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Nutritional restriction in lactating primiparous sows selectively affects female embryo survival and overall litter development

M. D. Vinsky[A], *S. Novak*[A], *W. T. Dixon*[A], *M. K. Dyck*[A] *and G. R. Foxcroft*[A,B]

[A]Department of Agricultural, Food and Nutritional Science, Swine Research and Technology Centre, University of Alberta, Edmonton, AB T6G 2P5, Canada.
[B]Corresponding author. Email: george.foxcroft@ualberta.ca

Abstract. This study explored the possibility of sex-specific effects on embryonic survival in primiparous sows subjected to restricted feed intake during the last week of lactation and bred after weaning (Restrict; $n = 16$), compared with control sows fed close to *ad libitum* feed intakes (Control; $n = 17$). Restrict sows were in a substantial negative net energy balance at weaning, and lost 13% of estimated protein and 17% of fat mass during lactation, yet the weaning-to-oestrous interval and ovulation rate were not different between treatments. However, embryonic survival at Day 30 of gestation was lower ($P < 0.05$) in Restrict than Control sows, and selectively reduced the proportion of female embryos surviving ($P < 0.01$). A decrease in weight and crown–rump length of surviving female ($P < 0.05$) and male ($P < 0.05$) embryos was seen in Restrict litters. The mechanisms mediating this sex-specific effect on embryonic loss in feed-restricted sows are unclear. The data presented here indicate that feed-restriction during the last week of lactation in primiparous sows causes a selective decrease in survival of female embryos and limits the growth of all surviving embryos.

Extra keywords: catabolism, imprinting, pig, sex ratio.

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 because 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*. 1997*a*) and oocyte quality (Zak *et al*. 1997*b*).

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*. 1997*b*). Furthermore, follicular fluid from these follicles is 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 ability of follicular fluid aspirated from these follicles to support *in vitro* oocyte maturation (Yang *et al*. 2000*a*; Clowes *et al*. 2003*a*).

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 sex-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 sex under normal conditions. Consistent with the evidence that *in utero* competition among littermates favours survival of the most advanced embryos (Pope 1994), Krackow (1995) suggested that asynchrony between uterine and embryonic (blastocyst) development before implantation may alter sex ratios in favour of male embryos. This

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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 would affect subsequent embryonic survival and the development of surviving embryos in a sex-specific manner.

Materials and methods

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 and Technology Centre (SRTC), University of Alberta. Within 48 h after farrowing, litter size was standardized to a minimum of nine piglets per sow by cross-fostering. On Day 14 of lactation, sows pre-selected for the trial were then paired on the basis of similar initial bodyweight 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 hours) with a standard lactation diet (14 MJ digestible energy kg^{-1}, 20% crude protein, 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 using 312 primiparous sows from the SRTC, in which it was found 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 bodyweight on subsequent productivity, heavier sows (greater than 220 kg at farrowing) were not included in the present experiment. Furthermore, in compliance with guidelines established under the Faculty Animal Policy and Welfare Committee approval for the present study, pre-selected sows losing more than 20 kg of bodyweight 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}, whereas 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, USA) and sow bodyweights 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 with *ad libitum* access to the same lactation diet.

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 sow's energy intake. Maintenance requirements were calculated using the equation: 106 Kcal metabolisable energy per kg of bodyweight$^{0.75}$ (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*. 2003*a*). Energy requirements for milk production were then calculated using the formula of Clowes *et al*. (2003*a*).

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. Sows were artificially inseminated with pooled semen (3×10^9 morphologically normal and motile spermatozoa per 50 mL dose) that was no more than 3 days old, and was 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.

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, chorioallantoic fluid volumes were measured, as a more practical and predictable estimate of placental development (S. Town, 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.

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 ~100 mg of tissue from individual embryos were transferred to pre-chilled, sterile 15-mL 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, Canada).

Sex-typing polymerase chain reaction

Embryos were sex-typed using a modified version of the polymerase chain reaction (PCR) protocol developed by Pomp *et al*. (1995). Primers specific to the SRY region of the Y-chromosome,

5′-TGAACGCTTTCATTGTGTGGTC-3′ (forward) and 5′-GCCAGT AGTCTCTGTGCCTCCT-3′ (reverse), were used. As a positive internal control, primers for the sex chromosomal *Zfy/Zfx* homologues were multiplexed in each reaction with 5′-ATAATCACATGGAGAGCCACA AGCT-3′ (forward) and 5′-GCACTTCTTTGGTATCTGAGAAAGT-3′ (reverse). With each PCR tube kept on ice, a master mix of 1.5 μL 10× buffer (ABI, Foster City, CA, USA), 0.9 μL of 25 mM $MgCl_2$ (ABI), 0.3 μL of 10 mM dNTP mix (Roche, Laval, Quebec, Canada), 1.5 μL 10× primer mix (1 μM SRY, 2 μM ZFY/ZFX), and 9.68 μL sterile MilliQ water, was added. After adding 1 μL of extracted genomic DNA, tubes were placed in a 96-well iCycler thermocycler (BioRad, Mississauga, ON, Canada) and the following PCR program was used: 94°C for 4 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and then 72°C for 1 min. Following this, a final extension phase of 72°C for 5 min and an indefinite hold at 4°C was used. Samples not used immediately for gel electrophoresis were stored at 4°C overnight or at −20°C until analysis. The PCR products, along with a 100-bp ladder (Invitrogen, Burlington, ON, USA) were separated on a 2% (w/v) agarose gel with a 1× Tris-buffered EDTA buffer run for 1 h at 90 V. The gel was stained with 0.2 mg mL^{-1} ethidium bromide (BioRad) and PCR amplicons were visualised and images were acquired using a BioRad Gel Doc System.

Statistical analysis

A total of 17 pairs of sows met all the criteria for inclusion in the present 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 was 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. The ESR was expressed as the percentage of ovulations proportional to the number of live embryos at Day 30 of gestation, and the sex ratio was calculated as the percentage of males within the litter. These percentage data were normalised using arcsin transformation before analysis.

All measurements of sow and litter characteristics were analysed using the same MIXED procedure of SAS described earlier. For the analyses of weight and backfat changes between Day 0 and 13 and Day 0 and 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 and 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-analysed by sex, to distinguish sex-specific treatment effects on embryonic development. Embryo weight, placental weight or allantochorionic volume, and crown–rump length (CRL) were analysed using the MIXED procedure of SAS, as a randomised block design, with blocks again based on sow pair. Observations were tested for a treatment effect, sex-specific effects, and any significant interactions between them. Because sow was the experimental unit, all individual measurements were averaged according to sex, within a litter, before statistical analysis.

Results

Feed intake, energy requirements and net energy balance

There was no difference in the number of piglets nursing during lactation between Control and Restrict sows (Table 1). There were no differences in mean litter birthweights 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 and Control sows with respect to estimated milk production from Day 0 to 13 (7.11 ± 0.27 kg, 7.47 ± 0.27 kg, respectively), but milk production during feed restriction from Day 14 to 21 (10.07 ± 0.32 kg, 11.96 ± 0.32 kg; $P < 0.001$), and overall milk production (8.59 ± 0.22 kg, 9.71 ± 0.22 kg; $P < 0.001$) was lower in the Restrict sows compared to Control sows.

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

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

*$P < 0.005$, **$P < 0.001$ compared with Control sows.

A comparison of 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 2).

Sow characteristics

There were no differences between the two treatment groups on Day 0 with respect to sow bodyweight or backfat (Table 2). Restrict sows were no different from Control sows with respect to weight loss (-5.89 ± 1.28 kg, -5.18 ± 1.28 kg, respectively), backfat loss (-0.35 ± 0.43 mm, -0.34 ± 0.43 mm), calculated body protein loss, or body fat loss (Table 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 and 21 in Restrict compared with 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 2). Overall, between farrowing and Day 21, bodyweight ($P < 0.001$), backfat ($P < 0.005$), and estimated body protein ($P < 0.001$) and body fat ($P < 0.001$) losses were greater in the Restrict compared with the Control sows (Table 2).

Table 2. Least square means ± s.e.m. for sow and litter characteristics during lactation used to estimate net energy balance, and changes in fat and lean tissue mass
ME, metabolisable energy; NE, net energy

Item	Control ($n = 17$)	Restrict ($n = 17$)
Characteristics for estimation of sow NE balance		
Estimated ME intake		
Day 0–13 (Mcal day^{-1})	15.02 ± 0.19	14.66 ± 0.19
Day 14–21 (Mcal day^{-1})**	15.20 ± 0.00	7.60 ± 0.00
Estimated energy requirements for milk production		
Day 0–13 (Mcal day^{-1})	11.97 ± 0.47	11.32 ± 0.47
Day 14–21 (Mcal day^{-1})**	19.86 ± 0.55	16.54 ± 0.55
Estimated sow net energy balance		
Day 0–13 (Mcal day^{-1})	−3.38 ± 0.42	−3.39 ± 0.42
Day 14–21 (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 0–13 of lactation		
Protein loss as % of parturition mass	2.72 ± 0.69	3.08 ± 0.69
Fat loss as % of parturition mass	3.16 ± 1.41	3.35 ± 1.41
Day 14–21 of lactation		
Protein loss as % of parturition mass**	2.61 ± 0.73	9.52 ± 0.73
Fat loss as % of parturition mass**	4.62 ± 1.28	13.94 ± 1.28

**$P < 0.001$ compared with Control sows.

Table 3. Least square means ± s.e.m. for sow reproductive performance and embryo survival data
Analysis of embryonic survival rate performed on arcsin-transformed 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 with Control sows.

Sow fertility, embryonic survival and development

The weaning-to-oestrous interval, pregnancy rate, day of gestation at necropsy, and ovulation rate were not different between treatments (Table 3). Correlation analysis established no relationships between ESR and net energy (NE) balance, protein loss or fat loss (Fig. 1). 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, the ESR ($P < 0.05$) and the number of live embryos ($P < 0.05$) were lower in Restrict than in Control sows. Based on clear distinctions between the PCR amplicons of male and female embryos (Fig. 2), the number of female embryos ($P < 0.01$) was also lower in Restrict sows compared with Controls, whereas no comparable difference was apparent in the number of male embryos. Overall, the sex ratios of embryos were 61% males for Restrict sows and 54% males for Controls. Allantochorionic volume and placental weight were not affected by treatment, sex, or by a treatment by sex interaction (Table 4). However, embryonic weight ($P < 0.005$) and CRL ($P < 0.05$) were lower in embryos recovered from Restrict sows. There was no sex-specific effect, or a treatment by sex interaction, for these measures of embryonic development.

Discussion

Most primiparous sows undergo a certain level of catabolism during lactation and, in the absence of imposed feed restriction, a decrease in bodyweight, 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 on subsequent reproductive performance. The present study used an established experimental model (Zak *et al*. 1997*a*), 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

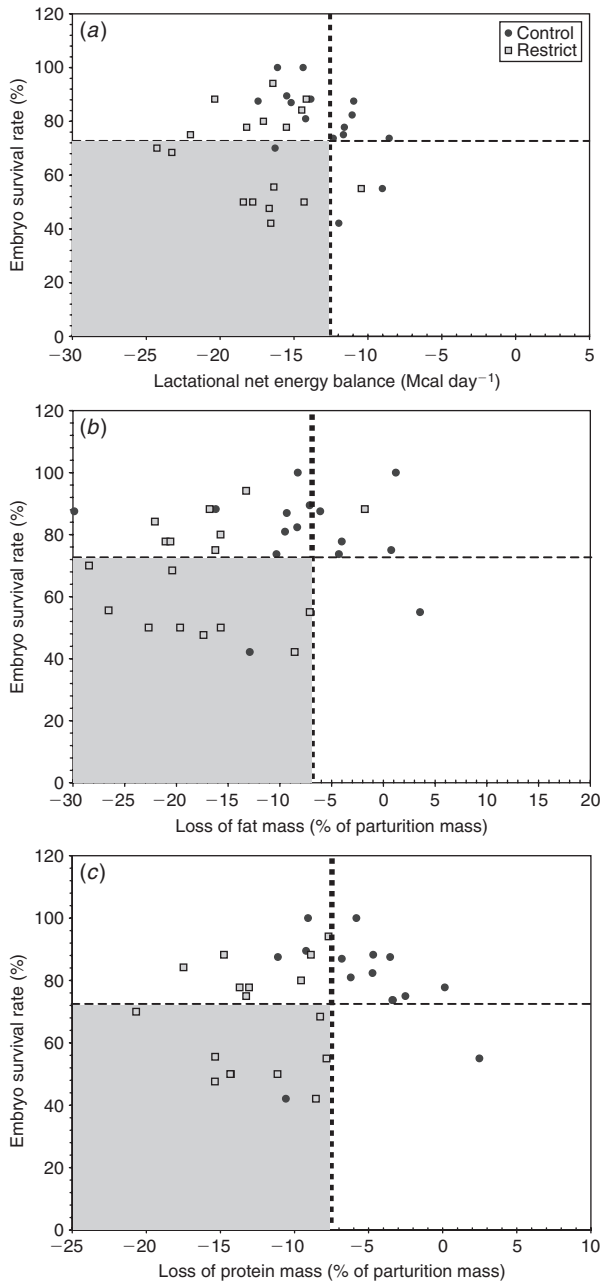

Fig. 1. (*a*) Relationship between Day 0 to 21 net energy balance and embryo survival rate (ESR). (*b*) Relationship between loss of fat mass from Day 0 to 21 as a percentage of parturition mass and ESR. (*c*) Relationship between loss of protein mass from Day 0 to 21 as a percentage of parturition mass 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 shaded quadrant illustrates the population of Restrict sows, which create the overall treatment effect.

relatively severe nutritional restriction in late lactation also produced detrimental effects on oocyte quality, measured using *in vitro* oocyte maturation assays (Zak *et al*. 1997*b*). This suggests that oocyte development is likely a contributing factor to poor embryonic survival in catabolic lactating

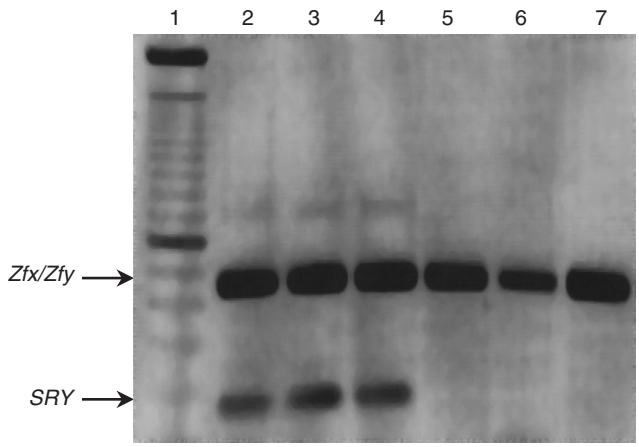

Fig. 2. Sex-typing polymerase chain reaction (PCR) amplicon run on a 2% (w/v) agarose gel at 100 V for 45 min. The amplified fragment of the *SRY* gene appears as a band at 163 bp, identifying the presence of the Y chromosome, and a band at 455 bp identifies the *Zfy/Zfx* genes, which was the positive PCR control. Lane 1 has a 100 bp ladder, lanes 2–4 identify male embryos, and lanes 5–7 identify female embryos.

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 oviducal and uterine environment in which fertilisation 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 oviducal 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 bodyweight, backfat, and estimated loss of body protein and fat mass were evident at the time of weaning in Restrict sows compared with Controls. The greater mobilisation of body protein and fat mass in the last week of lactation in the Restrict group was likely an attempt to meet the demands of milk production in the absence of adequate nutrient intake. However, as in previous studies using restriction of either total feed (Zak *et al*. 1997*a*), dietary protein (Clowes *et al*. 2003*a*, 2003*b*), or dietary lysine (Yang *et al*. 2000*a*), 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 mobilise 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

Table 4. Least-square means (± s.e.m.) for sex-specific embryo characteristics collected at Day 30 of gestation
Analysis of data was performed testing for differences in treatments, sex, and a treatment by sex interaction

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 ($n = 36$)

*$P < 0.05$, **$P < 0.005$ difference only between treatments.

may be protective against a lack of protein intake in lactating primiparous sows (Clowes *et al*. 2003*b*) 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 on 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 by Day 21 of lactation, starting from a parturition bodyweight of 189 kg. Consistent with the results of Clowes *et al*. (2003*a*), this resulted in no differences between treatment groups in weaning-to-oestrous interval or ovulation rate, yet impacted on embryonic survival in the Restrict sows. Correlation analysis established that there was no relationship between ESR and NE balance and 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 (-12.5 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*. (1997*b*), Yang *et al*. (2000*b*), and Clowes *et al*. (2003*a*) 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 presents 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 lactating sows.

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, because it postulates that healthy females with abundant resources would produce more females, because 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 to the uterus are limited. 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 sex, 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 detectible 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 that 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 via another mechanism. The lack of growth and development of embryos without effects on placental development suggests 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, the 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 the present 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 using mice has shown that the final stages of folliculogenesis, before the oocyte reaches metaphase II, are critical 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 the present 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 with *in vivo* oocytes (Gioia *et al*. 2005). This suggests that if the male pronucleus is not properly reprogrammed following fertilisation, 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 preimplantation embryogenesis are contributed from the oocyte (Ratnam *et al*. 2002); therefore, defects in an oocyte's ability to maintain imprints during the preimplantation 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 fetal development.

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 (Panning and Jaenisch 1996; Xue *et al*. 2002). Either a failure to establish methylation imprints in the oocyte, resulting in hypomethylation, or defects in the 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 chromatin 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 via the increased expression of the X chromosome specific transcript TSIX and CTCF protein, resulting in failure of dosage compensation, which is lethal to 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 ongoing studies.

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

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