Differential maternal and paternal genome effects on placental and fetal phenotype and gene expression at midgestation

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Declaration

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Abstract

Lifelong development is largely programmed prenatally. Genetic and epigenetic factors, such as mitochondrial (mt) DNA variation and parent-of-origin effects, significantly contribute to variation in important prenatal phenotypes that determine lifetime development, including placenta and fetal musculoskeletal system. Such effects initially impact on transcriptome expression levels and eventually give rise to altered phenotypic traits. However, data regarding the overall magnitude and specificity of maternal and paternal genome effects in mammalian prenatal development is lacking.

The present study aimed to dissect and quantify differential maternal and paternal genome effects on specific placental and fetal traits, and associated transcriptomic events which drive prenatal development. A large bovine fetal resource (n=73), consisting of both purebreds and reciprocal hybrids with *Bos taurus taurus* (Angus) and *Bos taurus indicus* (Brahman) (epi)genetics, was used in this study. We examined 41 gross- and histo-morphological placental and fetal traits, 51 fetal bone weight and geometry parameters, and 22 myofibre characteristics and muscle mass parameters using morphometrical and/or immunohistochemical methods. Expression of the long non-coding RNA H19 in fetal muscle was determined by real time quantitative PCR. Profiles of mRNA and microRNA expression were obtained with microarrays that contained 24,027 and 13,133 mammalian probe sets, respectively, to assess transcript abundances in fetal liver. Phenotypic data were analysed by Analysis of Variance (ANOVA) using general linear models with nested effects and transcriptome data were analysed with microarray ANOVA procedures.

The analyses identified 49 significant placental and fetal traits, including five principal components representing 51 bone parameters, and H19 gene expression levels in muscle, with

ANOVA model significance levels (P) ranging from 3×10^{-2}-9×10^{-17}. We showed that parental genomes contributed to the largest proportion of variation explained by linear models for a majority of placental and fetal traits. Fetal sex was the next most significant factor to explain variation in these traits and non-genetic maternal effects, such as post-conception weight gain and final maternal weight, explained the least amount of variation. Significant effects of the maternal genome ($P<5\times10^{-2}$-5×10^{-13}) predominantly contributed to genetic variation in:

(i) Gross- and histo-morphological placental traits and fetal organ weights (59.6−99.9%,); (ii) most extracted principle components (PCs) representing bone weight and geometry traits, including PC1/bone mass (74%), PC3/limb elongation (73%), PC4/flat bone elongation (74%) and PC5/axial skeletal growth (97%) and (iii) most myofibre characteristics including fast myofibre cross-sectional area (CSA, 93%), total cell CSA (82%), absolute mass of studied muscles (59-88%) and *H19* transcript abundance in fetal muscle (76%). Conversely, significant paternal genome ($P<4\times10^{-2}$-7×10^{-8}) predominantly contributed to genetic variation in:

(i) Fetal fluids weight (73%), umbilical cord weight and length (73%), maternal placenta (70%) and umbilical cord (83%) efficiencies; (ii) PC2/limb ossification (95%) and (iii) Relative mass of studied muscles to fetal weight (54-97%).

Further, using nested effects in ANOVA, we found that maternal genome strongly determined regressions between placental weights and umbilical cord traits ($P<4\times10^{-2}$-2×10^{-6}), whereas paternal genome and/or fetal sex determined regressions between weight of fetus, fetal organ and fetal fluid s and umbilical cord traits ($P<5\times10^{-2}$-10×10^{-8}).

For fetal liver transcription profiles, maternal genome strongly affected expression levels of:

(i) Twenty-four mRNA transcripts (false discovery rate, FDR adjusted $P<4\times10^{-2}$-10×10^{-6}), 13 of which were located in the mt genome and (ii) ten autosomal non-coding RNA transcripts

including mammalian *SNORD113-9*, small nucleolar (sno)RNA, *MIR187* and *MIR1973* microRNA (FDR adjusted $P<5\times10^{-2}$-8×10^{-3}).

Paternal genome moderately affected expression levels of:

(i) Forty-seven autosomal mRNA transcripts (FDR adjusted, $P<5\times10^{-2}$-4×10^{-2}) (ii) *MIR184* microRNA transcripts in five mammalian species (FDR adjusted, $P<5\times10^{-2}$-4×10^{-2}).

Two significant coexpression networks, between 86 significant mRNAs and non-coding RNA transcripts, were also identified for differential maternal and paternal genome effects.

Our results show, for the first time, that a wide range of phenotypic and molecular traits within the placental-fetal system are affected by differential maternal and paternal genome and fetal sex effects. Identified differential maternal and paternal genome effects on specific placental and fetal traits are consistent with expression patterns of parent-of-origin effects predicted by both conflict-of-interest and maternal-offspring coadapdation hypotheses, thereby providing important insights to accommodate both hypotheses that explain the evolutionary basis of genomic imprinting effects. Observed complex, and predominantly maternal genome, effects are suggested to result from interaction between epigenetic factors from nuclear and mt genomes via RNA interference. This is further evidence for complex epigenetic crosstalk and coordination that contributes to mammalian prenatal development. Identified morphological and transcriptional modules within the placental-fetal system help to provide a new level of understanding prenatal development, i.e., systematic integration of omics data. Detailed molecular profiles of all core tissues and organs are now required to elucidate genetic, epigenetic and non-genetic components and interactions that control variation in placental and fetal phenotype. Future studies linking genome and epigenome with phenome data covering the

complete placental-fetal system will provide a new multi-layer picture of understanding coordination for molecular and phenotypic events driving mammalian prenatal development.

Chapter 1: Literature Analysis

Chapter 1

1.1 Introduction

Lifelong development of a wide range of phenotypes is largely programmed prenatally. Stimuli or insults in the critical period of prenatal development can have lifetime consequences (Lucas *et al.* 1999). One outcome of prenatal development, birthweight, has been widely used as an indicator of postnatal development. Prenatally, mammalian development relies on nutrients provided by the placenta. Placental growth significantly affects birthweight (Salafia *et al.* 2007) and impacts on lifelong health (Lewis *et al.* 2006) and performance (Reynolds *et al.* 2010). Furthermore, the musculoskeletal system, that largely accounts for body mass and serves as an important endocrine organ to maintain metabolic homeostasis (Pedersen and Febbraio 2008; DiGirolamo *et al.* 2012), is programmed prenatally to a considerable extent (Sayer and Cooper 2005; Dennison *et al.* 2010). Such programming initiates at the transcriptome level (Cox *et al.* 2012; Sookoian *et al.* 2013), where the transcript abundance profile is a molecular phenotype (Jansen and Nap 2001) that eventually gives rise to variation in classical phenotypic traits. Thus, the placenta and prenatal musculoskeletal system, with associated transcriptome profiles, provide the foundation for postnatal development.

Factors significantly influencing placentogenesis and fetal musculoskeletal system programming, such as nutrition and environmental variation, are well documented (Dennison *et al.* 2010; Du *et al.* 2010; Wu *et al.* 2012). However, the study of genetic effects on placentogenesis and prenatal musculoskeletal development with associated transcriptional events, has gained much less attention, even though genetics is a major source of variation in mammalian lifelong development. For instance, previous data suggested that genetics accounted

for up to 56% of variation in placenta weight (Mesa *et al.* 2005; Buresova *et al.* 2006), up to 80% of variation in postnatal musculoskeletal mass (Smith *et al.* 1973; Gueguen *et al.* 1995; Arden and Spector 1997; Larzul *et al.* 1997) and up to 80% of variation in mRNA transcript abundance in human lymphoblastoid cell lines (Dixon *et al.* 2007). Furthermore, apart from Mendelian genetics, non-Mendelian genetic and epigenetic factors, caused by non-equivalence of maternal and paternal genomes, can significantly affect important prenatal traits. This includes genomic imprinting that refers to parent-of-origin specific patterns of gene expression (Reik and Walter 2001). Important imprinted genes such as insulin-like growth factor 2 (*IGF2*) are critical for placental (Constancia *et al.* 2002) and musculoskeletal development (Eggenschwiler *et al.* 1997; Jin-Tae Jeon 1999; Nezer *et al.* 1999).

Quantitative analyses of outbred mice identified various expression patterns of complex postnatal traits for genomic imprinting, which suggested different magnitudes of maternal and paternal contributions to genetic variation in specific traits (Wolf *et al.* 2008). More recently, statistical modelling quantified the magnitude of parent-of-origin effects attributed to genomic imprinting on specific postnatal mouse skeletal and bovine muscle traits (Leamy *et al.* 2008; Neugebauer *et al.* 2010; Neugebauer *et al.* 2010) as well as human global gene expression levels (Garg *et al.* 2012). However, such data is lacking for prenatal stages. This literature analysis firstly examines the lifetime impacts of development of placenta, fetal musculoskeletal system and associated molecular events. This is followed a review of genetic and epigenetic effects on traditional and molecular phenotypes in mammals including bovine, which leads to our research topic of differential maternal and paternal genome effects on phenotype of placenta, fetal musculoskeletal system and associated transcript abundance profiles.

1.2 Placenta

1.2.1 Role of placenta

Placenta, as a pivotal organ for prenatal development, maintains fetal homoeostasis by providing an immunological barrier between fetus and mother, and by mediating the transfer of respiratory gases, water, ions and nutrients. Furthermore, the placenta produces and/or metabolises maternally produced hormones, such as growth hormone, thyroid hormones and insulin-like growth factors (IGF)-I and -II (Fowden 2003; Murphy *et al.* 2006; Patel *et al.* 2011), which governs nutrient transfer, fetal growth and mother-fetus homeostasis. Nutrients and hormones traverse the specialised trophoblast cells of the placenta and are metabolised into forms most useful for fetal development (Bell and Ehrhardt 2002). Since the placenta is located between mother and fetus, and consists of maternal and fetal components, it is able to govern dynamic interactions between fetus and mother. Thus, placenta phenotype is identified as an important readout of fetal development and ultimately postnatal development (Khullar *et al.* 2004).

Along with the placenta, fetal umbilical cord and amniotic fluid significantly regulate fetal growth. Bioactive compounds from the mother move into fetal capillaries that culminate in umbilical cord vessels surrounded by Wharton's jelly, which contains gelatinous substance, fibroblasts and macrophages. Fetal amniotic fluid, produced by fetal lung secretion and urine, is also a reservoir of nutrients and hormones (Brace 1997; Underwood *et al.* 2005). It also facilitates and secures fetal growth (Underwood *et al.* 2005) and maintains mother-fetus water homeostasis and fluid circulation (Beall *et al.* 2007).

1.2.2 Placentogenesis and lifetime impact

Placentogenesis starts before implantation. First, the blastocyst forms the trophectoderm of the embryo, then trophoblast cells become the outer layer of placenta. With the completion of the amnion and allantois, as well as the vascularisation between chorion and amnion, the allantois and chorion combine to form chorioallantois (Schlafer *et al.* 2000), and the major structure of placenta is established. At the cellular level, the placenta is composed of up to six cell types depending on species, including maternal endothelium (capillary), connective tissue and epithelium, fetal chorionic epithelium (trophoblast), connective tissue and endothelium (capillary). Meanwhile, the umbilical cord develops from the yolk sac, and allantois and fetal fluids originate from maternal plasma (Wooding and Burton 2008).

As placenta mediates fetal nutrient supply, placental morphological features, namely placental phenotype, is crucial in determination of fetal development. Significant positive correlation identified between placental and fetal weight (Kloosterman 1970), is due to the involvement of placenta in fetal programming that contributes to variation in birthweight (Jansson and Powell 2007). Epidemiological data showed that a variety of adult diseases are significantly associated with altered placental phenotype, including placental weight/volume (**Table 1.1**). Data in animal models also showed that gross-placental traits, such as placentome/caruncle weight and number, largely determines placental capacity for glucose transport, thereby significantly affecting placental exchange and fetal growth (Owens *et al.* 1987; Dwyer *et al.* 2005), although specific measurements of gross-placental traits were lacking (Lewis *et al.* 2006). On the other hand, histo-morphological placental traits, including cell type composition of maternal and fetal components, surface area and barrier thickness of trophoblast and maternal epithelia, are primarily involved in nutrient transport and are strongly associated with placenta exchange capacity (Sibley *et al.* 1997; Belkacemi *et al.* 2010).

Table 1.1. Adult metabolic consequences resulting from altered placental phenotypes.

Consequences	Altered placenta phenotypes
↑ Blood pressure	↑ Placenta weight
↑ Coronary heart disease rates	↓ Placenta weight
↑ Stroke death rates	↓ Placenta weight
↑ Type-2 diabetes rates	↓ Placenta weight
↑ Plasma fibrinogen	↓ Placenta weight
↑ Blood pressure	↑ Placental ratio
↑ Impaired glucose tolerance	↑ Placental ratio
↑ Plasma fibrinogen	↑ Placental ratio
↑ Blood pressure	↓ Placental volume

↑: Increased. ↓: Decreased. (Adapted from Lewis *et al.* 2006)

The role of umbilical cord and fetal fluids in determining fetal growth has received little research attention. However, recent evidence showed that umbilical cord traits, such as cord/blood vessel size and quantity of Wharton's jelly, are significantly associated with birthweight (Di Naro *et al.* 2001), intra-uterine growth retardation (Ghezzi *et al.* 2005) and proportion of perinatal death (Bruch *et al.* 1997). Fetal amniotic fluid facilitates and secures fetal growth (Underwood *et al.* 2005) and maintains water homeostasis and fluid circulation (Beall *et al.* 2007). Fluid circulation enables mother-fetus hormonal crosstalk, such as cortisol from mother-to-fetus (Glover *et al.* 2009; Baibazarova *et al.* 2013) and insulin-like peptide 3 from fetus-to-mother, lead to mother-fetus physiological coadaptation (Ivell and Anand-Ivell 2009; Anand-Ivell *et al.* 2011). Thus, detailed and combined profiling of gross- and histo-placental phenotype with umbilical cord and fetal fluid traits will provide better understanding of placenta function in regulating fetal and lifetime development.

1.3 Skeleton

1.3.1 Role of skeleton

Bone is an important organ with mechanical and hematopoietic functions. A recent breakthrough revealed that bone is also an endocrine organ that secretes pivotal bone-derived hormones that maintain body homeostasis (Fukumoto and Martin 2009). Osteocyte-secreted fibre growth factor 23 (FGF23) acts on kidney to regulate phosphate homeostasis, while osteoblast-secreted osteocalcin acts on pancreatic ß-cells to increase insulin production and secretion, thereby regulating glucose metabolism and energy expenditure (Fukumoto and Martin 2009; DiGirolamo *et al.* 2012). Furthermore, bone was shown to contribute to regulation of male reproduction by mediating testosterone synthesis via osteocalcin (Oury *et al.* 2011). Therefore, skeletal phenotype not only represents itself development status per se, but also indicates the homeostasis state of the whole body.

1.3.2 Skeletogenesis and lifetime impact

The skeleton is developed from embryonic mesenchymal cells. Typically, the formation of the skeleton consists of two major processes: intramembranous and endochondral ossification. Flat bones, including most of the upper facial skeleton, parts of the mandible and the pelvis, are directly formed from conversion of undifferentiated mesenchymal cells to bone tissue. This process, without cartilage involvement, is defined as intramembranous ossification. In contrast, axial and appendicular bone is formed by a multi-step process that requires the sequential formation and degradation of cartilage that serve as a template (growth plate) for developing bones. This process, with cartilage involved, is referred to as endochondral ossification (Cooper *et al.* 2006).

Prenatal skeletal development, as one of the essential components of prenatal development, is a predictor of growth trajectory (Kobayashi *et al.* 2004). Prenatal skeletal development is important for ruminant postnatal growth, because ruminant neonates have to stand and walk soon after birth (Wrathall *et al.* 1974). Disrupted intrauterine growth leads to lower birthweight and increases the risk for various types of adult disease, including osteoporosis. Prenatal skeletal development can explain a large amount of variation in adult bone traits. Data from human population studies showed that birthweight, to which prenatal skeletal development significantly contributed, explained up to 19% and 24% of variation in adult whole body bone mineral content (BMC) and bone mineral density (BMD), respectively (**Table 1.2**). Furthermore, significant positive associations were identified between infancy weight and adult bone mass at age 65-70 (Cooper *et al.* 1997; Dennison *et al.* 2005). In addition, birthweight and infancy weight also predicted adult basal level of growth hormone and cortisol, which are key determinants of adult bone loss rate (Fall *et al.* 1998; Phillips *et al.* 1998; Dennison *et al.* 1999). Therefore, prenatal skeletal development is the critical stage that lays the foundation of lifelong skeletal growth.

Table 1.2. Variation in adult bone traits explained by birthweight.

Bone traits	Site	Amount of variation
Bone mineral content	Lumbar spine	0.15
	Femoral neck	0.12
	Whole body	0.19
Bone mineral density	Lumbar spine	0.12
	Femoral neck	0.12
	Whole body	0.24

(Adapted from Cooper *et al.* 2006)

1.4 Skeletal muscle

1.4.1 Role of skeletal muscle

Skeletal muscle accounts for up to half of mammalian body mass (Du *et al.* 2010). Furthermore, muscle is identified as an organ that produces various types of hormones, namely myokines, that regulate general metabolism (Pedersen and Febbraio 2008). For example, muscle-derived interleukin 6 (IL6), regulates insulin-stimulated glucose disposal and fatty acid oxidation through activation of AMP-kinase (Pedersen *et al.* 2007). IL6 also acts on liver to increase hepatic glucose production, and is involved in adipose tissue deposition (Pedersen and Fischer 2007). Muscle-derived IL15, stimulators of myogenic differentiation (Pedersen *et al.* 2007), together with insulin-like growth factors I (IGF1) and II (IGF2), key components of the IGF system and growth hormone – IGF axis, are major regulators of pre- and postnatal muscle development and growth (Adams 2002; Chang 2007; Pedersen and Febbraio 2008; Sawitzky *et al.* 2012). Hence, skeletal muscle, which serves as the largest endocrine organ, is another important indicator of body homeostasis status.

1.4.2 Myogenesis and lifetime impact

Similar to the skeleton, skeletal muscle originates from embryonic mesenchymal stem cells. Generally, prenatal muscle development involves several stages. Firstly, during embryonic development, mesenchymal stem cells develop into the progenitor of myoblasts (Relaix 2006), stimulated by important signals such as Pax3 and Pax7 (Kassar-Duchossoy *et al.* 2005). Subsequently, myoblasts undergo differentiation, which is regulated by fibroblast growth factor (Baird and Walicke 1989). In this process, myoblasts stop cell cycling while some muscle-specific genes are expressed (Du *et al.* 2010). Next, formation of myotubes is modulated by myogenin (Du *et al.* 2010). Then, cell fusion and the process of primary and secondary

myogenesis during embryonic and fetal phases (Picard *et al.* 2002) generates mature muscle fibres.

Skeletal muscle is developed from mesenchymal stem cells as described above, which also possess potential to form other cell types, such as adipocytes or fibroblasts (Zammit *et al.* 2004; Aguiari *et al.* 2008). Therefore, disruptions during prenatal muscle development can result in mesenchymal stem cell differentiation into adipocytes or fibroblasts, thereby replacing myogenesis (Du *et al.* 2010). This replacement leads to reduced prenatal deposition of myofibres, which is precursor to and determinant of postnatal muscle development (Picard *et al.* 2002; Du *et al.* 2010). In large mammals, including pig and cow, the total number of fibres for a lifetime is fixed at prenatal stages (90 days of gestation, 79% term for pig and 180 days of gestation, 63% term for cow (Picard *et al.* 2010)), and increase of postnatal muscle mass is achieved by enlargement or elongation of pre-deposited myofibres (Picard *et al.* 2002). Therefore, disrupted prenatal muscle development impairs lifetime muscular developmental potential and can cause adult muscle defects, such as loss of muscle force (Bayol *et al.* 2009), oxidative capacity (Zhu *et al.* 2006) and reduced muscle collagen (Karunaratne *et al.* 2005). Furthermore, substitution of prenatal muscle cells by adipocytes causes skeletal muscle insulin resistance, which significantly contributes to type 2 diabetes (Aguiari *et al.* 2008). Hence, prenatal muscle development programs not only lifetime muscle growth, but also adult body metabolism.

1.5 Transcriptome determination of phenotypic development

Phenotype is determined by expression of DNA information initiated by the transcription process. This process involves coordination of coding messenger RNAs (mRNAs) and non-protein coding RNAs, such as ribosomal RNA (rRNA), transfer RNA (tRNA), small nucleolar RNA (snoRNA) and microRNAs (miRNAs). The set of all these RNAs in a given tissue at a specific time point is the transcriptome. Expression levels of all RNA transcripts in a

transcriptome, the transcript abundance profile, can be quantified using microarray technology to describe a global picture of cellular function. This picture, which provides comprehensive information on pivotal molecular processes driving mammalian development, is defined as an important molecular phenotype (Jansen and Nap 2001). Molecular phenotype serves as a surrogate for traditional quantitative traits in which expression levels are closely related to traits (Schadt *et al.* 2003). While traditional quantitative traits represent gross biological measurements and the results of dynamic molecular events such as gene expression, transcript abundance profiles provide a detailed molecular picture of initial biological processes that give rise to traditional traits (Schadt *et al.* 2003), thereby capturing critical causative events. Using microarray profiling of transcriptome, differentially expressed mRNA and microRNA transcripts have been identified for development of human placenta (Sood *et al.* 2006; Mouillet *et al.* 2011), prenatal bovine and porcine muscle (Lehnert et al. 2007; Huang et al. 2008), human bone (Stokes *et al.* 2002; Gao *et al.* 2011) and liver (Yu *et al.* 2001; Tzur *et al.* 2009). Therefore, the transcript abundance profiling facilitates the identification of critical transcript groups in response to a specific phenotypic state.

1.6 Mendelian genetic effects on placenta, musculoskeletal system and associated transcript abundance profiles

Prenatal development, including placenta, musculoskeletal system and associated transcriptional events, are affected by various factors. Among these factors, parental nutritional and environmental variation has been extensively studied. A large amount of data demonstrated that altered maternal nutrition and lifestyle significantly impacted on placental phenotype (Clapp 2006; Belkacemi *et al.* 2010), thereby affecting growth of the fetus, including the musculoskeletal system (Javaid *et al.* 2006; Du *et al.* 2010) and associated transcription profiles (Miller *et al.* 2009; Lillycrop *et al.* 2010). Besides, Mendelian genetic factors, as an important

source of variation that influences lifetime development, have been widely studied in postnatal stages (**Table 1.3**). Previous studies demonstrated a large genetic basis in placental and postnatal musculoskeletal traits and mRNA transcript abundance profiles (**Table 1.3**). This strong genetic basis facilitates the identification of quantitative trait loci (QTLs) for placental and postnatal musculoskeletal traits and expression quantitative trait loci (eQTLs) for expression abundance profiles. For rat placental weight, there was a suggestive QTL mapped to chromosome 4 (Downing *et al.* 2012) and a significant QTL mapped to chromosome 15 (Buresova *et al.* 2006). Also in the rodent model, various QTLs were identified for postnatal bone mass (Klein *et al.* 1998; Beamer *et al.* 1999; Ishimori *et al.* 2006; Yu *et al.* 2007) and bone geometry parameters (Drake *et al.* 2001; Masinde *et al.* 2003; Lang *et al.* 2005; Kenney-Hunt *et al.* 2006). Using high-throughput genotyping and mRNA microarray profiling, over 6000 and 390 eQTLs were identified in postnatal human liver and brain tissue, respectively, which accounted for up to 90% of variations in specific mRNA expression levels (Schadt *et al.* 2008; Gibbs *et al.* 2010).

Table 1.3. Heritability (h^2) of placental and postnatal musculoskeletal traits.

Traits	h^2	Species	References
Placenta weight	25-56%	Pig, rat	(Mesa *et al.* 2005; Buresova *et al.* 2006)
Bone mineral density	50-90%	Human, rat	(Dequeker *et al.* 1987; Gueguen *et al.* 1995; Beamer *et al.* 1999; Deng *et al.* 1999; Yu *et al.* 2007)
Muscle mass	50-80%	Human, pig	(Seeman *et al.* 1996; Arden and Spector 1997; Larzul *et al.* 1997)
mRNA transcript abundance profiles	30-80%	Human adipose and lymphoblastoid cell lines	(Dixon *et al.* 2007; Emilsson *et al.* 2008; Visscher *et al.* 2008)

1.7 Non-Mendelian genetic and epigenetic factors in placenta, musculoskeletal system and associated transcript abundance profiles

1.7.1 Introduction to non-Mendelian genetics

Non-Mendelian genetics refer to patterns of inheritance where traits do not segregate in accordance with Mendel's laws. In classic Mendelian modes of inheritance, each parent contributes one of two possible alleles for a trait. Given the parental genotypes, Mendel's laws can be used to determine the distribution of phenotypes for the offspring. However, various exceptions have been identified where the phenotype of progeny did not match the prediction of Mendel's laws. Exceptions include cytoplasmic inheritance, sex-linked inheritance and epigenetic effects, particularly, genomic imprinting and parent-of-origin effects.

1.7.2 Non-Mendelian genetic and epigenetic effects on placenta and musculoskeletal system

1.7.2.1 Cytoplasmic inheritance and sex linkage

Non-Mendelian genetic effects such as cytoplasmic inheritance, due to extranuclear transmission of genes mostly located in mitochondria, play significant roles in placenta and the postnatal musculoskeletal system. In human, mitochondrial (mt) DNA mutations can cause placenta accreta (Aggarwal *et al.* 2001) and osteoporosis (Guo *et al.* 2011). In cattle, Mt DNA variation also significantly affected postnatal muscle mass parameters (Mannen *et al.* 1998). Another source of non-Mendelian genetic variation is sex-linkage, which is defined as the chromosomal-sex associated phenotypic expression of an allele, due to the transmission of X- or Y- linked genes. Previous studies showed that the X-chromosome-imprinted homeobox gene Esx1 is a key regulator of placental growth in rat (Li and Behringer 1998). For postnatal bone mass, X chromosome linked single nuclear polymorphisms (SNPs) (Estrada *et al.* 2012) and male-specific QTLs (Lagerholm *et al.* 2011) were identified to be significantly associated with human and mouse bone mineral density (BMD), respectively. For postnatal muscle mass, involvment of X- and Y-chromosome linked genetic effects were also reported in cattle (Engellandt and Tier 2002; Amen *et al.* 2007).

1.7.2.2 Genomic imprinting

As another major type of non-Mendelian modes of inheritance, genomic imprinting is one of the most important epigenetic modifications which changes gene expression, rather than altering the underlying DNA sequence, and impacts on phenotype. Specifically, genomic imprinting refers to the phenomenon where certain genes are expressed in a parent-of-origin specific manner, and specific alleles of these imprinted genes are either imprinted (silenced) or expressed (Moore and Haig 1991). In some imprinted genes, only the allele inherited from the mother is expressed, e.g., *H19* (Bartolomei *et al.* 1991), these genes are defined as maternally expressed/paternally imprinted genes. In other genes, only the allele inherited from the father is expressed, e.g., *IGF2 (*Giannoukakis *et al.* 1993*)*, these genes are paternally

expressed/maternally imprinted genes. *IGF2* is one of the most impressive imprinting QTL which explained up to 30% of phenotypic variation in pig muscle mass (Jin-Tae Jeon 1999; Nezer *et al.* 1999; Van Laere *et al.* 2003). The significant imprinting effects of *IGF*2 on muscle mass indicated the importance of non-Mendelian epigenetic factors in mammalian development, which has gained increasingly more attention in recent years.

To explain the evolutionary origin of imprinting, i.e., parent-of-origin effects, the hypothesis of parental tug-of-war or conflict of interest has been proposed (Moore and Haig 1991). This hypothesis attributes genomic imprinting to different interests of the male and female parent. Some gene expression is essential for conceptus nutrition and fetal demand is at the expense of maternal reproductive well-being. To have more offspring in her lifetime (increased genetic fitness), the control of expression of specific maternal alleles could reduce or control fetal demand. The opposite is true for paternal alleles of genes that extract nutrients from the mother. To maximise offspring survival and/or number of offspring from a particular female and pregnancy (genetic fitness), paternal expression of specific alleles increases fetal nutrient supply (Haig and Westoby 1989). In other words, paternally expressed imprinted genes tend to be growth promoting while maternally expressed imprinted genes tend to be growth limiting (Moore and Haig 1991). More recently, Wolf *et al.* (2006) proposed the maternal-offspring coadaptation hypothesis to explain the evolutionary basis of parent-of-origin effects. The gene expression patterns predicted by this hypothesisare consistent with the observation of overdominance of maternally expressed genes in those tissues where the mother and fetus were closely interacting, e.g., placenta. Imprinted genes have been investigated mainly in mouse (Monk *et al.* 2003; Williamson *et al.* 2006) and human, and to a lesser extent in domestic animals including pig, cow and sheep (Colosimo *et al.* 2009). As shown in **Figure 1.1**, the number of identified imprinted genes in large animals is much smaller than in human and mouse. This clearly indicates that genomic imprinting in large animals requires further investigation.

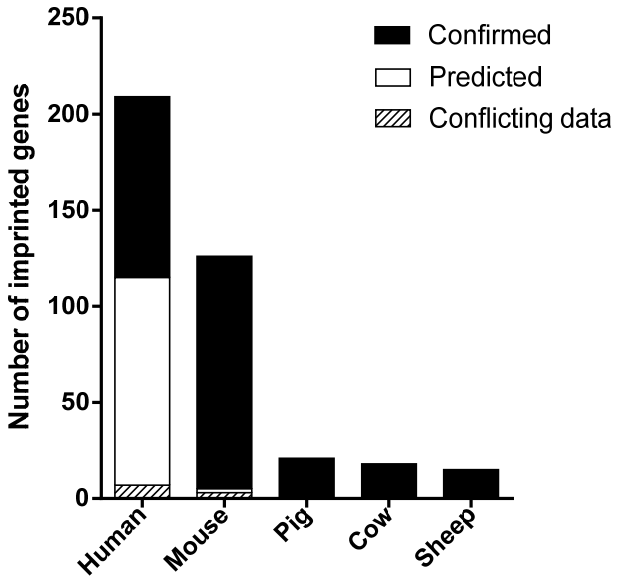

Figure 1.1 Number of imprinted genes reported to date.

Data from geneimprint database: http://www.geneimprint.com.

1.7.2.3 Parent-of-origin effects

Imprinted genes, together with other epigenetic mechanisms, such as microRNA (miRNA) interference, significantly contribute to types of non-Mendelian inheritance that have a parent-of-origin transmission pattern. One of the most interesting examples is polar overdominance, which was discovered in various types of tissues across different species. Polar overdominance was first discovered in sheep, where the callipyge (CLPG) mutation causes postnatal muscle hypertrophy only in heterozygous offspring and only when inherited through the paternal germline (Cockett *et al.* 1996). This polar overdominant mutation changes expression of a number of imprinted genes located in the *DLK1-DIO3* imprinted gene cluster at the distal end of chromosome 18 of sheep, including (i) paternally expressed *DLK1* and *PEG11*, and (ii) maternally expressed non-coding genes, *GTL2*, *PEG11as* and *MEG8* (Charlier *et al.* 2001; Davis *et al.* 2004; Vuocolo *et al.* 2007; Jason *et al.* 2008; Fleming-Waddell 2009). Subsequent studies

suggested that these maternally expressed non-coding genes host a large number of small C/D snoRNAs and miRNAs (Seitz *et al.* 2004), which targeted and exerted an inhibitory effect on paternally expressed *DLK1*, thereby generating the unique callipyge phenotype (Georges *et al.* 2003; Caiment *et al.* 2010). Individuals expressing the callipyge phenotype displayed higher absolute and relative weights of specific muscles and muscle groups of the torso (e.g. *M. longissimus lumborum*) and pelvic limb (e.g. *M. semimembranosus, M. quadriceps femoris*), but no muscle mass increase of the thoracic limb (e.g. *M. supraspinatus*) (Koohmaraie *et al.* 1995; Jackson *et al.* 1997). The increased muscle mass of CLPG sheep was later found to be caused by fast myofibre hypertrophy and higher glycolytic metabolism of affected muscles (Carpenter *et al.* 1996; Jason *et al.* 2008). These intriguing findings from callipyge sheep inspired various parallel studies in other species demonstrating the broad existence of such unique parent-of-origin dependent overdominant patterns of inheritance (see below).

Polar overdominance has also been reported in other animal models. In human, polar overdominance inheritance of postnatal obesity was found in French and German families (Wermter *et al.* 2008). In pig, a paternal polar overdominance effect was identified on postnatal myofibre characteristics, lean muscle mass and growth rate (Kim *et al.* 2004). In mouse, maternal deletion of *DLK-DIO3* locus, that is significantly associated with polar overdominance, resulted in placentomegaly (Rocha *et al.* 2008) and prenatal retarded bone development (Kagami *et al.* 2008). However, in bovine, such a unique pattern of inheritance due to genomic imprinting has not been reported. Recently, several studies in bovine addressed the relationship between imprinted genes/loci and economic traits using SNPs mapping (Imumorin *et al.* 2012). The findings included associations of SNPs within *PEG11*, *IGF2*, *CLPG* and *GNAS* loci with carcass weight (Berkowicz *et al.* 2011; Chen *et al.* 2011; Magee *et al.* 2011; Sikora *et al.* 2011), forelimb size (Chen *et al.* 2011) and stature (Sikora *et al.* 2011). However, these studies relied on known imprinted genes/loci and investigated phenotypes were limited to postnatal economically

important traits. Therefore, more data on genomic imprinting effects on a wider range of bovine developmental traits are needed.

More recently, using statistical modelling methods, detailed patterns of parent-of-origin effects were further dissected. Based on imprinting QTL estimation with an F3 generation mouse resource, Wolf *et al*. 2008 demonstrated a wide range of phenotypic patterns of genomic imprinting. In this study, apart from the traditional parental expression patterns with maternal and paternal expression assuming complete or partial monoallelic expression, dominance expression was further divided into two subtypes (**Figure 1.2**). These two subtypes, bipolar and polar dominance, were assigned based on the difference between heterozygotes and homozygotes. Polar dominance is further classified into the two subtypes of polar overdominance and polar underdominance, depending on the sign (positive or negative) of the difference between the affected heterozygote and the remaining three genotypes (see **Figure 1.2**). These classifications established the definitions of phenotypic expression of imprinting patterns, facilitating future studies for quantification of parent-of-origin effects and separation of maternal and paternal contributions to genetic variation in important traits for other animal species.

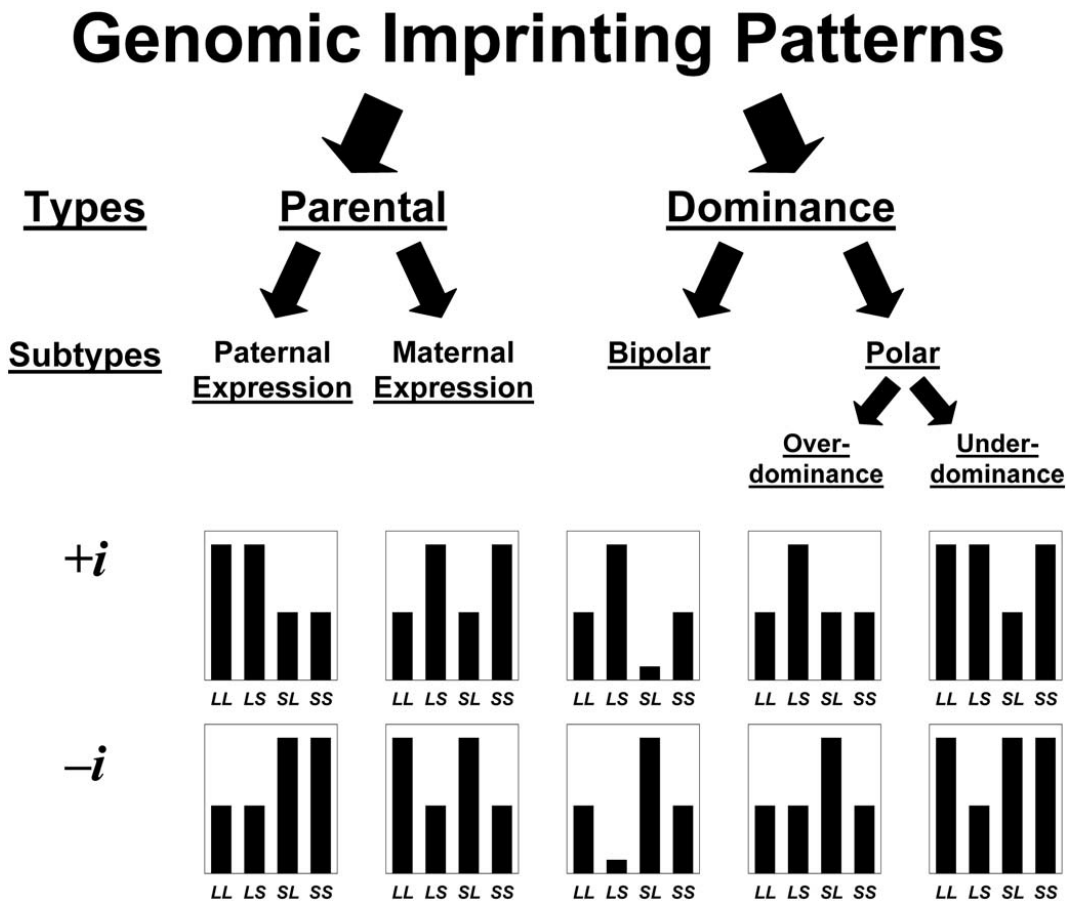

Figure 1.2. All possible phenotypic patterns of genomic imprinting according to Wolf et al. (2008).

Two principal patterns: Parental expression and dominance imprinting, male parent lists first. Parental expression: The allele is expressed either paternally or maternally. Dominance imprinting: Two homozygotes are the same while the heterozygotes are different from each other. Two subtypes of dominance imprinting: bipolar and polar. Bipolar: One heterozygote is larger than the homozygotes while the other heterozygote is smaller. Polar: One heterozygote is the same as the two homozygotes while the other heterozygote is not. Polar dominance may show overdominance, where the heterozygote differing from the other three genotypes is larger, or underdominance, where it is smaller. The plots give examples of the expected pattern of phenotypes for the four ordered genotypes when the sign of i is either positive or negative. Figure adapted from (Wolf *et al.* 2008).

Using QTL mapping, a handful of pivotal imprinted loci have been identified and contributions of parental imprinting to genetic variation in postnatal economic traits have been quantified in different species. In pig, up to 30% muscle mass variation was found to be

explained by the paternally expressed *IGF2* locus (Jin-Tae Jeon 1999; Nezer *et al.* 1999; Van Laere *et al.* 2003). More recently, a QTL displaying maternal polar overdominance and affecting pig pelvic limb muscle mass was located about 4 Mb from the *DLK1-GTL2* intergenic region (Boysen *et al.* 2010). This is in agreement with results from estimation of parental imprinting contribution to genetic variation in pig performance traits, where genetic variation in absolute and relative limb muscle mass was largely explained by maternal imprinting (Neugebauer *et al.* 2010). In sheep, similar to the *CLPG* locus, a QTL showing polar overdominance that affects postnatal muscle was mapped to ovine chromosome 18 (Matika *et al.* 2011). In bovine, a handful of studies identified imprinted QTLs affecting milk production (Kuehn *et al.* 2007), twinning and ovulation rate (Allan *et al.* 2009) and calving ease (Pausch *et al.* 2011). A recent study identified a large number of parent-of-origin QTLs, where six paternally expressed and 15 maternally expressed QTLs individually explained 1.4~5.1% of variance in birthweight and/or carcass weight in beef cattle (Imumorin *et al.* 2011). Neugebauer *et al*. (2010) estimated differential parental imprinting contribution to genetic variation in postnatal performance traits. In this study, up to 25% imprinting contribution to total genetic variation was identified, and there was a dominant maternal contribution to genetic variation in absolute muscle mass parameters and paternal contribution to genetic variation in relative muscle mass parameters (Neugebauer *et al.* 2010). The estimation of differential maternal and paternal contribution to genetic variation allowed the dissection of a multitude of parental genetic contributions to important phenotypes, due to the non-equivalence of maternal and paternal genome. However, studies have been limited to postnatal traits associated with economic importance.

1.7.3 Non-Mendelian genetic and epigenetic effects on transcription profiles

To our knowledge, non-Mendelian genetic effects on transcript abundance profiles in mammals remain largely uninvestigated. However, data from other species suggest the existence

of such effects. In *Drosophila*, significant Y chromosome effects on mRNA expression levels in testis were reported (Branco *et al.* 2013). In fish, significant mt type effects on gene expression level in fish liver were reported (Flight *et al.* 2011). Furthermore, using computational modelling with published human genomic and transcriptome data, putative imprinted eQTLs that display significant parent-of-origin effects on global gene expression levels were identified (Garg *et al.* 2012). These findings strongly suggest that (epi)genetic effects on transcript abundance profiles could provide novel insights into understanding complex biological processes driving mammalian development. Therefore, direct evidence for such effects in mammals is now required.

The accuracy of genetic analysis, while heavily reliant on well designed experiments and advanced genetic and genomic technology, is largely dependent on precise estimation of phenotype. Thus, phenotyping is emerging as the major operational bottleneck limiting the power of genetic analysis (Cobb *et al.* 2013). Furthermore, data from a wider range of traditional and molecular phenotyping will improve understanding of parent-of-origin effects and parental contribution to genetic variation in lifetime development. As discussed previously, lifetime development is largely programmed in prenatal stages, where genetic and epigenetic factors are dynamically involved (Faulk and Dolinoy 2011; Hochberg *et al.* 2011). Therefore, detailed analysis of genetic and epigenetic effects, including differential maternal and paternal genetic effects on a wide range of critical prenatal phenotypes, such as placenta, musculoskeletal system and associated molecular phenotypes, i.e., transcript abundance profiles that provide comprehensive pictures, can complete the picture of genetic and epigenetic regulation of mammalian development for critical traits over a lifetime.

1.8 Research aim

To dissect and quantify genetic and epigenetic effects on a comprehensive trait spectrum of the placenta, musculoskeletal system and associated transcriptional events, and in order to understand the mechanisms behind such effects driving prenatal development, we have generated a large collection of defined bovine fetuses consisting of both purebreds and reciprocal hybrids with Angus and Brahman genetics. The taurine (Angus) and indicine (Brahman) breeds represent subspecies of the domestic cow, currently named *Bos taurus taurus* and *Bos taurus indicus*, respectively (The Bovine Genome Sequencing Consortium 2009). Both subspecies originated from the wild aurochs (*Bos primigenius*) and are commonly referred to as *Bos taurus* and *Bos indicus* (*Linnaeus*, *1758*; *Bojanus*, *1827*; loc. cit. http://www.itis.gov) (Hiendleder *et al.* 2008). This unique intra-species resource with well defined divergent parental genomes allowed us to dissect maternal and paternal genome effects on traits of the placenta and fetus, fetal muscle, fetal bone and fetal liver transcriptome.

Specifically, we aimed to dissect and quantify midgestation differential maternal and paternal genome effects on:

(1) The placental-fetal system, including gross- and histomorphological placenta, umbilical cord and fetal traits, addressed in Thesis chapter two.

(2) Principal components of fetal skeletal system representing a variety of directly measured bone weight and geometry parameters, addressed in Thesis chapter three.

(3) Fetal myofibre characteristics, muscle weights and imprinted non-coding RNA H19 expression level in muscle, addressed in Thesis chapter four.

(4) Fetal liver phenotype and associated mRNA and non-coding miRNA transcript abundance profiles and corresponding mRNA and snoRNA/miRNA coexpression networks, addressed in Thesis chapter five.

Our results showed, for the first time, significant differential maternal and paternal genome effects on specific placental-, fetal-, musculoskeletal- and liver transcriptome traits. Furthermore, we found that identified important relationships between these traits were also controlled by differential maternal and paternal genome effects. Our results provide experimental evidence to support and accommodate controversial hypotheses that contribute to evolutionary origin of parent-of-origin effects. Moreover, our findings suggested the importance of bringing the understanding of (epi)genetic effects driving mammalian prenatal development to a system level, where coupling phoneme-wide with genome-wide data is necessary. Collectively, these findings extend our current understanding of the nature of parent-of-origin effects and point to a new direction for studying mammalian development.

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Chapter 2: Novel paternal and maternal genome effects on the placental-fetal system support both conflict-of-interest and maternal-offspring coadaptation

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Chapter 2

2.1 Abstract

The placenta facilitates maternal-fetal cross talk, is a major determinant of fetal growth and involved in programming of postnatal phenotype via genetic and epigenetic mechanisms. However, the magnitude and specificity of effects of maternal and paternal genomes on placental and fetal phenotype are unclear. Using an outbred bovine model with well defined maternal and paternal genetics, we generated purebred and reciprocal cross fetuses in growing adolescent mothers, to dissect and quantify effects of parental genomes, fetal sex and non-genetic maternal effects (maternal weight, post conception weight gain) on 41 gross- and histo-morphological placental-fetal phenotypes. Analyses of data from 73 fetuses recovered at midgestation (Day 153) by general linear models revealed that parental genomes explained significant proportions of variation (58.2−99.5%, P<0.05-0.0001) in 29 placental-fetal phenotypes. Fetal sex accounted for up to 32.2% (P<0.05-0.0001) of variation and non-genetic maternal effects for up to 25.2% (P<0.05-0.0001). Maternal genome predominantly contributed to variation (59.6−99.9%, P<0.001-0.0001) in gross- and histo-morphological placental phenotype, fetal weight and fetal organ weights, whereas paternal genome predominantly contributed to fetal fluids weight (73.0%, P<0.001), umbilical cord weight (73.9%, P<0.05) and length (73.2%, P<0.01), and maternal placental (69.6%, P<0.05) and umbilical cord (83.2%, P<0.0001) efficiency. The finding that maternal genome determined placental phenotype and paternal genome determined umbilical cord and fetal fluid phenotype, substantiates the predicted expression patterns of genomic imprinting effects by both maternal–offspring coadaptation and conflict-of-interest hypotheses in the placental-fetal system. Furthermore, maternal genome determined four regressions between placental weights and umbilical cord phenotype (P<0.05-0.0001), whereas paternal genome

and/or fetal sex determined 28 regressions between fetus-, organ- and fetal fluid weights and umbilical cord phenotype (P<0.05-0.0001). The finding of related placental and fetal phenotype merging in clusters differentiated by maternal and paternal genome effects suggests the existence of (epi)genetic-regulated morphological modules within the placental-fetal system.

2.2 Introduction

The placenta is at the intersection of cellular and molecular maternal-fetal communication (Lewis *et al.* 2012) and develops functional gross- and histo-morphological adaptations in response to fetal growth (Fowden *et al.* 2006). The placenta produces and/or metabolises maternal hormones pivotal for fetal growth and development, including growth hormone, thyroid hormones and insulin-like growth factor (IGF) -I and -II (Fowden 2003; Murphy *et al.* 2006; Patel *et al.* 2011), which regulate nutrient transfer, fetal growth and maternal-fetal homeostasis. As the critical organ located between mother and fetus, the placenta consists of maternal and fetal components and morphology varies in different species. In bovine, physiological exchange between mother and fetus occurs in button-like formations, i.e., placentomes, composed of interdigitating caruncular (maternal) and cotyledonary (fetal) tissues. Placentome size and number largely determines placental capacity for glucose transport, thereby significantly affecting placental exchange and fetal growth (Owens *et al.* 1987; Dwyer *et al.* 2005). At the cellular level, placenta is composed of up to six cell types depending on species, including maternal endothelium (capillary), connective tissue and epithelium, fetal chorionic epithelium (trophoblast), connective tissue and endothelium (capillary) (Wooding and Burton 2008). Composition of these cell types, along with surface area and barrier thickness of the exchange zone of placenta, primarily determine nutrient transport (Sibley *et al.* 1997). Thus, histo-placental phenotype is strongly associated with placental exchange capacity and ultimately, fetal growth (Belkacemi *et al.* 2010).

Along with placenta, fetal umbilical cord and fluids may also significantly affect fetal growth. Exchange of nutrients and bioactive compounds between placenta and fetus is largely carried out by umbilical cord blood vessels surrounded by Wharton's jelly; the composition of these structures is determined by fetal genetics. The role of the umbilical cord in determining fetal

growth so far has received little attention. Recent evidence shows that umbilical cord phenotype, such as cord and blood vessel size and quantity of Wharton's jelly, are significantly associated with birthweight (Di Naro *et al.* 2001), intra-uterine growth retardation (Ghezzi *et al.* 2005) and perinatal death (Bruch *et al.* 1997). Fetal amniotic and allantoic fluids, largely produced from fetal lung secretion and urine (Brace 1997), are a reservoir for nutrients and hormones (Sack *et al.* 1975; Underwood *et al.* 2005). Fetal fluids physically protect the fetus, promote fetal growth (Underwood *et al.* 2005) and maintain maternal-fetal water homeostasis and fluid circulation (Beall *et al.* 2007). The latter enables maternal-fetal hormonal communication, such as fetal developmental responses to maternal cortisol level (Glover *et al.* 2009; Baibazarova *et al.* 2013) and maternal physiological responses to fetal insulin-like peptide 3 level (Ivell and Anand-Ivell 2009; Anand-Ivell *et al.* 2011). Thus, placenta, fetus, umbilical cord and fetal fluids depend on each other and and coadapt to develop as a system in utero.

Although data is limited, it has been demonstrated that several components and parameters of the placental and fetal system have a genetic and epigenetic basis. Data from animal models and twin studies in human, showed that genetic factors explained 25-56% of variation in placenta weight (Mesa *et al.* 2005; Buresova *et al.* 2006), 18% of variation in placenta efficiency (fetus:placenta weight) (Mesa *et al.* 2005), and up to 40% of variation in umbilical cord phenotype, including 30% in cord length (Antoniou *et al.* 2011). Although data on Mendelian genetic effects on specific placental and fetal phenotypes are lacking, of effects factors following non-Mendelian modes of inheritance have been studied in model animals and human, including mitochondrial (mt) DNA (Marchington *et al.* 2006; Lattuada *et al.* 2008), X- (Ishikawa *et al.* 2003) and Y- (Hemberger *et al.* 2001) chromosomes and genomic imprinting (Coan *et al.* 2005; Nelissen *et al.* 2011; Buckberry *et al.* 2012). Genomic imprinting, as an important type of epigenetic modification, refers to parent-of-origin dependent allele-specific gene expression (Reik and Walter 2001) and has been extensively studied in mouse and human placenta. Several

imprinted genes have been functionally linked to placental and fetal growth, includings maternally expressed *IGF2R* (mouse only), *H19* and *PHLDA2*, and paternally expressed *IGF2*, *DLK1* and *PEG3* (Coan *et al.* 2005; Frost and Moore 2010; Nelissen *et al.* 2011). However, a holistic analysis of the magnitude and specificity of maternal and paternal genome effects on parameters of the placental-fetal system is lacking. Such data may provide important evidence to clarify controversial hypotheses on the evolutionary origin of genomic imprinting, in particular conflict-of-interest (Haig 2004) and maternal-offspring coadaptation (Wolf and Hager 2006), thereby improving understanding of the nature of parent-of-origin effects that determine placental and fetal growth (Wolf 2013).

To dissect and quantify (epi)genetic effects on a broad and comprehensive phenotypic spectrum of placental, fetal and umbilical cord and fetal fluid traits, we generated bovine fetuses with purebred and reciprocal hybrid Angus and Brahman genetics. The taurine (Angus) and indicine (Brahman) breeds represent subspecies of the domestic cow, currently named *Bos taurus taurus* and *Bos taurus indicus*, respectively (The Bovine Genome Sequencing Consortium 2009). Both subspecies originated from the wild aurochs (*Bos primigenius*) and are commonly referred to as *Bos taurus* and *Bos indicus* (*Linnaeus*, *1758*; *Bojanus*, *1827*; loc. cit. http://www.itis.gov) (Hiendleder *et al.* 2008). This best fit model (Bolker 2012) for understanding genetic and epigenetic effects in fetal development allowed us to unravel differential maternal and paternal genome effects on the placental-fetal system at mid-gestation. Furthermore, we demonstrated that critical relationships between umbilical cord and placental-fetal phenotype were determined by differential parental genome and fetal sex effects. Our findings provide experimental evidence that support the predicted expression patterns of genomic imprinting effects in placenta by both conflict-of-interest (Haig 2004) and maternal-offspring coadaptation (Wolf and Hager 2006) hypotheses. We also suggested a new integrated

approach for a better understanding of epi(genetic) relationships between complex phenotypes within the placental-fetal system.

2.3 Results

2.3.1 Proportion of variation explained by parental genomes, fetal sex and non-genetic maternal effects

Significant final statistical models for 11 gross-morphological and eight histo-morphological placental, seven fetal and four umbilical cord phenotypes, with adjusted R^2 values and significance levels of retained variables, are presented in **Table 2.1**. Parental genomes, fetal sex, and effects of maternal weight, caused by non-genetic variation and nested within maternal genomes (see methods), each contributed differentially to placental and fetal phenotype (**Figure 2.1**). Maternal and paternal genome together accounted for the largest proportion of explained variation in placental-fetal phenotype (58.2−99.5%), except for fetal brain weight, where more than half of the variation was explained by fetal sex and non-genetic maternal effects. Fetal sex explained 0.5%-32.2% of variation across placental and fetal phenotype, with larger contributions to variation in fetal organ weights and placental efficiency (22.7-32.2%). Non-genetic maternal effects explained up to 25.2% of variation in placental-fetal phenotype, with larger contributions to variation in gross- and most histo-morphological phenotype of placentomes (13.6-23.4%).

Table 2.1. Summary of the final general models (type III sums of squares) for placental and fetal traits with adjusted R^2 values and significance levels (*p*-values) of models and variables. Only *p*-values for factors, interactions and nested effects retained in the final model are shown.

Traits	R^2	Significance (*P-values*)							
		Model	*M*	*P*	*Sex*	*M*×*P*[b]	*M*×*S*[b]	FMW(*M*)[c]	MDG(*M*)[c]	*S*×FMW(*M*)[d]
Total placenta W	0.480	2.4E-10	1.8E-11	0.5496	0.0188					
Maternal placenta W	0.390	5.1E-08	1.8E-08	0.9869	0.0043					
Fetal placenta W	0.385	6.6E-08	3.0E-09	0.4072	0.1808					
Total caruncle no.	0.190	0.0018	0.0133	0.5388	0.4293				0.0165	
Mound caruncle W	0.269	0.0004	0.4093	0.1904	0.4234		0.0021	0.0812		0.0010
Mound caruncle %	0.233	0.0019	0.1941	0.2548	0.7073		0.0015	0.1280		0.0018
Largest caruncle W	0.252	0.0002	0.3066	0.1347	0.3968			0.0021		
Fetus W	0.619	1.1E-13	0.1088	0.0273	3.9E-06			0.0365		
Brain W	0.143	0.00845	0.1353	0.5029	0.0815			0.0358		
Heart W	0.512	2.0E-11	3.6E-10	0.0007	0.0270					
Lung W	0.499	2.3E-10	0.0000	0.2275	0.0006	0.0118				
Liver W	0.513	1.8E-11	0.0000	0.5983	0.0000					
Kidney W	0.517	3.2E-09	0.0003	0.2108	0.0903		0.1858	0.6212		0.0103
Fetal fluids W	0.278	0.0001	0.9335	0.0047	0.5656	0.0350			0.0280	
Umbilical cord W	0.104	0.0173	0.1319	0.0145	0.0309					
Umbilical cord L	0.117	0.0098	0.1053	0.0059	0.1127					
Umbilical artery D	0.407	5.5E-06	0.0046	0.0390	0.0009	0.0010		0.0298		
Umbilical vein D	0.415	7.0E-07	0.0448	2.3E-07	0.0002			0.0181	0.0001	

W: Weight. L: Length. D: Diameter. *M*: Maternal genome. *P*: Paternal genome. *S*: Fetal sex. FMN: Final maternal weight. MDG: Maternal daily gain. [b] Interaction effects. [c] Effects of final maternal weight or maternal daily gain nested within maternal genome. [d] Interaction between main effects of factor and nested effects. Table continued on next page.

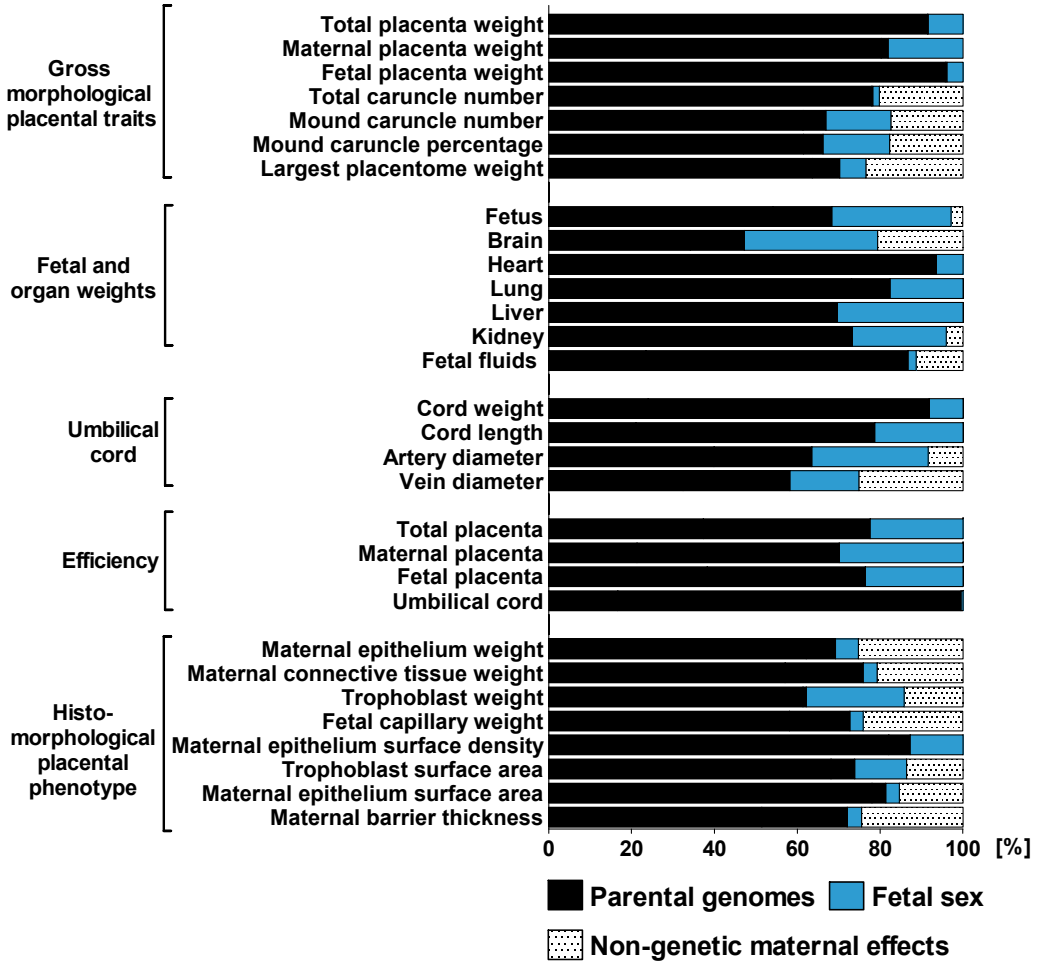

Figure 2.1. Relative contributions of parental genomes, fetal sex and non-genetic maternal effects to explained variation in placental and fetal phenotype.

Maternal and paternal genome, fetal sex and other significant effects were retained in final general linear models as presented in Table 2.1. Non-genetic maternal effect: Final maternal weight at mid-gestation and/or daily weight gain from conception to mid-gestation. Histo-morphological placental phenotype was determined for the largest placentome surrounding the fetus. Efficiency: Fetus weight divided by total, maternal and fetal placenta and umbilical cord weight.

Table 2.1 continued. Summary of the final general models (type III sums of squares) for placental and fetal traits with adjusted R^2 values and significance levels (*p*-values) of models and variables. Only *p*-values for factors, interactions and nested effects retained in the final model are shown.

Traits	R^2	Significance (*P-values*)								
		Model	*M*	*P*	*Sex*	*M*×*P*[b]	*M*×*S*[b]	*P*×*S*[b]	FMW(*M*)[c]	*P**FMW(*M*)[d]
Total placenta Eff	0.310	3.1E-06	0.0009	0.0045	0.0068					
Maternal placenta Eff	0.101	0.0247	0.1229	0.0402	0.3571			0.0462		
Fetal placenta Eff	0.273	1.7E-05	0.0018	0.0113	0.0101					
Umbilical cord Eff	0.215	0.0003	0.0566	0.0002	0.7548					
Maternal epitheilum W[a]	0.269	0.0001	0.1634	0.0762	0.7310				0.0010	
Maternal connective tissue W[a]	0.194	0.0019	0.4930	0.0362	0.9569				0.0150	
Trophoblast W[a]	0.230	0.0008	0.0948	0.2901	0.3241		0.0394		0.0283	
Fetal capillary W[a]	0.124	0.0184	0.2916	0.1072	0.9485				0.0341	
Maternal epithelium SD[a]	0.205	0.0016	0.0002	0.3115	0.3820					
Trophoblast SA[a]	0.246	0.0005	0.0383	0.0871	0.7490		0.0375		0.0256	
Maternal epithelium SA[a]	0.241	0.0003	0.0777	0.1918	0.7302				0.0202	
Maternal barrier thickness[a]	0.170	0.0114	0.8450	0.0062	0.9814	0.0666			0.0434	0.0171

W: Weight. Eff: Efficiency, calculated as fetus weight divided by total, maternal, fetal and umbilical cord weight. SD: Surface density. SA: Surface area. *M*: Maternal genome. *P*: Paternal genome. *S*: Fetal sex. FMW: Final maternal weight. MDG: Maternal daily gain. [a] Histo-morphological placental traits, determined for largest placentome surrounding the fetus. [b] Interaction effects. [c] Effects of final maternal weight or maternal daily gain nested within maternal genome. [d]Interaction between main effects of factor and nested effects.

Relative contributions of maternal and paternal genomes to total explained (epi)genetic variation in placental-fetal phenotypes are shown in **Figure 2.2**. Maternal genome predominantly contributed to genetic variation in gross- and histo-morphological placental phenotype, fetus and organ weights (59.6−99.9%), whereas paternal genome predominantly contributed to genetic variation in fetal fluids weight (73.0%), umbilical cord weight (73.2%) and length (73.9%), and maternal placental (69.6%) and umbilical cord efficiencies (83.2%). (Epi)genetic variation in diameter of umbilical vein, total and fetal placenta efficiencies were approximately equally explained by parental genomes (**Figure 2.2**).

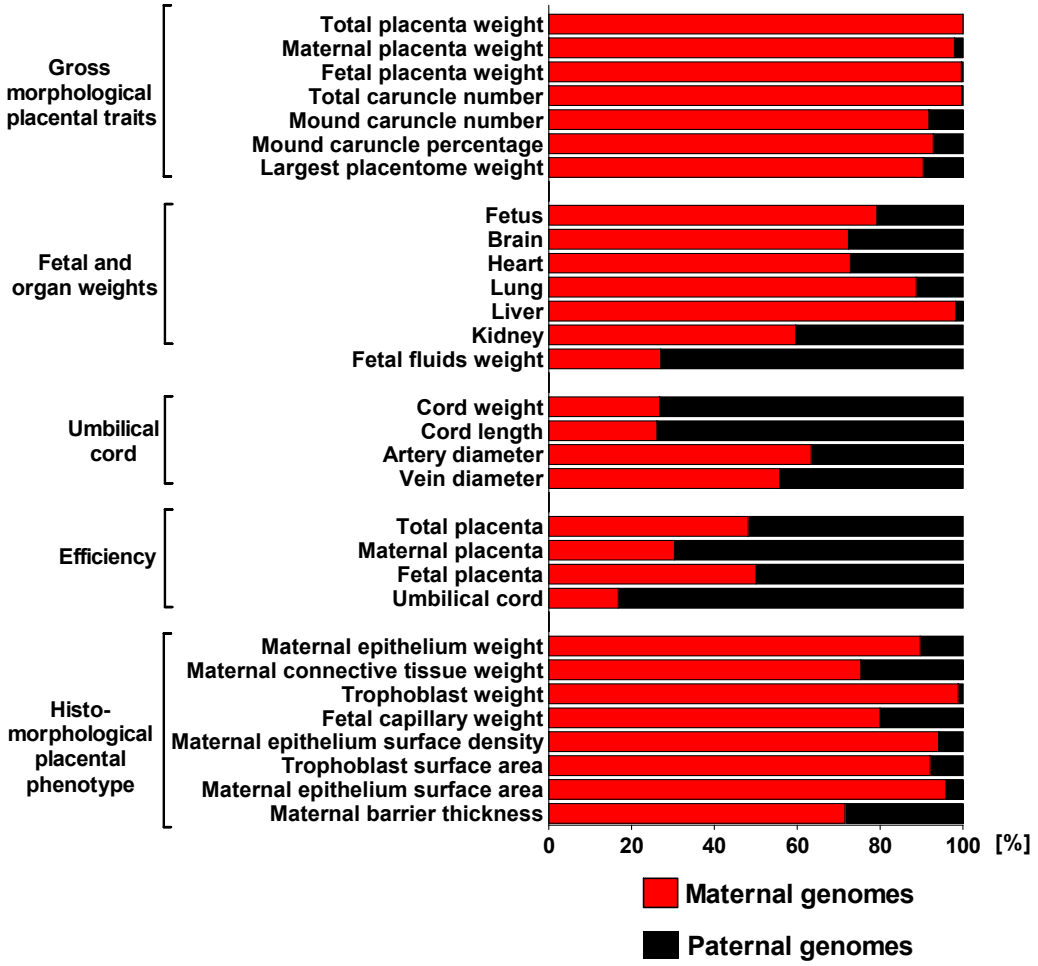

Figure 2.2. Relative contributions of maternal and paternal genome to genetic variation in placental and fetal phenotype.

Histo-morphological placental phenotype was determined for the largest placentome surrounding the fetus. Efficiency: Fetus weight divided by total, maternal and fetal placenta and umbilical cord weight.

2.3.2 Nested regression network between placental and fetal phenotype within parental genomes and fetal sex

For the above mentioned 30 significant placental and fetal phenotype, linear regressions nested within parental genome and/or fetal sex were identified between gross-placental phenotype, fetus and organ weights as response variables, and umbilical cord phenotype, fetal fluid weight and

histo-morphological placental phenotype as explanatory variables (**Figure 2.3-2.5**). Adjusted R^2 values and significance levels of models and retained nested explanatory umbilical cord phenotype, fetal fluids weight and histo-morphological placental phenotype are presented in **Table S2.1**.

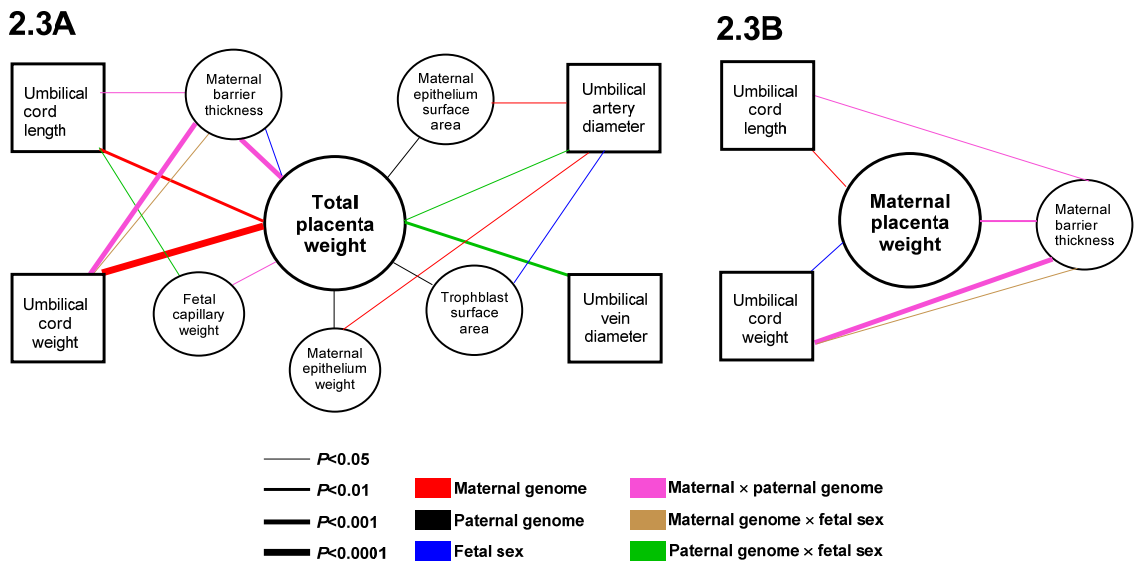

Figure 2.3. Regression network for total and maternal placenta weight determined by parental genomes and/or fetal sex.

Significant regressions of total placenta weight (**A**) and maternal placenta weight (**B**) on umbilical cord (square box) and histo-morphological placental (small circle) phenotype, nested within maternal genomes, paternal genomes, fetal sex or their interactions, presented as solid lines. Line thickness: Significance level (ANOVA) of the nested regression effects. Line colour: Main effects, or interaction between two main effects, within which the regression was nested. Histo-morphological placental phenotype determined for the largest placentome surrounding the fetus.

Strong maternal genome-, but weaker paternal genome-, and fetal sex-determined regressions between total, maternal and fetal placental weights and umbilical cord phenotype were identified (**Table S2.1**, **Figure 2.3**). Total placenta weight showed a strong maternal genome dependent

regression on umbilical cord weight ($P < 0.0001$) and length ($P < 0.01$), but significant paternal genome × fetal sex dependent (interaction) regressions on umbilical artery ($P < 0.05$) and vein diameters ($P < 0.01$) (**Figure 2.3A**). Fetal placenta showed strong maternal genome dependent regression ($P < 0.0001$) on umbilical cord weight (**Figure 2.4A**). However, fetal placenta also displayed significant paternal genome × fetal sex dependent regressions on umbilical artery ($P < 0.05$) and vein diameter ($P < 0.01$) (**Figure 2.4A**).

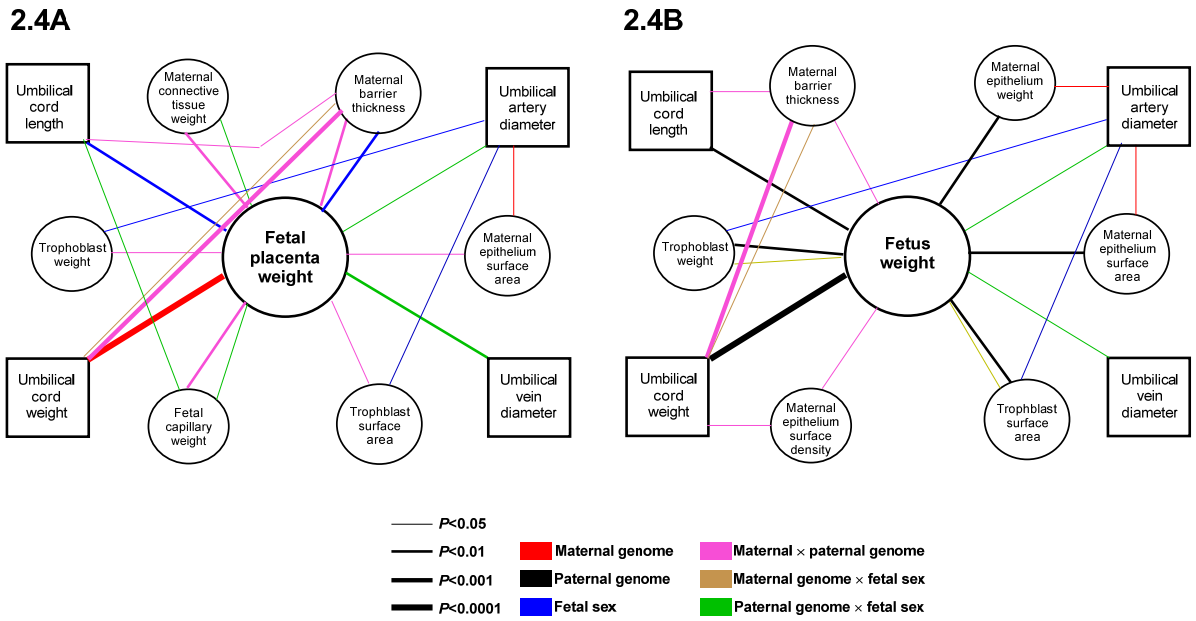

Figure 2.4. Regression network for fetal placenta and fetus weight determined by parental genomes and/or fetal sex.

Significant regressions of fetal placenta weight (**A**) and fetus weight (**B**) on umbilical cord (square box) and histo-morphological placental (small circle) phenotype, nested within maternal genomes, paternal genomes, fetal sex or their interactions, presented as solid lines. Line thickness: Significance level (ANOVA) of the nested regression effects. Line colour: Main effects, or interaction between two main effects, within which the regression was nested. Histo-morphological placental phenotype determined for the largest placentome surrounding the fetus.

Paternal genomes and/or fetal sex largely determined regressions between fetal and organ weights and umbilical cord phenotype (**Table S2.1, Figures 2.4, 2.5)**. Fetus weight showed

strong paternal genome dependent regressions on umbilical cord weight ($P < 0.0001$) and length ($P < 0.01$), and significant paternal genome × fetal sex dependent regressions on umbilical artery ($P < 0.05$) and vein diameter ($P < 0.05$) (**Figure 2.4B**). More than half of the regressions between fetus weight and histo-morphological placental phenotype were also strongly determined by paternal genome ($P<0.01$-0.0001, **Figure 2.4B**). Furthermore, paternal genome determined a number of significant regressions between (i) fetal heart weight and umbilical cord phenotype ($P<0.05$-0.001, **Figure 2.5B**), (ii) fetal lung weight with fetal fluids weight and histological placental phenotype ($P<0.05$, **Figure 2.5C**) and (iii) fetal liver weight and umbilical cord phenotype ($P<0.05$, **Figure 2.5D**). The only significant regression between fetal kidney weight and maternal epithelium weight ($P < 0.05$) was also determined by paternal genome (**Figure 2.5E**). Interestingly, fetal brain weight showed strong sex dependent regression on umbilical cord weight ($P < 0.001$) (**Figure 2.5A**). Fetal sex also determined regressions between (i) fetal heart weight and umbilical artery ($P<0.05$) and vein diameters ($P<0.01$, **Figure 2.5B**) and (ii) fetal liver weight and umbilical artery and vein diameters ($P<0.01$, **Figure 2.5D**).

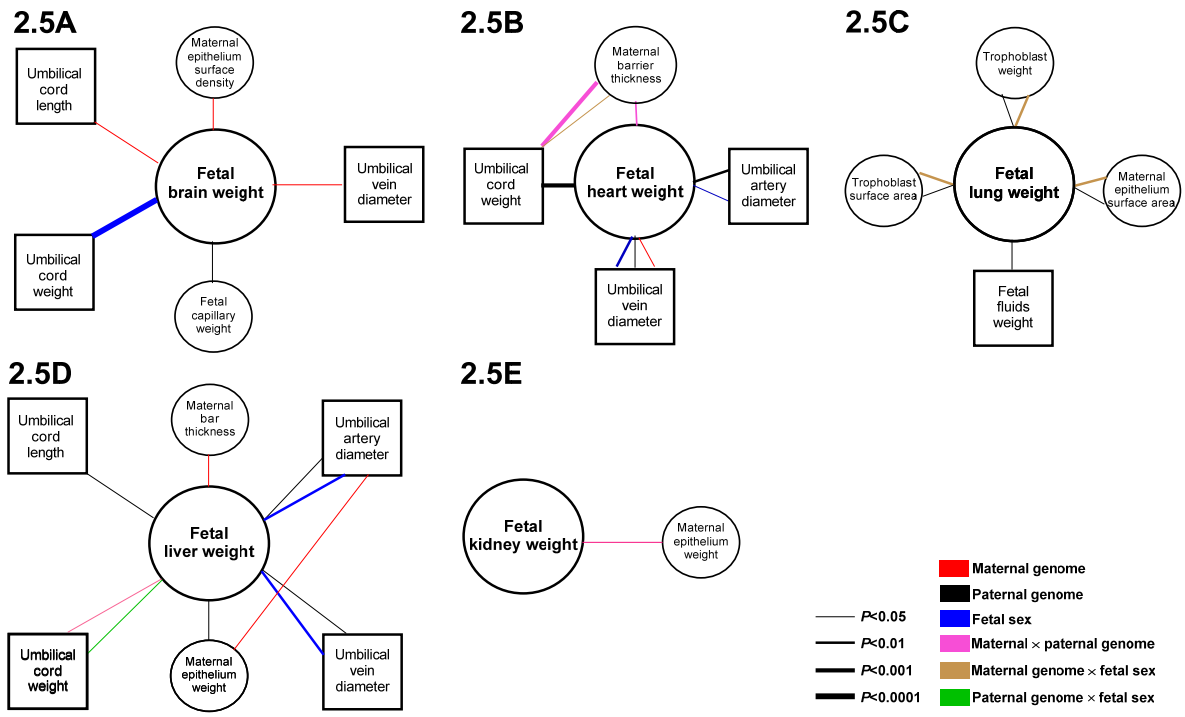

Figure 2.5. Regression network for fetal organ weights determined by parental genomes and/or fetal sex. Significant regressions of weight of fetal brain (**A**), heart (**B**), lung (**C**), liver (**D**) and kidney (**E**) on umbilical cord and fetal fluids (square box) and histo-morphological placental (small circle) phenotype, nested within maternal genomes, paternal genomes, fetal sex or their interactions, presented as solid lines. Line thickness: Significance level (ANOVA) of the nested regression effects. Line colour: Main effects, or interaction between two main effects, within which the regression was nested. Histo-morphological placental phenotype determined for the largest placentome surrounding the fetus.

Regressions between gross-morphological placental-fetal phenotype and histo-morphological placental phenotype were largely determined by maternal × paternal genome interaction (**Figures 2.3-2.5**, **Table S2.1**). Specifically, maternal barrier thickness showed significant maternal × paternal genome dependent regressions with weights of total- ($P < 0.001$), maternal ($P < 0.01$) and fetal placenta ($P < 0.01$), (**Figures 2.3, 2.4**), fetal heart ($P < 0.01$) and liver ($P < 0.05$) (**Figure 2.5B,D**). Maternal barrier thickness also showed significant maternal × paternal genome dependent regressions on umbilical cord weight ($P < 0.001$) and length ($P < 0.05$)

(**Figure 2.3**). In addition, histo-morphological placental phenotype showed strong maternal genome × fetal sex dependent regressions with fetal lung weight (**Figure 2.5C**).

2.3.3 Specific effects of Bt and Bi genomes, fetal sex and maternal weight

Least square means for specific effects of *Bos taurus taurus* (Bt) and *B. taurus indicus* (Bi) maternal and paternal genomes, fetal sex and regressions of non-genetic maternal effects of final maternal weight and daily weight gain, as detailed in statistical models for placental and fetal phenotype (**Table 2.1**), are presented in **Figures 2.6-2.10** and supplementary **Figures S2.1-2.5**. For those placental and fetal phenotype displaying significant maternal genome effects ($P <$ 0.001 – 0.0001), the Bt genome caused strikingly greater values than Bi genome (**Figures 2.6A-C, 2.7A,C, 2.10C**), except for efficiency phenotype, where the Bi genome caused significantly higher total ($P < 0.001$) and fetal ($P < 0.01$) placental efficiencies than Bt genome (**Figure 2.9A,C**). Similarly, for those phenotype where paternal genome was significant ($P <$ 0.05-0.0001), Bt genome resulted in greater values than Bi genome (**Figures 2.6D, 2.7A,D, 2.9A,D**), except for umbilical cord where Bi genome caused heavier ($P < 0.05$) and longer ($P < 0.01$) cord and larger cord vein diameter ($P < 0.0001$) (**Figures 2.8A,B,D**). Maternal × paternal genome interaction effects were significant for fetal lung ($P < 0.05$) and fluids weight ($P < 0.05$) and umbilical artery diameter ($P < 0.01$). Fetuses with Bt maternal (Bt_{mat}) × Bt paternal (Bt_{pat}) and $Bt_{mat} \times Bi_{pat}$ parental genomes had the heaviest lung weight ($P <$ 0.05-0.0001) (**Figure 2.7B**), fetuses with $Bt_{mat} \times Bi_{pat}$ had the greatest fetal fluid ($P <$ 0.05-0.0001, **Figure 2.7E**) and fetuses with $Bt_{mat} \times Bt_{pat}$ had the smallest umbilical artery diameter ($P <$ 0.05-0.0001, **Figure 2.8C**).

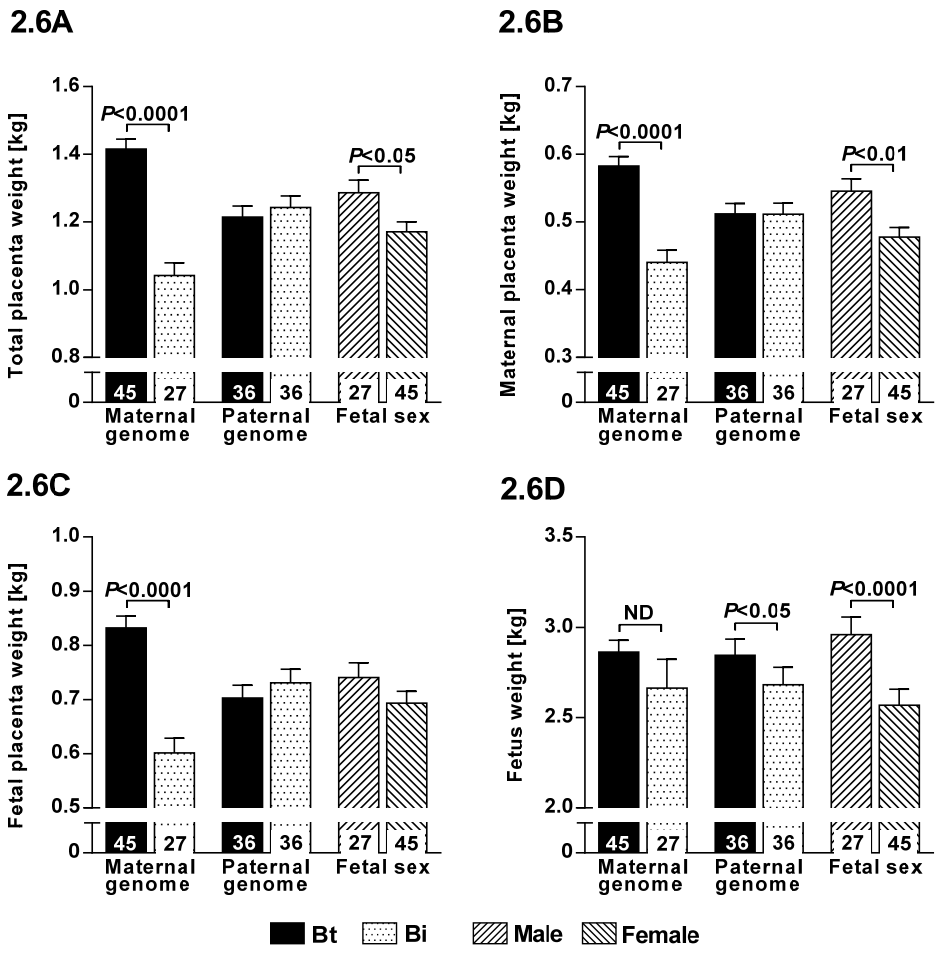

Figure 2.6. Specific effects of maternal genomes, paternal genomes and fetal sex on placenta and fetus weight at midgestation.

Least square means with standard errors of means and *P*-values for significant differences (*t*-test) between means for weight of total placenta (**A**), maternal placenta (**B**), fetal placenta (**C**) and fetus (**D**) are indicated. Bt: *Bos taurus taurus*, Angus breed. Bi: *Bos taurus indicus*, Brahman breed. Numbers for different maternal and paternal genomes and both sexes are indicated within bars.

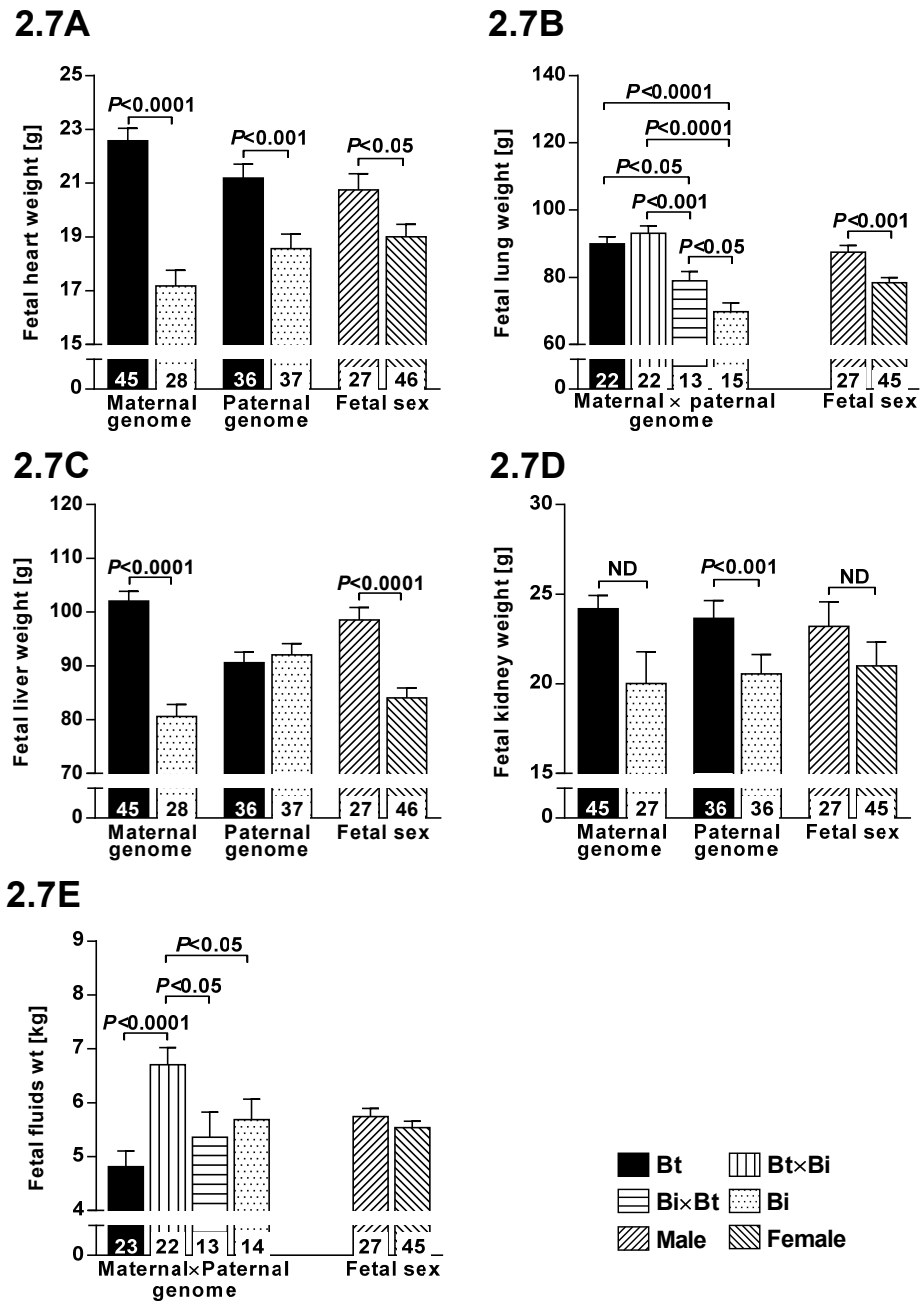

Figure 2.7. Specific effects of maternal genomes, paternal genomes and fetal sex on fetal organ and fluids weight at midgestation.

Least square means with standard errors of means and *P*-values for significant differences (*t*-test) between means for weight of fetal heart (**A**), lung (**B**), liver (**C**), kidney (**D**) and fluids (**E**) are indicated. Bt: *Bos taurus taurus*, Angus breed. Bi: *Bos taurus indicus*, Brahman breed. Numbers for different maternal and paternal genomes and both sexes are indicated within bars.

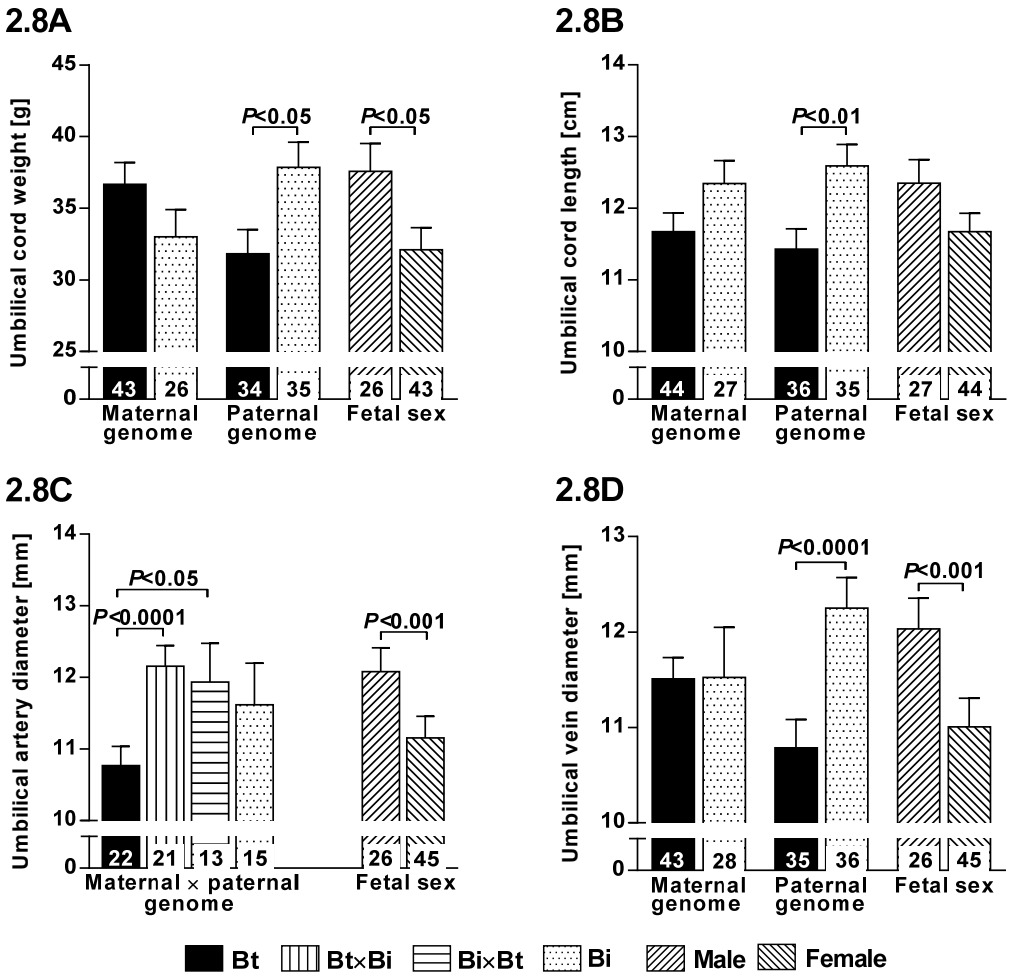

Figure 2.8. Specific effects of maternal genomes, paternal genomes and fetal sex on umbilical cord phenotype at midgestation.

Least square means with standard errors of means and *P*-values or significant differences (*t*-test) between means for umbilical cord weight (**A**), umbilical cord length (**B**), umbilical artery diameter (**C**) and umbilical vein diameter (**D**) are indicated. Bt: *Bos taurus taurus*, Angus breed. Bi: *Bos taurus indicus*, Brahman breed. Numbers for different maternal and paternal genomes and both sexes are indicated within bars.

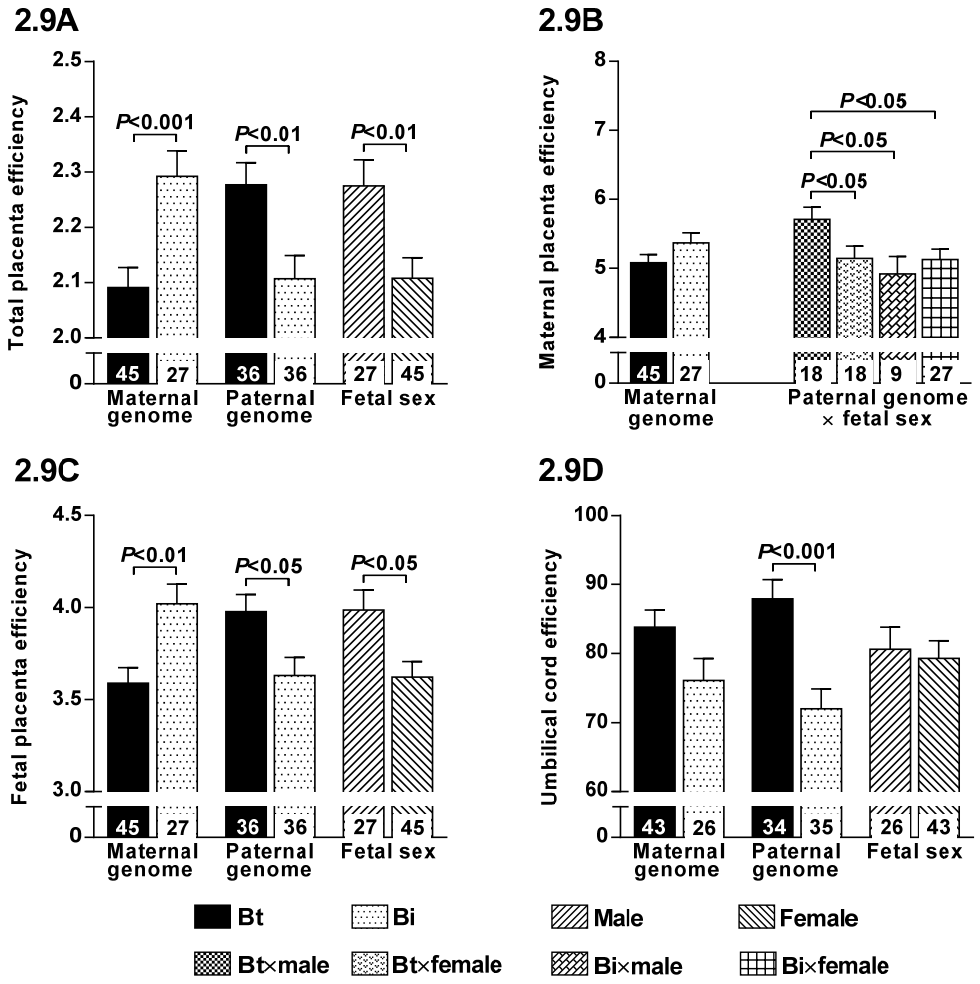

Figure 2.9. Specific effects of maternal genomes, paternal genomes and fetal sex on placental/umbilical cord efficiency at midgestation.

Least square means with standard errors of means and *P*-values for significant differences (*t*-test) between means for total placenta efficiency (**A**), maternal placenta efficiency (**B**), fetal placenta efficiency (**C**) and umbilical cord efficiency (**D**) are indicated. Efficiency: Fetus weight divided by total, maternal and fetal placenta and umbilical cord weight. Bt: *Bos taurus taurus*, Angus breed. Bi: *Bos taurus indicus*, Brahman breed. Numbers for different maternal and paternal genomes and both sexes are indicated within bars.

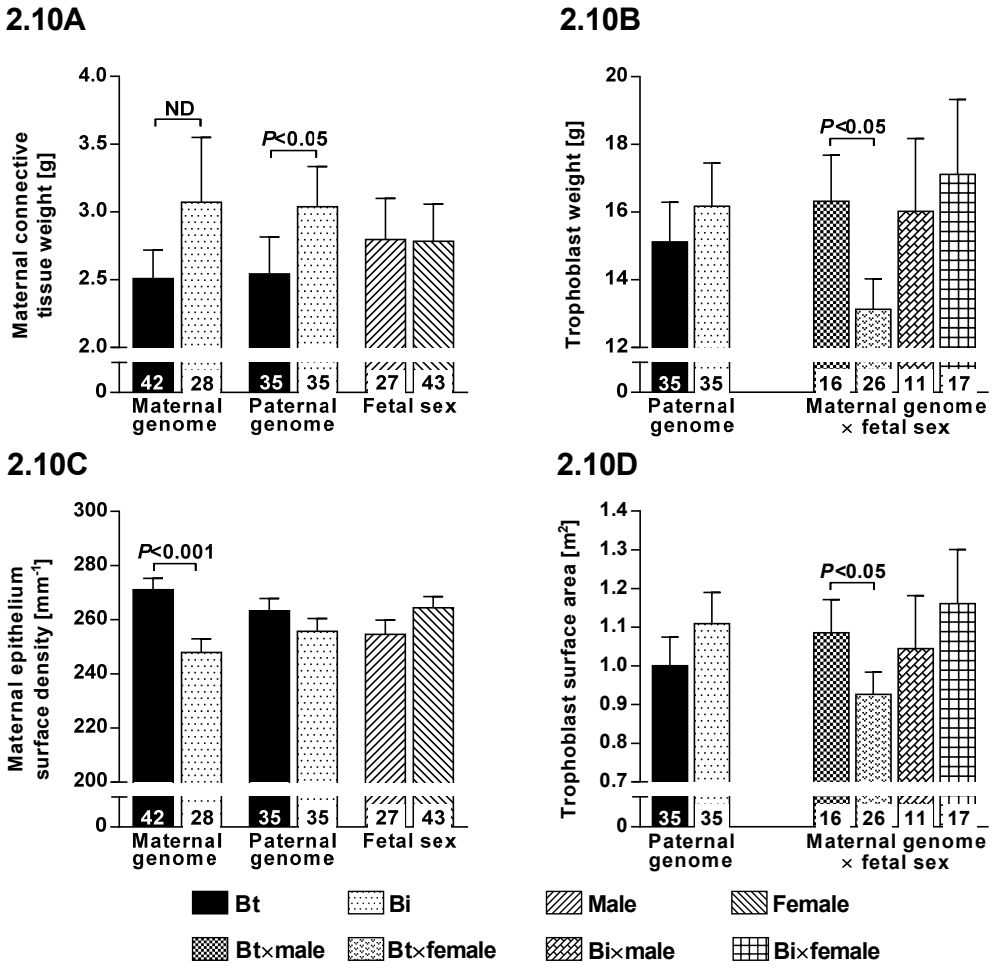

Figure 2.10. Specific effects of maternal genomes, paternal genomes and fetal sex on histo-morphological placental phenotype at midgestation.

Histo-morphological placental phenotype determined for the largest placentome surrounding the fetus. Least square means with standard errors of means and *P*-values for significant differences (*t*-test) between means for maternal connective tissue weight (**A**), trophoblast weight (**B**), maternal epithelium surface density (**C**) and trophoblast surface area (**D**) are indicated. Bt: *Bos taurus taurus*, Angus breed. Bi: *Bos taurus indicus*, Brahman breed. Numbers for different maternal and paternal genomes and both sexes are indicated within bars.

Fetal sex effects were significant for most placental and fetal phenotype, where male fetuses always had greater values, such as heavier maternal placenta weight ($P < 0.01$, **Figure 2.6B**), fetus weight ($P < 0.0001$, **Figure 2.6D**), liver weight ($P < 0.0001$, **Figure 2.7C**), larger umbilical

artery diameter ($P < 0.001$, **Figure 2.8C**) and higher total placenta efficiency ($P < 0.01$, **Figure 2.9A**). Maternal placenta efficiency displayed a significant paternal genome × fetal sex interaction effect ($P < 0.05$), where male fetuses with Bt paternal genome were the most efficient (**Figure 2.9B**). Maternal genome × fetal sex interaction effects were significant for trophoblast weight and surface area (both $P < 0.05$), where female fetuses had the lower weight and smaller area, compared to male fetuses with the Bt maternal genome (**Figure 2.10B,D**).

Most significant linear regressions of placental and fetal phenotype on non-genetic effects of final maternal weight and/or daily weight gain, nested within maternal genome, had positive slopes for Bt and Bi maternal genomes (see nested ANOVA *P*-values in **Figures S2.1-2.5**). However, regressions of umbilical cord phenotype showed opposite slopes for Bt and Bi maternal genomes (**Figure S2.2**). Regression of fetal kidney weight showed negative slope for Bt maternal genome and male fetus, but positive or no slope for other maternal genome and sex combinations (**Figure S2.1C**). Regressions of mound caruncle number and percentage displayed opposite slopes for different combinations of maternal genome and fetal sex (**Figure S2.3C,D**). For linear regressions of maternal barrier thickness, negative slope was identified for Bi maternal and Bt paternal genome (**Figure S2.5C**).

2.3.4 Parental genome and fetal sex-specific regressions between placental and fetal phenotype

Maternal genome significantly determined regressions between gross-morphological placental phenotype and umbilical cord phenotype (P<0.05-0.0001). Slopes of these regressions were generally positive, where regressions for Bt had higher intercepts and lower slopes than Bi (**Table S2.1, Figure 2.11A-D**). On the other hand, paternal genome largely determined regressions between fetus/organ weights and umbilical cord phenotype (P<0.05-0.0001). Slopes of these regressions were also generally positive, where regressions for Bt paternal genomes had

lower intercepts and higher slopes (**Table S2.1**, **Figure 2.12A-E**). In addition, paternal genome determined regressions between fetal kidney weight and fluids weight displayed opposite regression slopes for Bt and Bi (**Figure 2.12F**).

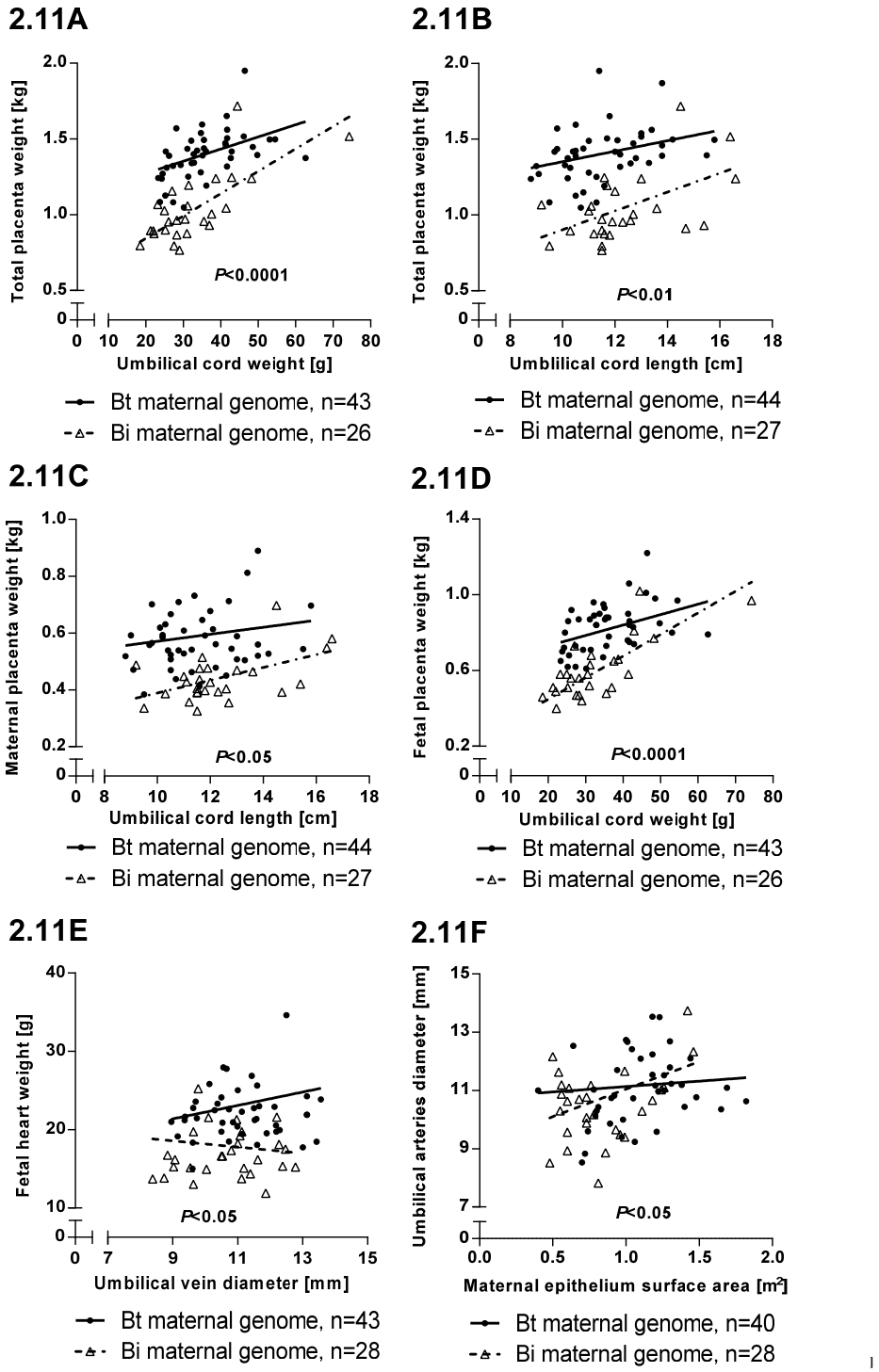

Figure 2.11. Specific linear regressions of gross-placental/fetal phenotype on umbilical cord/histo-placental phenotype nested within maternal genomes.

Representative significant regressions within maternal genetics were plotted with *P*-values (ANOVA), between total placenta and umbilical cord weight (**A**), total placenta weight and umbilical cord length (**B**), maternal placenta weight and umbilical cord length (**C**), fetal placenta and umbilical cord weight (**D**), fetal heart weight and umbilical

vein diameter (**E**), and umbilical artery diameter and maternal epithelium surface area (**F**). Histo-morphological placental phenotype determined for the largest placentome surrounding the fetus. Bt: *Bos taurus taurus*, Angus breed. Bi: *Bos taurus indicus*, Brahman breed. n: animal number.

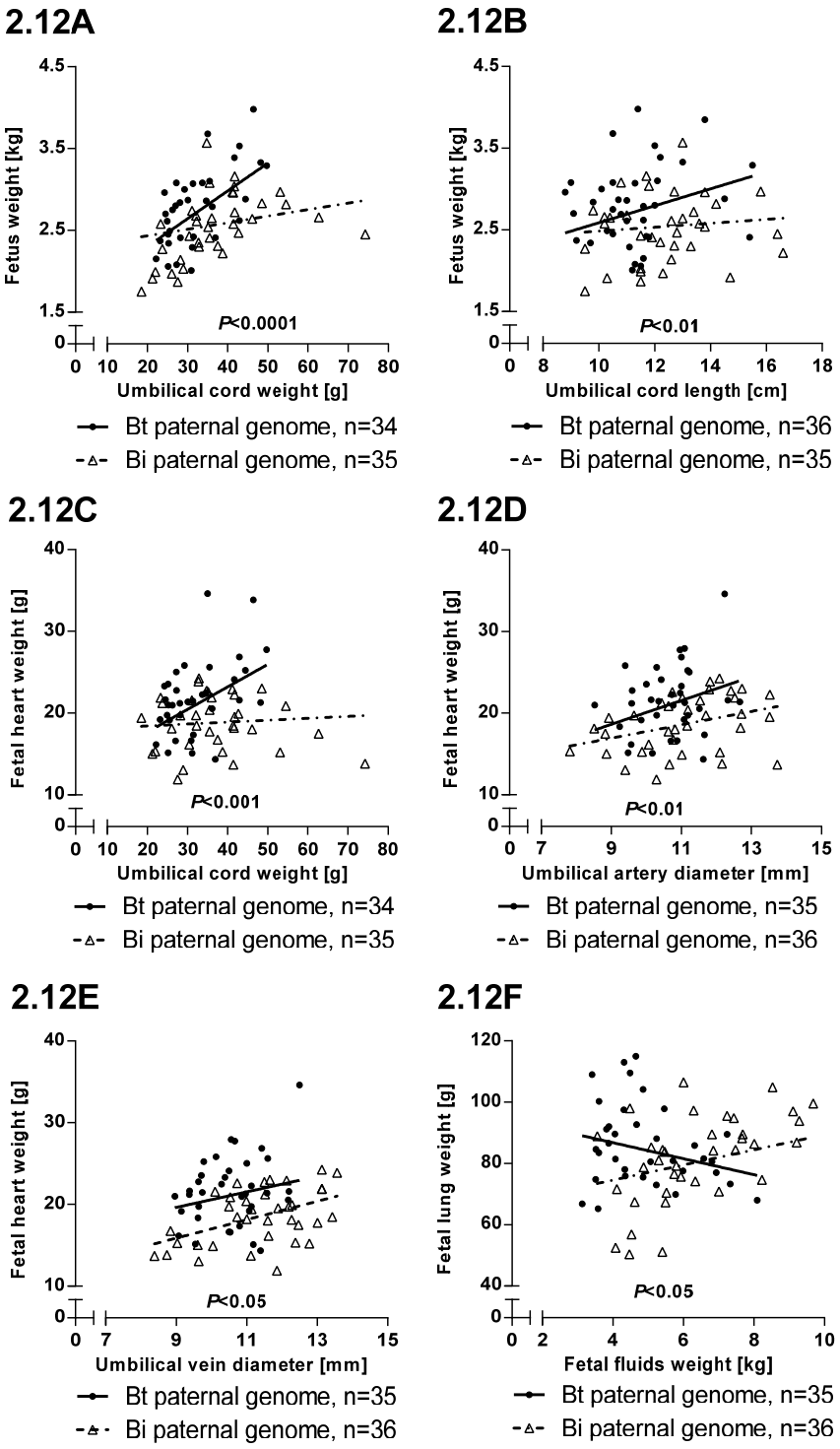

Figure 2.12. Specific regressions of fetal and organ phenotype on umbilical cord/fetal fluid phenotype nested within paternal genomes.

Representative significant regressions within paternal genetics were plotted with *P*-values (ANOVA), between fetus weight and umbilical cord weight (**A**), fetus weight and umbilical cord length (**B**), fetal heart weight and umbilical cord weight (**C**), fetal heart weight and umbilical artery diameter (**D**), fetal heart weight and umbilical vein diameter

(**E**), and fetal lung weight and fetal fluids weight (**F**). Bt: *Bos taurus taurus*, Angus breed. Bi: *Bos taurus indicus*, Brahman breed. n: animal number.

Fetal sex determined regressions between maternal/fetal placental weights and umbilical cord weight/length (P<0.05-0.01). Slopes of those regressions were generally positive, where male fetuses had lower regression intercepts and higher slopes (**Table S2.1**, **Figure 2.13A,B**). Fetal sex determined regressions between organ weights and umbilical cord phenotype showed positive regression slopes only for female fetuses (**Figure 2.13B-D**).

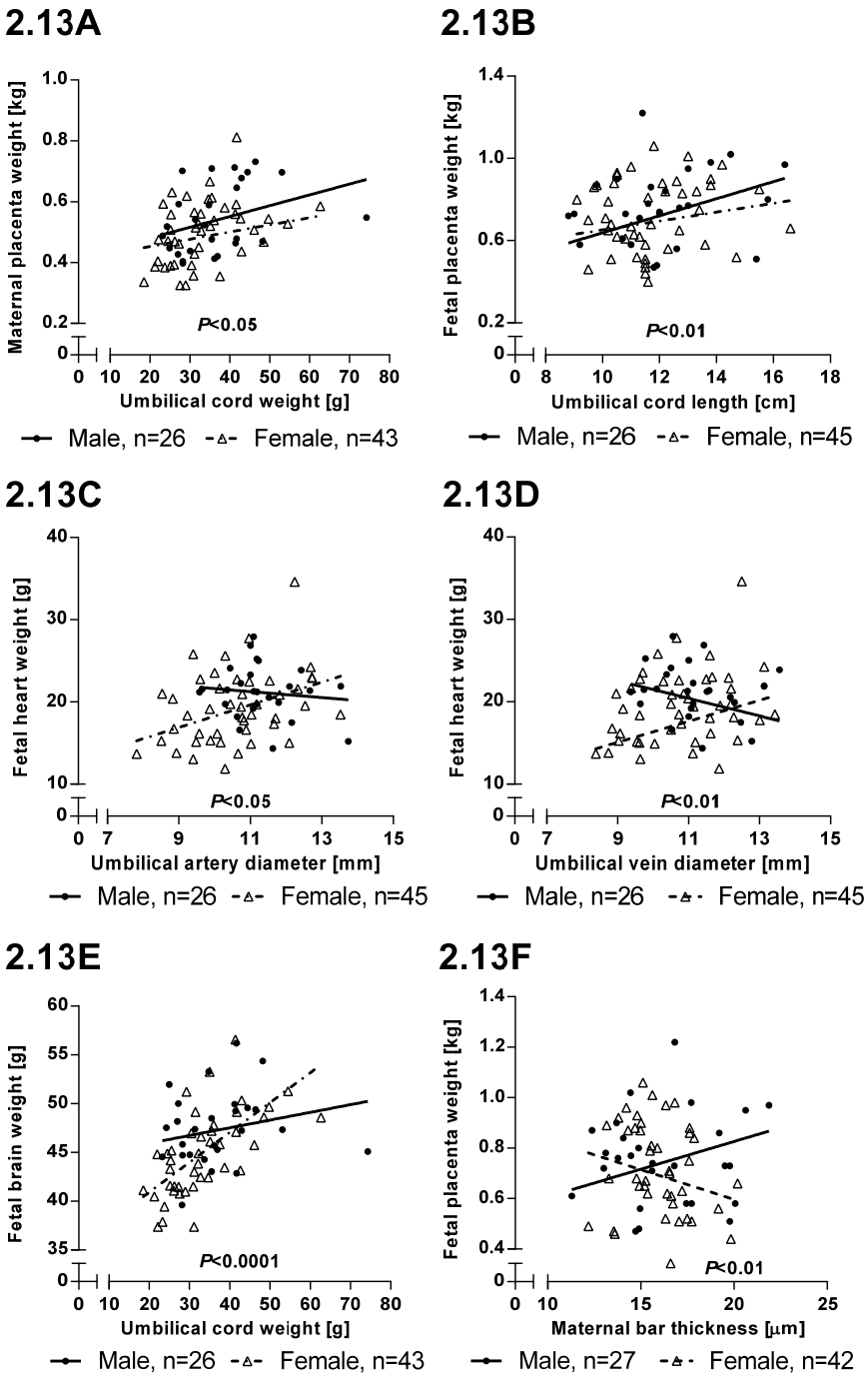

Figure 2.13. Specific regressions of gross-placental/fetal phenotype on umbilical cord/histo-placental phenotype nested within fetal sex.

Representative significant regressions within fetal sex were plotted with *P*-values (ANOVA), between maternal placenta weight and umbilical cord weight (**A**), fetal placenta weight and umbilical cord length (**B**), fetal heart weight and umbilical artery diameter (**C**), fetal heart weight and umbilical vein diameter (**D**), fetal brain weight and umbilical cord weight (**E**), and fetal placenta weight and maternal barrier thickness (**F**). Histo-morphological

placental phenotype were determined for the largest placentome surrounding the fetus. Bt: *Bos taurus taurus*, Angus breed. Bi: *Bos taurus indicus*, Brahman breed. n: animal number.

Maternal and paternal genome interaction largely determined regressions between maternal barrier thickness and placenta/fetus/organ weights (P<0.05, **Table S2.1**, **Figure S2.6**), and these regressions showed negative slopes only for fetuses with Bi maternal and Bt paternal genomes. Paternal genome and fetal sex interaction largely determined regressions between placenta/fetus weights and umbilical cord phenotype (**Table S2.1**, **Figure S2.7**), and these regressions showed negative slopes only for male fetus with Bt paternal genomes.

2.4 Discussion

For the first time, our results revealed a comprehensive picture of the magnitude of parental genome and fetal sex effects on placental-fetal phenotype at midgestation. Our results reveal a strong (epi)genetic basis (>47%) in placental and fetal phenotype (**Figure 2.1**), which is consistent with previous heritability estimates of 18-56% (Mesa *et al.* 2005; Buresova *et al.* 2006; Antoniou *et al.* 2011). The observed significant phenotypic differences between Bt and Bi parental genomes (**Figures 2.6-2.10**) likely result from allelic differences in genes with parent-of-origin effects controlling placental and fetal growth. Breed differences in placental and fetal phenotype were previously reported (Reynolds *et al.* 1980; Anthony *et al.* 1986; Bellows *et al.* 1993). However, detailed prenatal phenotypic profiling has, to our knowledge, not been previously reported. In fact, significant variation within Bt and Bi subspecies were included in our experiment to facilitate dissection of differential maternal and paternal (epi)gentic effects on the placental-fetal system.

One of our central novel findings is that while maternal genome predominantly contributed to genetic variation in all placental phenotype, fetus and organ weights, paternal genome predominantly contributed to genetic variation in fetal fluid weight, umbilical cord weight and length, and maternal placental and umbilical cord efficiencies (**Figure 2.2**). This provides experimental evidence for current hypotheses proposed to explain the evolutionary basis of genomic imprinting, i.e., maternal-off spring coadaptation (Wolf and Hager 2006) and genetic-conflict (Di Naro *et al.* 2001). Our finding of the predominant contribution of maternal genome to all placental phenotype supports the maternal–offspring coadaptation hypothesis proposed by Wolf and Hager (2006). Here, maternally expression of genes tends to dominate at the maternal fetal interface, i.e., placenta, where a majority of maternally expressed genes was previously observed (Wagschal and Feil 2006). In this context, coadaptation seemed to provide better prediction of imprinting effects on placenta, than conflict-of-interest which proposes coexistence of maternally and paternally expressed genes for restricting and promoting fetal growth, respectively (Haig 2004). Coadaptation was also supported by the large number of maternally expressed genes in placenta (e.g. *H19*, *PHLDA2*, *CDKN1C* and *GNA*S) (Coan *et al.* 2005; Frost and Moore 2010; Nelissen *et al.* 2011), which significantly affect placental and fetal growth.

The significant effects of maternal genes expressed in placenta on fetal development is in line with our second important finding, that fetus and organ weights, similar to placental phenotype, were also predominantly affected by maternal genome (**Figures 2.2, 2.7A-D**). However, ongoing studies have identified functionally important paternally expressed genes in placenta, such as *IGF2*, *DLK1* and *PEG10* (Frost and Moore 2010). Furthermore, dominance of paternally expressed imprinted genes was found in placenta of a horse-donkey species intercross (Wang *et al.* 2013). These findings seemed opposed to coadaptation theory in predicting imprinting effects in placenta, but may be explained by our third finding, that paternal genome predominantly

contributed to genetic variation in umbilical cord phenotype, placental efficiency phenotype and fetal fluids weight.

Although functionally important paternally expressed genes were found in placenta, the phenotypic effects of those genes might be exerted elsewhere on the placental-fetal system. The umbilical cord is responsible for transporting nutrient rich blood from placenta to fetal liver and brain, and cord parameters are associated with fetal growth (Di Naro *et al.* 2001). Also, fetal fluid is responsible for water and hormone circulation between mother and fetus (Ivell and Anand-Ivell 2009; Anand-Ivell *et al.* 2011). Thus, umbilical cord and fetal fluids largely represent nutrient exchange capacity and efficiency between mother, placenta and fetus. Therefore, control of these parameters by paternal genome may indicate the phenotypic effects of important paternal genes expressed in placenta on nutrient and/or hormone transfer. In fact, this is in agreement with the identification of a number of paternally expressed genes (e.g. *PEG10*, *IGF2* and *DLK1*) in human cord tissue and the effects of *PEG10* on birthweight (Lim *et al.* 2012).

Control of umbilical cord and fetal fluids by paternal genome also supports the conflict-of-interest hypothesis of genomic imprinting where paternally expressed genes tend to govern resource transfer between mother and fetus (e.g. *IGF2* and *DLK1*) (Haig 2004; Frost and Moore 2010). Our finding of significant paternal genome effects on the less studied umbilical cord and fetal fluids provides a novel view that could accommodate both existing hypotheses. Specifically, in the context of our results, we propose that (i) maternal-offspring coadaptation may be a better indicator of genomic imprinting pattern in placenta or fetus *per se* at midgestation, where predominant maternal genome effects were observed, (ii) conflict-of-interest also provides solid predictions when considering placenta and fetus as one system with comprehensive placental and fetal phenotype, where predominant maternal and paternal control of fetal growth coexist in

placenta/fetus and umbilical cord/fetal fluids, respectively; (iii) the ultimate effects of imprinted genes may be exerted elsewhere other than in the tissue where they were expressed. Varied predictions of the predominance of maternally expressed genes in placenta *per se* by maternal-offspring coadaptation and of balanced effects of maternal and paternal genes by conflict-of-interest in the placental-fetal system are also in agreement with the different tissue-specific expression pattern of genomic imprinting effects discussed by Wolf *et al*. (2008). The predominance of paternally expressed genes in the early stage of placental development (33-35 days after ovulation) in cross-bred equines was reported by Wang *et al*. (2013). The difference between this finding and our results of dominant maternal genome effects in the placenta along with prediction of maternal-offspring coadaptation may be explained by the species- and developmental stage- specificity of parent-of-origin effects (Wolf *et al.* 2008; Wolf 2013). Analyses of comprehensive placental-fetal phenotypes and tissues covering different time points or even different species will provide a better understanding of the nature of parent-of-origin effects on placental-fetal growth.

Another central finding of the present study is the strong maternal genome determined regressions between umbilical cord and placental phenotype, and paternal genome and/or fetal sex determined regressions between umbilical cord phenotype, fetal fluids weight and fetus and fetal organ weights (**Figures 2.3-2.5**). Along with emphasising the importance of umbilical cord and fetal fluids in placental and fetal growth, our findings suggest a novel morphological modularity of the placental-fetal system with (epi)genetic effects. Morphological modularity refers to the integration of a subset of developmentally and functionally correlated phenotype caused by modular pleiotropic effects, where this subset of correlated phenotype can be affected by a specific group of genes (Wagner *et al.* 2007). In addition to reported morphological modularity for postnatal mouse bone phenotype with pleiotropic (Wagner *et al.* 2007) and epistatic (gene-gene interaction) pleiotropic effects (Wolf *et al.* 2006; Pavlicev *et al.* 2008), our

findings suggest the existence of (i) a placenta-umbilical cord module predominantly affected by maternally expressed epigenetic factors and (ii) a fetus - umbilical cord - fetal fluids module largely affected by paternal- and/or sex- linked (epi)genetic effects.

Since imprinted genes commonly coordinate to regulate specific set of phenotype (Wolf 2013), we speculate that the differential (epi)genetic effects on specific morphological modules described above are exerted via maternally and paternally expressed genes. Such speculation is supported by previous reports where epigenetic factors, such as imprinted genes (Wagschal and Feil 2006; Frost and Moore 2010), microRNAs (Noguer-Dance *et al.* 2010) and sex-linked imprinted genes associated with X chromosome inactivation, tend to stay in clusters to differentially regulate placental and fetal growth (Raefski and O'Neill 2005; Wagschal and Feil 2006). Conversely, maternal barrier thickness showed a number of significant maternal × paternal genome dependent regressions with gross placental-fetal phenotype (**Figures 2.3-2.5**). Barrier thickness is a determinant of placental substrate transfer capacity (Amaladoss and Burton 1985), and is usually negatively correlated with fetal size (Roberts *et al.* 2001). Therefore, our finding indicates a parental genome coregulated bottleneck effect of maternal circulation on placental and fetal growth at midgestation. This indicates that maternal and paternal (epi)genetic factors not only differentiate, but can also cooperate to regulate the placental-fetal system. Together, our findings demonstrated the diversity and tissue-specificity of (epi)genetic factors regulating the placental and fetal system. A better understanding of epigenetic effects driving this system requires molecular analysis of specific morphological modules with corresponding epigenetic factors at the system level, together with placental-fetal phenotype.

Fetal sex explained a substantial amount of variation in placental and fetal phenotype, where males generally had significantly heavier placenta and umbilical cord, larger artery, and higher placental efficiencies, than females at midgestation (**Figures 2.1, 2.6-2.10**). This finding is novel,

although sex differences in fetal and birth phenotype have been previously reported (Reynolds *et al.* 1980; Anthony *et al.* 1986; Xiang *et al.* 2013). Our finding provides solid experimental data to support the sex specific placenta adaptation hypothesis where morphological evidence was lacking (Clifton 2010). Further, the finding of advanced male placental phenotype at midgestation indicates that natural selection favours male fetuses for nutrient supply, compared to female fetuses at midgestation. This can explain the proposed higher ability of male fetuses to adapt to an adverse intrauterine environment, which ultimately affects male development in postnatal stages (Clifton 2010). Moreover, having observed a large number of strong fetal sex determined regressions between fetal placenta weight and umbilical cord phenotype (**Figure 2.4A**), it is likely that sex specific placenta adaptation is mediated by fetal compartment of the placenta through the umbilical cord. This mediation can be carried out by hormonal dialogue between placenta and fetus (Geary *et al.* 2003; Anand-Ivell *et al.* 2011) and epigenetic mechanisms such as X chromosome inactivation (Looijenga *et al.* 1999; Migeon *et al.* 2005), sex-linked genes (Sood *et al.* 2006) and microRNAs (Clifton 2010). Detailed molecular profiles for specific fetal placenta and umbilical cord tissue will allow further identification of the drivers of sex specific placenta adaptation.

Our analyses identified relatively strong contributions of non-genetic final maternal weight and/or daily weight gain to variation in gross- and histo-morphological phenotype of the placenta, umbilical vein diameter and fetal brain weight compared to other investigated parameters (**Figure 2.1**). These non-genetic maternal effects were estimated within maternal genomes and can be interpreted as effects of pre-experimental environmental factors acting on dams, see methods and (Xiang *et al.* 2013). Significant pre-conception environmental effects on placenta and umbilical cord may be explained by the fact that they are responsible for nutrient and blood supply and closely tied to the extra-uterine environment. Such non-genetic effects of pre-conception environment of the dam on fetal brain have not been reported before. The estimated

regression coefficients suggested that the same mechanisms affected fetal brain weight in dams with Bt and Bi genome (**Figure S2.1B**).

In conclusion, we showed, for the first time, that the placental-fetal system is affected by differential maternal and paternal genome and fetal sex effects, which supports and accommodates both conflict-of-interest and maternal-offspring coadaptation hypotheses. Our findings also provide solid evidence to support the emerging sex-specific placenta adaptation hypothesis and highlight the important role of the umbilical cord and fetal fluids in the placental-fetal system. Furthermore, our analyses suggest the existence of morphological modules within the placental-fetal system, that can be distinguished by differential maternal/paternal and sex-linked (epi)genetic effects. These findings suggest the integration of epigenetic factors and placental-fetal growth. Thus, future research will require systematically coupling molecular profiles for genetic and epigenetic components at different tissue levels with comprehensive phenotype data. This approach will provide a broader understanding of mechanisms for genetic and epigenetic regulation of prenatal development.

2.5 Materials and methods

2.5.1 Animals

All animal experiments and procedures described in this study were approved by the University of Adelaide Animal Ethics Committee (No. S-094-2005 and S-094-2005A). We used animals and semen of Angus (*Bos taurus taurus*) and Brahman (*Bos taurus indicus*) cattle, both subspecies of domestic cow, that are commonly referred to as *Bos taurus* (Bt) and *Bos indicus* (Bi), respectively (Hiendleder *et al.* 2008; The Bovine Genome Sequencing Consortium 2009). Nulliparous Bt and Bi females of approximately 16–20 months of age were maintained on

pasture supplemented by silage. After an adjustment period of three to four weeks the animals received standard commercial estrous cycle synchronisation as described previously (Anand-Ivell *et al.* 2011). All fetuses were sired by two Bt and three Bi males. Dams were pregnancy tested by ultrasound scanning and fetuses recovered in an abattoir at Day 153±1 of gestation. Final maternal weight (FMW) was recorded and average maternal daily weight gain (MDG) was calculated as FMW minus weight at conception divided by gestation length (Supplementary **Figure S2.8**). We analysed 73 fetuses in total, including 23 Bt × Bt, 22 Bt × Bi, 13 Bi × Bt and 14 Bi × Bi (maternal genome listed first) with both sexes represented in each genetic group. The distribution of Bt and Bi maternal and paternal genomes, and number of females and males are shown in (**Table S2.2**).

2.5.2 Placental, fetal and umbilical cord gross-morphometry

After the heifers were killed, intact uteri were recovered and opened by longitudinal incision, from which fetuses were removed and weighed. Each fetus was eviscerated and fetal brain, heart, lung, liver and kidney weights were measured. Fetal umbilical cord was cut, measured for weight and length, and stored at -20 °C degree for later analysis. The amniotic and allantoic fluids were weighed together and defined as total fetal fluids. The largest placentome surrounding the fetus was cut at the base. This placetome was weighed, then placed in a petridish on ice, and cross-sectioned in the middle by parallel dual scalpels of 0.5cm distance. The 0.5cm thick placentome cross-section was placed into a 50 mL conical tube with ice cold 4% paraformaldehyde and 2.5% PVP-40 phosphate buffer solution. The section was washed four times with 1% phosphate buffer solution and stored in 70% ethanol until embedded in paraffin for immunohistochemistry, as described below. The same preservation procedure was applied to the umbilical cord.

Umbilical cords in 70% ethanol, and vacuum-packed uteri, were stored in a -20 °C cold room. In the laboratory, the diameter of the artery and veins of umbilical cord were measured using a

calliper. The uteri, on the other hand, were defrosted and both horns were fully opened. Membranes with cotyledon, defined as fetal placenta, were removed and weighed. For each uterus, individual caruncle was cut and weighed. The total caruncle number and total caruncle weight, i.e., maternal placenta weight, for each uterus were determined. Individual caruncle was bisected and classified into flat and mound types according to the degree of flatness. Number and percentage of each caruncle type were determined. Total, maternal and fetal placenta and umbilical cord efficiency was calculated as fetus weight divided by total placenta, maternal and fetal placenta and umbilical cord weight, respectively.

2.5.3 Placentome immunohistochemistry

Immunohistochemistry generally followed the prevsiously established protocol (Roberts *et al.* 2001; MacLaughlin *et al.* 2005). Briefly, an indirect double label immunohistochemistry was performed for three days. Antigen was retrieved using 10% Pronase (SIGMA, product code 1001254356, USA) for 15 mins at 37 °C. Following a 3% peroxidise block for 30 mins and protein block with 10% porcine serum and 1% bovine serum albumin in 1% PBS for 10 mins, the first anti-body, anti-Vimentin clone Vim 3B4 (DakoCytomation, code no. M7020, Denmark) in a 1:10 dilution, was applied to each section and left overnight. The next day, sections were washed in PBS three times before the biotinylated goat anti-mouse secondary antibody (DakoCytomation, code no. E0433, Denmark) was placed on the section for an hour. The sections were again washed with 1% PBS, followed by one hour incubation with 1:250 Strepavidin horseradish peroxidase (Rockland, P/N S000-03). Ammonium nickel (III) sulphate (SIGMA, A-1827, USA) was added to diaminobenzidine (SIGMA FAST, D4293-50, USA) solution. This allowed the maternal and fetal connective tissues and endothelial cells to develop a black stain. The second anti-body, mouse anti-cytokeratin AE1/AE3 (Millipore, cat no. MAB3412, USA) in 1:400 dilution, was placed on the section and left overnight. The same

protocol, as described above, was used on the third day, except that diaminabenzidine was used alone, thus, the cytoplasm or cell membrane of the trophoblast was stained brown. Hematoxylin (Accustain Hematoxylin Gill No. 2, SIGMA GHS232-1L, USA) and Eosin (Eosin Y Solution with Phloxine, SIGMA HT110332-1L, USA) were used as counterstains. Negative controls (without primary anti-bodies) were included in each batch.

2.5.4 Placentome histo-morphometry

High resolution images of the whole placentome section were generated through the NanoZoomer C9600 (Hamamatsu Photonics K.K., Japan) slide scanner (supplementary **Figure S2.9**). Ten fields at 20x magnification were selected in a random systematic order for each section, using the accompanying software, NDP.view (version 1.0.0, Hamamatsu Photonics K.K., Japan). The first field's location was chosen at random, followed by another field at 1 mm apart. Digital images of the fields were imported into Video Pro software (Leading Edge, Australia). Histo-morphometric assessment and computation for these bovine placentomes was similar to previously described protocol (Roberts *et al.* 2001; MacLaughlin *et al.* 2005). With the aid of the L-36 Merz grid transparency overlaying the monitor screen, a total of 360 points were counted for each section. Volume densities of the different placental cell types such as the maternal epithelium, maternal capillaries, maternal connective tissue, trophoblast, fetal capillaries, fetal connective tissue and "others" were then calculated. By multiplying volume density with placentome weight, the weight of each placental component was determined. Other morphometric parameters, such as the trophoblast and maternal epithelium surface density (cm^2/g), surface area (m^2), and barrier thickness (um), were estimated using intercept counting. For reproducibility of the method, a field was counted five times and the coefficient of variation was less than 7%.

2.5.5 Statistical analysis

All data were analysed by univariate Analysis of Variance (ANOVA) using the general linear model (GLM) procedure of R (v. 2.14 (Team. 2010)). Data was fitted into the following full linear model as described previously (Xiang *et al.* 2013), but excluding quadratic effects:

$$y_{ijk} = Intercept + M_i + P_j + S_k + gain(M_i) + weight(M_i) + F \times F + F \times F \times F + C \times C(M_i) + F \times C(M_i) + e_{ijk}$$

where y_{ijk} were fetal, umbilical cord, gross- and histo-morphological placenta phenotype, M_i was maternal genome effect (i = Bt, Bi), P_j was paternal genome effect (j = Bt, Bi), S_k was fetal sex effect (k = male, female), *gain* was post-conception daily weight gain and *weight* was final maternal weight. M_i, P_j, and S_k were fitted as fixed factors (F) and *gain* and *weight* were fitted as covariates (C). The covariates fitted in the model were nested within maternal genome (M_i), in order to adjust for effects *gain* and *weight* within each of the maternal genomes. Interactions between factors and covariates were tested as follows: $F \times F$ was 2-way interaction between factors, $M_i \times P_j$, $M_i \times S_k$ and $P_j \times S_k$, $F \times F \times F$ was 3-way interaction between factors, $M_i \times P_j \times S_k$; $F \times C(M_i)$ was 2-way interaction between factors and covariates nested within maternal genome, $P_j \times gain(M_i)$ and $S_k \times gain(M_i)$, $P_j \times weight(M_i)$, and $S_k \times weight(M_i)$.

Backward stepwise elimination was then used to reduce the full model based on type III sums of squares (SSIII) at significance level (P) of 0.05 as described previously (Xiang *et al.* 2013). Main effects of M_i, P_j and S_k were retained in the final model, irrespective of significance levels. Means for effects of factors and significant interactions and regression slopes for nested effects of covariates were plotted according to marginal means and estimated parameters obtained from the final model using GraphPad (GraphPad Software, Inc., CA, USA).

The final general linear model was used to analyse relative contributions of M_i, P_j and S_k and of significant non-genetic maternal effects to the explained variance of fetal, umbilical cord, gross- and histo-morphological placental phenotype, with type I sum of squares, as described previously (Xiang *et al.* 2013).

GLM was also used to determine relationships between fetal and gross-morphological placental phenotype with those umbilical cord and histo-mophological placental phenotype, which were significant in above described GLM analysis. Since parental genome and fetal sex effects were the primary research questions, linear regressions were estimated in GLM with adjustment for parental genomes and fetal sex effects as:

$$y_{ijk} = Incercept + F + F \times F + F \times F \times F + x(F) + x(F \times F) + x(F \times F \times F) + e_{ijk}$$

Where y_{ijk} was fetal and gross-morphological placental phenotype; F was main effects of factors described above, M_i, P_j and S_k; x was regression slope of umbilical cord and histo-morphological placental phenotype; $x(F)$ was the regression slopes nested within main effects, $x(M_i)$, $x(P_j)$ and $x(S_k)$; $x(F \times F)$ was the regression slopes nested within two-way interactions between main effects, $x(M_i \times P_j)$, $x(M_i \times S_k)$, $x(P_j \times S_k)$; $x(F \times F \times F)$ was the regression slopes nested within three-way interactions between main effects, $x(M_i \times P_j \times S_k)$. Backward elimination, as described above, was performed only for nested linear regression slopes, i.e., $x(F)$, $x(F \times F)$ and $x(F \times F \times F)$, until significant (P<0.05) nested effects remained. Significant nested regressions were plotted using Graphpad and regression networks were drawn for each fetal and gross-morphological placental phenotype, based on significance levels of the nested regressions slopes.

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Chapter 3: Widespread differential maternal and paternal genome effects on fetal bone phenotype at midgestation

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Chapter 3

3.1 Abstract

Parent-of-origin dependent genetic and epigenetic factors are important determinants of prenatal development and involved in programming of adult phenotype. However, data on the magnitude and specificity of effects of the maternal and paternal genome on fetal bone are lacking. We used an outbred bovine model with well defined maternal and paternal genetics and generated fetuses in growing adolescent mothers to dissect and quantify effects of parental genomes, fetal sex and non-genetic maternal effects (maternal weight, post conception weight gain) on the fetal skeleton at midgestation. Analysis of 51 direct morphometric measurements and weights of 10 bones each from 72 fetuses recovered at Day153 (54% term) of gestation identified six principal components (PC1-6) that explained 80% of the total variation in skeletal parameters. ANOVA of extracted PCs revealed that parental genomes accounted for most of the variation in bone mass (PC1, 72.1%), limb ossification (PC2, 99.8%), flat bone elongation (PC4, 99.7%) and axial skeletal growth (PC5, 96.9%). Limb elongation showed lesser effects of parental genomes (PC3, 40.8%) and the only PC with a significant non-genetic maternal effect (post conception weight gain, 29%). Further analyses revealed strong maternal genome effects on bone mass (PC1, $P<0.0001$) and axial skeletal growth (PC5, $P<0.001$), while effects of paternal genome were weak (bone mass/PC1, $P<0.05$) or non-significant (axial skeletal growth/PC5, $P>0.10$). Significant interactions between maternal and paternal genome affected limb ossification (PC2, $P<0.05$) and flat bone elongation (PC4, $P<0.05$), albeit with strikingly different phenotypic patterns. Fetal sex affected bone mass (PC1, $P<0.0001$) and limb elongation (PC3, $P<0.05$) only. Our results demonstrated for the first time complex patterns of maternal and paternal genome effects on specific components of the mammalian fetal skeleton. This provides

the basis for molecular dissection of genetic and epigenetic mechanisms that operate across pre- and postnatal development to determine adult bone phenotype.

Key words: Fetus, skeleton, parent-of-origin, genome, epigenetic.

3.2 Introduction

Postnatal skeletal growth and clinical parameters associated with bone disease have a moderate to strong genetic basis which facilitated identification of genome regions harbouring genes for postnatal bone mass (Klein *et al.* 1998; Beamer *et al.* 1999; Ishimori *et al.* 2006; Yu *et al.* 2007) and bone geometry parameters (Drake *et al.* 2001; Masinde *et al.* 2003; Lang *et al.* 2005; Kenney-Hunt *et al.* 2006) in animal models. However, prenatal skeletal development and fetal programming effects contribute significantly to postnatal bone phenotype and risk of adult bone disease such as osteoporotic bone fracture (Cooper *et al.* 2002; Cooper *et al.* 2006; Lanham *et al.* 2008). For example, fetal femur longitudinal growth rate from mid- to late-gestation accurately predicted skeleton size at age four (Harvey *et al.* 2010) and birth weight, which is highly correlated with prenatal skeletal development(Estêvão *et al.* 2012), explained a significant proportion of the variation in bone mass of adults in the seventh decade (Dennison *et al.* 2005). Furthermore, genetic factors interact with environmental factors in the early determination of postnatal bone phenotype (Karasik and Kiel 2008). For example, interaction between vitamin D receptor (*VDR*) genotype and birth weight strongly affected BMD in the elderly (Dennison *et al.* 2001). Similarly, interaction between growth hormone (*GH*) genotype and infant weight significantly affected bone loss rate at 61-73 years (Dennison *et al.* 2004). These data clearly showed that early life programming and interaction of genetic and non-genetic factors are pivotal in determining postnatal skeletal phenotype and risk of bone disease.

Genetic and epigenetic factors following non-mendelian modes of inheritance have emerged as an important source of variation in postnatal bone mass and bone geometry parameters. This includes effects of maternally inherited mitochondrial (mt) DNA (Guo *et al.* 2011), X- and Y-chromosomes (Lagerholm *et al.* 2011; Estrada *et al.* 2012) and genomic imprinting (Morison *et al.* 2005; Leamy *et al.* 2008; Lewis *et al.* 2012). The important functions of genes with genomic

imprinting (i.e., parent-of-origin dependent allele-specific gene expression) for pre- and postnatal skeletal development have been demonstrated in mouse models. Genes with complex transcript specific imprinting such as guanine nucleotide binding protein alpha stimulating (*GNAS*) (Sakamoto *et al.* 2005), or maternally expressed cyclin-dependent kinase inhibitor 1C (*CDKN1C*) (Yan *et al.* 1997) and paternally expressed insulin-like growth factor 2 (*IGF2*) (Eggenschwiler *et al.* 1997), are critical for bone formation and differentiation. Meta analysis of the combined human microarray gene expression dataset demonstrated that imprinted paternally expressed zinc-activated ligand-gated ion channel (*ZAC1*) regulates an imprinted gene network, including *GNAS* (Williamson *et al.* 2006), *CDKN1C* and *IGF2* (Varrault *et al.* 2006), that controls prenatal growth and bone ossification (Varrault *et al.* 2006).

The genetic and epigenetic mechanisms and effects described above suggested essential and complex but well defined roles of maternal and paternal genomes in determining pre- and postnatal skeletal development. However, fetal programming of the skeleton has exclusively been investigated from the perspective of environmental perturbations such as maternal malnutrition and lifestyle factors (Godfrey *et al.* 2001; Cole *et al.* 2009; Dennison *et al.* 2010). Data on the magnitude, specificity and complexity of genetic and epigenetic parent-of-origin effects on fetal skeletal parameters are lacking.

Previous genetic studies on bone phenotype in different species demonstrated that directly measured parameters are more informative than indirectly measured parameters such as BMD and can significantly improve the accuracy of genetic analyses of bone traits (Volkman *et al.* 2003; Leamy *et al.* 2008; Mao *et al.* 2008; Zheng *et al.* 2012). The bovine is outbred, carries a single fetus and has a gestation length and maturity at birth similar to human (Andersen and Plum 1965; Bebbere *et al.* 2013) . Furthermore, the bovine (*Bos taurus taurus*) genome sequence revealed a high conservation of gene structure with human (The Bovine Genome Sequencing

Consortium 2009). In the present study, we used well defined fetuses in four genetic groups that represented both purebred and reciprocal cross combinations of *Bos taurus taurus* and *Bos taurus indicus* genomes as best fit animal model (Bolker 2012) to dissect and quantify effects of maternal and paternal genomes and their interactions with non-genetic maternal effects on directly measured fetal skeletal parameters in growing adolescent mothers at mid-gestation. Using principal component regression analyses, we demonstrated for the first time widespread and specific impacts of parental genome, and effects of fetal sex and non-genetic maternal factors, on mammalian fetal bone phenotype at midgestation.

3.3 Material and methods

3.3.1 Animals

All animal experiments and procedures described in this study were approved by the University of Adelaide Animal Ethics Committee (No. S-094-2005 and S-094-2005A). We used animals and semen of Angus (*Bos taurus taurus*) and Brahman (*Bos taurus indicus*) cattle, subspecies of domestic cow and commonly referred to as *Bos taurus* (Bt) and *Bos indicus* (Bi), respectively (Hiendleder *et al.* 2008; The Bovine Genome Sequencing Consortium 2009). Nulliparous Bt and Bi females of approximately 16 – 20 months of age were maintained on pasture supplemented by silage. After an adjustment period of 3-4 weeks**,** the animals received standard commercial estrous cycle synchronization as described previously (Anand-Ivell *et al.* 2011). Fetuses were sired by 2 Bt or 3 Bi males. Dams were pregnancy tested by ultrasound scanning and fetuses recovered in an abattoir at Day 153±1 of gestation. Fetuses were removed from the uterus, eviscerated, vacuum packed and stored at - 20ºC until further processing. Final maternal weight (FMW) was recorded and average maternal daily weight gain (MDG) was

calculated as FMW minus weight at conception divided by gestation length (**Figure 3.1**). We analysed 72 fetuses in total, including 23 Bt × Bt, 22 Bt × Bi, 13 Bi × Bt and 14 Bi × Bi (maternal genome listed first) with both sexes represented in each genetic group. The distribution of Bt and Bi maternal and paternal genomes, and number of females and males are shown in Supplementary **Table S3.1.**

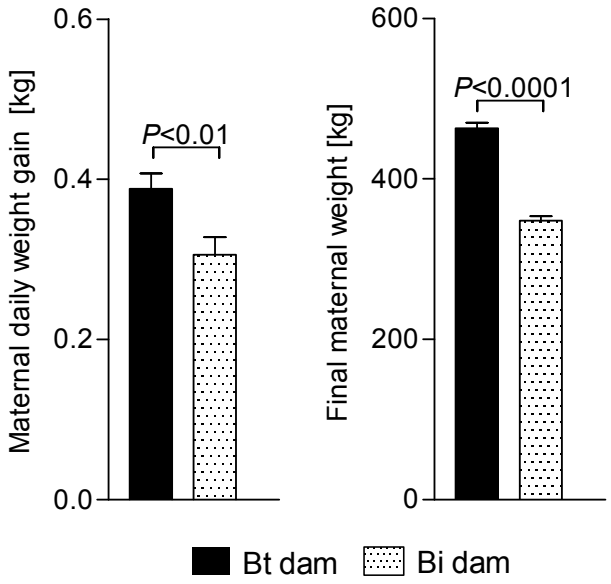

Figure 3.1. Post conception maternal daily weight gain and final weight for *Bos taurus taurus* and *Bos taurus indicus* dams.

Post conception maternal daily weight gain and final weight for *Bos taurus taurus* and *Bos taurus indicus* dams. (A) Post-conception maternal daily gain: Final maternal weight – weight at conception divided by 153 days of gestation. (B) Final maternal weight – weight before the animal was sacrificed on Day 153 of gestation. Means with standard deviations of means and *P*-values for significantly different means (*t*-test) are indicated. Bt – *Bos taurus taurus*, Bi – *Bos taurus indicus*.

3.3.2 Bone parameters

After thawing of fetuses, the following bones, with surrounding soft tissue, were removed: *Os mandibulare*, *Os scapulare*, *Os humeri*, *Os radiale*, *Os ulnare*, *Ossa metacarpalia*, *Os costale* VI, *Os pelvis*, *Os femoris*, *Os tibiale*, *Ossa metatarsalia*, *Columna vertebralis*. Each bone was immersed in water at 50 °C for two minutes, followed by a 10 % KOH-solution at room temperature for one minute and a final wash in milli-Q water to cease maceration. Remaining soft tissue was removed with scissors, forceps and a soft tooth brush. Bone was then blotted dry on absorbent paper for two minutes, weighed and measured to obtain geometry parameters (**Supplementary Figure S3.1)**. Weights were entire bone weights and calcified bone weights. Geometry parameters were entire bone lengths, calcified bone lengths, diameters and width. Geometry parameters were based on standard anatomical points of each bone (Budras and Robert 2003). Details of measurements and weights for each bone are described in **Supplementary Table S3.2**.

3.3.3 Principal component analysis

Principal component analysis (PCA)**,** as implemented in SPSS 17.0 (SPSS Inc., Chicago, IL, USA)**,** was used to analyse 51 bone weight and geometry parameters (Field 2009) and principal components (PCs) with eigenvalue ≥ 1 were extracted according to Kaiser criterion (Kaiser 1960). The oblique rotation method was used for PC extraction by considering measured parameters which were correlated (Field 2009). Bone weight and/or geometry parameters, with factor loading ≥ |0.4| in the 'pattern matrix', were identified as comprising an extracted PC and used to interpret the PC (Manly 2005). 'Factor score' was computed by using the regression method in SPSS for each PC to quantify respective variation. The factor scores were then analysed by general linear models (see below).

3.3.4 General linear models

Factor scores of extracted PCs were analysed with the linear model function (lm) in R (v. 2.14 (Team. 2010)). Data were fitted into the following general linear model with type III sums of squares as described (Xiang *et al.* 2013)

$$y_{ijk} = Intercept + M_i + P_j + S_k + gain(M_i) + weight(M_i) + F \times F + F \times F \times F + C \times C(M_i) + C^2(M_i) + F \times C(M_i) + F \times C \times C(M_i) + F \times C^2(M_i) + e_{ijk}$$

where y_{ijk} were extracted PCs, M_i was maternal genome effect (i = Bt, Bi), P_j was paternal genome effect (j = Bt, Bi), S_k was fetal sex effect (k = male, female), covariate of *gain* was post-conception daily weight gain and *weight* was final maternal weight. M_i, P_j, and S_k were fitted as factors (F) and *gain* and *weight* were fitted as covariates (C). The covariates fitted in the model were nested within maternal genome (M_i), in order to adjust for effects of *gain* and *weight* within each of the two types of maternal genomes used in the study. Interactions between factors and covariates were tested as follows: $F \times F$ was 2-way interaction between factors, $M_i \times P_j$, $M_i \times S_k$ and $P_j \times S_k$, $F \times F \times F$ was 3-way interaction between factors, $M_i \times P_j \times S_k$; $C \times C(M_i)$ was 2-way interaction of covariates nested within maternal genome, *gain* × weight(M_i); $C^2(M_i)$ was the quadratic term of covariates nested within maternal genome, $gain^2(M_i)$ and $weight^2(M_i)$; $F \times C(M_i)$ was 2-way interaction between factors and covariates nested within maternal genome, $P_j \times gain(M_i)$ and $S_k \times gain(M_i)$, $P_j \times weight(M_i)$, and $S_k \times weight(M_i)$; $F \times C \times C(M_i)$ was 3-way interaction between factors and the two covariates nested within maternal genome, $P_j \times gain \times weight(M_i)$ and $S_k \times weight \times gain(M_i)$; $F \times C^2$ was interaction between factors and quadratic terms of covariates nested within maternal genome, $P_j \times gain^2(M_i)$, $S_k \times gain^2(M_i)$, $P_j \times weight^2(M_i)$ and $S_k \times weight^2(M_i)$.

Backward stepwise elimination (Nelder 1994) was then used to reduce the model for each PC based on type III sums of squares at a significance level (P) of 0.05 as described (Xiang *et al.* 2013). Main effects of M_i, P_j and S_k were retained in the final model, irrespective of significance levels. The estimation of contribution of retained variables to explained variation in the model also followed previous procedures (Xiang *et al.* 2013). Means for effects of factors and significant interactions and regression slopes for nested effects of covariates were plotted according to marginal means and estimated parameters obtained from the final model using GraphPad (GraphPad Software, Inc., CA, USA).

3.4 Results

3.4.1 Principal components

Our analyses clearly partitioned the 51 measured bone weight and geometry parameters into six principal components (PC1-6) which explained 80% of the total variation (**Table 3.1**). Parameters assigned to PC1 represented entire and calcified weights of long bones and/or irregular bones and diameters of long bones and were interpreted as bone mass. Calcified long bone lengths were in PC2 and named limb ossification. PC3 largely consisted of entire lengths of long bones, except for humerus and femur, and was interpreted as limb elongation. PC4 contained entire lengths of flat bones plus femur and was described as flat bone elongation. The majority of parameters in PC5 represented lengths of cervical and lumbar vertebrae and weight of the vertebral column; this PC was interpreted as axial skeletal growth. PC6 contained only the length of thoracic vertebrae and named thoracic vertebral growth.

Table 3.1. Summary and interpretation of results of principal component (PC) analysis of 51 bone weight and geometry parameters.

PC[1]	*Bone parameters within PC*[2]	*Overall description of PC*	*Explained variance*[3]	*Cumulative variance*
1	Weight of entire mandible, scapula, humerus, radius, ulna, metacarpus, rib VI, pelvis, femur and tibia Weight of calcified scapula, humerus, radius, ulna, metacarpus, femur, tibia and metatarsus Diameter of humerus, radius, ulna, metacarpus, femur, tibia and metatarsus Length of entire scapula and thoracic vertebrae	Bone mass	0.58	0.58
2	Length of calcified humerus, radius, ulna, metacarpus, femur, tibia and metatarsus	Limb ossification	0.12	0.70
3	Length of entire radius, metacarpal, tibia, metarsus and weight of entire metatarsus	Limb elongation	0.03	0.73
4	Length of mandible1, mandible2, rib VI, pelvis, length of calcified scapula and width of entire scapula and entire femur	Flat bone elongation	0.03	0.76
5	Length of cervical vertebrae and lumbar vertebrae, weight of entire vertebral column and length of entire humerus	Axial skeletal growth	0.02	0.78
6	Length of thoracic vertebrae	Thoracic vertebrae growth	0.02	0.80

[1]PC: Principal components were identified by eigenvalue ≥ 1 criteria. [2]Bone parameters within PC: Bone parameters with factor loadings ≥ |0.4| are identified as comprising the PC. Explained variance[3]: Proportion of variance in complete set of bone parameters explained by each PC. Manible1: Distance from angle of mandible to condylar process. Mandible2: Distance from madibular notch to condylar process.

3.4.2 Proportion of variation explained by parental genomes, fetal sex and non-genetic maternal effects

The extracted PCs were analysed with a general linear model and significant final statistical models were obtained for PCs 1-5 but not PC6. Final models with adjusted R^2 values and significance levels of retained variables are presented in **Table 3.2**. Parental genomes were the most important source of variation for all PCs with significant statistical models. Maternal and paternal genomes together explained most of the variation in bone mass (PC1, 72.1%) and nearly all variation in limb ossification (PC2, 99.8%), flat bone elongation (PC4, 99.7%) and axial

skeletal growth (PC5, 96.9%). Parental genomes explained less variation in limb elongation (PC3, 40.8%), the only PC with a significant non-genetic maternal contribution (post conception weight gain, 29%). Significant contributions of fetal sex to variation in bone mass (PC1, 27.9%) and limb elongation (PC3, 30.4%) were of similar magnitude, but negligible (0.2 – 3.1%) for all other PCs (**Figure 3.2A**).

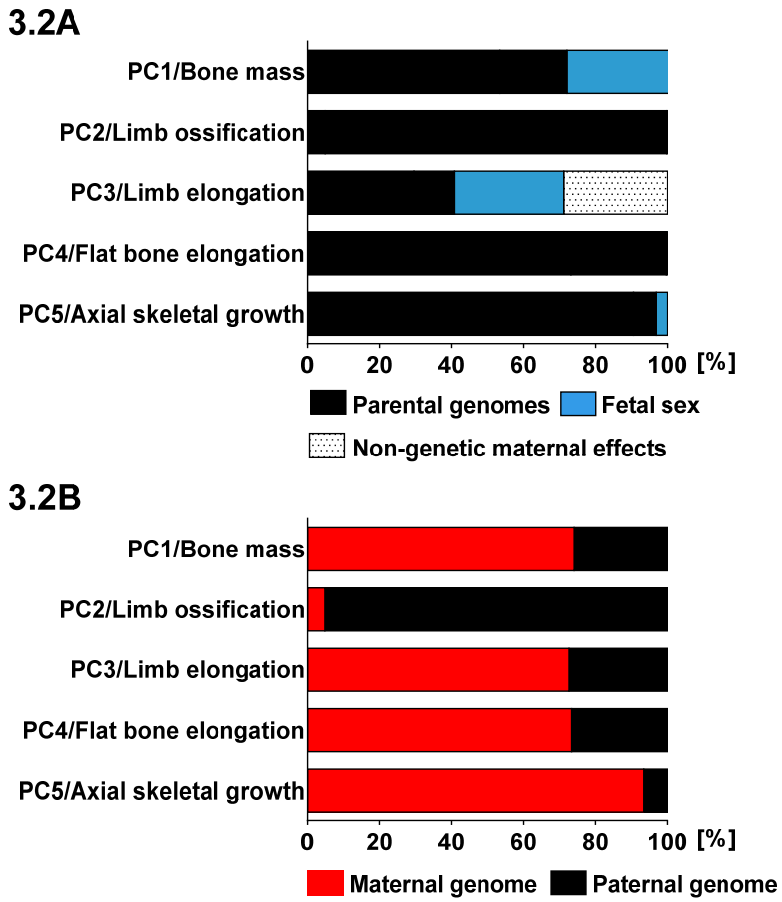

Figure 3.2. Relative contributions of genetic and non-genetic factors to variation explained in principal components for bone weight and geometry parameters.

(**A**) Contributions of parental genomes, fetal sex and non-genetic maternal effects of maternal daily weight gain. (**B**) Contributions of maternal and paternal genomes.

Table 3.2. Summary of the final general linear models (type III sums of squares) for principal components of bone weight and geometry parameters, with adjusted R^2 values and significance levels (*P*-values) of models and variables. Only *P*-values for factors, interactions and nested effects retained in the final model are shown. The model for PC6 was not significant (*P*>0.05).

Principal component	R^2	*P-values*					
		Model	*Maternal genome*	*Paternal genome*	*Fetal sex*	*Mat ×Pat*[1]	*MDG (Mat)*[2]
PC1/Bone mass	0.527	1.4E-11	3.7E-09	0.0199	1.1E-05		
PC2/Limb ossification	0.431	1.9E-08	0.5192	2.5E-08	0.7198	0.0323	
PC3/Limb elongation	0.118	0.0211	0.0125	0.0677	0.0239		0.0205
PC4/Flat bone elongation	0.439	1.2E-08	3.9E-08	4.3E-04	0.6662	0.0107	
PC5/Axial skeletal growth	0.177	0.0011	0.0001	0.4335	0.4580		

[1]Mat×pat: Maternal and paternal genome interaction effect. [2]MDG(mat): Effect of final maternal weight nested in maternal genetics.

More specifically, maternal genome accounted for most of the genetic variation in bone mass (PC1, 74.1%), limb elongation (PC3, 72.6%), flat bone elongation (PC4, 73.4%) and axial skeletal growth (PC5, 93.5%) while paternal genome explained nearly all genetic variation in limb ossification (PC2, 95.1%) (**Figure 3.2B**).

3.4.3 Specific effects of defined maternal and paternal genomes, fetal sex and non-genetic maternal factors

The use of clearly defined *B. t. taurus* (Bt) and *B. t. indicus* (Bi) maternal and paternal genomes also allowed quantification of specific effects of parental genomes. Further analysis of extracted PCs revealed strong maternal genome effects on bone mass (PC1, *P*<0.0001) and axial skeletal growth (PC5, *P*<0.001), while paternal genome effects were weak (bone mass/PC1, *P*<0.05) or non-significant (axial skeletal growth/PC5, *P*>0.05). Fetuses with Bt maternal genomes had higher bone mass and displayed increased axial skeletal growth (**Figure 3.3A,D**). Bone mass was also strongly affected by fetal sex (PC1, $P < 0.0001$), with higher bone mass in males (**Figure 3.3A**).

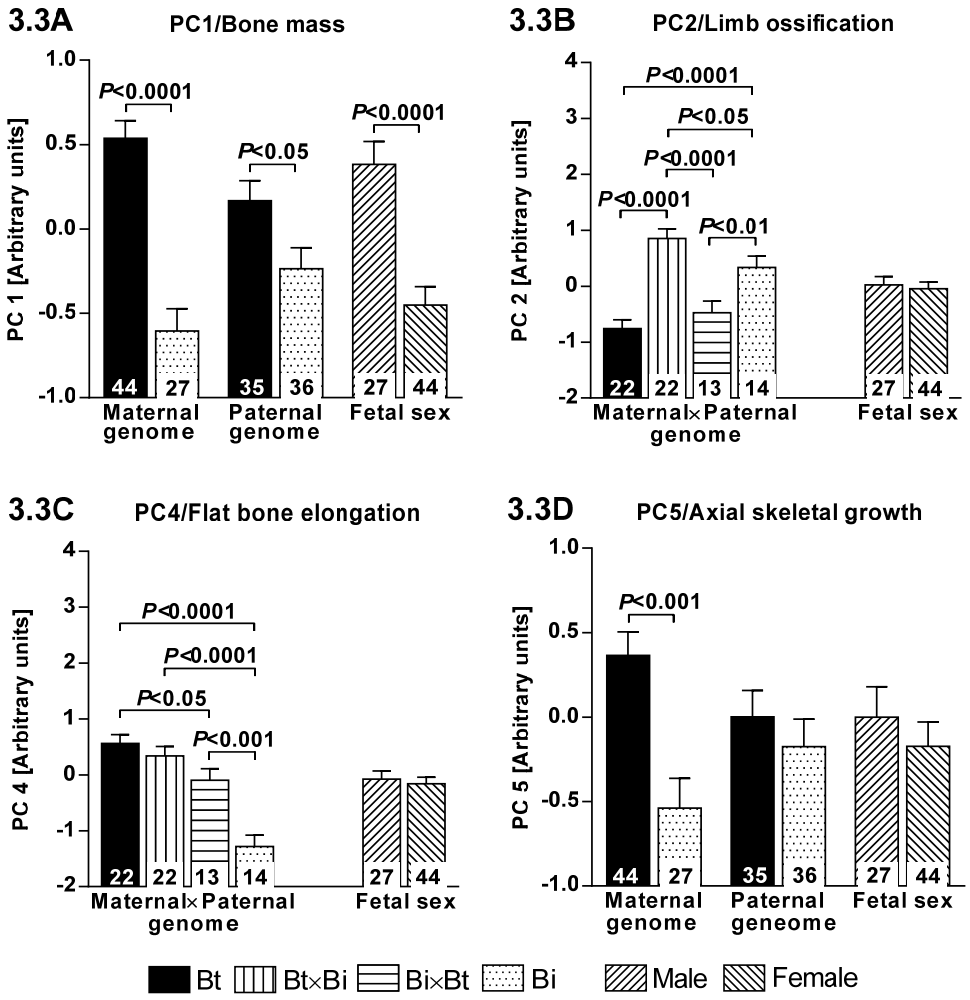

Figure 3.3. Specific effects of maternal genome, paternal genome and fetal sex on identified and extracted principal components (PC) of measured fetal bone geometry and weight parameters.

Least square means with standard errors of means are shown and *P*-values for significant differences (*t*-test) between means for factor scores of (**A**) PC1/bone mass, (**B**) PC2/limb ossification, (**C**) PC4/flat bone elongation and (**D**) PC5/axial skeletal growth. Bt: *Bos taurus taurus*, Angus breed. Bi: *Bos taurus indicus*, Brahman breed. Numbers for different maternal and paternal genomes and both sexes are indicated within bars.

There was a significant interaction ($P<0.05$) between maternal and paternal genomes on limb ossification (PC2) and flat bone elongation (PC4), but with strikingly different phenotypic patterns in the four maternal × paternal genome combinations (**Figure 3.3B,C**). Limb ossification was most advanced in Bt×Bi (maternal genome listed first) and Bi×Bi combinations,

indicating a strong positive effect of paternal Bi genome (**Figure 3.3B**). In contrast, the most advanced flat bone elongation was observed in Bt×Bt and Bt×Bi, followed by Bi×Bt, all of which differed from Bi×Bi fetuses (**Figure 3.3C**).

Analysis of limb elongation (PC3) revealed a significant non-genetic effect of post-conception maternal daily weight gain as nested within maternal genome ($P < 0.05$). There was a positive linear relationship between limb elongation and maternal weight gain in the Bi maternal genome and a negative linear relationship in the Bt maternal genome (**Figure 3.4B**). Limb elongation also differed between the sexes ($P < 0.05$), with greater limb elongation in females (**Figure 3.4A**).

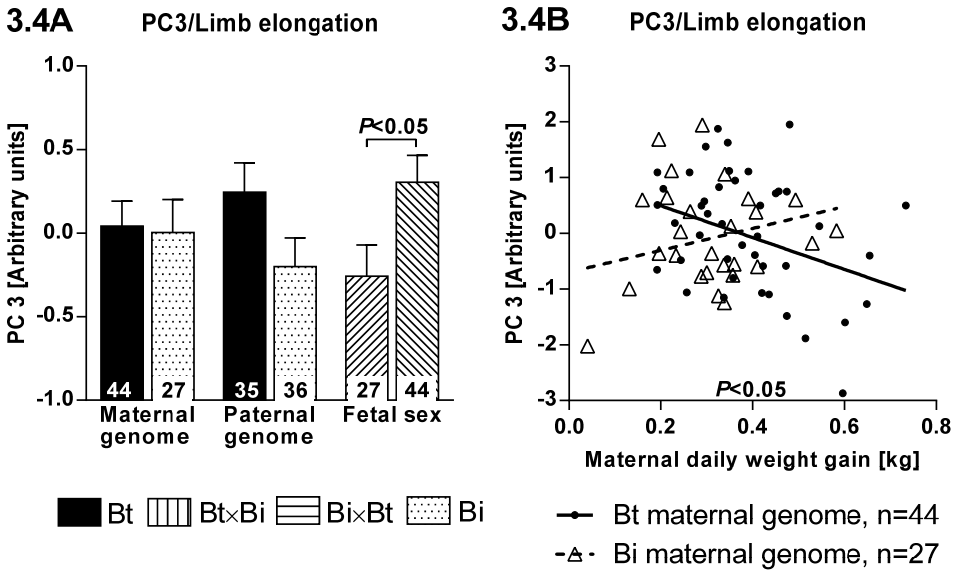

Figure 3.4. Effects of non-genetic effects of maternal daily weight gain nested within maternal genomes and fetal sex on PC3/limb elongation.

(**A**) Least square means with standard errors of means and *P*-values for significant differences (*t*-test) between means and (**B**) significant linear regressions within Bt and Bi maternal genetics for factor score of limb elongation/PC3 are shown. Bt: *Bos taurus taurus*, Angus breed. Bi: *Bos taurus indicus*, Brahman breed. Numbers for different maternal and paternal genomes and both sexes are indicated within bars.

3.5 Discussion

The aim of this study was to dissect and quantify effects of parental genomes, fetal sex, and non-genetic effects of maternal weight and post conception weight gain, on the fetal skeleton at mid-gestation. We showed for the first time that fetal bone phenotype in growing adolescent mothers is primarily determined by differential maternal and paternal genome effects. Fetal sex and post-conception weight gain had significant but lesser effects on specific bone groups.

3.5.1 Fetal bone phenotype at midgestation

Our analyses extracted six principle components (PCs) from 51 bone parameters. The PC concept (Field 2009) implies that parameters assigned to the same PC have shared properties while parameters in different PCs have distinct properties. All long bone diameters and length of entire scapula and thoracic vertebrae were assigned to bone mass together with bone weights. Other bone geometry parameters, including long bone lengths and cervical and lumbar vertebral column, were in PCs as diverse as limb ossification, limb elongation, flat bone elongation, axial skeletal growth and thoracic vertebrae growth (**Table 3.1**). The separation of long bone diameter from long bone length is a clear indication of the different bone growth properties represented by these two parameters and their opposite effects on bone strength (Rauch 2005).

3.5.2 Effects of maternal and paternal genomes

Parental genomes combined explained almost all variation in statistical models for fetal limb ossification, flat bone elongation and axial skeletal growth at mid-gestation. Furthermore, parental genomes accounted for most of the variation in fetal bone mass. This is consistent with previous reports of a significant genetic basis for bone mineral density in human (Smith *et al.*

1973; Gueguen *et al.* 1995) and shows that parental genome effects manifest early *in utero*. Interestingly, limb elongation deviated from this general pattern and was equally affected by parental genomes, fetal sex and non-genetic maternal effects.

Parental genome effects on fetal bone mass, limb and flat bone elongation, and axial skeletal growth were predominantly caused by the maternal genome. This contrasts with limb ossification, which was almost entirely controlled by the paternal genome (**Figure 3.2A,B** and **Figure 3.3,3.4**). Differential parental genetic effects on intrauterine skeletal growth based on significant correlations of parental birth weight and height of the father with neonatal bone mineral content were proposed earlier (Godfrey *et al.* 2001) and quantitative genetic modelling of parent-offspring data estimated that fetal and maternal genetic factors explained 31% and 19% of the variation, respectively, in newborn length in human [51]. The present designed experiment yielded much higher estimates of maternal (73-94%) and paternal genome (95%) effects on specific components of the fetal skeletal system, highlighting the importance of maternal and paternal (epi)genetic factors in prenatal skeletal growth and development. The observed strong maternal genome effects on axial skeletal growth (**Figure 3.2B, Figure 3.3D**) contrast with a report that used parental birth height to estimate significant paternal genetic effects on neonatal crown-heel length in human (Godfrey *et al.* 1997). However, a more recent study based on modelling of parent-offspring data also showed that neonatal crown-heel length was strongly affected by maternal genetics (Lunde *et al.* 2007).

The critical role of imprinted and maternally expressed genes such as *PHLDA2* (femur growth rate) (Lewis *et al.* 2012), *H19* (birth weight and size with correlated skeletal parameters) (Petry *et al.* 2011; Poole *et al.* 2011), *Gnas* (osteodystrophy, e.g. short stature and metacarpal length) (de Nanclares *et al.* 2007; Bastepe 2008) and *Dkn1c* (prenatal longitudinal limb growth) (Yan *et al.* 1997) in growth and development of bone tissue has been demonstrated. In addition to

epigenetic mechanisms such as imprinting, maternally inherited genetic components may also contribute to maternal genome effects on bone mass. A recent study in human indicated significant effects of common mtDNA variants on spine and hip bone mineral density (Guo *et al.* 2011) and the *B. t. taurus* and *B. t. indicus* specific mtDNA molecules of the fetus resource used in the present study differed by more than 230 SNPs (Hiendleder *et al.* 2008). Associations between X chromosome SNPs and differences in lumbar spine bone mineral density were found in human (Estrada *et al.* 2012).

Analysis of PCs demonstrated that variation in limb ossification was to a large extent controlled by paternal genome (**Figure 3.2A,B**), but with a significant interaction between maternal and paternal genomes. The phenotypic pattern of the four fetal combinations of parental genomes indicated a specific and positive effect of the paternal *B. t. indicus* genome on ossification (**Figure 3.3B**) that is consistent with paternal imprinting patterns that appear to be specific for different time points during ontogenesis (Wolf *et al.* 2008). This is further supported by known functions of paternally expressed genes such as *IGF2* (osteogenic cell differentiation) (Kang *et al.* 2011) and *Zac1* (fetal limb ossification) (Varrault *et al.* 2006).

The widespread specific and differential maternal and paternal genome effects discussed above are indicative of a major role of non-mendelian (epi)genetic effects on growth and development of bone tissue. Interestingly, we found an additional interaction effect between maternal and paternal genomes that indicated non-additive genetic effects on flat bone elongation. Unlike limb ossification, this parameter was characterized by strong maternal genome bias with a clear paternal contribution (**Figure 3.2B**) and the different parental genome combinations revealed hybrid vigour effects (Lamkey and Edwards 1998) in fetuses with a combination of *B.t.taurus* and *B.t.indicus* genomes (**Figure 3.3C**). It is not surprising that hybrid vigour or heterosis has very recently been associated with epigenetic mechanisms such as

miRNA interference (Chen 2013). Taken together, the present study suggests complex, non-mendelian and non-additive (epi)genetic effects on fetal bone phenotype.

It is notable that strong maternal genome effects identified in the present study are evident when fetuses enter the logarithmic phase of bone growth (Kan and Cruess 1987). The genetic conflict hypothesis for the evolution of genomic imprinting in placental mammals interprets maternally expressed genes as growth limiting and paternally expressed genes as growth promoting (Moore and Haig 1991). Accordingly, uterine environment can constrain fetal femur growth (Bonneau *et al.* 2011) and maternally expressed genes were found to suppress overall fetal bone growth or were negatively correlated with neonatal bone mineral content in human(Petry *et al.* 2011; Poole *et al.* 2011; Lewis *et al.* 2012). Thus, the present study provides further evidence for the important role of maternally expressed genes in control of prenatal skeletal development at a critical time point.

3.5.3 Fetal sex and non-genetic maternal effects

Sex-specific QTLs for bone mineral density and long bone length were identified in previous studies (Lagerholm *et al.* 2009; Lagerholm *et al.* 2011). Highly significant effects of fetal sex (P<0.0001) on fetal bone mass (**Figure 3.3A**) are consistent with previous reports of early sex differences in bone mass (Namgung and Tsang 2000; Wells 2007). However, effects of fetal sex on prenatal bone geometry parameters**,** as presented in limb elongation (**Table 3.1**, **Figure 3.4A**), have not previously been identified in human or animal models (Joffe *et al.* 2005; Wells 2007). Males are known to have overall larger skeletal size and longer bones at birth (Wells 2007) and the increased limb elongation in females of the present study (P<0.05) was unexpected. Additional studies are needed to assess if this could be a temporary effect at mid-gestation.

Non-genetic maternal factors such as lifestyle and nutrition status during pregnancy affect fetal (Godfrey *et al.* 2001; Mahon *et al.* 2010) and postnatal bone growth (Cooper *et al.* 2005; Tobias *et al.* 2005). The present data revealed a significant non-genetic effect of post-conception maternal weight gain on limb elongation (PC3) (**Figure 3.4A**). As this study was designed and standardized to test for parental genome and sex effects, recruited dams went through an adjustment period of 3-4 weeks prior to commencement of the experiment (see methods). Therefore, the observed non-genetic effect is best explained by differences in environmental factors acting on dams prior to recruitment for the experiment and indicates that pre-conception environment of females can affect fetal bone phenotype. Interestingly, direction and magnitude of these non-genetic maternal effects are maternal genome dependent (**Figure 3.4B**). This is reminiscent of previously reported significant interaction effects between specific genotypes and birth weight or infant weight on adult skeletal development (Dennison *et al.* 2001; Dennison *et al.* 2004; Karasik and Kiel 2008).

In conclusion, we have for the first time provided a comprehensive picture of differential maternal and paternal genome effects, in combination with fetal sex and non-genetic maternal effects, on directly measured fetal bone parameters at midgestation. Apart from genomic imprinting (Andrade *et al.* 2010) and/or interaction of miRNAs with target sites specific for parental alleles (Bae *et al.* 2012; Lian *et al.* 2012), other types of non-mendelian inheritance, such as maternally inherited mt DNA (Guo *et al.* 2011) and X- and Y-chromosome effects (Lagerholm *et al.* 2011; Estrada *et al.* 2012), are likely to contribute to widespread differential parental genome effects in the fetus. The present data provide the basis for molecular dissection of genetic and epigenetic mechanisms that operate across pre- and postnatal development to determine adult bone phenotype.

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Chapter 4: Maternal and paternal genomes differentially affect myofibre characteristics and muscle weights of bovine fetuses at midgestation

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Statement of Authorship continued

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By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

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Chapter 4

4.1 Abstract

Postnatal myofibre characteristics and muscle mass are largely determined during fetal development and may be significantly affected by epigenetic parent-of-origin effects. However, data on such effects in prenatal muscle development that could help understand unexplained variation in postnatal muscle traits are lacking. In a bovine model we studied effects of distinct maternal and paternal genomes, fetal sex, and non-genetic maternal effects on fetal myofibre characteristics and muscle mass. Data from 73 fetuses (Day153, 54% term) of four genetic groups with purebred and reciprocal cross Angus and Brahman genetics were analyzed using general linear models. Parental genomes explained the greatest proportion of variation in myofibre size of *Musculus semitendinosus* (80-96%) and in absolute and relative weights of *M. supraspinatus*, *M. longissimus dorsi*, *M. quadriceps femoris* and *M. semimembranosus* (82-89% and 56-93%, respectively). Paternal genome in interaction with maternal genome ($P<0.05$) explained most genetic variation in cross sectional area (CSA) of fast myotubes (68%), while maternal genome alone explained most genetic variation in CSA of fast myofibres (93%, $P<0.01$). Furthermore, maternal genome independently (*M. semimembranosus*, 88%, $P<0.0001$) or in combination (*M. supraspinatus*, 82%; *M. longissimus dorsi*, 93%; *M. quadriceps femoris*, 86%) with nested maternal weight effect (5-6%, $P<0.05$), was the predominant source of variation for absolute muscle weights. Effects of paternal genome on muscle mass decreased from thoracic to pelvic limb and accounted for all (*M. supraspinatus*, 97%, $P<0.0001$) or most (*M. longissimus dorsi*, 69%, $P<0.0001$; *M. quadriceps femoris*, 54%, $P<0.001$) genetic variation in relative weights. An interaction between maternal and paternal genomes ($P<0.01$) and effects of maternal weight ($P<0.05$) on expression of *H19*, a master regulator of an imprinted gene

network, and negative correlations between *H19* expression and fetal muscle mass ($P<0.001$), suggested imprinted genes and miRNA interference as mechanisms for differential effects of maternal and paternal genomes on fetal muscle.

4.2 Introduction

Skeletal muscle accounts for up to half of mammalian body mass (Du *et al.* 2010) and has important functions in metabolic homeostasis (Daniel *et al.* 1977; Wolfe 2006). It is a major source of endocrine factors, including insulin-like growth factors -I (IGF1) and -II (IGF2), key components of the insulin-like growth factor (IGF) system and growth hormone – IGF axis, which are major regulators of pre- and postnatal muscle development and growth (Adams 2002; Chang 2007; Pedersen and Febbraio 2008; Sawitzky *et al.* 2012). Skeletal muscle is composed of two major fibre types, type I (slow oxidative) fibres and type II (fast) fibres (Daniel *et al.* 1977). Myofibres originate from mesenchymal stem cells which differentiate into myoblasts during embryonic development (Relaix 2006). Myoblasts fuse to form myotubes which develop into myofibres at the fetal stage (Picard *et al.* 2002). In ruminants, myofibres differentiate during late fetal development into type I, type IIA (fast oxidative-glycolytic) and type IIX (fast glycolytic) myofibres (Scott *et al.* 2001; Greenwood *et al.* 2009). Thus, myofibre number is established during fetal development and postnatal skeletal muscle mass is largely determined prenatally (Picard *et al.* 2002; Du *et al.* 2010) by the interplay of a complex network of genetic and epigenetic factors (Brand-Saberi 2005; Baar 2010; Ge and Chen 2011; Bentzinger *et al.* 2012).

Studies on postnatal muscle tissue of human, porcine and bovine revealed that genetics explained up to 45% of variation in slow myofibre percentage (Simoneau and Bouchard 1995), up to 58% of variation in myofibre number (Larzul *et al.* 1997) and 74% of variation in myofibre size (Rehfeldt *et al.* 1999), respectively. Similarly, using proxies such as lean body mass and lean tissue percentage, studies in human (Seeman *et al.* 1996; Arden and Spector 1997) and porcine (Larzul *et al.* 1997) demonstrated that genetics accounted for approximately 50-80% of variation in postnatal muscle mass. Apart from genetic factors that follow Mendelian rules of inheritance, prenatal muscle development and postnatal muscle phenotype may be affected by

genetic and epigenetic factors with non-Mendelian modes of inheritance. This includes effects of mitochondrial (mt) genome (Mannen *et al.* 1998), X- and Y-chromosomes (Engellandt and Tier 2002; Amen *et al.* 2007), non-random X-inactivation (Amen *et al.* 2007), microRNA (miRNA) interference (Clop *et al.* 2006) and genomic imprinting (Engellandt and Tier 2002; Boysen *et al.* 2010; Neugebauer *et al.* 2010; Neugebauer *et al.* 2010). Genomic imprinting, i.e., parent-of-origin dependent allele-specific gene expression (Reik and Walter 2001), has been described for genes with pivotal roles in myogenesis, including *IGF2* and its receptor *IGF2R* (Nezer *et al.* 1999; Young *et al.* 2001). In porcine, mapping and gene expression studies demonstrated that *IGF2* alleles explained up to 30% of variation in postnatal muscle mass (Van Laere *et al.* 2003). The ovine callipyge (CLPG) mutation has provided an example of complex genetic and epigenetic effects on postnatal muscle phenotype. The CLPG mutation causes postnatal muscle hypertrophy only in heterozygous offspring and only when inherited through the paternal germline (Cockett *et al.* 1996). This polar overdominance changes imprinted gene expression, presumably by miRNA interference (Caiment *et al.* 2010), and affects absolute and relative weights of specific muscles and muscle groups of the torso (e.g. *M. longissimus lumborum*) and pelvic limb (e.g. *M. semimembranosus, M. quadriceps femoris*), but not of the thoracic limb (e.g. *M. supraspinatus*) (Koohmaraie *et al.* 1995; Jackson *et al.* 1997). The increased muscle mass of CLPG sheep is due to fast myofibre hypertrophy and results in higher glycolytic metabolism of affected muscles (Carpenter *et al.* 1996; Jason *et al.* 2008). A similar paternal polar overdominance effect on postnatal myofibre characteristics, muscle mass and growth has been described in porcine (Kim *et al.* 2004). Furthermore, the ovine Carwell locus, which exerts paternal effects on weight of *M. longissimus dorsi* and a shift from type IIA to type IIX myofibres, was mapped to the same chromosome region as the CLPG mutation (Nicoll *et al.* 1998; Cockett *et al.* 2005; Greenwood *et al.* 2006). More recently, statistical modelling revealed significant parent-of-origin effects attributed to genomic imprinting on postnatal absolute and

relative weights of specific muscles in porcine (Neugebauer *et al.* 2010) and bovine (Neugebauer *et al.* 2010).

Nutritional effects on prenatal myogenesis are well documented (Dwyer *et al.* 1994; Greenwood *et al.* 1999; Zhu *et al.* 2004; Du *et al.* 2010), but data on parental genetic and epigenetic effects are lacking. To our knowledge, only one previous study investigated genetic effects on mammalian prenatal muscle. This report described significant individual sire effects on bovine fetal biceps weight in the last trimester of gestation (Anthony *et al.* 1986). However, the study was designed to test only for effects of different sires and did not address differential effects of maternal and paternal genomes. In the present study, we generated the largest fetal resource to date for the study of (epi)genetic effects on mammalian prenatal muscle development. This collection of defined bovine fetuses consists of both purebreds and reciprocal hybrids with Angus and Brahman genetics. The taurine (Angus) and indicine (Brahman) breeds are subspecies of the domestic cow, currently named *Bos taurus taurus* and *Bos taurus indicus*, respectively (The Bovine Genome Sequencing Consortium 2009). Both subspecies originated from the wild aurochs (*Bos primigenius*) and are commonly referred to as *Bos taurus* and *Bos indicus* (*Linnaeus*, *1758*; *Bojanus*, *1827*; loc. cit. http://www.itis.gov) (Hiendleder *et al.* 2008). This unique intra-species model with well defined divergent parental genomes allowed us to dissect maternal and paternal genome effects on fetal myofibre characteristics and absolute and relative muscle weights at midgestation (Day153, 54% term). We show, for the first time, significant differential effects of parental genomes, independently or in combination with non-genetic maternal effects, on specific fetal muscles. Furthermore, we correlated expression of the imprinted non-coding RNA H19, which harbors miRNAs and is involved in regulation of *IGF2* and *IGF1R*, with fetal muscle mass, demonstrating that imprinted genes and miRNA interference provide plausible mechanisms for observed differential effects of parental genomes on fetal muscle phenotype.

4.3 Results

4.3.1 Proportion of variation explained by parental genomes, fetal sex and non-genetic effects

Myofibre characteristics determined in *M. semitendinosus* samples included number and cross-sectional area (CSA) of type I (slow) and type II (fast) myotubes and myofibres and total cell number and total cell CSA (Supplementary **Figure S4.1**). Wet weights were determined for *M. supraspinatus*, *M. longissimus dorsi*, *M. quadriceps femoris* and *M. semimembranosus*. Since the four fetal groups with specific combinations of *Bos taurus taurus* (Bt) and *Bos taurus indicus* (Bi) genomes showed significant differences in carcass weights (Supplementary **Figure S4.2**), relative muscle weights were analyzed in addition to absolute muscle weights to identify effects of parental genomes on muscle mass independent of fetal size.

Significant final statistical models for studied muscle parameters with adjusted R^2 values and significance levels of retained variables are presented in **Table 4.1**. Parental genomes, fetal sex, and effects of maternal weight, caused by non-genetic variation and nested within maternal genomes (see methods), each contributed differentially to muscle parameters (**Figure 4.1**). Parental genome was the most important source of variation for all studied traits with significant final statistical models. Maternal and paternal genomes together explained most of the variation in myofibre size (80-96%), absolute muscle weights (82-89%) and relative muscle weights (56-93%). Fetal sex contributed less to variation in myofibre characteristics (4-20%) and absolute (2-13%) and relative muscle weights (7-44%). Non-genetic maternal effects of final maternal weight accounted for some variation in absolute weights of *M. supraspinatus*, *M. longissimus dorsi* and *M. quadriceps femoris* (5-6%). Combined absolute and relative muscle weight showed parental genome contributions of 94% and 72%, respectively (**Figure 4.1**).

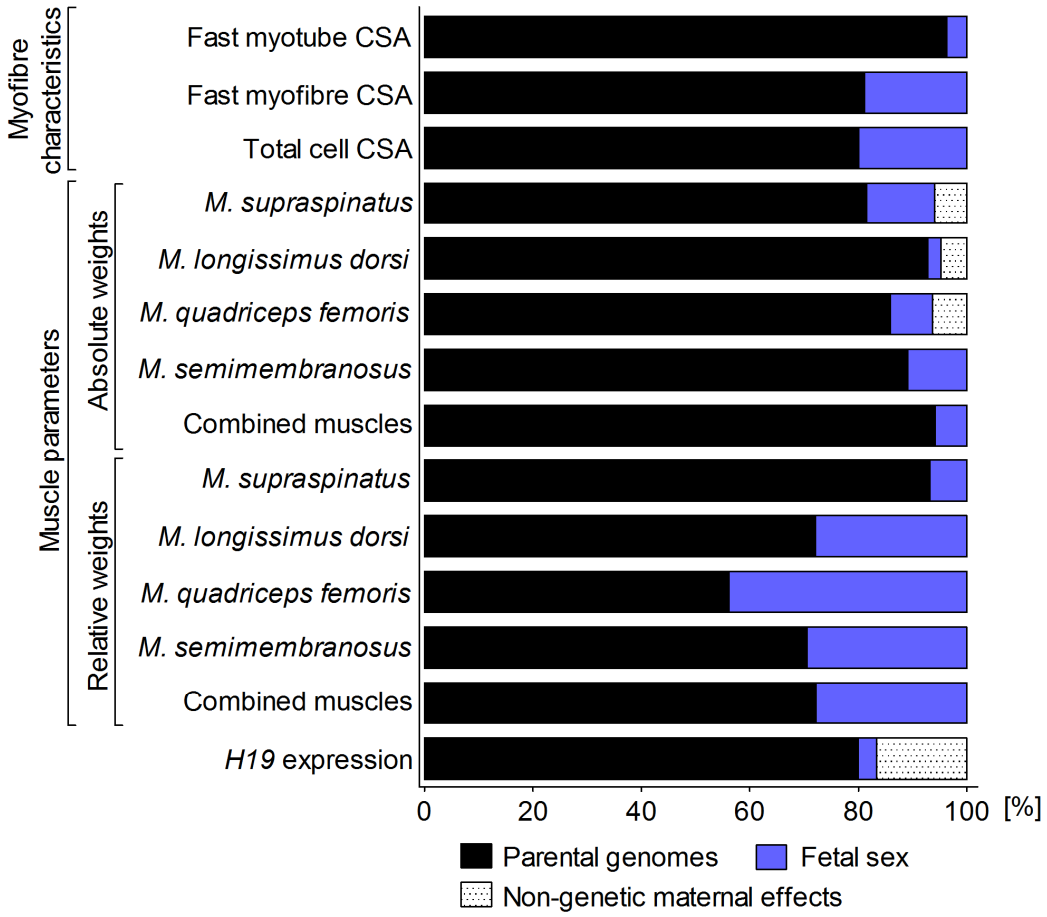

Figure 4.1 Relative contributions of parental genomes, fetal sex and non-genetic maternal effects to explained variation in fetal myofibre characteristics, absolute and relative muscle weights, and *H19* transcript abundance.

Myofibre characteristics were determined in *M. semitendinosus*. Maternal and paternal genome, fetal sex and other significant effects were retained in the final general linear models as presented in Table 4.1. Non-genetic maternal effect: Final maternal weight at mid-gestation. CSA: Cross-sectional area. Total cell: All myofibres measured regardless of cell type. Combined muscle weights: Sum of *M. supraspinatus, M. longissimus dorsi*, *M. semimembranosus* and *M. quadriceps femoris* weight. Relative muscle weight: Absolute muscle weight divided by decapitated and eviscerated fetal carcass weight.

Table 4.1. Summary of the final general models (type III sums of squares) for myofibre characteristics, muscle weight parameters and H19 gene expression with adjusted R^2 values and significance levels (*P*-values) of models and variables.

		***P*-values**					
Myofibre characteristics	R^2	**Model**	**Maternal genome**	**Paternal genome**	**Fetal sex**	**Maternal×Paternal genome**[b]	**Final maternal weight (Maternal genome)**[c]
Fast myotube CSA[a]	0.152	0.0043	ND	ND	0.4337	0.0129	
Fast myofibre CSA[a]	0.111	0.0117	0.0031	0.7345	0.1390		
Total cell CSA[a]	0.101	0.0160	0.0076	0.4280	0.1434		
Absolute muscle weights							
M. supraspinatus	0.689	8.7E-17	ND	2.3E-07	7.0E-04		0.0112
M. longissimus dorsi	0.649	1.2E-15	ND	6.9E-08	0.2828		0.0420
M. quadriceps femoris	0.666	1.0E-14	ND	2.1E-05	0.0457		0.0256
M. semimenbranosus	0.595	7.2E-12	5.1E-12	0.04974	0.0026		
Combined muscles	0.667	2.9E-14	5.0E-13	3.3E-05	0.0095		
Relative muscle weights							
M. supraspinatus	0.210	3.3E-04	0.5294	2.7E-05	0.2327		
M. longissimus dorsi	0.441	4.8E-09	0.0014	9.8E-08	1.6E-04		
M. quadriceps femoris	0.332	1.6E-06	0.0048	1.2E-04	1.4E-04		
M. semimenbranosus	0.136	0.0115	0.0176	0.4209	0.0637		
Combined muscles	0.517	2.1E-09	2.3E-04	2.2E-06	5.9E-06		
***H19* expression**	0.350	4.0E-06	ND	ND	0.1288	0.0051	0.0296

aTotal cell CSA: Average cross-sectional area of muscle cells irrespective of cell type. bMaternal × paternal genome: Effect of maternal and paternal genome interaction. cFinal maternal weight (maternal genome): Effect of final maternal weight nested in maternal genome. ND: Not determined because of significant interaction and/or nested effect of final maternal weight. Only P-values for factors, interactions and nested effects retained in the final model are shown.

The relative contributions of maternal and paternal genomes to total explained (epi)genetic variation in myofibre size and muscle weights are shown in **Figure 4.2**. Maternal genome explained most of the (epi)genetic variation in fast myofibre CSA (93%) whereas the paternal genome accounted for most of the variation in fast myotube CSA (68%). Maternal genome again explained most of the variation in total cell CSA (82%). Maternal genome also explained most of the genetic variation (59-88%) in all absolute muscle weights. Paternal genome, in contrast, explained most of the genetic variation (54-97%) in relative weights of *M. supraspinatus*, *M. longissimus dorsi* and *M. quadriceps femoris*. However, maternal genome accounted for 82% of genetic variation in relative weight of *M. semimembranosus.* Combined absolute muscle weight

was predominantly affected by maternal genome (73%) while combined relative muscle weight showed a stronger effect of paternal genome (63%). Overall, the data clearly showed a distinct pattern of effects of maternal and paternal genomes with an increase of maternal genome contributions (or conversely, a decrease of paternal genome contributions) to variation in absolute and relative weights of muscles from the thoracic limb (*M. supraspinatus*) to muscles from the torso (*M. longissimus dorsi*) and pelvic limb (*M. quadriceps femoris* and *M. semimembranosus*) (**Figure 4.2**).

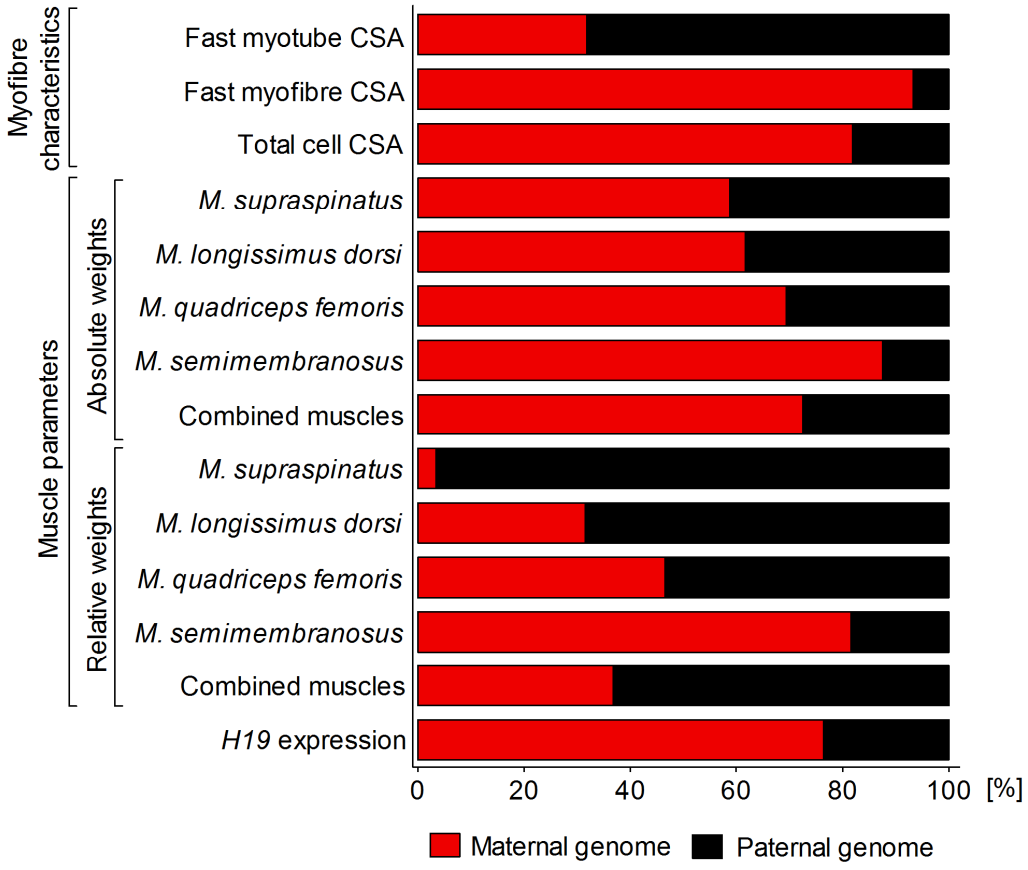

Figure 4.2. Relative contributions of maternal and paternal genome to genetic variation in fetal myofibre characteristics, absolute and relative muscle weights, and H19 transcript abundance.

Myofibre characteristics were determined in *M. semitendinosus.* CSA: Cross-sectional area. Total cell: All myofibres measured regardless of cell type. Combined muscle weights: Sum of *M. supraspinatus, M. longissimus*

dorsi, *M. semimembranosus* and *M. quadriceps femoris* weight. Relative muscle weight: Absolute muscle weight divided by decapitated and eviscerated fetal carcass weight.

4.3.2 Specific effects of Bt and Bi genomes, fetal sex and maternal weight

Least square means for specific effects of *Bos taurus taurus* (Bt, Angus) and *B. taurus indicus* (Bi, Brahman) maternal and paternal genomes, fetal sex and non-genetic maternal effects of final maternal weight, as detailed in statistical models for myofibre characteristics and muscle weights (**Table 4.1**), are presented in **Figure 4.3-4.6**. Fast myotube CSA was affected by a significant interaction between maternal and paternal genomes ($P<0.05$). Fetuses with Bt × Bt genomes had larger CSA ($P<0.05 – 0.01$) than fetuses of other genetic combinations (**Figure 4.3A**). Maternal genome significantly affected fast myofibre CSA and total cell CSA (both $P<0.01$) with Bt genomes causing larger CSA than Bi genomes (**Figure 4.3B,C**).

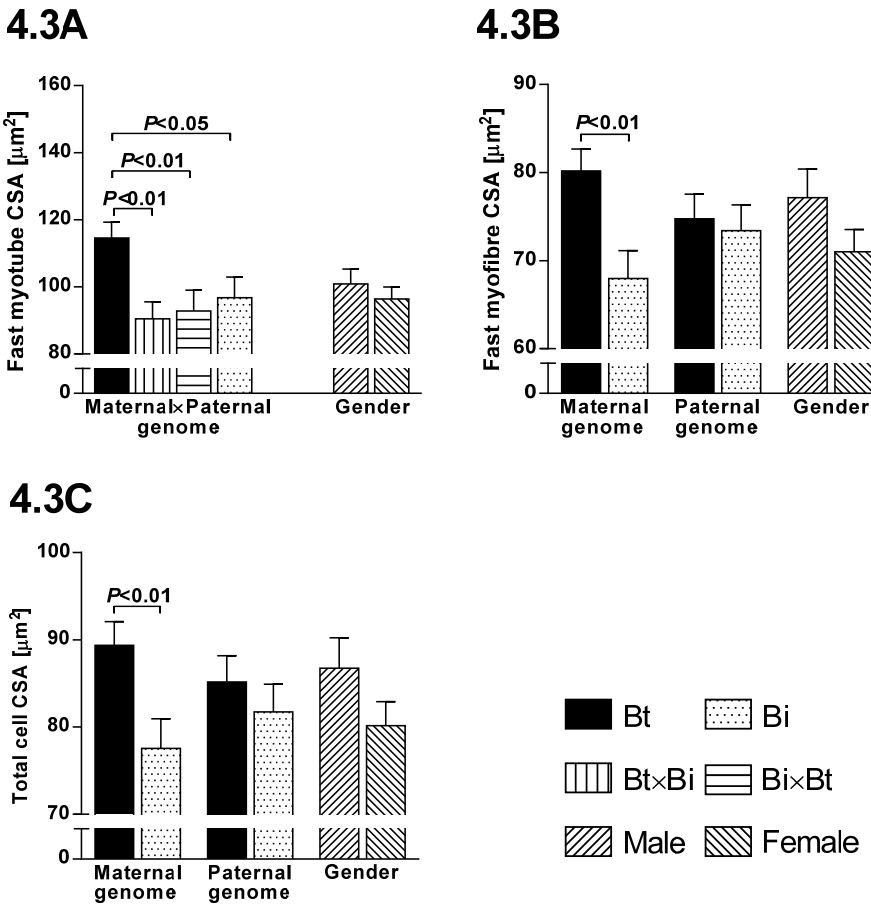

Figure 4.3. Specific effects of maternal genomes, paternal genomes and fetal sex on fetal myofibre characteristics of *M. semitendinosus* at midgestation.

Least square means with standard errors of means are shown and *P*-values for significant differences (*t*-test) between means for fast myotube CSA (A), fast myofibre CSA (B) and total cell CSA (C) are indicated. CSA: Cross-sectional area. Total cell: All myofibres measured regardless of cell type. Bt: *Bos taurus taurus*, Angus. Bi: *Bos taurus indicus*, Brahman.

Maternal genome significantly affected absolute weights of all muscles (**Figure 4.4A-D**), but *M. supraspinatus, M. longissimus dorsi* and *M. quadriceps femoris* also showed significant non-genetic effects of final maternal weight nested within maternal genome (all $P<0.05$, see below). Maternal genome effects, independent of maternal weight, were detected for *M. semimembranosus* ($P<0.0001$). Paternal genome, in contrast, independently and strongly

affected absolute weights of *M. supraspinatus, M. longissimus dorsi* and *M. quadriceps femoris* (all $P<0.0001$), but not *M. semimembranosus*, a muscle strongly affected by maternal genome (see above). Combined muscle weights showed significant effects of maternal and paternal genome that were stronger for the maternal genome. Irrespective of maternal or paternal origin Bt genome always increased, and Bi genome always decreased, absolute muscle weights. Fetal sex significantly affected absolute weights of *M. supraspinatus* ($P<0.001$), *M. quadriceps femoris* ($P<0.05$) and *M. semimembranosus* ($P<0.01$) with heavier muscles in males than in females (**Figure 4.4A,C,D**). Non-genetic effects of final maternal weight, nested within maternal genome, on absolute weights of *M. supraspinatus, M. longissimus dorsi* and *M. quadriceps femoris* ($P<0.05$) indicated positive linear relationships for Bi and Bt, but with a higher intercept and less slope in Bt (**Figure 4.5A-C**). Only one of the quadratic maternal weight effects tested yielded a significant result (*M. quadriceps femoris,* $P<0.01$). Examination of plotted curves with individual data points revealed that this was dependent upon two heavy dams with high leverage (see methods and supplementary **Figure S4.3**). Therefore, we fitted linear effects throughout. Nested effects of post conception maternal daily weight gain were not significant for any of the investigated muscle parameters.

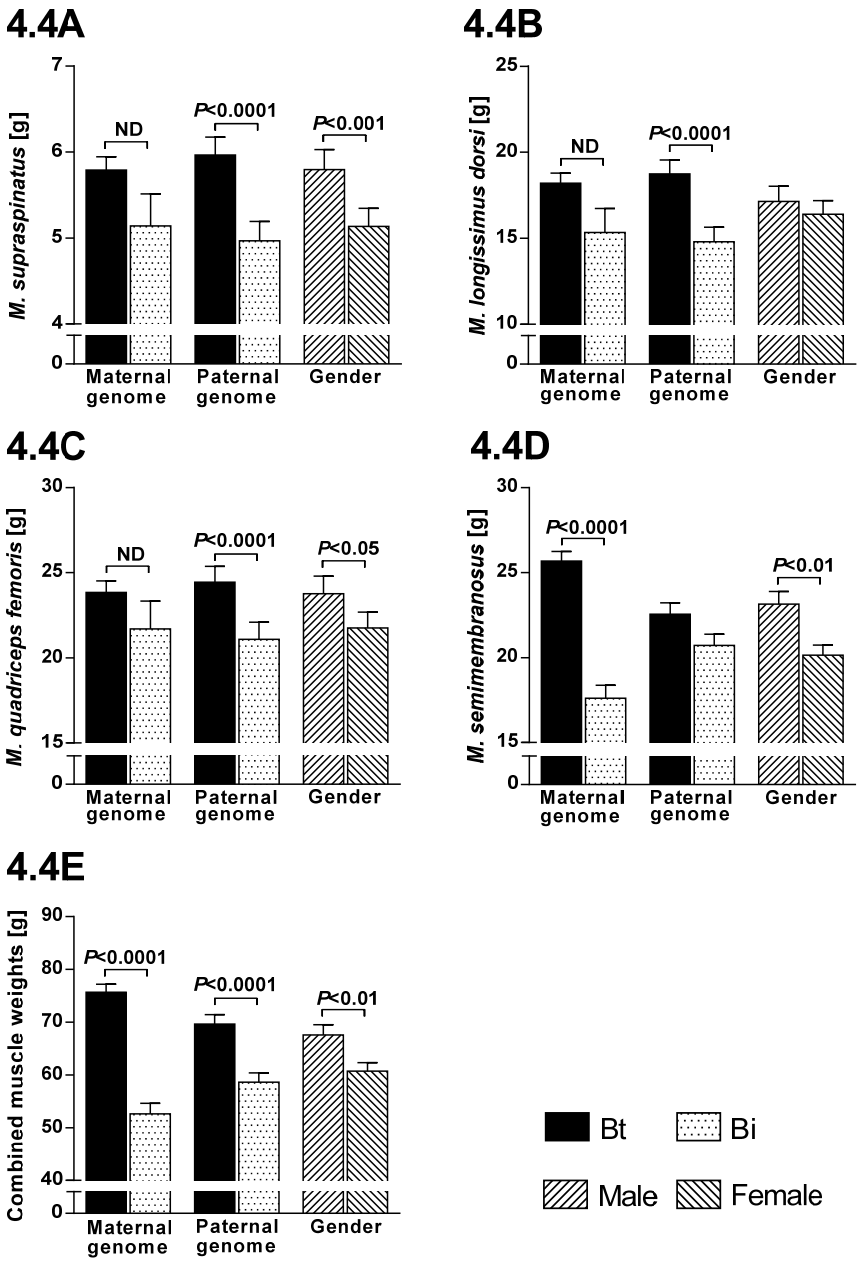

Figure 4.4. Specific effects of maternal genomes, paternal genomes and fetal sex on fetal absolute muscle weights at midgestation.

Least square means with standard errors of means are shown and *P*-values for significant differences (*t*-test) between means for *M. supraspinatus* (A), *M. longissimus dorsi* (B), *M. quadriceps femoris* (C), *M. semimembranosus* (D) and combined muscle weight (sum of weights of dissected muscles) (E) are indicated. ND: Not determined because of significant nested effect of final maternal weight (see Figure 5). Bt: *Bos taurus taurus*, Angus. Bi: *Bos taurus indicus*, Brahman.

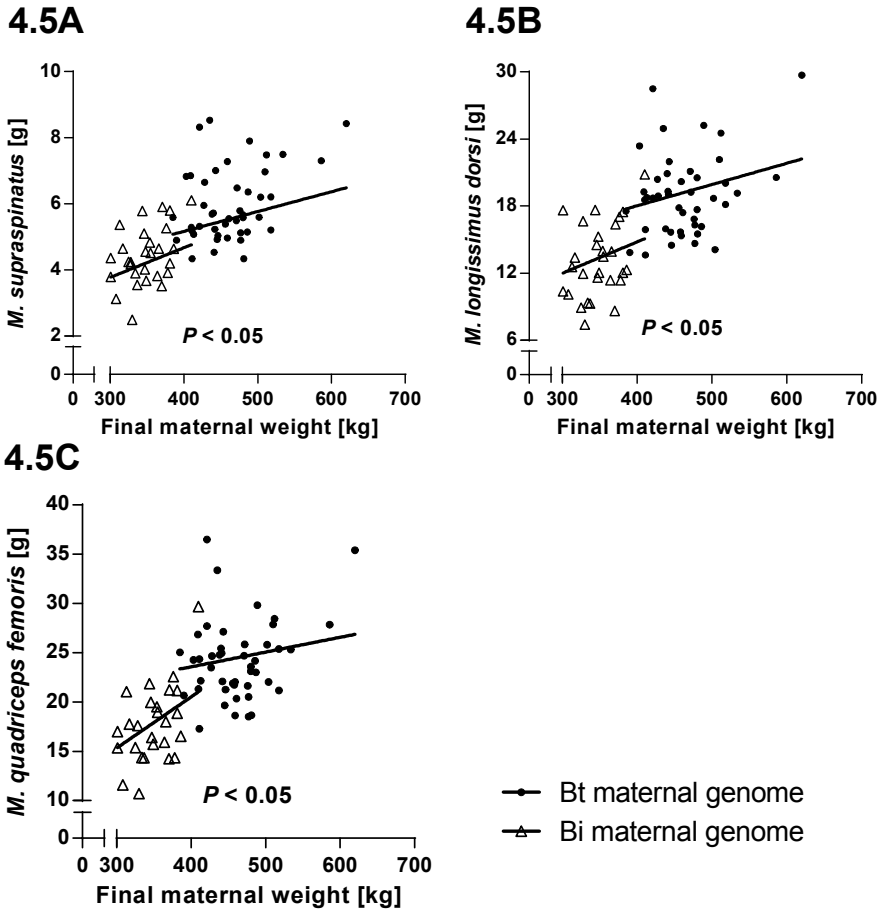

Figure 4.5. Effects of final maternal weight nested within maternal genomes on fetal absolute muscle weights at midgestation.

P-values (ANOVA) of significant linear regressions within Bt and Bi maternal genetics on absolute weights of *M. supraspinatus* (**A**), *M. longissimus dorsi* (**B**) and *M. quadriceps femoris* (**C**) are indicated. Bt: *Bos taurus taurus*, Angus. Bi: *Bos taurus indicus*, Brahman.

Maternal genome had moderate effects on relative weights of *M. longissimus dorsi* ($P<0.01$), *M. quadriceps femoris* ($P<0.01$) and *M. semimembranosus* ($P<0.05$), but not *M. supraspinatus*. Paternal genome showed strong effects on *M. supraspinatus* ($P<0.0001$), *M. longissimus dorsi* ($P<0.0001$) and *M. quadriceps femoris* ($P<0.001$), but not *M. semimembranosus*. Combined

relative muscle weight showed stronger effects of the paternal genome. Again, as for absolute muscle weights, Bt genome increased relative muscle weights irrespective of parental origin (**Figure 4.6A-D**). Strong fetal sex effects were present for relative weights of *M. longissimus dorsi* ($P<0.001$) and *M. quadriceps femoris* ($P<0.001$), with greater weights in females than in males (**Figure 4.6B,C**).

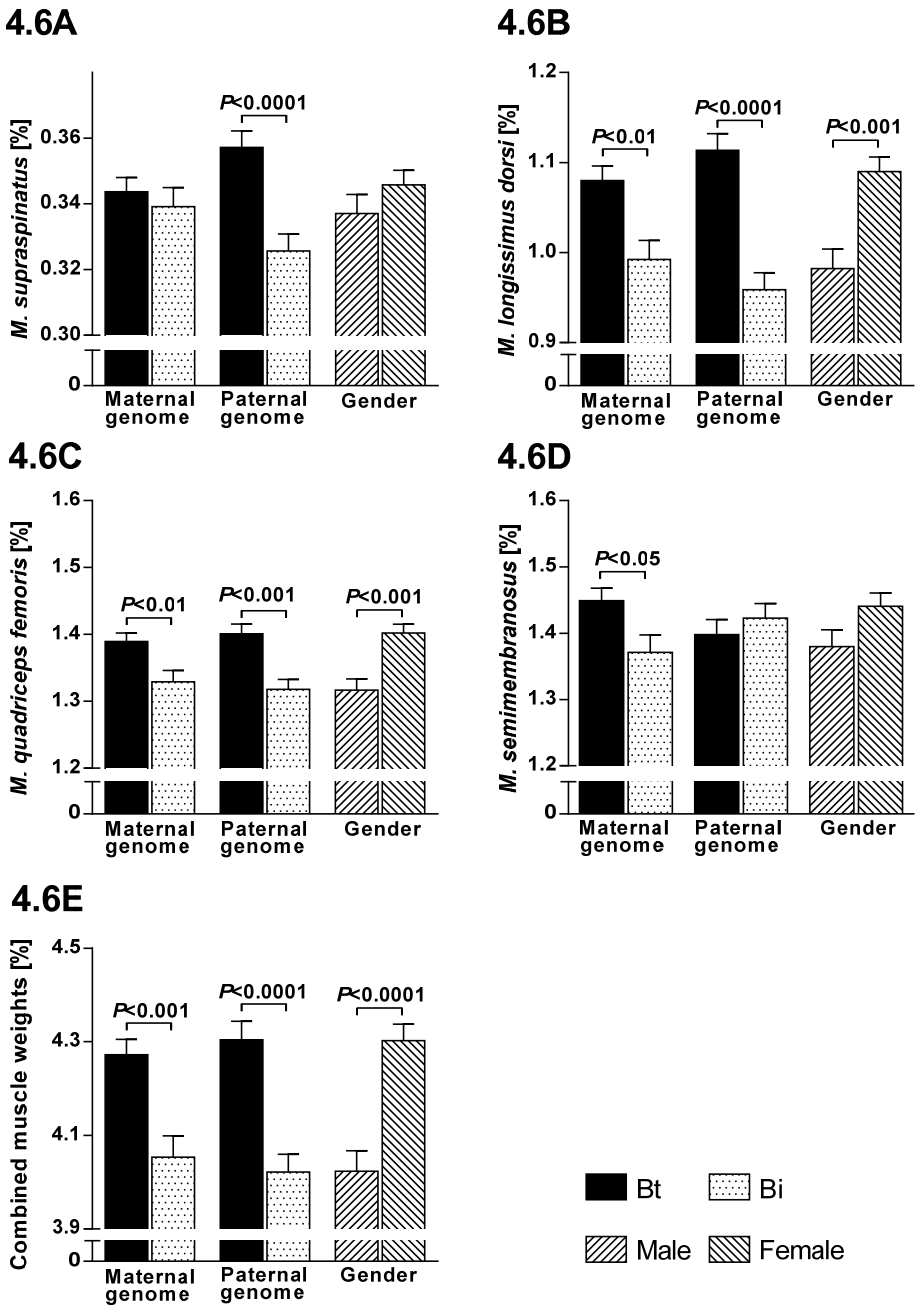

Figure 4.6. Specific effects of maternal genomes, paternal genomes and fetal sex on fetal relative muscle weights at midgestation.

Relative muscle weights were calculated as absolute muscle weight divided by fetal carcass weight. Least square means with standard errors of means and *P*-values for significant differences (*t*-test) between means for *M. supraspinatus* (A), *M. longissimus dorsi* (B), *M. quadriceps femoris* (C) and *M. semimembranosus* (D) are indicated. Combined relative muscle weight is the sum of relative weights of dissected muscles. Bt: *Bos taurus taurus*, Angus. Bi: *Bos taurus indicus*, Brahman.

4.3.3 Expression of the H19 lincRNA

Expression of the H19 large intergenic non-coding RNA (lincRNA) was measured by real-time quantitative PCR in *M. semitendinosus* samples. Transcript abundance was significantly affected by an interaction between maternal and paternal genomes ($P<0.01$) (**Table 4.1**). Fetuses with Bi × Bi genome showed higher levels of *H19* transcript ($P<0.01$) than fetuses of other genetic combinations (**Figure 4.7A**). Transcript abundance was also affected by final maternal weight ($P<0.05$) nested within maternal genome (**Figure 4.7B**). Subsequent regression analyses revealed significant negative relationships ($P<0.001$) between *H19* transcript abundance and combined absolute and relative muscle weight (**Figure 4.8A,B**).

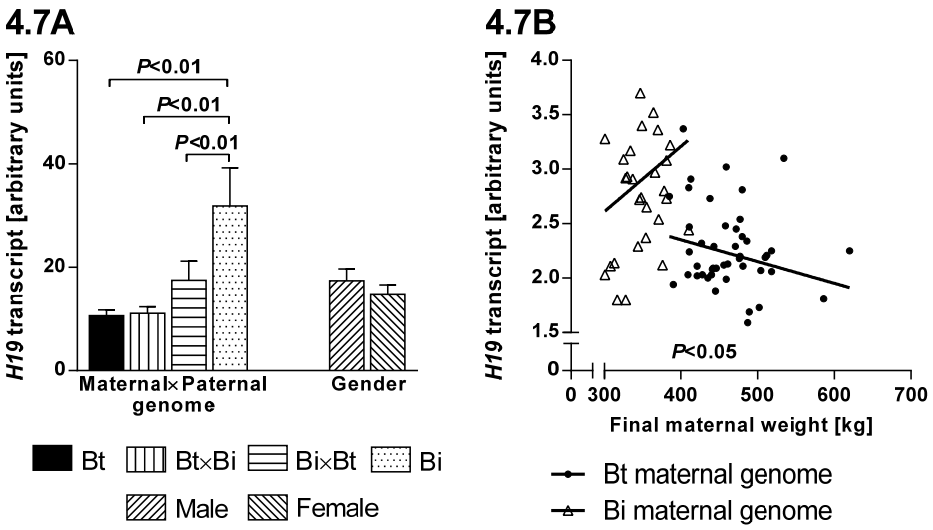

Figure 4.7. Effects of interaction of maternal and paternal genomes, fetal sex and final maternal weight nested within maternal genetics on *H19* transcript abundance in fetal *M. semitendinosus* at midgestation.

Least square means with standard error of means and *P*-values for significant differences (*t*-test) between means (A) and significant regressions of final maternal weight nested within Bt and Bi maternal genomes (B) are shown. Bt: *Bos taurus taurus*, Angus. Bi: *Bos taurus indicus*, Brahman.

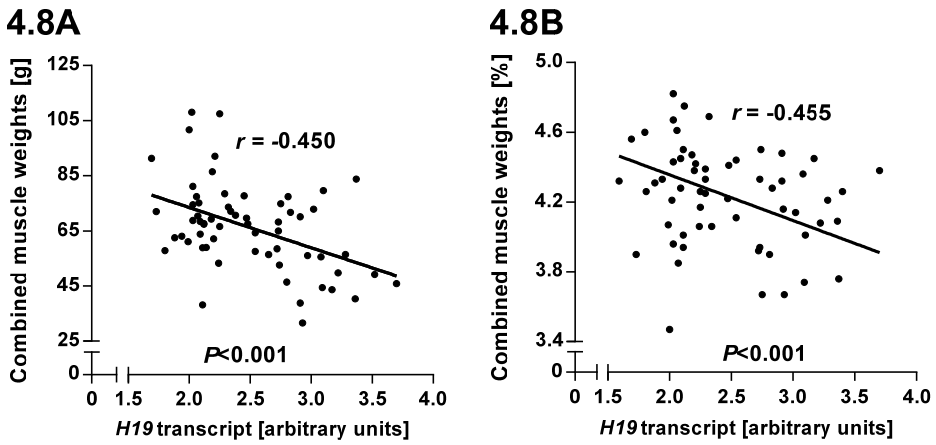

Figure 4.8. Regressions of fetal muscle mass at midgestation on *H19* transcript abundance.

(**A**) Absolute muscle mass and (**B**) relative muscle mass. Muscle mass is combined absolute and relative weights of *M. supraspinatus*, *M. longissimus dorsi*, *M. quadriceps femoris* and *M. semimembranosus*. *P*-values and Pearson correlation coefficients (*r*) are indicated.

4.4 Discussion

To our knowledge, this is the first study to examine effects of maternal and paternal genome on fetal myofibre characteristics and muscle mass. Our results showed that differential effects of parental genomes were the most important determinants of fetal muscle phenotype at midgestation. Fetal sex and non-genetic effects of final maternal weight had a significant but lesser impact on some investigated muscle parameters (**Figure 4.1**). Considering the fetal programming of skeletal muscle development (Picard *et al.* 2002; Du *et al.* 2010), these findings are consistent with generally medium to high heritabilities reported for postnatal myofibre size and muscle mass in mammals, including bovine (Larzul *et al.* 1997; Rehfeldt *et al.* 1999; Engellandt and Tier 2002; Smith *et al.* 2007; Mansan Gordo *et al.* 2012). Since myotubes are immature myofibres that decrease in size as myogenesis progresses (Martyn *et al.* 2004), both the predominant contribution of the paternal genome to variation in fast myotube cross sectional

area (CSA), and the predominant contribution of the maternal genome to variation in fast myofibre CSA (**Figure 4.2**), indicate specific roles of maternal and paternal genomes in myofibre differentiation and maturation.

The observed differences between *Bos taurus taurus* (Bt) and *Bos taurus indicus* (Bi) genomes likely result from allelic differences in genes with parent-of-origin effects controlling myofibre development. Evidence for subspecies differences in postnatal fibre type ratios and size, and in absolute postnatal muscle weights of Bt and Bi breeds has been reported previously (Whipple *et al.* 1990; Ferrell 1991; Strydom and Smith 2010). Differential parental effects were masked in total cell CSA, which was predominantly affected by maternal genome (**Figure 4.2**). Muscle specific differences in fibre type composition and size (Totland and Kryvi 1991) could explain some of the varying contributions of maternal and paternal genomes to different muscles. The present data suggest that maternal genes are important determinants of myofibre development and muscle mass.

Variation in the maternally inherited mt genome has been associated with effects on postnatal muscle mass (Mannen *et al.* 1998), but specific effects of maternal genes in myogenesis remain, to our knowledge, unexplored. The present results are in agreement with recent data obtained by statistical modelling and imprinted quantitative trait loci (QTL) analyses which suggested significant maternal parent-of-origin effects for postnatal muscle traits (Boysen *et al.* 2010; Neugebauer *et al.* 2010; Neugebauer *et al.* 2010). In contrast, paternally expressed genes with effects on myogenesis have been identified previously and were studied in detail. This includes the imprinted Delta-like 1 homolog (*DLK1*), which has been implicated in the commitment and/or proliferation of fetal myoblasts (Jason *et al.* 2008) and in increased postnatal myofibre diameter and muscle mass (Davis *et al.* 2004; Jason *et al.* 2008). Further examples of gene-specific genetic and epigenetic regulatory mechanisms that could explain effects of maternal and

paternal genomes on fetal muscle phenotype observed in the present study are found in the IGF1-AKT/PKB pathway (Schiaffino and Mammucari 2011). In the mouse embryo, paternally expressed IGF2 is required for fibre type specification (Merrick *et al.* 2007). This imprinted gene has been identified as a QTL for postnatal muscle mass (Jin-Tae Jeon 1999; Nezer *et al.* 1999) and encodes a miRNA in intron 2 that targets transcripts of the non-imprinted *IGF1* gene (Wang 2008). Several other genes in this pathway, including *PTEN*, a gatekeeper for the accretion of muscle mass (Sawitzky *et al.* 2012), are also targeted by miRNAs (Crist and Buckingham 2009; Ge and Chen 2011). The significance of allelic differences in miRNA target sequences for regulation of muscle mass by epistatic miRNA interference has been demonstrated with myostatin alleles in the ovine model (Clop *et al.* 2006). Genome sequences of *Bos taurus taurus* and *Bos taurus indicus* revealed genomic variation (The Bovine Genome Sequencing Consortium 2009; Canavez *et al.* 2012) that provides a basis for maternal and paternal (epi)genetic effects on myogenesis described in the present study.

The imprinted long intergenic non-coding (linc) RNA H19 is maternally expressed at high levels in embryonic and fetal tissues, including skeletal muscle (Lee *et al.* 2002; Gabory *et al.* 2006). The H19 gene is located immediately downstream of *IGF2* and involved in regulation of *IGF2* expression. More recently, *H19* has been identified as the master regulator of an imprinted gene network with important roles in growth and development (Gabory *et al.* 2010). The *H19* transcript was further shown to harbor a miRNA that suppresses *IGF1R* expression and prenatal growth (Cai and Cullen 2007; Keniry *et al.* 2012). Gene expression data generated in the present study demonstrated significant differences in *H19* transcript abundance of *M. semitendinosus* from fetuses with different parental combinations of Bt and Bi genomes (**Figure 4.7**). In human, *H19* expression is also affected by genetic background (Lin *et al.* 1999). Furthermore, *H19* expression was significantly negatively correlated with absolute and relative fetal muscle mass (**Figure 4.8**). This is consistent with the previously reported role of *H19* as a negative regulator

of prenatal growth and development (Keniry *et al.* 2012). Thus, imprinted gene expression and miRNA interference are plausible mechanisms for differential effects of maternal and paternal genomes observed in the present study.

Our data indicated predominant contributions of the maternal genome to variation in absolute fetal muscle weights and predominant contributions of the paternal genome to variation in relative fetal muscle weights (**Figure 4.2**). With respect to maternal genome, these results are in agreement with data available from an analysis of parent-of-origin effects on postnatal bovine muscle, where absolute muscle weights were predominantly affected by imprinted maternal genetic factors (Neugebauer *et al.* 2010). The genetic conflict hypothesis of genomic imprinting states that paternally expressed genes promote, and maternally expressed genes limit, fetal growth (Moore and Haig 1991). Accordingly, maternal genes are expected to control fetal size to avoid detrimental effects for the mother that are associated with higher nutrient transfer to the fetus and increased birthweight (Moore and Haig 1991). In the present study, fetuses with different maternal and paternal combinations of Bt and Bi genomes showed significant differences in carcass weight (Supplementary **Figure S4.2**) that are consistent with a phenotypic pattern of genomic imprinting for maternally expressed genes (see Figure 1 in (Wolf *et al.* 2008)) affecting fetal size. Correlations between absolute muscle weights and fetal carcass weight ranged from $r = 0.88$ (*M. longissimus dorsi*, $P<0.0001$) to $r = 0.95$ (*M. quadriceps femoris*, $P<0.0001$). Effects of the maternal genome on absolute muscle weights are, therefore, likely to be primarily correlated effects of maternal (epi)genetics on fetal size, presumably via imprinted genes (Moore and Haig 1991; Wolf *et al.* 2008) and/or epistatic interaction of miRNAs and their target sites (see above). However, mt DNA (Mannen *et al.* 1998; Hiendleder *et al.* 2004), or X-chromosome effects (Amen *et al.* 2007; Amen *et al.* 2007) could also contribute to Bt and Bi maternal (epi)genetic effects on muscle phenotype (**Figure 4.3,4.4**).

Predominance of parental genomic contributions to muscle weights varied from maternal for absolute weights to paternal for relative weights. An exception was *M. semimembranosus,* which showed only a weak maternal ($P<0.05$) and no paternal genome effect (**Figure 4.2, 4.4, 4.6**). Considering the genetic conflict hypothesis (Moore and Haig 1991), it appears that the full extent of paternal genome effects on muscle mass and shape should manifest postnatally, without causing detrimental effects to mother or fetus at parturition. Such effects could nevertheless be expected to be programmed prenatally (Picard *et al.* 2002; Du *et al.* 2010) and to be independent of absolute fetal muscle weights. This interpretation is consistent with the imprinting status of major regulators of fetal muscle development and growth in bovine e.g. paternally expressed growth promoting *IGF2* and maternally expressed growth inhibiting *IGF2R* (Dindot *et al.* 2004; Hiendleder *et al.* 2004). Imprinted gene effects with paternal mode of expression responsible for increased muscle mass in ovine (*DLK1*) and porcine (*IGF2*) manifest postnatally (Jin-Tae Jeon 1999; Nezer *et al.* 1999; Davis *et al.* 2004; Cockett *et al.* 2005).

Analyses of the proportion of parental contributions to muscle traits revealed that contributions of the maternal genome to absolute and relative fetal muscle mass increased (or conversely, contributions of the paternal genome decreased) from thoracic limb to torso and pelvic limb. This novel spatial effect of the maternal genome mirrored paternal effects on muscle mass observed in sheep with the polar overdominant callipyge mutation (Koohmaraie *et al.* 1995; Cockett *et al.* 1996; Jackson *et al.* 1997). Consistent with our findings, a recent study in porcine identified a quantitative trait locus (QTL) with maternal polar overdominance that affected postnatal pelvic limb muscle mass (Boysen *et al.* 2010). Moreover, statistical modelling of parent-of-origin effects on postnatal muscle mass in porcine and bovine also showed a preponderance of maternal effects attributed to genomic imprinting (Neugebauer *et al.* 2010; Neugebauer *et al.* 2010). The significant switch in gene expression, including imprinted transcripts from the *DLK1-DIO3* region, in ovine *M. longissimus dorsi* from fetus to neonate

(Byrne *et al.* 2010), could indicate developmental stage specific roles of maternal and paternal genomes in myogenesis. Interestingly, the imprinting status of genes can change from monoallelic to non-imprinted biallelic expression during development (Davies 1994; McLaren and Montgomery 1999; Goodall and Schmutz 2007). Statistical analyses of experimental data for postnatal growth and development in mouse identified multiple imprinted QTL with complex temporal patterns of parent-of-origin effects (Wolf *et al.* 2008). It is tempting to speculate that such effects could also be spatial.

Significant effects of sex on postnatal muscle mass of mammals, including bovine, have been reported (Seideman and Crouse 1986; Fortin *et al.* 1987; Uttaro *et al.* 1993; Larzul *et al.* 1997), but the present study is the first to examine sex effects in prenatal myogenesis. In agreement with fetal programming of postnatal muscle mass discussed above (see maternal and paternal genomes), sex explained greater proportions of variation in relative fetal muscle weights than in absolute muscle weights (**Figure 4.1**). Male fetuses had higher absolute muscle weights but lower relative muscle weights than females (**Figure 4.4, 4.6**). The latter findings are in agreement with results for postnatal muscle weights in porcine (Fortin *et al.* 1987) and ovine (Santos *et al.* 2007). In the present study, fetal sex had no effect on relative weight of *M. supraspinatus*, a shoulder muscle, but significantly affected the relative weights of *M. longissimus dorsi* (loin) and *M. quadriceps femoris* (pelvic limb) (**Figure 4.6**). This is again similar to results obtained for postnatal muscle mass in ovine (Santos *et al.* 2007), where sex had no effect on shoulder muscle percentage but significantly affected loin muscle percentage, with greater muscle percentage in females than in males. An explanation for these results could be that fetal shoulder muscle mass is under strong selection because of its relevance for birthing difficulties and thus survival. The loin and pelvic limb region of females may require a higher relative muscle weight to maintain sex-specific postnatal proportions and reproductive functions, which may be programmed during fetal development.

Our analyses identified significant contributions of final maternal weight (FMW) to variation in absolute fetal muscle weights and *H19* expression at midgestation (**Figure 4.1**). These non-genetic maternal effects were estimated as nested effects within maternal genetics using type I sums of squares in the final linear models, allowing the removal of maternal genetic contributions from effects of FMW (see methods). Non-genetic maternal components can be explained by differences in environmental factors acting on dams before they were recruited for the experiment. These environmental effects could not be erased during several weeks of

adjustment under a controlled environment prior to the start of the experiment. To our knowledge, pre-conception non-genetic maternal contributions to variation in fetal muscle mass have not been reported previously. The estimated regression coefficients suggested that the same mechanisms affect fetal muscle mass in dams with Bt and Bi genomes (**Figure 4.5, 4.7**).

In conclusion, we have shown for the first time, that fetal muscle development is differentially affected by maternal and paternal genome, independently, or in combination with non-genetic maternal effects. Our statistical analyses of effects of parental genomes, and molecular data for the imprinted maternally expressed lincRNA H19, suggested that imprinted gene networks (Gabory *et al.* 2010) and epistatic miRNA interference (Clop *et al.* 2006) could be major drivers of the observed parental effects on fetal muscle traits. Our conclusions are supported by results from statistical modelling of postnatal muscle traits (Engellandt and Tier 2002; Neugebauer *et al.* 2010; Neugebauer *et al.* 2010) which identified parent-of-origin effects attributed to imprinted genes as a major source of variation. Detailed molecular profiles are now required to elucidate genetic, epigenetic and non-genetic components and interactions that control variation in prenatal muscle traits. Our data further suggest that specific combinations of (epi)genetic and non-genetic factors can be used to optimise fetal, and therefore, postnatal muscle development and phenotype. Non-Mendelian (epi)genetic and non-genetic maternal effects can help understand unexplained variation in postnatal muscle traits. These traits may be highly variable within populations, even when genetics and environment are well controlled (Reverter *et al.* 2003; Greenwood *et al.* 2007).

4.5 Materials and Methods

4.5.1 Cattle and fetuses

All animal experiments and procedures described in this study were approved by The University of Adelaide Animal Ethics Committee (No. S-094-2005 and S-094-2005A). We used animals and semen of the Angus and Brahman breeds to study differential parental genome effects on fetal muscle phenotype at midgestation. The two breeds are subspecies of domestic cow, commonly referred to as *Bos taurus* and *Bos indicus*, respectively (Hiendleder *et al.* 2008; The Bovine Genome Sequencing Consortium 2009). Nulliparous Angus and Brahman dams which were approximately 16–20 months of age were purchased from farms in South Australia and Queensland and transferred to, and maintained at, Struan Agricultural Centre, South Australia. Animals were on pasture supplemented by silage. After an adjustment period of 3-4 weeks the animals received standard commercial estrous cycle synchronization as described previously (Anand-Ivell *et al.* 2011). All fetuses were sired by two Brahman and three Angus bulls. Dams were pregnancy tested by ultrasound scanning and fetuses recovered in an abattoir at Day 153±1 of gestation. Fetuses were removed from the uterus, eviscerated, vacuum packed and stored frozen at –20ºC until further processing. Final maternal weight (FMW) was recorded and average maternal daily weight gain (MDG) was calculated as FMW minus weight at conception divided by gestation length (Supplementary **Figure S4.4**). We analyzed 73 fetuses in total, including 23 Bt × Bt, 22 Bi × Bt, 13 Bt × Bi and 15 Bi × Bi (paternal genetics listed first) with both sexes represented in each genetic group. The distribution of Bt and Bi maternal and paternal genomes, and of females and males, are shown in Supplementary **Table S4.1.**

4.5.2 Muscle dissection and weights

Fetuses were thawed and the head removed by disarticulation between the *Os occipitale* and first cervical vertebra atlas. *Musculus supraspinatus, M. longissimus dorsi*, *M. semimembranosus* and *M. quadriceps femoris* (consisting of *M. rectus femoris, M. vastus medialis, M. vastus intermedius* and *M. vastus lateralis*) were dissected from both sides of the fetus. *M. longissimus dorsi* was defined from the 7th rib to the natural caudal end of the muscle, at the apophysis of the lumbosacral. The dissection protocol was based on Budras and Habel (Budras and Habel 2003) and muscle nomenclature according to Tucker (Tucker 1952). *M. semimembranosus* was obtained from 61 fetuses due to damage to some specimens from sampling adjacent *M. semitendinosus* for immunohistochemistry, described below. Dissected muscles from both sides of the fetus were weighed and absolute muscle weight was recorded as the mean weight for each muscle. Combined muscle weights were calculated as the sum of mean weight of each dissected muscle. Relative muscle weights, reflecting fetal muscle proportions, were calculated as muscle weight divided by the weight of the decapitated eviscerated fetus (see Supplementary **Figure S4.2**).

4.5.3 Muscle immunohistochemistry

At the time of fetus collection, a section of *M. semitendinosus* was cut from the centre of the muscle and mounted using gum tragacanth (Sigma Chemical Company, St. Louis, MO; prepared 5% wt/vol in distilled, deionized H_2O) onto a cork block, with muscle fibres running perpendicular to the cork block. Samples were frozen by immersion in iso-pentane cooled to approximately −160 °C in liquid nitrogen, before storage at −80 °C. Muscle tissue preparation and immunohistochemical staining followed the protocol by Greenwood et al. (Greenwood *et al.* 2009). Briefly, 10-µm-thick, serial cross-sections were cut from each frozen sample using a cryostat microtome (ThermoShandon AS 620 Cryostat SME, Thermotrace Ltd., Noble Park,

Victoria, Australia). After air-drying, cross-sections were stained against type I (slow) (clone WBMHC, Novocastra, Newcastle upon Tyne, UK; diluted 1:100 in PBS) and type II (fast) (clone MY-32, Sigma; diluted 1:400 in PBS) myosin heavy chain isoforms. Staining using these antibodies was previously shown to discern these myofibre types in ruminant fetal muscle (Greenwood *et al.* 1999). They were revalidated in bovine fetal muscle using myofibrillar ATPase staining for the present experiment. The stained sections were dehydrated and cleared using graded ethanols and xylenes to produce slides using a xylene-based mounting medium.

4.5.4 Myofibre Classification and Morphometry

Microscopic image analysis was used to classify and measure myofibres on stained slides. A Zeiss AxioPlan2 microscope fitted with Plan-Neofluar objectives (Carl Zeiss Pty. Ltd., Goettingen, Germany) and a Fujix colour digital camera (FUJIFILM Australia Pty. Ltd.) were used to produce images. Images were generated using a 40 × objective, and were captured using Analysis FIVE software (Soft Imaging System Corp. 12596 W. Bayaud Ave. Suite 300 Lakewood CO 80228, USA) and analysed using Image Pro Plus 6.0 software (Media Cybernetics, Inc. 4340 East-West Hwy, Suite 400 Bethesda, MD 20814-4411 USA). Fibre type was identified based on staining characteristics (Picard *et al.* 1998). Myotubes were defined as cells that appeared hollow in cross-section, the remainder were considered myofibres (Picard *et al.* 1994; Picard *et al.* 2002). Myofibres and myotubes were classified as type I (slow) myofibre, type I (slow) myotube, type II (fast) myofibre and type II (fast) myotube (Supplementary **Figure S4.1**).

Morphological measurements were conducted by manually tracing anti-laminin-stained (rabbit anti-laminin, affinity isolated antibody: Sigma; diluted 1:500 in PBS) margins of cells using the draw/merge object function of Image Pro Plus 6.0. For each fetus, the serial slow or fast stained myosin heavy chain slide with highest contrast was chosen to measure myofibre characteristics.

Three fields (40 × objective) of each chosen slide were analyzed. For each field, cross-sectional area (CSA) and number of type I (slow) myotubes and myofibres, type II (fast) myotubes and myofibres were measured. Furthermore, number and CSA were measured irrespective of cell type. All counted cells in the field comprised total cell number, and CSA of counted cells in the field was total cell CSA. For each myofibre characteristic an average was calculated of the three fields measured. For each fetus the average number of cells measured was 369, ranging from 152 to 705 cells. The average standard deviation between replicated fields for myofibre number was 1.3 for slow myotubes, 0.9 for slow myofibres, 5.1 for fast myotubes and 16.9 for fast myofibres. The average standard deviation between replicated fields for CSA was 43.3μm^2 for slow myotubes, 38.3μm^2 for slow myofibres, 19.7μm^2 for fast myotubes and 10.7μm^2 for fast myofibres.

4.5.5 Expression of *H19* in skeletal muscle

Samples from *M. semitendinosus* were collected into RNA later (Qiagen, Chadstone Centre, VIC, Australia) immediately after recovery of fetuses in the abattoir and stored at −80 °C after equilibration for 24 hours at 2-4 °C. Total RNA was extracted from *M. semitendinosus* of all fetuses by TRI Reagent® Solution (Ambion, Life Technologies™ Inc., Carlsbad, CA, USA) according to the manufacturer's instructions and RQ1-DNase treated (Promega, Madison, WI, USA). Reverse transcription was carried out using SuperScript™ III First-Strand synthesis system for RT-PCR (Invitrogen, Life Technologies™ Inc., Carlsbad, CA, USA) on 500 ng of total RNA with random hexamer oligonucleotides according to the manufacturer's instructions. Amplification of *H19* from cDNA was performed using a forward primer located at the junction of exons 3 and 4, and a reverse primer located within exon 5 (Supplementary **Table S4.2**). Total length of this amplicon was 171 bp. Real time quantitative PCR (qPCR) reactions were performed using Fast Start Universal SYBR Green Master (Roche Diagnostics GmbH,

Mannheim, Germany) in an Eppendorf Mastercycler® pro S thermal cycler (Eppendorf Inc., Hamburg, Germany) on 4µl of 40-fold diluted cDNA in a final volume of 12 µl with 6 µl of SYBR master mix (2×) at an annealing temperature of 60 ºC. Product specificity and integrity were confirmed using plots of melting curve and electrophoresis on a 2% agarose gel stained with GelRed™ Nucleic Acid Stain (Biotium Inc., Hayward, CA, USA). All qPCR experiments were performed in duplicate and the mean of both Cts used to calculate the amount of target transcript. We used the standard curve method with determination of PCR amplification efficiency. A two-fold serial dilution over eight data points was produced on a mixture of pooled cDNAs from all fetuses with equal proportions. Three replicates were used for each dilution of the cDNA template. Non-template control was included in all experiments. We determined relative expression levels of seven putative housekeeping genes including actin beta (*ACTB)*, ribosomal protein S9 (*RPS9*), ubiquitin B (*UBB*), H3 histone family 3A *(H3F3A)*, TATA box binding protein *(TBP)*, vacuolar protein sorting 4 homolog A (*VPS4A*) and cyclin G associated kinase (*GAK*) and used geNorm program version 3.5 (Vandesompele *et al.* 2002) to identify *GAK* and *VPS4A* (see supplementary **Table S4.2**) as the most stable genes for normalization of the target gene. Expression levels of *H19* were normalized to the geometric mean of the expression levels of the selected housekeeping genes. As the normalized expression data were not normally distributed, we performed statistical analysis after logarithmic transformation of the data. The results for least square means and standard errors of means were presented after back-transformation.

4.5.6 Statistical estimation of effects and means

All data were analyzed by Univariate Analysis of Variance (ANOVA) using the general linear model (GLM) procedure of SPSS 17.0 (SPSS Inc., Chicago, IL, USA). Initially, data were fitted to the following full linear model:

$$y_{ijk} = M_i + P_j + S_k + gain(M_i) + weight(M_i) + F \times F + F \times F \times F + C \times C(M_i) + C^2(M_i) + F \times C(M_i) + F \times C \times C(M_i) + F \times C^2(M_i) + e_{ijk}$$

where y_{ijk} were myofibre characteristics, muscle weights and transcript abundance, M_i was maternal genome effect (j = Angus, Brahman), P_j was paternal genome effect (i = Angus, Brahman), S_k was fetal sex effect (k = male, female), *gain* was post-conception daily weight gain and *weight* was final maternal weight. M_i, P_j and S_k were fitted as fixed factors (F) and *gain* and *weight* were fitted as covariates (C). The covariates fitted in the model were nested within maternal genome (M_i) in order to adjust for effects of *gain* and *weight* within each of the two dam breeds. Interactions between factors and covariates were tested as follows: $F \times F$ was 2-way interaction between factors, $M_i \times P_j$, $M_i \times S_k$ and $P_j \times S_k$, $F \times F \times F$ was the 3-way interaction between factors, $M_i \times P_j \times S_k$; $C \times C(M_i)$ was the 2-way interaction of covariates nested within maternal genome, *gain*×weight(M_i); $C^2(M_i)$ was the quadratic term of covariates nested within maternal genetics, $gain^2(M_i)$ and $weight^2(M_i)$; $F \times C(M_i)$ was the 2-way interaction between factors and covariates nested within maternal genetics, $P_j \times gain(M_i)$ and $S_k \times gain(M_i)$, $P_j \times weight(M_i)$ and $S_k \times weight(M_i)$; $F \times C \times C(M_i)$ was the 3-way interaction between factors and the two covariates nested within maternal genetics, $P_j \times gain \times weight(M_i)$ and $S_k \times weight \times gain(M_i)$; $F \times C^2$ was the interaction between factors and quadratic terms of covariates nested within maternal genetics, $P_j \times gain^2(M_i)$, $S_k \times gain^2(M_i)$, $P_j \times weight^2(M_i)$ and $S_k \times weight^2(M_i)$.

Backward stepwise elimination was used to reduce the model for each measured parameter based on type III sums of squares (SSIII) at significance level (P) of 0.05. Type III sums of squares are independent of the order that effects are fitted in the model (Shaw and Mitchell-Olds 1993). Specifically, elimination started with the least significant (largest P value) interaction or effect. Insignificant variables were removed stepwise according to marginality rules (Nelder 1994) i.e. independent variables cannot be eliminated until after the interaction is eliminated due

to insignificance, and lower order interactions cannot be eliminated until after the corresponding higher order interaction is eliminated. Main effects were also considered to be marginalized by corresponding nested effects of covariates. Elimination continued until only significant effects and interactions remained, or had to be retained to maintain the marginality requirements. Main effects of M_i, P_j and S_k were retained in the final model, irrespective of the significance levels. This approach retained factors of the experimental design and produced models with relatively large coefficients of determination (R^2). R^2 values, model significance levels and significance levels of factors and nested covariates in the final model for each measured parameter are shown in **Table 4.1**. Means for effects of factors and interactions (with *P*-values from *t*-tests of the contrast, **Figures 4.3, 4.4, 4.6, 4.7**) and regression slopes for nested effects of covariates (**Figure 4.5, 4.7** and supplementary **Figure S4.3**) were plotted according to marginal means and estimated parameters obtained from the final model. *P*-values of maternal and/or paternal genome effects on fast myotube CSA, absolute weights of *M. supraspinatus, M. longissimus dorsi and M. quadriceps femoris*, and *H19* transcript abundance were not determined. The significant effects of final maternal weight nested within maternal genetics and/or significant interaction effects of maternal and paternal genome, would have biased *P*-values for corresponding main effects estimated with type III sums of squares (**Table 4.1**, **Figure 4.3, 4.4, 4.7**).

Only one nested quadratic effect was significant when tested; $weight^2(M_i)$ explained a significant ($P = 0.007$) amount of variation in absolute *M.quadriceps femoris* weight. However, examination of plotted curves with individual data points revealed that this effect was dependent upon two heavy dams with high leverage. Therefore, this quadratic effect was removed from the model and the linear effect retained. The graph for the initial quadratic effect is presented in supplementary **Figure S4.3.**

The contribution of maternal genome (M_i), paternal genome (P_j), fetal sex (S_k) and significant interaction and nested effects (P<0.05) to explained variation in myofibre characteristics, muscle weights and *H19* transcript abundance, was calculated from type I sums of squares (SSI). Type I sums of squares are dependent on the order in which effects are fitted in the model and sum to the total model SS (Shaw and Mitchell-Olds 1993; Nelder 1994) (**Figures 4.1, 4.2)**.

Final maternal weight (FMW) may contain both genetic and non-genetic effects as a function of breed and permanent environmental effect from origin of dam. Dams were sourced from different properties and had, therefore, been subject to different environments prior to recruitment for the experiment. By using SSI and fitting the maternal genome effect before *weight* in the model, we apportioned all the maternal genetic effect to maternal breed (M_i) and left only environmental effects attributable to *weight*. Specifically, variables and/or interactions were fitted into the final SSI model in the following order:

1) $M_i, P_j, S_k, F \times F$ and $C(M_i)$ (M_i before P_j)
2) $P_j, M_i, S_k, F \times F$ and $C(M_i)$ (P_j before M_i)

The SSI values of P_j and M_i were averaged from both models, assuming equal importance of maternal and paternal genomes. SSI values of other variables and interactions were identical for models 1 and 2. The SSI contribution of an interaction was apportioned equally to each component of the interaction. The contributions of maternal genetics (M_i), paternal genetics (P_j), fetal sex (S_k) and final maternal weight (*weight*) to myofibre characteristics, muscle weights and transcript abundance were calculated from the SSI of M_i, P_j, S_k and *weight* as a percentage of total SSI, respectively (**Figure 4.1**). The contribution of *weight* was defined as the non-genetic maternal effect, since the estimation of SSI values of *weight* were independent of maternal genome. The relative proportions of maternal and paternal genomes to total genetic variation in

myofibre characteristics, muscle weights and transcript abundance were calculated by totalling respective contributions (**Figure 4.2**).

The regressions and Pearson correlation coefficients (*r*) for absolute and relative combined muscle weights and *H19* transcript abundance were estimated in SPSS 17.0 (SPSS Inc., Chicago, IL, USA).

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Chapter 5: Differential maternal and paternal genome effects on fetal liver transcriptome expression and coexpression network at midgestation

Chapter 5

5.1 Abstract

Transcriptome expression profiles are molecular phenotypes that describe global activities of RNAs and capture critical molecular events which give rise to phenotypic traits. Similar to phenotypic traits, transcriptome expression profiles display a significant genetic basis and putative parent-of-origin effects. To further dissect (epi)genetic effects on molecular phenotype, including mechanisms behind differential parental genome and fetal sex effects driving fetal development, we profiled the liver transcriptome of 24 bovine fetuses at midgestation with mRNA and microRNA (miRNA) microarrays. mRNA and miRNA transcript abundences in four genetic groups with purebred and reciprocal cross Angus and Brahman genetics were analysed by ANOVA and bioinformatic approaches. Maternal genome predominantly contributed to genetic variation in fetal liver weight (79.4%, P<0.0001) and strongly affected expression level of 24 mRNA transcripts (False discovery rate (FDR) adjusted P<0.05-0.0001), 13 of which were located in the mitochondrial (mt) genome. Paternal genome explained 20.6% (P<0.05) of genetic variation in fetal liver weight and moderately affected abundances of 47 mRNA transcripts (FDR adjusted P<0.05-0.035) from autosomes. Fetal sex strongly affected liver weight (P<0.01) and expression level of 26 mRNA transcripts (FDR adjusted P<0.05-0.0001), 18 of which were located in sex-linked chromosomes. Furthermore, maternal genome significantly affected expression levels of mammalian *SNORD113-9*, small nucleolar (sno)RNA, *MIR187* and *MIR1973* transcripts (FDR adjusted P<0.05-0.01), while paternal genome significantly affected expression level of mammalian *MIR184* transcripts (FDR adjusted P<0.05-0.035). Differentially expressed mRNAs and miRNAs were identified in five significant biological pathways largely involved in mitochondrial energy production and two coexpression networks. Network one

included 53 coexpression events between a majority of mitochondrial (mt) mRNA and miRNA/snoRNA transcripts significantly affected by maternal genome; network two consisted of 29 coexpression events between autosomal mRNA and *MIR184* transcripts significantly affected by paternal genome. These findings suggest that non-Mendelian (epi)genetic crosstalk between nuclear and mt genome involves microRNA/snoRNA interference as a major mechanism behind significant differential parental genome effects driving fetal development.

5.2 Introduction

Phenotype is determined by expression of DNA information, which initiates from transcription processes. This involves coordination of coding messenger RNAs, mRNAs and non-protein coding RNAs, such as ribosomal RNA (rRNA), long non-coding RNA (lincRNA), transfer RNA (tRNA), small nucleolar RNA (snoRNA) and microRNAs. The activities of all these RNAs, i.e., the transcriptome, describe a global picture of cellular function that can be measured. Microarray technology, provides transcript abundance profiles for a large number of RNA, which are defined as a 'molecular phenotypes' (Jansen and Nap 2001). Molecular phenotype serves as a surrogate for traditional quantitative traits in which expression levels are closely related to traits (Schadt *et al.* 2003). Unlike traditional quantitative traits that represent gross biological measurements and consequences of dynamic molecular events, transcript abundance profiles provide a detailed picture of biological processes that give rise to traditional traits (Schadt *et al.* 2003) and are likely to capture critical causative events. Using microarray profiling of the transcriptome, a large number of critical mRNA and/or miRNA groups have been identified for mammalian prenatal development, including human placenta (Sood *et al.* 2006; Mouillet *et al.* 2011), cattle and pig prenatal muscle (Lehnert et al. 2007; Huang et al. 2008), human bone (Stokes *et al.* 2002; Gao *et al.* 2011) and liver (Yu *et al.* 2001; Tzur *et al.* 2009). Therefore, transcript abundance profiles, as a highly informative trait, facilitate the unravelling of pivotal biological processes driving mammalian prenatal development.

Similar to traditional quantitative traits, molecular phenotypes, such as transcript abundance profiles, including mRNA expression levels, showed a large classical Mendelian genetic basis. In human, average broad-sense heritability of mRNA transcript expression levels was approx 0.3 in blood and adipose tissue based on large population studies (Emilsson *et al.* 2008) and in lymphoblastoid cell lines from twin studies (Visscher *et al.* 2008). Estimates for narrow-sense

(i.e., additive genetic) heritability of transcript expression levels in human lymphoblastoid cell lines was reported to be as high as 0.8 (Dixon *et al.* 2007). Expression quantitative trait loci (eQTL) studies have mapped loci that collectively accounted for up to 78% and 90% of variation in expression levels of mRNAs in postnatal human liver (Schadt *et al.* 2008) and brain (Gibbs *et al.* 2010), respectively. However, Mendelian genetic analysis of transcript abundance profiles, similar to traditional quantitative traits, considers the two alleles from parental genomes to be functionally equivalent. This assumption may be invalid when non-Mendelian modes of inheritance, such as mitochondrial (mt) DNA, sex-chromosomes and genomic imprinting (i.e., parent-of-origin dependent allele-specific gene expression) are present.

To our knowledge, non-Mendelian genetic effects on transcript abundance profiles in mammals remain largely uninvestigated. However, data from other model species, such as Y chromosome effects on testis-specific gene expression in *Drosophila* (Branco *et al.* 2013) and mt haplotype effects on gene expression levels in fish liver (Flight *et al.* 2011) suggested the existence of a non-Mendelian (epi)genetic basis of transcript abundance variation in mammals. Indeed, using computational modelling with published human genomic and transcriptome data, putative imprinted eQTLs that display significant parent-of-origin effects on global gene expression levels were identified (Garg *et al.* 2012). Therefore, more direct evidence is required to demonstrate potential non-mendelian (epi)genome effects on transcript abundance profiles, that could provide novel insights into understanding complex biological processes driving mammalian growth and development.

To further dissect parent-of-origin specific (epi)genome effects associated with fetal development, we profiled the transcriptome of midgestation liver for a subset of individuals from a previously generated bovine fetal resource (see methods in Xiang *et al.* (2013) and previous Chapters in this thesis). This subgroup again consisted of both purebreds and reciprocal hybrids

with *Bos taurus* (Angus), and *Bos indicus* (Brahman), genetics, which allowed us to dissect differential parental genome effects in fetal development at the phenotypic and transcriptional levels. We show, for the first time, significant differential effects of parental genomes on fetal liver phenotype and associated profiles of mRNA and non-coding miRNA transcription. Identified differentially expressed mRNA and miRNA/snoRNA transcripts, and corresponding coexpression networks, suggested that the interplay of multiple non-mendelian (epi)genomic mechanisms, i.e., mt genome effects, miRNA interference and genomic imprinting, is responsible for differential parental genomic effects that drive mammalian prenatal development.

5.3 Materials and methods

5.3.1 Animals and tissue preparation

All animal experiments and procedures described in this study were approved by the University of Adelaide Animal Ethics Committee (No. S-094-2005 and S-094-2005A). Twenty-four fetuses, six each of Bt × Bt, Bt × Bi, Bi × Bt and Bi × Bi (maternal genetics listed first; Bt, *Bos taurus taurus*, Angus; Bi, *Bos taurus indicus*, Brahman) with three males and three females in each genetic group were recovered at Day 153±1 of gestation, as described in previous Chapters and Xiang *et al*. (2013). Fetal livers were weighed and a 40-50 mg tissue sample from the *Lobus hepaticus sinister* was immediately placed in RNA-later® (Qiagen, Chadstone Centre, VIC, Australia), stored for 24 hours at 2–4 °C and preserved at -80 °C until further processing.

5.3.2 RNA extraction

Liver tissue was homogenised by PRECELLYS®24 lyser / homogeniser (Bertin Technologies, Saint Quentin en Yvelines Cedex, France), with 1 ml of TRIzol reagent (Ambion, Life Technologies™, Inc., Carlsbad, CA, USA) in tubes containing 1.4 mm ceramic beads (Mo Bio Laboratories, Inc., Carlsbad, CA, USA). Homogenisation was performed at 6500 rpm in two

cycles. Total RNA was extracted from fetal tissues using TRI Reagent® (Ambion, Life Technologies™, Inc., Carlsbad, CA, USA). After removing insoluble materials from the homogenate by centrifugation at 12,000 g for 10 minutes at 2 to 8 °C and incubation of the supernatant on ice for 5 minutes, 0.2 ml of chloroform was added, followed by incubation on ice for 3 minutes and centrifugation for 20 minutes at 12,000 g (4 °C). The aqueous phase was separated and mixed with 0.6ml isopropyl alcohol, and incubated for overnight at -20° C, followed by centrifugation at 13,000 g (4 °C). After removing the supernatant, the pellet was washed by adding 1.2 ml of 75% ethanol, and re-pelleted by centrifugation at 12,000 g for 5 minutes (4 °C). The RNA pellet was dissolved in 50-100 µl of nuclease-free water (GIBCO UltraPure™ Distilled Water, Invitrogen™, Inc., Auckland, NZ). RNA quantity and integrity was assessed by a NanoDrop ND-1,000 spectrophotometer (Thermo Scientific, Inc., Wilmington, DE, USA) and Agilent RNA 6,000 Nano Kit with Bioanalyzer 2,100 (Agilent Technology, Inc., Santa Clara, CA, USA). The mean RIN (RNA Integrity Number) value for fetal liver was 8.05. Tissue samples were provided by Prof. Stefan Hiendleder and RNA was extracted by Dr. Ali Javadmanesh.

5.3.3 Microarray procedure

Liver RNA samples were delivered to Australian Genome Research Facility Ltd (Adelaide Microarray Centre, Adelaide, Australia) for microarray analysis, where reverse transcription reactions for cDNA were performed. The cDNAs of 24 fetal liver tissues, including six samples for each genetic group, Bt × Bt, Bt × Bi, Bi × Bt and Bi × Bi (maternal genome listed first) with both sexes represented, were analysed for mRNA expression profiles by Affymetrix GeneChip® Bovine Genome Array platform (Affymetrix, Santa Clara, CA). Each mRNA microarray contained 24,027 probe sets representing over 23,000 transcripts and included approximately 19,000 annotated UniGene clusters. The same cDNA resource was also analysed for microRNA

(miRNA) expression by GeneChip® miRNA 3.0 Array platform (Affymetrix, Santa Clara, CA). Each miRNA array contained 19,724 probe sets for pre- and mature miRNA, small nucleolar RNAs (snoRNAs) and small cajal body-specific RNAs (scaRNAs) covering 153 organisms. Each cDNA sample was processed and hybridised to an individual slide. The target labelling, hybridisations, fluidics, chip scanning, were performed by the Australian Genome Research Facility Ltd (Adelaide Microarray Centre, Adelaide, Australia). All microarray images and quality control measurements were within recommended limits. mRNA and miRNA microarray raw data were also processed and normalised by Australian Genome Research Facility Ltd (Adelaide Microarray Centre, Adelaide, Australia) following recommended standard procedure, using Bioconductor project (http://www.bioconductor.org), an extension for bioinformatics of the statistical language/software R (http://r-project.org). Normalised transcript expression intensity values were returned to the lab for further analyses. The miRNA microarray data were analysed for mammalian miRNAs, 13,133 probe sets, only.

5.3.4 Data analysis

Fetal liver weight was analysed by univariate Analysis of Variance (ANOVA) using the general linear model (GLM) procedure of SPSS 17.0 (SPSS Inc., Chicago, IL, USA) with the following full linear model:

$$y_{ijk} = M_i + P_j + S_k + M_i \times P_j + e_{ijk} \qquad (1)$$

where y_{ijk} were fetal liver weight, M_i was maternal genome effect (j = Angus, Brahman), P_j was paternal genetic effect (i = Angus, Brahman), S_k was fetal sex effect (k = male, female), and $M_i \times P_j$ was effect of maternal and paternal genome interaction. Backward stepwise elimination was used only to reduce $M_i \times P_j$, based on type III sums of squares (SSIII) at significance level (P) of 0.05 as described previously (Xiang *et al.* 2013). Main effects of M_i, P_j and S_k were retained in

the final model, irrespective of significance levels. Means for effects of factors and significant interactions and regression slopes for nested effects of covariates were plotted according to marginal means and estimated parameters obtained from the final model using GraphPad (GraphPad Software, Inc., CA, USA) (**Figure 5.1**). The final general linear model was also used to analyse the relative contributions of M_i, P_j and S_k to the explained variance in liver weight, with type I sum of squares (SSI) as described previously (Xiang *et al.* 2013).

Transcript expression intensities were analysed by fixed model ANOVA procedure using R/maanova (v 1.16.0, http://churchill.jax.org/software/rmaanova.shtml), a package implemented in statistical language/software R (http://r-project.org). Initially, to test the effect of parental genome interaction on transcript expression, array data were fitted in (1), where y_{ijk} were transcript expression intensities, M_i, P_j, S_k and $M_i \times P_j$ were the same as described above. For microarray fixed model ANOVA procedure, all explanatory variables (M_i, P_j and S_k) were fitted as fixed factors, and tested with 1,000 permutations by F_s statistics methods for its greatest power (Cui and Churchill 2003; Cui *et al.* 2005). All significance levels (*P*-values) in microarray ANOVA were adjusted for false discovery rate using Storey's *q*-value approach (selecting "jsFDR" in maanova), for its strong power in controlling type I errors in multiple-hypothesis testing (Storey 2002). Subsequently, a second fixed model following above microarray ANOVA procedures was tested without $M_i \times P_j$ for independent main effects of maternal genome, paternal genome and fetal sex on transcript expression as follow:

$$y_{ijk} = M_i + P_j + S_k + e_{ijk} \qquad (2)$$

Significant independent maternal and paternal genome effects on individual transcript expression were retained when the transcript showed no significant interaction effects of maternal and paternal genome. This procedure considered interaction effects as a higher order variable according to the marginality rule and the concept of backward elimination model

development (Nelder 1994; Xiang *et al.* 2013). Significant transcripts for effects of maternal genome, paternal genome and fetal sex were plotted based on the $\log_2$ fold change of transcript abundance from *Bos taurus,* Angus (Bt) to *Bos indicus,* Brahman (Bi) or male to female, by considering transcript abundance in Bt or male as reference groups and scaling the transcript abundance to one (**Figure 5.2B,C,D**). Annotation detail with $\log_2$ fold change and adjusted *P* values for significant transcripts is shown in **Table 5.1-5.5**.

Significant transcripts identified by the microarray ANOVA procedure were used for biological pathways using enrichment analysis in the Database for Annotation, Visualization and Integrated Discovery (DAVID, http://david.abcc.ncifcrf.gov/) (Huang *et al.* 2009; Huang *et al.* 2009), with a background containing all transcripts present on the bovine Affymetrix microarray. Unannotated probe sets were not included in the analysis. Enrichment analysis was performed choosing 'Functional Annotation Clustering' function with 'highest classification stringency'. Annotation clusters with enrichment score above one, i.e., each pathway within that cluster with Benjamini-corrected Fisher Exact *P*-value below 0.05, were identified as significant. For a significant annotation cluster, where pathways consisted of the same transcript members, the pathway with the best *P*-value was chosen, are shown in **Figure 5.4** and **Table S5.1**.

Significant transcripts for the ANOVA procedure described above were analysed for coexpression events, i.e., correlations of expression intensity between transcripts from mRNA and miRNA microarrays, by CoExpress software (v1.5, http://www.bioinformatics.lu/CoExpress/) using the statistical language/software R as background processor. The mRNA and miRNA expression intensities for significant transcripts were loaded in CoExpress, and analysed using Pearson correlation as a metrics parameter with threshold |coexpression score|>0.7, to identify significant coexpressed transcript pairs from mRNA and miRNA microarrays. Identified significant coexpressed transcript pairs, i.e.,

coexpression events, were validated by bootstrapping and false discovery rate (<0.01), with 1,000 runs and permutations. Significant coexpression events were also classified for coexpression networks with threshold |coexpression score|>0.7, as illustrated in **Figure 5.5**, using Cytoscape software (v2.8.3, http://www.cytoscape.org/). Affymetrix IDs of coexpressed transcripts with computed coexpression score were loaded and analysed in Cytoscape, with transcript IDs from mRNA array being 'source interaction', transcript IDs from miRNA array being 'target interaction' and coexpression score being 'interaction type'. Negative coexpression score values, i.e., negative correlation, were labelled in black and positive coexpression score values were labelled in red.

For significant miRNA transcripts from the ANOVA procedure, predicted target mRNAs were searched in TargetScanHuman (v 6.2, http://www.targetscan.org/vert_61/) database for both conserved and nonconserved miRNA sites. Identified predicted target mRNAs were matched with significant mRNA transcripts identified in our experiment by ANOVA procedures. Expression intensity values of matched significant miRNA and predicted target mRNA transcripts were analysed for by linear regression and Pearson correlation coefficient (r) in SPSS 17.0 (SPSS Inc., Chicago, IL, USA) and are shown in **Figure 5.6**.

5.4 Results

5.4.1 Differential parental genomes and fetal sex effects

Parental genome accounted for the majority (79.4%) of explained variation in fetal liver weight (R^2 = 0.665, model P<0.0001), with 20.6% of variation explained by fetal sex (**Figure 5.1A**). Maternal genome predominantly contributed (79.4%) to genetic variation in liver weight, with 20.6% of genetic variation explained by paternal genome (**Figure 5.1B**). Liver weight of fetuses with Bt maternal genome had greater liver weight than fetuses with Bi maternal genome

($P < 0.0001$). Paternal genome effects on liver weight were moderate ($P < 0.05$), where fetuses with Bt paternal genome had lower liver weight than fetuses with Bi paternal genome. Fetal sex effects on liver weight were also strong ($P < 0.01$), with greater liver weight in male fetuses (**Figure 5.1C**).

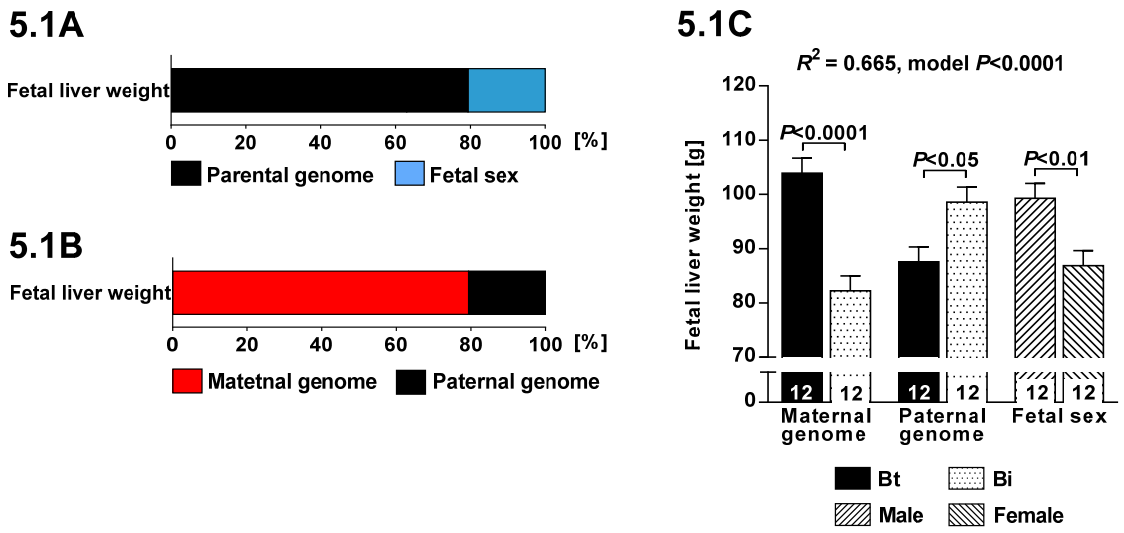

Figure 5.1. Significant parental genomes and fetal sex effects on fetal liver weight at midgestation.

(**A**): Relative contributions of parental genome and fetal sex to variation explained by statistical model in fetal liver weight. (**B**): Relative contributions of maternal and paternal genome to genetic variation in fetal liver weight. (**C**): Specific effects of maternal genome, paternal genome and fetal sex on liver weight. R^2 value of final model, least square means with standard errors of means, *P*-values for model (ANOVA) and significant differences (*t*-test) between means are indicated. Bt: *Bos taurus taurus*, Angus. Bi: *Bos taurus indicus*, Brahman.

Microarray ANOVA identified 97 (16 unannotated) and 15 differentially expressed transcripts from mRNA and miRNA microarray profiling, respectively, for parental genomes and/or fetal sex (**Figure 5.2A**, **Table 5.1-5.5**). No transcripts showed significant effects of parental genome interaction, or more than one significant independent maternal genome, paternal genome and fetal sex effect. Maternal genome strongly affected (FDR adjusted $P < 0.05$-0.001) about 25% of differentially expressed mRNA transcripts (24/97), where transcript abundances varied by $2^{2.39}$-$2^{-0.39}$ fold from fetuses with Bt maternal genome to fetuses with Bi maternal genome (**Figure 5.2B**, **Table 5.1**). Most transcripts (20/24) affected by maternal genome showed higher abundance in fetuses with Bt maternal genome. Over half of the transcripts up-regulated in

fetuses with Bt maternal genome (13/20) were located in the mt genome, whereas one transcript (tafazzin, *TAZ*) down-regulated in fetuses with Bt maternal genome was located on the X chromosome (**Figure 5.2B**, **Table 5.1**). Paternal genome also had moderate effects (FDR adjusted $P < 0.05$-0.035) on about 50% of differentially expressed mRNA transcripts (47/97), where transcript abundances changed by $2^{1.9}$-$2^{-1.66}$ fold from fetuses with Bt paternal genome to fetuses with Bi paternal genome (**Figure 5.2C**, **Table 5.2**). About 50% of the transcripts (22/47) affected by paternal genome displayed higher transcript abundance in fetuses with Bt paternal genome, and the remainder (25/47) showed lower transcript abundances in fetuses with Bt paternal genome. All annotated mRNA transcripts affected by paternal genome were from autosomes (**Figure 5.2C**, **Table 5.2**). Fetal sex strongly affected (FDR adjusted $P < 0.05$-0.0001) approximately 25% of differentially expressed mRNA transcripts (26/97), where transcript abundances differed by $2^{4.93}$-$2^{-7.65}$ fold between male and female fetuses (**Figure 5.2D**, **Table 5.3**). *XIST* displayed a dramatic $2^{7.65}$ fold increase in transcription level from male to female fetuses. More than 50% of the transcripts (18/26) affected by fetal sex were more highly expressed in males than in females. The majority of transcripts (17/26) affected by fetal sex were located on sex-chromosomes (**Figure 5.2D**, **Table 5.3**).

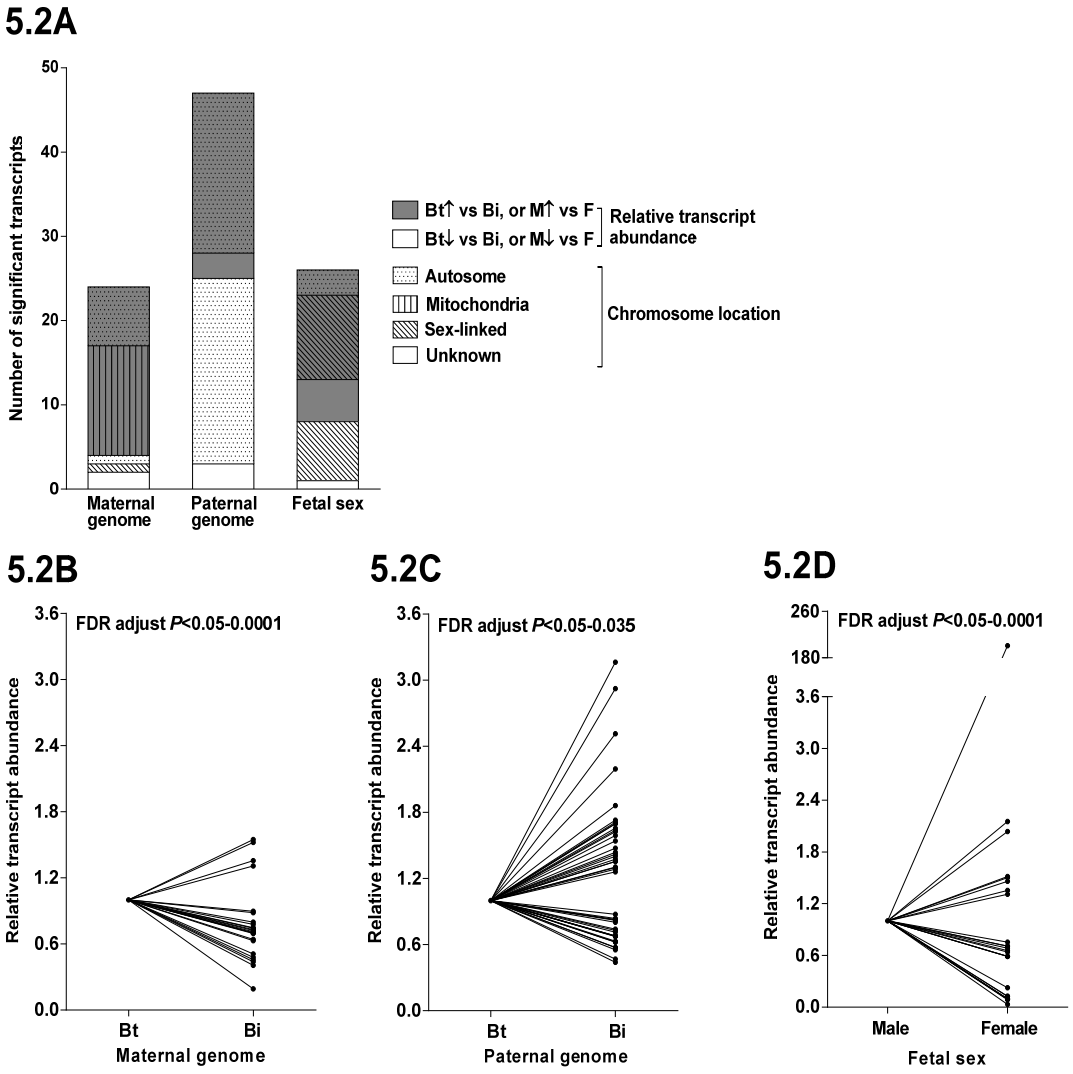

Figure 5.2. Significant parental genome and fetal sex effects on mRNA transcript abundances in fetal liver at midgestation.

(**A**): Number and chromosomal location of differentially expressed mRNA transcripts for maternal genome, paternal genome and fetal sex. Bt↑/Bt↓ vs Bi and M↑/M↓ vs F: Higher/lower transcript abundances in Bt (*Bos taurus taurus*, Angus) compared to Bi (*Bos taurus indicus*, Brahman), and higher/lower transcript abundances in M (male) compared to F (female). (**B-D)**: Visualisation of fold change for differentially expressed mRNA transcripts from Bt to Bi, or male to female, with false discovery rate (FDR) adjusted *P*-values, for significant effects of maternal genome (**B**), paternal genome (**C**) and fetal sex (**D**).

Table 5.1. Annotation of 24 mRNA transcripts for significant maternal genome effects, with $\log_2$ fold change (Log FC) from Bt (*Bos taurus taurus*, Angus) to Bi (*Bos taurus indicus*, Brahman) and false discovery rate (FDR) adjusted *P*-values.

	AffyID	**Gene symbol**	**Chromosome**	**Log FC Bt-Bi**	***P***
Bt>Bi (20 transcripts)	12915607	12S rRNA	Mt	0.5242	<0.0001
	12910847	ATP8	Mt	1.1329	<0.0001
	12910851	COX3	Mt	0.5095	<0.0001
	12910839	CYTB	Mt	0.6429	<0.0001
	12910831	ND1	Mt	0.9744	<0.0001
	12910853	ND3	Mt	1.1897	<0.0001
	12910835	ND5	Mt	0.4549	0.0003
	12910849	ATP6	Mt	0.4265	0.0010
	12910843	COX1	Mt	0.1567	0.0010
	12910841	ND2	Mt	0.4666	0.0010
	12766147	LOC788205	19	0.5183	0.0053
	12910837	ND6	Mt	1.0603	0.0091
	12825628	DEFB5	27	2.3862	0.0138
	12766536	DLG4	19	0.4227	0.0141
	12910855	ND4L	Mt	0.4922	0.0174
	12910833	ND4	Mt	0.1788	0.0191
	12683292	ECE2	1	0.3615	0.0335
	12835606	LOC100300995	3	0.3260	0.0337
	12859128	C1R	5	1.3025	0.0489
	12864647	-	5	0.6696	0.0335
Bt<Bi (4 transcripts)	12904062	TAZ	X	-0.4397	0.0383
	12911953	-	-	-0.6054	0.0043
	12895924	-	8	-0.6299	0.0087
	12766919	-	-	-0.3883	0.0383

Bt>Bi or Bt<Bi: Higher or lower transcript abundance in Bt than Bi. AffyID: Probe set ID in Affymetrix database for corresponding transcripts. Mt: Mitochondrial genome. Missing cells: Currently unannotated in bovine genome.

Table 5.2. Annotation of 47 mRNA transcripts for significant paternal genome effects, with $\log_2$ fold change (Log FC) from Bt (*Bos taurus taurus*, Angus) to Bi (*Bos taurus indicus*, Brahman) and false discovery rate (FDR) adjusted *P*-values.

	AffyID	**Gene symbol**	**Chromosome**	**Log FC Bt-Bi**	***P***
Bt>Bi (22 transcripts)	12891664	ERMP1	8	0.560	0.0340
	12896617	NT5E	9	0.800	0.0340
	12681528	BACE2	1	0.563	0.0389
	12680249	DNAJC19	1	0.560	0.0389
	12843233	AMY2A	3	0.666	0.0389
	12856688	GRIP1	5	0.489	0.0389
	12857285	HSP90B1	5	0.458	0.0389
	12887583	SPINK1	7	0.805	0.0389
	12899267	NT5E	9	0.684	0.0389
	12703781	XDH	11	0.680	0.0389
	12714336	ISM1	13	0.435	0.0389
	12794153	PRKAR2A	22	0.276	0.0389
	12806044	ZNF451	23	0.256	0.0410
	12893658	LOC513555	8	0.192	0.0413
	12899189	LOC100298760	9	0.274	0.0413
	12800321	KCTD6	22	0.285	0.0413
	12678626	ALCAM	1	0.316	0.0449
	12747078	SLC5A4	17	1.093	0.0462
	12804538	GSTA1	23	0.564	0.0465
	12732926	-	-	1.189	0.0357
	12678067	-	-	0.858	0.0389
	12678099	-	-	0.569	0.0399

Bt>Bi: Higher transcript abundance in Bt than Bi. AffyID: Probe set ID in Affymetrix database for corresponding transcripts. Missing cells: Currently unannotated in bovine genome. Table continued on next page.

Table 5.2 continued. Annotation of 47 mRNA transcripts for significant paternal genome effects, with $\log_2$ fold change (Log FC) from Bt (*Bos taurus taurus*, Angus) to Bi (*Bos taurus indicus*, Brahman) and false discovery rate (FDR) adjusted *P*-values.

	AffyID	**Gene symbol**	**Chromosome**	**Log FC Bt-Bi**	***P***
Bt<Bi (25 transcripts)	12745085	THOC7	22	-1.662	0.0340
	12682346	LOC614619	1	-0.765	0.0340
	12791531	LOC512150	18	-0.896	0.0340
	12860923	MMP19	5	-0.712	0.0381
	12718011	TGM2	13	-0.669	0.0389
	12829004	MGC151567	28	-0.335	0.0389
	12891710	MGC133950	8	-0.378	0.0389
	12893631	KIF24	8	-0.437	0.0389
	12901429	FRMD1	9	-0.504	0.0389
	12842122	RUSC1	3	-0.361	0.0399
	12878930	MUM1	7	-0.762	0.0399
	12721568	LOC618482	14	-0.525	0.0399
	12750220	LOC506868	18	-1.547	0.0399
	12800396	PXK	22	-0.791	0.0413
	12796427	CIDEC	22	-0.729	0.0413
	12865592	CD27	5	-0.562	0.0413
	12792456	LRRK1	21	-0.437	0.0433
	12895640	AQP3	8	-0.775	0.0438
	12881221	PGBD2	7	-0.485	0.0446
	12890668	LOC523509	8	-0.383	0.0446
	12751825	LOC515600	18	-1.133	0.0471
	12914799	-	-	-0.700	0.0340
	12677543	-	-	-1.330	0.0389
	12912489	-	-	-0.459	0.0389
	12723781	-	14	-0.623	0.0465

Bt<Bi: Lower transcript abundance in Bt than Bi. AffyID: Probe set ID in Affymetrix database for corresponding transcripts. Missing cells: Currently unannotated in bovine genome.

Table 5.3. Annotation of 26 mRNA transcripts for significant fetal sex effects, with $\log_2$ fold change (Log FC) from M (male) to F (female) and false discovery rate (FDR) adjusted *P*-values.

	AffyID	Gene Symbol	Chromosome	Log FC M-F	*P*
M>F (18 transcripts)	12910493	DDX3Y	Y	3.5456	<0.0001
	12906413	EIF2S3Y	X	3.3993	<0.0001
	12863039	LOC784883	5	2.9891	<0.0001
	12915307	OFD1Y	X	3.3854	<0.0001
	12911065	USP9Y	Y	3.3494	<0.0001
	12910802	-	Y	2.9729	<0.0001
	12914463	ZFY	Y	2.1560	<0.0001
	12910799	SHROOM2	X	0.4979	0.0006
	12907746	CD99	X	0.4048	0.0021
	12748258	ZNF280B	17	0.6081	0.0360
	12913877	SEPT10	11	0.4981	0.0373
	12906425	-	X	4.9342	<0.0001
	12915591	-	X	0.6468	<0.0001
	12902201	LOC100337085	-	0.5441	0.0006
	12901774	LOC786753	-	0.7612	0.0330
	12901752	LOC781463	-	0.7612	0.0330
	12915335	-	-	3.3912	<0.0001
	12915301	-	-	0.7648	<0.0001
M<F (8 transcripts)	12906978	KDM6A	X	-0.4371	<0.0001
	12908136	EIF2S3	X	-0.5461	<0.0001
	12907005	ZFX	X	-0.5994	<0.0001
	12909363	-	X	-1.0271	<0.0001
	12906927	TBC1D8B	X	-1.1066	<0.0001
	12903084	XIST	X	-7.6504	<0.0001
	12908839	LOC523644	X	-0.3896	0.0004
	12907015	-	-	-0.5874	<0.0001

M>F or M<F: Higher or lower transcript abundance in male than female fetuses. AffyID: Probe set ID in Affymetrix database for corresponding transcripts. Missing cells: Currently unannotated in bovine genome.

For 15 differentially expressed transcripts from the miRNA microarray, maternal genome had strong effects on *SNORD113-9* transcript (FDR $P < 0.01$), a human C/D box small nucleolar RNA (snoRNA) and eight *MIR187* transcripts (FDR $P < 0.05$-0.01) from a variety of mammalian species including human, rat and bovine. *SNORD113-9* and *MIR187* displayed lower transcript abundance in fetuses with Bt maternal genome than in fetuses with Bi paternal genome by $2^{0.6402}$-$2^{1.85}$ fold. Maternal genome also had moderate effects (FDR $P < 0.05$) on human *MIR1973* transcript, but *MIR1973* showed higher transcript abundance in fetuses with Bt

maternal genome than fetuses with Bi maternal genome by $2^{0.5663}$ fold (**Figure 5.3A**, **Table 5.4**). All miRNA and snoRNA transcripts affected by maternal genome were located on autosomes. Conversely, paternal genome had moderate effects (FDR adjusted $P < 0.05$-0.035) on five *MIR184* transcripts from human, rat, mouse, bovine and equine, where all transcripts showed higher abundance in fetuses with Bt paternal genome than fetuses with Bi paternal genome by $2^{1.4}$-$2^{1.66}$ fold. All miRNA transcripts affected by paternal genome were located on autosomes (**Figure 5.3B**, **Table 5.5**). No transcripts from the miRNA microarray showed significant fetal sex effects.

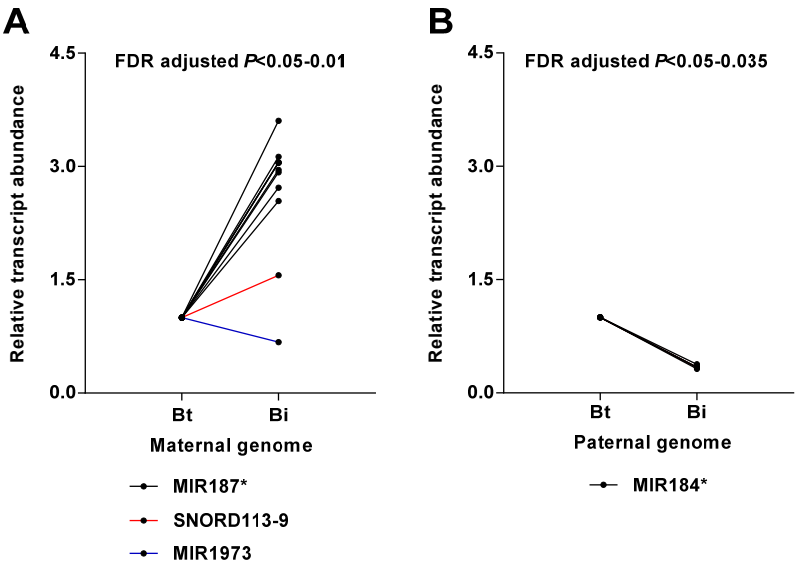

Figure 5.3. Significant parental genome effects on non-coding RNA transcript abundances in fetal liver at midgestation.

Visualisation of fold change for differentially expressed non-coding RNA transcripts from Bt to Bi, with false discovery rate (FDR) adjusted *P*-values, for significant effects of maternal genome (**A**) and paternal genome (**B**). *Multiple transcripts from different mammalian species. MIR: microRNA. SNORD: small nucleolar RNA. Bt: *Bos taurus taurus*, Angus. Bi: *Bos taurus indicus*, Brahman.

Table 5.4. Annotation of 10 miRNA transcripts for significant maternal genome effects, with $\log_2$ fold change (Log FC) from Bt (*Bos taurus taurus*, Angus) to Bi (*Bos taurus indicus*, Brahman) and false discovery rate (FDR) adjusted *P*-values.

	AffyID	Gene symbol	Species	Chr	Log FC Bt-Bi	*P*
Bt>Bi (1 transcripts)	hsa-miR-1973_st	MIR1973	*Homo sapiens*	4	0.5663	0.0223
Bt<Bi (9 transcripts)	14qI-9_x_st	SNORD113-9	*Homo sapiens*	14	-0.6402	0.0074
	cfa-miR-187_st	MIR187	*Canis familiaris*	7	-1.8497	0.0074
	bta-miR-187_st	MIR187	*Bos taurus*	24	-1.6099	0.0083
	ppa-miR-187_st	MIR187	*Pan paniscus*	-	-1.3459	0.0091
	mml-miR-187_st	MIR187	*Macaca mulatta*	18	-1.5627	0.0109
	rno-miR-187_st	MIR187	*Rattus norvegicus*	18	-1.6453	0.0115
	eca-miR-187_st	MIR187	*Equus caballus*	8	-1.4433	0.0178
	ssc-miR-187_st	MIR187	*Sus scrofa*	6	-1.5479	0.0190
	hsa-miR-187_st	MIR187	*Homo sapiens*	18	-1.6083	0.0424

Bt>Bi or Bt<Bi: Higher or lower transcription abundance in Bt than Bi. AffyID: Probe set ID in Affymetrix database for corresponding transcripts. Chr: Chromosome. Missing cells: Currently unannotated in bovine genome.

Table 5.5. Annotation of 5 miRNA transcripts for significant paternal genome effects, with $\log_2$ fold change (Log FC) from Bt (*Bos taurus taurus*, Angus) to Bi (*Bos taurus indicus*, Brahman) and false discovery rate (FDR) adjusted *P*-values.

AffyID	Gene symbol	Species	Chromosome	Log FC Bt-Bi	*P*
bta-miR-184	MIR184	*Bos taurus*	21	1.5683	0.0342
eca-miR-184	MIR184	*Equus caballus*	1	1.6591	0.0463
hsa-miR-184	MIR184	*Homo sapiens*	15	1.5679	0.0463
mmu-miR-184	MIR184	*Mus musculus*	9	1.5520	0.0463
rno-miR-184	MIR184	*Rattus norvegicus*	8	1.4016	0.0474

AffyID: Probe set ID in Affymetrix database for corresponding transcripts.

5.4.2 Biological pathways for differentially expressed transcripts

Eighty-one annotated differentially expressed transcripts for maternal and paternal genetic and fetal sex effects, were analysed and five significant biological pathways, largely involved in energy production in mitochondria, were identified (**Figure 5.4**, **Table S5.1**). These pathways included phosphorylation (ten transcripts, $P < 0.001$), respiratory chain (eight transcripts, $P < 0.0001$), mitochondrial inner membrane (seven transcripts, $P < 0.001$), NADH dehydrogenase

(ubiquinone) activity (seven transcripts, $P < 0.0001$) and hydrogen ion transmembrane transporter activity (four transcripts, $P < 0.01$). Most transcripts involved in these pathways were located in the mt genome (**Figure 5.4**, **Table S5.1**).

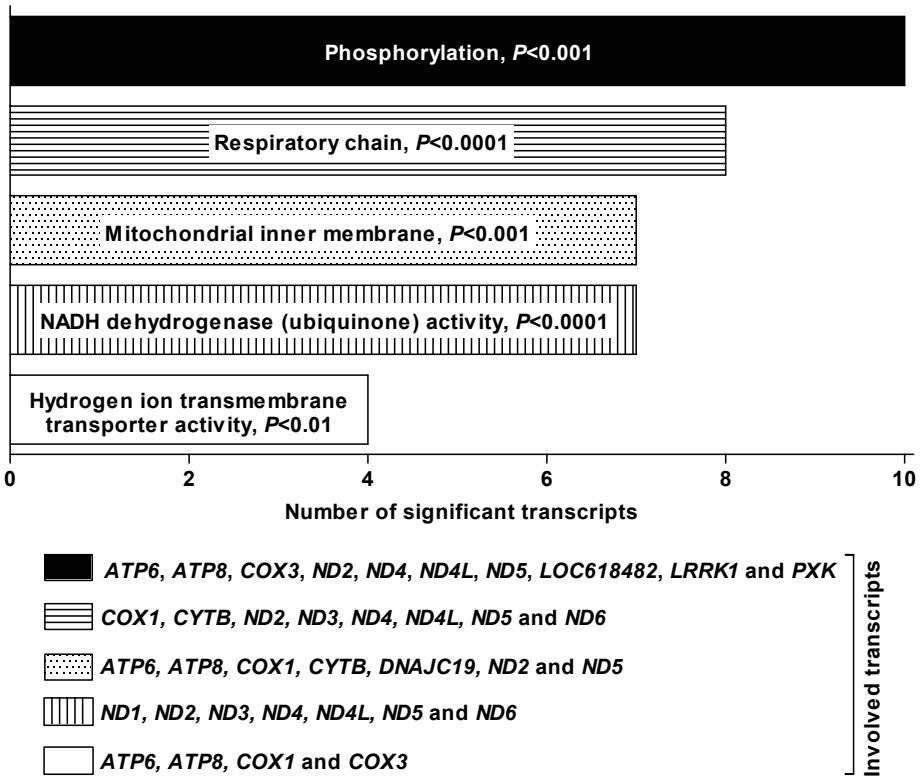

Figure 5.4. Biological pathways identified for differentially expressed transcripts in fetal liver at midgestation.

P-value for Fisher exact test of enrichment analysis and name of involved transcripts for each pathway is indicated.

5.4.3 Transcript coexpression differentiated by parental genome effects

Our analysis identified two transcript coexpression networks (**Figure 5.5**, **Table S5.2-5.3**). Network one included 53 coexpression events between mRNA and miRNA/snoRNA transcripts significantly affected by maternal genome, and a majority of these coexpression events (40/53) were negative relationships (**Figure 5.5A**, **Table S5.2**). For mRNA transcripts involved in

network one, TAZ showed the largest number of coexpression events (6) with miRNA transcripts, all of which were positive relationships. For miRNA/snoRNA transcripts involved in network one, *SNORD113-9* displayed the largest number of coexpression events (16) with mRNA transcripts, with most these events also being negative relationships (14/16). The miRNA transcript, *MIR1973* showed the largest number of positive coexpression events (5) with mRNA transcripts (**Figure 5.5A**, **Table S5.2**). Coexpression network two consisted of 29 coexpression events between mRNA and *MIR184* transcripts significantly affected by paternal genome, and the majority of the coexpression events were negative relationships (**Figure 5.5B**, **Table S5.3**). Positive coexpression events (5) were largely shown between pancreatic alpha-amylase, *AMY2A*, and *MIR184* transcripts (**Figure 5.5B**, **Table S5.3**).

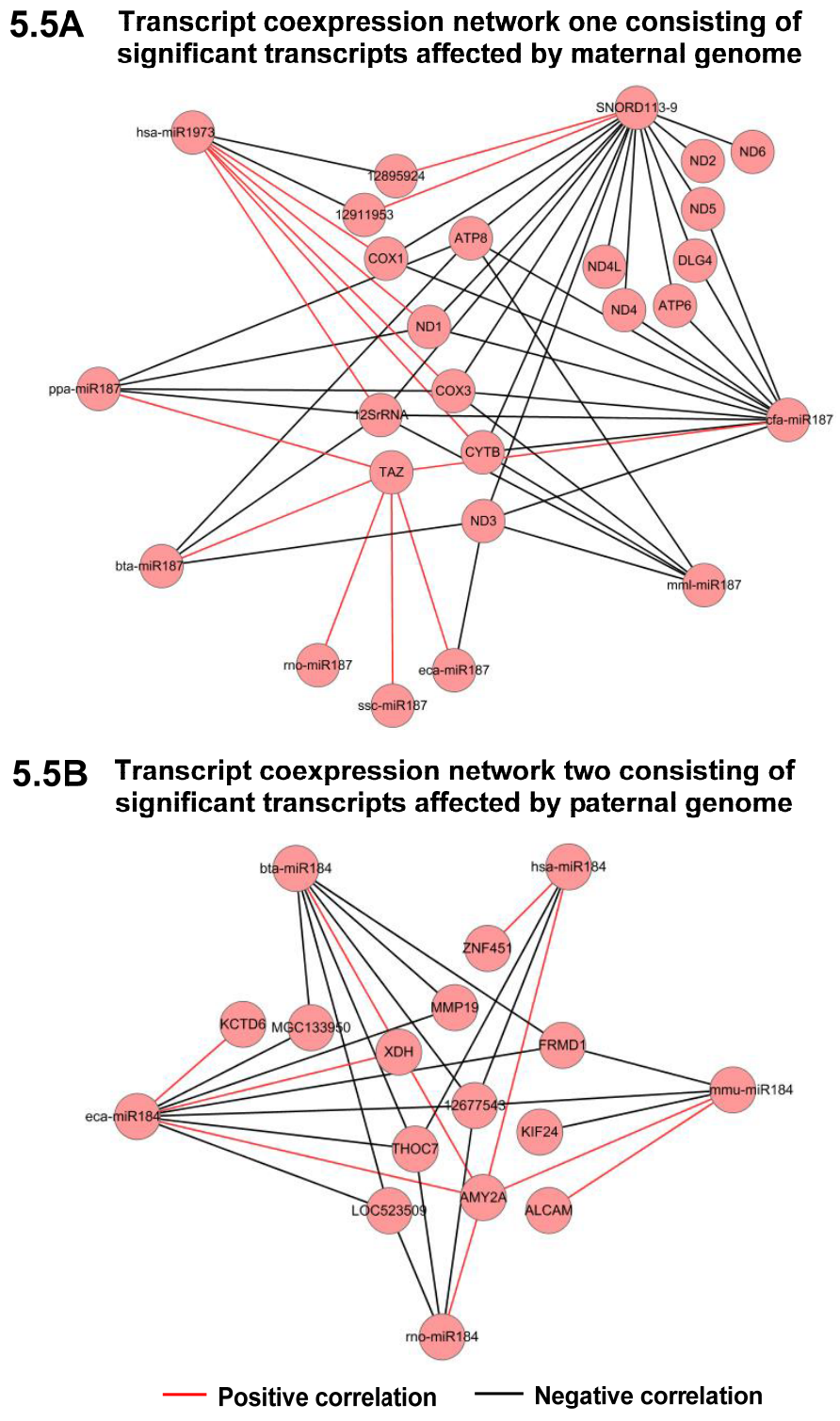

Figure 5.5. Transcript coexpression networks differentiated by maternal and paternal genome effects in fetal liver at midgestation.

Two coexpression networks are composed of mRNA and non-coding RNA transcripts significantly affected by maternal genome (A) and paternal genome (B). miR: microRNA. SNORD: small nucleolar RNA. bta: *Bos taurus*. cfa: *Canis*

familiaris. eca: *Equus caballus*. hsa: *Homo sapiens*. mml: *Macaca mulatta*. ppa: *Pan paniscus*. rno: *Rattus norvegicus*. ssc: *Sus scrofa*.

Predicted target mRNAs were searched in TargetScanHuman database for differentially expressed miRNA transcripts and were matched with differentially expressed mRNA transcripts in our results. This approach identified two mRNA and miRNA pairs: a) *ECE2* and bovine *MIR187* transcripts both significantly affected by maternal genome, and b) *MMP19* and bovine *MIR184* transcripts both significantly affected by paternal genome (**Figure 5.6**). Strong negative Pearson correlations were identified for transcription intensity value between a) *ECE2* and bovine *MIR187* transcripts (r = -0.57, P < 0.01), and b) *MMP19* and bovine *MIR184* transcripts (r = -0.74, P <0.0001) (**Figure 5.6**).

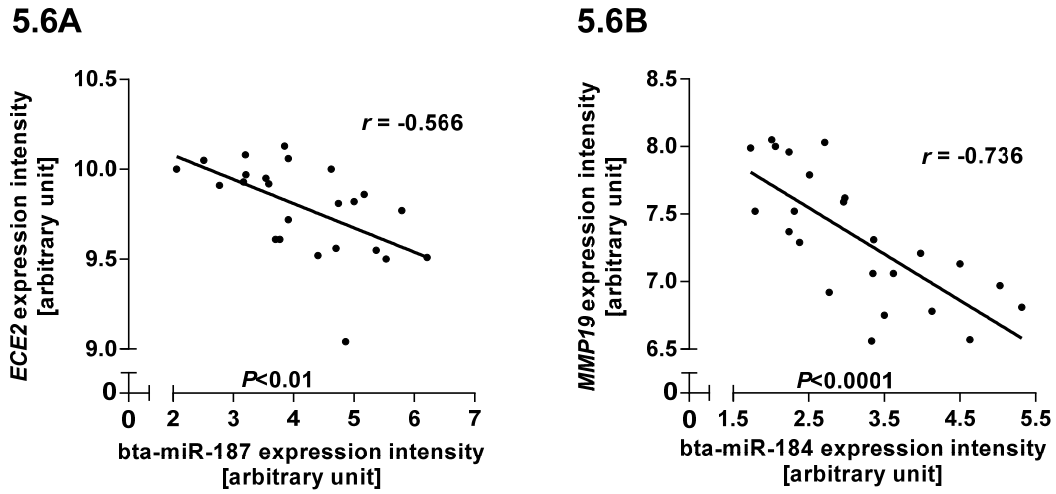

Figure 5.6. Pearson correlation regressions of microarray expression intensity values between significant miRNAs and predicted target mRNAs.

P-values and coefficients (*r*) of Pearson correlation between ECE2 mRNA and bovine MIR187 microRNA transcripts (A) and between MMP19 mRNA and bovine (bta) MIR184 microRNA (B) are indicated.

5.5 Discussion

We demonstrated, for the first time, significant differential effects of parental genomes on prenatal phenotype and associated mRNA and miRNA transcription profiles in a large mammal. Biased parental genome effects on fetal liver weight at midgestation were extended to the transcriptome level, where such effects were exerted in different mRNA, miRNA/snoRNA transcript groups.

5.5.1 Complex maternal genome effects

At the phenotypic level, the observation of dominant maternal genome effects on fetal liver weight (**Figure 5.1B,C**) agreed with our previous finding using a larger sample size (Chapter 2, **Figure 1.1**). In line with these results, at the transcriptome level, maternal genome had strong effects (P < 0.05-0.0001) on mRNA, miRNA and snoRNA abundances (**Figure 5.2B, 5.3A**). Maternal genome significantly affected expression levels of a large number of mt transcripts (**Figure 5.2A**, **Table 5.1**), which predominantly contributed to the biological pathways involved in mitochondrial energy production (**Figure 5.4**, **Table S5.1**). Physiologically, liver is vital for maintaining metabolic homeostasis and is critical to fetal development. Therefore, enhanced advanced mitochondrial activity in fetuses with Bt maternal genome can contribute to higher liver weight in fetuses with Bt maternal genome, as observed in our study. Non-coding RNAs also contributed to mitochondrial metabolism, where *SNORD113-9* and *MIR187* displayed strong suppressing effects on mitochondrial gene expression levels (**Figure 5.5A, 5.6A**), and lower expression levels in fetuses with Bt maternal genome (**Figure 5.3A**). Conversely, *MIR1973* generally increased mt gene expression (**Figure 5.5A**), with higher expression levels in fetuses with Bt maternal genome (**Figure 5.3A**). Our findings indicate the complexity of strong maternal genome effects which include interplay of multiple genetic and epigenetic mechanisms. According to our experimental design, that focused on differential (epi)genomic effects resulting

from Bt and Bi genome interaction, we propose two possible mechanisms that contribute to predominant maternal genome effects on liver phenotype and gene expression levels, as follows.

Firstly, significant maternal genome effects on mt transcripts and liver weight may be largely due to variations between Bt and Bi maternal mt genomes, transmitted from dam to fetus through ooplasm. Previously, the critical role of maternal mt genome variation in determining postnatal biological and clinical traits has been demonstrated, including for ruminant muscle mass (Mannen *et al.* 1998), body weight and height (Derr *et al.* 2012), human blood pressure (Liu *et al.* 2012) and insulin sensitivity (Fall *et al.* 2012). However, we found that the maternal mt genome, rather than the nuclear genome, is a strong determinant in prenatal mRNA transcription and liver development. This is not surprising considering previously reported significant effects of Bt and Bi mtDNA on cellular respiration, development and phenotype of transmitochondrial cloned cattle fetuses generated with Bi or Bt mtDNA and the same Bt nuclear donor (Hiendleder *et al.* 2004). Such dramatic effects on gene expression may be due to significant differences between Bt and Bi mt genome sequences that contribute to phenotypic diversity in these two subspecies (Hiendleder *et al.* 2008). Secondly, when considering transcript abundance as a phenotype affected by maternal genome effects, it is possible to attribute significant maternal genome effects in mt transcript expression to maternally expressed (epi)genetic factors, e.g., maternally expressed genes, according to our previous interpretations (Xiang *et al.* 2013). Such specific epigenetic effects on mt transcript expression level seem to be completely novel. However, epigenetic effects, such as allele-specific parent-of-origin expression, have been previously identified in the mt ribosomal protein gene (MRPL48) in mouse brain (Gregg *et al.* 2010). Furthermore, recent data demonstrated that epigenetic factors, such as DNA methyltransferase 1, which is strongly associated with DNA methylation and genomic imprinting, regulated mt gene transcription in human and mouse (Shock *et al.* 2011). As the nuclear genome closely interacts

with the mt genome to fulfil biological functions (Poyton and McEwen 1996), it is possible that imprinted genes contribute to regulation of mt gene transcription.

We have observed significant maternal genome effects on miRNA and snoRNA transcripts, including *MIR1973*, *MIR187* and *SNORD113-9* (**Figure 5.3A**). Firstly, *SNORD113-9* is a transcript member of the human C/D box snoRNA family, most of which are reported to guide epigenetic methylation of substrate RNAs (Galardi *et al.* 2002). Furthermore, *SNORD113-9* is located in the *DLK1-DIO3* imprinting domain on chromosome 14 (Valleron *et al.* 2012), which also indicates involvement in genomic imprinting (Cavaillé *et al.* 2002). This is in line with our results where *SNORD113-9* transcript was significantly affected by biased maternal genome effects (**Figure 5.3A**). Moreover, *SNORD113-9* transcript was closely coexpressed with a number of mt transcripts, which were also significantly affected by maternal genome effects (**Figure 5.5A**). This is consistent with suggested strong association between nuclear small RNAs and mt genome expression level (Das *et al.* 2012; Sripada *et al.* 2012) and can be another clue to support our hypothesis that genomic imprinting mechanisms may be associated with regulation of mt gene transcription.

Our results that differentially expressed miRNA transcripts for maternal genome effects are coexpressed with a number of mt transcripts indicate that epigenetic effects on mt gene transcription may be carried out by miRNA interference. This is supported by previously reported data where miRNAs were identified to be expressed in mt genome and regulators of mt gene expression (Burchard *et al.* 2010; Bandiera *et al.* 2011; Barrey *et al.* 2011). Furthermore, our result showing a large number of strong negative coexpression events between mt mRNA and miRNA/snoRNA transcripts (**Figure 5.5A,5.6A**) is highly consistent with recent findings where mammalian microRNAs predominantly act to decrease target mRNA expression levels (Guo *et al.* 2010; Mukherji *et al.* 2011). Although no differentially expressed predicted target mt

mRNAs for miRNA transcripts were identified, we believe that future research with larger sample size and precise cell biology technology will allow us to identify and validate such epigenetic nuclear-mt genome interaction.

The observed strong maternal genome effects on fetal liver development are likely due to maternal genome determination of mitochondrial energy production. The complex mechanisms of predominant maternal genome effects may be due to either transmission of maternal mt variation to fetal genome, epigenetic factors such as imprinted genes expressed from the nuclear genome, or combined effects. The possible epigenetic crosstalk between nuclear and mt genomes could be facilitated by non-coding RNA interference. However, the mechanism for such crosstalk is not completely understood and further experimental confirmation is required (Sripada *et al.* 2012)..

5.5.2 Paternal genome effects

All mRNA transcripts affected by paternal genome effects are from genes on autosomes and showed participation in pathways representing mitochondrial energy production (**Figure 5.3**). Furthermore, we found that the mRNA-miRNA coexpression network is affected by paternal genome effects which indicates that *MIR184* (**Figure 5.5B**), as a posttranscriptional regulator of mRNA expression (Cullen 2004), had repression effects on a variety of autosome genes (**Figure 5.5B**). Repression effects were further demonstrated by the highly significant negative regression between bovine *MIR184* and its predicted target mRNA transcript, *MMP19* (**Figure 5.6B**), which is responsible for breakdown of the extracellular matrix in prenatal development processes (Yu *et al.* 2012). The mRNA transcripts positively coexpressed with *MIR184* showed higher abundances in fetuses with Bt paternal genome, and mRNA transcripts negatively coexpressed with *MIR184* showed lower transcript abundances in fetuses with Bt paternal genome, compared

to fetuses with Bi paternal genome. This indicates that *MIR184* plays an important role in prenatal development of liver.

5.5.3 Fetal sex effects

We identified strong fetal sex effects on transcript abundance of mRNAs, most of which (17/26) were from sex-linked chromosomes. This indicates that sex dimorphism in midgestation liver weight is likely due to sex differentiation at the transcriptome level. Strong sex effects on bovine transcriptome were previously reported in blastocysts of day-7 embryos, although sex differentiation on corresponding phenotype was not evident (Bermejo-Alvarez *et al.* 2010). In our study, at midgestation transcriptome sex differentiation is demonstrated by markedly higher abundances of Y-chromosome transcripts, i.e., *DDX3Y*, *USP9Y* and *ZFY*, in male fetuses and landmark X-chromosome transcript, X-inactivation gene, XIST, in female fetuses (**Figure 5.2C**, **Table 5.3**). Furthermore, we also identified fetal sex effects on expression level of transcripts from autosomes, with higher abundances in males, i.e., *LOC784883*, *ZNF280B* and *SEPT10*, suggesting widespread sex effects on the transcriptome. Therefore, our results provide further evidence for the hypothesis that sex effects on prenatal phenotype have a substantial transcriptome basis in mammals, independent of sex-specific hormonal effects (Bhasin *et al.* 2008; Bermejo-Alvarez *et al.* 2010).

In conclusion, we show for the first time, significant differential maternal and paternal genome, and fetal sex, effects on phenotypic and transcriptomic characteristics in midgestation fetal liver. Dissection of such effects suggests that non-Mendelian (epi)genetic crosstalk between nuclear and mt genome could be a major mechanism driving fetal growth. Identified associations between miRNA and mRNA expression, consistent with previous data where nuclear miRNA regulates mt gene expression (Das *et al.* 2012; Sripada *et al.* 2012), suggest the miRNA interference may contribute to such crosstalk. Future research with precise molecular targeting

techniques can further validate differentially expressed mRNAs, non-coding RNAs and corresponding coexpression relationships. Our work extends the knowledge of interplay of epigenetic mechanisms driving mammalian development (Vaissière *et al.* 2008) and provides novel insights into understanding mendelian and nonmendelian genome and epigenome communication.

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Chapter 6: General discussion

Chapter 6

General Discussion

6.1 Introduction and overview

Lifelong development is largely programmed prenatally. During prenatal development, the placenta controls nutrient transfer between mother and fetus and impacts on lifelong health of the offspring (Lewis *et al.* 2006). The musculoskeletal system is another core tissue (Pedersen and Febbraio 2008; DiGirolamo *et al.* 2012) programmed to a considerable extent during prenatal developmental stages (Sayer and Cooper 2005; Dennison *et al.* 2010). Programming starts at the genome level with epigenetic modification (Cox *et al.* 2012; Sookoian *et al.* 2013) which alters transcript abundance profiles and consequently giving rise to variation in associated cell types and phenotypic traits (Jansen and Nap 2001). Previous data has demonstrated a large Mendelian genetic basis of placental and musculoskeletal traits, including placenta weight (Mesa *et al.* 2005; Buresova *et al.* 2006) and postnatal musculoskeletal mass (Smith *et al.* 1973; Gueguen *et al.* 1995; Arden and Spector 1997; Larzul *et al.* 1997). Transcript abundance profile, defined as a molecular phenotype (Jansen and Nap 2001), also displayed a significant Mendelian genetic basis (Dixon *et al.* 2007). Furthermore, significant additional, non-Mendelian genetic components, including mitochondrial (mt) DNA variation, sex-chromosome linked effects (Engellandt and Tier 2002; Amen *et al.* 2007; Guo *et al.* 2011; Estrada *et al.* 2012) and epigenetic factors such as parent-of-origin effects caused by genomic imprinting (Jin-Tae Jeon 1999; Nezer *et al.* 1999; Van Laere *et al.* 2003; Wolf *et al.* 2008), were previously reported to contribute to variation in postnatal musculoskeletal traits. However, comprehensive data on such effects on placental and fetal traits, including gross and/or histological parameters of important

tissues and associated molecular traits, were lacking. Such data can provide novel insights into the magnitude of maternal and paternal genome contribution to mammalian development and experimental evidence to support the hypothesis of evolutionary origin of parent-of-origin effects driving prenatal development.

In the present study, we used a resource collection of defined bovine fetuses consisting of purebreds and reciprocal hybrids with *Bos taurus*, Angus (Bt) and *Bos indicus*, Brahman (Bi) genetics, and thus well defined divergent parental genomes, to dissect maternal and paternal genome effects on comprehensive traits of the placental-fetal system including umbilical cord, fetal fluids and organs, musculoskeletal system and liver transcriptome. We have provided answers to the four research questions proposed in Chapter 1 by clearly demonstrating significant differential maternal and paternal genome effects on:

(1) the placental-fetal system, including umbilical cord, fetal fluid and organ characteristics, Chapter 2.

(2) the fetal bone system, representing a variety of directly measured weight and geometry parameters, Chapter 3.

(3) fetal myofibre characteristics and muscle weights with effects of imprinted non-coding RNA H19 in muscle, Chapter 4.

(4) fetal liver phenotype and mRNA and miRNA transcript abundance profiles, Chapter 5.

Along with these novel findings, we identified phenotypic coregulation and transcript coexpression networks which can be also differentiated by maternal and paternal genome effects. These findings bring our understanding of (epi)genomic effects driving prenatal development to a system level.

6.2 Parental genome effects

Maternal and paternal genome explained the highest proportion of variation, as determined by general linear models, in a majority of investigated phenotypic traits, i.e., 58.2−99.5% of variation in placenta, fetal organs, umbilical cord and fetal fluids weights, 19−99.5% of variation in principle components representing bone geometry and bone weight parameters and 56−96% of variation in myofibre characteristics and muscle mass. These findings are in line with previously reported significant genetic effects in placental and postnatal traits of the musculoskeletal system (Dequeker *et al.* 1987; Gueguen *et al.* 1995; Seeman *et al.* 1996; Arden and Spector 1997; Larzul *et al.* 1997; Beamer *et al.* 1999; Deng *et al.* 1999; Mesa *et al.* 2005; Buresova *et al.* 2006; Yu *et al.* 2007). Observed strong parental genome effects on transcript abundance profiles are also in agreement with reported substantial heritability estimates for mRNA expression levels (Dixon *et al.* 2007; Emilsson *et al.* 2008; Visscher *et al.* 2008).

6.2.1 Complex maternal genome effects

We identified strong and predominantly maternal genome effects on a majority of traits in the placental-fetal system (Chapter 2), fetal musculoskeletal system (Chapter 3 and 4) and in liver transcript abundance profile (Chapter 5) at midgestation. This indicates that the maternal genome plays a dominant role in driving fetal development at midgestation. In the context of our experimental design, which focused on genetic variation in fetal development, such predominant maternal genome effects may be due to the combined effects of multiple mechanisms including mt genome effects and maternally expressed genes with genomic imprinting. The assumption that the mt genome contributed to the maternal genome effect is supported by our results in Chapter 5, where maternal genome significantly affected fetal liver weight and expression levels of a significant number of mt genes. Previously, significant mt genome effects were reported in placenta (Aggarwal *et al.* 2001), postnatal musculoskeletal system in mammals (Mannen *et al.*

1998; Guo *et al.* 2011), and transcript abundance profile in fish liver (Flight *et al.* 2011). Studies in transmitochondrial cloned cattle also identified significant effects of Bt and Bi mt DNA on cellular respiration, including hepatocytes, and embryo-fetal development at prenatal stages (Hiendleder *et al.* 2004). Along with the finding that significant variation in maternal mt genome sequence between Bt and Bi likely contribute to the significant phenotypic diversity in these two subspecies (Hiendleder *et al.* 2008), it is highly likely that the mt genome effects accounted for a large proportion of non-Mendelian maternal genome effects that predominantly affect midgestation placental and fetal phenotype. Therefore, future research will include testing maternal genome effects on expression levels of mt genes in other tissues where predominant maternal genome effects were also identified, such as placental and fetal tissue including the musculoskeletal system.

It is possible to attribute observed maternal genome effects partly to maternally expressed imprinted genes, considering that previous reports in mammals showed that maternally expressed *GNAS* (Sikora *et al.* 2011) and *H19* (Gabory *et al.* 2010) are critical to growth and development. This assumption is supported by our results where midgestation muscle mass showed strong negative correlations with maternally expressed *H19* transcript abundance (Chapter 4). Furthermore, previous findings suggested that *H19* is the master regulator of an imprinted gene network with important roles in growth and development (Gabory *et al.* 2010) harbouring a miRNA that suppresses *IGF1R* expression and prenatal growth (Cai and Cullen 2007; Keniry *et al.* 2012). Thus, imprinted gene expression and miRNA interference are both additional plausible mechanisms for maternal genome effects. In fact, we found that non-coding RNA transcripts, including *SNORD113-9*, *MIR187* and *MIR1973*, are differentially expressed for significant maternal genome effects (Chapter 5). This finding gives further support for the involvement of non-coding RNA interference mechanisms in significant maternal genome effects. Moreover, *SNORD113-9* is a member of C/D box snoRNA family, most of which guide

epigenetic substrate RNAs methylation (Galardi et al. 2002) and is located in the *DLK1-DIO3* imprinted domain (Valleron et al. 2012). This indicates that *SNORD113-9* is involved both in genomic imprinting and mRNA expression interference (Cavaillé et al. 2002). Therefore, our findings suggest that the coordination of genomic imprinting and miRNA interference contribute to significant maternal genome effects.

Multiple non-Mendelian (epi)genetic mechanisms may contribute to the significant maternal genome effects and it is likely that these mechanisms interact. This speculation is supported by our results where non-coding RNA transcripts, including *SNORD113-9* from the imprinted *DLK1-DIO3* domain, strongly coexpressed with mt genes, and these transcripts are also significantly affected by maternal genome effects (Chapter 5). Previous reports demonstrated that genomic imprinting and miRNA inference regulated mt gene expression level. For instance, DNA methyltransferase 1, that is strongly associated with DNA methylation and genomic imprinting, regulated mt gene transcription in human and mouse (Shock *et al.* 2011). Several miRNA transcripts were identified to be expressed in the mt genome and shown to regulate mt gene expression (Burchard *et al.* 2010; Bandiera *et al.* 2011; Barrey *et al.* 2011). Furthermore, nuclear miRNAs were recently found to translocate into mitochondria and regulate mt gene expression in human heart (Das *et al.* 2012). Such miRNA-mt genome interaction is presumably carried out by the signalling complex at the outer mitochondrial membrane (Sripada *et al.* 2012). These findings suggested that epigenetic factors from the nuclear genome interact with the mt genome via non-coding RNA interference to regulate mammalian development. Along with our findings where aforementioned epigenetic mechanisms were identified within significant maternal genome effects, we propose that:

Strong maternal genome effects on fetal development are likely due to the interaction between mt genome effects and maternally expressed epigenetic factors from the nuclear genome via non-coding RNA interference.

Our finding provides further evidence to support that epigenetic crosstalk involves multiple epigenetic factors contributing to mammalian development. To validate such interactive epigenetic effects, the first step is to design an additional experiment that includes maternal mt genome effects and imprinting effects in the same crossbred animal herd. Precise subspecies-specific genetic markers, including mt DNA variation and nuclear genome DNA variants located in imprinted loci, must be included to allow us to separate and quantify these two types of maternal genome effects. In parallel, phenotypic data from a wide range of prenatal traits is necessary for correlation analyses with molecular traits, in order to compare the magnitude of differential maternal nuclear and mt effects. Corresponding transcriptome data from tissues that display significant maternal genome effects will also be required to determine expression status of maternally expressed genes and mt genes. Quantitative polymerase chain reaction and pyrosequencing techniques can be used to further validate differentially expressed mRNA and non-coding RNA transcripts and parent-of-origin expression pattern of imprinted genes. This future research will allow us to unravel the nature of strong maternal genome effects driving fetal development.

6.2.2 Parental genome and evolutionary basis of parent-of-origin effects

In contrast to the predominantly maternal genome effects described above, paternal genome effects accounted for less variation in the traits examined. However, traits that did display strong paternal genome effects provided novel insights into understanding the evolutionary origin of parent-of-origin effects. In our study, traits showing predominantly paternal genome effects included fetal fluid weights, umbilical cord weight and length, maternal placenta and umbilical

cord efficiencies (Chapter 2), limb ossification as a principle component representing calcified long bone length (Chapter 3), and muscle mass relative to fetal weight (Chapter 4). These findings along with aforementioned significant maternal genome effects suggest that paternal genome affects specific fetal traits and seems to have a different 'interest' in these traits compared to the maternal genome. This appears to fit hypotheses for the evolutionary basis for parent-of-origin effects, i.e., conflict-of-interest (Di Naro *et al.* 2001) and maternal-offspring coadaptation (Wolf and Hager 2006). The conflict-of-interest hypothesis states the coexistence of (imprinted) maternally and paternally expressed genes for suppressing and promoting fetal growth for their own interests in terms of genetic fitness, respectively (Haig 2004). However, in some cases, genomic imprinting was predominantly observed for maternally expressed genes; this includes the dominance of maternally expressed genes in placenta (Wagschal and Feil 2006). Therefore, Wolf *et al.* (2008) proposed the maternal-offspring coadaptation hypothesis which predicts that at the interface where mother and offspring are closely interacting, e.g., placenta, expression of maternal genes tends to be predominant for the sake of fetal development.

Our finding of predominant maternal genome effects on a majority of placental and fetal traits, as discussed above, is in agreement with the coadaptation hypothesis. However, considering all examined phenotypic traits together as a system, i.e., umbilical cord, fetal fluids, and relative muscle mass, strong paternal genome effects are still evident. As described earlier, umbilical cord is responsible for blood exchange between the mother and fetal liver, heart and brain via the placenta and is significantly associated with fetal growth (Di Naro *et al.* 2001). Fetal fluid is responsible for water and hormone circulation between mother and fetus (Sack *et al.* 1975; Ivell and Anand-Ivell 2009; Anand-Ivell *et al.* 2011). These traits largely represent the nutrient exchange capacity and efficiency between the mother, placenta and fetus. Therefore, determination by the paternal genome of these traits may represent the phenotypic effects of important paternal genes expressed at the placenta-fetus interphase on nutrient and/or hormone

transfer. This is also in line with the conflict-of-interest hypothesis of parent-of-origin effects where paternally expressed genes tend to govern resource transfer between mother and fetus (e.g. *IGF2* and *DLK1*) (Haig 2004; Frost and Moore 2010). Thus, we propose that:

a) Maternal-offspring coadaptation may be a better prediction of expression patterns for parent-of-origin effects in placenta or fetus *per se* at midgestation

b) Conflict-of-interest provides additional predictions of expression patterns for parent-of-origin effects when considering placenta and fetus as a system of comprehensive placental and fetal traits.

Varied predictions of the dominance of maternal or paternal imprinted genes by the maternal-offspring coadaptation and conflict-of-interest hypotheses at different phenotypic levels is also in agreement with the different tissue-specific expression pattern of parent-of-origin effects discussed by Wolf *et al.* (2008). Conversely, Wang *et al.* (2013) identified the dominance of paternally expressed genes at an early stage (33-35 days after ovulation) of placental development of cross-bred equines. The difference between this finding and our results of dominant maternal genome effects in the placental system along with prediction of maternal-offspring coadaptation may be explained by species and developmental stage specificity of parent-of-origin effects (Wolf *et al.* 2008; Wolf 2013).

Predominant paternal genome effects on limb ossification and relative muscle mass are harder to explain. Given the fact that paternally expressed genes, such as *IGF2* (Kang *et al.* 2011) and *Zac1* (Varrault *et al.* 2006), are critical for bone formation and ossification, and *IGF2* and *DLK1* are important for pre- and postnatal muscle development (Jin-Tae Jeon 1999; Nezer *et al.* 1999; Davis *et al.* 2004; Cockett *et al.* 2005), it is likely that genomic imprinting mechanisms may contribute to observed significant paternal genome effects. Therefore, investigation of imprinting

status of these genes in umbilical cord, fetal fluids, muscle and calcified bone tissue, and the association between expression levels of these genes with corresponding phenotypic or molecular traits, will improve the understanding of mechanisms involved in paternal genome effects.

6.2.3 Independent effects and interaction effects of parental genomes

We observed significant phenotypic differences between Bt and Bi parental genomes in traits of the placenta, fetus, fetal organs and the fetal musculoskeletal system, where the majority of these traits are increased in Bt compared to Bi. Breed differences in placental and fetal traits were previously reported (Reynolds *et al.* 1980; Anthony *et al.* 1986; Bellows *et al.* 1993), but our finding of significant breed phenotypic differences at midgestation is novel. Furthermore, we found several traits that display significant maternal and paternal interaction effects, such as fetal lung and fluid weights and umbilical artery diameters (Chapter 2), PC2/fetal limb ossification and PC4/flat bone elongation which are principle components representing calcified and flat bone length, respectively (Chapter 3), fast myotube CSA (cross-sectional area) and muscle *H19* expression level (Chapter 4). As summarised in **Figure 6.1**, the parental genome interaction effects on these traits displayed strikingly different expression patterns. Furthermore, these traits displayed predominant maternal genome contribution to genetic variation, except fetal fluids weight and PC2/limb ossification which showed predominant paternal genome contribution (see previous chapters). PC2/limb ossification showed a specific and positive effect of the paternal Bt genome (**Figure 6.1D**) and its phenotypic values in the four fetus groups are consistent with paternal imprinting patterns that appear to be specific for different time points during ontogenesis (Wolf *et al.* 2008). This paternal lineage-specific effect, together with other observed patterns of parental genome effects, demonstrates the specificity of (epi)genomic effects on different placental and fetal traits. According to Wolf *et al* (2008)'s interpretation for

the complex parent-of-origin expression patterns (**Figure 1.2**), we postulate that the different phenotypic patterns observed in our study indicate the existence of unique modes of epigenetic inheritance for placental and/or fetal traits, which are similar to the polar overdominance mode identified in sheep (Cockett *et al.* 1996), pig (Kim *et al.* 2004) and human (Wermter *et al.* 2008).

According to the classic definition of hybrid vigour effects (Lamkey and Edwards 1998), fetal fluids weight and PC2/limb ossification showed better-parent heterosis for Bt×Bi fetuses (maternal genome listed first, **Figure 6.1C,D**). To our knowledge, heterotic effects on prenatal bone and umbilical traits for Bt×Bi genotype at midgestation are novel, although such heterotic effects on birthweight of Bt×Bi was reported (Brown *et al.* 2000). This suggested the existence of complex non-Mendelian and non-additive genetic effects, which contribute to heterosis effects (Carlborg and Haley 2004; Melchinger *et al.* 2007), on specific fetal and placental traits. Future research will focus on validation of these tissue-specific effects by identification of imprinted genes, miRNAs or other genetic and epigenetic factors that account for large variation in phenotypic traits, which may lead to discovery of novel epigenetic inheritance modes and epigenetic mechanism that account for heterosis.

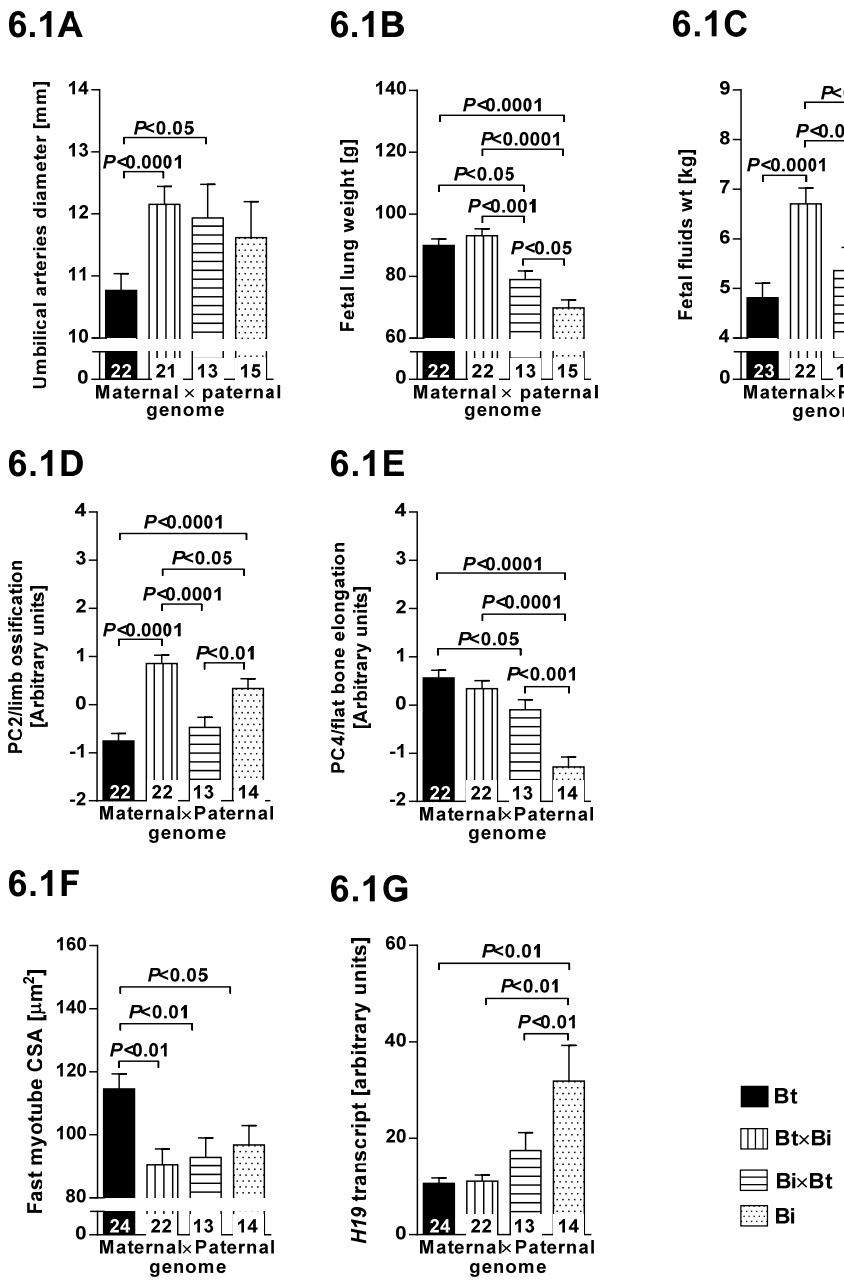

Figure 6.1. Effects of interaction of maternal and paternal genomes on placental and fetal traits at midgestation. Least square means with standard error of means and *P*-values for significant differences (*t*-test) between means are indicated. Bt: *Bos taurus taurus*, Angus. Bi: *Bos taurus indicus*, Brahman.

6.3 Fetal sex effects

We identified significant fetal sex effects on a majority of phenotypic traits and expression levels of a large number of liver mRNA transcripts at midgestation (Chapter 2-5). To our knowledge, evidence of sex effects on phenotypic and molecular traits at midgestation in mammalian development is novel, although differentially expressed mRNA transcripts for male and females were previously identified in Day 7 cattle embryos (Bermejo-Alvarez *et al.* 2010). In our study, male fetuses displayed advanced placental traits, which provides solid experimental data to support the sex specific placenta adaptation hypothesis, where morphological evidence was previously lacking (Clifton 2010). Such fetal sex and placental coadaptation can be carried out by hormonal dialogue between placenta and fetus (Geary *et al.* 2003; Anand-Ivell *et al.* 2011) and epigenetic mechanisms such as X-chromosome inactivation (Looijenga *et al.* 1999; Migeon *et al.* 2005), sex-linked genes (Sood *et al.* 2006) and microRNAs (Clifton 2010). Since advanced male fetal traits were identified along with advanced male placental traits, our finding also indicates that natural selection favours male fetuses at midgestation for nutrient supply, compared to female fetuses. This can explain the proposed higher ability of male fetuses at prenatal stage to adapt to adverse intrauterine environment, which ultimately affects male development in postnatal stages (Clifton 2010). Detailed molecular profiles for specific compartments of placenta, i.e., maternal and fetal layers, and umbilical cord tissue will allow further identification of the drivers of sex specific placenta adaptation.

In our study, transcriptome sex differentiation is demonstrated by the remarkably higher transcript abundance of Y-chromosome transcripts, i.e., *DDX3Y*, *USP9Y* and *ZFY*, in male fetuses and the landmark X-chromosome transcript, X-inactivation gene, XIST, in female fetuses (Chapter 5). The identification of significant fetal sex effects on autosome transcripts, such as *LOC784883*, *ZNF280B* and *SEPT10*, suggests widespread sex effects on the transcriptome.

These findings provide further evidence for a transcriptome basis of sex differentiation which is independent of sex hormones (Bhasin *et al.* 2008; Bermejo-Alvarez *et al.* 2010).

6.4 Non-genetic maternal effects

We have identified a substantial number of non-genetic maternal effects on placental and fetal traits including gross- and histo-morphological traits of placentomes (Chapter 2), limb elongation, a principle component representing total long bone length (Chapter 3), and muscle weights (Chapter 4). These non-genetic maternal effects were estimated independently of maternal genomes and represent pre-experimental environmental factors acting on dams (Xiang *et al.* 2013). These environmental effects could not be erased during approximately four weeks of adjustment under a controlled environment prior to the start of the experiment. Considering previous reports that pre-conception non-genetic effects, such as maternal nutrition status and life style, affect fetal bone growth (Godfrey *et al.* 2001; Mahon *et al.* 2010), it is not surprising that such effects are present in placental and fetal muscle traits as well. Different regression slopes between non-genetic maternal weights and fetal traits suggest that specific combinations of (epi)genetic and non-genetic factors can be used to optimise fetal development. Thus, it is tempting to explore the molecular basis of such combined effects. In the current study, due to limited sample size, we did not estimate the non-genetic effects on transcript abundance profile. Therefore, future studies with a larger sample size will allow us to elucidate genetic, epigenetic and non-genetic components and interactions that control corresponding phenotypic variation.

6.5 Phenotypic and transcriptional modules

In the present study, we identified differential maternal genome, paternal genome and/or fetal sex effects determined regression networks between placental and fetal phenotypic (Chapter 2) and transcriptional (Chapter 5) traits. This indicates the existence of morphological and

molecular modules under (epi)genetic control. Generally, the concept of modules refers to patterns of connectedness in which elements are grouped into highly connected subsets, with these subsets being more loosely connected to each other. The connectedness, mathematically speaking, is the correlation between elements, i.e., phenotypic or molecular traits, within the subset (Wagner *et al.* 2007). In our study, at the phenotypic level, maternal genome, paternal genome or fetal sex alone or together, determined various regressions between traits within the placental-fetal system. Further investigation of our results showed that strong maternal genome determined regressions between umbilical cord and placental traits, and paternal genome and/or fetal sex determined regressions between umbilical cord traits/fetal fluids weight and fetus/fetal organ weights (Chapter 2). This finding not only emphasises the importance of umbilical cord and fetal amniotic fluids in placental and fetal growth, but also suggests a novel morphological modularity of placental and fetal system with (epi)genetic effects.

Morphological modules are formed due to a coordination specific group of genes, and such effects refer to modular pleiotropic effects (Wagner *et al.* 2007). In addition to such genetic effects, our results of a) the placenta and umbilical cord module being predominantly affected by maternally expressed epigenetic factors; and b) the fetus/umbilical cord/fetal fluids module largely affected by paternal- and/or sex- linked (epi)genetic effects, indicates novel modular (epi)genetic effects. Our speculation of differential (epi)genetic effects on specific morphological modules within the placental-fetal system is likely to be correct, as imprinted genes, one of the most important epigenetic factors, commonly coordinate to regulate a specific set of traits (Wolf 2013). This assumption is also supported by other previous reports, where epigenetic factors, such as imprinted genes (Wagschal and Feil 2006; Frost and Moore 2010), microRNAs (Noguer-Dance *et al.* 2010) and sex-linked imprinted genes associated with X chromosome inactivation (Raefski and O'Neill 2005; Wagschal and Feil 2006), tend to be localised in clusters to differentially regulate placental and fetal growth. Another morphological module may exist

between maternal barrier thickness, a histo-placental trait, and a number of gross placental/fetal traits affected by maternal and paternal genome interaction. Barrier thickness is a determinant of placental substrate transfer capacity (Amaladoss and Burton 1985), and is usually negatively correlated with fetal size (Roberts *et al.* 2001). Therefore, our finding indicates a parental genome coregulated bottleneck effect of maternal circulation on placental and fetal growth at midgestation. This suggests that maternal and paternal (epi)genetic factors, can also cooperate to regulate the placental and fetal system.

At the transcriptome level, coexpressed mRNA and non-coding RNA transcripts are differentiated by significant maternal and paternal genome effects (Chapter 5). Considering transcript abundance as a trait and the fact that coexpression refers to correlated expression levels, our finding also indicates the existence of transcript networks and molecular modules that display epigenetic effects. In a module, correlated traits, function together and perform a similar biological task (Wagner *et al.* 2007). Thus, our finding of differential maternal and paternal genome effects on molecular modules describes the process in which transcriptome molecule clusters, including mt mRNAs, miRNA and snoRNAs, coordinate to receive and transmit differential parental genome effects to phenotype. Along with our finding of regression networks at the phenotype level, the data suggest that systematic integration of a wide range of phenotypic and/or molecular traits will provide improved understanding of complex placental-fetal system that significantly determine individual lifetime performance. Recent studies have already shown that inclusion of data from genome, phenome and corresponding epigenome, i.e., comprehensive 'omics' data, is making new breakthroughs in understanding human disease and improving animal breeding outcomes (Ge *et al.* 2003; Berry *et al.* 2011; Jain *et al.* 2013). By using this holistic approach, we may further understand the complex mechanisms behind (epi)genetic effects on mammalian prenatal development that contribute to human health and animal production.

6.6 General conclusions

In summary, we show for the first time, that a wide range of phenotypic and molecular traits within the placental-fetal system are affected by widespread differential maternal and paternal genome and fetal sex effects. We showed that predominant maternal genome effects on a majority of phenotypic and transcriptional traits are due to combined effects of maternal mt DNA variation and maternally expressed imprinting factors. Interaction between epigenetic factors from nuclear and mt genomes via non-coding RNA interference can also contribute to significant maternal genome effects, which provides additonal evidence for the coordination of multiple epigenetic mechanisms that regulate mammalian prenatal development. Differential maternal and paternal genome effects on specific placental and fetal phenotype provide important insights which can accommodate existing evolutionary hypotheses for parent-of-origin effects, such as conflict-of-interest (Moore and Haig 1991) and maternal-offspring coadapdation (Wolf and Hager 2006) hypotheses, and emphasises the importance of previously less studied umbilical cord and fetal fluids in placental and fetal development. Significant advances in the knowledge of developmental status of male placentae and fetuses at midgestation, provides solid evidence to support the sex specific placenta adaptation hypothesis (Clifton 2010), and demonstrated sex-specific crosstalk between fetus and placenta. Significant non-genetic maternal effects on placental and fetal traits suggest that specific combination of genetic and non-genetic factors can optimise fetal development. Next, separation of effects of maternal mitochondrial and maternally expressed genes in maternal genome effects on placental and fetal traits is required. This can be achieved by quantifying expression levels of mitochondrial and maternally expressed genes in tissues displaying significant maternal genome effects. Detailed molecular profiles are also required to elucidate genetic, epigenetic and non-genetic components and interactions that control variation in corresponding tissues. Identified morphological and transcriptional modules suggest the next level, i.e., systematic integration of omics data, of

understanding prenatal development. Considering emerging breakthroughs in studying human diseases and animal breeding with an integrated omics approach (Ge *et al.* 2003; Berry *et al.* 2011; Jain *et al.* 2013), coupling genome, epigenome with phenome data covering the complete placental-fetal system will provide a multi-layer picture of the coordination of molecular and phenotypic events driving mammalian prenatal development.

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Supporting figures and tables

Supporting figures:

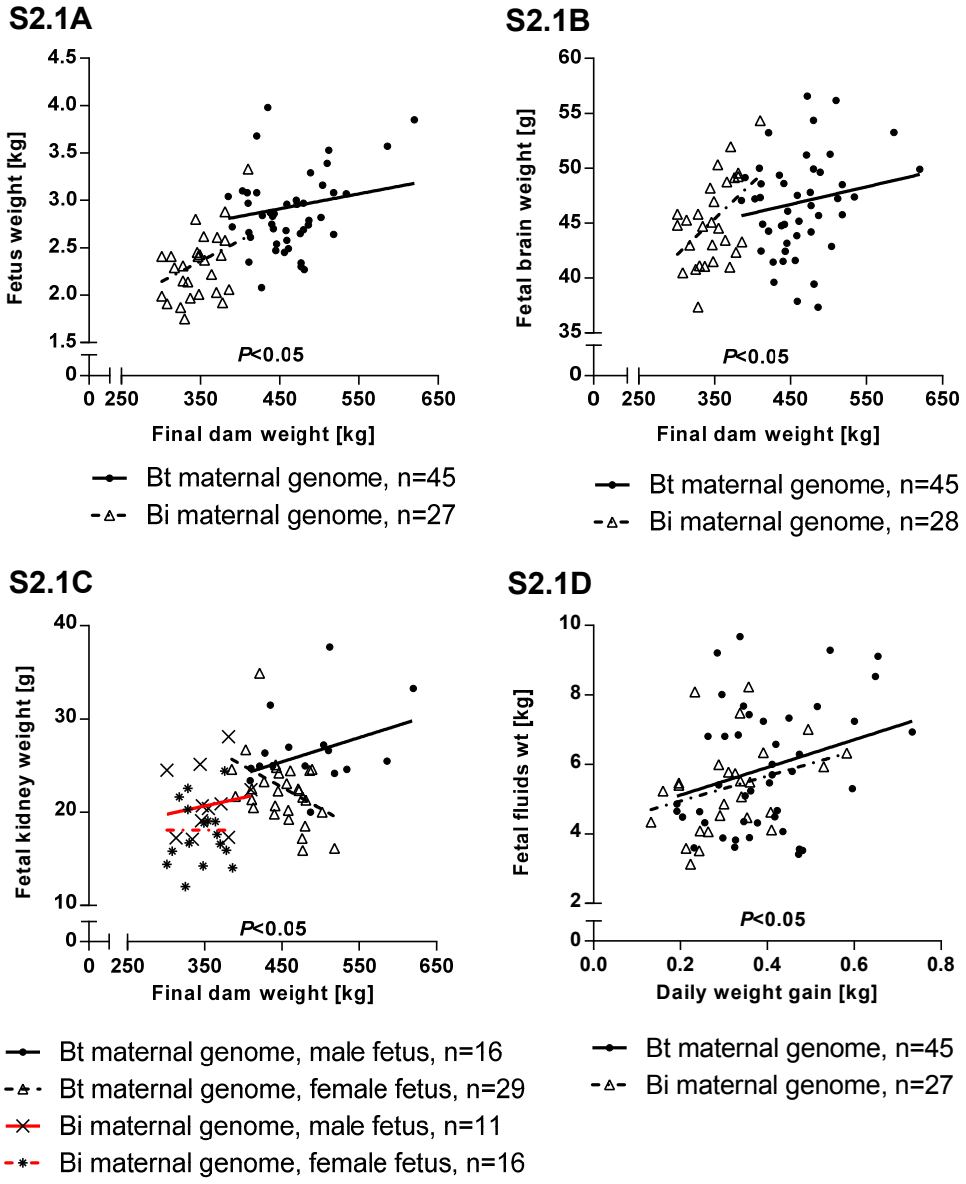

Figure S2.1. Effects of final maternal weight or daily weight gain nested within maternal genomes on fetal and organ weights weights at midgestation.

P-values (ANOVA) of significant linear regressions within Bt and Bi maternal genetics on weight of fetus (A), brain (B), kidney (C) and fetal fluids (D) are indicated. Bt: *Bos taurus taurus*, Angus breed. Bi: *Bos taurus indicus*, Brahman breed. n: animal number.

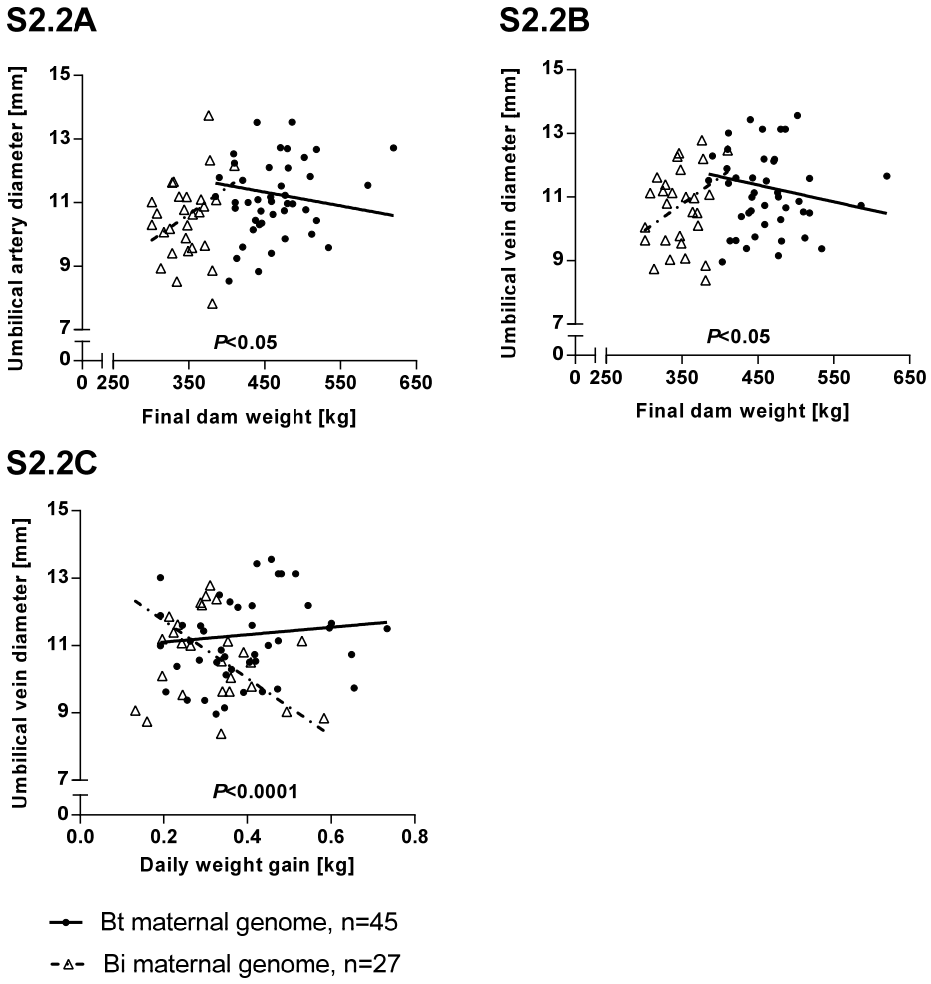

Figure S2.2. Effects of final maternal weight or daily weight gain nested within maternal genomes on umbilical artery/vein diameter at midgestation.

P-values (ANOVA) of significant linear regressions within Bt and Bi maternal genetics on weight of umbilical artery diameter (A) and umbilical vein diameter (B,C) are indicated. Bt: *Bos taurus taurus*, Angus breed. Bi: *Bos taurus indicus*, Brahman breed. n: animal number.

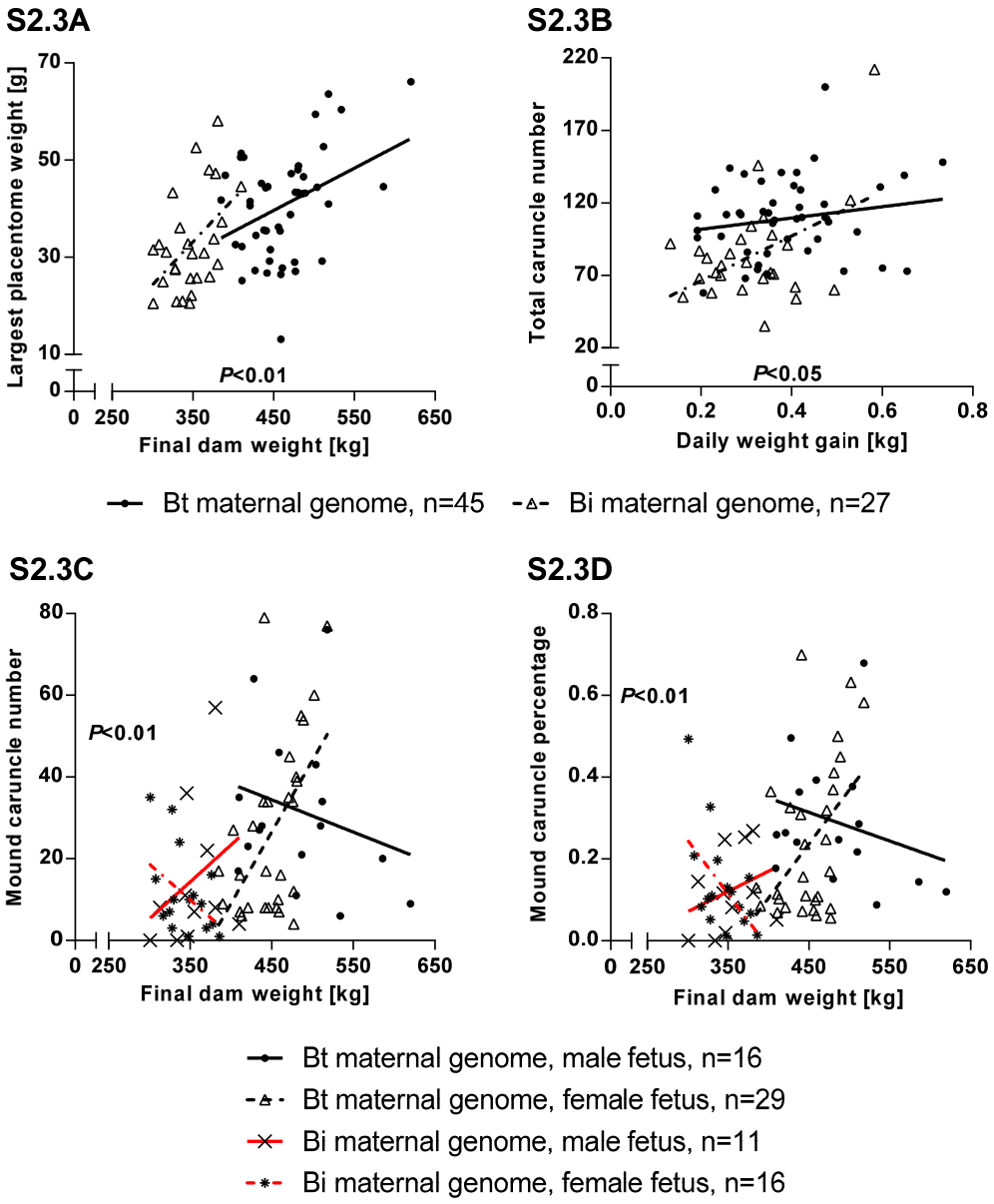

Figure S2.3. Effects of final maternal weight or daily weight gain nested within maternal genomes on gross-morphological placentome phenotype at midgestation.

P-values (ANOVA) of significant linear regressions within Bt and Bi maternal genetics on largest placentome weight (A), total caruncle number (B), mound carunle number (C) and percentage (D) are indicated. n: animal number. Bt: *Bos taurus taurus*, Angus breed. Bi: *Bos taurus indicus*, Brahman breed. n: animal number.

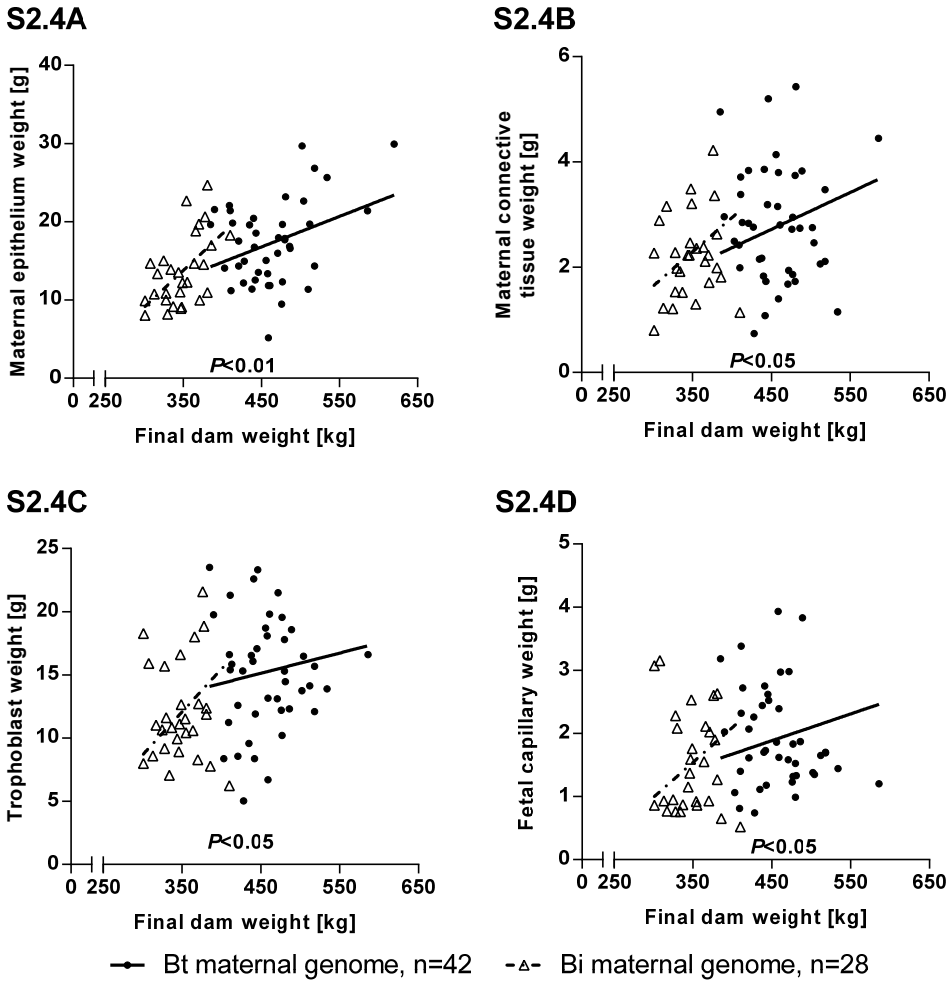

Figure S2.4. Effects of final maternal weight or daily weight gain nested within maternal genomes on histo-morphological placental phenotype at midgestation.

P-values (ANOVA) of significant linear regressions within Bt and Bi maternal genetics on maternal epithelium weight (A), maternal connective tissue weight (B), trophoblast weight (C) and fetal capillary weight (D) are indicated. Histo-morphological placental phenotype were determined for the largest placentome surrounding the fetus. Bt: *Bos taurus taurus*, Angus breed. Bi: *Bos taurus indicus*, Brahman breed. n: animal number. Figure continued in Figure S5.

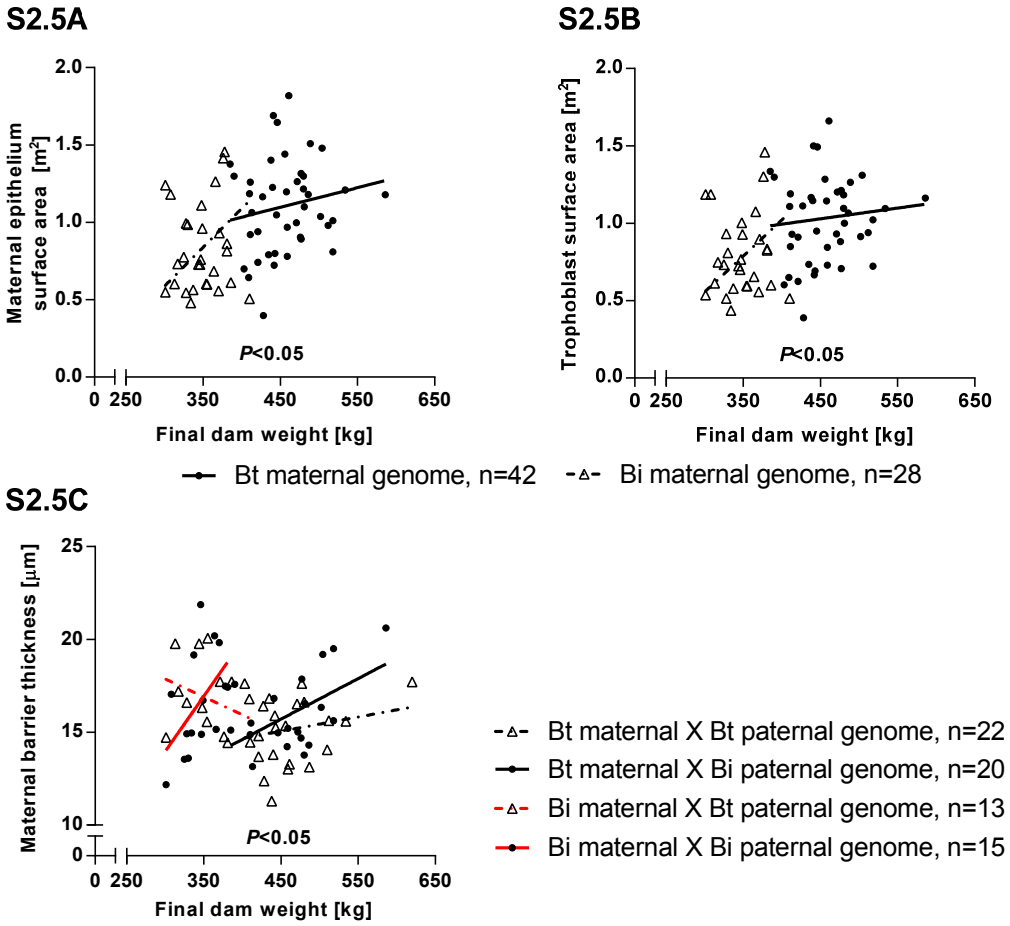

Figure S2.5. Effects of final maternal weight or daily weight gain nested within maternal genomes on histo-morphological placental phenotype at midgestation continued.

P-values (ANOVA) of significant linear regressions within Bt and Bi maternal genetics on maternal epithelium surface area (A), trophoblast surface area (B), and maternal barrier thickness (C) are indicated. Histo-morphological placental phenotype were determined for the largest placentome surrounding the fetus. Bt: *Bos taurus taurus*, Angus breed. Bi: *Bos taurus indicus*, Brahman breed. n: animal number.

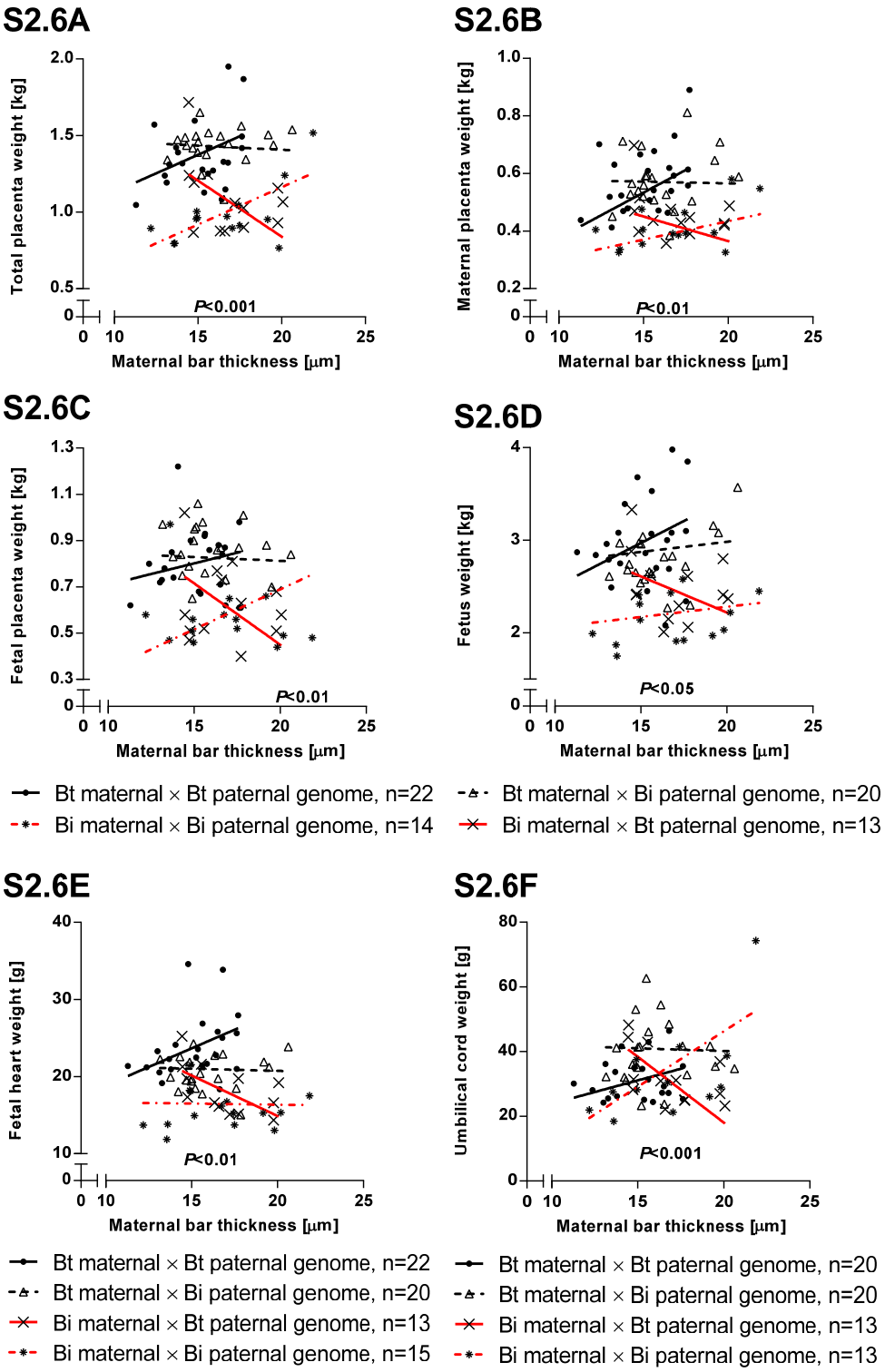

Figure S2.6. Specific regressions of gross placental/fetal phenotype on maternal barrier thickness nested within maternal and paternal genome interaction.

Representative significant regressions within maternal and paternal genetic interaction were plotted with *P*-values (ANOVA), of total placenta weight (A), maternal placenta weight (B), fetal placenta weight (C), fetus weight (D), fetal heart weight (E), and umbilical cord weight (F). Histo-morphological placental phenotype were determined for

the largest placentome surrounding the fetus. Bt: *Bos taurus taurus*, Angus breed. Bi: *Bos taurus indicus*, Brahman breed. n: animal number.

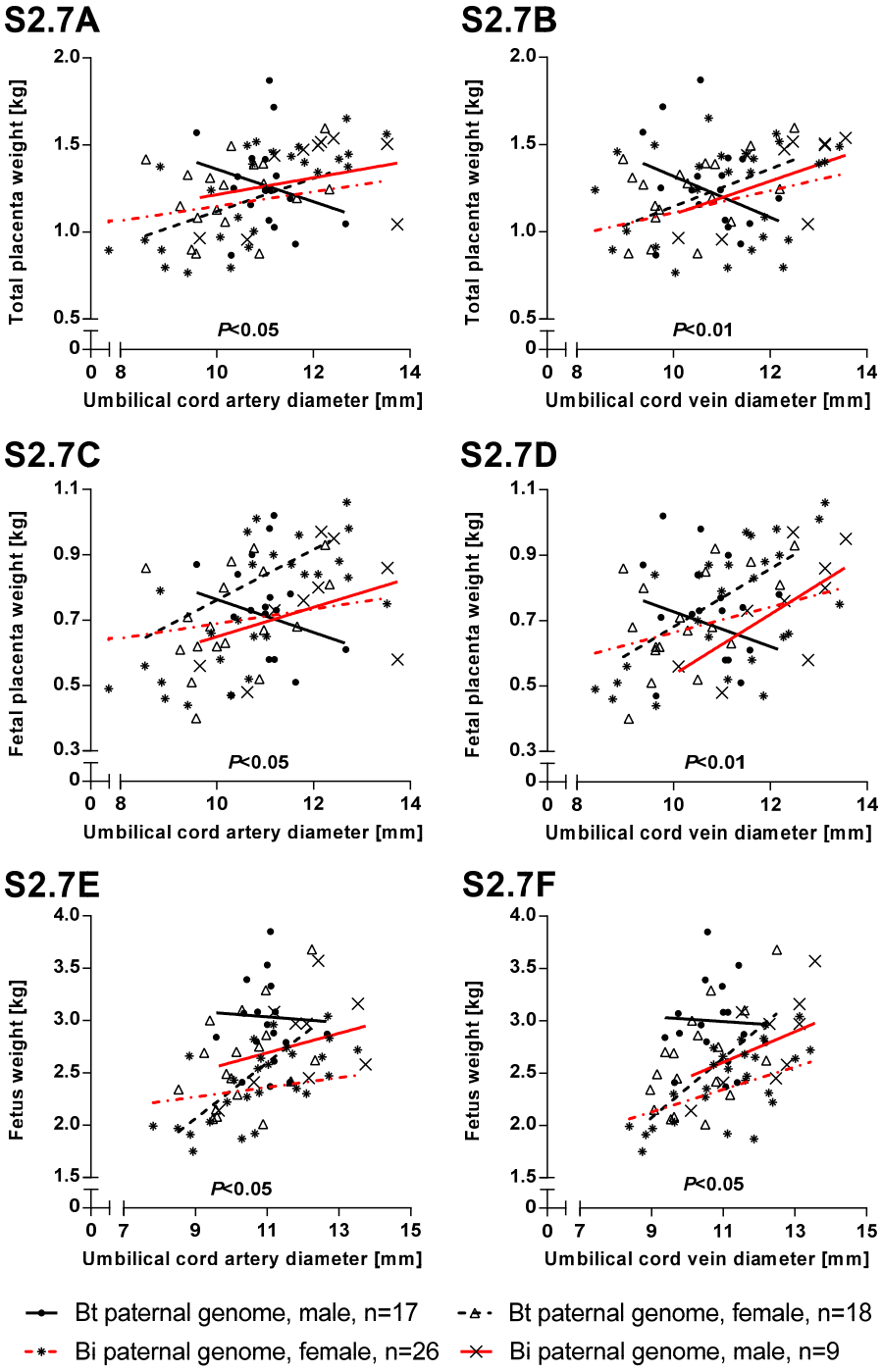

Figure S2.7. Specific regressions of gross placental/fetal phenotype on umbilical artery/vein diameter nested within paternal genome and fetal sex interaction.

Representative significant regressions within paternal genome and fetal sex interaction were plotted with *P*-values (ANOVA), between total placenta weight and umbilical artery diameter (A), total placenta weight and umbilical vein diameter (B), fetal placenta weight and umbilical artery diameter (C), fetal placenta weight and umbilical vein diameter (D), fetus weight and umbilical artery diameter (E), and fetus weight and umbilical vein diameter (F). Histo-morphological placental phenotype were determined for the largest placentome surrounding the fetus. Bt: *Bos taurus taurus*, Angus breed. Bi: *Bos taurus indicus*, Brahman breed. n: animal number.

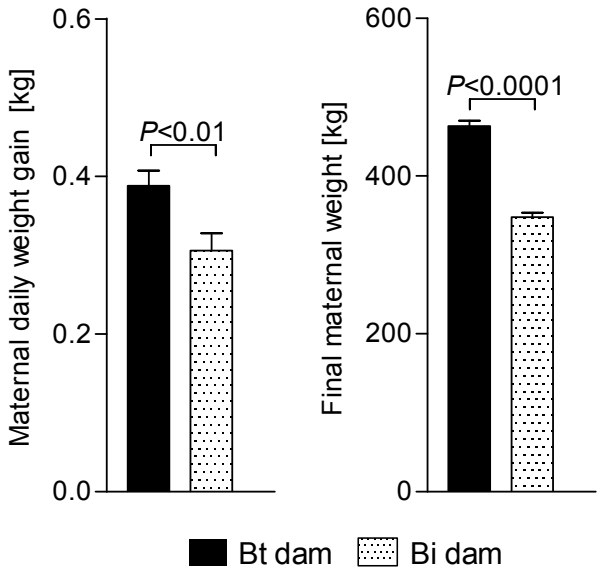

Figure S2.8. Daily weight gain and final weight for *Bos taurus taurus* and *Bos taurus indicus* dams.

(A) Post-conception maternal daily gain: Final maternal weight minus weight at conception divided by days of gestation. (B) Final maternal weight: Weight before slaughter on Day 153 of gestation. *P*-values for significantly different means (t-test) are indicated. Bt: Bos taurus taurus, Angus. Bi: Bos taurus indicus, Brahman.

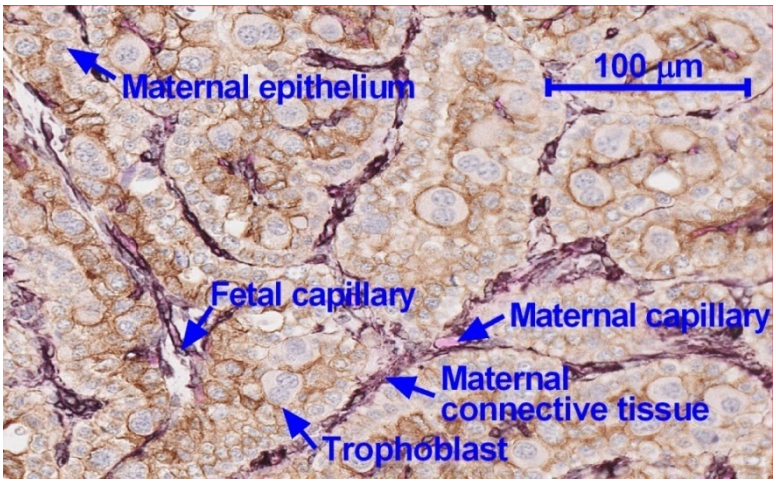

Figure S2.9. Example of immunohistochemical staining for fetal placentome at midgestation.

Arrows indicate placental cell types, including maternal epithelium, maternal capillary, maternal connective tissue, trophoblast and fetal capillaries.

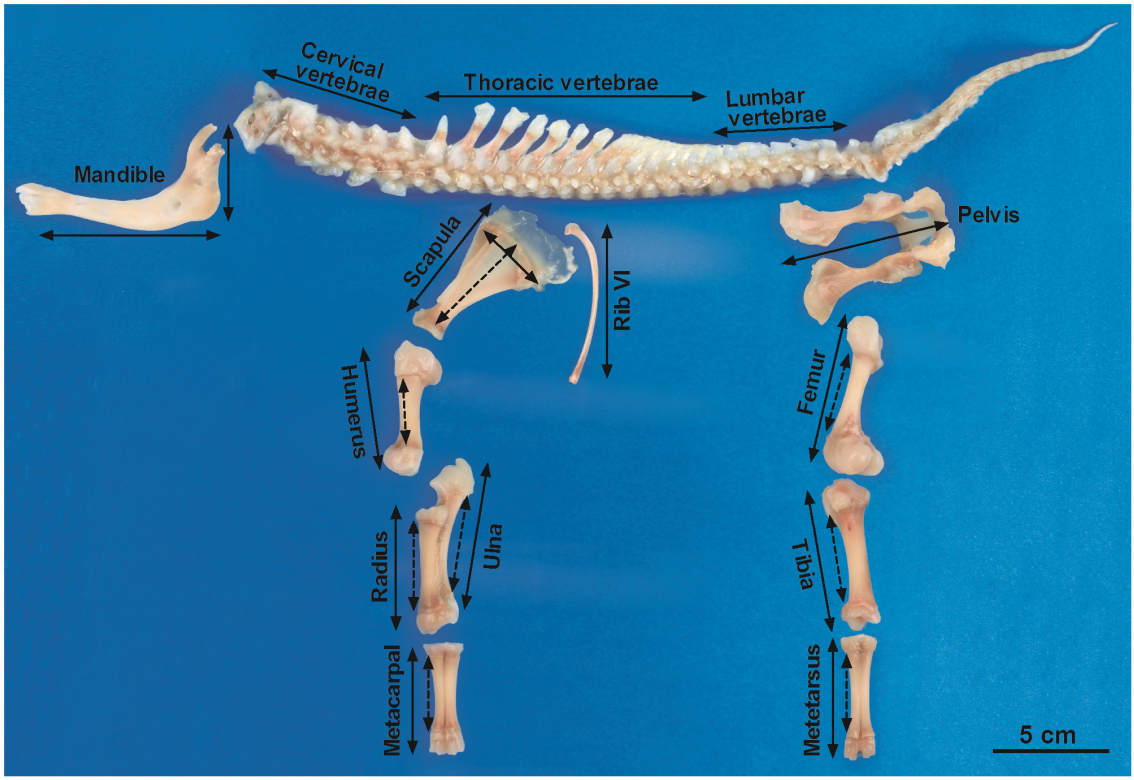

Figure S3.1. Example of set of Day 153 fetal bones with measurements of bone geometry parameters indicated.

Solid arrows: Entire bone length and/or width. Dashed arrows: calcified long bone length. Anatomical reference points used to measure calcified bone length are given in **Table S3.2**.

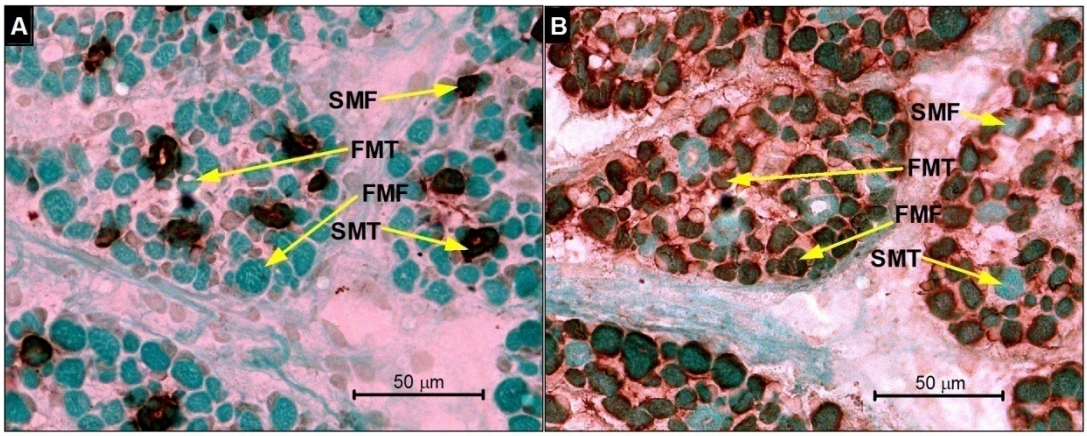

Figure S4.1. Example of immunohistochemical staining for fetal slow and fast myofibres in *M. semitendinosus* at midgestation.

(**A**) and (**B**) show serial stained sections of muscle tissue from one fetus against slow and fast myosin heavy chain isoforms, respectively. Arrows indicate slow myotubes (SMT), slow myofibres (SMF), fast myotubes (FMT) and fast myofibres (FMF).

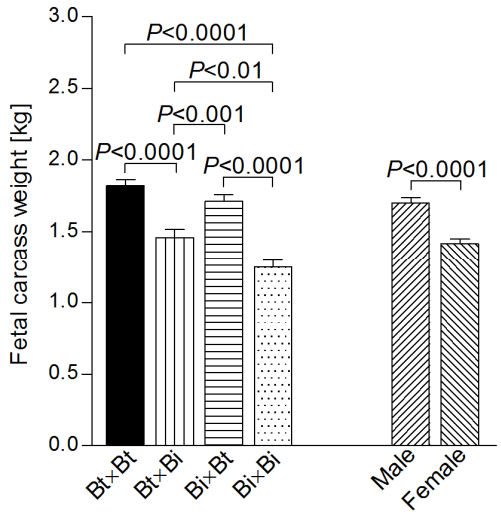

Figure S4.2. Fetal carcass weights for the four different combinations of maternal and paternal genomes and fetal sex at midgeststion.

Least square means with standard errors of means and *P*-values for significant differences (*t*-test) between means are indicated. Data were analysed with a general linear model in SPSS 17.00 that included the factors fetal genetic group *i*, *i* = Bt × Bt, Bt × Bi, Bi × Bt, Bi × Bi (paternal genetics given first) and fetal sex *j*, *j* = male, female. The interaction between fetal genetic group and fetal sex was included in the model but removed as it was not significant (*P*>0.05).

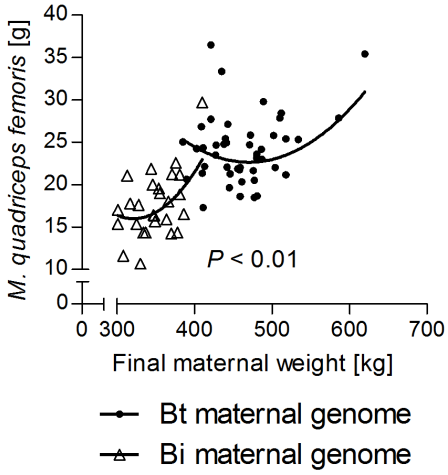

Figure S4.3. Quadratic effects of final maternal weight nested within maternal genomes on absolute weight of fetal *M. quadriceps femoris* at midgestation.

The P-value (ANOVA) of this nested effect is indicated. Bt: Bos taurus taurus, Angus. Bi: Bos taurus indicus, Brahman.

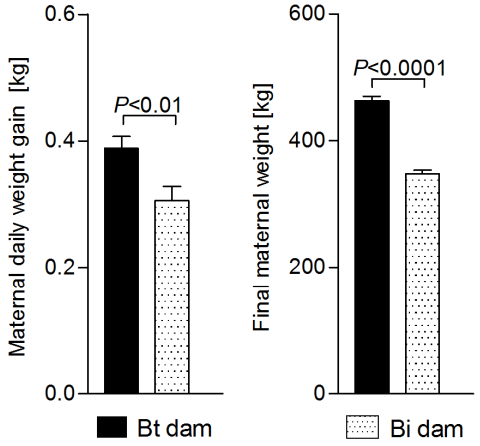

Figure S4.4. Daily weight gain and final weight for *Bos taurus taurus* and *Bos taurus indicus* dams.

(A) Post-conception maternal daily gain: Final maternal weight minus weight at conception divided by days of gestation. (B) Final maternal weight: Weight before slaughter on Day 153 of gestation. *P*-values for significantly different means (t-test) are indicated. Bt: Bos taurus taurus, Angus. Bi: Bos taurus indicus, Brahman.

Supporting tables:

Table S2.1 Summary of adjusted R^2 value, significance levels of models and nested regressions between gross-morphological placental and fetal traits (response variables) and umbilical traits and histo-morphological placental traits (explanatory variables). Only *P*-values for significant nested regressions retained in the final model are shown.

Response traits	R^2	Model[a] *P-value*	Significance (*P*-values) for nested regressions				
			Explanatory traits	Explanatory traits nested within:			
				M	***P***	***Sex***	***M*×*P*[b]**	***P*×*S*[b]**
Total placenta W	0.659	1.2E-12	Umbilical cord W	1.9E-06				
	0.560	6.9E-10	Umbilical cord L	0.0020				
	0.547	9.4E-09	Umbilical artery D					0.0463
	0.622	7.5E-11	Umbilical vein D					0.0050
	0.530	8.7E-09	Maternal epithelium W[c]		0.0150			
	0.515	7.8E-08	Fetal capillary W[c]				0.0289	
	0.522	1.3E-08	Maternal epithelium SA[c]		0.0241			
	0.515	2.0E-08	Trophoblast SA[c]		0.0375			
	0.595	1.5E-09	Maternal barrier THN[c]			0.0223	0.0002	
Maternal placenta W	0.451	5.7E-07	Umbilical cord W			0.0308		
	0.436	6.9E-07	Umbilical cord L	0.0355				
	0.456	1.4E-06	Maternal barrier THN[c]				0.0060	

W: Weight. L: Length. D: Diameter. SA: Surface area. THN: Thickness. *M*: Maternal genome. *P*: Paternal genome. *S*: Fetal sex. [a] Models were adjusted for main effects, all two interactions and three way interactions for maternal and paternal genomes and fetal sex, which were not included in the backward elimination process. [b] Two way interaction. [c] Histo-morphological placental traits determined for largest placentome surrounding the fetus. Table continued on next page.

Table S2.1 continued. Summary of adjusted R^2 value, significance levels of models and nested regressions between gross-morphological placental and fetal traits (response variables), and umbilical traits and histo-morphological placental traits (explanatory variables). Only *P*-values for significant nested regressions retained in the final model are shown.

Response traits	R^2	Model[a] *P-value*	Significance (*P*-values) for nested regressions				
			Explanatory traits	Explanatory traits nested within:			
				M	***P***	***Sex***	***M*×*P*[b]**	***P*×*S*[b]**
Fetal placenta W	0.605	7.5E-11	Umbilical cord W	1.6E-06				
	0.473	1.1E-07	Umbilical cord L			0.0069		
	0.483	3.0E-07	Umbilical artery D					0.0312
	0.565	3.3E-09	Umbilical cord vein D					0.0030
	0.480	1.4E-06	Maternal connective tissue W[c]				0.0092	0.0251
	0.447	2.2E-06	Trophoblast W[c]				0.0137	
	0.493	7.5E-07	Fetal capillary W[c]				0.0053	0.0295
	0.461	1.2E-06	Maternal epithelium SA[c]				0.0393	
	0.462	1.1E-06	Trophoblast SA[c]				0.0369	
	0.512	1.7E-07	Maternal barrier THN[c]			0.0090	0.0014	

W: Weight. L: Length. D: Diameter. SA: Surface area. THN: Thickness. *M*: Maternal genome. *P*: Paternal genome. *S*: Fetal sex. [a] Models were adjusted for main effects, all two interactions and three way interactions for maternal and paternal genomes and fetal sex, which were not included in the backward elimination process. [b] Two way interaction. [c] Histo-morphological placental traits determined for largest placentome surrounding the fetus. Table continued on next page.

Table S2.1 continued. Summary of adjusted R^2 value, significance levels of models and nested regressions between gross-morphological placental and fetal traits (response variables), and umbilical traits and histo-morphological placental traits (explanatory variables). Only *P*-values for significant nested regressions retained in the final model are shown.

Response traits	***R*²**	**Model[a] *P-value***	**Significance (*P*-values) for nested regressions**					
			Explanatory traits	**Explanatory traits nested within:**				
				M	***P***	***Sex***	***M*×*P*[b]**	***M*×*S*[b]**	***P*×*S*[b]**
Fetus W	0.728	1.9E-15	Umbilical cord W		9.6E-08				
	0.621	9.2E-12	Umbilical cord L		0.0092				
	0.651	8.5E-12	Umbilical artery D						0.0285
	0.711	4.7E-14	Umbilical cord vein D						0.0157
	0.659	1.2E-12	Maternal epithelium W[c]		0.0010				
	0.672	6.4E-12	Trophoblast W[c]		0.0012			0.0204	
	0.606	3.5E-10	Maternal epithelium SD[c]				0.0332		
	0.643	4.2E-12	Maternal epithelium SA[c]		0.0038				
	0.661	1.5E-11	Trophoblast SA[c]		0.0022			0.0193	
	0.625	9.1E-11	Maternal barrier THN[c]				0.0224		
Fetal brain W	0.303	2.6E-04	Umbilical cord W			6.7E-05			
	0.173	1.2E-02	Umbilical cord L	0.0138					
	0.156	2.1E-02	Fetal capillary W[c]		0.0123				
	0.150	2.0E-02	Maternal epithelium SD[c]	0.0115					

W: Weight. L: Length. D: Diameter. SD: Surface density. SA: Surface area. THN: Thickness. *M*: Maternal genome. *P*: Paternal genome. *S*: Fetal sex. [a] Models were adjusted for main effects, all two interactions and three way interactions for maternal and paternal genomes and fetal sex, which were not included in the backward elimination process. [b] Two way interaction. [c] Histo-morphological placental traits,determined for largest placentome surrounding the fetus. Table continued on next page.

Table S2.1 continued. Summary of adjusted R^2 value, significance levels of models and nested regressions between gross-morphological placental and fetal traits (response variables), and umbilical traits and histo-morphological placental traits (explanatory variables). Only *P*-values for significant nested regressions retained in the final model are shown.

Response traits	R^2	Model[a] *P-values*	Significance (*P*-values) for nested regressions				
			Explanatory traits	Explanatory traits nested within:			
				M	***P***	***Sex***	***M*×*P*[b]**	***M*×*S*[b]**
Fetal heart W	0.599	1.7E-04	Umbilical cord W					
	0.606	6.9E-11	Umbilical artery D		0.0051	0.0146		
	0.642	1.1E-11	Umbilical vein D	0.0365	0.0236	0.0013		
	0.559	4.8E-09	Maternal barrier THN[c]				0.0064	
Fetal lung W	0.550	8.3E-10	Fetal fluids W		0.0105			
	0.563	1.0E-08	Trophoblast W[c]		0.0331			0.0035
	0.552	2.0E-08	Maternal epithelium SA[c]		0.0170			0.0071
	0.555	1.7E-08	Trophoblast SA[c]		0.0124			0.0063

W: Weight. L: Length. D: Diameter. SA: Surface area. THN: Thickness. *M*: Maternal genome. *P*: Paternal genome. *S*: Fetal sex. [a] Models were adjusted for main effects, all two interactions and three way interactions for maternal and paternal genomes and fetal sex, which were not included in the backward elimination process. [b] Two way interaction. [c] Histo-morphological placental traits determined for largest placentome surrounding the fetus. Table continued on next page.

Table S2.1 continued. Summary of adjusted R^2 value, significance levels of models and nested regressions between gross-morphological placental and fetal traits (response variables), and umbilical traits and histo-morphological placental traits (explanatory variables). Only *P*-values for significant nested regressions retained in the final model are shown.

Response traits	***R*²**	**Model[a] *P-values***	**Significance (*P*-values)s for nested regressions**					
			Explanatory traits	**Explanatory traits nested within:**				
				M	***P***	***Sex***	***M*×*P*[b]**	***M*×*S*[b]**	***P*×*S*[b]**
Liver W	0.694	2.4E-12	Umbilical cord W				0.0149		0.0292
	0.548	1.5E-09	Umbilical cord L		0.0224				
	0.605	7.4E-11	Umbilical artery D		0.0330	0.0085			
	0.646	3.2E-12	Umbilical cord vein D		0.0224	0.0011			
	0.539	3.7E-09	Maternal epithelium W[c]		0.0458				
	0.543	2.8E-09	Maternal epithelium SD[c]	0.0332					
	0.574	1.9E-09	Maternal barrier THN[c]					0.0130	
Kidney W	0.473	6.6E-07	Maternal barrier THN[c]				0.0254		

W: Weight. L: Length. D: Diameter. SD: Surface density. THN: Thickness. *M*: Maternal genome. *P*: Paternal genome. *S*: Fetal sex. [a] Models were adjusted for main effects, all two interactions and three way interactions for maternal and paternal genomes and fetal sex, which were not included in the backward elimination process. [b] Two way interaction. [c] Histo-morphological placental traits determined for largest placentome surrounding the fetus. Table continued on next page.

Table S2.1 continued. Summary of adjusted R^2 value, significance levels of models and nested regressions between gross-morphological placental and fetal traits (response variables), and umbilical traits and histo-morphological placental traits (explanatory variables). Only *P*-values for significant nested regressions retained in the final model are shown.

Response traits	***R*²**	**Model[a] *P-values***	**Significance (*P*-values) for nested regressions**					
			Explanatory traits	**Explanatory traits nested within:**				
				M	***P***	***Sex***	***M*×*P*[b]**	***M*×*S*[b]**	***P*×*S*[b]**
Umbilical cord W	0.225	0.0074	Maternal epithelium SD[c]				0.0118		
	0.376	0.0002	Maternal barrier THN[c]				0.0002	0.0133	
Umbilical cord L	0.233	0.0050	Fetal capillary W[c]						0.0228
	0.242	0.0039	Maternal barrier THN[c]				0.0139		
Umbilical artery D	0.363	3.3E-05	Trophoblast W[c]			0.0229			
	0.359	3.8E-05	Maternal epithelium SA[c]	0.0266					
	0.364	3.1E-05	Trophoblast SA[c]			0.0210			

W: Weight. L: Length. Di: Diameter. SD: Surface density. THN: Thickness. *M*: Maternal genome. *P*: Paternal genome. *S*: Fetal sex. [a] Models were adjusted for main effects, all two interactions and three way interactions for maternal and paternal genomes and fetal sex, which were not included in the backward elimination process. [b] Two way interaction. [c] Histo-morphological placental traits determined for largest placentome surrounding the fetus.

Table S2.2. Summary for distribution of maternal and paternal genomes and sex of fetuses.

		n
Maternal genome	Angus	45
	Brahman	28
Paternal genome	Angus	36
	Brahman	37
Fetal sex	Male	27
	Female	46

Table S3.1. Summary for distribution of maternal and paternal genomes and sex of fetuses.

		n
Maternal genome	Angus	45
	Brahman	28
Paternal genome	Angus	36
	Brahman	37
Fetal sex	Male	27
	Female	46

Table S3.2. Summary of measurements of bone weight and geometry parameters.

	Weight parameters		*Geometry parameters*[1]		
	Entire bone weight	*Calcified bone weight*	*Entire bone length*[2]	*Calcified bone length*	*Width or diameter*[3]
Mandible:	Entire bone weight		Angle of mandible to condylar process and madibular notch to condylar process		
Scapula:	Entire bone weight	Calcified bone weight	Lateral most distal point of cartilage to ventral angle	Lateral border mid-point to ventral angle	Caudal angle to cranial angle (width)
Humerus:	Entire bone weight	Diaphysis weight	Between most distal points on epiphysis	Craniomedial calcified body of humerus	Diameter[2]
Radius:	Entire bone weight	Diaphysis weight	Between most distal points on epiphysis	Craniomedial calcified body of radius	Diameter[2]
Ulna:	Entire bone weight	Diaphysis weight	Between most distal points on epiphysis	Calcified olecranon to lateral styloid process	Diameter[2]
Metacarpal:	Entire bone weight	Diaphysis weight	Between most distal points on epiphysis	Calcified case to intercapital notch	Diameter[2]
Rib VI:	Entire bone weight		Between articulaio costo-vertebrae and calcified end		
Femur:	Entire bone weight	Diaphysis weight	Between most distal points on epiphysis	Craniomedial calcified body of femur	Diameter[2]
Tibia:	Entire bone weight	Diaphysis weight	Between most distal points on epiphysis	Calcified beginning of cranial border to calcified end of tibial cochlea	Diameter[2]
Metatarsus:	Entire bone weight	Diaphysis weight	Between most distal points on epiphysis	Craniomedial calcified base to head	Diameter[2]
Pelvis:	Entire bone weight		Distal end of tuber coxae to distal end of ischial tuber		
Vertebral column	Entire bone weight		Cervical, thoracic and lumbar vertebrae		

[1]: Distances between listed anatomic points on each bone (Budras and Robert 2003). [2]: Entire length of long bone or distance between most distal anatomic points available in (Budras and Robert 2003) and identifiable for irregular bone. [3]: Determined at the mid-shaft of long bone.

Table S4.1. Summary for distribution of maternal and paternal genomes and sex of fetuses.

		n
Maternal genome	Angus	45
	Brahman	28
Paternal genome	Angus	36
	Brahman	37
Fetal gender	Male	27
	Female	46

Table S4.2. Primer sequences used for quantitative real time polymerase chain reaction of *H19* and housekeeping genes.

Primer name	Sequence (5' to 3')	Annealing temperature	Fragment size
H19-F	TCAAGATGACAAGAGATGGTGCTA	60 ºC	171 bp
H19-R	GGTGTGGGTCGTCCGTTC	60 ºC	171 bp
VPS4A-F	GAAGACAGAAGGCTACTCGGGTG	60 ºC	106 bp
VPS4A-R	ACAGACCTTTTTGAAGTGTGTTGCT	60 ºC	106 bp
GAK-F	CACGACCATCTCACACTACCCA	60 ºC	128 bp
GAK-R	AGTTTGAGTACAAGTCCACAATTTCC	60 ºC	128 bp

Table S5.1. Summary of pathway analysis for differentially expressed transcripts, with enrichment score and modified Fisher Exact *P*-value for each pathway and corresponding transcripts information.

Pathways	**Annotation method**	**Enrichment Score:**	***P***	**Involved transcripts**	**Chr**	**AffyID**
Phosphorylation	GOTERM_ BP_FAT	3.080	2.9E-04	ATP6	Mt	12910849
				ATP8	Mt	12910847
				COX3	Mt	12910851
				LOC618482	14	12721568
				LRRK1	21	12792456
				ND2	Mt	12910841
				ND4	Mt	12910833
				ND4L	Mt	12910855
				ND5	Mt	12910835
				PXK	22	12800396
Respiratory chain	GOTERM_ CC_FAT	7.777	6.6E-09	COX1	Mt	12910843
				CYTB	Mt	12910839
				ND2	Mt	12910841
				ND3	Mt	12910853
				ND4	Mt	12910833
				ND4L	Mt	12910855
				ND5	Mt	12910835
				ND6	Mt	12910837

Pathway analysis was performed by using DAVID (http://david.abcc.ncifcrf.gov/), where enrichment score and modified Fisher Exact *P*-value were determined. AffyID: Probe set ID in Affymetrix database for corresponding transcripts. Table continued on next page.

Table S5.1 continued. Summary of pathway analysis for differentially expressed transcripts, with enrichment score and modified Fisher Exact *P*-value for each pathway and corresponding transcripts information.

Pathways	**Annotation method**	**Enrichment Score:**	***P***	**Involved transcripts**	**Chr**	**AffyID**
Mitochondrial inner membrane	GOTERM_CC_FAT	2.874	3.5E-04	ATP6	Mt	12910849
				ATP8	Mt	12910847
				COX1	Mt	12910843
				CYTB	Mt	12910839
				DNAJC19	1	12680249
				ND2	Mt	12910841
				ND5	Mt	12910835
NADH dehydrogenase (ubiquinone) activity	GOTERM_MF_FAT	9.024	5.3E-10	ND1	Mt	12910831
				ND2	Mt	12910841
				ND3	Mt	12910853
				ND4	Mt	12910833
				ND4L	Mt	12910855
				ND5	Mt	12910835
				ND6	Mt	12910837
Hydrogen ion transmembrane transporter activity	GOTERM_MF_FAT	2.251	0.0041	ATP6	Mt	12910849
				ATP8	Mt	12910847
				COX1	Mt	12910843
				COX3	Mt	12910851

Pathway analysis was performed by using DAVID (http://david.abcc.ncifcrf.gov/), where enrichment score and modified Fisher Exact *P*-value were determined. AffyID: Probe set ID in Affymetrix database for corresponding transcripts. Table continued on next page.

Table S5.2. Summary of transcript coexpression (CE) network one identified between significant mRNA and non-coding RNA transcripts for microarray ANOVA.

mRNA affyID	mRNA symbol	Chr	miRNA affyID	Non-coding RNA symbol	Species	Chr	CE score
12895924	-	8	14qI-9_x_st	SNORD113-9	*Homo sapiens*	14	0.7859
12911953	-	-	14qI-9_x_st	SNORD113-9	*Homo sapiens*	14	0.7075
12910855	ND4L	Mt	14qI-9_x_st	SNORD113-9	*Homo sapiens*	14	-0.7046
12766536	DLG4	19	14qI-9_x_st	SNORD113-9	*Homo sapiens*	14	-0.7068
12910843	COX1	Mt	14qI-9_x_st	SNORD113-9	*Homo sapiens*	14	-0.7678
12910833	ND4	Mt	14qI-9_x_st	SNORD113-9	*Homo sapiens*	14	-0.7746
12910853	ND3	Mt	14qI-9_x_st	SNORD113-9	*Homo sapiens*	14	-0.7848
12910849	ATP6	Mt	14qI-9_x_st	SNORD113-9	*Homo sapiens*	14	-0.8061
12910837	ND6	Mt	14qI-9_x_st	SNORD113-9	*Homo sapiens*	14	-0.8075
12910835	ND5	Mt	14qI-9_x_st	SNORD113-9	*Homo sapiens*	14	-0.8206
12915607	12S rRNA	Mt	14qI-9_x_st	SNORD113-9	*Homo sapiens*	14	-0.8281
12910841	ND2	Mt	14qI-9_x_st	SNORD113-9	*Homo sapiens*	14	-0.8294
12910839	CYTB	Mt	14qI-9_x_st	SNORD113-9	*Homo sapiens*	14	-0.8294
12910851	COX3	Mt	14qI-9_x_st	SNORD113-9	*Homo sapiens*	14	-0.8335
12910831	ND1	Mt	14qI-9_x_st	SNORD113-9	*Homo sapiens*	14	-0.8437
12910847	ATP8	Mt	14qI-9_x_st	SNORD113-9	*Homo sapiens*	14	-0.8549
12904062	TAZ	X	bta-miR-187_st	MIR187	*Bos taurus*	24	0.7262
12910847	ATP8	Mt	bta-miR-187_st	MIR187	*Bos taurus*	24	-0.7054
12915607	12S rRNA	Mt	bta-miR-187_st	MIR187	*Bos taurus*	24	-0.7067
12910853	ND3	Mt	bta-miR-187_st	MIR187	*Bos taurus*	24	-0.7071

Transcript coexpression network and score were determined using CoExpression v1.5 (http://www.bioinformatics.lu/CoExpress/). AffyID: Probe set ID in Affymetrix database for corresponding transcripts. Chr: Chromosome location. Missing cells: Currently unannotated in bovine genome. SNORD: small nucleolar RNA. MIR: microRNA. Table continued on next page.

Table S5.2 continued. Summary of transcript coexpression (CE) network one identified between significant mRNA and non-coding RNA transcripts for microarray ANOVA.

mRNA affyID	mRNA symbol	Chr	miRNA affyID	Non-coding RNA symbol	Species	Chr	CE score
12904062	TAZ	X	cfa-miR-187_st	MIR187	*Canis familiaris*	7	0.7473
12910835	ND5	Mt	cfa-miR-187_st	MIR187	*Canis familiaris*	7	-0.7105
12766536	DLG4	19	cfa-miR-187_st	MIR187	*Canis familiaris*	7	-0.7118
12910833	ND4	Mt	cfa-miR-187_st	MIR187	*Canis familiaris*	7	-0.7136
12910831	ND1	Mt	cfa-miR-187_st	MIR187	*Canis familiaris*	7	-0.7200
12910843	COX1	Mt	cfa-miR-187_st	MIR187	*Canis familiaris*	7	-0.7278
12910849	ATP6	Mt	cfa-miR-187_st	MIR187	*Canis familiaris*	7	-0.7290
12910839	CYTB	Mt	cfa-miR-187_st	MIR187	*Canis familiaris*	7	-0.7290
12910851	COX3	Mt	cfa-miR-187_st	MIR187	*Canis familiaris*	7	-0.7523
12910847	ATP8	Mt	cfa-miR-187_st	MIR187	*Canis familiaris*	7	-0.7548
12915607	12S rRNA	Mt	cfa-miR-187_st	MIR187	*Canis familiaris*	7	-0.7615
12910853	ND3	Mt	cfa-miR-187_st	MIR187	*Canis familiaris*	7	-0.7696
12904062	TAZ	X	eca-miR-187_st	MIR187	*Equus caballus*	8	0.7151
12910853	ND3	Mt	eca-miR-187_st	MIR187	*Equus caballus*	8	-0.7157
12910843	COX1	Mt	hsa-miR-1973_st	MIR1973	*Homo sapiens*	4	0.7459
12910831	ND1	Mt	hsa-miR-1973_st	MIR1973	*Homo sapiens*	4	0.7168
12910839	CYTB	Mt	hsa-miR-1973_st	MIR1973	*Homo sapiens*	4	0.7081
12915607	12S rRNA	Mt	hsa-miR-1973_st	MIR1973	*Homo sapiens*	4	0.7072
12910851	COX3	Mt	hsa-miR-1973_st	MIR1973	*Homo sapiens*	4	0.7060
12895924	-	8	hsa-miR-1973_st	MIR1973	*Homo sapiens*	4	-0.7119
12911953	-	-	hsa-miR-1973_st	MIR1973	*Homo sapiens*	4	-0.7784
12910839	CYTB	Mt	mml-miR-187_st	MIR187	*Macaca mulatta*	18	-0.7010
12910853	ND3	Mt	mml-miR-187_st	MIR187	*Macaca mulatta*	18	-0.7142
12910851	COX3	Mt	mml-miR-187_st	MIR187	*Macaca mulatta*	18	-0.7165
12915607	12S rRNA	Mt	mml-miR-187_st	MIR187	*Macaca mulatta*	18	-0.7195
12910847	ATP8	Mt	mml-miR-187_st	MIR187	*Macaca mulatta*	18	-0.7439
12904062	TAZ	X	ppa-miR-187	MIR187	*Pan paniscus*	-	0.7110
12910831	ND1	Mt	ppa-miR-187	MIR187	*Pan paniscus*	-	-0.7071
12910851	COX3	Mt	ppa-miR-187	MIR187	*Pan paniscus*	-	-0.7134
12910847	ATP8	Mt	ppa-miR-187	MIR187	*Pan paniscus*	-	-0.7418
12915607	12SrRNA	Mt	ppa-miR-187_st	MIR187	*Pan paniscus*	-	-0.7110
12904062	TAZ	X	rno-miR-187	MIR187	*Rattus norvegicus*	18	0.7065
12904062	TAZ	X	ssc-miR-187	MIR187	*Sus scrofa*	6	0.7092

Transcript coexpression network and score were determined using CoExpression v1.5 (http://www.bioinformatics.lu/CoExpress/). AffyID: Probe set ID in Affymetrix database for corresponding transcripts. Chr: Chromosome location. Missing cells: Currently unannotated in bovine genome. MIR: microRNA.

Table S5.3. Summary of transcript coexpression (CE) network two identified between significant mRNA and non-coding RNA transcripts for microarray ANOVA.

mRNA affyID	mRNA symbol	Chr	miRNA affyID	miRNA symbol	Species	Chr	CE score
12843233	AMY2A	3	bta-miR-184_st	MIR184	*Bos taurus*	21	0.7268
12890668	LOC523509	8	bta-miR-184_st	MIR184	*Bos taurus*	21	-0.7129
12901429	FRMD1	9	bta-miR-184_st	MIR184	*Bos taurus*	21	-0.7290
12891710	MGC133950	8	bta-miR-184_st	MIR184	*Bos taurus*	21	-0.7334
12745085	THOC7	22	bta-miR-184_st	MIR184	*Bos taurus*	21	-0.7353
12860923	MMP19	5	bta-miR-184_st	MIR184	*Bos taurus*	21	-0.7363
12677543	12677543	-	bta-miR-184_st	MIR184	*Bos taurus*	21	-0.7571
12843233	AMY2A	3	eca-miR-184_st	MIR184	*Equus caballus*	1	0.7301
12800321	KCTD6	22	eca-miR-184_st	MIR184	*Equus caballus*	1	0.7098
12703781	XDH	11	eca-miR-184_st	MIR184	*Equus caballus*	1	0.7016
12860923	MMP19	5	eca-miR-184_st	MIR184	*Equus caballus*	1	-0.7036
12890668	LOC523509	8	eca-miR-184_st	MIR184	*Equus caballus*	1	-0.7070
12901429	FRMD1	9	eca-miR-184_st	MIR184	*Equus caballus*	1	-0.7070
12891710	MGC133950	8	eca-miR-184_st	MIR184	*Equus caballus*	1	-0.7077
12745085	THOC7	22	eca-miR-184_st	MIR184	*Equus caballus*	1	-0.7327
12677543	12677543	-	eca-miR-184_st	MIR184	*Equus caballus*	1	-0.7707
12806044	ZNF451	23	hsa-miR-184_st	MIR184	*Homo sapiens*	15	0.7700
12843233	AMY2A	3	hsa-miR-184_st	MIR184	*Homo sapiens*	15	0.7345
12677543	12677543	-	hsa-miR-184_st	MIR184	*Homo sapiens*	15	-0.7166
12745085	THOC7	22	hsa-miR-184_st	MIR184	*Homo sapiens*	15	-0.7175
12843233	AMY2A	3	mmu-miR-184_st	MIR184	*Mus musculus*	9	0.7261
12678626	ALCAM	1	mmu-miR-184_st	MIR184	*Mus musculus*	9	0.7136
12893631	KIF24	8	mmu-miR-184_st	MIR184	*Mus musculus*	9	-0.7116
12901429	FRMD1	9	mmu-miR-184_st	MIR184	*Mus musculus*	9	-0.7145
12677543	12677543	-	mmu-miR-184_st	MIR184	*Mus musculus*	9	-0.7383
12843233	AMY2A	3	rno-miR-184_st	MIR184	*Rattus norvegicus*	8	0.7074
12890668	LOC523509	8	rno-miR-184_st	MIR184	*Rattus norvegicus*	8	-0.7211
12677543	12677543	-	rno-miR-184_st	MIR184	*Rattus norvegicus*	8	-0.7242
12745085	THOC7	22	rno-miR-184_st	MIR184	*Rattus norvegicus*	8	-0.7461

Transcript coexpression network and score were determined using CoExpression v1.5 (http://www.bioinformatics.lu/CoExpress/). AffyID: Probe set ID in Affymetrix database for corresponding transcripts. Chr: Chromosome location. Missing cells: Currently unannotated in bovine genome. MIR: microRNA.

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