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Uterine crowding in the sow affects litter sex ratio, placental development and embryonic myogenin expression in early gestation

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Abstract. Uterine crowding in the pig results in intrauterine growth restriction (IUGR), and permanently affects fetal muscle fibre development, representing production losses for the commercial pig herd. The present study sought to understand how different levels of uterine crowding in sows affects muscle fibre development in the early embryo at the time of muscle fibre differentiation and proliferation. Sows either underwent surgical, unilateral oviduct ligation (LIG; $n = 10$) to reduce the number of embryos in the uterus, or remained as intact, relatively-crowded controls (CTR; $n = 10$). Embryos and placentae were collected at Day 30 of gestation, and myogenic regulatory factor (MRF) transcript abundance was determined using real-time PCR for both myogenin (*MYOG*) and myoblast differentiation 1 (*MYOD1*). Unilateral tubal ligation resulted in lower numbers of embryos *in utero*, higher placental weights and a higher male : female sex ratio ($P < 0.05$). Relative *MYOD1* expression was not different, but *MYOG* expression was higher ($P < 0.05$) in the LIG group embryos; predominantly due to effects on the male embryos. Relatively modest uterine crowding therefore affects MRF expression, even at very early stages of embryonic development, and could contribute to reported differences in fetal muscle fibre development, birthweight and thus post-natal growth performance in swine.

Introduction

In the pig, as in other mammals, the total number of muscle fibres formed determines total muscle mass, and myogenesis during embryonic development follows a biphasic pattern (Wigmore and Stickland 1983). A population of primary fibres is used for the subsequent attachment and fusion of myoblasts to form secondary fibres, and muscle fibre hyperplasia is complete by approximately Day 90 of gestation (Wigmore and Stickland 1983). Reduced muscle fibre development *in utero* can affect commercial pig production in many ways. First, the total muscle secondary fibre number in piglets can impact their future growth rate (van der Lende and de Jager 1991), as runted piglets need 23 more days to reach market weight compared with non-runts (Hegarty and Allen 1978). Furthermore, as litter uniformity becomes an important contributing factor to the productivity of all-in all-out grower to finish systems, the concept of producing normally sized and uniform litters has economic advantages to commercial pork production in terms of carcass and meat quality and post-natal performance (see review of Foxcroft *et al.* 2007).

Even though muscle fibre hyperplasia is complete by late gestation, the determination of the total number of muscle fibres occurs much earlier and is sensitive to nutritional status and the uterine environment. Doubling the amount of a standard commercial diet fed to sows during gestation resulted in a higher number of secondary fibres in their offspring (Dwyer *et al.* 1994); however, this effect was only observed when nutrition was manipulated between Day 25 and 50 of gestation, a time period that precedes the onset of secondary muscle fibre formation (Wigmore and Stickland 1983). The observation that administration of porcine somatotropin (pST) to sows from Day 10 to 24, but not after Day 50 of gestation, resulted in significantly higher numbers of muscle fibres in the semitendinosus muscle of the fetus (Rehfeldt *et al.* 1993) is also consistent with this concept. Maternal under-nutrition in early gestation can negatively impact the number of total muscle fibres in guinea-pigs (Dwyer *et al.* 1995) and in sheep (Quigley *et al.* 2005). Lower secondary to primary muscle fibre ratios in developing sheep fetuses have been observed in response to peri-conceptional maternal underfeeding (Quigley *et al.* 2005) or with asynchronous embryo transfer (Maxfield *et al.* 1998). Similarly, fewer but enlarged fast twitch fibres were also observed in sheep that were underfed through early gestation (Fahey *et al.* 2005).

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Recent studies in commercial dam-line sows suggest that inadvertent intrauterine crowding during gestation resulted in classic measures of intrauterine growth restriction (IUGR), including a negative impact on the number of muscle fibres (Town *et al.* 2005). In addition, irrespective of crowding, the examination of muscle fibre development in pig fetuses demonstrated a lower secondary to primary muscle fibre ratio in those that were small *v.* average for gestational age at 100 days of gestation (Tilley *et al.* 2007).

Town *et al*. (2004) used unilateral oviduct ligation to study the effects of the number of embryos *in utero* on prenatal development. They reported lower placental and fetal weights, and fewer secondary muscle fibres, in Day 90 fetuses recovered from control sows, as compared with fetuses from the relatively less-crowded uteri of the oviduct-ligated sows. In the same study, an analysis at Day 30 of gestation also revealed that conceptuses of oviduct-ligated sows had higher average placental weights than control sows, in the absence of any difference in embryonic weight. These results again suggest that the effects of uterine crowding in determining secondary muscle fibre numbers at Day 90 of gestation may be due to effects on myogenic development during early gestation, mediated by limited placental development.

The myogenic regulatory factors (MRFs) myogenic factor 5 (MYF5), myoblast differentiation factor 1 (MYOD1), myogenin (MYOG), and myogenic factor 6 (MYF6; also known as MRF4), are critical transcription factors involved in the development of muscle (for review see Arnold and Braun 2000). Although initially identified by their common ability to convert non-muscle cells into muscle cells *in vitro*, knockout studies in mice have identified distinct roles for each MRF in myogenesis. MYF5 and MYOD1 act to direct pluripotent cells into myoblast precursor cells, whilst myogenin initiates terminal differentiation of myoblasts into muscle cells. Although MYF6 was also initially thought to be a factor involved in terminal differentiation, recent evidence suggests that it acts to regulate *MYOD1* expression (Kassar-Duchossoy *et al*. 2004).

The purpose of the present study was to investigate whether the effects of uterine crowding on the determination of secondary muscle fibre numbers reported by Town *et al*. (2004) was mediated by changes in expression of MRFs at Day 30 of gestation, when placental weight was already lower in control sows than in the less-crowded uteri of oviduct-ligated females. Since Day 25 to 50 of gestation is a critical period for the determination of secondary fibre numbers, we hypothesised that a decrease in *MYOD1* and *MYOG* expression would be observed at Day 30, which might be the basis for the reduced secondary fibre numbers observed in Day 90 fetuses. Furthermore, since gender appears to influence both the individual development of an embryo and the development of the litter as a whole, the gender-specific expression of *MYOD1* and *MYOG* was assessed.

Materials and methods

Animals

Day 30 embryos from the study of Town *et al*. (2004) were used in this study. The experiment was conducted in accordance with the Canadian Council on Animal Care guidelines and with the approval of the Faculty Animal Policy and Welfare Committee (Protocol 200134D). Briefly, the present study involved 30 parity three lactating sows weaned at Day 23 of lactation. Sows were randomly assigned to undergo unilateral oviduct ligation (LIG; $n = 15$) approximately three days after their first post-weaning oestrus, or to be intact controls (CTR; $n = 15$). A subset of 10 LIG and 10 CTR sows were taken for the present study and embryos analysed for MRF expression and sex-typing were obtained from sows slaughtered at Day 30 of gestation. Individual ovulation rate, available ovulation rate for LIG sows, total embryo number and viable embryo number were recorded. Viable embryos were assessed as embryos that appeared to be healthy rather than necrotic, and whose crown–rump length was considered normal (Vinsky *et al.* 2006). Embryo survival rate was estimated based on viable embryo number *in utero* as a proportion of the available ovulation rate.

Collection and paraformaldehyde fixation of embryos

Embryos were collected within one hour of slaughter and treated with paraformaldehyde in order to preserve the cellular morphology for possible subsequent analysis using *in situ* hybridisation. Whole Day 30 embryos were pierced at the base of the presumptive skull with a 27-gauge needle to allow easier perfusion of paraformaldehyde. Embryos were then immersed in 4% (w/v) buffered paraformaldehyde (85 mM PIPES, 25 mM HEPES, 5 mM EGTA, pH 7.0), incubated at 4°C overnight, dehydrated with increasing concentrations of methanol and then stored at −30°C until further analysis. In total, 251 viable embryos from all 20 sows were used for analysis of *MYOD1* and *MYOG* expression, comprising a total of 93 embryos from the LIG group, and 158 embryos from the CTR group, respectively. Paraformaldehyde fixation of embryos was originally intended to allow subsequent *in situ* hybridisation studies, however, having failed to establish acceptable semiquantitative analysis of MRF expression using *in situ* hybridisation techniques, RNA was extracted from the paraformaldehyde-fixed tissues for RT–PCR analysis.

Quantitative real-time PCR (RT–PCR)

RNA extraction

Extraction of paraformaldehyde-fixed tissues was performed using a modified proteinase K protocol described by Masuda *et al*. (1999). This protocol was found to be effective for RNA extraction of fixed tissue destined for real-time PCR analysis (Koch *et al.* 2006). 1.0 mL homogenisation buffer (200 mM TRIS-HCl, 200 mM NaCl, 1.5 mM $MgCl_2$, 2% (w/v) SDS, pH 7.5) was added for every 100 mg of whole embryo tissue, and homogenised. 500 μg proteinase K (Sigma, Oakville, ON, Canada) was added to 1.0-mL aliquots of the resulting tissue lysate and then incubated at 45°C for 1 h. 1.0 mL of buffer-saturated phenol (pH 4.3)/chloroform/isoamyl alcohol (25 : 24 : 1), followed by 1.0 mL chloroform, was then used for total RNA extraction. RNA was precipitated using 2.0 mL isopropanol, and the RNA pellet was then dissolved in DEPC-treated RNase-free water (Qbiogene, Irvine, CA, USA) and diluted to a concentration of $\sim 1\ \mu g\ \mu L^{-1}$. Total RNA was electrophoretically separated on a 1% (w/v) agarose gel and stained with ethidium bromide to verify RNA integrity (results not

Table 1. Oligonucleotides and TaqMan MGB probes

Gene	Primers	Sequence 5′ → 3′	Expected RNA amplification size	Expected DNA amplification size
Cyclophilin	Sense	AAT GCT GGC CCC AAC ACA		
	Antisense	TCA GTC TTG GCA GTG CAA ATG	55 bp	450 bp
	Probe	CAC AAA CGG TTC CCA GTT TTT		
MYOD1	Sense	ACT CAG ACG CAT CCA GCC C		
	Antisense	GTA ATA GGT GCC GTC GTA GCA GT	107 bp	598 bp
	Probe	CGG CAT GAT GGA TTA T		
MYOG	Sense	GGC CCC AAC CCA GGG		
	Antisense	GGA GTG CAG ATT GTG GGC A	63 bp	682 bp
	Probe	ATC ATC TGC TCA CAG CTG		

shown). All RNA samples showed the strong presence of both intact 18S and 28S rRNA, indicating that RNA extraction from paraformaldehyde-fixed tissues was successful. DNAse I treatment using DNA-free kit (Ambion Inc., Austin, TX, USA) was performed after RNA extraction to ensure removal of residual genomic DNA.

Quantitative RT–PCR

Quantitative real-time two-step RT–PCR was used to measure expression levels of the MRF genes *MYOD1* and *MYOG* in all 251 embryos. Approximately 1 μg of total RNA was reverse-transcribed to cDNA for real-time analysis using MMLV RT (Invitrogen, Burlington, ON, Canada) and an oligo-dT^{15} primer. Prior to reverse transcription, the RNA was heat-denatured at 70°C for 5 min to remove secondary structures and also for removal of methylol groups formed during the fixation technique (Masuda *et al.* 1999). Real-time PCR reactions for each gene were run in duplicate in 96-well optical plates in an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Primers and TaqMan MGB probe sets for porcine cyclophilin A (*PPIA*), *MYOD1*, and *MYOG* were designed using Primer Express software (Applied Biosystems) and amplicons spanned an exon–exon boundary to further exclude real-time amplification of genomic DNA (Table 1). Primer specificity was determined by agarose gel electrophoresis. RT–PCR products produced under real-time amplification conditions were run on a 2% (w/v) agarose gel and stained with ethidium bromide (data not shown). Gels were placed over a UV lamp and examined for the presence of amplification products from target mRNAs, as well as the possible presence of amplification products from DNA contamination.

Analysis of RT–PCR data

Relative transcript abundance was determined by the comparative C_T method (Livak and Schmittgen 2001), however, data were expressed and analysed as ΔC_T (Yuan *et al.* 2006). The ΔC_T for each sample was the C_T value for the gene of interest minus the C_T value for cyclophilin in that sample. The threshold was set above the measured baseline and in the logarithmic phase of amplification. The threshold cycle value (C_T), defined as the cycle number at which detected fluorescence passes the fixed threshold value, was determined for each gene. Amplification

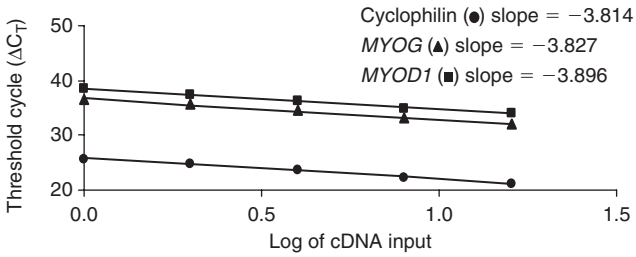

Fig. 1. Real-time PCR efficiency determination of the reference gene cyclophilin, the target gene *MYOD1*, and the target gene *MYOG*. C_T at threshold cycle is plotted *v.* the log of relative input amounts of cDNA.

efficiencies for each gene were determined using a serial dilution in order to establish the suitability of the comparative C_T method for gene analysis (Fig. 1). The slopes for the serial dilutions are linear and thus show that the method using fixed tissue as a source for real-time analysis is appropriate and yields quantitative results. Furthermore, the slopes for *MYOD1*, *MYOG* and cyclophilin are similar, suggesting that each gene tested was equally amplified by the procedure. Amplification efficiencies were measured to be between 85.6 to 87.4% for all genes, and the deviation from an ideal 100% efficiency was likely due to the use of fixed rather than fresh tissue. No correction was made for efficiency and thus the transformations of ΔC_T values to fold differences using the $2^{-\Delta\Delta CT}$ were not made.

Sex-typing PCR

DNA extraction

1.0-mL aliquots of tissue lysate were supplemented with 500 μg proteinase K (Sigma) and incubated at 45°C for 5 days. DNA extraction was performed using phenol (pH 7.9)/chloroform/isoamyl alcohol (25 : 24 : 1), followed by further extraction with 1.0 mL chloroform. DNA was precipitated using 2 mL of 100% (v/v) ethanol and 100 μL of 6 M ammonium acetate and incubated overnight at −20°C. This was followed by a wash of 75% (v/v) ethanol. DNA pellets were then dissolved in DEPC-treated H_2O to a final concentration of ~1 μg μL^{-1}. Gel electrophoresis of extracted DNA samples was performed on a 1% (w/v) agarose gel to verify the integrity of the DNA sample.

Table 2. Embryo and placental characteristics, and embryo *MYOD1* and *MYOG* expression levels in control (CTR) and unilateral oviduct-ligated sows (LIG)

Both *MYOD1* and *MYOG* expression are expressed as ΔC_T (normalised to cyclophilin), which represents the number of cycles required to amplify the transcript to a threshold level, therefore a higher ΔC_T reflects the lower gene expression in that sample

Parameter	CTR ($n = 10$) 'relatively-crowded'	LIG ($n = 10$) 'non-crowded'	Pooled s.e.m.	*P*-value
Total ovulation rate	20.1	21.2	0.93	0.415
Available ovulation rate	20.1	10.4	0.96	<0.0001
Embryo survival (%)	83.4	91.4	3.73	0.145
Number of live embryos	16.5	9.6	0.90	<0.0001
Embryo weight (g)	1.145	1.248	0.07	0.285
Placental weight (g)	19.60	26.08	1.49	0.007
Male : female ratio	51.99	61.83	2.81	0.023
MYOD1 (ΔC_T)	9.10	8.92	0.67	0.565
MYOG (ΔC_T)	5.59	4.85	0.27	0.015

The sex of the 251 Day 30 embryos was determined using a previously-developed PCR protocol (Pomp *et al.* 1995). One primer set was designed to amplify a 157-base pair segment of the Y chromosome-specific region (SRY) and another primer set was designed to amplify a 445-base pair segment of the X chromosome-specific region (ZFX). The primer sets are described in detail in Vinsky *et al.* (2006). DNA samples were co-amplified with both primer sets, and PCR products were resolved using a 2% (w/v) agarose gel. PCR of samples resulting in the amplification of both the 157-base pair fragment and the 445-base pair fragment were identified as males, while those showing only the 445-base pair fragments were identified as females. The PCR protocol was repeated with samples of DNA isolated from adult animals whose sex was known, in order to verify the results of amplification of the samples (Vinsky *et al.* 2006).

Statistical analysis

All data were verified for normal distribution and then analysed using the mixed-classification model of SAS (SAS Inst., Cary, NC, USA). Average relative gene expression (ΔC_T) of *MYOD1* and *MYOG*, and average placental weight within the embryos in each litter, litter sex ratio, ovulation rate and embryo survival, as well as the number of viable embryos at Day 30 of gestation were the measured variables, with sow considered to be the experimental unit. Treatment was used as a fixed factor, and the sow was included as a random factor. Significance was considered as $P < 0.05$ and the results are presented as least-squares means ± s.e.m.

Correlations were performed across all sows to determine whether possible relationships existed between expression level and sex ratio, embryo weight, placental weight, or viable embryo number.

Relative ΔC_T values of *MYOD1* and *MYOG*, and embryonic and placental weights, were also examined with respect to the sex of the embryos analysed. Mixed-models analysis was used with treatment, sex, and treatment by sex interaction, as fixed factors in the model, and with sow as a random factor. Embryos were also grouped by gender, and analysis was also performed to determine the effect of the treatment within each gender, using treatment as a fixed factor, and the sow as a random factor.

Results

Embryonic and placental characteristics of CTR and LIG sows

For the 20 sows chosen at random, there were large differences between CTR and LIG sows with respect to viable embryo number and placental weight, but not embryo weights (Table 2). In addition, treatment affected the sex ratio, with a higher male : female ratio observed in the LIG sows than in the relatively-crowded CTR sows (Table 2). Although there were differences in the original 30 sows for embryo survival as reported by Town *et al.* (2004), there were no differences ($P = 0.145$) in the 20 sows chosen for the present study (Table 2). There was no effect of embryo gender on embryo weight or placental weight, and there were no interactions ($P = 0.41$) between sex and treatment (Table 3). Placental weight was greater in both male and female embryos in the LIG compared with CTR sows.

Irrespective of treatment or sex of the embryos, there was a negative relationship ($r = -0.630$; $P = 0.003$) between the number of viable embryos and placental weight across all sows.

Sex-typing PCR

PCR analysis of DNA extracted from individual Day 30 embryo samples resulted in easily-identifiable bands representing the SRY and ZFX genes (data not shown), allowing the identification of the sex of the embryo. Banding patterns of embryos were compared with the banding patterns produced by PCR of porcine DNA from pigs of known gender.

Analysis of MYOD1 and MYOG expression in embryos

The mean relative expression (ΔC_T) of *MYOG*, but not *MYOD1*, was different ($P < 0.05$) between CTR and LIG sows (Table 2). Furthermore, *MYOG* expression, as reflected by a lower ΔC_T value, was higher in male but not in female embryos in the LIG

Table 3. Influence of sex and level of uterine crowding on embryo weight, placental weight, and embryo *MYOD1* and *MYOG* expression

Both *MYOD1* and *MYOG* expression are expressed as ΔC_T, which represents the number of cycles required to amplify the transcript to a threshold level, therefore a higher ΔC_T reflects the lower gene expression in that sample

Parameter	CTR 'relatively-crowded'		LIG 'non-crowded'	
	Males	Females	Males	Females
Embryo weight (g)	1.20 ± 0.05	1.16 ± 0.05	1.27 ± 0.07	1.19 ± 0.06
Placental weight (g)	19.67 ± 1.03^{x}	19.39 ± 1.00^{x}	27.08 ± 1.73^{y}	24.33 ± 1.98^{y}
MYOD1 ΔC_T	9.15 ± 0.45	8.96 ± 0.69	8.84 ± 0.82	8.98 ± 0.77
MYOG ΔC_T	5.77 ± 0.23^{x}	5.35 ± 0.32^{x}	4.71 ± 0.33^{y}	5.02 ± 0.30^{xy}

^{x,y}Means with different superscripts within row are significantly different ($P < 0.05$).

compared with the CTR sows ($P < 0.01$; Table 3), demonstrating an interaction ($P < 0.05$) between treatment and embryo gender for myogenin expression. When analysed across all sows, no correlations were established between mean relative expression of either *MYOD1* or *MYOG*, and mean embryonic or placental weights. However, the number of viable embryos at Day 30 was negatively correlated with the average *MYOG* expression for each sow, and this correlation is mainly due to the effect of number of embryos on male *MYOG* expression, as there were no associations established between female *MYOG* expression and embryo number (Fig. 2). On the contrary, *MYOD1* expression was not related to number of viable embryos but to the sex ratio. This relationship was not sex-dependent as both female and male *MYOD1* expression was influenced by sex ratio to a similar extent (Fig. 3).

Discussion

Unilateral surgical oviduct ligation in sows to reduce embryo crowding resulted in higher placental weights, a higher male : female ratio and increased embryonic *MYOG* expression at Day 30 of gestation. Also, although there were overall effects of treatment on *MYOG* expression, this was predominately due to an increase in expression in male Day 30 embryos in the non-crowded uterine environment, compared with the relatively-crowded environment in intact control sows. The higher *MYOG* expression observed in non-crowded embryos is consistent with the suggestion from earlier studies, that effects of uterine crowding on the number of secondary muscle fibres in Day 90 fetuses (Town *et al.* 2004) may be due to effects on myogenesis during a critical period earlier in gestation, which predetermines the number of secondary muscle fibres (Rehfeldt *et al.* 1993; Dwyer *et al.* 1994).

Regardless of the gender of the embryos observed in the present study, significantly higher placental weights but no differences in embryo weights were observed in LIG sows at Day 30. However, this model did result in differences in both placental and fetal weights, along with differences in secondary muscle fibre numbers, by Day 90 of gestation (Town *et al.* 2004). Differences in myogenesis in the present study also had significantly higher placental weights. Although there were no significant differences in embryo weights at Day 30, this model did result in observed differences in fetal weights by Day 90 of gestation (Town *et al.* 2004), along with reduced secondary muscle fibre numbers. Differences in myogenesis mediated by altered placental development are indicative of intrauterine growth restriction (IUGR), and the present study was performed to examine the events in earlier gestation, at a time when the embryos are sensitive to crowding, which results in fetal IUGR at a later stage.

Previous studies of the roles of MRFs indicate that *MYOD1* is responsible for the determination of myoblasts, while the role of *MYOG* is to initiate terminal differentiation (reviewed by Arnold and Braun 2000). The observations from the present study suggest that regulation of relative *MYOG* expression is affected by the degree of intrauterine crowding of embryos at Day 30 of gestation in the pig. The lack of differences in *MYOD1* expression suggests that the effects of uterine crowding may act through differentiation of a pre-existing population of myoblasts, rather than on proliferation of myoblasts.

Our results are consistent with evidence suggesting that the determination of secondary muscle fibre numbers occurs during the time period coinciding with the formation of primary muscle fibres (Dwyer *et al.* 1994). In an analysis of the prenatal development of small and large littermates, primary fibres in small littermates, although equal in number to the large littermates, were smaller and may therefore impose limits on the surface area available for the establishment of secondary fibres (Wigmore and Stickland 1983). Therefore, in the pig, primary fibre numbers may be the result of a relatively fixed genetic component, while secondary fibre numbers are sensitive to environmental influences (Handel and Stickland 1987; Dwyer and Stickland 1991). Studies with *MYOG* knockout mice have determined that *MYOG* is not essential for the attachment of primary fibres, although differentiation of these fibres is delayed (Venuti *et al.* 1995). Therefore, it is possible that the observed differences in secondary muscle fibres in Day 90 fetuses from LIG and CTR sows (Town *et al.* 2004) may be due to the effect of *MYOG* expression at Day 30 on the differentiation of primary fibres.

Myogenin expression levels at early stages of development may also lead to direct effects on the development of secondary fibres. Studies in avian models have shown that the myoblasts that have an ability to form primary and secondary fibres are predetermined early in development (Stockdale 1992).

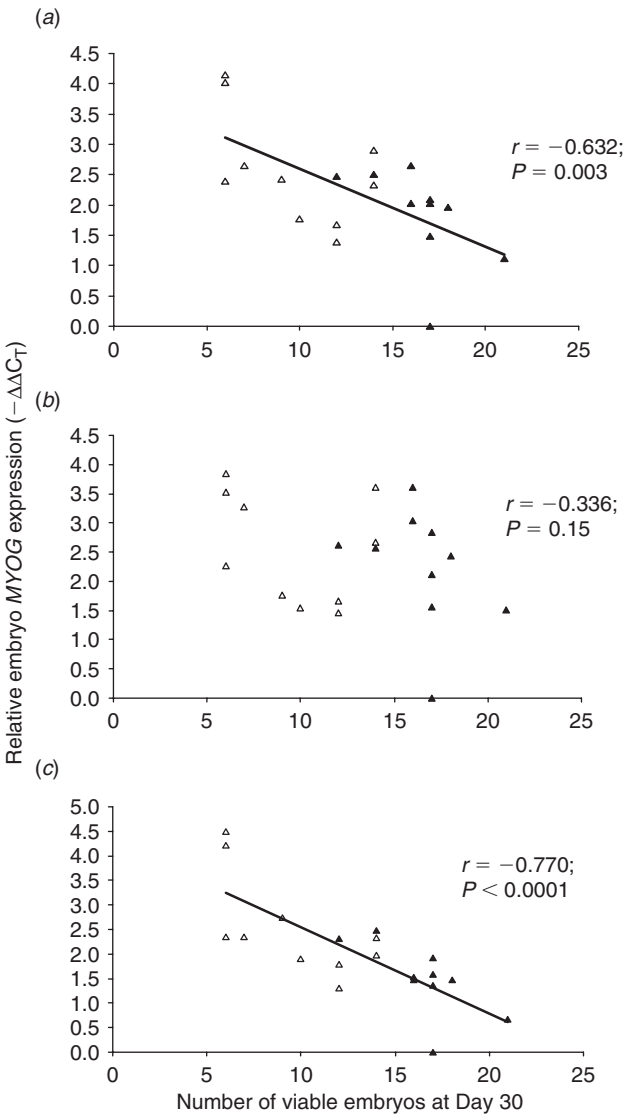

Fig. 2. Negative correlation between relative expression levels ($-\Delta\Delta C_T$) of myogenin and viable embryo numbers at Day 30 of gestation within each sow (*a*) from both CTR (▲) and LIG (Δ) treatment groups. Since a low ΔC_T represents a higher expression, for presentation of the relationships in the figure, the ΔC_T values were subtracted from the lowest expression (lowest ΔC_T) for the group to obtain relative expression as $-\Delta\Delta C_T$. As the number of embryos within a sow increases, the relative *MYOG* expression decreases across all embryos (*a*). There is no relationship between *MYOG* expression and number of viable embryos in female embryos (*b*), but there is a strong relationship between *MYOG* expression and number of viable embryos in male embryos (*c*).

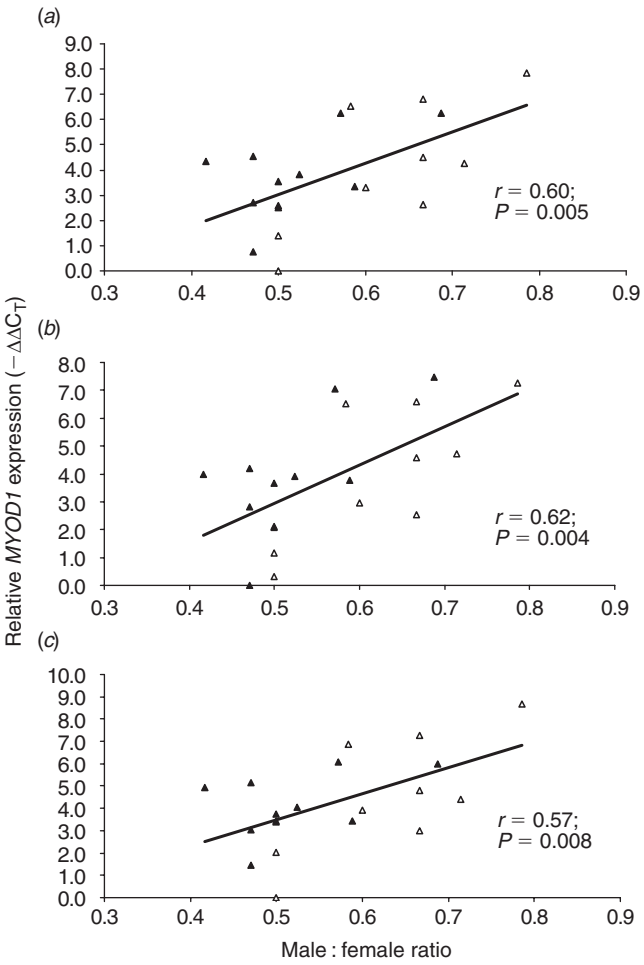

Fig. 3. Relationship between embryo *MYOD1* expression ($-\Delta\Delta C_T$) and male : female ratio in CTR (▲) and LIG (Δ) treatments. Since a low ΔC_T represents a higher expression, for presentation of the relationships in the figure, the ΔC_T values were subtracted from the lowest expression (lowest ΔC_T) for the group to obtain relative expression as $-\Delta\Delta C_T$. As the number of males compared with the number of females increases, *MYOD1* expression increases in all embryos (*a*). There is also a strong positive relationship in female (*b*) and male (*c*) embryo *MYOD1* expression.

A study of muscle cells in the human limb further suggests that cells expressing *MYOG* are present before primary fibre formation and are involved directly in the formation of secondary fibres later in development (Edom-Vovard *et al.* 1999). Furthermore, Fahey *et al*. (2005) determined that in sheep, peak *MYOG* expression occurred at Day 65, which preceded the majority of muscle fibre formation that occurred at Day 85 of gestation, again supporting the concept that peak *MYOG* expression occurs before muscle fibre formation. Similarly, in a recent study demonstrating differences in secondary : primary muscle fibres in small-sized compared with average-sized fetuses in the pig, there were no differences in *MYOG* expression at Day 65 (Tilley *et al.* 2007), again suggesting that differences in *MYOG* would be observed earlier in gestation at a time when muscle fibre development is sensitive to environmental influences. Therefore it is possible that lower *MYOG* expression at Day 30 in the present study in CTR sows may represent a reduction in the number of cells available for the development of secondary fibres at later stages of gestation. However, effects on both fibre types are not necessarily mutually exclusive.

No relationship between the average relative *MYOG* expression of all the embryos and either average embryo weight or

placental weight was established. However, a significant correlation was observed between relative *MYOG* expression and the total number of viable embryos at Day 30. The relative state of uterine crowding has been shown to negatively affect embryo weight at Day 90 of gestation, as well as placental weight at both Day 30 and Day 90 of gestation (Town *et al.* 2004). Together, these results suggest that although uterine crowding may affect the growth and development of the embryo and the placenta, the establishment of myoblasts may not be affected by overall growth and development, or by the state of crowding. Additionally, relative *MYOG* expression also appears to be independent of both embryonic and placental weight, but appears to be influenced by the presence of higher numbers of embryos. However, when each gender was examined individually, only males showed a significant correlation with viable embryo number, while female *MYOG* expression did not demonstrate a relationship. Thus, the results from the present study suggest that although differences in *MYOG* expression occur due to the relative state of uterine crowding, this difference may be largely attributed to an effect on male embryos.

In the pig, the gender of an embryo can play a significant role in its growth and development during gestation. Wise and Christenson (1992) observed that as early as Day 70 of gestation, male pig fetuses and placentae are heavier than in their female counterparts, and that a male fetus surrounded by two females, or vice versa, was significantly lighter at Day 104 of gestation. It is possible that the faster-growing male embryos and placentae are more sensitive to uterine crowding than females. The gender-specific differences observed in *MYOG* expression may be due to mechanisms altered by uterine crowding to which male embryos are more sensitive. Interestingly, we observed a difference in sex ratio in this study, in which the more-crowded uterine environment selected against males, as a roomy environment showed 62% males, as compared with a 52% males in the crowded, control sows. The embryonic survival rate was lower in the more-crowded environment (Town *et al.* 2004), and thus one could argue that those embryos that were lost in the more-crowded environment were more likely to have been male. This is consistent with earlier studies demonstrating that male embryos occupied more space in less crowded uteri than female embryos, and that as uterine space per embryo became more limited, the sex ratio decreased in favour of females (Chen and Dziuk 1993). Taken together, this suggests that a relationship exists between the degree of uterine crowding, resultant skewing of the sex ratio in favour of females in a crowded environment and an associated strong reduction in male embryo gene *MYOG* expression in this environment.

Kochhar *et al*. (2001) proposed that the sex chromosomes provide a protective mechanism for increased survival in a more-crowded uterine environment. The Y chromosome is believed to encode transcription factors that may accelerate growth, while the X chromosome encodes genes that restrain growth. Therefore, before X-inactivation in females, higher expression of X chromosome-encoded genes may help mitigate the effects of environmental stress (Kochhar *et al.* 2001). If such a protective mechanism does, in fact, exist, it may also act to limit any possible benefits of reduced crowding on myogenesis. Thus, the effect of uterine crowding on *MYOG* expression may be somewhat independent of individual embryo growth *in utero*, or access of that embryo to nutrients through increased materno-fetal exchange provided by a greater placental mass.

Unlike *MYOG*, *MYOD1* expression was not associated with the number of viable embryos *in utero*; however, it was highly correlated with the litter sex ratio. As the male : female sex ratio of the litter increased, the relative *MYOD1* expression increased, and this effect was uniform across both male and female embryos within the litter. This increase in *MYOD1* was not correlated with any other parameter, such as embryo weight, placental weight or embryo number, which does not offer support for the concept that increased *MYOD1* may be related to larger placental weights or lower embryo numbers observed in the non-crowded sows. The mechanism that selected against female embryos in the non-crowded sows to increase the sex ratio of males to females, has also impacted *MYOD1* expression in all of the embryos, irrespective of sex in these litters.

In summary, the results from the present study indicate that uterine crowding affects the expression of genes related to myogenesis, and is presumably a key factor in determining the reduction in total numbers of secondary muscle fibres reported at Day 90 of gestation. Therefore, critical consideration may be needed when genetic selection is used to increase litter size, as selection criteria that favour large litters may, at the same time, be compromising the postnatal potential for muscle development. Strategies that effectively increase muscle differentiation may be effective in alleviating the negative effects of uterine crowding on the development of muscle fibre numbers. The dynamics of prenatal loss in modern commercial dam-lines, driven by high ovulation rates in mature sows, drives excessive uterine crowding in early gestation (for review see Foxcroft *et al.* 2007) and is associated with low birthweight litters which bear all of the hallmarks of IUGR, including reduced weight of the semitendinosus muscles. Further investigation into the mechanisms which reduce secondary fibre numbers and the specific timing of the effect would be beneficial in optimising muscle development in this meat-producing species. Continued study into whether or not the effect of uterine crowding on secondary muscle fibre numbers remains consistent at Day 90 of gestation, with respect to gender, would help support the idea of a gender-specific response. Based on the results of the present study, strategies that target males may not only help to mitigate the effects of increased uterine crowding on the determination of secondary muscle fibre numbers, but may also be of interest in increasing muscle fibre numbers in situations in which uterine crowding is not an issue.

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References

Arnold, H. H., and Braun, T. (2000). Genetics of muscle determination and development. *Curr. Top. Dev. Biol.* **48**, 129–164.

Chen, Z. Y., and Dziuk, P. J. (1993). Influence of initial length of uterus per embryo and gestation stage on prenatal survival, development, and sex ratio in the pig. *J. Anim. Sci.* **71**, 1895–1901.

Dwyer, C. M., and Stickland, N. C. (1991). Sources of variation in myofibre number within and between litters of pigs. *Anim. Prod.* **52**, 527–533.

Dwyer, C. M., Stickland, N. C., and Fletcher, J. M. (1994). The influence of maternal nutrition on muscle fiber number development in the porcine fetus and on subsequent postnatal growth. *J. Anim. Sci.* **72**, 911–917.

Dwyer, C. M., Madgwick, A. J., Ward, S. S., and Stickland, N. C. (1995). Effect of maternal undernutrition in early gestation on the development of fetal myofibres in the guinea-pig. *Reprod. Fertil. Dev.* **7**, 1285–1292. doi:10.1071/RD9951285

Edom-Vovard, F., Mouly, V., Barbet, J. P., and Butler-Browne, G. S. (1999). The four populations of myoblasts involved in human limb muscle formation are present from the onset of primary myotube formation. *J. Cell Sci.* **112**(Pt 2), 191–199.

Fahey, A. J., Brameld, J. M., Parr, T., and Buttery, P. J. (2005). The effect of maternal undernutrition before muscle differentiation on the muscle fiber development of the newborn lamb. *J. Anim. Sci.* **83**, 2564–2571.

Foxcroft, G., Bee, G., Dixon, W. T., Hahn, M., Harding, J., Patterson, J., Putman, T., Sarmento, S., Smit, M., Tse, W.-Y., and Town, S. (2007). Consequences of selection for litter size on piglet development. In 'Paradigms in Pig Science'. (Eds J. Wiseman, M. A. Varley, S. McOrist and B. Kemp.) pp. 207–229. (Nottingham University Press: Thrumpton, Nottingham.)

Handel, S. E., and Stickland, N. C. (1987). Muscle cellularity and birth weight. *Anim. Prod.* **44**, 311–317.

Hegarty, P. V., and Allen, C. E. (1978). Effect of pre-natal runting on the post-natal development of skeletal muscles in swine and rats. *J. Anim. Sci.* **46**, 1634–1640.

Kassar-Duchossoy, L., Gayraud-Morel, B., Gomes, D., Rocancourt, D., Buckingham, M., Shinin, V., and Tajbakhsh, S. (2004). Mrf4 determines skeletal muscle identity in Myf5:Myod1 double-mutant mice. *Nature* **431**, 466–471. doi:10.1038/NATURE02876

Koch, I., Slotta-Huspenina, J., Hollweck, R., Anastasov, N., Hofler, H., Quintanilla-Martinez, L., and Fend, F. (2006). Real-time quantitative RT–PCR shows variable, assay-dependent sensitivity to formalin fixation: implications for direct comparison of transcript levels in paraffin-embedded tissues. *Diagn. Mol. Pathol.* **15**, 149–156. doi:10.1097/01.PDM.0000213450.99655.54

Kochhar, H. P., Peippo, J., and King, W. A. (2001). Sex-related embryo development. *Theriogenology* **55**, 3–14. doi:10.1016/S0093-691X(00)00441-6

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)). *Methods* **25**, 402–408.

Masuda, N., Ohnishi, T., Kawamoto, S., Monden, M., and Okubo, K. (1999). Analysis of chemical modification of RNA from formalin-fixed samples and optimization of molecular biology applications for such samples. *Nucleic Acids Res.* **27**, 4436–4443. doi:10.1093/NAR/27.22.4436

Maxfield, E. K., Sinclair, K. D., Dunne, L. D., Broadbent, P. J., Robinson, J. J., Stewart, E., Kyle, D. G., and Maltin, C. A. (1998). Temporary exposure of ovine embryos to an advanced uterine environment does not affect fetal weight but alters fetal muscle development. *Biol. Reprod.* **59**, 321–325. doi:10.1095/BIOLREPROD59.2.321

Pomp, D., Good, B. A., Geisert, R. D., Corbin, C. J., and Conley, A. J. (1995). Sex identification in mammals with polymerase chain reaction and its use to examine sex effects on diameter of Day 10 or 11 pig embryos. *J. Anim. Sci.* **73**, 1408–1415.

Quigley, S. P., Kleemann, D. O., Kakar, M. A., Owens, J. A., Nattrass, G. S., Maddocks, S., and Walker, S. K. (2005). Myogenesis in sheep is altered by maternal feed intake during the peri-conception period. *Anim. Reprod. Sci.* **87**, 241–251. doi:10.1016/J.ANIREPROSCI.2004.11.005

Rehfeldt, C., Fiedler, I., Weikard, R., Kanitz, E., and Ender, K. (1993). It is possible to increase skeletal muscle fibre number *in utero. Biosci. Rep.* **13**, 213–220. doi:10.1007/BF01123503

Stockdale, F. E. (1992). Myogenic cell lineages. *Dev. Biol.* **154**, 284–298. doi:10.1016/0012-1606(92)90068-R

Tilley, R. E., McNeil, C. J., Ashworth, C. J., Page, K. R., and McArdle, H. J. (2007). Altered muscle development and expression of the insulin-like growth factor system in growth-retarded fetal pigs. *Domest. Anim. Endocrinol.* **32**, 167–177. doi:10.1016/J.DOMANIEND.2006.02.003

Town, S. C., Putman, C. T., Turchinsky, N. J., Dixon, W. T., and Foxcroft, G. R. (2004). Number of conceptuses *in utero* affects porcine fetal muscle development. *Reproduction* **128**, 443–454. doi:10.1530/REP.1.00069

Town, S. C., Patterson, J. L., Pereira, C. Z., Gourley, G., and Foxcroft, G. R. (2005). Embryonic and fetal development in a commercial dam-line genotype. *Anim. Reprod. Sci.* **85**, 301–316.

van der Lende, T., and de Jager, D. (1991). Death risk and preweaning growth rate of piglets in relation to the within-litter weight distribution at birth. *Livest. Prod. Sci.* **28**, 73–84. doi:10.1016/0301-6226(91)90056-V

Venuti, J. M., Morris, J. H., Vivian, J. L., Olson, E. N., and Klein, W. H. (1995). Myogenin is required for late but not early aspects of myogenesis during mouse development. *J. Cell Biol.* **128**, 563–576. doi:10.1083/JCB.128.4.563

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

Wigmore, P. M., and Stickland, N. C. (1983). Muscle development in large and small pig fetuses. *J. Anat.* **137**(Pt 2), 235–245.

Wise, T. H., and Christenson, R. K. (1992). Relationship of fetal position within the uterus to fetal weight, placental weight, testosterone, estrogens, and thymosin beta 4 concentrations at 70 and 104 days of gestation in swine. *J. Anim. Sci.* **70**, 2787–2793.

Yuan, J. S., Reed, A., Chen, F., and Stewart, C. N., Jr (2006). Statistical analysis of real-time PCR data. *BMC Bioinformatics* **7**, 85. doi:10.1186/1471-2105-7-85

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