University of Alberta

EPIGENETIC REGULATION OF EMBRYOGENESIS IN CATABOLIC PRIMIPAROUS LACTATING SOWS

by

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ABSTRACT

This study explored the role of epigenetics in mediating gender-specific effects on embryogenesis in primiparous sows feed restricted during the last week of lactation. In the first experiment embryos in Restrict sows had decreased weight and crown-rump-length ($P<0.05$), and fewer female embryos survived to Day 30 ($P<0.01$). In the second experiment, variance in DNA methylation decreased for both Restrict male ($P<0.001$) and female embryos ($P<0.06$), reflecting the loss of abnormal embryos. Variance tended to decrease in *Igf2r* in Restrict female embryos ($P<0.07$), while *Xist* decreased in Restrict male embryos ($P<0.08$). The third experiment sought to establish the ontogeny of these epigenetic affects. Leukocyte DNA of Restrict sows exhibited decreased methylation ($P<0.05$), but no changes in epigenetics or embryogenesis were evident at Day 6 of gestation. A subset of Restrict litters was identified as sensitive to latent epigenetic mechanisms that trigger gender-specific loss of embryos by Day 30 of gestation.

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TABLE OF CONTENTS

CHAPTER 1

CHAPTER 2

LITERATURE REVIEW

CHAPTER 3

NUTRITIONAL RESTRICTION IN LACTATING PRIMIPAROUS SOWS SELECTIVELY AFFECTS FEMALE EMBRYO SURVIVAL AND OVERALL LITTER DEVELOPMENT.

CHAPTER 4

ALTERED EPIGENETIC VARIANCE IN SURVIVING LITTERS FROM NUTRITIONALLY RESTRICTED LACTATING PRIMIPAROUS SOWS.

CHAPTER 5

CHAPTER 6

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Air	Antisense *Igf2r*
BORIS	Brother of the regulator of imprinted sites
BW	Body weight
CpG	Cytosine-guanine. repeating region
CRL	Crown-rump length
Ct	Cycle threshold
CTCF	CCCTC-Binding factor
DE	Digestible energy
Dlk1- Gtl2	Drosophila homolog-like / Gene trap locus 2
DMR	Differentially methylated region
DNA	Deoxyribonucleic acid
DNMT	DNA methyl-transferase
ESR	Embryonic survival rate
FAPWC	Faculty animal policy welfare committee
GVBD	Germinal vesicle breakdown
HDAC	Histone deactylase
ICM	Inner cell mass
ICR	Imprinting control region
Igf-1	Insulin-like growth factor 1
Igf2	Insulin-like growth factor 2
Igf2as	Igf2 antisense
Igf2r	Insulin-like growth factor 2 receptor
Impact	Imprinted and ancient gene
Ins	Insulin
IUGR	Intrauterine growth retardation
IVF	*In vitro* fertilization
IVM	*In vitro* maturation
LDL	Low density lipoprotein
LOI	Loss of imprinting
LOS	Large offspring syndrome

LSM	Least-squared means
MCal	Mega calories
ME	Metabolizable energy
MJ	Mega joules
NE	Net energy
NRC	National Research Council
PCR	Polymerase chain reaction
Peg1/Mest	Paternally expressed gene 1 / mesoderm specific transcript
Peg3	Paternally expressed gene 3
QTL	Quantitative trait loci
RIA	Radioimmunoassay
RP-HPLC	Reverse-phase high performance liquid chromatography
SAM	S-adenosyl-methionine
SAS	Statistical analysis system
SEM	Standard error of the mean
SINE	Short interspersed element
Slc22	Solute carrier family 22 (organic cation transporter)
SRTC	Swine Research Technology Centre
Sry	Sex determining region on the Y chromosome
THF	Tetrahydrofolate
Tsix	X-inactivation specific transcript antisense
VFI	Voluntary feed intake
Xist	X-inactivation specific transcript
Zfx/Zfy	Zinc finger protein (X or Y linked)

CHAPTER 1

GENERAL INTRODUCTION

Increased catabolism during lactation in the primiparous sows is a common occurrence, and in moderation has no lasting effects on reproductive performance. However, depending on the timing and severity of catabolism, sow productivity can be affected. Previous studies have show the last week of lactation to be a critical period, during which increased catabolism beyond normal limits, decreases ovulation rate and embryonic survival (Foxcroft 1997; Zak *et al.* 1997a). Additional studies using *in vitro* maturation assays have shown that that increased catabolism during lactation affects the quality of oocytes produced within the pre-ovulatory cohort of follicles (Zak *et al.* 1997b, Yang *et al.* 2000, Clowes *et al.* 2003). Overall this suggests that increased lactational catabolism can hinder the development of pre-ovulatory follicles in a way which pre-programs the pattern of embryonic survival and development to Day 28.

In a broader context, such effects fit within the more general theory of fetal programming, also referred to as the Barker hypothesis, which covers a broad range of metabolic, endocrine, and genetic changes during embryonic and fetal development which can be perpetuated through the lifetime of the individual (Barker 1998).

Although there are a number of mechanisms by which this programming of embryonic development can be achieved, epigenetic defects in the early embryonic genome have received considerable attention (Young 2001). Epigenetics is a heritable change in gene expression that is not dependent on the DNA sequence itself, but rather on factors that regulate gene expression. DNA methylation, specifically of cytosine, has been established as the most important regulator of epigenetics as it can permanently alter

gene expression, such as with imprinted genes, or temporarily turn non-imprinted genes off by the formation of heterochromatin (Reik and Walter 2001). In particular, the regulation of imprinted gene expression is vital to embryonic development, as all imprinted genes are either paternally or maternally expressed, and these patterns of expression are established by DNA methylation during gametogenesis (Moore and Haig 1991).

As such, factors which alter the establishment of these imprints during gametogenesis are passed on to the subsequent embryo and may result in defects in embryonic development. As the experimental paradigm investigated in the following chapters examines the effect of nutritional restriction on increasing lactational catabolism, the deficiency of nutrients essential for DNA methylation may cause changes to fetal programming. The most critical nutrients for DNA methylation are folate, vitamin B_{12}, vitamin B_6, methionine, and choline (Davis and Uthus 2004). The focus of Chapter two is to provide a literature review of epigenetic regulation, nutrients critical for DNA methylation, and their relation to the Barker hypothesis.

In previous work using an earlier iteration of the same experimental paradigm, involving excessive tissue catabolism resulting from feed restriction in late lactation in the sow, embryonic survival to Day 30 was compromised (Zak *et al.* 1997a). However, it was not known if there was any effect on embryonic growth and development. Also, it has been suggested that epigenetic defects during gestation could alter sex ratios, by altering embryonic survival in a sex-dependent manner (Young 2001). Therefore, in Chapter three, the focus of this study was to determine if lactational feed restriction in primiparous sows could alter survival, development, and sex ratios of embryos recovered

from these sows at Day 30 of gestation. The results of this study confirmed that embryonic survival was decreased in sows with increased lactational catabolism, and that embryonic development was delayed as measured by weight and crown-rump length, without affecting placental size. Furthermore, there was sex-dependent survival of embryos, as fewer female embryos survived to Day 30 of gestation.

These results suggested a fetal programming effect that was connected to epigenetic defects, as indicated by the changes in sex ratios. Therefore, in Chapter four, analysis of epigenetic defects in the surviving population of embryos at Day 30 of gestation was carried out. As there were developmental delays in the Day 30 embryos, defects in imprinted genes which regulate embryonic development, such as *Igf2* and *Igf2r* were determined (Amarger *et al.* 2002; Killian *et al.* 2001). However, as it was likely that increased developmental delays already would have resulted in increased embryonic mortality by Day 30 the variance in epigenetic traits were hypothesized to reflect the pattern of embryonic survival at this time. For instance, the expression of *Xist*, a gene whose regulation is controlled by DNA methylation, and is essential for female embryonic survival, was quantified in surviving embryos (Lee 2000; Young 2001). Analysis of the variance for each epigenetic trait was especially performed to determine if the variance in expression of these traits was affected in the treatment group, on the hypothesis that loss of embryos with defects important for survival would reduce the variation in epigenetic markers in these litters. This was similar to the approach taken by Archer *et al.* (2003), who used this technique to determine if cloned pigs had greater variance in epigenetic traits compared to naturally bred controls. In addition to the expression profiles of *Igf2*, *Igf2r* and *Xist*, the global methylation of embryonic DNA was

quantified using RP-HPLC (Rameshoye 2002). The results of this study indicated that although the mean DNA methylation and epigenetic gene expression was not different in embryos surviving to Day 30, the variance surrounding these traits was altered.

Having found evidence that variance in epigenetic traits was altered in Day 30 embryos due to increased lactational catabolism, the next step was to determine if epigenetic defects could be found before embryonic survival became a factor, and the experiment described in Chapter five explored this possibility. According to the research of Kang *et al.* (2001) the blastocyst stage of embryonic development in the pig had the lowest level of non-imprinted DNA methylation: Therefore, any changes in global methylation at this stage would indicate changes in the methylation of imprinted genes. Furthermore, this pre-implantation stage was assumed to precede the appearance of developmental delays that could influence embryonic survival (Geisert and Schmitt 2002). To determine if lactational catabolism decreased nutrients essential for DNA methylation, sow plasma folate and B_{12} were measured. Maternal leukocyte and embryonic DNA methylation were also quantified to see if there were treatment effects. Results indicated a decrease in maternal DNA methylation during feed restriction; however, neither plasma folate nor B_{12} decreased, indicating that another nutrient must be involved. Although DNA methylation was not altered in the blastocysts recovered, there were noticeable developmental delays in embryos in specific litters across treatment groups. Based on the results in Chapter three, indicating that sows would overlap in treatment response, we re-analyzed the data irrespective of treatment. By dividing the sows litters into those with high levels of embryonic development and those with low embryonic development, we were able to look for factors which would influence

diversity in development. Examination of these results showed that as the level of development of recovered embryos increased, embryo recoveries also improved. Furthermore, a moderate decrease in net energy balance throughout lactation was associated with improved ovulation rates and embryonic development, although it is currently not understood why this occurred.

For the first time in the pig, this thesis provides evidence that increased catabolism during lactation results in developmental delays and decreased female embryo survival, as a result of epigenetic defects. A summary of the results, and potential implications of the evidence provided by Chapters three through five are discussed in the final chapter, along with directions for future studies.

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CHAPTER 2

LITERATURE REVIEW

2.1 Introduction to epigenetics

Epigenetics is a heritable change in gene expression that is not dependent on the DNA sequence itself, but rather on factors that regulate gene expression. Gene imprinting is an epigenetic modification of genes, whereby a given allele is identified as having been inherited from the maternal or paternal parent. This modification results in the silencing of one allele and in the differential expression of a gene or chromosomal region according to the parental origin of inheritance.

Genomic imprinting was discovered in 1984 when the results of two mouse experiments were analyzed (Figure 2.1) (Lyle 1997). Surani *et al.* (1984) used nuclear transplantation to make embryos that had only one of the two sets of parental chromosomes (uniparental embryos), while McGrath and Solter (1984) used other more sophisticated genetic techniques to make embryos that inherited specific chromosomes from one parent only (uniparental disomy). Both experimental methods led to the conclusion that mammalian genes could function differently depending on whether they came from the mother or the father (Cattanach and Kirk 1985; Mann and Lovell-Badge 1984).

In 1991 the first imprinted genes were described, and were indeed expressed differently on maternal and paternal chromosomes (Barlow *et al.* 1991; Bartolomei *et al.* 1991; DeChiara *et al.* 1991). Furthermore, it became clear that DNA methylation was the key to understanding the molecular mechanism of imprinting. Methylation of the 5' position of cytosine marks the imprinted genes differently in egg and sperm, and

inheritance of these epigenetic marks leads to differential gene expression (Reik *et al.* 1987).

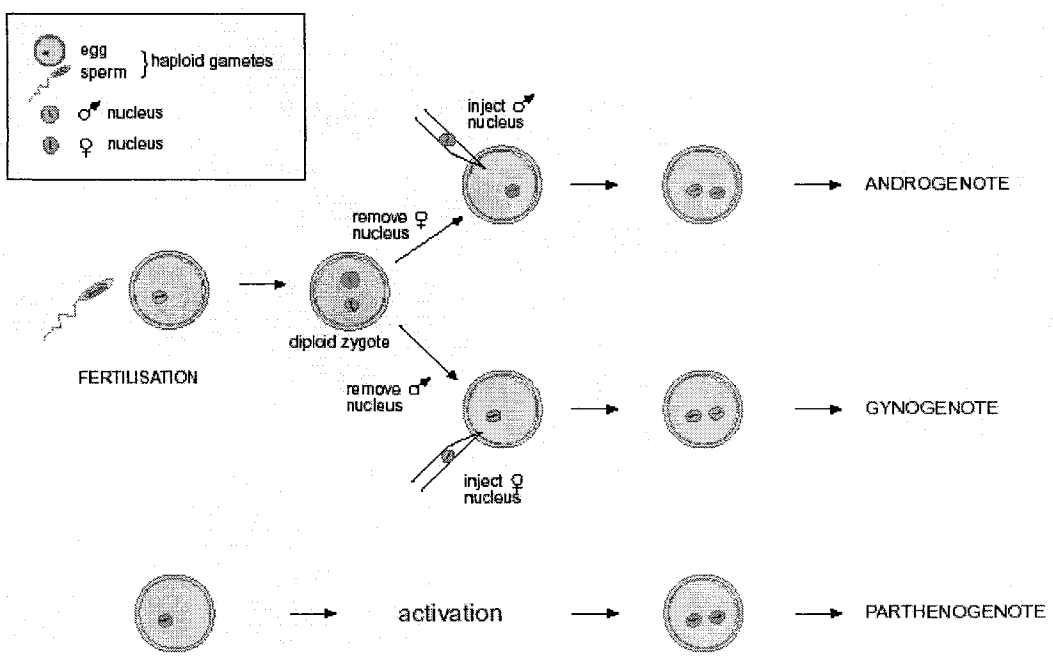

Figure 2.1 Production of uniparental embryos (Androgenote and Gynogenote) by nuclear transplatation. Gynogenotes tend to have large placentae and smaller fetuses, while androgenotes produce larger fetuses with more normal placentae. Uniparental disomic embryos were produced by chemical activation that caused disjunction (Parthenogenote). (Modified from Lyle 1997)

The methylation patterns on the maternal and paternal chromosomes were completely different, resulting in differential gene expression. Two of the first imprinted genes discovered, insulin-like growth factor 2 (*Igf2)* and *Igf2* receptor (*Igf2*r), illustrate how imprinted genes are differentially expressed. Although both genes regulate fetal growth and development, *Igf2* was expressed from the paternal allele and increased fetal growth, while the maternal allele was silent. Conversely, *Igf2*r was only maternally expressed, and caused a reduction in the fetal growth rate.

This reciprocal relationship between paternal and maternal alleles was discovered to be the basic mechanism underlying the actions of all imprinted genes. Deeper examination of the interactions between paternally imprinted *Igf2* and maternally imprinted *Igf2r* determined that IGF2r binds to IGF2, causing it to be internalized into the cell for degradation; thus, maternal IGF2r reduces fetal and placental growth by binding to paternal IGF2, and then internalizes it into a lysosome for degradation (O'Dell and Day 1998). This relationship explained the respective growth inducing or reducing effects of paternally imprinted *Igf2* and maternally imprinted *Igf2r*. Other imprinted genes were described as having similar activity, with the paternally imprinted genes enhancing and maternal imprinted genes suppressing fetal growth (Haaf 2001). From these observations the genetic conflict hypothesis was developed, that a struggle over maternal resources by the genome of the fetus was the driving force in the evolution of imprinting (Moore and Haig 1991). Paternally expressed imprinted genes are, therefore, selected to extract more resources from the mother to benefit health and survival of the offspring, whereas maternally expressed imprinted genes tend to conserve resources, to protect the health of the mother and to maximize reproductive performance of the female (Moore and Haig 1991).

The genetic conflict theory is of particular importance to research projects that strive to show a connection between gene imprinting, embryo survival, and fetal development. As imprinted genes are only expressed from the genetic contribution of one parent, embryonic survival and fetal development can be impaired if the regulation of gene expression from either parent is altered. However, in order to examine the relationships between gene imprinting and embryonic survival and fetal development, it

is important to understand the characteristics of, and the mechanisms that regulate, imprinted genes.

2.2 Imprinted Genes

Imprinted genes have a number of unique characteristics that distinguish them from other genes or regions of methylated DNA, which are described in detail in Reik and Walter (2001), and can be summarized into the following key points. Imprinted genes show a high amount of linkage, as about 80% show clustering in regions that appear to be evolutionarily conserved, and reflect coordinated regulation. Inside these clusters are often found Imprinting Control Regions (IC or ICR) that regulate the methylation status of imprinted genes as far away as 1MB, as seen by the alteration of these methylation patterns by deletion of an ICR. Imprinted gene clusters are also usually rich in CpG islands and 88% of imprinted genes have CpG islands, compared to 47% in the rest of the genome. In addition to CpG islands, imprinted genes commonly have clustered direct repeats; however the sequences of these repeats are not unique to imprinted genes, and can be found in other regions of the genome.

An additional distinguishing characteristic of imprinted genes is that nearby regulatory regions often show unusual patterns of DNA methylation. These regions are called DMR's, or differentially methylated regions, and can demonstrate a variety of methylation patterns associated with activation or inactivation of the genes they regulate (Murrell *et al.* 2001). In some cases the DMR remains methylated throughout development, in other instances methylation of a DMR can be highly variable, showing methylation at some stages, and demethylation at others (Li *et al.* 2004). Thus

methylation of a DMR can either activate or inhibit gene expression: Therefore, it is impossible to tell how the methylation of a DMR will affect gene expression unless the sequence and regulatory function of the DMR is known.

Imprinted genes also show two regional distinguishing characteristics. The first is asynchronous replication during S-phase of the cell cycle, whereby the paternal allele is replicated before the maternal allele, although the reasons for this are not understood it is believed to be involved in timing of gene expression during different stages of embryonic development and/or in different cell types (Simon *et al.* 1999). The second is that near imprinted clusters, different frequencies of meiotic recombination are found, with a particularly elevated rate of recombination during male meiosis (Paldi *et al.* 1995). It is still unclear how regional epigenetic features function in the regulation of imprinted gene expression, but fuller understanding of these regional features will enable us to better interpret the role of imprinted genes in fetal development.

2.3 Cycle of Imprinting

It is still a mystery how all the characteristics that distinguish imprinted genes help to coordinate and regulate their activity. However, three stages of the cycle of gene imprinting: erasure, establishment, and maintenance have been described by Reik and Walter (2001) (Figure 2.2), although many of the underlying mechanisms involved have yet to be determined.

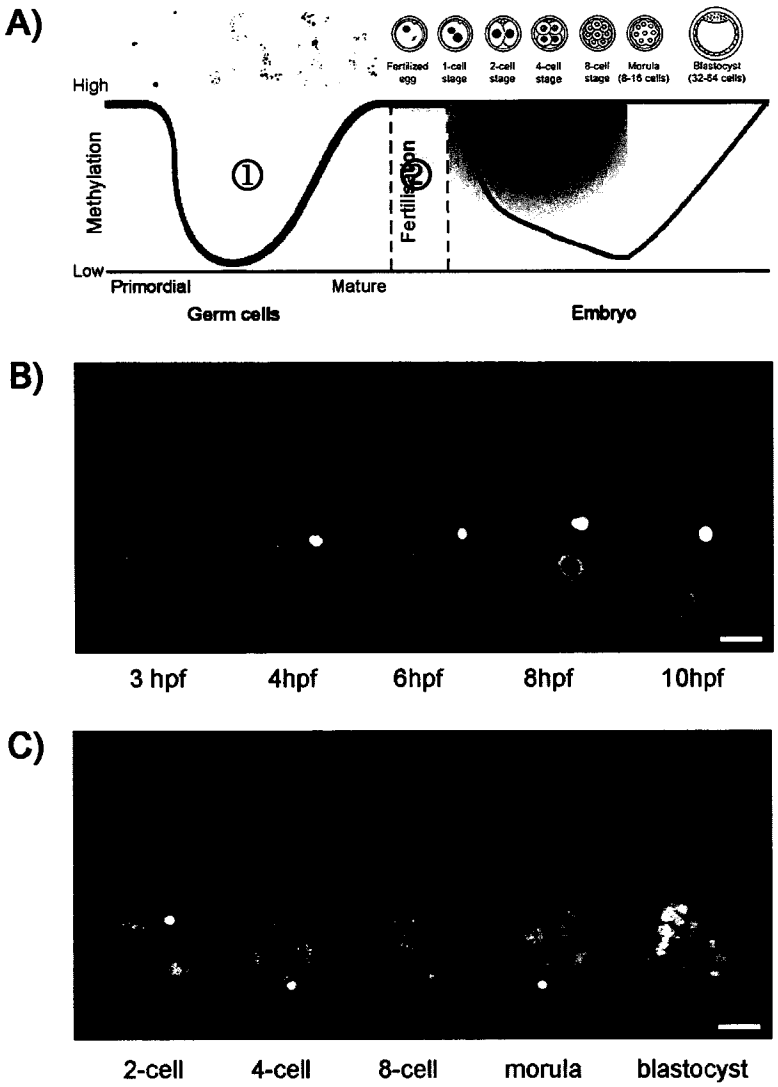

Figure 2.2 Cycle of methylation as seen in mouse gametes and zygotes (Modified from Santos and Dean 2004).

(A) Highly methylated primordial germ cells enter the germinal ridge and undergo loss and reacquisition of methylation during their expansion phase. Examples of these cells (day 11.5, 13.5 and 14.5) stained for alkaline phosphatase, a primordial germ cell marker, are pictured above. The horizontal time axis and the vertical axis indicating the relative methylation levels are not to scale. 1) Erasure 2) Establishment 3) Maintenance

(B) Active demethylation: the first cell cycle following fertilization shows a rapid and asymmetric loss of DNA methylation (Antibody for 5MeC - red signal) in the male (green in lower merge panel) but not in the female pronucleus. Genome-wide loss of DNA methylation starts at sperm decondensation (left) and continues until it is undetectable in the paternal compartment (right), the process taking about 6 h. Lower panels show a merge (yellow) between the DNA methylation (red) and DNA stain (green). Hpf = hours post fertilisation. Scale bar 25 μm

(C) Passive demethylation phase. From the 2-cell stage (left) to the morula (right) the DNA methylation (red) is passively lost due to the exclusion of DNA methyltransferase 1 (Dnmt1) from the nucleus. By the blastocyst stage lineage-specific de novo methylation is apparent with the inner cell mass (ICM) being highly methylated (red) while the trophectoderm remains hypomethylated. Lower panels show a merge (yellow) between the DNA methylation (red) and DNA stain (green). Scale bar 25 μm.

Erasure is the first step in setting down imprints in a newly developing germline. A genome-wide demethylation effectively erases both paternal and maternal imprints. This demethylation is non-distinguishing, and it is unknown if it is by passive or active mechanisms. However, three basic mechanisms which have been proposed to be involved in demethylation as discussed by Santos and Dean (2004). The first is the direct removal of the methyl group from the major groove by breaking the carbon bond and the producing methanol (Bhattacharya *et al.* 1999; Bird 2002). The second hypothesis is that an enzyme would perform a base substitution, either specifically removing the methyl cytosine and replacing it with a cytosine (Klimasauskas *et al.* 1994) or removing the CpG dinucleotide by nucleotide excision. A third possibility is hydrolytic deamination, converting 5-methyl-cytosine to thymine that would be replaced by a cytosine in the next replication cycle by mismatch repair enzymes (Santos and Dean 2004). Several candidates have been suggested for the active demethylase in early embryonic development, but so far the best candidate is methyl binding domain protein 2 (MBD2) which has been shown *in vitro* to have CpG demethylation activity in a human embryonic kidney cell line (Bhattacharya *et al.* 1999).

Establishment of gene imprints then follows erasure to reflect the sex of the developing fetus, male germlines will program growth enhancing imprinted genes and female germlines will program growth repressing genes for expression in future offspring during embryonic development. At this time DMR's are also methylated in order to properly regulate expression and timing of their imprinted gene(s). Establishment occurs in mouse oocytes during the phase of oocyte growth that follows the first meiotic arrest

(Obata *et al.* 1998), between metaphase of the first meiotic division, and metaphase of the second meiotic division. In contrast, during mouse spermatogenesis establishment occurs between the leptotene and pachytene stages (Lucifero *et al.* 2002); Figure 2.3). Although it is not yet known which enzymes are responsible for establishment in male germ cells, the various isoforms of Dmnt1 (DNA methyl-transferase 1), Dnmt3a and Dnmt3b are possible candidates based on their ability to methylate DNA during embryonic development (Okano *et al.* 1999). However, a DNA methyl-transferase, Dnmt3L, was identified as a *de novo* methylase that establishes the imprints in the oocytes of female mice (Hata *et al.* 2002). In a similar manner, a zinc-finger binding protein named BORIS that is up-regulated during erasure in the testis of male mice, and is believed to be involved in re-methylation, following erasure (Loukinov *et al.* 2002).

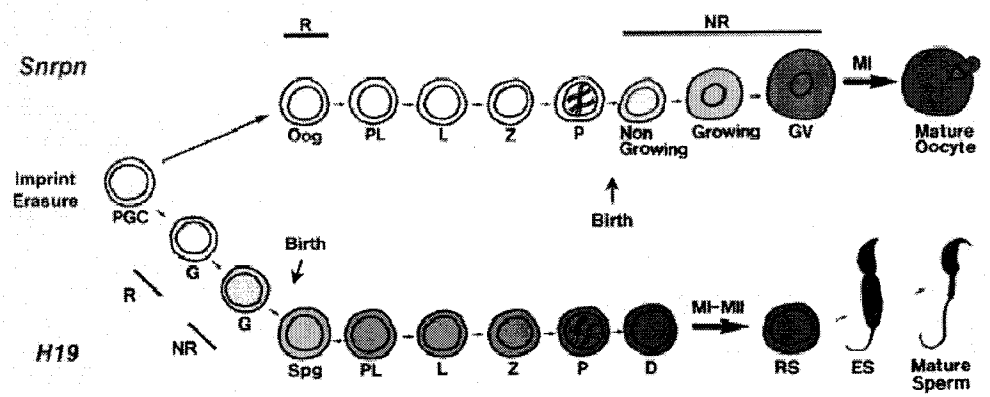

Figure 2.3 Establishment in mouse oocytes has been shown to be completed by metaphase of the 2nd meitotic division in most cases. For this figure the female imprinted small nuclear riboprotein N is shown to be increasingly methylated as shading darkens. In spermatogenesis imprinting is completed between leptotene and pachytene, as seen in the case of H19. R= replicating cells; NR= non-replicating cells; Oog= oogonia; PL= preleptotene; L= leptotene; Z= zygotene; P= pachytene; GV= germinal vesicle stage oocyte; D= diplotene; RS= round spermatid; ES= elongating spermatid. (Modified from Lucifero *et al.* 2002)

Heterochromatic imprinting patterns are maintained during successive rounds of cellular replication throughout the life of the organism through the activity of specific methylases. This maintenance phase of the imprinting cycle prevents the reprogramming of imprints inherited on both the paternal and maternal chromosomes. The major enzyme responsible for maintenance of imprinted genes is DNMT1 and its various isoforms (Hermann, *et al.* 2004). During cellular replication as new DNA is synthesized in a eukaryote approximately 40 million CpG islands are converted into a hemi-methylated state, as the original DNA remains methylated, but the newly synthesized strand is unmethylated. These hemi-methylated CpG sites must be fully methylated in order to maintain the original DNA methylation pattern. DNMT1 is located at the replication fork and methylates newly biosynthesized DNA strands immediately following replication (Hermann, *et al.* 2004). Unique maintenance methylases have been identified in germ cells, such as DNMT1o in growing oocytes and DNMT1p in pachytene spermatocytes (Ko *et al.* 2005). Interestingly, DNMT1o is inherited in the cytoplasm of the oocyte and remains in the cellular cytoplasm following fertilization until implantation of the embryo, at which time it enters the nucleus. In contrast, DNMT1p inhibits the biosynthesis of DNMT1 during spermatogenesis, which may be essential for preventing maintenance methylation during erasure. It has been suggested that DNMT1p may survive spermatogenesis and following fertilization may be modified to act as a functional DNMT1 involved in maintenance of imprints during embryogenesis.

Once imprints are established, maintenance is required following fertilization and implantation, when there is genome-wide demethylation and *de novo* methylation of non-imprinted genes (Li 1999). The imprinted genes and their respective DMRs need

protection from demethylation, which can occur by both active and passive mechanisms, but it is not understood how this protection is conferred. Although it has yet to be studied, I believe it is highly probable that a unique histone modification at imprinted regions would protect them from demethylation. When the non-imprinted genes of the paternal genome are demethylated it occurs by an active mechanism only hours after fertilization when the parental genomes are still separate in the pronuclei (Mayer *et al.* 2000). However the maternal genome is largely demethylated passively by failure to maintain methylation during DNA replication, potentially by excluding DNMT1 from the nucleus during cleavage divisions (Mayer *et al.* 2000).

In a recent study Dean *et al.* (2001) examined the maintenance of imprinted genes in different mammalian species during the wave of active and passive demethylation in the newly fertilized oocyte, by comparing the methylation status of pig, rat, mouse, and bovine zygotes from the time of fertilization up to the 16-cell stage (Figure 2.4). All species showed conservation of the active demethylation of the heavily methylated paternal genome before the two-cell stage. Although it is yet unproven, I believe that because sperm DNA is also condensed via protamines and unique histone modifications (Rousseaux *et al.* 2005) that enzymes within the cytoplasm of the oocyte target it for demethylation preferentially over the maternally methylated DNA in the earliest stages of embryonic development. From the two-cell stage to the eight-cell stage, the methylation status of the paternal DNA is further reduced by passive demethylation which also reduced the methylation of maternal DNA (Dean *et al.* 2001). By the end of the eight-cell stage in cattle, the morula stage in mice (Dean *et al.* 2001), and the blastocyst stage in pigs (Kang *et al.* 2001) only imprinted genes remained methylated, which I believe are

protected by specially modified histones yet to be identified, in a manner similar to the modifications seen in sperm histones (Rousseaux *et al.* 2005). In the bovine embryo *de novo* methylation occurs from the eight-cell stage right up to the 16-cell stage (Dean *et al.* 2001). However, mouse embryos remain demethylated right up to four cell divisions after the 16-cell stage, at which point the inner cell mass (ICM) of the blastocyst begins *de novo* methylation. Therefore, although basic methylation events are conserved across mammalian species, the timing of such events has been altered over the course of evolution. However, it still is a mystery how imprinted DMRs are maintained during this extensive passive/active demethylation process.

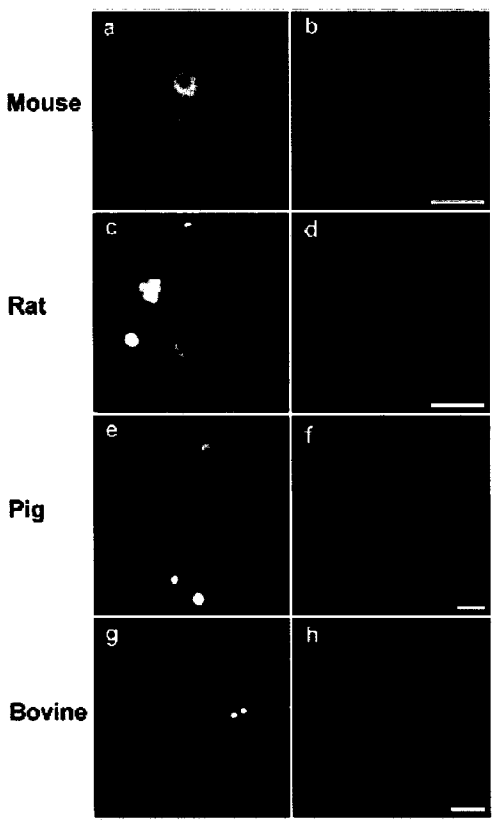

Figure 2.4 Indirect immunofluorescence with the use of an antibody to 5-methyl cytosine (in red) shows that the male pronucleus is selectively demethylated immediately after fertilization, whereas the female pronucleus remains methylated, in mouse (*a* and *b*), rat (*c* and *d*), pig (*e* and *f*), and cow (*g* and *h*). DNA staining is in blue. The larger of the two pronuclei is the male, except in cows, where they are of the same size. (Scale bar 19 μm.) (Dean *et al.* 2001)

2.4 Regulatory Mechanisms

After imprints are erased and established, they are maintained throughout development of the embryo and even during gene transcription. Imprinted genes are unique in that they are only expressed from one active allele while the other allele is functionally silenced. The regulation of this differential gene expression occurs in a number of differing ways, although the basic mechanism is the same. DNA methylation is produced by methyltransferases which work in concert with two other factors, histone deacetylases and methyl-binding proteins, as described in a review by Li (1999). This review describes how after methylation occurs, a methyl-binding protein such as MeCP2 is complexed with a histone deacetylase (HDAC) and binds to the site of methylation. Upon binding the HDAC deacetylates the nearby histones, compacting the DNA from a relaxed euchromatin formation into a condensed heterochromatin formation (Figure 2.5). Thus, any genes or regulatory elements nearby are silenced, as no enhancers, transcriptional promoters, or polymerases can properly bind to the DNA through the condensed heterochromatin.

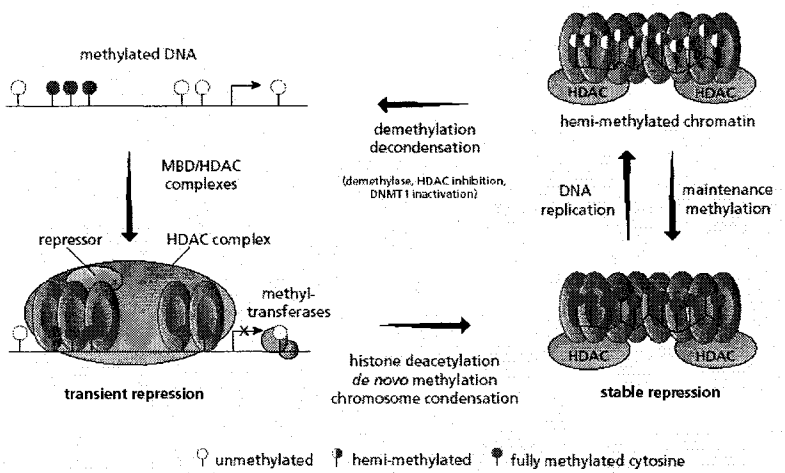

Figure 2.5 DNA is first methylated (*top left*), therefore methyl-binding proteins (MBD, methyl-binding domain) and HDAC complex to deacetylate the histones (*bottom left*). This forms heterochromatin and silences gene activity (*bottom right*). During replication the newly synthesized strand is in an unmethylated state, therefore maintenance must occur in order to preserve the imprinted gene patterns. (Modified from Li 1999)

Four regulatory systems have been discovered to date that use DNA methylation to regulate expression of imprinted genes: promoter methylation, antisense transcripts, boundary elements, and promoter silencing as reviewed by Reik and Walter (2001) (Figure 2.6). All of these mechanisms have been shown to exist in a well-studied region of the *Igf2/H19* genes (Kaffer *et al.* 2001).

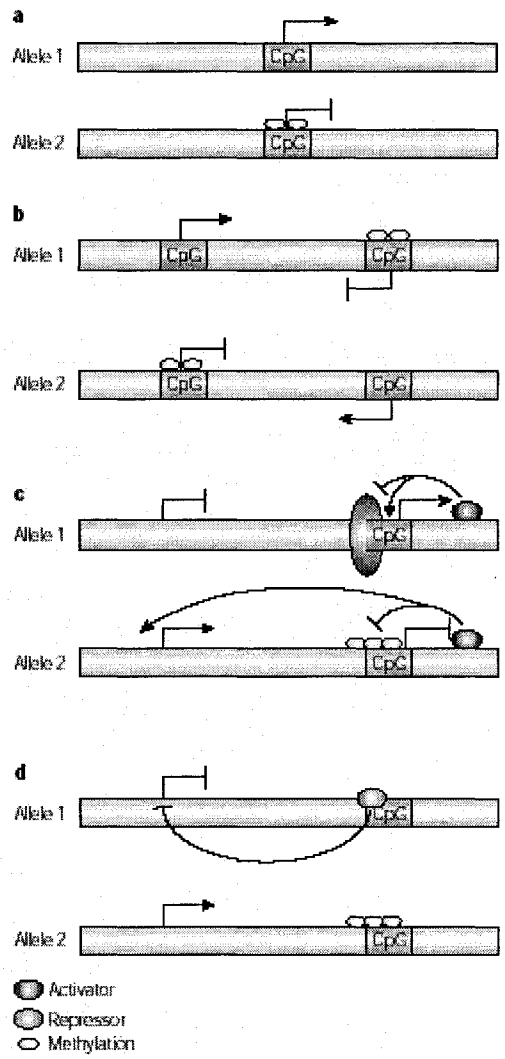

Figure 2.6 Regulatory mechanisms that control imprinted gene expression. Each uses DNA methylation in slightly different manners to control the activation or silencing of an imprinted gene. a) Promoter methylation b) Antisense transcripts c) Boundary Elements d) Promoter silencing (Reik and Walter 2001)

Promoter methylation has been demonstrated in the four upstream DMRs of *Igf2*, which are actually four different promoters that regulate *Igf2* expression in specific tissues, with varying intensities (Moore *et al.* 1997) (Figure 2.6a). For example, the P0 promoter expresses *Igf2* in the placenta, and is methylated in all tissues except the placenta, in which the expression of *Igf2* induces placental growth and development (Constancia *et al.* 2002). The methylation of specific promoters induces the formation of

chromatin which blocks the binding of RNA polymerases and thus prevents gene expression.

DNA methylation can also regulate gene expression by the binding of boundary elements (Figure 2.6c). The regulatory action of boundary elements is best described in the case of the CTCF binding protein that forms a physical barrier between *Igf2* and its tissue specific enhancers (Bell and Felsenfeld 2000). CTCF has an eleven-zinc finger-binding domain that allows binding of the protein to an unmethylated insulator region just upstream of *H19*. Once bound the protein blocks the conformational changes required to bring the enhancers into contact with the upstream promoters of *Igf2*, and instead forces them into contact with *H19*. However, if the insulator region, which is also an ICR, is methylated the CTCF protein cannot bind, and thus the enhancers can activate *Igf2* promoters and regulate expression of the *Igf2* gene. In mammalian conceptuses, the insulator region is methylated on the paternal chromosome allowing expression of *Igf2*, and unmethylated in the female homolog, which instead expresses *H19*.

The search for more DMRs, like the insulator region between *Igf2* and *H19*, has led to the discovery of yet another type of regulatory element that controls imprinted gene expression called a promoter silencer (Figure 2.6d). When the silencer DMR is unmethylated, repression factors bind and in a mechanism opposite to that of an enhancer, repress the expression of an imprinted gene. However, when the DMR is methylated the repressor cannot bind and thus the gene becomes actively transcribed. An example of a silencer is found between *Igf2* and *H19*, and specifically represses maternal expression of *Igf2* in the tongue and skeletal muscle (Ainscough *et al.* 2000).

The best-characterized example of antisense silencing is the *Air* transcript (Figure 2.6b). This paternally expressed antisense transcript is non-coding, with sequences overlapping those of the *Igf2*r promoter (Sleutels *et al.* 2002). *Air* specifically silences expression of three maternal genes, the upstream *Igf2*r gene that its sequence overlaps, plus *Slc22a2* and *Slc22a3* that are located upstream which do not have overlapping sequences. Thus, by some unknown mechanism the *Air* antisense transcript acts to methylate and thus silence three genes, *Slc22a2*, *Slc22a3*, and *Igf2*r.

These four mechanisms of imprinted gene regulation, promoter methylation, antisense transcripts, boundary elements, and promoter silencing, are vital for regulating the timing and tissue specific expression of imprinted genes. In the context of fetal growth and development, it is essential to understand how these regulatory mechanisms function, and if they can be altered by factors such as nutritional deficiency.

2.5 The Archetype of imprinted genes: Insulin Growth Factor 2 (Igf2)

Current estimates suggest that there are between 100 and 500 imprinted genes within the total 100,000 genes in the human genome with similar numbers likely existing in all mammals (Young 2001). Of these, only 50 have been identified, and many have not been fully cloned or their functions identified. However, a substantial proportion of the imprinted genes that have been characterized are involved in the control of fetal growth and placental development (Ferguson-Smith and Surani 2001; Young 2001). For instance, deletion of the paternally expressed *Igf2*, *Peg1/Mest, Peg3* or *Ins1/Ins2* genes results in intrauterine growth restriction (IUGR) (Cattanach and Kirk 1985; Duvillie *et al.*

1997; Lefebvre *et al.* 1998; Li *et al.* 1999). However, of these fetal growth and development genes, *Igf2* has been the best-studied so far.

In 1991 *Igf2* was identified by DeChiara *et al.* (1991) as one of the first imprinted genes. This gene is paternally expressed and maternally silent. When expressed, the gene produces growth promoting effects in both the placenta and fetus by binding to IGF1r (Constancia *et al.* 2002; O'Dell and Day 1998). IGF binding proteins both facilitate transport of IGF2 to the receptors and regulate their activities. In the mouse, *Igf2* expression induces this growth promoting effect unchallenged for the first 13.5 days of gestation (Morali *et al.* 2000), at which point maternally-expressed IGF2r is produced. IGF2r binds IGF2 with a higher affinity than IGF2r or Insulin type-A receptor (Figure 2.7). When IGF2 is bound by IGF2r, it is internalized into the cell for degradation; thus, maternally expressed IGF2r represses fetal growth by preventing paternally expressed IGF2 from producing growth-enhancing effects.

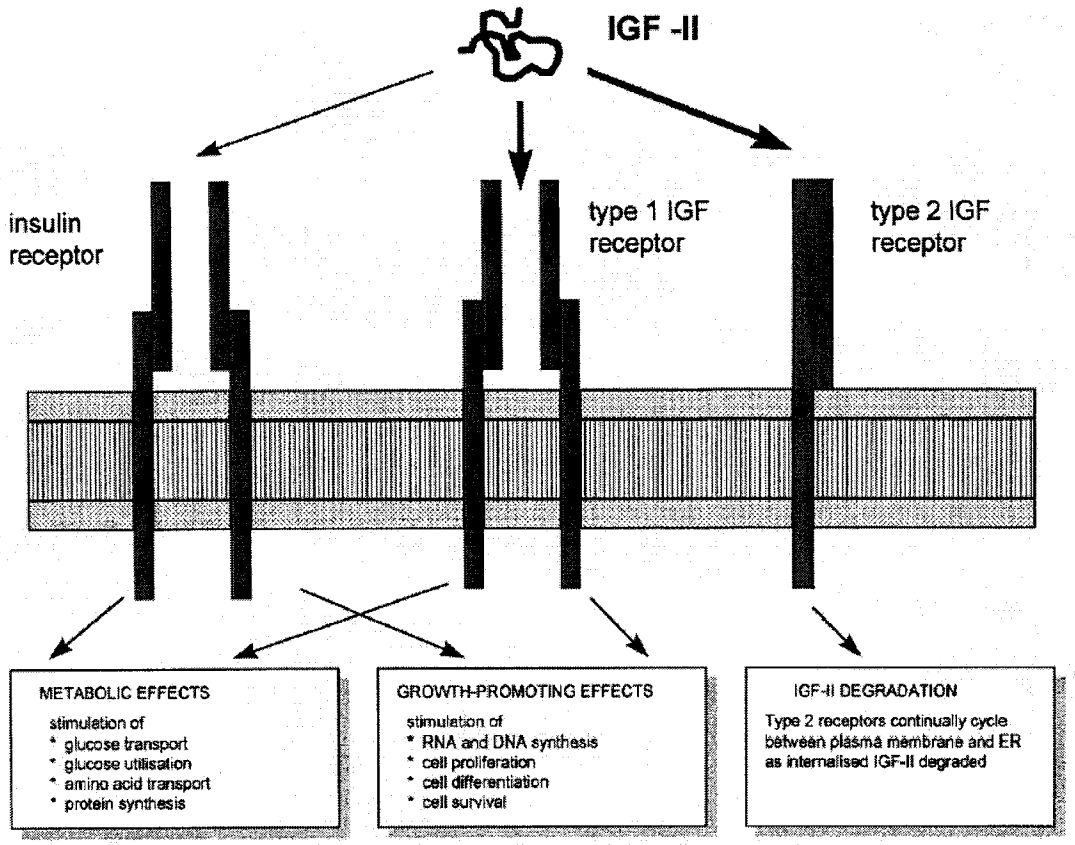

Figure 2.7 Paternally expressed IGF2 binds to IGF1r for the first 13.5 days of gestation in the mouse, promoting cellular growth and differentiation. After that time, IGF2 begins binding to maternally expressed IGF2r that internalizes IGF2 into the cell for degradation, and thus reduces the fetal growth rate.(O'Dell and Day 1998)

Igf2 has growth-enhancing effects in the placenta, producing a unique placental 4.7kb mRNA transcript that is regulated by a single promoter called P0 (Constancia *et al.* 2002). In the hemochorial placenta of the mouse, IGF2 is specifically expressed in the labyrinthine trophoblast and if knocked-out causes reduced placental growth. This reduction in placental mass results in growth retarding effects on fetal development as early as day 12 of gestation in the knock-out mouse, when the mean placental weight was 76% of normal (Constancia *et al.* 2002). This was caused by a decrease in passive permeability of nutrients in the mutant placenta. Initially secondary active placental amino acid transport is upregulated, compensating for the decrease in passive

permeability. Later, however, the compensation fails and fetal growth restriction ensues. Thus, imprinted gene action in the placenta directly controls the supply of maternal nutrients to the fetus, and therefore supports the genetic conflict hypothesis of imprinting (Moore and Haig 1991). From this research, it is hypothesized that *Igf2* and perhaps other imprinted genes, control both the placental supply of, and the genetic demand for, maternal nutrients to the mammalian fetus (Young 2001).

Although *Igf2* plays an important role in placental development, its activity as a fetal growth and development factor is better understood. *Igf2* Knockout mice are born only 60% of normal body weight and also show reduced pre-natal and post-natal growth rates (DeChiara *et al.* 1991). In normal mice, *Igf2* is paternally expressed in all fetal tissues, but is most highly expressed in the tongue, liver, and somites. Although in most fetal tissues *Igf2* is only paternally expressed, in the cases of the choroid plexus, leptomeninges and brain, neither allele is imprinted and expression is therefore termed biallelic. It should also be noted that in the adult liver, *Igf2* expression also becomes biallelic, which means methylation remodeling occurs as tissues differentiate during development (Vu and Hoffman 1994).

If imprinting methylation is established at the single cell stage, and is then altered in different cell lineages as development progresses, there must be an unknown mechanism by which imprints can be remodeled. Moreover, it might be possible that during embryonic or fetal development, the methylation of imprinted genes may be altered due to other factors such as environmental conditions in the womb.

2.6 Nutrition and DNA Methylation

The impact of nutrition on DNA methylation has been studied in detail, particularly in cancer and cloning research. As methylation can both silence and activate gene expression, understanding its effect on cellular growth, development, and differentiation is an important goal in these fields. Cancer research has determined the major nutrients that specifically alter DNA methylation in adult tissues when feeding a restricted diet. In particular, dietary restriction of methionine, choline, folate, zinc, Vitamin B12 and B6 result in a decrease in DNA methylation in adult tissues (Friso and Choi 2002). All of these nutrients play a role in single carbon biosynthesis, the biochemical process by which methyl-groups are transferred to a methyl-donor amino acid called S-adenosyl-methionine (SAM). In this pathway, a carbon unit from serine or glycine is transferred to tetrahydrofolate (THF) to form methylene-THF. This molecule is then reduced to methyl-THF which is used to methylate homocysteine to form methionine, a reaction which is catalyzed by a B_{12}-containing methyltransferase. Much of the methionine that is formed in this manner is converted to S-adenosyl-methionine (SAM), the universal donor of methyl groups for the methylation of both imprinted and non-imprinted DNA (Figure 2.8).

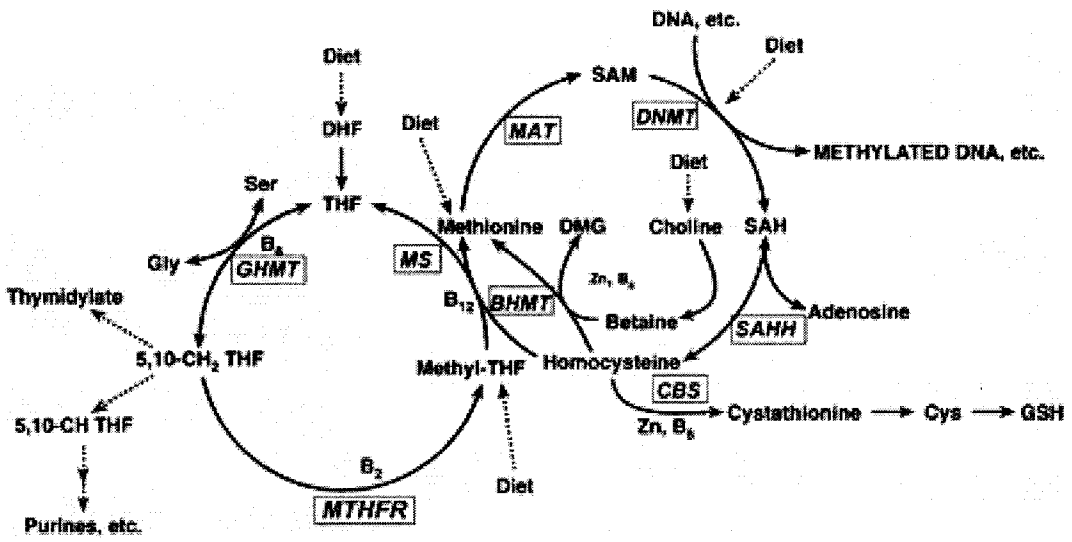

Figure 2.8 Through this pathway all the various nutritional components act together to generate and regenerate SAM. A DMNT can then transfer the methyl-group to an available cytosine to produce imprinted regions of methylated DNA.

Enzymes are shown in italics with a box around them. These include glycine hydroxymethyltransferase (GHMT); methylenetetrahydrofolate reductase (MTHFR); 5-methyltetrahydrofolate: omocysteine S-methyltransferase (methionine synthase of MS); betaine-homocysteine S-methyltransferase (BHMT); methionine adenosyltransferase (MAT); DNA methyltransferase (DNMT); S-adenosyl-homocysteine hydrolase (SAHH); and cystathionine-b-synthase (CBS).
Abbreviations: dihydrofolate (DHF); serine (Ser); glycine (Gly); Cysteine (Cys); tetrahydrofolate (THF); vitamin B6 or pyridoxine (B6); vitamin B12 or cobalamin (B12); vitamin B2 or riboflavin (B2); 5,10-methyltetrahydrofolate (5,10-CH2 THF); 5,10-methylenetetrahydrofolate (5,10-THF); 5-methyltetrahydrofolate (methyl-THF); zinc (Zn); dimethylglycine (DMG); S-adenosylmethionine (SAM); S-adenosylhomocysteine (SAH); glutathione (GSH).
(Davis and Uthus 2004)

A family of enzymes called DNA Methyl-Transferases (DNMT) facilitate the transfer of the methyl-group from SAM to specific cytosine residues whose neighboring histones become deacetylated by a histone deacetylase/methyl-binding protein complex, compacting the methylated region of DNA, and forming a condensed region of heterochromatin (Li 1999). Thus, any genes or regulatory elements in the heterochromatin region are silenced, as no enhancers, transcriptional promoters, or polymerases can properly bind to the DNA through the condensed heterochromatin.

Afterwards the methyl-group is regenerated on the used SAM through the enzymes and nutritional co-factors of single carbon biosynthesis. Thus, by reducing the amount of nutrients required for DNA methylation, the activity of DNMT can be reduced. Experiments in cancer research have shown that reducing the dietary intake of nutrients essential for DNA methylation can reduce DNA methylation in adult tissues.

For instance zinc deficiency can reduce the efficiency of methyl-transfer from SAM in rat liver, resulting in genomic DNA hypomethylation as well as histone hypomethylation (Dreosti 2001; Wallwork and Duerre 1985). Moreover, animals fed diets deficient in the methyl donors methionine and choline, have hypomethylated DNA (Locker *et al.* 1986; Tsujiuchi *et al.* 1999; Wainfan *et al.* 1989). Further studies have shown that both global (Sibani *et al.* 2002) and gene specific methylation (Bhave *et al.* 1988) are affected by these mechanisms. The additional restriction of dietary vitamins B_{12} and B_6 can also result in demethylation, as both vitamins act as coenzymes that participate in one carbon biosynthesis (Selhub 2002).

Manipulation of dietary folate supply has provided the most compelling data for the interaction of nutrients and DNA methylation. The coenzymatic forms of folate, particularly 5-methyltetrahydrofolate (methyl THF), serve as a cofactor and substrate for the regeneration of methionine from homocysteine in single carbon biosynthesis. This rate-limiting step is essential for altering the availability of methionine that can then be adenylated to produce SAM, the universal methyl donor essential for DNA methylation. Therefore, not only can dietary folate depletion decrease genomic DNA methylation in both human (Jacob *et al.* 1998; Rampersaud *et al.* 2000) and animal models (Balaghi and

Wagner 1993), but, as described in the study by (Rampersaud *et al.* 2000), a folate replete diet also may restore DNA methylation status.

2.7 Fetal programming and the Barker Hypothesis

Researchers in fetal nutrition have speculated that during starvation of the dam, the expression of specific genes could be programmed to regulate the physiological changes the fetus undergoes to adapt to starvation (Moore and Reik 1996). Moreover, permanent phenotypic changes as a result of starvation *in utero* could be produced from epigenetic defects, as speculated by Young (2001). Many studies have been conducted on data collected from women who suffered nutritional restriction during pregnancy which indicate that nutritionally-restricted fetuses can develop a wide variety of diseases and conditions in adult life. These include coronary heart disease, hypertension, type 2 diabetes, insulin resistance, altered cholesterol metabolism, problems with blood coagulation, polycystic ovary syndrome, and negative effects on fetal growth and placental size (Godfrey and Barker 2000). All of these adult diseases can be associated with deficits in fetal nutrition, as predicted by the theory of fetal programming (Barker 1998).

The theory of fetal programming, also referred to as the Barker Hypothesis, is based on Barker's statistical analysis of a variety of data from all over the world. One of the best known analysis was on data collected in the early 1900's in the south east of England, where a correlation between low birth weight data and a higher incidence of cardiovascular disease in adults was discovered (Barker and Osmond 1986). The results of this study suggested that events in early childhood could influence the occurrence of

coronary heart disease in adult life. Since then, similar studies in Sweden, Wales, the USA, and India have shown the same relationship between low birth weigh and increased risk of coronary heart disease (Barker 1998). The hypothesis is that at certain times in early life, the fetus or infant may be susceptible to these adverse conditions, which then produce life-long effects on organ structure and function; this is termed "fetal programming" or "metabolic imprinting".

There are many factors which could influence the development of a fetus and result in a change in fetal programming; however, fetal nutrition is the best documented. The best case study for suggesting a connection between fetal programming and nutrition examined the outcomes of the Dutch famine during the Second World War (Roseboom *et al.* 2001). It was discovered that the timing of nutrient restriction during gestation had greater effects on organs and systems developing at that time. Additionally, maternal malnutrition in early gestation was found to permanently affect adult health, without necessarily affecting the size of the baby at birth. Those individuals who were subjected to nutrient deprivation during early gestation had increased LDL cholesterol, body mass index, and higher risk of coronary heart disease. Individuals exposed to famine in mid gestation had increased prevalence of obstructive airways disease, and those exposed in late gestation had reduced glucose tolerance.

2.8 The Barker Hypothesis and Epigenetics

The fetal programming theory encompasses a number of different biological mechanisms that act together in a synergistic fashion to alter fetal growth and development. The concept of gene imprinting is entirely consistent with the theories of fetal programming

or metabolic imprinting (Young 2001). Gene imprinting would be one mechanism synergizing with many other regulatory mechanisms in the developing embryo, to regulate later fetal growth and development. The effects of nutrition on fetal growth are far more complex than simply those of substrate deprivation, and gene imprinting is just one mechanism by which this complex effect is seen (McKay *et al.* 2004). Although regulation at a genetic level undoubtedly alters fetal growth and development there are indications that gene imprinting and DNA methylation plays a role in this.

There are reports that suggest nutrition can alter gene imprinting after fertilization due to a failure to maintain imprinting patterns. Errors have been linked in embryonic gene methylation to a number of human diseases such as Prader-Willi, Angelman, Beckwith–Wiedemann, and Silver-Russel syndromes (Paulsen and Ferguson-Smith 2001). A loss of imprinting (LOI), involving failure to methylate and thus suppress gene activity, can also result in Wilms Tumors, embryonic tumors of the liver, and many other forms of cancer in both newborns and adults (Richardson 2002). Another error in DNA methylation arises during nuclear transfer in the cloning of mammals, whereby a failure to erase, or "reset" the methylation pattern to an embryonic stage of development causes premature abortions. This failure to reset methylation patterns to earlier stages of development has been seen in the high embryonic fatality rate in the cloning of sheep, cattle, mice, goats, and pigs (Humpherys *et al.* 2001; Polejaeva *et al.* 2000). There have also been indications that failure during either establishment or maintenance of imprinted genes in sheep and cattle oocytes during IVM and/or IVF result in large offspring syndrome (LOS) as reviewed by Young *et al.* (1998). In this review, much of the evidence for LOS is described as anecdotal, coming from embryo transfer companies

rather than published scientific reports, and as such, the phenotypes of these LOS are not well characterized. At this time, no perturbing environmental factors have yet been identified that cause LOS, however, when sheep embryos are cultured in chemically-defined media, there is a noticeable reduction in the incidence of LOS (Young *et al.* 1998). At present researchers are looking for a way to screen LOS embryos *in vitro* and to identify a link between LOS and imprinted genes. I believe that a disruption in reprogramming and/or maintenance of imprinted growth related genes might be the cause of LOS. Oddly, LOS has never been seen in IVM/IVF mice, hamsters, rabbits and pigs, and the various reasons for this are described in Young *et al.* (1998). In brief summary, mice must be cultured in serum free media because unlike livestock species, embryonic development is inhibited by serum. In addition, rodents, rabbits, and pigs are litter bearing species which may make it more difficult to detect increases in birth weight when growth may be limited by placental crowding. Alternatively, IVF human embryos do not show LOS because they are only cultured to the 4-cell stage, while IVF livestock embryos are usually cultured for five extra days to reach the blastocyst stage. During those extra days in cattle and sheep, I believe that an error in maintenance occurs, and the methylation of imprinted genes is lost during progressive rounds of replication. Therefore, the current theory is that the type of media, acting as the nutritional source for the embryo, can result in errors that affect gene imprinting during establishment or maintenance of methylation.

There is some speculation that epigenetic defects resulting in altered fetal programming may also originate from the oocyte. For instance, full-sibling embryos cultured in the same drop of culture serum produced both normal and abnormal offspring,

indicating that the oocyte was a factor (Sinclair *et al.* 2000). A more recent study was also able to show that IVM oocytes had a higher incidence of epigenetic defects when compared to *in vivo* matured oocytes (Gioia *et al.* 2005). It is entirely possible that the extent of epigenetic defects seen in these oocytes may depend on the timing of disruptive influences. This critical window for influencing epigenetic mechanisms in the oocyte would be very similar to the effects seen in the Dutch famine, where the timing of nutrient deprivation in women had variable outcomes in adult life (Roseboom *et al.* 2001). As seen in the mouse, the final progression of oocyte maturation from the germinal vesicle stage to arrest at metaphase II is a critical period for gene imprinting, when DNA methylation is established (Lucifero *et al.* 2002). It is most likely that, in cases of IVM, the environment provided by the media is unsuitable for the progression of immature oocytes through the final establishment of DNA methylation of imprinted genes if the oocytes are not epigenetically competent. It still remains to be determined what serum components or nutritional factors may influence the epigenetic development of the oocyte.

2.9 Opportunities for epigenetic research in swine

To date there is no evidence that undernutrition can impact methylation of maternally imprinted genes in swine, and alter growth and development of a porcine embryo. An initial goal is, therefore, to determine if nutrition can alter establishment of maternal gene imprinting, by restricting the total feed intake of the sow in the critical period of late follicular development that has been shown in previous studies to produce latent effects on embryonic survival in both the gilt (Almeida *et al.* 2000) and sow (Zak *et al.* 1997a).

As the mechanisms of imprinting are complex and multifactorial, this broad approach seems advisable. Moreover, it has been shown in previous studies that limiting feed intake during this period will also result in developmentally retarded oocytes (Yang *et al.* 2000; Zak *et al.* 1997b), coinciding with the critical initial period of establishment when imprinting begins to occur as seen in other species (Lucifero *et al.* 2002). During this period, maternal constituents are transmitted to the cytoplasm to influence the maturation of the oocyte, and the final stages of gene imprinting establishment. This period of feed reduction is associated with a decrease in follicular fluid IGF-1, a factor required for cumulus cell development (Zak *et al.* 1997a; Yang *et al.* 2000 ; Clowes *et al.* 2003): This alters the unique oviductal environment in which *in vivo* follicular and early embryonic development take place (Novak *et al.* 2002). Indirect effects of inadequate follicular maturation on early embryonic development may also be mediated by metabolic effects on the process of luteinization and differences in progesterone synthesis (Mao *et al.* 2001). These effects on the environment of the uterus and oviduct may also fail to provide the developing embryo with key nutrients for maintenance of DNA methylation of imprinted genes.

We have previously reviewed the integrated mechanisms that may contribute to early embryonic survival and development, and responses influenced by changes in nutrient and metabolic state in swine (Foxcroft *et al.* 2000; Foxcroft *et al.* 2006). However, to date no research has been done in swine to establish a connection between DNA methylation and nutrition. Although studies on the affects of dietary folate on DNA methylation have yet to be conducted in the pig, a recent experiment by Guay *et al.* (2002) in sows showed that reducing folic acid and vitamin B_{12} in early pregnancy

decreased available homocysteine and methyl-THF *in utero*. This reduced embryonic growth and development would be expected to have an impact on DNA methylation in the tissues of the sow, and on the methylation of imprinted genes in the implanting and developing embryo. Furthermore, it has been shown that if a sow is provided folate supplements between weaning and sixty days after mating there are on average 1.5 more piglets/litter (Matte *et al.* 1984). Although these authors hypothesized that the folate was needed as a limiting nutrient for DNA/RNA synthesis in embryogenesis, this effect could also be due to improved establishment and/or maintenance of the methylation imprints on genes critical for embryonic survival. Embryonic death and decreased litter sizes from this effect are seen in IVF nuclear transfer studies conducted in most domesticated animals, including the pig (Kang *et al.* 2001). Moreover, in studies where the sow's diet is restricted, the maturation of oocytes is reduced (Zak *et al.* 1997b), and would likely result in increased developmental delays and/or increased embryonic mortality. Although these effects were associated with altered hormonal levels in the sow and reduced nutrient availability for the embryos, further studies may show that DNA methylation, and more specifically, gene imprinting is affected by maternal nutrition in swine.

In pigs the global methylation status of DNA has never been assessed from the time the single oocyte reaches maturity after germinal vesicle breakdown (GVBD). However, one report illustrated the changes in global methylation from fertilization through to the blastocyst stage in swine (Kang *et al.* 2001; Figure 2.9). Using the short interspersed element (SINE) sequence Pre-1 and centromeric satellite sequences in pig embryos, these authors showed that the changes in methylation were similar to those seen in other mammalian species, with the exception that the most dramatic demethylation of

non-imprinted genes occurred at the blastocyst stage of development. Although this technique only looked at changes in methylation surrounding for two specific non-coding regions, they are a good indicator of global methylation as a whole. These sequences have also been used in subsequent bisulphite PCR studies to identify increased epigenetic variation in cloned swine (Archer *et al.* 2003).

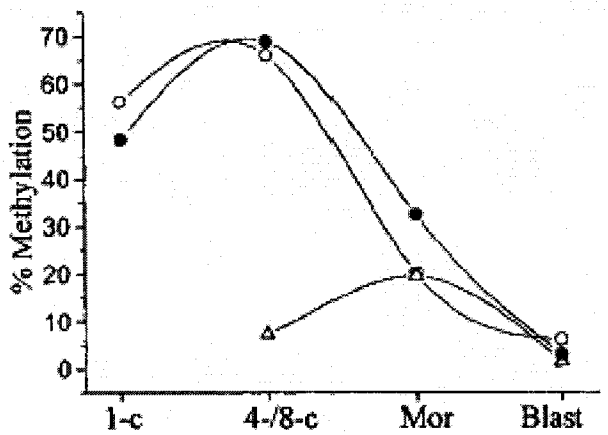

Figure 2.9 Gradual demethylation patterns in satellite sequences of nuclear transfer (NT) pig embryos at various stages of development. Methylation changes in accordance with the developmental stage of *in vivo* (*open triangle*), IVF (*open circle*), and NT (*closed circle*) pig embryos. *1-c* = 1-cell egg; *4-/8-c* = 4- to 8-cell embryos; *Mor* = morulae; *Blast* = blastocysts. In all cases, DNA methylation of satellite markers reaches maximum demethylation at the blastocyst stage of development, and although not shown, the DNA becomes remethylated beyond this point (Kang *et al.* 2001).

Although these sequences have proven useful, further sequencing needs to be performed in the pig to fully understand the roles that imprinted and otherwise methylated genes have in porcine embryonic growth and fetal development. To date, there are few imprinted genes fully sequenced in the pig (Table 2.1). However *Igf2* has been sequenced in the pig and Van Laere *et al.* (2003) identified a mutation in *Igf2* that provides enhanced muscle growth in certain pigs. Aside from *Igf2*, few other imprinted genes have been sequenced, making research into the area of imprinted gene expression

much more difficult, and this lack of information needs to be resolved. Without more candidate imprinted genes available with which to quantify biological differences between sows, research in this area will remain limited. Statistical analysis has indicated several qualitative trait loci (QTL) in swine that have differences between maternal and paternal inheritance, indicating that there may be many more imprinted genes to be mapped and sequenced in the pig, with great relevance to the swine genetics industry and subsequently to producers (Table 2.1). Once identified, these imprinted genes may also be found to have essential roles in prenatal development and possibly embryonic survival in swine.

Table 2.1 Summary of imprinted genes sequenced in the pig and quantitative trait loci identified in the pig of importance to swine industry.

Item	Classification	Reference

IMPACT	Sequenced imprinted gene	(Okamura *et al.* 2005)
IGF2-INS-H19	Complete regional sequence	(Amarger *et al.* 2002)
IGF2R	Sequenced imprinted gene	(Killian *et al.* 2001)
IGF2AS	Sequenced imprinted gene	(Braunschweig *et al.* 2004)
DLK1- GTL2 (Callipyge)	Sequenced imprinted gene	(Kim *et al.* 2004)
Number of nipples	QTL	(Holl *et al.* 2004)
Backfat	QTL	(Thomsen *et al.* 2004)
Muscle thickness	QTL	(de Koning *et al.* 2000)
Loin muscle area	QTL	(Thomsen *et al.* 2004)
Meat color	QTL	(de Koning *et al.* 2001)
Mummified pigs	QTL	(Holl *et al.* 2004)
Still born pigs	QTL	(Holl *et al.* 2004)
Birth weight	QTL	(Holl *et al.* 2004)
Ovulation rate	QTL	(Holl *et al.* 2004)
Age at puberty	QTL	(Holl *et al.* 2004)

With sequencing data being limited in the pig, researchers have begun to turn towards broader approaches to uncover the relationships between DNA methylation, embryonic survival, and fetal development. The antibody for 5-methyl-cytosine, which is non-species specific, allows for sensitive detection of changes in global methylation in the male and female pronuclei of fertilized zygotes (Gioia *et al.* 2005; Santos and Dean 2004). This technique could be used to examine the quality of sperm and oocytes before IVF, providing an additional level of quality control which may help avoid epigenetic defects (Gioia *et al.* 2005).

Research into swine epigenetics is still in its infancy. As molecular techniques become more automated and able to handle higher throughput, research in this area is likely to grow rapidly. To date, most of the swine epigenetic research is focused on cloning technologies, however, swine nutrition and reproduction will inevitably become amalgamated into the study of swine epigenetics to better control variability in the quality of swine production. As such, it becomes important to investigate the relationships between established experimental paradigms with known effects on sow metabolism during lactation, reproductive performance, and embryonic survival, and epigenetics. Previous studies which increased lactational catabolism in sows have shown decreased embryo survival (Zak *et al.* 1997a) and changes in reproductive performance (Zak *et al.* 1997a, Zak *et al.* 997b, Clowes *et al.* 2003). As such, the experiments in the following chapters attempt to measure these characteristics in order to establish a relationship to this experimental paradigm. Furthermore, it has been suggested that epigenetic defects may result in increased intrauterine growth retardation (IUGR) and increased male or female mortality (Young *et al.* 2001), which may be observable by day 30 of gestation. The sequences for a few candidate genes are also available in the pig, specifically *Igf2*, *Igf2r*, and *Xist* and, if defective in embryonic expression, could result in IUGR and / or embryonic mortality (Young *et al.* 2001). Furthermore, *Igf2* is paternally-imprinted (Amarger *et al.* 2002) while *Igf2r* is maternally-imprinted (Killian *et al.* 2001), therefore changes in expression patterns of either would indicate whether epigenetic defects originated from the maternal genome, or from failure to maintain imprinting status during embryogenesis. *Xist* expression is regulated by maternal methylation and has been linked to female-biased embryonic mortality (Panning and Jaenisch 1996; Xue *et al.* 2002).

Therefore quantifying the expression of these genes may reveal epigenetic defects in embryonic development. Furthermore, quantification of the methylation status of the embryonic genome using reverse-phase high performance liquid chromatography (Ramsahoye 2002) may also provide some insight into genome wide epigenetic defects. However, if defects in any of these epigenetic traits reduce embryo survival before day 30 of gestation then the surviving embryos would not carry these defects, and therefore would not show any changes in these traits. However, an analysis of the variance of these traits, such as performed by Archer *et al.* (2003), may identify which are essential for embryonic survival. Assuming embryo survival is reduced by day 30 of gestation as seen in Zak *et al.* 1997a, it would be invaluable to conduct a second animal trial using the same experimental paradigm, but collecting embryos at day 5 of gestation before embryonic survival becomes a factor (Geisert and Schmitt, 2002) could allow identification of specific defective epigenetic traits. Furthermore, blood sampling before, during, and following lactational feed restriction in the sow would enable the identification of which nutrients are essential for DNA methylation (Davis and Uthus, 2004) and may lead to embryonic epigenetic defects. Methylation analysis of the sow's leukocyte DNA using a dot blotting technique (Park *et al.* 2005) may also provide some insight into the relationship between nutrient status, DNA methylation, and presumptive embryonic epigenetic defects originating from the sow.

The following chapters will detail these investigations in determining if increased catabolism during the last week of lactation in primiparous sows has any effect on epigenetic traits in embryos from subsequent litters.

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CHAPTER 3

NUTRITIONAL RESTRICTION IN LACTATING PRIMIPAROUS SOWS SELECTIVELY AFFECTS FEMALE EMBRYO SURVIVAL AND OVERALL LITTER DEVELOPMENT. [1]

3.1 Introduction

The consequences of maternal nutrition on embryonic and fetal development in mammals in relation to gene imprinting (Young 2001) remains poorly understood. The severity and timing of nutritional restriction in the lactating primiparous sow provides an interesting model to study latent metabolic effects on embryonic development and survival since such restriction appears to exert selective effects on subsequent reproductive performance (Foxcroft 1997). In situations of severe maternal catabolism, litter size in the subsequent parity is limited by both a reduced ovulation rate and decreased embryonic survival. However, the timing of feed restriction can have variable effects on embryonic survival (Zak *et al.* 1997a) and oocyte quality (Zak *et al.* 1997b).

Lactational catabolism exerts a detrimental effect on the maturity of oocytes recovered from the presumptive pre-ovulatory follicles after weaning when tested using *in vitro* oocyte maturation assays (Zak *et al.* 1997b). Furthermore, follicular fluid from these follicles was less able to support the *in vitro* maturation of pools of oocytes recovered from prepubertal gilts. These results were supported by later studies that established a relationship between increased protein catabolism in lactation and a decrease in the size, number and maturity of follicles at the time of weaning, and the

[1] This chapter comprises the text of a paper that has been published in Reproduction, Fertility and Development, 2006, **18**, 347-355

ability of follicular fluid aspirated from these follicles to support *in vitro* oocyte maturation (Yang *et al.* 2000a; Clowes *et al.* 2003a).

Reviews by Pope *et al.* (1990), Hunter (2000), and Geisert and Schmitt (2002) discuss evidence linking variability in oocyte developmental competence to asynchronous embryonic development, as a key factor in determining embryonic survival in the pig. Previous studies of developmental variability within litters have explored possible mechanisms that decrease embryonic survival for the less developed embryos. However, these studies did not address how this might skew the sex ratio of the surviving embryos. Although there are reports that litter sex ratios in the pig can be affected by paternal breed (Gorecki 2003), uterine capacity (Chen and Dziuk 1993), and oocyte glutathione concentration (Yoshida, *et al.* 1993), few have explored the possibility that metabolically-induced changes in embryonic survival may also be gender-specific in the pig. Normal populations of swine show no consistent differences in sex ratios (Gray and Katanbaf 1985; Brooks, *et al.* 1991), indicating no underlying survival advantage for a specific gender under normal conditions. Consistent with the evidence that *in utero* competition among littermates favors survival of the most advanced embryos (Pope 1994), Krackow (1995) suggested that asynchrony between uterine and embryonic (blastocyst) development prior to implantation may alter sex ratios in favor of male embryos. This suggestion was based on evidence that male embryos develop faster to the blastocyst stage than females, making them more likely to implant first (Cassar *et al.* 1994). Therefore, if uterine development was synchronized with the earlier implanting and developmentally more advanced male blastocysts, male-biased litters would result. However, if uterine development is not advanced, then survival of the later implanting,

less developmentally mature, female embryos might be favoured, and female-biased litters would result.

Therefore, in the present study we examined the possibility that feed-restriction of primiparous sows in late lactation affects subsequent embryonic survival and development of surviving embryos in a gender-specific manner.

3.2 Materials and methods

3.2.1. Animals and treatments

This study was performed in accordance with the Canadian Council on Animal Care guidelines and with the approval of the Faculty Animal Policy and Welfare Committee (Protocol #2003-09), and involved 34 primiparous F1 sows (Genex Hybrid; Hypor, Regina, SK, Canada) which farrowed normally and were managed according to standardized and approved protocols at the Swine Research & Technology Centre (SRTC), University of Alberta. Within 48 h after farrowing, litter size was standardized to a minimum of 9 piglets per sow by cross-fostering. On Day 14 of lactation, sows pre-selected for trial were then paired on the basis of similar initial body weight and backfat at farrowing (Day 0), and similar loss in weight and backfat between Day 0 and 14 of lactation. Within a pair, sows were then randomly allocated to one of two treatments (Restrict or Control).

All sows were fed three times daily (0800, 1200 and 1500) with a standard lactation diet (14 MJ DE kg^{-1}, 20% CP, 1.02% lysine) from Day 0 to 14 of lactation using a standardized step-up feeding regimen, which encouraged sows to increase their voluntary feed intake (VFI) to reach a pre-set maximum of 5.0 kg day^{-1} as soon as

possible after farrowing. This pre-set maximum feed allowance was based on previous data collected on 312 primiparous sows from the SRTC, indicating that a VFI of 5.0 kg day^{-1} was marginally below the recorded maximum VFI (5.07±0.27 kg day^{-1}) for these primiparous sows in late lactation. Feed intakes were monitored daily throughout the experiment using standardized feed weigh-back procedures. In an effort to ensure that sows were not being underfed relative to their metabolic requirements, and to remove confounding effects of sow body mass on subsequent productivity, heavier sows (greater than 220 kg at farrowing) were not included in this experiment. Furthermore, in compliance with guidelines established under the FAPWC approval for this study, pre-selected sows losing over 20 kg of body weight by Day 14 were removed from trial, on the basis that further feed restriction would be a welfare concern; replacement sows were incorporated as needed. From Day 14 to 21 of lactation, Control sows continued to be fed a maximum of 5.0 kg day^{-1}, while Restrict sows were limited to 2.5 kg day^{-1} of the same diet; daily feed allowances were again equally divided between the three feeding times. Water was freely available for sows and litters throughout the experiment and creep feed was not provided to the litters.

Sow backfat was measured by real-time ultrasonography using a 5 Mhz real-time linear probe (Scanprobe II, Scano, Ithaca, NY) and sow body weights were measured within 24 h after farrowing (Day 0), and on Day 14 and Day 21 of lactation. Sow weight and backfat measurements were used to estimate total sow body protein and fat mass using the equations of Whittemore and Yang (1989). After weaning and until breeding, sows were provided *ad libitum* access to the same lactation diet.

3.2.2 Calculations of net energy balance and energy requirements

Net energy balance (Mcal) was estimated as the sow's energy requirements for maintenance and milk production (Mcal) subtracted from the sows energy intake. Maintenance requirements were calculated using the equation; 1.86 Mcal ME kg^{-1} $BW^{0.75}$ (NRC 1998). Litters were weighed after litter-size adjustments within 24 h after farrowing, on Day 14 and Day 21 of lactation, and whenever there was a litter size adjustment. Total weight gain of the litter was then used to estimate milk production based on the assumption that 3.88 g of milk production was equivalent to 1 g day^{-1} of litter gain (Clowes *et al.* 2003a). Energy requirements for milk production were then calculated using the formula of Clowes *et al.* (2003a).

3.2.3 Sow management after weaning

Sows were checked twice daily at 0800 and 2000 for onset of first standing oestrus after weaning, using back-pressure testing during fence-line contact with a mature, high libido boar. Sows were artificially inseminated with pooled semen (3 x 10^9 morphologically-normal, and motile, spermatozoa $50mL^{-1}$ dose) that was no more than 3 days old, and collected on-site from the same three Genex Large White boars (Hypor, Regina, SK, Canada) designated for use in the experiment. Sows were inseminated 12 h after onset of standing heat and every 24 h thereafter, as long as a good standing heat reflex was observed, with the quality of the insemination procedure recorded. After insemination, sows were fed a standard gestation diet according to standard SRTC procedures. Gestational day was based on the day of onset of oestrus being designated as Day 0 of pregnancy.

3.2.4 Ovulation rate and embryonic survival

All sows were slaughtered on-site by qualified staff using approved necropsy procedures on Day 28 ± 3 of pregnancy. Immediately after slaughter, the reproductive tract was recovered from each sow, the number of corpora lutea (ovulation rate), and the number, apparent viability, length, and weight of all embryos *in utero* were recorded using standard procedures (Almeida *et al.* 2000). Placental wet weight was initially recorded (n=14 sows) as a measure of placental development using established procedures (Almeida *et al.* 2000). For the remaining 20 sows, chorio-allantoic fluid volumes were measured, as a more practical and predictable estimate of placental development (unpublished data). Immediately after dissection, all embryos were wrapped in foil, flash frozen in liquid nitrogen, and transported in dry ice for storage at -70°C until further analysis.

3.2.5 DNA extraction

Individual embryos were carefully removed from their foil wrappings using pre-chilled tweezers, while immersed in liquid nitrogen in a pre-chilled mortar packed in dry ice. Embryos were then pulverized and ground to a fine powder using a pre-chilled pestle. Aliquots of approximately 100mg of tissue from individual embryos were transferred to pre-chilled, sterile 15mL Falcon tubes and stored at -70°C until extraction. DNA was isolated using a standard phenol/chloroform/isoamyl alcohol extraction protocol (Sambrook and Russell 2001) with all reagents purchased from Sigma (Oakville, ON).

3.2.6 Sex typing PCR

Embryos were sex typed using a modified version of the protocol developed by Pomp *et al.* (1995). Primers specific to the SRY region of the Y-chromosome 5′-TGAACGCTTTCATTGTGTGGTC-3' (forward) and 5'-GCCAGTAGTCTCTGTGCCTCCT-3' (reverse) were used. As a positive internal control, primers for the sex chromosomal ZFX/ZFY homologs were multiplexed in each reaction with 5'-ATAATCACATGGAGAGCCACAAGCT-3' (forward) and 5'-GCACTTCTTTGGTATCTGAGAAAGT-3' (reverse). With each PCR tube kept on ice a master mix of 1.5µl 10xBuffer (ABI, Foster City, CA), 0.9µl of 25mM $MgCl_2$ (ABI), 0.3µl of 10mM dNTP mix (Roche, Laval, Quebec), 1.5µl 10x Primer Mix (1µM SRY, 2µM ZFX/ZFY), and 9.68µL sterile MilliQ water, was added. After adding 1µl of extracted genomic DNA, tubes were placed in a 96-well iCycler thermocycler (BioRad, Mississauga, ON) and the following PCR program was utilized: 94°C for 4 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1min, and then 72°C for 1 min. Following this, a final extension phase of 72°C for 5min and an indefinite hold at 4°C was carried out. Samples not used immediately for gel electrophoresis were stored at 4°C overnight or at -20°C until analyzed. PCR products, along with a 100bp ladder (Invitrogen, Burlington, ON) were separated on a 2% (w/v) agarose gel with a 1xTBE buffer run for 1 h at 90v. The gel was stained with 0.2 mg/mL ethidium bromide (Bio-Rad) and PCR amplicons were visualized and images were acquired using a BioRad Gel Doc System.

3.2.7 Statistical Analysis

A total of 17 pairs of sows met all the criteria for inclusion in this study and data from these sows was used in the final analysis. Sow was used as the unit of measurement for determining treatment effects on ovulation rate (number of corpora lutea), number of live embryos, embryonic survival rate (ESR), number of males, number of females, and sex ratios. Data were analyzed using the MIXED procedure of SAS (SAS Inst., Inc., Cary, NC) as a randomized block design, with blocks based on sow pairs. The Kenwardroger approximation was used for the denominator degrees of freedom. ESR was expressed as the percentage of ovulations proportionate to the number of live embryos at Day 30 of gestation, and sex ratio was calculated as the percentage of males within the litter. These percentage data were normalized using arcsin transformation before analysis.

All measurements of sow and litter characteristics were analyzed using the same MIXED procedure of SAS described above. For the analyses of weight and backfat changes between Day 0 to 13 and Day 0 to 21, Day 0 weight and backfat were used as covariates where they improved the goodness of fit. For the analysis of weight and backfat changes between Day 14 to 21 weight and backfat, respectively at Day 14 were used as covariates. All statistics performed on data related to Day 14 and 21 sow protein and fat composition used Day 0 and 14 measurements, respectively, as covariates to adjust for pre-treatment differences. No covariates showed significant interactions with treatment, and were therefore, independent.

After sex typing, embryo characteristics were re-analyzed by gender, to distinguish gender-specific treatment effects on embryonic development. Embryo weight, placental weight or allantochorionic volume, and crown-rump length (CRL) were

analyzed using the MIXED procedure of SAS, as a randomized block design, with blocks again based on sow pair. Observations were tested for a treatment effect, gender-specific effects, and any significant interactions between them. As sow was the experimental unit, all individual measurements were averaged by gender, within a litter, before statistical analysis.

3.3 Results

3.3.1 Feed intake, energy requirements and net energy balance

The number of piglets nursing during lactation were not different between Control and Restrict sows (Table 3.1). There were no differences in mean litter birth weights between Control and Restrict sows, or litter weight gain from Day 0 to 13. However, during feed restriction from Day 14 to 21, litters of Restrict sows had lower average weight gain than litters of Control sows, resulting in a lower total weight gain in litters of Restrict sows from Day 0 to 21. There was no difference between Restrict sows compared to Control sows in estimated milk production from Day 0 to Day 13 (7.11 ± 0.27 kg, 7.47 ± 0.27 kg), but milk production (10.07 ± 0.32 kg, 11.96 ± 0.32 kg, $P < 0.001$) during feed restriction from Day 14 to 21, and overall milk production was lower (8.59 ± 0.22 kg, 9.71 ± 0.22 kg $P < 0.001$) in respective treatment groups.

Comparing the energy requirements for sow maintenance and milk production during lactation, with the energy derived from sow lactation feed intakes, indicated that the estimated overall net energy balance was not different between treatment groups at Day

13 of lactation but was very different between treatments in week 3 ($P < 0.001$), when feed restriction was applied (Table 3.2).

3.3.2 Sow characteristics

There were no differences between the two treatment groups on Day 0 in sow body weight or backfat (Table 3.2). Restrict sows were no different than Control sows in weight loss (-5.89 ± 1.28 kg, -5.18 ± 1.28 kg) backfat loss (-0.35 ± 0.43 mm, -0.34 ± 0.43 mm), calculated body protein loss, or in body fat loss (Table 3.2) from Day 0 to 13 of lactation. However, both weight loss (-16.48 ± 1.41 kg, -4 ± 1.41 kg, $P < 0.001$) and backfat loss (-2.3 ± 0.38 mm, -1.03 ± 0.38 mm, $P < 0.01$) were greater between Day 14 to 21 in Restrict compared to Control sows. Calculations of body composition changes indicate an increased loss of body protein and body fat as a percentage of parturition mass, during feed restriction from Day 14 to 21 of lactation (Table 3.2). Overall, between farrowing and Day 21, body weight ($P < 0.001$), backfat ($P < 0.005$), and estimated body protein ($P < 0.001$) and body fat ($P < 0.001$) losses, were greater, in the Restrict compared to Control sows (Table 3.2).

3.3.3 Sow fertility, embryonic survival and development

Weaning-to-oestrous interval, pregnancy rate, day of gestation at necropsy, and ovulation rate were not different between treatments (Table 3.3). Correlation analysis established no relationships between ESR and NE balance, protein loss or fat loss (Figure 3.2). Of the 34 sows allocated to this experiment, data from one Control sow was removed from

embryo analysis because abnormal embryo survival rates were associated with recorded breeding problems.

At Day 30 of gestation, embryonic survival rate ($P < 0.05$) and the number of live embryos ($P < 0.05$) were lower in Restrict than in Control sows at Day 30 of gestation. Based on clear distinctions between the PCR amplicons of male and female embryos (Figure 3.1), the number of female embryos ($P < 0.01$) was also lower in Restrict sows compared to Controls, whereas no comparable difference was apparent in the number of male embryos. Overall, the sex ratios were 61% males in Restrict sows and 54% males in Controls. Allantochorionic volume and placental weight were not affected by treatment, gender, or by a treatment by gender interaction (Table 3.4). However, embryonic weight ($P < 0.005$) and CRL ($P < 0.05$) were lower in embryos recovered from Restrict sows. There was no gender-specific effect, or a treatment by gender interaction for these measures of embryonic development.

3.4 Discussion

Most primiparous sows undergo a certain level of catabolism during lactation and, in the absence of imposed feed restriction, a decrease in body weight, backfat, body protein, and body fat is expected and considered normal (Aherne and Williams 1992). However, the timing and severity of this loss of body condition can substantially impact subsequent reproductive performance. This study used an established experimental model (Zak *et al.* 1997a), in which reduced feed intake during the last week of lactation in primiparous sows results in detrimental effects on subsequent embryonic survival. In the same experimental paradigm, the relatively severe nutritional restriction in late lactation also

produced detrimental effects on oocyte quality, measured using in vitro oocyte maturation assays (Zak *et al.* 1997b). This suggests that oocyte development is likely a contributing factor to poor embryonic survival in catabolic lactating sows. However, feed-restriction can also affect the endocrine function of the pre-ovulatory follicles and differentiating corpora lutea, which in turn produce deleterious effects on the oviductal and uterine environment in which fertilization and embryonic development take place (Foxcroft *et al.* 2000, 2003). In previously catabolic sows, this would intensify the competitive environment in which the already compromised embryos are expected to survive. It is unclear how the balance between the latent effects of catabolism on follicular maturation and the priming of the oviductal and uterine environment would affect the relative rate of male and female embryonic development and how this might bias the litter sex ratio.

Although both Restrict and Control sows had a negative net energy balance by Day 13 of lactation, significant differences in body weight, backfat, and estimated loss of body protein and fat mass, were evident at the time of weaning in Restrict sows compared to Controls. The greater mobilization of body protein and fat mass in the last week of lactation in the Restrict group was likely the attempt to meet the demands of milk production in the absence of adequate nutrient intakes. However, as in previous studies using restriction of either total feed (Zak *et al.* 1997a), dietary protein (Clowes *et al.* 2003a, 2003b), or dietary lysine (Yang *et al.* 2000a), there was still a significant reduction in milk output in the present study, as measured by reduced growth rate of the litters of Restrict sows. Therefore, feed restriction during lactation not only affects the

condition of the sow by increasing catabolism, but also has detrimental effects on the growth of the litter.

Given this increased propensity to mobilize body protein to meet the requirements for milk synthesis during periods of feed restriction in contemporary commercial dam-line sows, the concept that increased maternal protein mass at farrowing may be protective against a lack of protein intake in lactating primiparous sows (Clowes *et al.* 2003b) is of particular interest. The loss of more than 12 % of sow protein mass at farrowing appears to significantly reduce the fertility of sows after weaning, but a lower protein mass at farrowing puts sows at greater risk of reduced ovarian follicular development. Therefore, with these concepts in mind, the level of total dietary restriction imposed in the present study was intended to create a deficit in dietary protein and energy that would significantly impact fertility after weaning in terms of reduced embryonic survival, while at the same time ensuring a maternal body mass at farrowing that was intended to avoid differences in ovulation rate as a potential confounding factor. In the present study, Restrict sows lost 12.6% of their estimated parturition protein and 17.3% of their parturition fat mass at farrowing, and had a body weight at farrowing of 189 kg. Consistent with the results of Clowes *et al.* (2003a), this resulted in no differences between treatment groups in weaning-to-oestrous interval or ovulation rate, yet impacted embryonic survival in the Restrict sows. Correlation analysis established no relationships between ESR and NE balance, protein loss or fat loss. However, presentation of these relationships using the approach of van den Brand *et al.* (2000), suggests that thresholds of NE balance (-5 Mcal day^{-1}), and estimated loss of fat (-7%) and protein (-7.5%) in

relation to tissue mass at farrowing may exist, below which the risk of sows having marked reductions in embryonic survival is substantially greater.

As a period of feed restriction imposed during the last week of lactation appears to have the most detrimental effect on subsequent embryonic survival, and results in increasing catabolism during the final stages of oogenesis, it is very probable that feed restriction also involves defects in oocyte maturation as another cause of increased embryonic loss. The studies of Zak *et al.* (1997b), Yang *et al.* (2000b), and Clowes *et al.* (2003a) used oocyte *in vitro* maturation techniques to demonstrate detrimental effects of nutrient restriction and sow catabolism on oocyte maturation and suggested that this would increase embryonic loss. Studies in sheep have also demonstrated the latent effects of maternal nutrition before ovulation on oocyte quality and embryonic development *in vitro* (McEvoy *et al.* 1995; Lozano *et al.* 2003). This opens the possibility that genetic imprinting of the oocyte may be an integral part of the overall nutritional effects of reduced feed intake on ovarian function and embryo survival in the lactating sow.

By identifying the sexes of the surviving embryos, the present study provides the first evidence for a selective pressure against the survival of female embryos in sows subjected to restricted feed intake at critical stages of follicular development. This effect in swine is in contrast to generally accepted concepts of nutrient-restricted sex skewing seen in other mammalian species (Trivers and Willard 1973). However, the Trivers and Willard hypothesis applies to populations of offspring produced from monotocous species, and not to groups of individuals produced in litters from a single polytocous female. As suggested by Gorecki (2003), the local resource competition model (Clark 1978, modified by Silk 1983) is better suited to polytocous species such as swine, as it

postulates that healthy females with abundant resources would produce more females, as sows are territorial and would benefit from these resources. Conversely, if resources are poor, sows would favour male offspring, which disperse upon reaching maturity and move into new ranges. Gorecki (2003) supported this theory by demonstrating that under normal conditions perinatal mortality is female-biased. Studies examining how nutritional status of the sow can alter litter sex ratios have provided results indicating that sows may select males over females when nutrients are limited to the uterus. For instance, Mendl *et al.* (1995) reported that sows with increased access to feed produced a higher proportion of females than males, which would be consistent with the converse effect seen in the present study. It is also generally accepted that male embryos require a greater maternal investment as measured by piglet weight (Fernandez-Llario *et al.* 1999) and uterine space at Day 35 of gestation (Chen and Dziuk 1993). However, our data shows that irrespective of gender, there was a treatment effect in Restrict sows that was limiting the growth and development of embryos at Day 30 of gestation. As there was no detectable change in either placental weight or allantochorionic volume, and the ratio between placental and embryonic weight remains comparable to normal, non-growth restricted embryos from the same genotype (Town *et al.* 2004), it can be reasoned that all embryos had an equal opportunity to receive maternal nutrition and uterine crowding was not a major factor. In addition, even though there was increased uterine space from increased embryo loss in the Restrict sows, embryos from these sows were still growth-retarded, indicating an effect on embryo development by another mechanism. The lack of growth and development of embryos without effects on placental development suggest that this mechanism selecting for male-biased litters is not likely due to

nutritional limitations during gestation. In our situation, a male-biased ratio is likely resulting from an effect of maternal nutrition on oocyte quality and subsequently early post-zygotic development.

In pig production, a situation resulting in male-biased litters is not considered desirable, as gilts born in litters with 67% or more males have been shown to exhibit poorer reproductive performance (Drickamer, *et al.* 1997). Consequently, trend towards male-biased litters in the Restrict sows in the present study (61% males in Restrict sows v. 54% in Controls), indicates that if the sows from our study had eventually farrowed, the female piglets from the Restrict group might have had poorer lifetime reproductive performance. In accordance with the Barker hypothesis (Young 2001), this may indicate that the imposed feed restriction not only affects the quality of the oocytes in the sow, but also influences the fertility of her female offspring. However the mechanism(s) of these selective effects on sex ratios are not fully understood.

One possible explanation for the selection against female embryos in this study is that an epigenetic defect in DNA methylation due to feed restriction could have altered patterns of gene imprinting, resulting in the improper expression of imprinted genes, and subsequent female-specific lethality. Research in the mouse has shown that the final stages of folliculogenesis, before the oocyte reaches metaphase II, are a critical period for establishing DNA methylation on imprinted genes that affect embryogenesis (Lucifero, *et al.* 2002). A deficiency in the nutrients essential for DNA methylation is normally associated with embryonic hypomethylation during gestation (Wu *et al.* 2004). However, it is equally likely that nutrient deprivation during the oestrous cycle, and more specifically during oocyte maturation, could result in hypomethylation of maternally

methylated imprinted genes within the oocyte. Defects in the enzymes that regulate DNA methylation can result in hypomethylation, and have been linked to embryonic growth retardation and increased mortality (Sun *et al.* 2004). Genes such as antisense IGF2r (AIR) are regulated by maternal methylation, and if these imprints were hypomethylated within the oocyte, intrauterine growth retardation could occur in embryogenesis (Sleutels *et al.* 2002). This would be consistent with the effects on embryo growth seen in our study. Furthermore, oocytes of reduced quality have been shown *in vitro* to have reduced epigenetic competence, as manifested by a reduced ability to form and actively demethylate the sperm pronucleus when compared to *in vivo* oocytes (Gioia *et al.* 2005). This suggests that if the male pronucleus is not properly reprogrammed following fertilization, many methylated genes could have defective expression and result in impaired embryogenesis. Also, it can be presumed that all the mechanisms required for maintenance of imprints during pre-implantation embryogenesis are contributed from the oocyte (Ratnam *et al.* 2002); therefore, defects in an oocyte's ability to maintain imprints during the pre-implantation period could result in abnormally imprinted gene expression and subsequent growth-restricted embryos. Although it has yet to be determined if genomic imprinting plays a role in the current study, it is a likely candidate to explain how feed-restriction during lactation can restrict conceptus growth and development in the subsequent breeding.

This hypothesis may also explain why, in addition to embryonic developmental delay, there is also a selective loss of female embryos preferentially over male embryos before Day 30. Many imprinted genes reside on the X-chromosome, which, without proper methylation, may result in failure of dosage compensation to inactivate the X-

chromosome and lead to a decrease in female embryo survival rates (Xue *et al.* 2002; Panning and Jaenisch 1996). Either a failure to establish methylation imprints in the oocyte, resulting in hypomethylation, or defects in maintenance of methylation imprints during embryogenesis, may explain this effect. It has been shown that expression of X-inactivation specific transcript (*Xist*), leading to inactivation of the maternal X-chromosome via methylation and heterochromatin formation on the X-chromosome, results in *Xist*-mediated silencing of the maternal X chromosome, and this causes increased lethality towards male embryos as seen in cases of Skewed X-inactivation (Lanasa *et al.* 2001). However, the opposite could be occurring in the present study, as decreased methylation would result in suppression of *Xist* by the increased expression of the X-chromosome specific transcript *Tsix* and CTCF protein, resulting in failure of dosage compensation which is lethal towards females, but not males (Lee 2000). Improper methylation of imprinted genes on the X-chromosome could explain how feed restriction during the last week of lactation results in a decrease in the number of female embryos at Day 30. We are presently investigating these possibilities in our ongoing studies.

The data in this study does however provide the first evidence of a link between well-established latent effects of catabolism during folliculogenesis in the pig, and gender-specific effects on embryonic development and survival, that are indicative of gene imprinting seen in other species.

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Table 3.1 Least square means ± SEM for litter weights during a 21 day lactation.

Item	Control (n=17)	Restrict (n=17)
Litter data		
Litter size (piglets)	9.5 ± 0.2	9.5 ± 0.2
Initial weight (kg)	14.0 ± 0.6	12.8 ± 0.6
Weight gain Day 0 to 13 (kg)	27.0 ± 1.0	25.6 ± 1.0
Weight gain Day 14 to 21 (kg)**	**21.6 ± 0.6**	**18.7 ± 0.6**
Total weight gain (kg)*	**48.5 ± 0.6**	**43.8 ± 0.6**

* $P < 0.005$ compared to Control sows ** $P < 0.001$ compared to Control sows

Table 3.2 Least square means ± SEM for sow and litter characteristics during lactation used to estimate net energy balance, and changes in fat and lean tissue mass.

Item	Control (n=17)	Restrict (n=17)
Characteristics for estimation of sow NE balance		
Estimated ME intake		
Day 0 to 13 (Mcal day^{-1})	15.02 ± 0.19	14.66 ± 0.19
Day 14 to 21 (Mcal day^{-1}) **	**15.20 ±0 .00**	**7.60 ± 0.00**
Estimated energy requirements for milk production		
Day 0 to 13 (Mcal day^{-1})	11.97 ± 0.47	11.32 ± 0.47
Day 14 to 21 (Mcal day^{-1}) **	**19.86 ± 0.55**	**16.54 ± 0.55**
Estimated sow net energy balance		
Day 0 to 13 (Mcal day^{-1})	-3.38 ± 0.42	-3.39 ± 0.42
Day 14 to 21 (Mcal day^{-1}) **	**-9.93 ± 0.55**	**-14.04 ± 0.55**
Characteristics for estimation of fat and protein loss		
Day 0 of lactation		
Farrow weight (kg)	189.8 ± 3.24	189.1 ± 3.24
Farrow backfat (mm)	19.8 ± 0.72	20.5 ± 0.72
Body fat at farrow (kg)	49.2 ± 1.41	50.1 ± 1.41
Body protein at farrow (kg)	29.4 ± 0.67	29.1 ± 0.67
Day 0 to 13 of lactation		
Protein loss % of parturition mass	2.72 ± 0.69	3.08 ± 0.69
Fat loss % of parturition mass	3.16 ± 1.41	3.35 ± 1.41
Day 14 to 21 of lactation		
Protein loss % of parturition mass **	**2.61 ± 0.73**	**9.52 ± 0.73**
Fat loss % of parturition mass **	**4.62 ± 1.28**	**13.94 ± 1.28**

* $P < 0.05$ compared to Control sows. ** $P < 0.001$ compared to Control sows

Table 3.3 Least square means ± SEM for sow reproductive performance and embryo survival data.

Item	Control (n=16)	Restrict (n=17)
Wean-to-oestrous interval (days)	5.3 ± 0.3	5.4 ± 0.3
Ovulation rate	18.3 ± 0.7	18.2 ± 0.6
Pregnancy rate (% of sows bred)	100	100
Day of gestation at slaughter	30.3 ± 0.2	30.1 ± 0.2
Number of live embryos*	**14.4 ± 0.8**	**12.3 ± 0.8**
Embryonic survival rate (%)*	**79.2 ± 4.0**	**67.9 ± 3.9**
Number of males	7.7 ± 0.6	7.5 ± 0.6
Number of females*	**6.5 ± 0.6**	**4.7 ± 0.6**

* $P < 0.05$ compared to Control sows. Analysis of Embryonic survival rate performed on arcsin transformed data

Table 3.4 Least-square means (±SEM) for gender specific embryo characteristics collected at Day 30 of gestation

Characteristic	Control males (n=124)	Restrict males (n=128)	Control females (n=104)	Restrict females (n=80)
Embryo weight (g) **	**1.55 ± 0.07**	**1.39 ± 0.07**	**1.51± 0.07**	**1.37 ± 0.07**
Crown-rump length (mm) *	**23.87 ± 0.44**	**23.21 ± 0.43**	**23.75 ± 0.44**	**23.13 ± 0.43**
Allantochorion volume (mL)	251.31 ± 17.86 (n=59)	225.96 ±17.29 (n=58)	235.88 ± 17.86 (n=46)	233.11 ± 17.29 (n=39)
Placental weight (g)	22.87 ± 1.89 (n=75)	23.38 ± 1.89 (n=44)	22.31 ± 1.89 (n=49)	22.13 ± 1.89 (n=36)

* $P < 0.05$ difference only between treatments. ** $P < 0.005$ difference only between treatments. Analysis of data was performed testing for differences in treatments, gender, and treatment by gender interaction. Brackets designate the number of samples in each analysis.

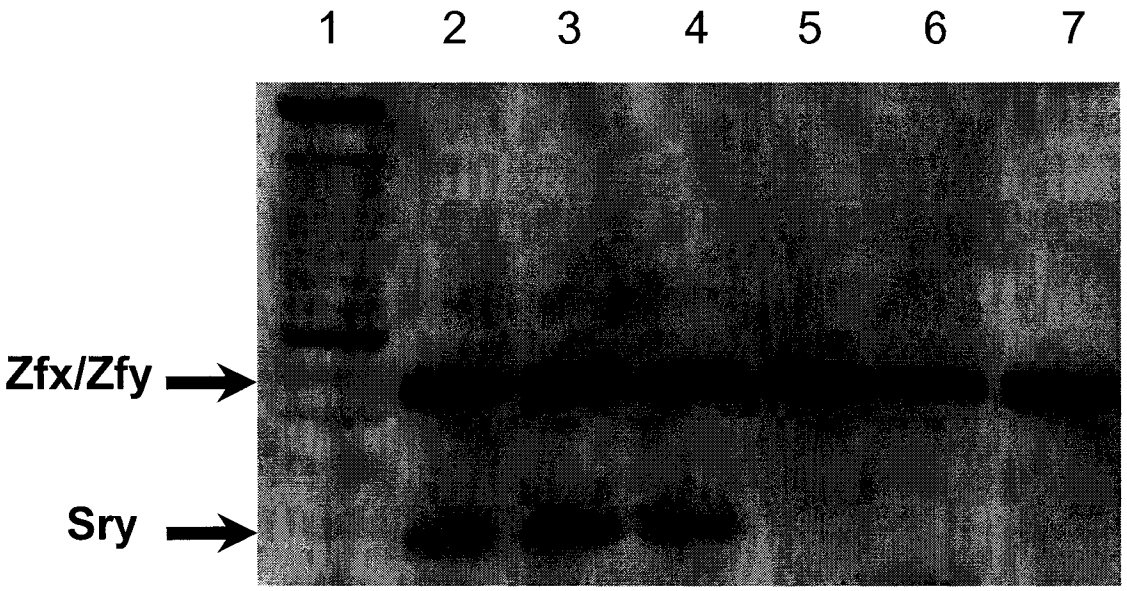
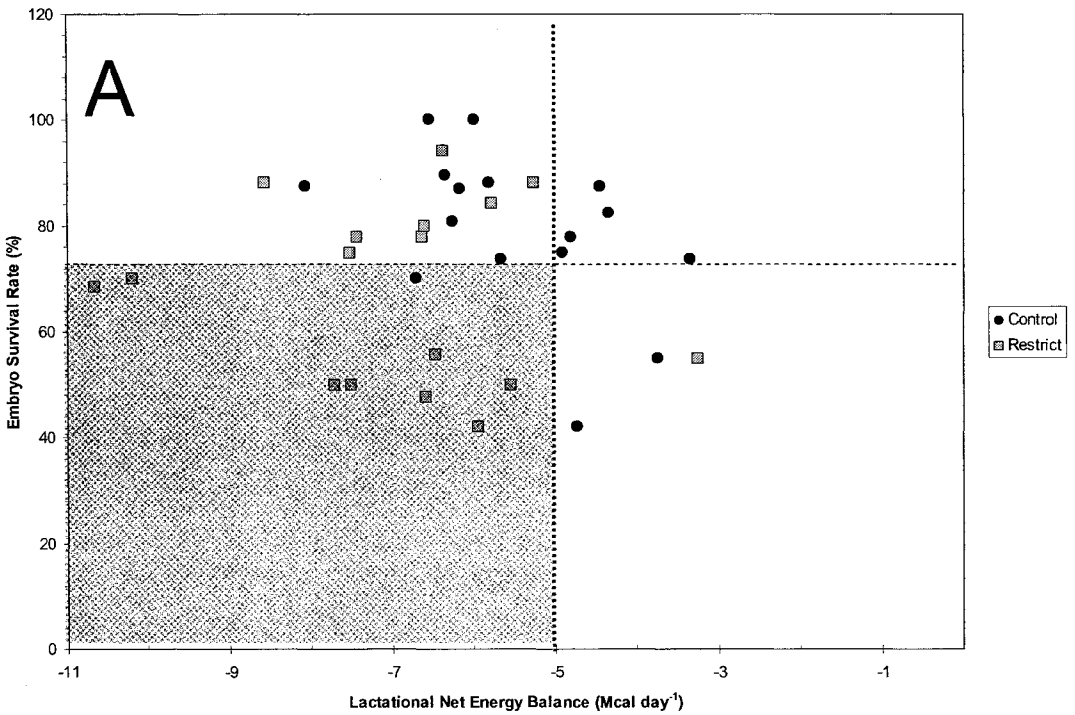
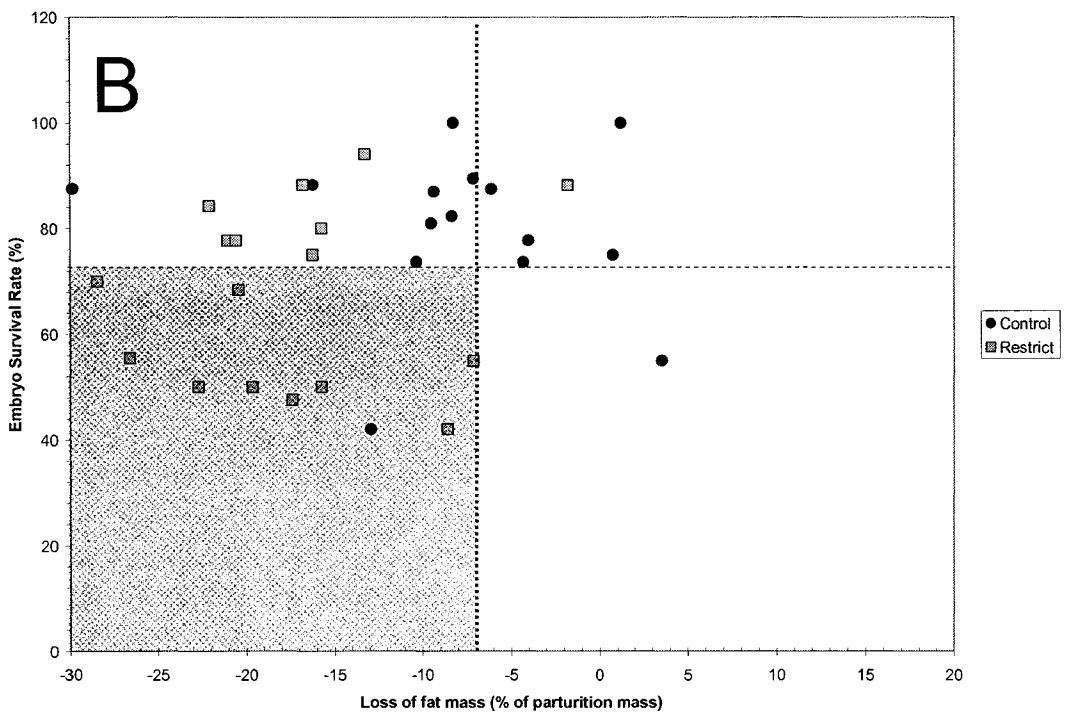


Figure 3.1 Sex typing PCR amplicon run on a 2% (w/v) agarose gel at 100v for 45 min. Amplification of the SRY gene appears as a band at 163bp, identifying the presence of the Y-chromosome, while a band at 455bp identifies the Zfx/Zfy gene which is the positive PCR control. Lane 1 has a 100bp ladder, lanes 2-4 identify male embryos, and lanes 5-7 identify female embryos.





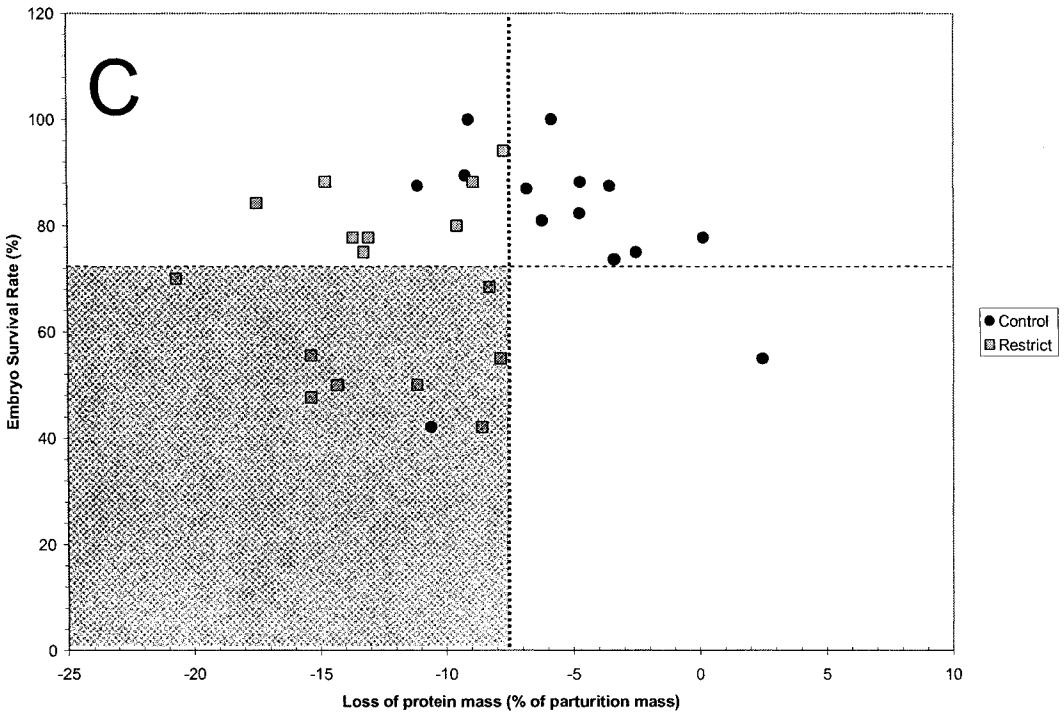


Figure 3.2 A) The relationship between Day 0 to 21 net energy balance and embryo survival rate (ESR). B) The relationship between loss of fat mass from Day 0 to 21 as a percentage of parturition mass and ESR. C) The relationship between loss of protein mass from Day 0 to 21 as a percentage of parturition mass and ESR. The horizontal line is the average ESR of all sows (73%) and the vertical line is arbitrarily drawn. In all three figures the hatch marked quadrant illustrates the population of Restrict sows which create the overall treatment effect.

CHAPTER 4

ALTERED EPIGENETIC VARIANCE IN SURVIVING LITTERS FROM NUTRITIONALLY RESTRICTED LACTATING PRIMIPAROUS SOWS.[2]

4.1 Introduction

Feed restriction of sows during the last week of lactation has been associated with increased catabolism of both protein and fat tissues and, following return to estrus and successful breeding, decreased embryonic survival (Zak *et al.* 1997a, Vinsky *et al.* 2006). Furthermore, sex-typing has demonstrated that the decrease in embryonic survival is mainly associated with the loss of female embryos in this experimental model (Vinsky *et al.* 2006). This reduction in female embryo survival could be due to maternally-inherited epigenetic defects, as feed restriction occurs at the time that oocytes are entering the final stages of maturation (Foxcroft 1997), coincident with the establishment phase of methylation imprints reported in other species (Lucifero *et al.* 2002). The present study investigated the possibility that epigenetic defects originating from increased lactational catabolism in sows may lead to overall delays in embryonic development and the proportional increase in embryonic loss in female embryos reported previously (Vinsky *et al.* 2006).

Heterogeneity in oocyte maturation has been suggested as a primary factor in determining embryonic survival (Geisert and Schmitt, 2002; Pope *et al.* 1990). This suggests that embryos which survive to Day 30 should be of better quality and more homogeneous than embryos which failed to survive. Therefore, an analysis using the F-test on variance of survivors would identify traits which are important for embryonic

[2] This chapter comprises the text of a paper that has been submitted for review to Reproduction, Fertility and Development (August, 2006)

survival. Archer *et al.* (2003) used this technique to establish greater variability in cloned swine than in naturally bred control counterparts. The same underlying concept was used in the present study in which we hypothesized that epigenetic defects in embryos recovered from sows nutritionally-restricted during late lactation would reduce the variance in embryonic development within the surviving littermates at Day 30 of gestation compared to litters in Control sows.

Three approaches were taken to study the involvement of epigenetic mechanisms. 1) A genome-wide approach to detect large scale epigenetic changes in methylation state involved analysis of DNA methylation by reverse-phase high performance liquid chromatography (RP-HPLC) (Ramsahoye 2002). 2) Gene expression for insulin-like growth factor 2 (*Igf2*) and its receptor (*Igf2r*) were used as candidate imprinted genes for detecting epigenetic defects that are important for normal embryonic growth and development, and with known sequences in the pig (Amarger *et al.* 2002; Killian *et al.* 2001). 3) To determine why female embryos are preferentially lost over males in Restrict sows, the expression of the X-chromosome specific transcript (*Xist)* was analyzed: *Xist* is regulated by maternal methylation, with higher expression in female than in male embryos, and has been linked to female-specific in utero mortality (Panning and Jaenisch 1996; Xue *et al.* 2002).

4.2 Materials and Methods

4.2.1 Animals and treatments

The experimental paradigm used in this study was described previously (Vinsky *et al.* 2006). In brief, 34 primiparous F1 sows (Genex Hybrid; Hypor, Regina, SK, Canada) had

litters standardized to a minimum of nine piglets per sow by cross-fostering within 48 h after farrowing. Sows pre-selected for trial were paired on the basis of similar changes in body condition from Day 0 to Day 14 of lactation, and, within each pair, sows were assigned to be fed a standard lactation diet (Table 4.1) limited to 2.5 kg day^{-1} (Restrict) or fed to a maximum of 5.0 kg day^{-1} (Control) from Day 14 to 21 of lactation. After weaning and until breeding, all sows were provided *ad libitum* access to the same lactation diet. After insemination, sows were fed a standard gestation diet based on NRC requirements. Gestational day was based on the day of the onset of estrus being designated as Day 0 of pregnancy. The primary data on sow reproductive performance, and embryonic survival and development, were again reported previously (Vinsky *et al.* 2006).

4.2.2 DNA extraction, sex-typing polymerase chain reaction and DNA methylation analysis

Preparation of embryonic tissues, and isolation, purification, and sex-typing of DNA used for further analysis in the present study were described by Vinsky *et al.* (2006). Aliquots of stored DNA were analyzed for DNA methylation using reverse-phase high performance liquid chromatography (RP-HPLC) following the guidelines provided by (Ramsahoye, 2002). Samples were analyzed in batches, with each treatment pair being analyzed on the same day to avoid interassay variation between pairs. A Supelcosil LC-18-T reverse phase column and a Supelcosil LC-18-T guard column (Sigma Aldrich, Oakville, ON, Canada) were used on a Varian pump with a Varian autosampler (Varian, Palo Alto, CA). Nucleotides were detected using a UV detector set at 260nm (Bio-Rad,

Spring Valley, CA, USA). The mobile phase consisted of 100mM ammonium phosphate pH 6.0 (Sigma) pumped at 1.0 ml/min for a runtime of 30 minutes. UV absorbance data was stored and analyzed using the Shimadzu Class VP computer software (Shimadzu, Columbia, MD, USA). Ratios for each nucleotide measured between 250/260 nm and 280/260 nm were compared to previously determined values from (Dawson, 1986). To confirm the accuracy of the E_{max} values, samples of lambda DNA (Invitrogen, Burlington, ON, Canada) were run through the system and the ratios of nucleotides compared to the values obtained from the fully sequenced genome (NC001416).

4.2.3 RNA isolation, cDNA synthesis and Realtime PCR

For RNA analysis, a 50 mg aliquot of previously powdered embryonic tissue was placed into a pre-chilled 1.5 mL tube in dry ice. RNA was then isolated using a Trizol procedure (Invitrogen). Total RNA, was reconstituted in 50 µl of DEPC water (Qbiogene, Montreal, QC, Canada) and stored at -70°C. RNA samples were treated with DNase using the Ambion DNase easy kit (Austin, TX, USA). Samples were quantified by UV absorbance at 260 nm using a 96-well SpectraMax200® spectrophotometer plate reader and Softmax PRO® software (Sunnyvale, CA, USA). A 1 µg/µl working stock for each RNA sample was prepared. Oligo-dT 15 primed cDNA synthesis was performed in a Bio-Rad iCycler (Bio-Rad, Mississauga, ON, Canada) using 2 µg of RNA in the presence of 1 µl of RNase Out (Invitrogen). Realtime PCR was performed using an ABI 7700 thermocycler on 1µl of cDNA, in duplicate (Foster City, CA, USA). Primers and Taqman-MGB probes (Table 4.2) were designed using the Primer express® software v2.0 (ABI) using sequences found in GENBANK for porcine cyclophilin (AY008846), *Xist* (AJ429140),

and *Igf2r* (AF339885). The *Igf2* primers sequences were previously reported in Van Laere *et al.* (2003). Real-time semi-quantification of the "gene of interest" mRNA for each individual sample was performed and the Ct value was subsequently normalized against its respective mean cyclophilin (endogenous control) Ct value using the standard ΔCt method (Pfaffl, 2001). Inter-assay variation was evaluated by repeated analysis of a known sample on each 96 well plate, and confirmed to be negligible.

4.2.4 Statistical analysis

A total of 17 pairs of sows met all the criteria for inclusion in the present study and data from these sows were used in the final analysis. Sow reproductive data, embryo characteristics, and litter data was analyzed using the MIXED procedure of SAS (SAS Institute, Cary, NC, USA) as previously described (Vinsky *et al.* 2006). As the sow is the experimental unit, all individual DNA methylation and real-time gene expression data collected on each embryo was averaged within each litter, before analysis. These means were analyzed using the MIXED procedure of SAS, with blocks based on sow pair, testing for both treatment and sex-specific effects. Variance in embryo characteristics, DNA methylation and real-time gene expression was calculated for each treatment and sex using the MEAN procedure of SAS. Appropriate comparisons between the calculated variances for each treatment and sex was determined using an F-test, with differences being significant at $P < 0.05$ (Archer *et al.* 2003).

4.3 Results

4.3.1. Variance in physical parameters

The variance for physical characteristics by treatment and sex is depicted in Fig. 4.1. The variance in embryo weights at Day 30 was much lower in Restrict than Control female embryos ($P < 0.001$) and in Restrict than Control male embryos ($P < 0.01$). Furthermore, Restrict male embryos showed greater variance in embryo weight than Restrict female embryos ($P < 0.01$). Variance in crown-rump length (CRL) also tended to be higher in Restrict male than Restrict female embryos ($p<0.06$), but there were no differences between male and female Control embryos.

4.3.2 DNA methylation

Across treatments, average DNA methylation was ~3.8% of all cytosines. No difference in mean embryonic global methylation was detected between treatment groups, regardless of sex (Fig. 4.2). The variance in global methylation tended to be greater in Control female ($P < 0.06$) and was greater in Control male embryos ($P < 0.001$), than in Restrict female and male counterparts, respectively (Fig. 4.3). Restrict male embryos also had a tendency towards lower variance than Restrict female embryos ($P < 0.09$). Variance in global methylation also tended to be correlated with the variance of embryo weights ($R = 0.42$, $P < 0.10$) in Restrict, but not Control embryos.

4.3.3 Gene expression

As expected there were no differences between treatments in the expression of the endogenous control cyclophilin. Differences in *Igf2* and *Igf2r* gene expression were not observed between Control and Restrict embryos (Fig. 4.2). In Control embryos only *Igf2* was correlated with embryo weight ($R = 0.28$, $P < 0.001$) and CRL ($R = 0.26$, $P < 0.005$). Variance in *Igf2r* expression was greater in Control female embryos ($P < 0.04$), and tended to be greater in Restrict female embryos ($P < 0.08$), than in their respective male littermates (Fig. 4.3). *Xist* showed higher mean expression ($P < 0.001$) in female embryos compared to males, irrespective of treatment (Fig. 4.2). Males from Control sows had a tendency towards increased variance in *Xist* expression as compared to males from Restrict sows ($P < 0.08$) (Fig. 4.3). However, across treatments, female embryos from either Restrict or Control sows had less variance in *Xist* expression than their male counterparts ($P < 0.001$).

4.4 Discussion

In a previous study of the effects of increased lactational catabolism in the sow on subsequent fertility and embryo survival to Day 30, Vinsky *et al.* (2006) reported that both male and female embryos from Restrict sows weighed less at Day 30 than embryos from Control sows. However, as in the study of Archer *et al.* (2003), the changes in variance of litter traits was predicted to better identify important changes in litter dynamics than a simple analysis of the trait means. Indeed, in the present study, further analysis revealed a dramatic decrease in the variance of embryo weights in Restrict compared to Control sows (see Fig 4.1). This is consistent with the prediction that those

embryos which died before Day 30 were more diverse and extreme in weight. Furthermore, the sex-specific decrease in female embryo survival also resulted in decreased variance in Restrict female embryo weights when compared to Restrict male embryo weights, providing further evidence of a sex-specific effect on embryonic survival and development. A sex-specific effect on embryo development was also evident in a tendency for lower variance in the length of Restrict female embryos compared to male littermates. In contrast to earlier conclusions based simply on embryo trait means (Vinsky *et al.* 2006), the difference in the variance in embryo traits indicate that embryo survival has not compromised the development of male embryos to the same extent as female littermates. These analyses illustrate how decreased, and sex-specific changes in, embryonic survival can be reflected in decreased variance for a trait connected to embryonic growth and development.

Analysis of the global methylation of Day 30 embryos were very similar to the levels of methylation seen in adult sow tissue samples (Vanyushin *et al.* 1970) and indicated no differences in mean methylation between litters from Restrict and Control sows (see Fig. 4.2). However, the variance in methylation state changed drastically, with greater variance in Control male and female embryos than in Restrict male and female counterparts (see Fig. 4.3). We again interpret these data as indicating that this difference in variance is due to the lower embryonic survival in the Restrict group of sows, removing embryos with more extreme global DNA methylation, and thus reducing litter heterogeneity for methylation state. This outcome is consistent with estimates of the nutrients available to the sows in the various treatment groups based on NRC requirements (1998) (see Table 4.1), as Restrict sows were greatly deprived of the

nutrients essential for DNA methylation (Davis and Uthus, 2004). Restrict male embryos also had a tendency towards lower variance in global methylation than Restrict female embryos; as Restrict males do not show decreased survival at Day 30, this could be part of their survival advantage which results in the sex ratio skewing previously reported (Vinsky *et al.* 2006). Changes in the variance of global methylation tends to correlate to changes in the variance of embryo weight, which again supports the conclusion that feed restriction reduces population variance for traits that directly affect survival, while adjusting the mean for other traits where variation is not directly affecting survival to Day 30.

After establishing that changes in the variance of global DNA methylation were associated with reduced embryo survival to Day 30, it was appropriate to examine specific candidate genes which may be affected by changes in methylation. Mean *Igf2* and *Igf2r* expression did not differ between treatments (see Fig. 4.2): However, mean *Igf2* expression did correlate with embryo weight and crown-rump length in Control, but not in Restrict litters, suggesting that some other factor is responsible for decreasing embryonic development and/or survival in Restrict sows. There were also no differences in variance of *Igf2* between treatments, regardless of sex, which further implies that *Igf2* expression in Day 30 embryos is not the cause of reduced embryonic development or survival. As the imprinting and methylation of *Igf2* is paternal, it is not unexpected that the treatment would have no effect on this gene.

Changes in the variance of *Igf2r* expression did however indicate that maternal malnutrition may be altering maternally-imprinted genes. *Igf2r* had greater variance across treatments in female embryos than males: However, this difference in variance

was only significant in Control embryos. The relative decrease in *Igf2r* variance in Restrict female embryos may again, therefore, be connected to increased female mortality. In the context of the genetic conflict hypothesis (Moore and Haig 1991) this would imply that over expression of *Igf2r* would result in reduced embryonic growth and development (O'Dell and Day 1998) as seen in Restrict embryos, and that under-expression of *Igf2r* would result in increased mortality (Lau *et al.* 1994).

To further explore the possibility of female-specific lethality due to epigenetic defects in gene expression, *Xist* expression was quantified. *Xist* had the typically higher expression (lower ΔC_t) in females than males (Fig. 4.2). However, there was a tendency towards decreased variance in *Xist* expression in Control male embryos compared to males from Restrict sows (Fig. 4.3), indicating that males from Control sows have tighter regulation of *Xist* expression, which may be a factor in maintaining male survival to Day 30 even though growth is otherwise compromised. Furthermore, the across treatment differences between the sexes in the variance of *Xist* indicates that females generally need tighter regulation of *Xist* expression than males, as it plays a larger role in regulating female development and survival.

The data in the present study provides evidence that epigenetic mechanisms may mediate the latent effects of increased catabolism during the last week of lactation in the sow on embryonic development and survival to Day 30.

4.5 References

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Table 4.1 Estimated composition of lactating sow diet and formulation of nutrients essential to reproduction as fed.

	Composition per kg[x]	Control Diet (5 kg day^{-1})	Restrict Diet (2.5 kg day^{-1})	NRC requirements[y] (5 kg day^{-1})
DE (kcal)	3159	15793	7896	15790
ME (kcal)	3039	15196	7598	15158
CP (kg)	0.21	1.03	0.51	0.69
Lysine (g)	10.5	50.8	25.4	27.4
Zinc (mg)	39	195	97	250
Selinium (mg)	0.29	1.47	0.74	0.75
B12 (mg)	0.01	0.04	0.02	0.07
B2 (mg)	1.96	9.78	4.89	18.7
B6 (mg)	4.26	21.32	10.66	5.0
Folate (mg)	0.48	2.41	1.20	6.5
Biotin (mg)	0.14	0.72	0.36	1.0
Choline (g)	13	65	33	53

[x] Composition was 45.3% wheat, 25% barley, 21.5% soybean meal, 2.2% canola oil, 2% herring fish meal (Formulated based on NRC 1998 values) and 4% breeder #4 (Consultant Feeds Ltd. Calmar, AB)

[y] Calculated using NRC swine requirements software V2.03 based on feed composition, and Control sow averages for farrow weight (189 kg), piglets per litter (10), piglet gain (120g day^{-1}), and lactation (21 days).

Table 4.2 Porcine specific real-time PCR primers and probes

Target	Forward	Reverse	Probe
Cyclophilin	5′-AATGCTGGCCC CAACACA-3′	5′-TCAGTCTTGGC AGTGCAAATG-3	5′-VIC-ACGGTTCCCAGTTT T-MGBNFQ-3′
Igf2	5′-CAAGTCCGAG AGGGACGTGT-3′	5′-CCAGGTGTCAT AGCGGAAGAA-3′	5′-FAM-CCGACCGTGCTTCCGGAC AACTT-MGBNFQ-3′
Xist	5′-TGGGACAGAG TGAGGGAGGT-3′	5′-GGCCACTACTA TGAGCAGGGAG-3′	5′-FAM-CCAAGGAGATAGCCT G-MGBNFQ-3′
Igf2r	5′-CAGCTTTCATT CCGTGGGA-3′	5′-TGTTGAATTCC AGAAGAGATCTGG-3′	5′-FAM-TCTGTTTTGAAAAGTGC C-MGBNFQ-3′

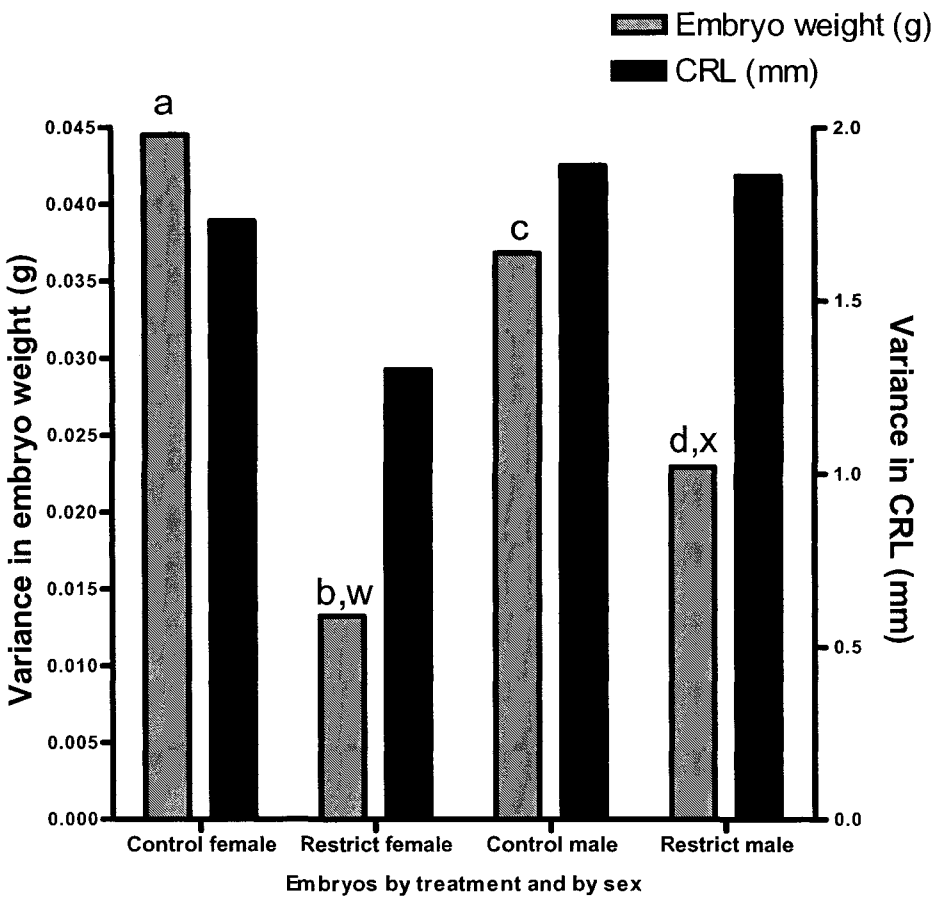

Figure 4.1 Variance in the physical development for Day 30 male and female embryos. Superscripts *a,b* and *c,d* indicates differences between treatments within sex at $P < 0.001$ and $P < 0.01$, respectively. Superscripts *w,x* indicates differences between sexes within treatment at $P < 0.01$.

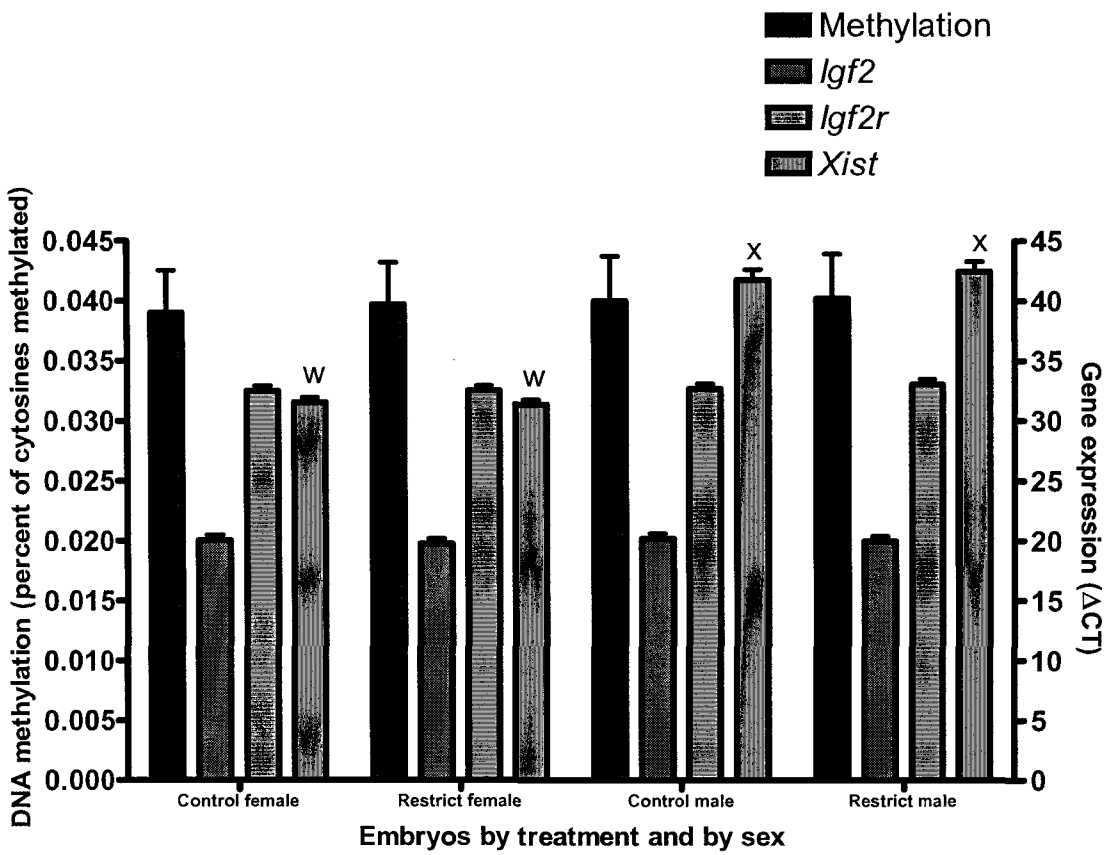

Figure 4.2 The means for global methylation and gene expression of Day 30 embryos. Superscripts *w,x* indicates differences between sexes within treatment at $P < 0.001$.

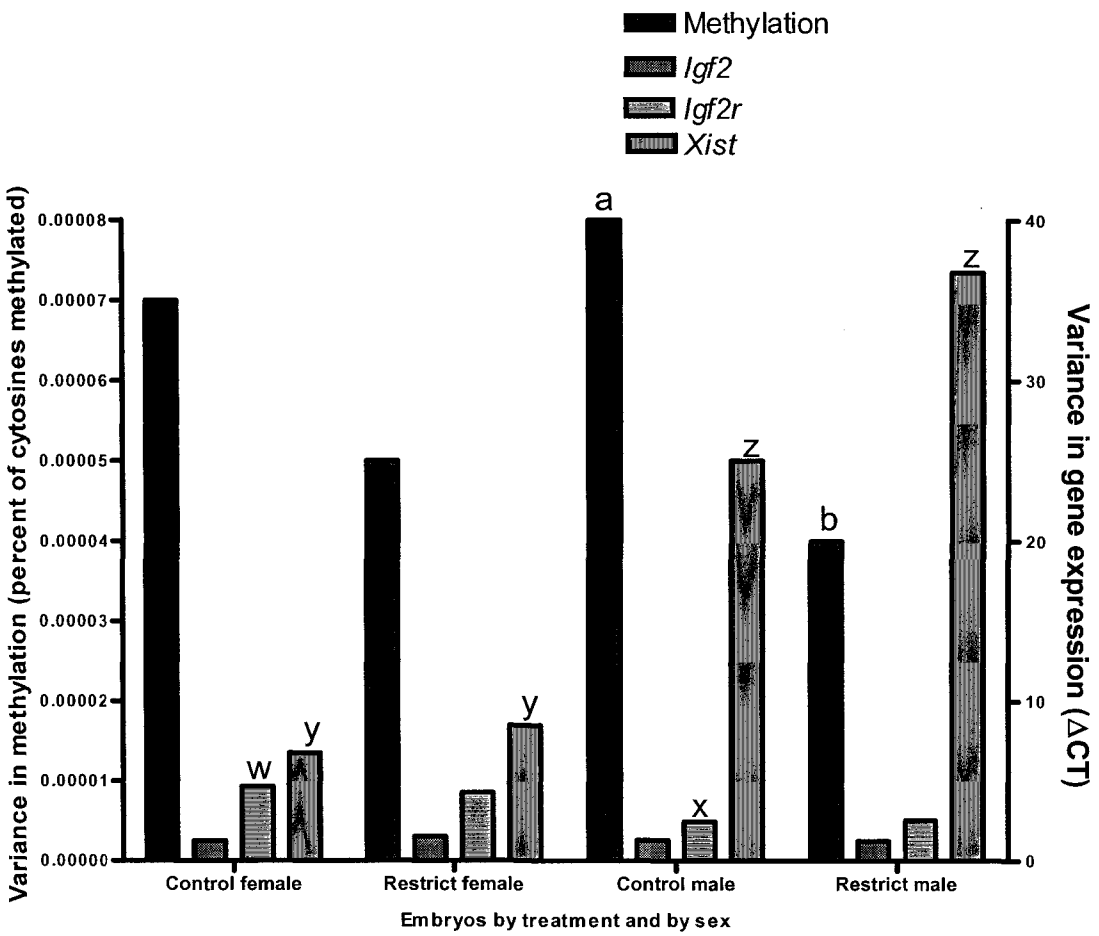

Figure 4.3 Variation in molecular data from Day 30 male and female embryos. Superscripts *a,b* indicates differences between treatments within sex at $P < 0.001$. Superscripts *w,x* and *y,z* indicates differences between sexes within treatment at $P < 0.04$ and $P < 0.001$, respectively.

CHAPTER 5

ONTOGENY OF METABOLIC EFFECTS ON EMBRYONIC DEVELOPMENT IN LACTATING AND WEANED PRIMIPAROUS SOWS.[3]

5.1 Introduction

Increased catabolism during the last week of lactation in primiparous sows is known to reduce embryonic survival and development to Day 30 of gestation in the subsequent litter (Foxcroft, 1997). Using a refinement of the experimental paradigm reported by Zak *et al* (1997*a*), Vinsky *et al.* (2006*a*) confirmed a detrimental effect of catabolism in late lactation on embryonic survival. The greater deficit in overall net energy balance in feed-restricted sows led to increased loss of both protein and fat mass during lactation. The thresholds of maternal tissue loss, above which there was an increased likelihood of embryonic loss and developmental delays, were consistent with thresholds reported by Clowes *et al.* (2003*a,b*) for effects on ovarian follicular development in lactating and weaned sows subjected to different nutritional regimens in gestation and lactation. Furthermore, Vinsky *et al.* (2006*a*) used sex-typing polymerase chain reaction (PCR) to demonstrate that the increased loss of embryos before Day 30 of gestation was female-biased. A reduction in female embryonic survival as a response to nutritional restriction in the sow is consistent with the local resource competition model (Clark 1978, modified by Silk 1983).

The etiology of this selective loss of female embryos before Day 30 of gestation is unknown. Vinsky *et al.* (2006*a*) suggested that female embryo loss could be due to epigenetic defects originating from the oocyte, as the timing of feed restriction coincides

[3] This chapter comprises the text of a paper that has been submitted for review to Reproduction, Fertility and Development (August, 2006)

with the final stages of oocyte maturation (Foxcroft 1997) and with epigenetic establishment seen in other species (Lucifero *et al.* 2002). In support of this suggestion, Vinsky *et al.* (2006*b*) reported that reduced embryo development and decreased female embryo survival were associated with differences in the variance of epigenetic traits in the surviving litters at Day 30 of gestation. Analysis of the variance in global DNA methylation and *Xist* expression suggested that a sub-population of embryos within a proportion of litters from nutritionally-restricted sows were epigenetically defective and lost before Day 30 of gestation and surviving embryos in these sows were also developmentally retarded. Heterogeneity and competition within litters is believed to be a primary cause of decreased litter survival before Day 30 of gestation, and has been suggested to have an epigenetic component (Geisert and Schmitt 2002). By studying epigenetic traits of early stage embryos it should be possible to determine the ontogeny of epigenetic defects leading to changes in embryonic survival and development. Furthermore, information on the circulating levels of nutrients essential for DNA methylation, such as folate and B_{12} (Mason 2003), and extent of methylation of sow leukocyte DNA, could provide insights into the connection between nutrient deprivation in the sow and epigenetic defects in blastocysts.

The present study used the experimental paradigm described by Vinsky *et al.* (2006*a*,*b*) to examine associations between changes in sow leukocyte DNA methylation, plasma folate and B_{12} concentrations in the sow during feed restriction, and changes in DNA methylation and developmental delays in sex-typed embryos at the early blastocyst stage of development. In the context of the earlier study of Day 30 embryos, evidence from a number of mammalian species, including the pig, suggest that global

demethylation of non-imprinted genes occurs at the blastocyst stage (Kang *et al.* 2001). Therefore, the main objectives of this study were, therefore: 1) To determine if methylation state of embryos would be suggestive of the involvement of imprinted genes as mediators of the epigenetic effects of sow catabolism on the gender-specific loss of embryos; and 2) To explore the etiology of heterogeneity in litter development that appears to underlie effects of maternal catabolism on embryonic survival in the sow.

5.2 Materials and Methods

5.2.1 Animals and treatments

This study was performed using a previously described model (Vinsky *et al.* 2006*a*) in accordance with the Canadian Council on Animal Care guidelines and with the approval of the Faculty Animal Policy and Welfare Committee. This study involved 34 primiparous F1 sows (Genex Hybrid; Hypor, Regina, SK, Canada) which farrowed normally and were managed according to standardized and approved protocols at the Swine Research & Technology Centre (SRTC), University of Alberta. Within 48 h after farrowing, litter size was standardized to a minimum of 9 piglets per sow by cross-fostering. On Day 14 of lactation sows pre-selected for trial were paired on the basis of similar body condition from Day 0 to 14 of lactation, and within each pair sows were assigned to either Restrict or Control treatment group. Sows were fed three times daily with a standard lactation diet (Table 5.1) from Day 0 to 14 of lactation, to a pre-set maximum of 5.0 kg day^{-1}. From Day 14 to 21 of lactation Control sows continued to be fed a maximum of 5.0 kg day^{-1}, whereas Restrict sows were limited to 2.5 kg day^{-1}. After weaning and until breeding, sows were provided with *ad libitum* access to the same

lactation diet. After insemination, sows were fed a standard gestation diet according to SRTC procedures.

Within 24 h after farrowing (Day 0), on Day 14 and 21 of lactation, at estrus, and immediately before slaughter on Day 6 of gestation, sow backfat and loin-depths were measured by real-time ultrasonography using a 5 Mhz real-time linear probe (Scanprobe II, Scano, Ithaca, NY) and sow body weights were recorded. Sow weight and backfat measurements were used to estimate total sow body protein and fat mass using the equations of Whittemore and Yang (1989). Litters were weighed after litter-size adjustments within 24 h after farrowing, on Day 14 and 21 of lactation, and whenever there was a litter size adjustment. Changes in relative tissue loss and net energy balance of sows were estimated as described previously (Vinsky *et al.* 2006a).

5.2.2 Blood sampling and Folate/B_{12} Radioimmunoassay

Blood was sampled from ear veins of sows using acute venepuncture during brief periods of nose-snare restraint in the morning prior to feeding on Day 13 and 21 of lactation, and during first post-weaning estrus. Blood was collected into an EDTA vacutainer (Becton Dickinson, Franklin Lakes, NJ, USA) and centrifuged for 10 min at 2000 x*g*. Plasma was then aspirated and both the plasma and remaining blood cells placed in separate tubes and frozen at -70°C. Assays of plasma folate and B_{12} was performed using Dualcount solid phase, no boil, Radioimmunoassay (RIA) (DPC / Intermedico, Markham, ON, Canada) using 200 µl of plasma. Validation of the Dualcount assay kit for folate indicated an intra- and inter-assay CV of 5.47 % and 0.68 %, respectively. Folate assay sensitivity, defined as 85 % of total binding, was 0.62 ng ml^{-1}. Cold recovery of folate was 104 ± 1 %

and a serial dilution of porcine plasma showed parallelism to the standard curve. Validation of the Dualcount assay kit for B_{12} indicated an intra- and inter-assay CV of 7.52 % and 28.22 %, respectively. B_{12} assay sensitivity, defined as 96 % of total binding, was 71 pg ml^{-1}. Cold recovery of B12 was 116 ± 2 % and a serial dilution of porcine plasma showed parallelism to the standard curve.

5.2.3 Sow management after weaning

Sows were checked twice daily at 0800 and 2000 for onset of first standing oestrus after weaning, using back-pressure testing during fence-line contact with a mature, high libido boar. From 24 h after onset of standing heat and every 6 h until ovulation occurred, real-time ultrasonography was performed using a Pye Medical 200 (Pye Medical Scanner 200, model 41480, Canmedical, Kingston, Ontario) with a 7.5 Mhz transcutaneous probe to determine the timing of ovulation. Estimated time of ovulation was defined as previously described by Almeida *et al.* (2001). Sows were inseminated 12 h after onset of standing heat and every 24 h thereafter, as long as a good standing heat reflex was observed, with pooled semen (3 x 10^9 morphologically-normal and motile spermatozoa per $50mL^{-1}$ dose) that was no more than 3 days old. Semen was collected and processed on-site from the same three Genex Large White boars (Hypor, Regina, SK, Canada) designated for use in the experiment. Gestation day was based on the day of ovulation being designated as Day 1 of pregnancy: Precise stages of gestation at which embryos were recovered as based on estimated time of ovulation within Day 1 and the known time of slaughter of the sows.

5.2.4 Ovulation rates and blastocyst collection

All sows were slaughtered on-site by qualified staff using approved necropsy procedures between Day 5 and 7.5 of pregnancy. Immediately after slaughter, the reproductive tract was recovered from each sow and the number of corpora lutea (ovulation rate) was recorded. The broad ligament was removed and embryos flushed from each uterine horn using 50 ml of phosphate buffered saline (PBS). Embryos were collected in petri dishes, washed in PBS, and their stage of development was identified under a binocular dissecting microscope (Wild M3, Wild Heerbrugg, Switzerland). Blastocysts with few cells and small blastocoels were termed early blastocysts, those with many trophoblast cells but still zona-encased were termed blastocysts, while those without zona were termed hatched blastocysts. After classification all embryos were transferred to individual 0.5 mL tubes, frozen in dry ice, and stored at -70°C.

5.2.5 DNA extraction and sex-typing polymerase chain reaction

Isolation and purification of embryonic DNA was performed using a Dynabead DNA Direct Universal Kit (Invitrogen, Burlington, ON, Canada). All embryos were sex-typed using a 45-cycle, touch-down PCR protocol that was otherwise unchanged from the PCR protocol described by Vinsky *et al.* (2006*a*).

Maternal sow DNA was isolated from leukocytes using a standard phenol/chloroform/isoamyl alcohol extraction protocol (Sambrook and Russell 2001), with materials purchased from Invitrogen.

5.2.6 Dot blotting methylation analysis

Dot blotting of embryonic and maternal DNA was performed as described by Park *et al.* (2005). DNA was dotted onto a Zeta-Probe® nylon membrane (Bio-Rad Laboratories, Hercules, CA, USA) using a Bio-Dot Microfiltration Apparatus (Bio-Rad Laboratories) and baked for 30 min in a vacuum at 80°C. Mouse monoclonal antibodies for 5-methylcytosine were used as the primary antibody at a 1:2000 dilution (Aviva, San Diego, CA, USA) and goat anti-mouse Alexa Fluor® 680 antibodies (Invitrogen) were used as the secondary antibody at a 1:2000 dilution. The quantity of genomic DNA was assessed using SYBR® DX Blot Stain (Invitrogen) and fluorescence was measured using a Typhoon 9410 (GE Healthcare, Piscataway, NJ, USA). The relative degree of DNA methylation was expressed as a ratio of Alexa Fluor® 680 fluorescence to the fluorescence of SYBR® DX Blot Stain.

5.2.7 Statistical analysis

A total of 33 sows met all criteria for inclusion in this study and data from these sows was used in the final analysis. Sow was used as the unit of measurement for determining treatment effects on ovulation rate (number of corpora lutea), number of embryos recovered, percent blastocysts, number of males, number of females, and DNA methylation. Data was analyzed using the MIXED procedure of SAS (SAS Inst., Inc., Cary, NC) as a randomized block design, with blocks based on sow pairs. The Kenwardroger approximation was used for the denominator degrees of freedom.

All measurements of litter characteristics were analyzed using the same MIXED procedure of SAS. Sow characteristics were analyzed in the MIXED procedure of SAS

using a repeated measurement model, with a significant interaction between treatments and time point ($P < 0.001$). Normalcy of data was tested using the Shapiro-Wilks test in the UNIVERIATE procedure of SAS. Protein and fat loss were calculated as a percent loss of parturition mass. Plasma Folate and B_{12} were analyzed using the MIXED procedure of SAS using a repeated measurements model.

Embryonic DNA methylation averaged by sow was analyzed using in a one-tailed t-test and the MIXED procedure of SAS. Sow leukocyte DNA methylation analyzed in a one-tailed t-test using the MIXED procedure of SAS using a repeated measurements model.

5.2.8 Scoring of embryos

Scoring of embryos within a litter was used as a measure of the quality and homogeneity of embryonic development. The aggregate score for a litter was derived as follows: blastocysts and hatched blastocysts were given a score of 10, early blastocysts scored 8, morulae scored 6, any earlier stage cleaved embryos scored 4, and one cell embryos scored 2. The 25^{th} percentile and the 75^{th} percentile across all the scores were calculated and sows were placed into either a high scoring group if above the 75^{th} percentile (H) (n = 9), or the low scoring group if below the 25^{th} percentile (L) (n = 16). Statistical analysis based on these categories was performed as previously described for each treatment group, using the same statistical methods, but with no blocked pairs. In the absence of any treatment effect, characteristics of the sows with H and L litters were considered, irrespective of treatment.

5.3 Results

5.3.1 Feed intake, energy requirements and net energy balance

The number of piglets nursing throughout lactation was not different between Control and Restrict sows (Table 5.1). There were no differences in initial litter weight or litter weight gain from Day 0 to 13 between Control and Restrict sows. During feed restriction from Day 14 to 21, litters of Restrict sows had lower average weight gain than litters of Control sows ($P < 0.005$); consequently, estimated daily milk production was lower in Restrict sows from Day 14 to 21 than in control sows (11.5 ± 0.3 kg vs. 10.6 ± 0.3 kg, respectively; $P < 0.005$)

Comparing the energy requirements for sow maintenance and milk production during lactation, with the energy derived from sow lactation feed intakes, indicated that the estimated overall net energy balance was not different between treatment groups by Day 13 of lactation but was very different between treatments from Day 14 to 21 ($P < 0.001$), when feed restriction was applied (Table 5.2).

Analyzing the same data based on H/L scoring indicated differences between H and L groups in Day 0 to 21 ME intake (11.1 ± 0.5 Mcal $day^{-1}$and 12.7 ± 0.4 Mcal day^{-1}, $P < 0.05$, respectively) and in Day 0 to 21 net energy balance (-7.8 ± 0.7 and -5.1 ± 0.6, $P < 0.01$, respectively).

5.3.2 Sow characteristics

Measurements of average sow weight, backfat and loin depth are reported in Fig. 5.1. There were no differences between treatment groups on Day 0 in sow body weight,

backfat and loin depth. There were no differences in weight loss between Control and Restrict sows except from Day 14 to 21 of lactation (-6.2 ± 1.2 kg and -19.9 ± 1.3 kg, respectively; $P < 0.001$) and from Day 21 of lactation to heat (-6.0 ± 1.7 kg and 0.3 ± 1.7 kg, respectively; $P < 0.05$). Backfat loss only differed between Control and Restrict sows from Day 0 to 13 of lactation (-0.5 ± 0.5 mm and -1.9 ± 0.5 mm, respectively; $P < 0.05$) and from estrus to Day of slaughter (1.2 ± 0.5 mm and -1.1 ± 0.5 mm, respectively; $P < 0.005$). Differences in loin depth were not observed between treatments at any time points; however, loin depth did correlate with protein mass (R = 0.42; $P < 0.001$) and loin depth loss with protein mass loss (R = 0.47; $P < 0.001$). Estimated protein and fat mass were not different between treatments at Day 0 of lactation (Table 5.2). Both loss of protein and fat mass was greater in Restrict sows compared to Control sows throughout lactation and from Day 14 to 21 of lactation (see Table 5.2). From weaning to estrus, estimated protein mass decreased in Control sows, while increasing in Restrict sows. From estrus to Day of slaughter, estimated protein mass increased faster in Restrict than in Control sows. Comparing across all time points within treatment groups Control sow's estimated protein mass decreased between Day 14 to 21, then increased from estrus to Day of slaughter, while estimated fat mass increased between estrus and day of slaughter compared to any previous time point. In Restrict sows both change in estimated protein and fat decreased dramatically from Day 14 to 21 of lactation, then increased from Day 21 of lactation to estrus. From estrus to Day of slaughter only the change in estimated protein mass significantly increased in Restrict sows.

Analyzing the same data based on H/L scoring indicated no differences in mean or loss of weight, backfat, loin depth, protein or fat mass throughout the experiment (data not shown).

5.3.3 Plasma Folate/B12 RIA

No differences were observed between Control and Restrict sows in plasma folate measured at Day 13 of lactation (19.7 ± 1.3 ng ml^{-1} and 19.6 ± 1.3 ng ml^{-1}, respectively), Day 21 of lactation (15.2 ± 1.7 ng ml^{-1} and 17.8 ± 1.5 ng ml^{-1}, respectively), or during estrus (18.6 ± 1.7 ng ml^{-1} and 19.5 ± 1.6 ng ml^{-1}, respectively). Analyzing the same data based on H/L scoring indicated no differences between H and L sows in folate levels (data not shown).

There were also no differences between Control and Restrict sows in B_{12} levels measured at Day 13 of lactation (606.3 ± 122.9 pg ml^{-1} and 697.3 ± 129.9 pg ml^{-1}, respectively), Day 21 of lactation (384.7 ± 69.3 pg ml^{-1} and 354.9 ± 65.0 pg ml^{-1}, respectively), or during estrus (3189 ± 1078.5 pg ml^{-1} and 1625.8 ± 897.6 pg ml^{-1}, respectively). Analyzing the same data based on H/L scoring indicated no differences between H and L groups in B12 levels (data not shown).

4.3.4 Sow fertility and embryonic development

Of the 34 sows allocated to this experiment, data from one Control sow was removed from the trial because of a failure to return to heat. Of the animals included in the analysis, pregnancy rate, day of gestation at necropsy, ovulation rate and estrus-to-

ovulation interval were not different between treatments (Table 5.3). However weaning-to-estrus interval ($P < 0.05$) was different. At slaughter between Day 5 and 7.5 of gestation, there was no difference in embryo recovery rates between treatments (Fig. 5.2). Irrespective of treatment, there was a correlation between overall embryo recovery rate, irrespective of stage of development, and the number of blastocysts and hatched blastocysts recovered ($r = 0.75$, $P < 0.001$) (Fig 5.3).

Analyzing the same data based on H/L scoring indicated a difference between H and L groups in ovulation rate (20 ± 0.7 and 17.1 ± 0.5, $P < 0.005$, respectively). Regardless of scoring classification, ovulation rate tended to correlate to number of blastocysts and hatched blastocysts ($r = 0.33$, $P < 0.06$) (Fig 5.4).

5.3.5 Sex ratios and DNA methylation

There was no difference in the sex-ratios between treatment groups. Restrict sows had 49% males in their litters compared to 51% males in Control sows.

The methylation of embryonic DNA was not different between Control and Restrict male (6.5 ± 2.7% and 6.5 ± 3.3%, respectively) or female (4.4 ± 2.8% and 5.6 ± 2.6%, respectively) embryos. Also, there were no differences in the variance of methylation between Control and Restrict male (2.8 ± 1.7% vs. 3.3 ± 2.2%, respectively) or female (1.8 ± 1.6% vs, 1.9 ± 2.1%, respectively) embryos. Analyzing the same data based on H/L scoring revealed no differences in methylation between H and L male (7.6 ± 3.6% and 6.3 ± 3.9%, respectively) or female (5.0 ± 3.4% and 5.0 ± 4.0%, respectively) embryos. Also there were no differences in the variance between H and L male (3.1 ±

2.3% and 4.5 ± 3.0%, respectively) or female (1.6 ± 2.2% and 5.2 ± 3.2%, respectively) embryos.

The methylation of sow leukocyte DNA was not different between treatment groups measured at Day 13 of lactation (Restrict sows 20.1 ± 5.1% vs. Control sows 36.8 ± 6.7%). However, at Day 21 of lactation leukocyte DNA methylation in Restrict sows was lower than Control sows (17.9 ± 6.4% vs. 36.3 ± 6.4%, $P < 0.05$, respectively), and remained lower at the time of estrus (21.2 ± 6.5% vs. 40.5 ± 7.1%, $P < 0.05$, respectively). Analyzing the same data based on H/L scoring indicated no differences between H and L sows for leukocyte DNA methylation at any given time-point.

5.3 Discussion

The goal of this experiment was to determine ontogeny of the effect of increased lactational catabolism on embryonic development and epigenetic traits, using the experimental paradigm from earlier studies of embryonic survival and development at Day 30 of gestation (Vinsky *et al.* 2006a).

5.3.1 Metabolic and reproductive responses to treatment

As in the previous experiment feed restriction during the last week of lactation, and a corresponding decrease in net energy balance in Restrict sows compared to Controls, were associated with decreased litter weight gain during the last week of lactation (Vinsky *et al.* 2006a). Estimated sow protein and fat loss during lactation were similar to the previous experiment: The greater estimated fat loss in Control Sows

compared to the previous study likely reflected the increase of 0.5 pigs per litter suckling the sow, resulting in sows mobilizing more fat to compensate for increased milk production. The increased catabolism of tissues in Restrict sows during the last week of lactation are likely the most critical factor determining reproductive performance following weaning (Clowes *et al.* 2003b) and exceeded the thresholds suggested for negative impacts on subsequent sow fertility (Clowes *et al.* 2003b; Vinsky *et al.* 2006a)

The continued weight and estimated protein loss in weaned Control sows is likely associated with involution of the mammary glands. However, by Day 6 of gestation Control sows appeared to have entered an anabolic state and were gaining estimated protein mass. The apparent switch to an anabolic state in Restrict sows by the day of estrus may be confounded by increased feed intakes in these sows after their period of restriction (Booth *et al.* 1994). However the greater gain in estimated protein between estrus and slaughter in Restrict sows occurs at a time when feed intakes would have been comparable. This observation, together with a change in measured backfat between treatments at slaughter, is consistent with suggestions from previous studies that in the post-weaning period, renewed lean tissue growth creates greater energy demands that are met at the expense of fat deposits (Clowes *et al.* 1994).

The reproductive characteristics of the sows after weaning were also essentially the same as in a previous study using the same experimental paradigm (Vinsky *et al.* 2006a), with no differences between treatments for ovulation rate or pregnancy rate (see Table 5.3). Although the Restrict sows had the same weaning-to-estrus interval as in the previous experiment, Control sows in the present study had shorter weaning-to-estrus interval compared to pair-matched Restrict sows. However, estrus-to-ovulation interval

was not different between treatments, indicating that although Control sows came into heat sooner, the physiological mechanisms regulating the timing of ovulation within the estrus period were consistent between treatment groups.

Overall the metabolic changes and reproductive characteristics of weaned sows in the present study were very consistent with the earlier study involving recovery of day 30 embryos (Vinsky *et al.* 2006a), suggesting that mechanisms mediating the latent effects of catabolism on early embryonic development would have been activated in the Restrict sows in the present experiment.

5.3.2 Methylation state

DNA collected from the leukocytes of Restrict sows at weaning on Day 21 of lactation and at estrus was less methylated than the DNA from Control sows, indicating a limitation of nutrients essential for DNA methylation during lactational feed restriction. Two nutrients important for DNA methylation are folate and vitamin B_{12} (Davis and Uthus 2004). However, plasma concentrations of these nutrients were not different at Day 21 of lactation, or during estrus. Across treatments, the plasma levels of B_{12} are within the expected range for lactating sows and weaned sows (Guay *et al.* 2002). However, estrus plasma concentrations were relatively high, and this has been attributed to post-weaning atrophy of the mammary gland which releases vitamin B_{12} back into the plasma pool (Girard *et al.* 1994). Generally, folate levels were lower than expected (Guay *et al.* 2002). However, as the Control sows had excellent reproductive performance, the lower circulating plasma folate must not have exerted detrimental effects on reproductive performance.

As folate and B_{12} levels were not affected by lactational feed restriction, the link to hypomethylation in sow leukocyte DNA remains to be established. Decreased availability of methionine, an amino acid essential for DNA methylation, may be involved, as a decrease in available methionine has been indirectly linked to cases of hypomethylation in lymphocyte DNA (Yi *et al.* 2000). It has also been shown that fasting can lower methionine levels to the point where the transulfuration of homocysteine decreases, thus reducing glutathione levels (Sakata *et al.* 2005). Glutathione is essential for male pronucleus formation in the porcine oocyte (Yoshida *et al.* 1993), and this process is regulated by DNA methylation (Gioia *et al.* 2005) and essential for proper embryonic development. Unfortunately, an attempt to measure methionine concentrations in the sows plasma in this experiment failed for technical reasons and the limited sample volumes prevented re-analysis of plasma methionine.

Using the same experimental paradigm of feed restriction in lactating sows, (Vinsky *et al.* 2006b) presented evidence for epigenetic defects as an underlying cause of female-biased lethality and developmental delays seen in Day 30 embryos. Analysis at the blastocyst stage of development in the present study established no apparent differences in relative DNA methylation using a fluorescent dot-blot technique. It may be that the dot-blot technique is not sensitive enough to detect changes in the methylation state of embryos, as only imprinted genes would be methylated at the blastocyst stage (Kang *et al.* 2001). As adult sow DNA methylation is equivalent to the levels seen at Day 30 of gestation (Vinsky *et al.* 2006b), and the relative level of blastocyst DNA methylation was lower than sow leukocytes, it can be concluded that blastocysts have lower methylation than at Day 30 of gestation. This adds to the growing body of evidence

that the levels of methylation seen at the blastocyst stage are significantly lower than those seen in later embryonic development and in adulthood (Kang *et al.* 2001, Vinsky *et al.* 2006b, Vanyushin *et al.* 1970).

5.3.3 Embryonic recovery and embryo development

There were no differences in embryo recovery rates, nor in the sex ratios of embryos within litters between the two treatment groups in the present study. The overall recovery rates of around 70% were close to the 73% embryo survival rate at Day 30 previously reported across treatment groups (Vinsky *et al.* 2006a). It can, therefore, be assumed that the gender-specific loss of embryos in Restrict sows must occur after Day 5 to 7 of gestation. This is consistent with previous research into embryonic survival in the pig, which concluded that most of the embryonic loss is between Day 9 and Day 30 of gestation (Dziuk 1987). Furthermore, the lack of any phenotypic changes in embryos at Day 6 of gestation, and no measured difference in variance in methylation between the sexes and treatment groups, suggests that gender-specific epigenetic defects have not yet influenced embryonic survival (Vinsky *et al.* 2006b).

Pope (1990) suggested that an underlying cause of embryonic loss was the developmental asynchrony of embryos in the pre-implantation period, reflecting variation in follicular maturation and the duration of ovulation in these follicles. However, subsequent research using real time ultrasonography to study the pattern of follicular development failed to confirm that the duration of ovulation affects embryonic diversity between 77 to 110 hours following ovulation (Soede 1992). This suggests that other factors must contribute to embryonic diversity. As seen in Fig 5.2. there was a range of embryonic development in both treatment groups, regardless of stage of gestation.

Furthermore, it appeared that sows with the greatest embryonic development also had the highest recovery rates, regardless of treatment (Fig 5.3.). It is unclear why this occurred, as the embryos were flushed from the entire length of the uterine horn, as recommended for embryos between Day 5 and 7 of gestation (Polge 1982). We, therefore, concluded that sows at this stage of pregnancy are exhibiting differences in embryonic development irrespective of treatment, which may be similar to the variation in response to treatment seen in the break-point analysis presented by Vinsky *et al.* (2006a). This distribution of embryonic survival confirms the observation from many previous studies, that a proportion of both Control and Restrict fed sows have almost 100% embryonic survival to Day 30.

It seems likely that these sows are the H subgroup identified as the 75th percentile on the basis of the embryo scoring system used in this study. To better explain the metabolic and reproductive responses of lactating sows we scored each sow based on the number and maturity of embryos recovered. Using the same logic it appears that gender-specific loss of embryos in the remaining Restrict sows later in gestation will occur in the L classification group, characterized by both poorer recovery after uterine flushing and a greater asynchrony in development within litter-mate embryos. Using the H/L classification, the embryo data were, therefore, further analyzed to determine factors that might place the Restrict L-type litters at risk. At the early blastocyst stage of development, no unique identifying characteristics were evident for the Restrict L-type litters. H and L type litters were then compared, irrespective of treatment, to try and characterize the origins of the differences in developmental synchrony within litters.

In these subsets of data, H-type sows had lower ME intakes and net energy balances throughout lactation, and higher ovulation rates compared to L-type sows. In comparison, L-type sows had a much higher net energy balance throughout lactation, comparable to net energy balance in all sows during Day 0 to 13 of lactation (see Table 5.2), and yet had lower ovulation rates than H-type sows. As seen in Fig. 5.4, an increase in ovulation rate tends to correlate to increased development to the blastocyst stage, which may be an indicator of the sow's overall reproductive performance.

The functional link between these characteristics is unclear. However, one interpretation is that sows with the highest inherent voluntary feed intakes up to Day 13 of lactation would be subjected to a greater restriction relative to their maximum feed intake threshold, regardless of whether they were allocated to the Restrict or Control treatment from Day 14 to weaning. How this translates into differences in the central and local ovarian mechanisms affecting gonadotropin secretion and/or mechanisms controlling ovarian sensitivity to gonadotropins, merits further investigation. Regardless of the mechanisms that determine the sub-set of H-type sows, characterized by the most synchronized and developed embryos at the hatched blastocyst stage, and high ovulation rate, the experimental paradigm used in this study dictates that the Restrict sow litters are at risk of gender-specific loss of embryos by Day 30, and must be in the L-type subgroup. Applying the quadrant type depiction of litter characteristics in Fig. 5.3 and 5.4, as in the previous study of Day 30 embryos (Vinsky *et al.* 2006a), it appears that the risk factors for subsequent embryonic loss would be lower ovulation rates, poor recovery of embryos when flushing the uterus, and greater asynchrony in those embryos recovered. By the same token, the same risk factors do not apparently result in gender-specific loss of 10 to

15% of embryos present in Control L-type litters by Day 30. The present study has, therefore, effectively identified the key litter sub-groups that appear to make the biggest contribution to increased embryonic loss in previously catabolic sows. The characteristics of this subset of sows and litters should be a key focus in future experiments using this experimental paradigm. Finally, from the perspective of advancing the development of non-surgical embryo transfer technologies for the swine industry, the H-type litters appear to represent the best potential donor embryos for transfer.

5.3 References

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Table 5.1 Least square means ± SEM for litter weights during a 21 day lactation.

Item	Control (n = 16)	Restrict (n = 17)
Litter data		
Litter size (piglets)	10.0 ± 0.1	10.1 ± 0.1
Initial weight (kg)	13.2 ± 0.7	14.9 ± 0.7
Weight gain Day 0 to 13 (kg)	24.9 ± 1.4	25.0 ± 1.4
Weight gain Day 14 to 21 (kg)	20.8 ± 0.6 [a]	19.1 ± 0.5 [b]
Total weight gain (kg)	45.8 ± 1.3	44.1 ± 1.3

Weight at Day 13 was used as a significant covariate for weight gain from Day 14 to 21 ($P < 0.05$). Treatment means with *a,b* superscripts differ within rows by $P < 0.005$.

Table 5.2 Least square means ± SEM for estimated net energy balance (ME, metabolizable energy; NE, net energy balance)

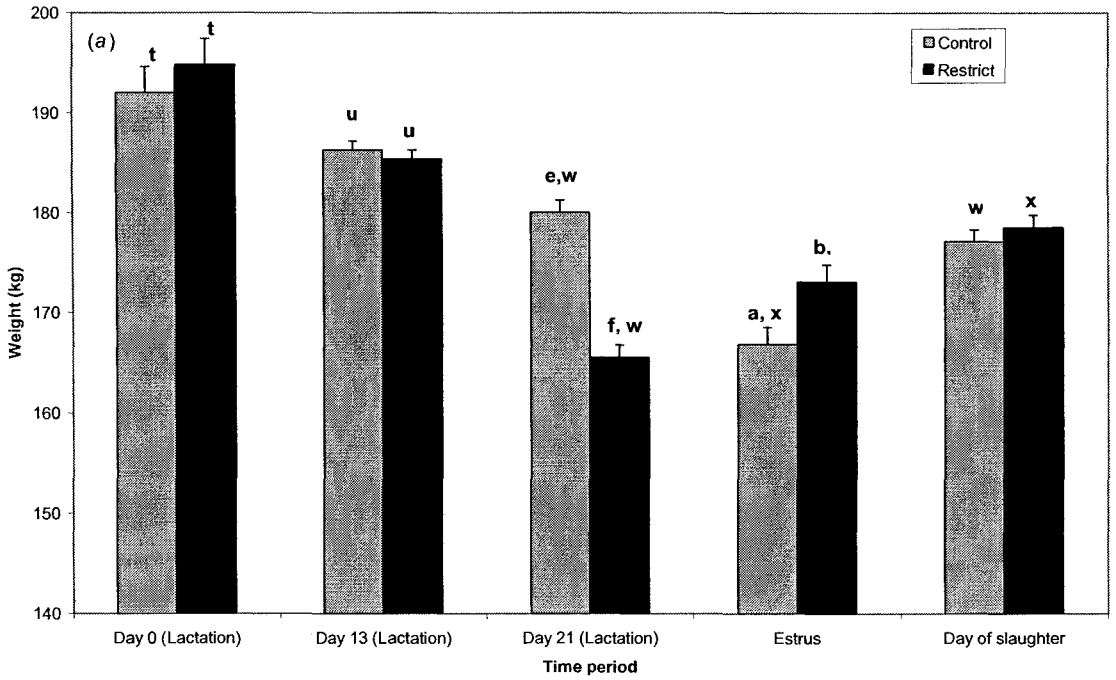
Item	Control (n = 16)	Restrict (n = 17)
Estimated sow net energy balance		
Day 0 to 13 (Mcal day^{-1})	-4.2 ± 0.8	-4.6 ± 0.8
Day 14 to 21 (Mcal day^{-1})	-6.7 ± 0.5 ^{e}	-9.5 ± 0.5 ^{f}
Day 0 to 21 (Mcal day^{-1})	-5.1 ± 0.6 ^{c}	-7.3 ± 0.6 ^{d}
Estimation of change in fat and protein mass		
Day 0 of lactation		
Body fat at farrow (kg)	55.8 ± 1.3	55.7 ± 1.3
Body protein at farrow (kg)	28.9 ± 0.5	29.6 ± 0.5
Day 0 to 13 of lactation		
Fat % of parturition mass	-4.2 ± 1.5 ^{w}	-8.1 ± 1.5 ^{w}
Protein % of parturition mass	-4.4 ± 0.6 ^{w}	-4.3 ± 0.6 ^{w}
Day 14 to 21 of lactation		
Fat % of parturition mass	-9.3 ± 1.3 ^{aw}	-13.8 ± 1.3 ^{bx}
Protein % of parturition mass	-1.4 ± 0.9 ^{ex}	-11.0 ± 0.9 ^{fx}
Day 0 to 21 of lactation		
Fat % of parturition mass	-13.6± 1.8 ^{a}	-21.6 ± 1.8 ^{b}
Protein change % of parturition mass	-5.8 ± 1.0 ^{e}	-15.4 ± 1.0 ^{f}
Day 21 of lactation to estrus		
Fat % of parturition mass	-4.4 ± 1.9 ^{w}	0.6 ± 1.9 ^{y}
Protein % of parturition mass	-4.4 ± 1.1 ^{cw}	1.1 ± 1.1 ^{dy}
Estrus to Day of slaughter		
Fat % of parturition mass	5.3 ± 1.7 ^{x}	0.4 ± 1.9 ^{y}
Protein % of parturition mass	4.1 ± 0.8 ^{ay}	6.8 ± 0.9 ^{bz}

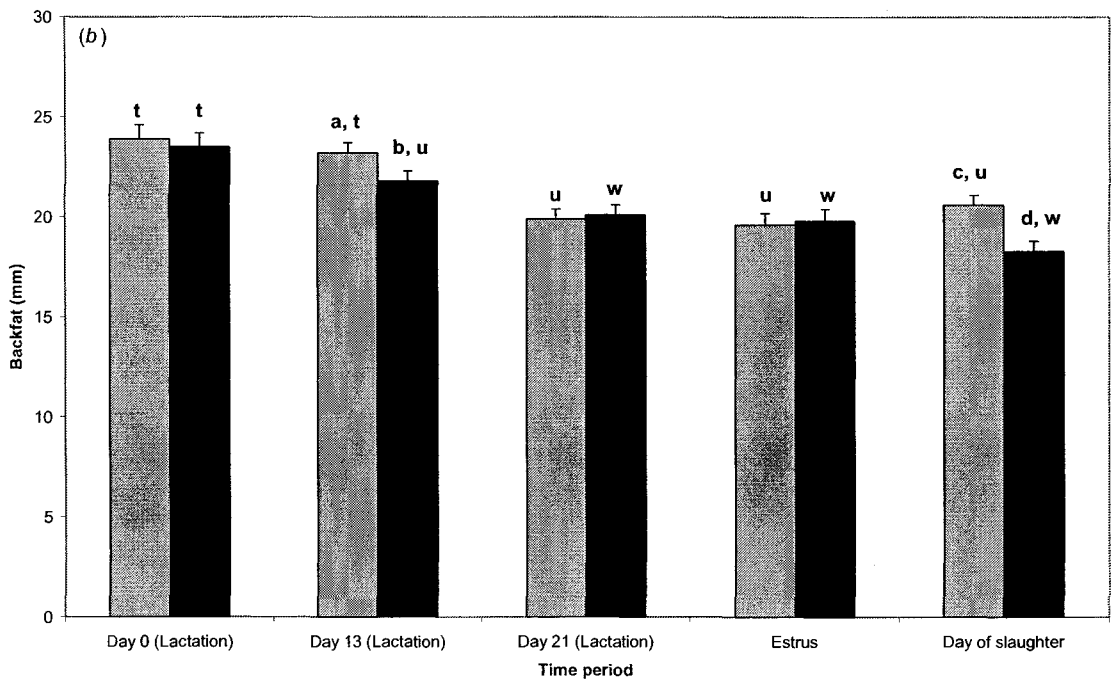
Treatment means with different *a,b; c,d; e,f* superscripts differ within rows by $P < 0.05$, $P < 0.005$, $P < 0.001$, respectively. Treatment means with different *w, x, y, z* superscripts differ within columns by $P < 0.05$.

Table 5.3 Least square means ± SEM for sow reproductive performance and embryo survival data.

Item	Control (n = 16)	Restrict (n= 17)
Wean-to-estrus interval (days)	4.7 ± 0.2 [a]	5.3 ± 0.2 [b]
Estrus-to-ovulation interval (hours)	34.5 ± 4.8	45.2 ± 4.6
Ovulation rate	18.1 ± 0.6	18.4 ± 0.6
Pregnancy rate (% of sows bred)	100	100
Days of gestation at slaughter	6.4 ± 0.1	6.4 ± 0.1
Percent of embryos recovered (%)	70.4 ± 5	74.4 ± 5

Treatment means with different *a,b* superscripts differ within rows by $P < 0.05$. Analysis of percent of embryos recovered and pregnancy rate performed on arcsin transformed data.





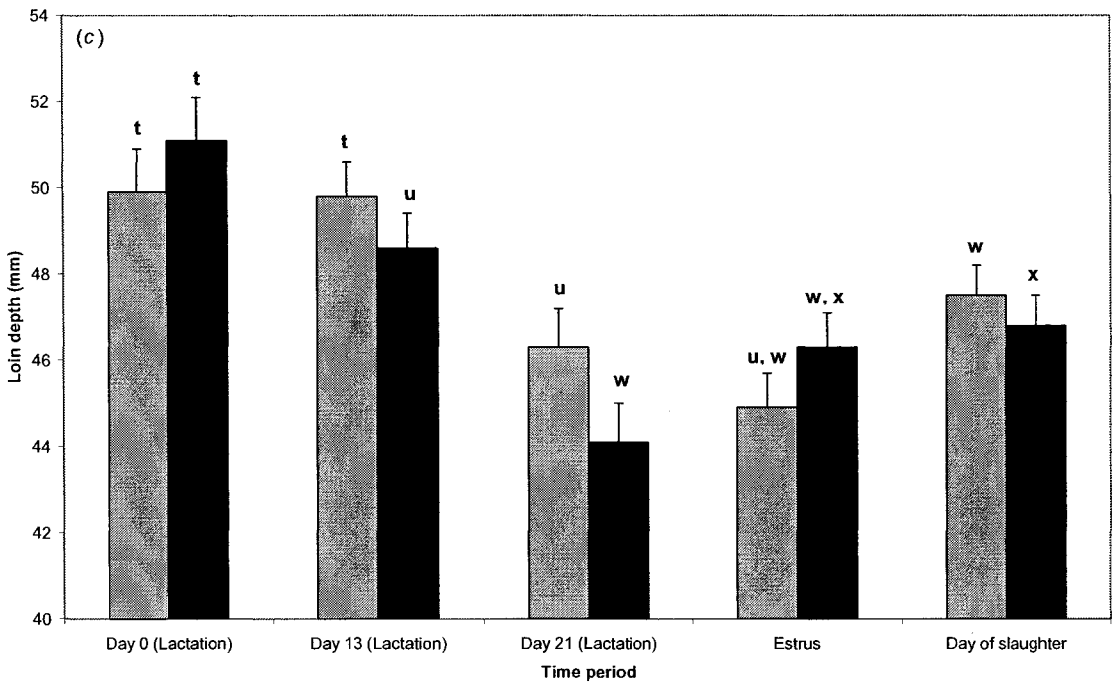


Figure 5.1 Sow characteristics during lactation, at standing heat (estrus), and at Day of slaughter. The bars show the least square means ± SEM changes in weight (*a*), fat (*b*), and loin depth (*c*). Treatment means with superscripts *a,b c,d and e,f* indicate differences between treatments within periods, significant at $P < 0.05$, $P < 0.005$, $P < 0.001$, respectively. Treatment means with different *t, u, w, x* superscripts indicate differences between periods within treatments, significant at $P < 0.05$.

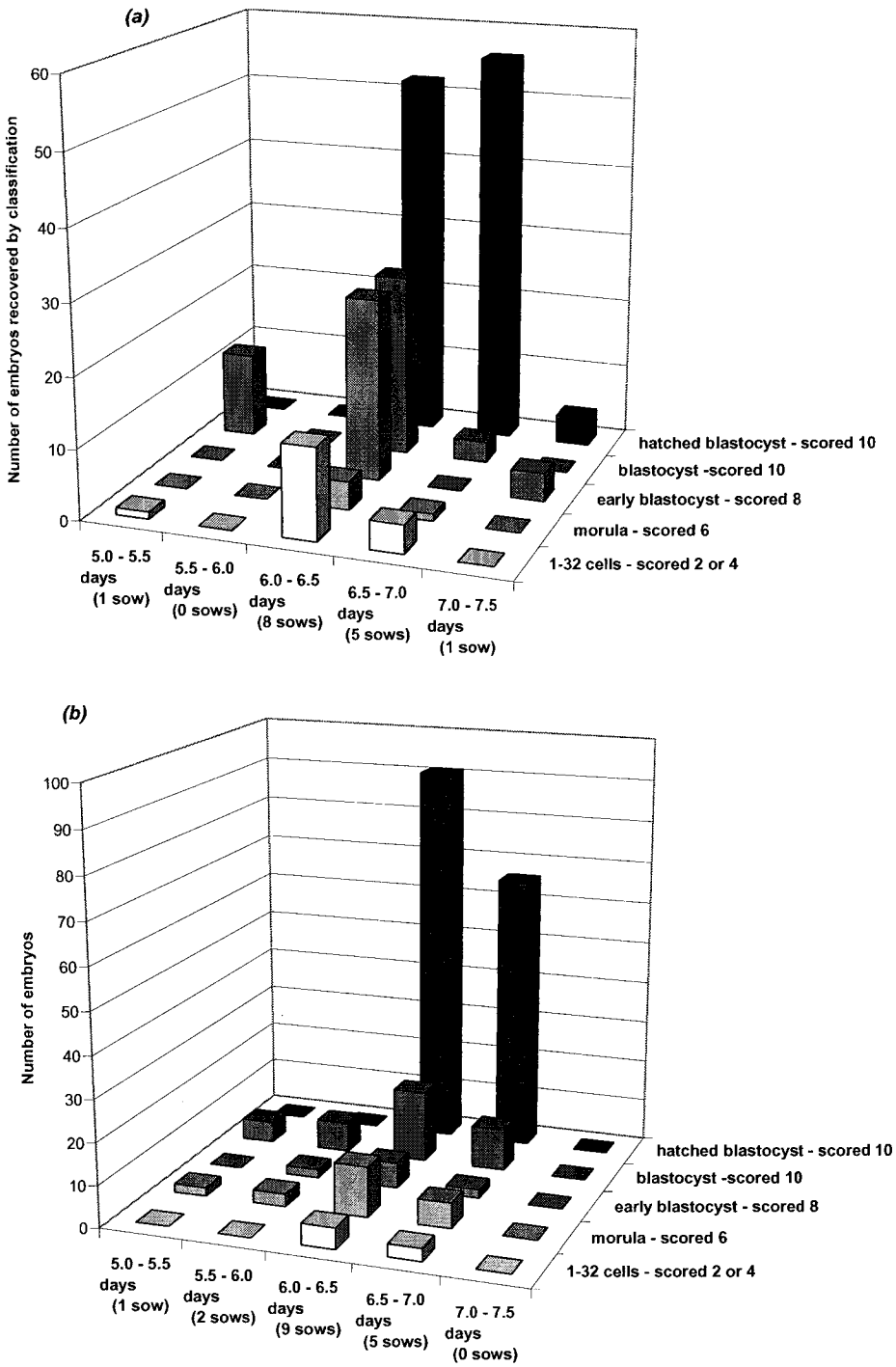


Figure 5.2 Relationship between development of embryos recovered and exact Day of gestation at recovery for Control sows (*a*) and Restrict sows (*b*). Number of sows are shown in parentheses below each day.

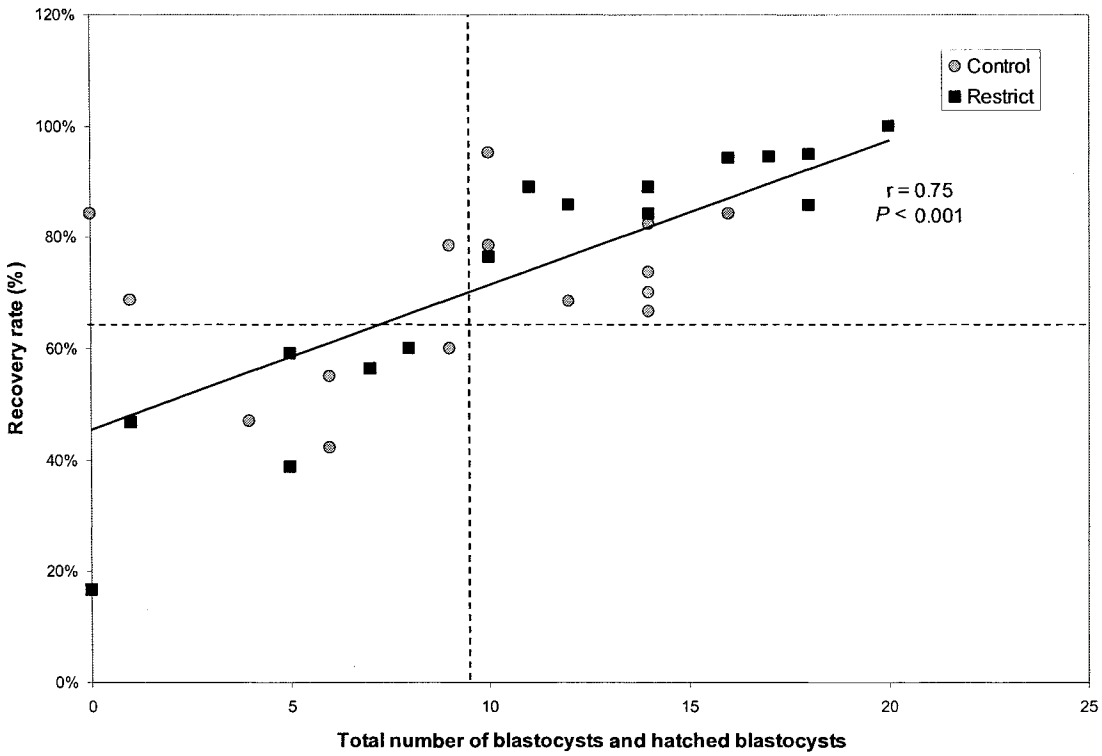


Figure 5.3 Relationship between the number of blastocysts and hatched blastocysts, and the overall rate of embryo recovery. Across both treatment groups, broken lines suggest thresholds for risk factors leading to gender specific loss of embryos by Day 30 in Restrict litters.

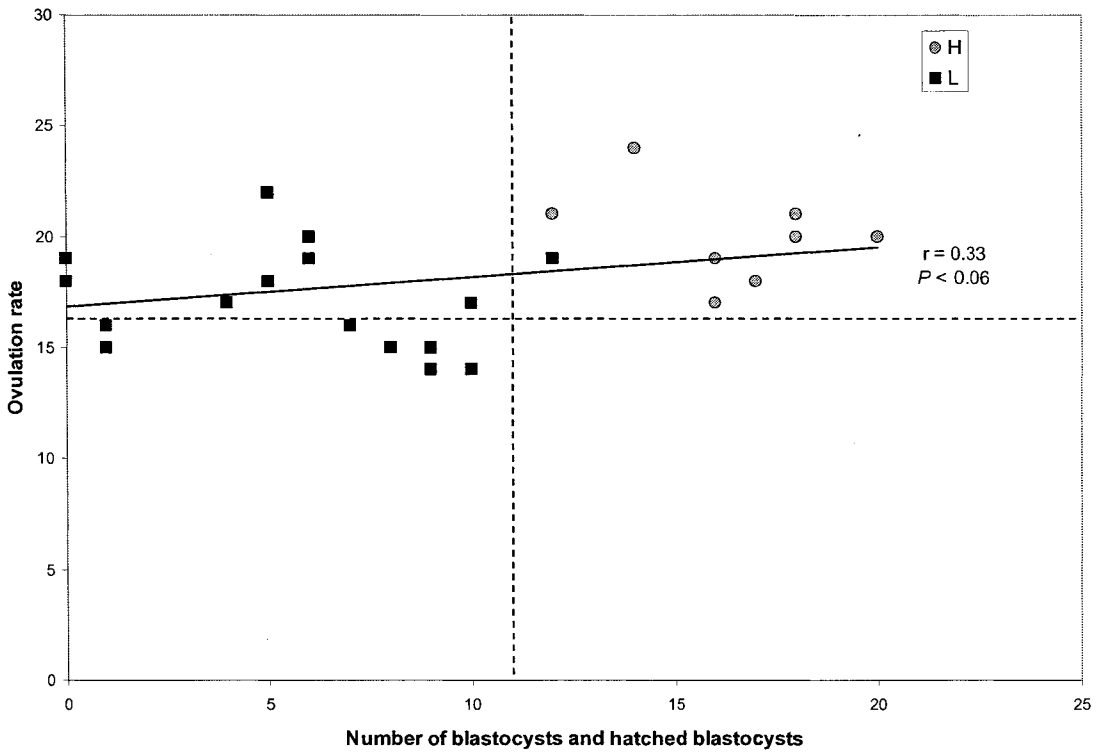


Figure 5.4 Relationship between the number of blastocysts and hatched blastocysts recovered, and ovulation rate, in H and L type litters. Broken lines suggest threshold for risk factors leading to gender-specific loss of embryos anticipated by Day 30 in Restrict, L-type litters.

CHAPTER 6

GENERAL CONCLUSIONS

The primiparous lactating sow provides a unique model for studying the latent effects of feed restriction on fetal programming because in the absence of major changes in reproductive characteristics in the immediate weaning-to-estrus interval in current dam-lines, embryonic development is compromised and is the key determinant of second litter size (Foxcroft *et al.* 2005). Although this model has been previously used to observe changes in embryonic survival (Zak *et al.* 1997a) and oocyte maturation (Zak *et al.* 1997b), it was important to examine in greater detail effects on fetal/embryonic programming. There are many ways a fetus may be programmed during development (Foxcroft *et al.* 2006); however, the most compelling involves epigenetics, as it has the potential to have large effects on embryonic growth and survival (Young 2001). Therefore the overall goal of these studies was to determine if using the previously established experimental paradigm of the catabolic lactating and weaned primiparous sows could produce epigenetic defects that would alter embryonic development and survival.

The most established time-point for examining embryonic development and survival in the pig is at Day 30 of gestation, representing the period after developmentally defective embryos are lost, but before uterine crowding becomes a factor (Town *et al.* 2004). In Chapter three embryonic survival decreased in sows feed restricted during the last week of lactation, as seen previously in this experimental model (Zak *et al.* 1997a). However, we also found decreased embryonic weight and crown-rump length at Day 30 of gestation, which had not been seen previously. Even though the sows were supplied

with *ad libitum* feed following weaning and embryonic crowding should not be a factor, these results implied some form of programming had altered the development of embryos from feed-restricted sows. It is unclear by what mechanism this embryonic reprogramming was achieved; however, in the same experimental paradigm, nutritional restriction in late lactation also produced detrimental effects on oocyte quality, measured using in vitro oocyte maturation assays (Zak *et al.* 1997b). This suggests that defects in oocyte maturation could be the origin of these developmental delays later in gestation. Later studies supported these results by establishing a relationship between increased protein catabolism in lactation and a decrease in the size, number and maturity of follicles at the time of weaning, and the ability of follicular fluid aspirated from these follicles to support *in vitro* oocyte maturation (Yang *et al.* 2000; Clowes *et al.* 2003).

However, feed restriction can also affect the oviductal and uterine environment by altering endocrine function of the pre-ovulatory follicles and differentiating corpora lutea (Foxcroft *et al.* 2000, 2003). As proper synchronization between the embryos and the environment of the oviduct and uterus is essential for proper development, any changes in this relationship could intensify embryonic loss of the most delayed embryos, leaving those which survive at a growth disadvantage compared to Controls (Dziuk 1987).

What makes these results of even more interest is that the number of female embryos surviving to Day 30 decreased, while the number of males did not (see Chapter three). There are reports that litter sex ratios in the pig can be affected by paternal breed (Gorecki 2003), uterine capacity (Chen and Dziuk 1993), and oocyte glutathione concentration (Yoshida, *et al.* 1993); however, none of these studied established connections between nutrient deprivation during lactation and sex-specific embryonic

survival in the pig. Researchers have, however, examined how increased nutritional status of the sow during pregnancy can alter litter sex ratios and provided results indicating that sows may select males over females if nutrients are limiting. For instance, Mendl *et al.* (1995) reported that sows with increased access to feed produced a higher proportion of females than males, which would support our results if the converse was true. It is also generally accepted that male embryos require a greater maternal investment as measured by piglet weight (Fernandez-Llario *et al.* 1999) and uterine space at Day 35 of gestation (Chen and Dziuk 1993), possibly making female embryos a poorer investment for the sow when resources are limited. Normally in populations of swine there are no differences in sex ratios (Gray and Katanbaf 1985; Brooks, *et al.* 1991), suggesting that what we are seeing in Chapter three is a real treatment effect. In naturally occurring populations of swine, the local resource competition model explains this treatment effect by postulating that healthy females with abundant resources would produce more females, as sows are territorial and would benefit from these resources (Clark 1978, modified by Silk 1983). Conversely, if resources are poor, sows would favour their male offspring, which upon reaching maturity become nomadic. In fact under normal circumstances perinatal mortality has been shown to be female-biased (Gorecki 2003).

It has also been suggested that that asynchrony between uterine and embryonic development prior to implantation may alter sex ratios in favor of male embryos (Krackow 1995). This suggestion was based on evidence that male embryos develop faster to the blastocyst stage than females, making them more likely to implant first and possibly synchronized with the uterine environment (Cassar *et al.* 1994). As embryonic development is delayed in both sexes at Day 30 of gestation, it is possible that this same

affect occurred in pre-implantation development, but to a greater extent in female embryos of feed-restricted sows which caused greater female embryonic mortality.

As both embryonic development and female-specific lethality were affected by feed restriction during the last week of lactation, these results suggested that they have possible connections to epigenetic defects in fetal programming. We knew, based on research in the mouse, that the period before the oocyte reaches metaphase II is a critical period for establishing DNA methylation on imprinted genes that affect embryogenesis (Lucifero, *et al.* 2002). This critical time point should be the same in the pig, and begins at the same time as we induce lactational feed restriction (Foxcroft 1997). Although there are many nutrients which are essential for DNA methylation (Davis and Uthus 2004), a major deficiency in any one of these nutrients could result in defects in epigenetic programming of the oocyte and/or the resulting embryo depending on the time required after weaning to recover from nutritional insult. These defects could manifest themselves as a failure to properly establish methylation imprints, as an inability to maintain imprints throughout development, or as a defect in reprogramming of the embryonic genome during normal pre-implantation development (Kang *et al.* 2001). Hypomethylation of embryonic DNA has been shown to result when enzymes that regulate DNA methylation are defective, resulting in IUGR and increased mortality (Sun *et al.* 2004). Failure to epigenetically reprogram the embryonic genome has also been shown to occur when oocytes are of reduced quality (Gioia *et al.* 2005), which may result in defective gene expression and result in impaired embryogenesis. Also, as it is presumed that all the mechanisms required for epigenetic maintenance are contributed to the zygote from the oocyte (Ratnam *et al.* 2002), defects in these mechanisms could result in abnormal

imprinted gene expression and the outcome seen in Chapter three. Defects in any of these critical stages could also result in failure to inactivate the second X-chromosome in female embryos, which would lead to a decrease in female embryo survival rates (Xue *et al.* 2002; Panning and Jaenisch 1996).

It was therefore, of great importance for us to determine if epigenetic defects could have resulted in decreased embryonic development and reduced female embryonic survival. In Chapter four we sought to answer these questions by examining the embryos surviving to Day 30 for changes in DNA methylation and epigenetic gene expression. Since we believed that delays in embryonic development were responsible for decreased female embryo survival, we also presumed that embryos which survive to Day 30 should be healthier and more homogeneous than embryos which failed to survive. Although we still hoped to find epigenetic defects in survivors that might influence growth and development, but not be essential for embryonic survival, we hypothesized that most of the embryos lost would have had the greatest developmental delays with the worst epigenetic defects. Therefore, even if the means for each epigenetic trait were unchanged, differences in the variance for each trait would indicate changes in the litters of Day 30 embryos that were of importance for survival. This analysis is based on the work of Archer *et al.* (2003), who used this technique to establish that there was greater variability in the epigenetic traits of cloned swine than in naturally bred control counterparts. To first analyze these changes in the Day 30 embryos, we chose a genome-wide approach to detect large scale epigenetic changes in DNA methylation by reverse-phase high performance liquid chromatography (RP-HPLC) (Ramsahoye 2002). This technique showed no differences in the mean levels of global methylation, but did

however prove that the levels of methylation within Day 30 embryos were consistent with those found in adult porcine cells (Vanyushin *et al.* 1970). This also suggests that global methylation is not responsible for the developmental delay seen in Restrict embryos surviving to Day 30. Analyzing the variance in global methylation indicated decreased variance in the Restrict embryos for either sex compared to Control counterparts. This implies that a defect in global DNA methylation during early embryogenesis could be connected to decreased embryonic survival at Day 30. As expected, Restrict female embryos had more variance in DNA methylation than Restrict male embryos, which would indicate a failure to properly regulate the methylation of Restrict female embryos during development and possibly leading to greater embryo loss. Performing the same analysis using real-time PCR we analyzed the expression of genes regulated by DNA methylation, *Igf2*, *Igf2r*, and *Xist*. Both *Igf2* and *Igf2*r are imprinted genes which are important for regulating embryonic growth and development in the pig (Amarger *et al.* 2002; Killian *et al.* 2001). However, no changes in the expression of these genes in the embryos surviving to Day 30 could be detected, although variance did tend to decrease in *Igf2r* expression in Restrict female embryos. As *Igf2* is paternally methylated, changes in its expression would be unlikely. However, since *Igf2r* is maternally methylated, this further confirms that a defect in methylation could have resulted in over-expression of *Igf2r* which in turn would have resulted in decreased embryonic growth and development possibly above a threshold for which survival was impossible (O'Dell and Day 1998). Analysis of *Xist* expression was hoped to further explain these results, as its expression is regulated by DNA methylation and is essential for female embryo survival (Young 2001; Lee 2000). Although neither the mean or

variance showed a difference in female embryos between treatment groups, the variance in Restrict male expression tended to be higher than Control male embryos. Since the methylation of *Xist* changes during embryogenesis it was not surprising that its expression is not different between female embryos since epigenetic defects vital to early embryonic survival would have removed epigenetically-defective embryos long before methylation of *Xist* would have contributed. The tendency for higher variance in Restrict males does however imply that there is a defect in the regulatory mechanisms that methylate *Xist* which, while it might not result in male lethality, could influence early female survival if compounded with other additional defects.

Overall, these results suggested that epigenetic defects during embryogenesis were causing reduced embryonic development and female embryonic survival at Day 30 of gestation. However these results failed to identify the origin and timing of these defects in embryogenesis. Therefore in Chapter five we examined blastocysts recovered from animals subjected to the same experimental paradigm. The blastocyst stage of embryonic development provided a unique stage of development since Kang *et al.* (2001) have shown that methylation of non-imprinted genes should be at a minimum at this time, therefore any changes in methylation status would reflect changes in imprinted genes. Furthermore, it is believed that embryonic mortality is not a factor before Day 9 of gestation in the pig (Geisert and Schmitt 2002), then embryos present between Days 5 and 7 of gestation should contain the defects which lead to decreased development and survival later in gestation. Sex typing PCR confirmed that there was no difference in sex ratios at this stage of development and since average recovery rates were the same as average embryo survival rates in Chapter three we concluded that the embryos collected

in this study should be representative of the embryos lost in Chapter three. However, using a dot blotting technique we were unable to identify differences in global methylation status between embryos from either treatment. We also tested blood samples from the sows before treatment (Day 13), after treatment (Day 21) and upon returning to estrus to determine if decreases in sow plasma folate and B_{12} might be causing the epigenetic defects in subsequent embryos. The results suggested that folate and B_{12} levels were not affected by treatment and post-weaning atrophy of the mammary gland likely released more B_{12} back into circulation, ensuring a surplus supply during the post ovulatory period (Girard *et al.* 1996). However, sow leukocyte DNA was lower in Restrict than Control sows, suggesting that although folate and B_{12} might not be in deficit, other nutritional factors important for DNA methylation may be lacking.

Our interest in developmental delays in embryogenesis led us to explore the diversity in embryonic development across treatment groups. Regardless of treatment or day of slaughter, embryos within litters were observed to be at various stages of development. As the duration of ovulation may not exert critical effects on embryonic diversity (Soede 1992), it was concluded that this embryonic diversity must be related to the maturation of the pre-ovulatory follicles in some manner. Furthermore, there was a positive correlation between recovery rates and stage and synchrony of development, regardless of treatment. In Chapter three we determined using scatter-plots that there was always some overlap between sow responses to different treatments; Therefore, we examined these results in greater detail, irrespective of treatment, to see if there was some underlying cause of these developmental differences. Each sow was scored based on the number of embryos recovered and stage of embryonic development, and the top 25% of

sows were classified as high (H) and compared with the bottom 25% of low scoring (L) sows. Re-analyzing the data revealed that H sows had lower ME intakes and net energy balances throughout lactation. However, H sows also have higher ovulation rates when compared to the treatment model results (see Chapter three; Chapter four), but when compared to the metabolic results in the treatment model (Chapter three; Chapter four there is no reason to assume that the lower level of feed intake see in H sows would positively influence post-weaning reproductive performance. Furthermore, L sows have a much higher net energy balance throughout lactation, achieving almost the same levels as seen during the two weeks before feed restriction. However L sows have lower ovulation rates compared to H sows. One interpretation of this is that sows with the highest inherent voluntary feed intakes up to Day 13 of lactation would have greater restriction relative to their maximum voluntary feed intake, regardless of allocation to the Restrict or Control treatment from Day 14 to weaning. Also, in these H/L sows, an increase in ovulation rate tends to correlate to increased development to the blastocyst stage, which suggests that perhaps the H sows inherently have better synchronization between the uterus, oviduct and timing of ovulation than L sows.

The results of these experiments provide us with the opportunity to consider further investigation into this experimental paradigm. Although analysis of Day 30 embryos provides insight into programming defects which may alter embryonic development and future litter performance, it is clear that in order to understand the mechanisms that mediate these effects on embryonic survival and development, defective embryos must be recovered at an earlier time point. There are two stages at which to assess epigenetic traits in this experimental paradigm, either in the oocyte or in the pre-

implantation embryo. As seen in studies such as Gioia *et al.* (2005) oocyte quality can be measured using immunofluorescence for DNA methylation either before or after fertilization. Although this would provide information of genome-wide epigenetic defects, it may not be sensitive enough to identify smaller changes in critical regions. Furthermore, sex-typing of fertilized embryos would be extremely problematic, hence the reason for attempting sex-typing PCR and dot blotting in Chapter five. Although dot blotting failed to show differences in methylation status, the use of a fluorescent probe designed to target the Y chromosome (Kawarasaki *et al.* 2000) and the use of radiolabeled methyl incorporation assay (RMI) on dot blotted embryonic DNA may provide greater sensitivity (Piyathilake *et al.* 2000). However, the technique with the greatest chance for success for detecting epigenetic defects in early embryogenesis, would be to assess gene expression in the pre-implantation phase of gestation. As mentioned previously, embryos recovered before Day 9 of gestation can be developmentally-delayed and yet still be present *in utero* because developmental asynchrony does not influence survival until implantation (Geisert and Schmitt 2002). The amount of RNA recovered from each individual embryo would be of sufficient quantity for sex determination based on expression of *Sry* or a similar male-specific gene. Alternatively, sex determination could be achieved when analyzing the patterns of gene expression using a large-scale screening method, such as a microarray (Whitworth *et al.* 2005). This approach would better identify all the genes, both imprinted and non-imprinted, that are involved in fetal programming in this experimental paradigm.

In conclusion, the results presented in the thesis provide compelling evidence that feed restriction during the last week of lactation in the primiparous sow results in

epigenetic defects in subsequent litters. Future studies arising from these results may be able to better explain how treatment alters specific nutrients within the sow and how these changes relate to the programming of the oocyte and developing embryo.

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