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Ontogeny of metabolic effects on embryonic development in lactating and weaned primiparous sows

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Abstract. Using an established experimental paradigm, feed restriction during the last week of lactation in primiparous sows reduces embryonic growth and development and produces female-specific embryonic mortality by Day 30 of gestation. Because this gender-specific loss of embryos at Day 30 was associated with changes in the variation of markers of epigenetic imprinting, the present study sought to establish the ontogeny of such epigenetic affects. Leucocyte DNA of restrict-fed sows exhibited decreased global methylation during the last week of lactation and during the return to oestrus ($P < 0.05$), but no associated changes in plasma folate and vitamin B_{12}. Furthermore, no changes in methylation of blastocyst DNA, embryonic sex ratios or development were evident at Day 6 of gestation that would characterise the underlying defects that reduced female embryo survival by Day 30. However, regardless of treatment, embryo recovery rates and synchrony in embryonic development were associated with the stage of development of the recovered embryos ($r = 0.68$; $P < 0.001$). The subset of sows classified as bearing litters with superior embryonic development had lower net energy balance over lactation ($P < 0.01$) and higher ovulation rates ($P < 0.005$) compared with sows classified as having poorer embryonic development. Collectively, these data suggest that a subset of litters within restrict-fed sows will be most sensitive to the latent epigenetic mechanisms that ultimately trigger gender-specific loss of embryos by Day 30 of gestation, but that these selective mechanisms are not evident by Day 6 of gestation.

Introduction

Increased catabolism during the last week of lactation in primiparous sows is known to reduce embryonic survival and development by Day 30 of gestation in the subsequent litter (Foxcroft 1997). Using a refinement of the experimental paradigm reported by Zak *et al*. (1997), Vinsky *et al*. (2006) 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*, 2003*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) 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 aetiology of this selective loss of female embryos before Day 30 of gestation is unknown. Vinsky *et al*. (2006) suggested that female embryo loss could be due to epigenetic defects originating from the oocyte, because the timing of feed restriction coincides 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*. (2007) reported that reduced embryonic development and decreased female embryonic 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 was epigenetically defective and lost before Day 30 of gestation, with surviving embryos in these sows being 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 vitamin B_{12} (Mason 2003), and the extent of methylation of sow leucocyte 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, 2007) to examine associations between

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changes in sow leucocyte DNA methylation, plasma folate and vitamin 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 several mammalian species, including the pig, suggests that global demethylation of non-imprinted genes occurs at the blastocyst stage (Kang *et al*. 2001). As such, changes in the residual methylation at the blastocyst stage would indicate that changes in imprinted regions before embryonic mortality would be a factor (Geisert and Schmitt 2002). Therefore, the main objectives of this study were: (*1*) to determine whether the 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 by Day 30; and (*2*) to explore the aetiology of heterogeneity in litter development that appears to underlie effects of maternal catabolism on embryonic survival in the sow.

Materials and methods

Animals and treatments

The present study was performed using a previously described model (Vinsky *et al*. 2006) in accordance with the Canadian Council on Animal Care guidelines and with the approval of the Faculty Animal Policy and Welfare Committee. The study involved 34 primiparous F_1 sows (Hypor, Regina, SK, Canada) which farrowed normally and were managed according to standardised and approved protocols at the Swine Research & Technology Centre (SRTC), University of Alberta. Within 48 h after farrowing, litter size was standardised to a minimum of nine piglets per sow by cross-fostering. On Day 14 of lactation, sows preselected for trial were paired on the basis of similar body condition from Days 0 to 14 of lactation; within each pair, sows were assigned to either Restrict or Control treatment groups. Sows were fed three times daily with a standard lactation diet from Days 0 to 14 of lactation, to a preset maximum of 5.0 kg day^{-1}. From Days 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}. Immediately after weaning and until breeding, sows were provided with *ad libitum* access to the same lactation diet. After insemination until slaughter, sows were fed a standard gestation diet according to National Research Council (NRC 1998) requirements.

Within 24 h after farrowing (Day 0), on Days 14 and 21 of lactation, at oestrus and immediately before sows were killed, sow back fat and loin depths were measured by real-time ultrasonography using a 5-MHz real-time linear probe (Scanprobe II; Scano, Ithaca, NY, USA) and sow bodyweight was recorded. Sow weight and back fat 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 Days 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*. 2006).

Blood sampling and folate/vitamin B_{12} radioimmunoassay

Blood was sampled from ear veins of sows using acute venepuncture during brief periods of nose-snare restraint in the morning before feeding on Days 13 and 21 of lactation and during first post-weaning oestrus. Blood was collected into an EDTA vacutainer (Becton Dickinson, Franklin Lakes, NJ, USA) and centrifuged for 10 min at 2000*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 vitamin B_{12} were performed using Dualcount solid phase, no boil, Radioimmunoassay (RIA; DPC/Intermedico, Markham, ON, Canada) using 200 μL plasma. Validation of the Dualcount assay kit for folate indicated intra- and interassay coefficients of variation (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 vitamin B_{12} indicated intra- and interassay CV of 7.52% and 28.22%, respectively. Vitamin B_{12} assay sensitivity, defined as 96% of total binding, was 71 pg mL^{-1}. Cold recovery of vitamin B_{12} was 116 ± 2% and a serial dilution of porcine plasma showed parallelism to the standard curve.

Sow management after weaning

Sows were checked twice daily at 0800 and 2000 hours 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, ON, Canada) with a 7.5-MHz transcutaneous probe to determine the timing of ovulation. Estimated time of ovulation was defined as described previously 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 (3×10^9 morphologically normal and motile spermatozoa per 50 mL 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) 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 was based on estimated time of ovulation within Day 1 and the known time of slaughter of the sows.

Ovulation rates and blastocyst collection

All sows were killed on-site by qualified staff using approved necropsy procedures between Days 5 and 7.5 of pregnancy. Immediately after sows were killed, 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 phosphate-buffered saline (PBS). Embryos were collected in Petri dishes, washed in PBS and their stage of development identified under a binocular dissecting microscope (Wild M3; Wild Heerbrugg,

Gais, Switzerland). Blastocysts with few cells and small blastocoels were termed early blastocysts, those with many trophoblast cells but still zona encased were termed blastocysts and those without zona were termed hatched blastocysts. After classification, all embryos were transferred to individual 0.5-mL tubes, frozen on dry ice and stored at −70°C.

DNA extraction and sex-typing PCR

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).

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

Dot blotting methylation analysis

Dot blotting of embryonic and maternal DNA was performed as described by Park *et al*. (2005). The 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.

Statistical analysis

A total of 33 sows met all criteria for inclusion in the present study and data from these sows were 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, percentage blastocysts, number of males, number of females and DNA methylation. Data were analysed using the MIXED procedure of SAS (SAS Institute, Cary, NC, USA) as a randomised 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 analysed using the same MIXED procedure of SAS. Sow characteristics were analysed 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 UNIVARIATE procedure of SAS. Protein and fat loss were calculated as a percentage loss of parturition mass. Plasma folate and vitamin B_{12} were analysed using the MIXED procedure of SAS using a repeated-measurements model.

Table 1. Least square mean ± s.e.m. for litter weights over a 21-day lactation

Litter data	Control ($n = 16$)	Restrict ($n = 17$)
Litter size (no. 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 Days 14 to 21 ($P < 0.05$).

Treatment means with differenct superscript letters within rows differ significantly ($P < 0.005$).

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

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 were scored as 8; morulae were scored as 6; any earlier-stage cleaved embryos were scored as 4; and one-cell embryos were scored as 2. The 25th and 75th percentiles across all scores were calculated and sows were placed into either a high-scoring group if above the 75th percentile (H; $n = 9$) or the low-scoring group if below the 25th percentile (L; $n = 16$). Statistical analysis based on these categories was performed as described above 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.

Results

Feed intake, energy requirements and net energy balance

The number of piglets nursing throughout lactation did not differ between Control and Restrict sows (Table 1). There were no differences in initial litter weight or litter weight gain from Days 0 to 13 between Control and Restrict sows. During feed restriction from Days 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 Days 14 to 21 than in Control sows (11.5 ± 0.3 *v.* 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, the estimated overall net energy balance was not different between treatment groups by Day 13 of lactation, but was very different between treatments from Days 14 to 21 ($P < 0.001$), when feed restriction was applied (Table 2).

Table 2. Least square mean ± s.e.m. for estimated net energy balance

Item	Control ($n = 16$)	Restrict ($n = 17$)
Estimated sow net energy balance (Mcal day^{-1})		
Days 0–13	-4.2 ± 0.8	-4.6 ± 0.8
Days 14–21	-6.7 ± 0.5^{e}	-9.5 ± 0.5^{f}
Days 0–21	-5.1 ± 0.6^{c}	-7.3 ± 0.6^{d}
Estimates of changes 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
Days 0–13 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}
Days 14–21 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}
Days 0–21 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 oestrus		
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}
Oestrus 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 superscript letters (a, b; c, d; e, f) differ within rows by $P < 0.05$, $P < 0.005$ and $P < 0.001$, respectively. Treatment means with different superscript letters (w, x, y, z) differ within columns by $P < 0.05$.
ME, metabolisable energy; NE, net energy balance.

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

Sow characteristics

Measurements of average sow weight and loin depth are shown in Fig. 1. There were no differences between treatment groups on Day 0 in sow bodyweight, back fat and loin depth. There were no differences in weight loss between Control and Restrict sows, except from Days 14 to 21 of lactation (-6.2 ± 1.2 and -19.9 ± 1.3 kg, respectively; $P < 0.001$) and from Day 21 of lactation to heat (-6.0 ± 1.7 and 0.3 ± 1.7 kg, respectively; $P < 0.05$). The back fat of Control and Restrict sows was different on Day 13 of lactation (23.2 ± 0.5 *v.* 21.8 ± 0.5, respectively; $P < 0.05$) and on the day of slaughter (20.6 ± 0.5 *v.* 18.3 ± 0.5, respectively; $P < 0.005$). However, there was no difference in sow back fat between Control and Restrict groups on Day 0 of lactation (23.9 ± 0.7 *v.* 23.5 ± 0.7, respectively), Day 21 of lactation (19.9 ± 0.5 *v.* 20.1 ± 0.5, respectively) and at oestrus (19.6 ± 0.6 *v.* 19.8 ± 0.6, respectively). Back fat loss only differed between Control and Restrict sows from Days 0 to 13 of lactation (-0.5 ± 0.5 and -1.9 ± 0.5 mm, respectively; $P < 0.05$) and from oestrus to the day of slaughter (1.2 ± 0.5 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 on Day 0 of lactation (Table 2). Loss of both protein and fat mass was greater in Restrict sows compared with Control sows throughout lactation and from Days 14 to 21 of lactation (see Table 2). From weaning to oestrus, estimated protein mass decreased in Control sows, whereas it increased in Restrict sows. From oestrus to the day of slaughter, estimated protein mass increased faster in Restrict than Control sows. Comparing across all time points within treatment groups, the estimated protein mass in Control sows decreased between Days 14 and 21, then increased from oestrus to the day of slaughter, whereas estimated fat mass increased between oestrus and the day of slaughter compared with any previous time point. In Restrict sows changes in both estimated protein and fat decreased markedly from Days 14 to 21 of lactation and then increased from Day 21 of lactation to oestrus. From oestrus to the day of slaughter, only the change in estimated protein mass increased significantly in Restrict sows.

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

Plasma folate and vitamin B_{12} RIA

No differences were observed between Control and Restrict sows in plasma folate measured on Day 13 of lactation

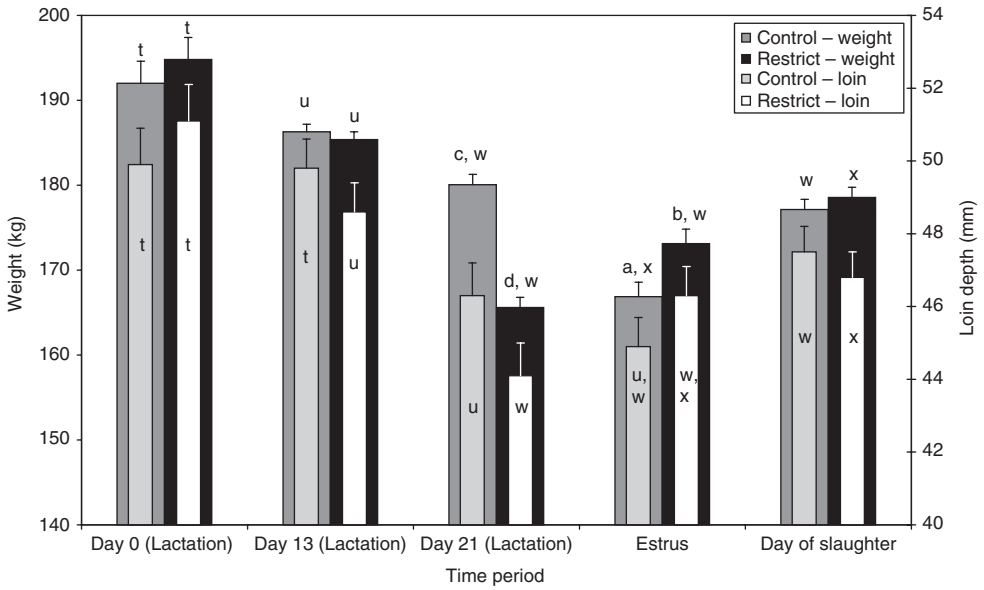

Fig. 1. Sow characteristics during lactation, at standing heat (oestrus) and on the day of slaughter. Bars show the least square mean ± s.e.m. changes in weight and loin depth. Treatment means with superscripts a, b and c, d indicate differences between treatments within periods, significant at $P < 0.05$ and $P < 0.001$, respectively. Treatment means with different t, u, w, x superscripts indicate differences between periods within treatments, significant at $P < 0.05$.

(19.7 ± 1.3 *v.* 19.6 ± 1.3 ng mL^{-1}, respectively), Day 21 of lactation (15.2 ± 1.7 *v.* 17.8 ± 1.5 ng mL^{-1}, respectively) or during oestrus (18.6 ± 1.7 *v.* 19.5 ± 1.6 ng mL^{-1}, respectively). Analysing 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 vitamin B_{12} levels measured on Day 13 of lactation (606.3 ± 122.9 *v.* 697.3 ± 129.9 pg mL^{-1}, respectively), Day 21 of lactation (384.7 ± 69.3 *v.* 354.9 ± 65.0 pg mL^{-1}, respectively) or during oestrus (3189.0 ± 1078.5 *v.* 1625.8 ± 897.6 pg mL^{-1}, respectively). Analysing the same data based on H/L scoring indicated no differences between H and L groups in vitamin B_{12} levels (data not shown).

Table 3. Least square mean ± s.e.m. for sow reproductive performance and embryo survival data

Item	Control ($n = 16$)	Restrict ($n = 17$)
Weaning-to-oestrus interval (days)	4.7 ± 0.2^{a}	5.3 ± 0.2^{b}
Oestrus-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
% Embryos recovered	70 ± 5	74 ± 5

Treatment means with different superscript letters (a, b) differ within rows by $P < 0.05$. Analysis of the percentage of embryos recovered and pregnancy rate was performed on arcsin-transformed data.

Sow fertility and embryonic development

Of the 34 sows allocated to this experiment, data from one Control sow were 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 oestrus to ovulation interval were not different between treatments (Table 3). However, the weaning to oestrus interval ($P < 0.05$) was longer in Restrict sows compared with controls. At slaughter between Days 5 and 7.5 of gestation, there was no difference in embryo recovery rates between treatments (Table 3). Embryos recovered from Control and Restrict sows were found to be between one and 32 cells ($n = 17$ and 8, respectively), morula ($n = 5$ and 18, respectively), early blastocyst ($n = 26$ and 8, respectively), blastocyst ($n = 29$ and 27, respectively) and hatched blastocyst ($n = 108$ and 157, respectively) stages of development. Irrespective of treatment, there was a correlation between overall embryo recovery rate between Days 5.5 and 7.0 and the number of blastocysts and hatched blastocysts recovered ($r = 0.68$; $P < 0.001$; Fig. 2).

Analysing the same data based on H/L scoring indicated a difference between H and L groups in ovulation rate (20.0 ± 0.7 and 17.1 ± 0.5, respectively; $P < 0.005$). Regardless of scoring classification, ovulation rate tended to correlate with the number of blastocysts and hatched blastocysts ($r = 0.33$; $P < 0.06$), irrespective of treatment or day of gestation. Restricting analysis to embryos recovered within the 24-h period designated as Day 6, a greater proportion of normal and hatched blastocysts was contributed from H-scoring sows (Fig. 3).

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 with 51% males in Control sows.

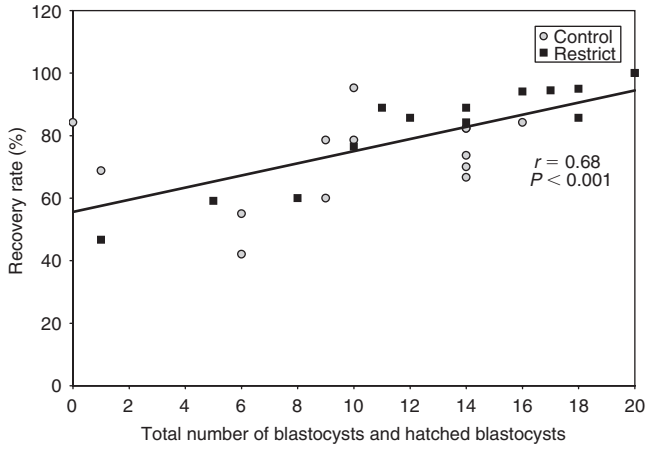

Fig. 2. Relationship between the number of blastocysts and hatched blastocysts and the overall rate of embryo recovery between Days 5.5 and 7.0 of gestation.

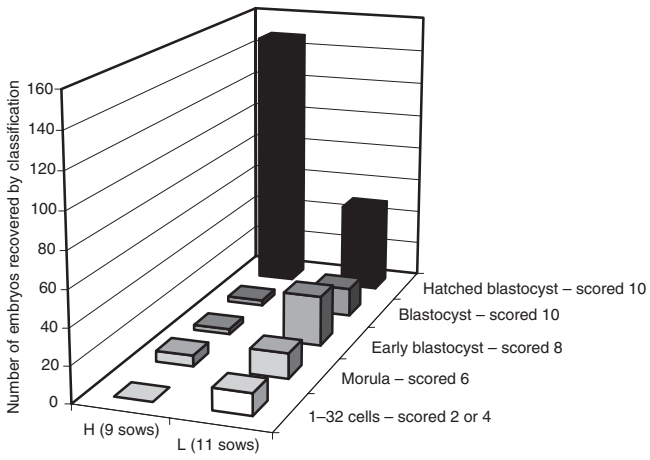

Fig. 3. Relationship between development of embryos recovered on Day 6 of gestation and score, regardless of treatment. The number of sows is shown in parentheses beside each score.

The methylation of embryonic DNA was not different between Control and Restrict males emabryos (6.5 ± 2.7% and 6.5 ± 3.3%, respectively) or female embryos (4.4 ± 2.8% and 5.6 ± 2.6%, respectively). In addition, there were no differences in the variance of methylation between Control and Restrict male embryos (2.8 ± 1.7% *v.* 3.3 ± 2.2%, respectively) or female embryos (1.8 ± 1.6% *v.* 1.9 ± 2.1%, respectively). Analysing the same data based on H/L scoring revealed no differences in methylation between H and L male embryos (7.6 ± 3.6% and 6.3 ± 3.9%, respectively) or female embryos (5.0 ± 3.4% and 5.0 ± 4.0%, respectively). In addition, there were no differences in the variance between H and L male embryos (3.1 ± 2.3% and 4.5 ± 3.0%, respectively) or female embryos (1.6 ± 2.2% and 5.2 ± 3.2%, respectively).

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

Discussion

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

As in the previous study, feed restriction during the last week of lactation and a corresponding decrease in net energy balance in Restrict sows compared with Controls were associated with decreased litter weight gain during the last week of lactation (Vinsky *et al*. 2006). Estimated sow protein and fat loss during lactation was similar to that in the previous experiment: the greater estimated fat loss in Control sows compared with that reported in the previous study likely reflected the increase of 0.5 pigs per litter suckling the sow, resulting in sows mobilising more fat to compensate for increased milk production in the present study. The increased catabolism of tissues in Restrict sows during the last week of lactation is likely the most critical factor determining reproductive performance following weaning (Clowes *et al*. 2003*b*) and exceeded the thresholds suggested for negative impacts on subsequent sow fertility (Clowes *et al*. 2003*b*; Vinsky *et al*. 2006).

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 oestrus may be confounded by increased feed intake in these sows after their period of restriction (Booth *et al*. 1994). However, the greater gain in estimated protein between oestrus and slaughter in Restrict sows at a time when feed intakes would have been comparable is characteristic of classic compensatory growth after periods of severe restriction. This observation, together with a change in measured back fat 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*. 2006), with no differences between treatments for ovulation rate or pregnancy rate (see Table 3). Although the Restrict sows had the same weaning to oestrus interval as in the previous experiment, Control sows in the present study had a shorter weaning to oestrus interval compared with pair-matched Restrict sows. However, the oestrus 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 oestrous period were consistent between treatment groups.

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

DNA collected from the leucocytes of Restrict sows at weaning on Day 21 of lactation and at oestrus was less methylated than the DNA from Control sows, indicating a limitation of nutrients essential for DNA methylation during lactational feed restriction. This lower methylation in Restrict sows carries over into oestrus, which is most likely due to longer-lived leucocytes, such as granulocytes, produced during the last week of lactation persisting to oestrus (Swenson 1993). 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 on Day 21 of lactation or during oestrus. Across treatments, the plasma levels of vitamin B_{12} are within the expected range for lactating sows and weaned sows (Guay *et al*. 2002). However, oestrus plasma concentrations of vitamin B_{12} 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*. 1996). Generally, folate levels were lower than expected (Guay *et al*. 2002). However, because the Control sows had excellent reproductive performance, the lower circulating plasma folate must not have exerted detrimental effects on reproductive performance.

Because folate and vitamin B_{12} levels were not affected by lactational feed restriction, the link to hypomethylation in sow leucocyte DNA remains to be established. Decreased availability of methionine, an essential amino acid for DNA methylation, may be involved, because 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 sow plasma in the present study failed for technical reasons and the limited sample volumes prevented re-analysis of plasma methionine. However, the fact that increased catabolism of protein as an energy source occurs in Restrict sows suggests that methionine deficiencies are likely.

Using the same experimental paradigm of feed restriction in lactating sows, Vinsky *et al*. (2007) 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 because only imprinted genes would be methylated at the blastocyst stage (Kang *et al*. 2001). However, by choosing a technique that measures global methylation, we were able to make comparisons between the present study and our previous results on global DNA methylation at Day 30 of gestation (Vinsky *et al*. 2007). Because adult sow DNA methylation is equivalent to levels seen at Day 30 of gestation (Vinsky *et al*. 2007) and the relative level of blastocyst DNA methylation was lower than sow leucocytes, it can be concluded that blastocysts have lower methylation than on 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 (Vanyushin *et al*. 1970; Kang *et al*. 2001; Vinsky *et al*. 2007).

There were no differences in embryo recovery rates or in the sex ratios of embryos within litters between the two treatment groups in the present study. The overall recovery rates of approximately 70% were close to the 73% embryo survival rate on Day 30 reported previously across treatment groups (Vinsky *et al*. 2006). Therefore, it can be assumed that the gender-specific loss of embryos in Restrict sows must occur after Days 5–7 of gestation. This is consistent with previous research into embryonic survival in the pig, which concluded that most of the embryonic loss is between Days 9 and 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*. 2007).

Pope *et al*. (1990) suggested that an underlying cause of embryonic loss was the developmental asynchrony of embryos in the preimplantation 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 and 110 h following ovulation (Soede *et al*. 1992). This suggests that other factors must contribute to embryonic diversity. As seen in Fig. 3, there was a range of embryonic development in both treatment groups on Day 6 of gestation, with a greater proportion of normal and hatched blastocysts being contributed from the H-scored sows. Furthermore, it appeared that sows with the greatest embryonic development also had the highest recovery rates, regardless of treatment (Fig. 2). It is unclear why this occurred, because the embryos were flushed from the entire length of the uterine horn, as recommended for embryos between Days 5 and 7 of gestation (Polge 1982). We therefore conclude 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*. (2006). This distribution of embryonic survival confirms the observation from many previous studies, namely that a proportion of both Control and Restrict 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 the present 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, characterised 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 further analysed to determine factors that may 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. Then, the H- and L-type litters were compared, irrespective of treatment, to try to characterise 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, as well as higher ovulation rates, compared with 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 over the period Days 0–13 of lactation (see Table 2), and yet had lower ovulation rates than H-type sows. Because an increase in ovulation rate tends to correlate with increased development to the blastocyst stage, this 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 subset of H-type sows characterised by the most synchronised and developed embryos at the hatched blastocyst stage and high ovulation rate, the experimental paradigm used in the present 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. The depiction of litter characteristics in Fig. 2 and correlation analysis of ovulation rate *v.* blastocyst recovery suggests that 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 10–15% of embryos present in Control L-type litters by Day 30, indicating that other factors must influence embryonic survival in Control sows following implantation. Therefore, the present study has effectively identified the key litter subgroups 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.

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References

Almeida, F. R., Mao, J., Novak, S., Cosgrove, J. R., and Foxcroft, G. R. (2001). Effects of different patterns of feed restriction and insulin treatment during the luteal phase on reproductive, metabolic, and endocrine parameters in cyclic gilts. *J. Anim. Sci.* **79**, 200–212.

Booth, P. J., Craigon, J., and Foxcroft, G. R. (1994). Nutritional manipulation of growth and metabolic and reproductive status in prepubertal gilts. *J. Anim. Sci.* **72**, 2415–2424.

Clark, A. B. (1978). Sex-ratio and local resource competition in a prosimian primate. *Science* **201**, 163–165. doi:10.1126/SCIENCE.201.4351.163

Clowes, E. J., Aherne, F. X., and Foxcroft, G. R. (1994). Effect of delayed breeding on the endocrinology and fecundity of sows. *J. Anim. Sci.* **72**, 283–291.

Clowes, E. J., Aherne, F. X., Foxcroft, G. R., and Baracos, V. E. (2003*a*). Selective protein loss in lactating sows is associated with reduced litter growth and ovarian function. *J. Anim. Sci.* **81**, 753–764.

Clowes, E. J., Aherne, F. X., Schaefer, A. L., Foxcroft, G. R., and Baracos, V. E. (2003*b*). Parturition body size and body protein loss during lactation influence performance during lactation and ovarian function at weaning in first-parity sows. *J. Anim. Sci.* **81**, 1517–1528.

Davis, C. D., and Uthus, E. O. (2004). DNA methylation, cancer susceptibility, and nutrient interactions. *Exp. Biol. Med.* **229**, 988–995.

Dziuk, P. J. (1987). Embryonic loss in the pig: an enigma. In 'Proceedings of the Inaugural Conference of the Australian Pig Science Association, Werribee, Victoria, Australia: Manipulating Pig Production I'. (Ed. J. L. Barnett.) pp. 28–39. (Frankland: Melbourne.)

Foxcroft, G. R. (1997). Mechanisms mediating nutritional effects on embryonic survival in pigs. *J. Reprod. Fertil. Suppl.* **52**, 47–61.

Geisert, R. D., and Schmitt, R. A. M. (2002). Early embryonic survival in the pig: can it be improved? *J. Anim. Sci.* **80**(Suppl. 1), E54–E65.

Gioia, L., Barboni, B., Turriani, M., Capacchietti, G., Pistilli, M. G., Berardinelli, P., and Mattioli, M. (2005). The capability of reprogramming the male chromatin after fertilization is dependent on the quality of oocyte maturation. *Reproduction* **130**, 29–39. doi:10.1530/REP.1.00550

Girard, C. L., Robert, S., Matte, J. J., Farmer, C., and Martineau, G. P. (1996). Serum concentrations of micronutrients, packed cell volume, and blood hemoglobin during the first two gestations and lactations of sows. *Can. J. Vet. Res.* **60**, 179–185.

Guay, F., Jacques Matte, J., Girard, C. L., Palin, M. F., Giguere, A., and Laforest, J. P. (2002). Effects of folic acid and vitamin B12 supplements on folate and homocysteine metabolism in pigs during early pregnancy. *Br. J. Nutr.* **88**, 253–263. doi:10.1079/BJN2002653

Kang, Y. K., Koo, D. B., Park, J. S., Choi, Y. H., Kim, H. N., Chang, W. K., Lee, K. K., and Han, Y. M. (2001). Typical demethylation events in cloned pig embryos. Clues on species-specific differences in epigenetic reprogramming of a cloned donor genome. *J. Biol. Chem.* **276**, 39 980–39 984. doi:10.1074/JBC.M106516200

Lucifero, D., Mertineit, C., Clarke, H. J., Bestor, T. H., and Trasler, J. M. (2002). Methylation dynamics of imprinted genes in mouse germ cells. *Genomics* **79**, 530–538. doi:10.1006/GENO.2002.6732

Mason, J. B. (2003). Biomarkers of nutrient exposure and status in one-carbon (methyl) metabolism. *J. Nutr.* **133**(Suppl. 3), 941S–947S.

NRC (1998). 'Nutritional Requirements of Swine.' (National Academy Press: Washington, DC.)

Park, I. Y., Sohn, B. H., Choo, J. H., Joe, C. O., Seong, J. K., Lee, Y. I., and Chung, J. H. (2005). Deregulation of DNA methyltransferases and loss of parental methylation at the insulin-like growth factor II (Igf2)/H19 loci in p53 knockout mice prior to tumor development. *J. Cell. Biochem.* **94**, 585–596. doi:10.1002/JCB.20263

Polge, C. (1982). Embryo transplantation and preservation. In 'Control of Pig Reproduction'. (Eds D. J. A. Cole and G. R. Foxcroft.) pp. 277–291. (Butterworths Scientific: London.)

Pope, W. F., Xie, S., Broermann, D. M., and Nephew, K. P. (1990). Causes and consequences of early embryonic diversity in pigs. *J. Reprod. Fertil. Suppl.* **40**, 251–260.

Sakata, S. F., Okumura, S., Matsuda, K., Horikawa, Y., Maeda, M., Kawasaki, K., Chou, J. Y., and Tamaki, N. (2005). Effect of fasting on methionine adenosyltransferase expression and the methionine cycle in the mouse liver. *J. Nutr. Sci. Vitaminol. (Tokyo)* **51**, 118–123.

Sambrook, J., and Russell, D. (2001). 'Molecular Cloning, A Laboratory Manual.' (Cold Spring Harbor Laboratory Press: New York.)

Silk, J. B. (1983). Local resource competition and facultative adjustment of sex-ratios in relation to competitive abilities. *Am. Nat.* **121**, 56–66. doi:10.1086/284039

Soede, N. M., Noordhuizen, J. P., and Kemp, B. (1992). The duration of ovulation in pigs, studied by transrectal ultrasonography, is not related to early embryonic diversity. *Theriogenology* **38**, 653–666. doi:10.1016/0093-691X(92)90028-P

Swenson, M. J. (1993). Physical properties and cellular and chemical constituents of blood. In 'Dukes Physiology of Domestic Animals'. (Eds M. J. Swenson and W. O. Reece.) pp. 22–48. (Cornell University Press: Ithaca, NY.)

Vanyushin, B. F., Tkacheva, S. G., and Belozersky, A. N. (1970). Rare bases in animal DNA. *Nature* **225**, 948–949. doi:10.1038/225948A0

Vinsky, M. D., Novak, S., Dixon, W. T., Dyck, M. K., and Foxcroft, G. R. (2006). Nutritional restriction in lactating primiparous sows selectively affects female embryo survival and overall litter development. *Reprod. Fertil. Dev.* **18**, 347–355. doi:10.1071/RD05142

Vinsky, M. D., Murdoch, G. K., Dixon, W. T., Dyck, M. K., and Foxcroft, G. R. (2007). Altered epigenetic variance in surviving litters from nutritionally restricted lactating primiparous sows. *Reprod. Fertil. Dev.* **19**, 430–435. doi:10.1071/RD06082

Whittemore, C. T., and Yang, H. (1989). Physical and chemical composition of the body of breeding sows with differing body subcutaneous fat depth at parturition, differing nutrition during lactation and differing litter size. *Anim. Prod.* **48**, 203–212.

Yi, P., Melnyk, S., Pogribna, M., Pogribny, I. P., Hine, R. J., and James, S. J. (2000). Increase in plasma homocysteine associated with parallel increases in plasma *S*-adenosylhomocysteine and lymphocyte DNA hypomethylation. *J. Biol. Chem.* **275**, 29 318–29 323. doi:10.1074/JBC.M002725200

Yoshida, M., Ishigaki, K., Nagai, T., Chikyu, M., and Pursel, V. G. (1993). Glutathione concentration during maturation and after fertilization in pig oocytes: relevance to the ability of oocytes to form male pronucleus. *Biol. Reprod.* **49**, 89–94. doi:10.1095/BIOLREPROD49.1.89

Zak, L. J., Cosgrove, J. R., Aherne, F. X., and Foxcroft, G. R. (1997). Pattern of feed intake and associated metabolic and endocrine changes differentially affect postweaning fertility in primiparous lactating sows. *J. Anim. Sci.* **75**, 208–216.

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