthermore, the uterine epithelium was described to contain about 2% binucleate cells (Wooding and Wathes, 1980) which appears to be the feto-maternal syncytium or remnants of the pyknotic binucleate cell. In the present study, relative to the total number of trophoblast cells, the percentage of mononucleate cells, trophoblast giant cells, and feto-maternal syncytia averaged 75%, 22% and 3%, respectively at Day 153 of gestation. Preliminary analysis also showed that the number and proportion of different trophoblast cell types did not differ between the two placentome types and were similar for both fetal sexes.

5.2 Sex-specific placental and umbilical cord phenotype mediate sex differences in fetal growth at mid-gestation

We report the novel finding that normal female concepti at mid-gestation exhibit hallmarks of intrauterine growth restriction (Chapter 3). While we focused on Day 153 concepti to build on our hypothesis that sexual dimorphism in placenta and umbilical cord at this important developmental stage mediates female-specific growth resembling IUGR, previous data have shown that male bovine fetuses are heavier as early as Day 100 of

gestation (Eley et al., 1978). This led us to explore the effects of sex on a smaller number of conceptus traits using embryos recovered at Day 48 to better understand what may have contributed to divergent growth between males and females at Day 153. Interestingly, we found that in addition to being already heavier, male Day 48 embryos also had heavier umbilical cords while their *Placenta fetalis* weight was not significantly different from females (**see Appendix 2**). These preliminary results suggest that the umbilical cord, which provides the conduit for nutrient transfer from the placenta to the fetus, contributes to sex differences in embryo weight early on and that umbilical blood flow differs between males and females as early as Day 48 of gestation. Further, our results at Day 153 of gestation indicate that sex differences in umbilical cord parameters persist as gestation progresses and precede changes in placental weight and relative growth of fetal organs. At mid-gestation, where placental weight is lower compared to fetal weight, indicating a shift to higher fetal demand relative to placental supply (Schneider, 1996), the umbilical cord phenotype of females has a stronger impact on fetal growth than that of males.

The present study has identified a significant role of the placenta and umbilical cord in mediating sex-specific fetal growth patterns. Further studies to investigate the mechanisms responsible for this sexual dimorphism could determine the effects of sex on expression of growth factors, such as the vascular endothelial growth factor which is involved in angiogenesis and the development of the vascular system including the placental microvasculature and umbilical cord (Krukier and Pogorelova, 2006, Pfarrer et al., 2006, Arroyo and Winn, 2008). While the majority of human IUGR cases are associated with fetal hypoxia, it was not possible to examine oxygen and carbon dioxide levels in cord blood in the current study. Sex differences in these blood gases and haemoglobin levels may be considered for future work. This would provide further information on the association between hypoxia and γ-glutmayl transferase (GGT) activity or components related to

glutathione synthesis as reported in fetal guinea pigs and young rats (Šťastný et al., 1985, Oh et al., 2008).

Females are more often born with IUGR compared to males (Borzsonyi et al., 2011). However, even in newborns with very low birth weights, neonatal morbidity and mortality rate are still higher in males than females (Stevenson et al., 2000). Findings of the present study have implications for human medicine where we show that female-specific adaptation to constrained nutrient supply are already established by mid-gestation and may explain greater resilience of females to insults later in pregnancy. This further supports the need for development of customized fetal sex-specific prenatal care in human.

5.3 Polar overdominance of paternal genome on umbilical cord phenotype and maternal genome effects on placenta drive heterosis *in utero*

In Chapter 4, we identified significant fetal genetic effects on twenty-one conceptus traits including imprinted transcripts of the IGF system, which displayed phenotypic expression patterns consistent with maternal- and paternal imprinting or polar over- and underdominance, as described in mouse (Wolf et al., 2008). Our study strongly indicates that polar overdominance in combination with maternal and paternal genome effects on placental and umbilical cord phenotype drives heterosis *in utero*.

The specific parental genome effects, where superior maternal A genome effect is evident in placental traits and polar overdominance of paternal B genome is evident in umbilical cord phenotype and fetal fluid volume, clearly concur with the genetic conflict hypothesis (Moore and Haig, 1991). In light of this hypothesis and our findings, we interpret our data such that the maternal genome regulates fetal growth through the placenta, while the paternal genome supports heterotic fetal growth through its positive polar overdominance effects on umbilical cord and fetal fluid volume, which facilitate greater resource extraction from the mother. A different statistical modelling approach to dissect the magnitude of paternal and maternal genome effects on fetal muscle and bone phenotypes (Xiang et al.,

2013, Xiang et al., 2014) was also applied to our data to quantify maternal and paternal genome effects on placental and umbilical cord phenotype (Estrella et al., 2013, **see Appendix 3**). The results obtained from this analysis are consistent with the maternal-, paternal-, and polar overdominance patterns reported and conclusions drawn in Chapter 4. Future investigation of the association of paternally expressed genes with the umbilical cord phenotype is warranted.

Here and in Chapter 3, significant contribution of the umbilical cord phenotype to differences in fetal growth between genetic groups and sex is evident. This strongly suggests that in addition to placenta as a fetal programming agent, the umbilical cord is a likely candidate to have long-term effects on postnatal health and performance. Studies leading to the development of biomarkers for umbilical cord vessel diameter to predict postnatal risk for poor performance will be useful in human medicine and animal production alike.

5.4 A significant role for imprinted *IGF2/H19* and *IGF2R/AIRN* in placental development and heterosis

Effects of fetal sex and genetics on tissue-specific transcript abundances of IGF system components were reported in Chapters 3 and 4. Differences in transcript levels of *IGF2* in fetal brain and heart between sexes and between fetal genotypes in *Placenta fetalis* at mid-gestation, as well as lower circulating level of IGF2 in females compared to males, clearly indicate the more significant role of IGF2 compared to IGF1 at mid-gestation. The female-specific negative relationship between circulating levels of IGF2 and total placental weight (Chapter 3), as well as the negative correlation between *IGF2* and placental weight, and the positive correlation between *IGF2* and the number and proportion of syncytia (Chapter 4), further support a role of IGF2 in placental development by predominantly promoting trophoblast invasion over that of proliferation (Dunk et al., 2009).

Furthermore, transcript abundance of *IGF1R* in placenta was below the detection limit, indicating that effects of IGF2 in placenta are largely mediated by IGF2R. Polar

overdominance in placental *IGF2R* expression of the B×A hybrid, together with intermediate *IGF2* expression reported in Chapter 4, suggest that IGF2 action on trophoblast migration and syncytium formation is through interaction with IGF2R. This concurs with a previous study which indicated that IGF2 effects on migration of extravillous trophoblast cells were mediated by IGF2R *in vitro* (McKinnon et al., 2001). While the bulk of *IGF2R* mRNA in bovine fetal tissues is transcribed from the maternally inherited allele, a more recent report demonstrated substantial expression of transcript from the paternal allele in bovine at Day 80 of gestation (Bebbere et al., 2013). Thus, analysis of the imprinting status of *IGF2R* in the placenta at Day 153 would contribute to further understanding of the maternal and paternal contributions to polar overdominance expression. Polymorphic and variable imprinting of *IGF2R* has been demonstrated in human placenta (reviewed in Bebbere et al., 2013). The expression patterns of *H19* and *AIRN* were highly similar to expression of *IGF2* and *IGF2R*, respectively. This further demonstrates the co-regulation and/or interaction of these imprinted genes and their associated long non-coding (lnc) RNAs. It also points to important roles of these lnc RNAs in placental growth and development. For example, *H19*'s anti-proliferative function as a tumour suppressor (Yoshimizu et al., 2008) and negative regulator of placental growth (Keniry et al., 2012) is consistent with results of the present study where transcript abundance of *H19* was in a negative relationship with *Placenta fetalis weight.* On the other hand, *AIRN* plays an important role in regulating imprinted expression of *IGF2R* (Wutz et al., 2001, Sleutels et al., 2002) but its direct effects on placental development remain unclear. Future studies on fetal genetic effects could extend to miRNAs and other genes involved in placental and fetal growth including imprinted transcripts from the Delta-like 1 homologue – Iodothyronine deiodinase type 3 (DLK1-DIO3) imprinted gene cluster.

5.5 General Conclusions

Using *Bos taurus taurus* (Angus, A) and *Bos taurus indicus* (Brahman, B) purebred and reciprocal cross concepti recovered at mid-gestation (Day 153), and a similar resource of concepti recovered at the late embryo stage (Day 48), the current study has examined developmental changes in the placenta that could impact fetal growth and focused on effects of fetal sex and genetics on conceptus traits, specifically gross morphology and histomorphology of the placenta, morphology of the umbilical cord, and transcript abundance of genes from the IGF system.

The main findings of this research project include (i) the significant contribution of convex placentomes to fetal growth as supported by their exclusive positive relationships with embryo and fetal weights and their high number and average weight in B×A and A×A concepti; (ii) fetal sex effects on placental and umbilical cord phenotypes that mediate sex-specific fetal growth, where females resemble an IUGR phenotype; (iii) significant fetal genetic effects on placental and umbilical cord phenotypes and imprinted transcripts of the IGF system, with the superior B×A hybrid birth weight mirrored in observed polar overdominance patterns caused by the combination of paternal *Bos taurus indicus* and maternal *Bos taurus taurus* genomes; and (iv) differences in circulating IGF2 and transcript abundance of *IGF2* in fetal brain and heart between sexes and differences in transcript abundances of *IGF2*, *IGF2R*, *H19*, *AIRN* in placenta between fetuses with different maternal and paternal genome combinations demonstrate the important role of IGF2/IGF2R and associated lncRNAs at this stage of fetal development.

In conclusion, the results of this study support the hypothesis that differences in placental and umbilical cord phenotypes and expression of genes in the IGF system between males and females and between purebreds and reciprocal cross hybrids determine variation in intrauterine growth at mid-gestation and contribute to programming of postnatal performance and health.

5.6 Literature Cited

AIRES, M. B., DEGAKI, K. Y., DANTZER, V. & YAMADA, A. T. 2014. Bovine placentome development during early pregnancy. *In:* MENDEZ-VILLAS, A. (ed.) *Microscopy: Advances in Scientific Research and Education.* Spain: Formatex Research Center.

ARROYO, J. A. & WINN, V. D. 2008. Vasculogenesis and angiogenesis in the IUGR placenta. *Seminars in Perinatology,* 32**,** 172-177.

BEBBERE, D., BAUERSACHS, S., FÜRST, R. W., REICHENBACH, H.-D., REICHENBACH, M., MEDUGORAC, I., ULBRICH, S. E., WOLF, E., LEDDA, S. & HIENDLEDER, S. 2013. Tissue-specific and minor inter-individual variation in Imprinting of IGF2R is a common feature of *Bos taurus* concepti and not correlated with fetal weight. *PLOS ONE,* 8**,** e59564.

BORZSONYI, B., DEMENDI, C., NAGY, Z., TOTH, K., CSANAD, M., PAJOR, A., RIGO, J. J. & JOO, J. G. 2011. Gene expression patterns of insulin-like growth factor 1, insulin-like growth factor 2 and insulin-like growth factor binding protein 3 in human placenta from pregnancies with intrauterine growth restriction. *Journal of Perinatal Medicine,* 39**,** 701-707.

DUNK, C., HUPPERTZ, B. & KINGDOM, J. 2009. Development of the placenta and its circulation. *In:* RODECK, C. (ed.) *Fetal Medicine: Basic Science and Clinical Perspective.* Second ed.: Elsevier Limited.

ELEY, R. M., THATCHER, W. W., BAZER, F. W., WILCOX, C. J., BECKER, R. B., HEAD, H. H. & ADKINSON, R. W. 1978. Development of the conceptus in the bovine. *Journal of Dairy Science,* 61**,** 467-473.

ESTRELLA, C., XIANG, R., FITZSIMMONS, C., KRUK, Z., BURNS, B., ROBERTS, C. T. & HIENDLEDER, S. Novel paternal genome effects on placental and fetal phenotype at midgestation. 8th World Congress on Developmental Origins of Health and Disease, 2013 Singapore.

IGWEBUIKE, U. M. 2006. Trophoblast cells of ruminant placentas—a minireview. *Animal Reproduction Science,* 93**,** 185-198.

KENIRY, A., OXLEY, D., MONNIER, P., KYBA, M., DANDOLO, L., SMITS, G. & REIK, W. 2012. The H19 lincRNA is a developmental reservoir of miR-675 that suppresses growth and Igf1r. Nature Cell Biology, 14, 659-665

KRUKIER, I. I. & POGORELOVA, T. N. 2006. Production of vascular endothelial factor and endothelin in the placenta and umbilical cord during normal and complicated pregnancy. *Bulletin of Experimental Biology and Medicine,* 144**,** 216-218.

MCKINNON, T., CHAKRABORTY, C., GLEESON, L. M., CHIDIAC, P. & LALA, P. K. 2001. Stimulation of human extravillous trophoblast migration by IGF-II is mediated by IGF Type 2 receptor involving inhibitory G protein(s) and phosphorylation of MAPK. *The Journal of Clinical Endocrinology & Metabolism,* 86**,** 3665-3674.

MOORE, T. & HAIG, D. 1991. Genomic imprinting in mammalian development: a parental tug-of-war. *Trends in Genetics,* 7**,** 45-49.

OH, C., DONG, Y., HARMAN, C., MIGHTY, H. E., KOPELMAN, J. & THOMPSON, L. P. 2008. Chronic hypoxia differentially increases glutathione content and γ-glutamyl cysteine synthetase expression in fetal guinea pig organs. *Early Human Development,* 84**,** 121-127.

PFARRER, C. D., RUZIWA, S. D., WINTHER, H., CALLESEN, H., LEISER, R. & SCHAMS, D. 2006. Localization of vascular endothelial growth factor (VEGF) and its receptors VEGFR-1 and VEGFR-2 in bovine placentomes from implantation until term. *Placenta,* 27.

SCHMIDT, S., GERBER, D., SOLEY, J. T., AIRE, T. A. & BOOS, A. 2006. Histo-morphology of the uterus and early placenta of the African Buffalo (*Syncerus caffer*) and comparative placentome morphology of the African Buffalo and cattle (*Bos taurus*). *Placenta,* 27**,** 899-911.

SCHNEIDER, H. 1996. Ontogenic changes in the nutritive function of the placenta. *Placenta,* 17**,** 15-26.

SLEUTELS, F., ZWART, R. & BARLOW, D. P. 2002. The non-coding Air RNA is required for silencing autosomal imprinted genes. *Nature,* 415**,** 810-813.

ŠŤASTNÝ, F., LISÝ, V., TOMÁŠOVÁ, H. & TROJAN, S. 1985. Effects of short-term and prolonged aerogenic hypoxia on gamma-glutamyl transpeptidase activity in the brain, liver, and biological fluids of young rats. *Neurochemical Research,* 10**,** 819-828.

STEVENSON, D. K., VERTER, J., FANAROFF, A. A., OH, W., EHRENKRANZ, R. A., SHANKARAN, S., DONOVAN, E. F., WRIGHT, L. L., LEMONS, J. A., TYSON, J. E., KORONES, S. B., BAUER, C. R., STOLL, B. J. & PAPILE, L.-A. 2000. Sex differences in outcomes of very low birthweight infants: the newborn male disadvantage. *Archives of Disease in Childhood - Fetal and Neonatal Edition,* 83**,** F182-F185.

WOLF, J. B., HAGER, R. & CHEVERUD, J. M. 2008. Genomic imprinting effects on complex traits: A phenotype-based perspective. *Epigenetics,* 3**,** 295-299.

WOODING, F. B. & WATHES, D. C. 1980. Binucleate cell migration in the bovine placentome. *Journal of Reproduction and Fertility,* 59**,** 425.

WOODING, F. B. P. & BURTON, G. 2008. Synepitheliochorial placentation: ruminants (ewe and cow). *In:* WOODING, F. B. P. & BURTON, G. (eds.) *Comparative Placentation.* Berlin, Hiedelberg: Springer-Verlag.

WUTZ, A., THEUSSL, H. C., DAUSMAN, J., JAENISCH, R., BARLOW, D. P. & WAGNER, E. F. 2001. Non-imprinted Igf2r expression decreases growth and rescues the Tme mutation in mice. *Development*, 128**,** 1881-1887.

XIANG, R., GHANIPOOR-SAMAMI, M., JOHNS, W. H., EINDORF, T., RUTLEY, D. L., KRUK, Z. A., FITZSIMMONS, C. J., THOMSEN, D. A., ROBERTS, C. T., BURNS, B. M., ANDERSON, G. I., GREENWOOD, P. L. & HIENDLEDER, S. 2013. Maternal and paternal genomes differentially affect myofibre characteristics and muscle weights of bovine fetuses at midgestation. *PLOS ONE,* 8**,** e53402.

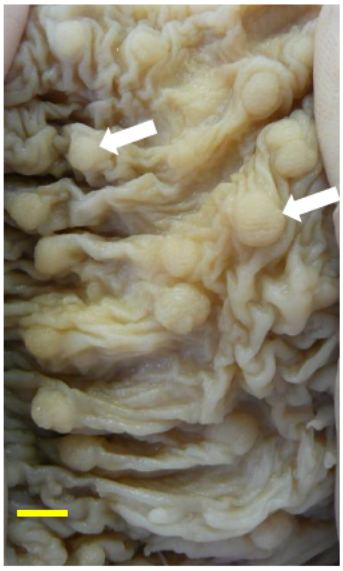
XIANG, R., LEE, A. M. C., EINDORF, T., JAVADMANESH, A., GHANIPOOR-SAMAMI, M., GUGGER, M., FITZSIMMONS, C. J., KRUK, Z. A., PITCHFORD, W. S., LEVITON, A. J., THOMSEN, D. A., BECKMAN, I., ANDERSON, G. I., BURNS, B. M., RUTLEY, D. L., XIAN, C. J. & HIENDLEDER, S. 2014. Widespread differential maternal and paternal genome effects on fetal bone phenotype at mid-gestation. *Journal of Bone and Mineral Research,* 29**,** 2392–2404

YOSHIMIZU, T., MIROGLIO, A., RIPOCHE, M.-A., GABORY, A., VERNUCCI, M., RICCIO, A., COLNOT, S., GODARD, C., TERRIS, B., JAMMES, H. & DANDOLO, L. 2008. The H19 locus acts in vivo as a tumor suppressor. *Proceedings of the National Academy of Sciences of the United States of America,* 105**,** 12417-12422.

Appendices

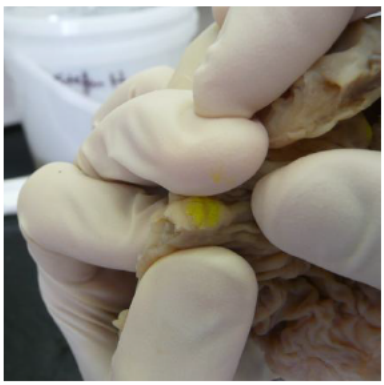
Appendix 1 Caruncles at Day 48 (late embryo stage).

Gravid horn



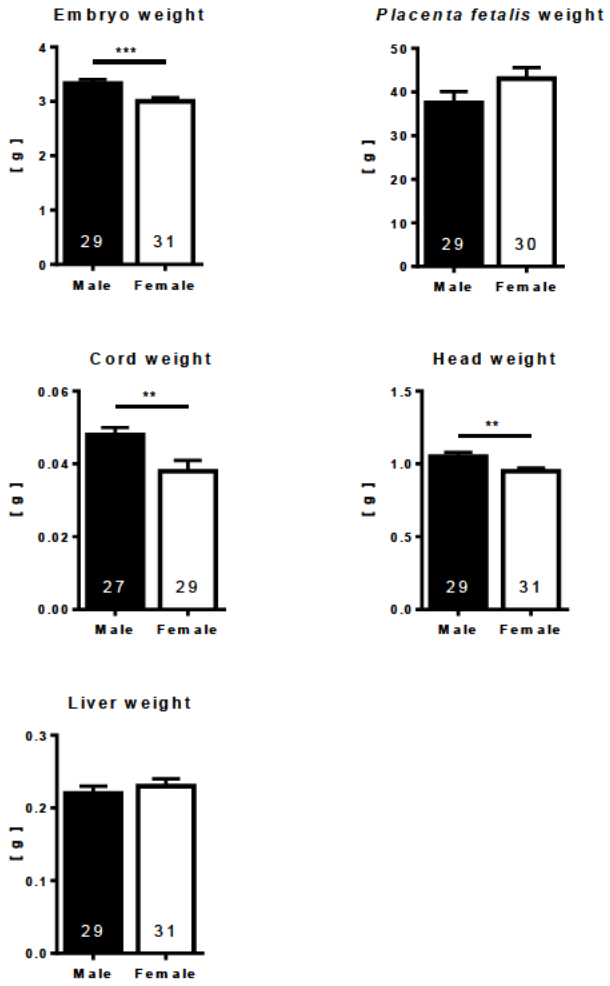
Non-Gravid horn

Caruncle with uneven surface



Developed caruncles with signs of interdigitation are found in the gravid horn but not in the non-gravid horn at Day 48 (late embryonic stage). Developed caruncles measured approximately 1-7 mm, while some caruncles presented with an irregular and/or uneven surface. Scale bar is approximately 3 mm. Arrows point to developed caruncles.

Appendix 2 Sex differences in selected concepti traits at Day 48 (late embryo stage).



Preliminary analyses of some concepti characteristics at Day 48 of gestation. Data adjusted for fetal genetics and presented as least square means ± SEM. ***P<0.001, **P<0.01

Appendix 3 Paternal genome effects on placental phenotype at mid-gestation (poster presented at the 8th World Congress on Developmental Origins of Health and Disease, 2013).

DOHAD2013

Epigenetics

DOHaD13-1691

NOVEL PATERNAL GENOME EFFECTS ON PLACENTAL AND FETAL PHENOTYPE AT MIDGESTATION

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Background: Inequality of maternal and paternal genomes due to genomic imprinting and mitochondrial DNA is well established. However, the magnitude of maternal and paternal genome effects on variation in placental and fetal traits remains to be investigated.

Method: We used Bos taurus and Bos indicus dams and sires to generate both purebred and reciprocal cross fetuses (n=73) that were collected at day 153 of gestation. Weights and measurements of placenta, umbilical cord, fetal fluids and fetal organs were analysed by general linear models with main effects of maternal and paternal genome and fetal sex.

Results: Our results show that parental genomes explained significant amounts of variation (58-100%, P<0.05-0.0001) in twenty-two placental and fetal traits. Specifically, while maternal genome largely explains genetic variation in placental traits (90%) and fetal organ

weights (60-98%), the paternal genome predominantly contributes to variation in umbilical cord weight and length (73%), placenta (70%) and umbilical cord (83%) efficiencies, and total fetal fluids weight (73%). Moreover, there are significant paternal genome-determined correlations between a) fetus and umbilical cord weight ($P<0.0001$), b) fetal heart and umbilical cord weight ($P<0.001$), c) fetal heart weight and umbilical artery ($P<0.01$) and vein diameter ($P<0.05$), d) fetal lung and total fetal fluids weight ($P<0.05$). The umbilical cord is a channel for maternal resource transfer to the fetus. Another reservoir of bioactive compounds the fetus has immediate access to are the fetal fluids. Both characteristics are influenced by the paternal genome, apparently directed to enhancing fetal growth. On the other hand, the maternal genome, possibly via maternally expressed genes, regulates resource allocation to the fetus through changes in placental morphology and weight.

Conclusion: In conclusion, our study shows for the first time, the extent of maternal and paternal genome contributions to placental and fetal traits. Our data support co-existence of parental genetic conflict and maternal co-adaptation as explanations for parent-of-origin effects at the phenotypic level.

References: Haig D (2004) Genomic Imprinting and Kinship: How Good is the Evidence? Annual Review of Genetics 38: 553-85.

Wolf JB, Hager R (2006) A Maternal–Offspring Coadaptation Theory for the Evolution of Genomic Imprinting. PLoS Biology 4: e380.