Short Communication

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Altered epigenetic variance in surviving litters from nutritionally restricted lactating primiparous sows

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Abstract. Feed restriction of primiparous sows during the last week of lactation has been shown to decrease embryonic growth and female embryo survival to Day 30 of gestation. This study sought to determine whether global DNA methylation and epigenetic gene expression of the candidate genes *Igf2*, *Igf2r*, and *Xist* were associated with these treatment effects. Given that these epigenetic traits are expected to be important for embryo viability, changes in variance for these traits at Day 30 were predicted to be reflected in the loss of abnormal embryos at this time. Consistent with this prediction, variance in DNA methylation was reduced ($P < 0.001$) in Restrict male embryos, and there was a tendency for reduced variance ($P < 0.06$) in Restrict female embryos. Variation in DNA methylation tended to be correlated ($R = 0.42$, $P < 0.1$) with the difference in variance of embryo weights between treatments ($P < 0.01$), suggesting a relationship between epigenetic changes and embryonic development. Variance in *Igf2r* expression tended to decrease ($P < 0.07$) in Restrict female embryos while variance in *Xist* expression tended to decrease in Restrict male embryos ($P < 0.08$), suggesting that maternally inherited epigenetic defects may cause female embryonic loss and reduced growth before Day 30 of gestation.

Additional keywords: catabolism, imprinting, pig, real-time polymerase chain reaction, reverse-phase high performance liquid chromatography.

Introduction

Feed restriction of sows during the last week of lactation has been associated with increased catabolism of both protein and fat tissues and decreased embryonic survival in sows bred at first oestrus after weaning (Zak *et al.* 1997; Vinsky *et al*. 2006). Furthermore, the decrease in embryonic survival was mainly associated with the loss of female embryos in this experimental model (Vinsky *et al*. 2006). Reduced survival of female embryos 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 delay embryonic development and mediate the sex-specific loss of embryos reported previously (Vinsky *et al*. 2006).

Heterogeneity in oocyte maturation has been suggested as a primary factor in determining embryonic survival (Pope *et al*. 1990; Geisert and Schmitt 2002). Therefore, embryos surviving to Day 30 should be of better quality and more homogeneous than embryos which failed to survive, and analysis of the variance within surviving littermates would identify traits which are important for embryonic survival. Archer *et al*. (2003) used this approach to analyse the greater variability in cloned swine than in individuals derived from natural matings. Using the same underlying concept, we hypothesised 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 with 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 (Killian *et al*. 2001; Amarger *et al*. 2002); (3) to determine why female embryos are preferentially lost over males in Restrict sows, the expression of the X-chromosome specific transcript (*Xist*) was analysed: *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).

Materials and methods

Animals and treatments

The experimental paradigm used was described previously (Vinsky *et al*. 2006) and was conducted in accordance with

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Table 1. Estimated composition of lactating sow diet and formulation of nutrients essential to reproduction and DNA methylation, as fed

	Composition per kg[A]	Control diet (5 kg day^{-1})	Restrict diet (2.5 kg day^{-1})	NRC requirements[B] (5 kg day^{-1})
DE (kcal)	3159	15 793	7896	15 790
ME (kcal)	3039	15 196	7598	15 158
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

[A]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, Calmar, AB, Canada).
[B]Calculated using NRC swine requirements software v. 2.03 based on feed composition, and Control sow averages for farrow weight (189 kg), piglets per litter (10), piglet gain (120 g day^{-1}), and lactation (21 days).

the Canadian Council on Animal Care guidelines and with the approval of the Faculty Animal Policy and Welfare Committee (Protocol #2003-09). Briefly, 34 primiparous F_1 sows (Hypor, Regina, SK, Canada) had litters standardised to a minimum of nine piglets per sow by cross-fostering within 48 h after farrowing. Sows were paired on the basis of similar changes in body condition from Day 0 to 14 of lactation, and within each pair sows were assigned to be fed a standard lactation diet (Table 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. The day on which standing oestrus was first observed was designated as Day 0 of pregnancy. Background data on sow reproductive performance, and embryonic survival and development, were reported previously (Vinsky *et al*. 2006).

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

Preparation of embryonic tissues, and isolation and purification of DNA used for further analysis in the present study were described by Vinsky *et al*. (2006). Aliquots of stored DNA were analysed for DNA methylation using RP-HPLC following the guidelines provided by (Ramsahoye 2002). Samples were analysed in batches, with each treatment pair being analysed 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, USA). Nucleotides were detected using a UV detector set at 260 nm (Bio-Rad, Spring Vally, CA, USA). The mobile phase consisted of 100 mM ammonium phosphate pH 6.0 (Sigma) pumped at 1.0 mL min^{-1} for a runtime of 30 min. UV absorbance data were stored and analysed 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 with previously determined values by 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 with the values obtained from the fully sequenced genome (NC001416).

RNA isolation, cDNA synthesis and real-time polymerase chain reaction

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 SpectraMax190 spectrophotometer plate reader and Softmax PRO software (Sunnyvale, CA, USA). A 1 μg μL^{-1} 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). Real-time polymerase chain reaction was performed using an ABI 7700 thermocycler on 1 μL of cDNA, in duplicate (Foster City, CA, USA). Primers and Taqman-MGB probes (Table 2) were designed using the Primer express software v. 2.0 (ABI) using sequences found in Genbank for porcine cyclophilin (AY008846), *Xist* (AJ429140), and *Igf2r* (AF339885). The *Igf2* primers sequences were previously reported by Van Laere *et al*. (2003). Real-time semi-quantification of the 'gene of interest' mRNA for each individual sample was performed and the C_t

Table 2. Porcine specific real-time polymerase chain reaction primers and probes

Target	Forward	Reverse	Probe
Cyclophilin	5′-AATGCTGGCCCCAACACA-3′	5′-TCAGTCTTGGCAGTGCAAATG-3	5′-VIC-ACGGTTCCCAGTTTT-MGBNFQ-3′
Igf2	5′-CAAGTCCGAGAGGGACGTGT-3′	5′-CCAGGTGTCATAGCGGAAGAA-3′	5′-FAM-CCGACCGTGCTTCCGGACAACTT-MGBNFQ-3′
Xist	5′-TGGGACAGAGTGAGGGAGGT-3′	5′-GGCCACTACTATGAGCAGGGAG-3′	5′-FAM-CCAAGGAGATAGCCTG-MGBNFQ-3′
Igf2r	5′-CAGCTTTCATTCCGTGGGA-3′	5′-TGTTGAATTCCAGAAGAGATCTGG-3′	5′-FAM-TCTGTTTTGAAAAGTGCC-MGBNFQ-3′

value was subsequently normalised against its respective mean cyclophilin (endogenous control) C_t value using the standard ΔC_t 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.

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 were analysed using the MIXED procedure of SAS (SAS Institute, Cary, NC, USA) as previously described (Vinsky *et al*. 2006). As sow was the experimental unit, all individual DNA methylation and real-time gene expression data collected on each embryo were averaged within each litter, before analysis of the means. These means were analysed 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 were calculated for each treatment and sex using the non-averaged litter data and the MEAN procedure of SAS. Appropriate comparisons between the calculated variances for each treatment and sex were determined using an *F*-test, with differences being significant at $P < 0.05$ (Archer *et al*. 2003).

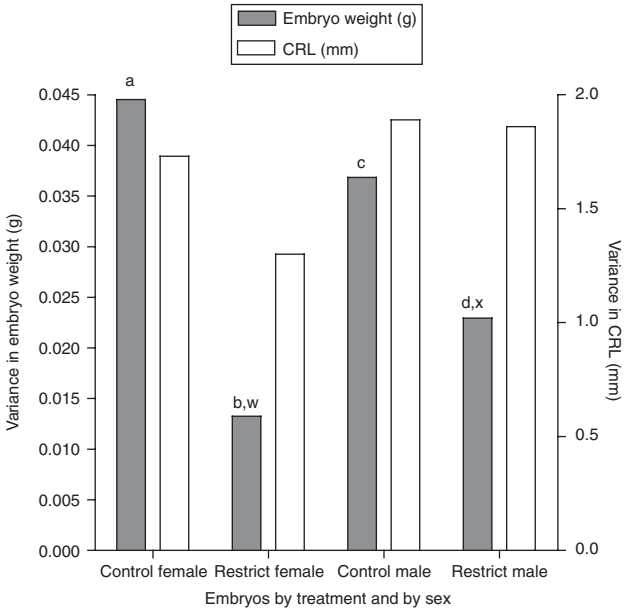

Fig. 1. Variance in the physical development for Day 30 male and female embryos. [a–b,c–d]Differences between treatments within sex ($P < 0.001$ and $P < 0.01$, respectively). [w,x]Differences between sexes within treatment ($P < 0.01$). CRL, crown–rump length.

Results

Variance in physical parameters

The variance for physical characteristics by treatment and sex is depicted in Fig. 1. The variance in embryo weights at Day 30 was much lower in Restrict than Control female ($P < 0.001$), and in Restrict than Control male ($P < 0.01$), embryos. Furthermore, Restrict male embryos showed greater variance in embryonic 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. The distribution of the embryonic weights calculated as the percent of embryos in each weight category within a litter and averaging those values by treatment and sex, is shown in Fig. 2. Control female and Control male embryonic weights are slightly skewed to the right, and by comparison Restrict females and Restrict males have less skewing.

DNA methylation

Across treatments, average DNA methylation was estimated as 3.8% of all cytosines. No difference in mean embryonic global methylation was detected between treatment groups, regardless of sex (Fig. 3). 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). 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.

Gene expression

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. 3). 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 ($P < 0.05$), and tended to be greater in Restrict female embryos ($P < 0.08$), than in their respective male littermates (Fig. 4). *Xist* showed higher mean

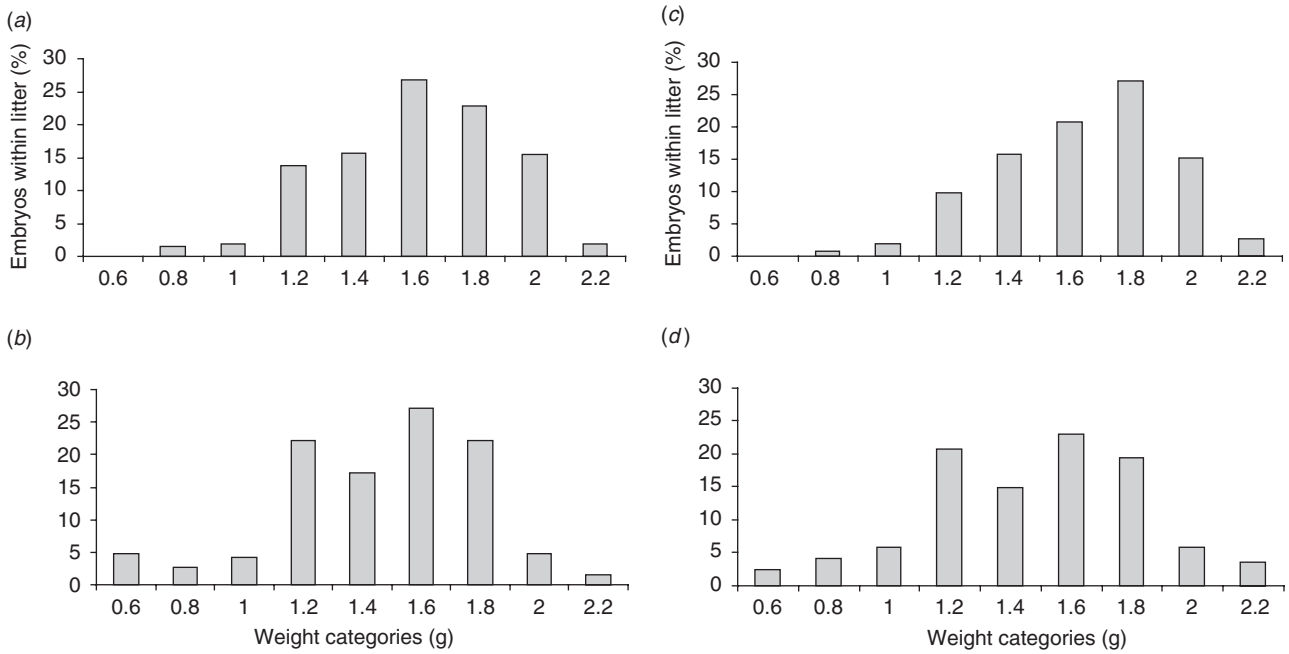

Fig. 2. The distribution of embryonic weights, calculated as the percentage of embryos in each weight category within a litter, and then averaged by treatment and sex. Control female (*a*) and Control male (*b*) embryonic weights are slightly skewed to the right; the distribution of weights is less skewed in Restrict female (*c*) and Restrict male (*d*) litters, which have a decrease in the proportion of heavier embryos, and a lower mean embryonic weight (Vinsky *et al*. 2006).

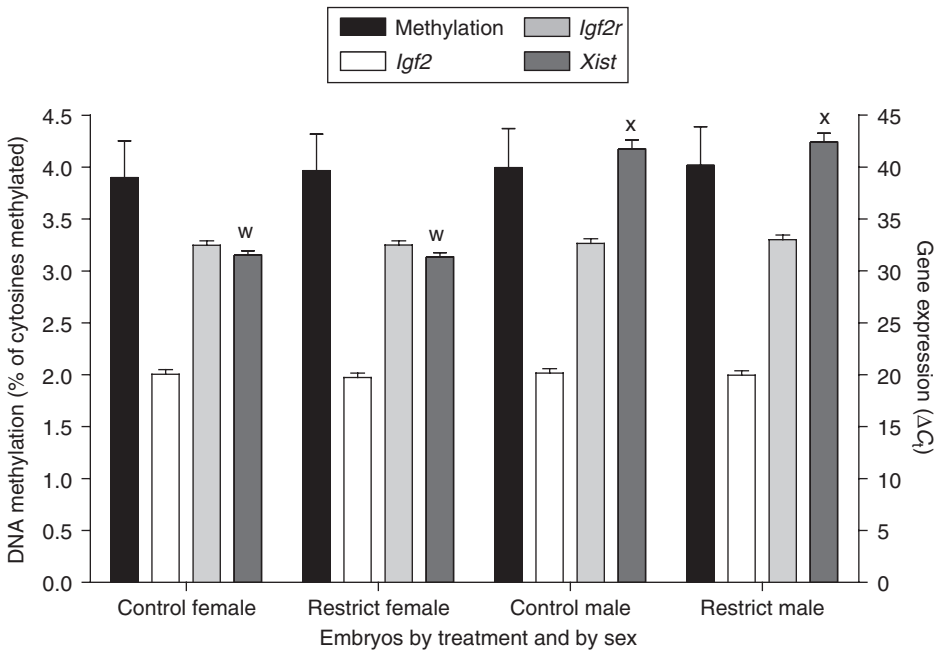

Fig. 3. Mean (+s.e.m.) global methylation and gene expression in Day 30 embryos. ΔC_t values are inversely related to the level of gene expression (Pfaffl 2001). ^{w,x}Differences between sexes within treatment ($P < 0.001$).

expression ($P < 0.001$), as reflected by a lower ΔC_t value, in female embryos compared with males, irrespective of treatment (Fig. 3). Males from Control sows had a tendency towards increased variance in *Xist* expression as compared with males from Restrict sows ($P < 0.08$) (Fig. 4). However, across treatments, female embryos from either Restrict or Control sows had less variance in *Xist* expression than their male counterparts ($P < 0.001$).

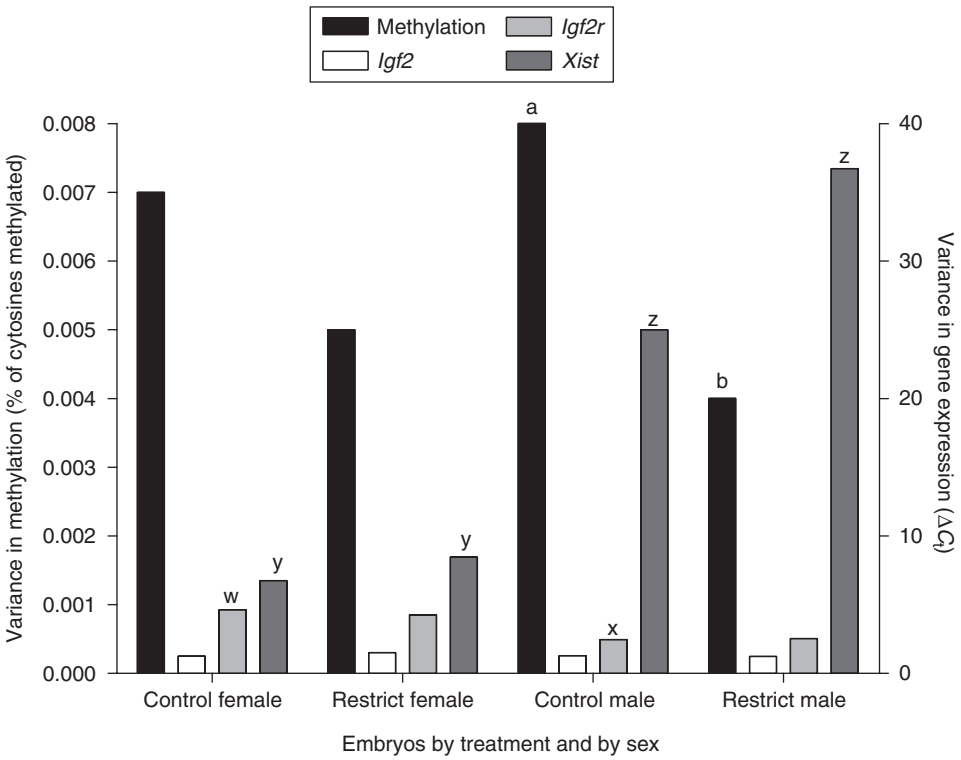

Fig. 4. Variation in global methylation and gene expression in Day 30 male and female embryos. [a,b]Differences between treatments within sex ($P < 0.001$). [w–x,y–z]Differences between sexes within treatment ($P < 0.04$ and $P < 0.001$, respectively).

Discussion

We predicted that changes in the variance of litter traits would better identify important changes in litter dynamics than a simple analysis of the trait means. Consistent with this concept, in addition to having lower mean weights, there was a dramatic decrease in the variance in embryonic weights in Restrict compared with Control sows, and also less variance in Restrict female than Restrict male embryos (see Fig. 1). Considered in the context of the data presented in Fig. 2, showing decreased skewing towards embryos with heavier weights in Restrict compared with Control embryos, these results suggest that the loss of embryos before Day 30 in Restrict sows likely occurred in litters that were characterised by more diverse and also retarded embryonic development. Furthermore, the further decrease in variance in embryonic weight in the surviving female embryos suggests a sex-specific link between the increased diversity in the development of female embryos and their susceptibility to undergo embryonic loss compared with developmentally delayed but surviving male littermates. Estimated nutrient availability in the various sow treatment groups (see Table 1) suggested that Restrict sows were greatly deprived of the nutrients essential for DNA methylation (Davis and Uthus 2004), which have been suggested to result in abnormal embryonic DNA methylation (Waterland and Jirtle 2004). Global DNA methylation of embryos surviving to Day 30 was very similar to that seen in adult sow tissue samples (Vanyushin *et al*. 1970) and was not different between litters from Restrict and Control sows (see Fig. 3).

However, there was a dramatic reduction in the variance in methylation state in Restrict compared with Control embryos (see Fig. 4). Again, we interpret these data as indicating that the lower embryonic survival in Restrict sows, removes embryos with more extreme global DNA methylation, thus, reducing litter heterogeneity, and hence variance, for methylation state without altering the mean.

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 imprinted genes that may be affected by changes in methylation state. As the imprinting and methylation of *Igf2* is paternal, the lack of a treatment effect on either the mean or variance in *Igf2* expression was not unexpected. In contrast, greater variance in *Igf2r* expression in Control female compared with Control male embryos, but a lack of such differences in variance between Restrict female and male embryos, is consistent with the expectation that maternal malnutrition may alter maternally-imprinted genes and the relative decrease in *Igf2r* variance in Restrict female embryos may again be connected to increased female

embryo mortality. In the context of the genetic conflict hypothesis (Moore and Haig 1991) this would imply that decreased methylation of *Igf2r* would result in its reduced gene expression (Wutz *et al*. 1997) and would result in increased mortality of abnormally larger embryos (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 in females than males (Fig. 3). However, there was a tendency towards decreased variance in *Xist* expression in Control male embryos compared with males from Restrict sows (Fig. 4), indicating that males from Control sows have tighter regulation of *Xist* expression, which may be a factor favouring 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 provide 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.

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