Number of conceptuses *in utero* affects porcine fetal muscle development

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Abstract

Unmodified, third parity, control sows (CTR; $n = 30$) or sows subjected to unilateral oviduct ligation before breeding (LIG; $n = 30$), were slaughtered at either day 30 or day 90 of gestation and used to determine the effects of numbers of conceptuses *in utero* on prenatal, and particularly muscle fibre, development. Ovulation rate, number of conceptuses *in utero*, placental and fetal size, and (day 90 sows) fetal organ and semitendinosus muscle development were recorded. Tubal ligation reduced ($P < 0.05$) the number of viable embryos at day 30 and fetuses at day 90. Placental weight at day 30 and day 90, and fetal weight at day 90, were lower ($P < 0.05$) in CTR sows. All body organs except the brain were lighter, and the brain:liver weight ratio was higher in CTR fetuses ($P < 0.05$), indicative of brain sparing and intrauterine growth restriction in fetuses from CTR sows. Muscle weight, muscle cross-sectional area and the total number of secondary fibres were also lower ($P < 0.05$) in CTR fetuses. The number of primary fibres, the secondary:primary muscle fibre ratio, and the distribution of myosin heavy chain-Iβ, -IIa, fetal and embryonic isoforms did not differ between groups. Thus, even the relatively modest uterine crowding occurring naturally in CTR sows negatively affected placental and fetal development and the number of secondary muscle fibres. Consequences of more extreme crowding *in utero* on fetal and postnatal development, resulting from changing patterns of early embryonic survival, merit further investigation.

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Introduction

In a species domesticated for commercial meat production like the pig, both the developmental competence and number of offspring born are critical. Earlier studies of uterine crowding in the gilt suggested that when the number of embryos exceeded 14, intrauterine crowding is a limiting factor for litter size born (Dziuk 1968), and Bazer *et al.* (1969a,b) suggested that increased embryonic loss was due to maternal limitations (uterine capacity) and not to inherent limitations of the embryo. Although competition among embryos in the pre-implantation period may reduce within-litter variation in development by selectively removing the least developed embryos (van der Lende *et al.* 1990), uterine capacity can affect both litter size and the average birth weight of the litter, even in gilts with 'normal' ovulation rates (Père *et al.* 1997).

Days 30 to 40 of gestation are the critical period when uterine capacity exerts its effects (Fenton *et al.* 1970, Knight *et al.* 1977, Vallet 2000). Furthermore, Vallet *et al.* (2003) suggested that fetal growth rate is less sensitive to intrauterine crowding than placental growth rate and that, as in the prolific Meishan female (Ford & Youngs 1993), an increase in placental efficiency may initially protect the developing fetus from a limitation in placental size. However, conclusions based only on a consideration of fetal weight may overlook critical effects on fetal development established early in gestation.

The pathology of 'runted' or intrauterine growth retarded (IUGR) offspring has been described previously in the pig (Adams 1971, Widdowson 1971, Cooper *et al.* 1978, Hegarty & Allen 1978, Flecknell *et al.* 1981), and effects on the developmental potential of IUGR pigs could not be entirely explained on the basis of their lower birth weight (van der Lende & de Jager 1991). Furthermore, the extent of within-litter IUGR was already established at days 27 to 35 of gestation and the largest litters *in utero* generally included one or more IUGR fetuses (van der Lende *et al.* 1990).

Pre-implantation embryonic losses are still considered to be the largest proportion of prenatal loss in the pig (see

Ashworth & Pickard 1998), and in commercial practice this generalisation likely reflects the situation in gilts and in weaned, first parity, sows. However, the dynamics of prenatal loss in some dam-line populations may be changing and Foxcroft (1997) suggested that several generations of selection for prolificacy may have increased the discrepancy between ovulation rate and the number of conceptuses surviving post-implantation, and uterine capacity. Even in gilts, embryonic survival rate to day 28 of gestation can be 100% in individuals with >20 ovulations, and the number of conceptuses *in utero* at day 28 was inversely related to placental volume (Almeida *et al.* 2000). In higher parity females, the situation may be even more extreme. Although embryonic survival to day 25 to 30 of gestation across three studies in commercial dam-line sows was only around 60%, mean ovulation rates of 26.9 ± 1.4 (Orzechowski 1998), 26.6 ± 0.4 (Vonnahme *et al.* 2002) and 24.7 ± 0.4 (Town 2004) resulted in 16.4, 15.8 and around 15.0 embryos *in utero* respectively, between days 25 and 30 of gestation. At this stage the number of conceptuses *in utero* was again negatively related to placental size and a proportion of these conceptuses were then lost before days 50 to 55 of gestation (Vonnahme *et al.* 2002, Town 2004). Although 'functional' uterine capacity may increase in higher parity sows (Town 2004), it is still likely that the number of surviving embryos initially exceeds functional uterine capacity in a substantial proportion of these sow herds.

Although uterine crowding and reduced placental size did not affect the size and weight of the embryo in the immediate post-implantation period (Almeida *et al.* 2000, Vonnahme *et al.* 2002, Town 2004), potential impacts on fetal development need careful study. If the placental compensatory mechanisms described by Biensen *et al.* (1998) are not adequate, crowding may still affect fetal development. This raises important questions for both fetal and postnatal development, particularly with respect to the development of fetal muscle fibres, which start to differentiate around day 35 of gestation in the pig (Wigmore & Stickland 1983). Therefore, in contrast to situations in which IUGR is limited to a discrete subpopulation of 'runt' fetuses (Royston *et al.* 1982, Wooton *et al.* 1983), we hypothesize that a pattern of prenatal loss that results in uterine crowding in the post-implantation stage of gestation will produce a more uniform effect on placental and fetal development.

Dwyer *et al.* (1992) suggested that a reduction in placental size might be the mechanism mediating negative effects of maternal undernutrition on birth weight and the number of secondary muscle fibres in the offspring (Handel & Stickland 1987, Dwyer *et al.* 1994). Consistent with the earlier data of Hegarty and Allen (1978) indicating that runts have reduced muscle growth potential and needed 23 days longer to reach a weight of approximately 105 kg, Dwyer *et al.* (1993) established a positive correlation between the total number of muscle fibres and postnatal growth potential. Furthermore, the effect of

maternal nutrition was identified as occurring between days 25 and 50 of gestation, the period immediately preceding secondary muscle fibre hyperplasia (Dwyer *et al.* 1994). This, and the more recent study of Clelland and Stickland (2001), led to the central hypothesis tested in the present study, that, 'by detrimentally affecting placental size in early gestation, uterine crowding will also affect fetal organ development and the number and type of muscle fibres, analogous to the situation of IUGR in nutritionally challenged sows'. Preliminary data from an initial experiment supported this hypothesis, in that even when the number of conceptuses *in utero* did not affect birth weight, 'crowding' nevertheless resulted in measurable IUGR in the fetus (Town *et al.* 2002). The present study evaluated an alternative experimental approach to test further this central hypothesis.

Materials and Methods

Animals

The experiment was conducted at the Swine Research and Technology Centre at the University of Alberta, in accordance with the guidelines of the Canadian Council for Animal Care and under authorisation from the University of Alberta Animal Policy and Welfare Committee (approval # 200134D). Sixty Hybrid F1 third parity sows (Genex Swine Group, Regina, Saskatchewan, Canada) were housed in a controlled environment barn, managed and fed as per standard protocols during gestation and lactation and were weaned at 23 days after farrowing. Average sow body weight and back fat measurements at post-weaning oestrus were 210 ± 2.01 kg and 17.4 ± 0.33 mm respectively. Sows were randomly allocated to one of two groups. The experimental group ($n = 30$) underwent unilateral oviduct ligation surgery (LIG) approximately 3 days after the end of their first post-weaning oestrus. The purpose of this surgery was to reduce the number of embryos *in utero* by preventing the oocytes ovulated from the ovary ipsilateral to the ligated oviduct from being fertilised and entering the uterus. The remaining animals ($n = 30$) did not undergo surgery and formed the control group (CTR). Half the animals from each group were killed at day 30 of gestation to determine the number of conceptuses *in utero*. The remaining animals were killed at day 90 of gestation to determine the effects of crowding on fetal development. Day 90 was chosen since total number of muscle fibres is considered to be definitively established by approximately day 90 in the pig (Wigmore & Stickland 1983). Anaesthesia was induced using an intravenous short acting general anaesthetic (5% solution of sodium thiopental, Pentothal, Merial Ltd, Iselin, NJ, USA; dosage 6.6 ml kg^{-1} body weight) via an ear vein. Anaesthesia was maintained with a closed circuit system of inhalation of the general anaesthetic halothane (Fluothane), in combination with nitrous oxide and oxygen via a facemask. Post surgical care was provided using approved procedures.

Heat checking and breeding

All sows were bred by artificial insemination at their second post-weaning oestrus. Heat detection was carried out every 12 h (0700 and 1900 h) using the back pressure test during periods of fence-line contact with a mature vasectomised boar starting on day 18 of the oestrous cycle. All sows were bred by artificial insemination using pooled semen from the same group of three fertile boars, using 3 billion sperm per dose, at 12 h and 36 h after the onset of standing heat and then every 24 h until the animals were no longer in standing heat (i.e. 12 h, 36 h, 60 h etc.). Signs of a return to oestrus were recorded between days 18 and 22 post-insemination and pregnancy was confirmed at day 25 of gestation using real time ultrasound (RTU).

Slaughter and necropsy procedure

Sows were shipped to a local abattoir and reproductive tracts were recovered and dissected within 1 h after slaughter. Ovulation rate and the number of viable embryos or fetuses *in utero* were recorded for all sows. At day 30 of gestation, embryonic and placental weights were also measured. At day 90, fetal and placental weights were recorded and all fetuses were necropsied to determine various body organ weights including the brain, heart, lungs, liver, kidneys and spleen. After removal and weighing of the internal organs, the empty carcass was also weighed. The brain:liver weight ratio was then used as an estimate of disproportionate changes in organ development, indicative of the occurrence of IUGR. Results were averaged within litter. Relative piglet organ weights (actual organ weight divided by the actual body weight)

were also calculated to further examine the pattern of organ growth in fetuses with different body weights. Again the average measurement from each litter was used for analysis. Two day-90 fetuses closest to the mean body weight within each litter were chosen for removal of the semitendinosus muscles, which were dissected, weighed, mounted on aluminium foil in a slightly stretched position and frozen in melting isopentane cooled in liquid nitrogen ($-156\,°C$). Samples were stored at $-80\,°C$ until used for immunohistochemical and electrophoretic analyses.

Antibodies

Table 1 lists the monoclonal antibodies directed against various myosin heavy chain (MHC) isoforms used in this study.

Immunohistochemical staining

Serial sections (10 µm) of fetal day 90 semitendinosus muscle were collected on poly-L-lysine-coated slides (Electron Microscopy Sciences, Fort Washington, PA, USA) and stored at $-80\,°C$ for later analysis. Adult triceps brachii tissue was also collected to confirm reactivity of the antibodies with porcine tissue. Frozen muscle sections were air-dried, washed once in phosphate-buffered saline with 0.1% (v/v) Tween 20 (PBS-Tween), twice in PBS, and incubated for 15 min in 3% (v/v) H_2O_2 in methanol. The avidin-biotin-peroxidase technique (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA, USA) was used to visualise immunoreactivity according to the manufacturer's protocol with modifications according to Putman *et al.* (2003) and using the antibody dilutions in Table 1.

Table 1 Antibodies and their respective dilutions in immunohistochemistry (IHC) and In-Gel immunodetection (IG) assays.

	Assay		
Reagents	**IHC**[b]	**IG**	**Source/Reference**
Anti-MHC (all isoforms) (MF-20)[a]	Not used	1:10	Developmental Studies Hybridoma Bank, University of Iowa, Ames, IO, USA
Anti-MHCIβ (NOQ7.5.4D)	1:4000	1:7000	Sigma-Aldrich, St Louis, MO, USA
Anti-MHCIβ (BA-D5)[a]	1:100	Not used	Schiaffino *et al.* (1989)
Anti-MHC1α (F88.12F8)	1:10	Not used	Biocytex, Marseilles, France
Anti-MHC (developmental and all fast forms) (MY-32)[a]	1:1000	1:160 000	Sigma-Aldrich
Anti-MHCIIa (SC-71)[a]	1:50	1:200	Schiaffino *et al.* (1989)
Anti-MHCIIb (BF-F3)[a]	1:50	1:200	Schiaffino *et al.* (1989)
Anti-MHC (all isoforms except MHCIId(x) (BF-35)	0.1 µg/ml	1:1000	Schiaffino *et al.* (1989)
Anti-MHC (embryonic) (BF-45)	1:1000	1:2000	Schiaffino *et al.* (1988)
Anti-MHC (embryonic) (NCL-d)[a]	1:20	1:40	Novocastra Laboratories, Newcastle, UK
Anti-MHC (neonatal) (NCL-n)[a]	1:10	1:20	Novocastra Laboratories
Anti-mouse IgG (biotinylated) (raised in horse – rat pre-adsorbed and affinity purified)	1:500	1:500	Vector Laboratories, Burlingame, CA, USA
Anti-mouse IgM (biotinylated) (used with BF-F3)	1:500	1:500	Vector Laboratories
Normal mouse IgG (control serum)	1:2000	Not used	Santa Cruz Biochemical, Santa Cruz, CA, USA

[a] Hybridoma culture supernatant; [b] for immunohistochemistry, a blocking solution containing horse serum (BS-1) was used for all dilutions of IgG antibodies while a goat serum-containing blocking solution (BS-2) was used in the dilutions of BF-F3 (IgM).

Control sections were processed in parallel incubations in which the primary antibody was omitted or substituted with non-specific control mouse IgG. After dehydration in ethanol, sections were cleared with xylene and mounted with Entellan (Merck, Darmstadt, Germany).

Image acquisition was carried out using a motorised scanning stage Zeiss Axioplan IIM Universal Microscope (Carl Zeiss Jena GmbH, Jena, Germany) and a Photometrics CoolSNAP HQ Camera (Roper Scientific, Tucson, AZ, USA) in conjunction with the Metamorph Imaging System (Universal Imaging Corporation, Downingtown, PA, USA). Images of the entire cross section of each muscle were obtained and saved to CD in montage format for Metamorph Image analysis. Serial sections of day 90 tissue stained for the various MHC isoforms were examined to determine which muscle fibre isoforms were present. Sections stained with the NOQ7.5.4D antibody (anti-MHCIβ) were used to determine semitendinosus muscle total cross-sectional area (CSA), muscle fibre type (i.e. primary or secondary), and fibre CSA. Two distinct areas of the muscle were examined. For each individual muscle, five fields were selected at random from the deep red portion and five from the superficial white portion of the muscle. Results from both portions of the muscle were averaged, and the resulting 10 fields per muscle encompassed a total area of $1.48\,mm^2$. An average of $10\,410 \pm 981$ fibres (mean$\pm$ S.D.) were examined per muscle. Mean CSA of primary and secondary fibres was measured from at least 300 fibres of each type. Total numbers of primary and secondary fibres were calculated for each muscle by extrapolating the mean number of fibres of each type per mm^2 to the actual CSA of the entire semitendinosus muscle determined by measurement using the Metamorph Imaging System. Two piglets were analysed per litter and the results were averaged within sow to examine the effects of uterine crowding on muscle fibre development.

Myosin extraction

Frozen muscles were pulverised and the myosin fraction was extracted according to the protocols of Putman *et al.* (2003). Protein concentrations were determined using the Bradford procedure (Bio-Rad Laboratories, Hercules, CA, USA).

Standard sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The MHC complement of whole muscle extracts was analysed by SDS-PAGE using a slightly modified version of the method described by Hämäläinen and Pette (1996), which has been described elsewhere (Putman *et al.* 2003). MHC isoforms were separated at 10 °C for 24 h at 275 V (constant voltage) and visualised by silver staining. The relative MHC isoform contents were quantified densitometrically using the Syngene Chemigenius gel documentation system and GeneTools gel analysis software

(Syngene, Cambridge, Cambs, UK). The scheme used for identifying the various MHC isoforms was validated by In-Gel immunodetection and by comparison with rodent muscles.

In-Gel immunodetection

In-Gel immunodetection of the various MHC isoforms was carried out using the Unblot In-Gel Chemiluminescent Detection Kit for Biotinylated Antibody Probes (Pierce Biotechnology, Rockford, IL, USA). Following standard SDS-PAGE, gels were pretreated with 50% (v/v) isopropyl alcohol and then washed in ultrapure water. Gels were then reacted for 1 h at room temperature with monoclonal antibodies against various MHC isoforms at the dilutions indicated in Table 1 and processed with the appropriate biotinylated secondary antibody (biotinylated horse anti-mouse IgG or biotinylated goat anti-mouse IgM (BF-F3)) and streptavidin-horse radish peroxidase according to the manufacturer's protocol. The gel was then placed between two cellophane sheets and exposed to a charge-coupled device camera to detect the chemiluminescent signal using the Syngene Chemigenius gel documentation system (Syngene).

Statistical analysis

To determine the effects of treatment on ovulation rate, number of viable embryos, embryonic survival rate, placental and embryo/fetal weights, placental efficiency, fetal organ weights, fetal brain:liver weight ratio, muscle weight and CSA, fibre number, secondary:primary fibre ratio and MHC isoform distribution, data were analysed as appropriate for a completely randomised design. Sow was used as the experimental unit for analysis, and fetal weights, placental weights, organ and muscle parameters were averaged within each reproductive tract (sow) before analysis. Data were analysed using the General Linear Model (GLM) procedure of the Statistical Analysis System (SAS 1990, SAS Inst. Inc., Cary, NC, USA). The model included treatment group (CTR or LIG). Significance was considered as $P < 0.05$. The results are presented as means$\pm$ S.E.M. Important associations among characteristics measured were examined across treatment groups using correlation analysis (INSIGHT procedure; SAS 1990). These included relevant correlations within gestational age between number of viable embryos/fetuses, embryo/fetal weight, placental weight, fetal organ weights (day 90) and brain:liver weight ratio.

Results

General results

Of the 60 sows slaughtered, 58 were confirmed pregnant by the presence of viable conceptuses. Removals from final analysis were due to missing data as a result of damage during tract removal and tissue collection (missing ovaries, etc). Overall ovulation rate for this sow population

Table 2 Reproductive characteristics (means± S.E.M.) of control (CTR) and unilaterally oviduct ligated (LIG) sows (*n* = 59).

	Treatment group		
Parameter	CTR (*n* = 30) 'Relatively crowded'	LIG (*n* = 29) 'Non-crowded'	*P*
Overall ovulation rate	19.2 ± 0.5 (*n* = 30)	20.6 ± 0.5 (*n* = 28)	0.054
Ovulation rate available for fertilization	19.2 ± 0.5	10.5 ± 0.6	<0.001
Number of sows at day 30 (*n*)	15	15	
Number of viable embryos (day 30)	15.1 ± 0.8	9.3 ± 0.8	<0.001
Embryonic survival to day 30 (%)	79 ± 3	91 ± 2	0.006
Average placental weight (g) at day 30	19.2 ± 1.0	26.2 ± 1.4	<0.001
Average embryo weight (g) at day 30	1.15 ± 0.05	1.22 ± 0.05	0.34
Placental efficiency at day 30	0.071 ± 0.005	0.050 ± 0.003	0.001
Number of sows at day 90 (*n*)	15	14	
Number of viable fetuses (day 90)	14.4 ± 0.5	9.4 ± 0.7	<0.001
Fetal survival to day 90 (%)	76 ± 3	84 ± 3	0.029
Average placental weight (g) at day 90	219 ± 8	274 ± 14	0.003
Average fetal weight (g) at day 90	588 ± 18	679 ± 18	0.002
Placental efficiency at day 90	2.84 ± 0.09	2.64 ± 0.12	0.20

was 19.90 ± 0.36. Data derived from dissection of reproductive tracts at day 30 and day 90 are presented in Table 2. In addition to an unexpected and marginal difference in ovulation rate between CTR (19.23 ± 0.48) and LIG (20.61 ± 0.50) sows, ovulation rate available for fertilisation was substantially reduced by the ligation procedure. As a consequence, both the number of viable embryos at day 30 and the number of fetuses at day 90 differed between treatments. Embryo survival to day 30 and fetal survival to day 90 were both higher in the LIG group.

Average placental weight was lighter in CTR animals at day 30 and day 90 of gestation. Average embryo weight was not different between groups at day 30; however, average fetal weights at day 90 were lighter in CTR sows. Placental efficiency, calculated as the embryo weight:placental weight ratio, was higher in the CTR group at day 30; however, this relationship was lost in the day 90 fetuses in which placental efficiency was not different between the two groups.

Average placental weight was positively correlated with average embryo weight at day 30 of gestation (*r* = 0.56; *P* < 0.01; Fig. 1a) and with average fetal weight at day 90 of gestation (*r* = 0.68; *P* < 0.01; Fig. 1b). Furthermore, average placental weight was negatively correlated with the number of viable embryos at day 30 (*r* = −0.61; *P* < 0.01; Fig. 2a) and the number of viable fetuses at day 90 (*r* = −0.67; *P* < 0.01; Fig. 2b). Although average embryo weight was not correlated with the number of viable embryos at day 30, a negative relationship was established between the number of fetuses and fetal weight at day 90 (*r* = −0.61; *P* < 0.01; Fig. 3).

Placental efficiency was not correlated with average embryonic or fetal weight at either day 30 or day 90 of gestation, although it was weakly correlated with the number of viable embryos at day 30 (*r* = 0.37; *P* < 0.05) and the number of viable fetuses at day 90 (*r* = 0.35; *P* = 0.06). However, placental efficiency showed a strong negative correlation with average placental weight
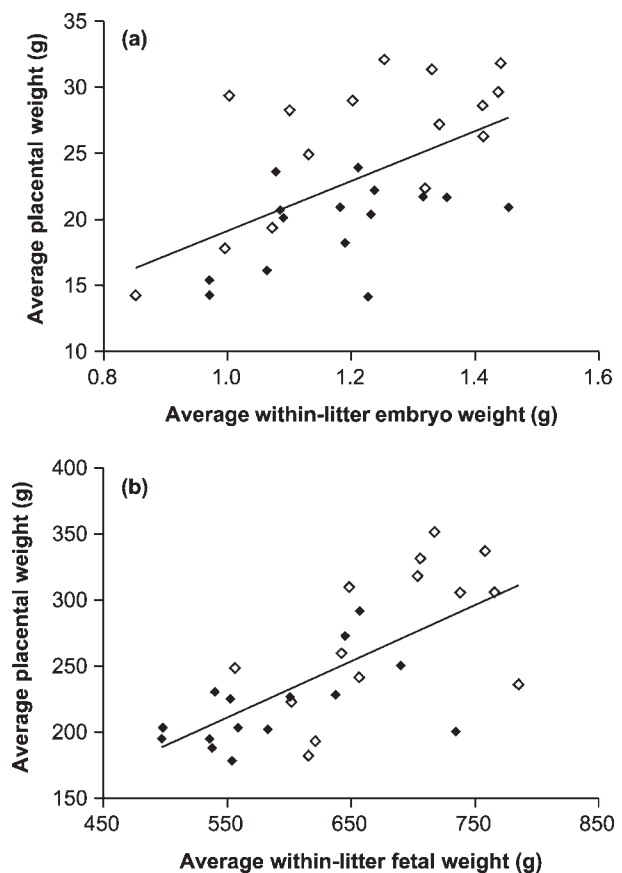

Figure 1 Correlation between average placental weight and (a) average within-litter embryo weight at day 30 of gestation (r^2 = 0.31; *P* = 0.0014) and (b) average within-litter fetal weight at day 90 of gestation (r^2 = 0.46; *P* < 0.0001) in LIG animals (◇) and CTR animals (◆).

at day 30 (*r* = −0.65; *P* < 0.0001) and day 90 (*r* = −0.75; *P* < 0.0001).

Day 90 necropsy data

Fetal organ weight data at day 90 of gestation are shown in Table 3. The brain was the only organ for
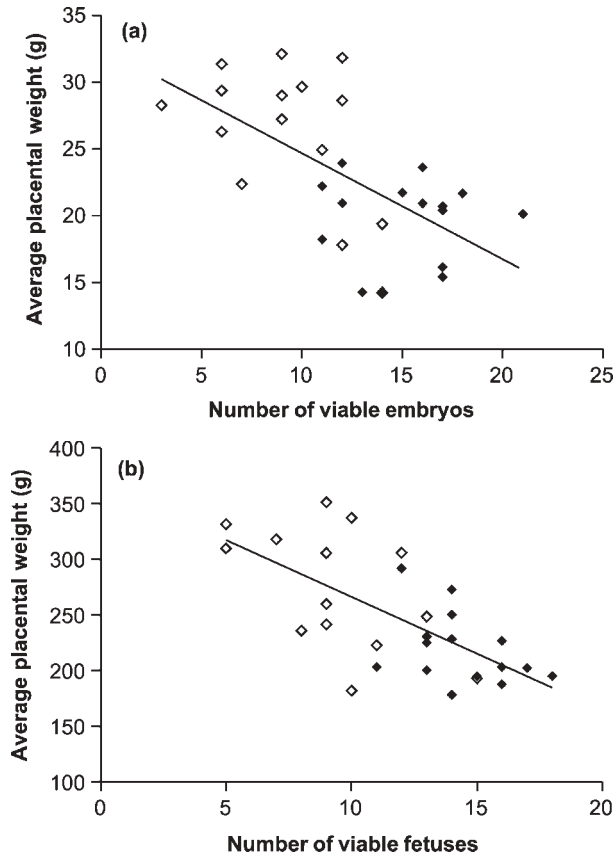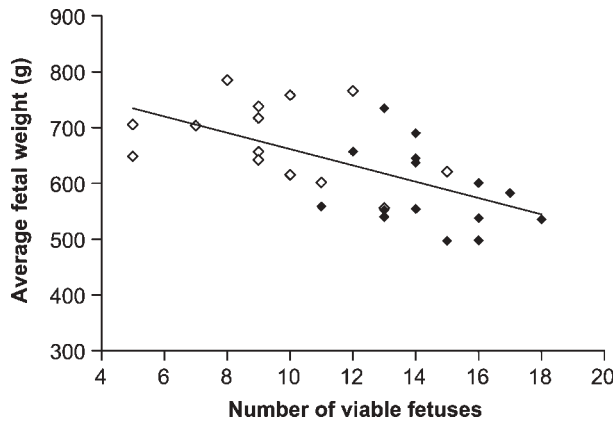
Figure 2 Correlation between average placental weight and (a) number of viable embryos at day 30 of gestation ($r^2 = -0.37$; $P = 0.0003$) and (b) number of viable fetuses at day 90 of gestation ($r^2 = -0.45$; $P < 0.0001$) in LIG animals (◇) and CTR animals (◆).

which there was no difference between treatments. All other organs were heavier in fetuses from LIG sows ($P < 0.05$). Furthermore, the brain:liver weight ratio was higher in the fetuses from CTR litters, and the brain: semitendinosus muscle weight ratio and the ratio of the brain:total number of secondary muscle fibres, were also higher in the CTR sows.

Figure 3 Correlation between average fetal weight and number of viable fetuses at day 90 of gestation ($r^2 = -0.37$; $P = 0.0005$) in LIG animals (◇) and CTR animals (◆).

Table 3 Average empty carcass and body organ weights and brain:organ weight ratios (means ± S.E.M.) in fetuses from control (CTR) and unilaterally oviduct ligated (LIG) sows at day 90 of gestation.

Parameter	CTR ($n = 15$) 'Relatively crowded'	LIG ($n = 14$) 'Non-crowded'	P
Spleen (g)	0.84 ± 0.04	1.07 ± 0.09	0.015
Liver (g)	17.81 ± 0.63	21.48 ± 0.94	0.003
Heart (g)	4.09 ± 0.15	4.65 ± 0.15	0.013
Lungs (g)	17.55 ± 0.64	19.61 ± 0.64	0.031
Kidneys (g)	6.33 ± 0.18	7.35 ± 0.25	0.002
Brain (g)	19.65 ± 0.33	20.02 ± 0.41	0.48
Empty carcass (g)	464.7 ± 13.5	536.4 ± 14.3	0.001
Brain:liver weight ratio	1.17 ± 0.04	0.97 ± 0.04	0.002
Brain:ST muscle weight ratio	10.49 ± 0.43	9.25 ± 0.33	0.031
Brain:total secondary fibre ratio	$(62 ± 3.5)^{10^{-5}}$	$(52 ± 2.3)^{10^{-5}}$	0.021

ST, semitendinosus.

Fetal weight was positively correlated to absolute weight of the fetal liver ($r = 0.91$; $P < 0.0001$; Fig. 4a), brain ($r = 0.56$; $P < 0.002$; Fig. 4b), heart ($r = 0.91$; $P < 0.0001$), lungs ($r = 0.81$; $P < 0.0001$), spleen ($r = 0.67$; $P < 0.0001$)
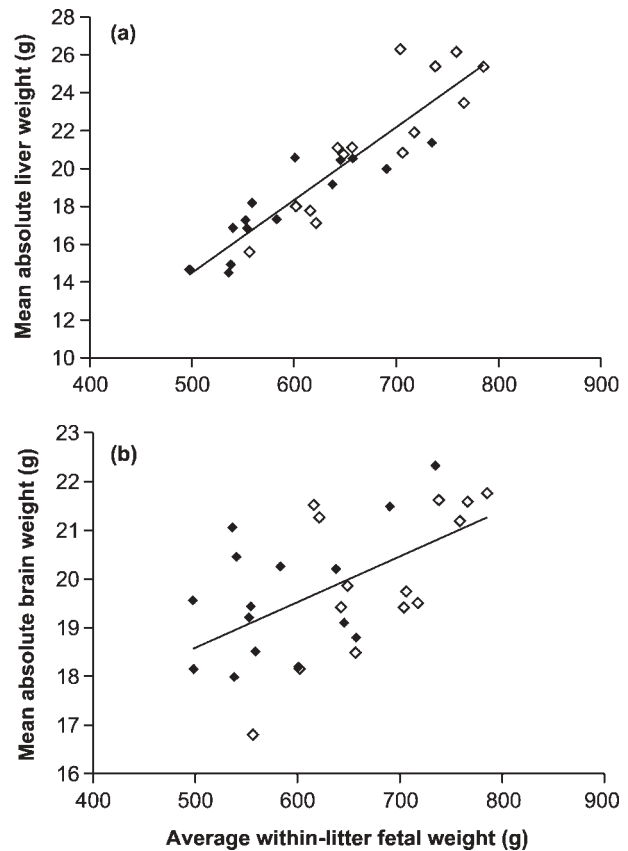

Figure 4 Correlation between (a) mean absolute liver weight ($r^2 = 0.83$; $P < 0.0001$) and (b) mean absolute brain weight ($r^2 = 0.31$; $P = 0.0017$) and average within-litter fetal weight at day 90 of gestation in LIG animals (◇) and CTR animals (◆).
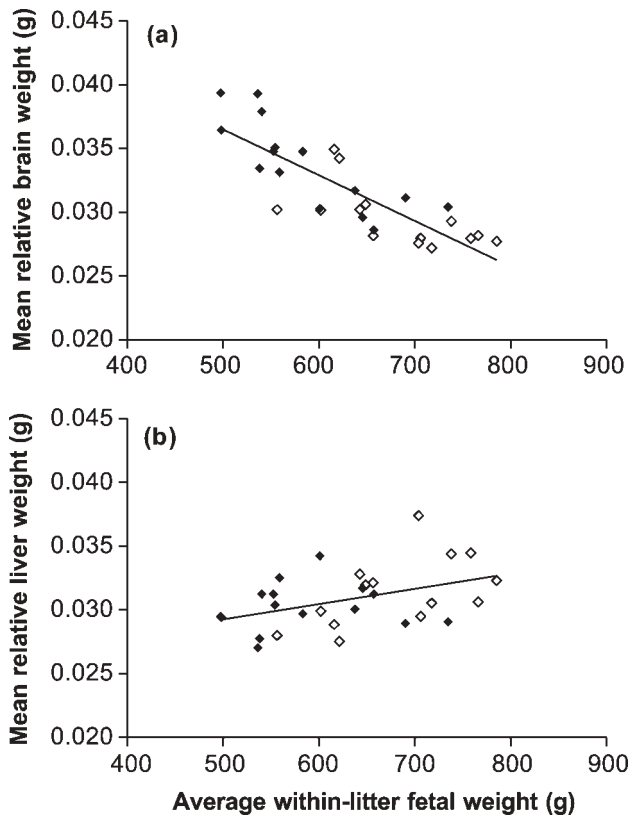
Figure 5 Correlation between (a) mean relative brain weight ($r^2 = -0.68$; $P < 0.0001$) and (b) mean relative liver weight ($r^2 = 0.18$; $P = 0.0231$) and average within-litter fetal weight at day 90 of gestation in LIG animals ($\diamond$) and CTR animals ($\blacklozenge$).

Figure 6 Correlation between mean brain:liver weight ratio and (a) average within-litter fetal weight ($r^2 = -0.58$; $P < 0.0001$) and (b) average within-litter placental weight ($r^2 = -0.55$; $P < 0.0001$) at day 90 of gestation in LIG animals ($\diamond$) and CTR animals ($\blacklozenge$).

Figure 7 Correlation between mean brain:liver weight ratio and number of viable fetuses at day 90 of gestation ($r^2 = 0.53$; $P < 0.0001$) in LIG animals ($\diamond$) and CTR animals ($\blacklozenge$).

and semitendinosus muscles ($r = 0.79$; $P < 0.0001$) and to semitendinosus CSA ($r = 0.74$; $P < 0.0001$). When relative organ weights were calculated as the absolute organ weight:body weight ratio, mean relative brain weight showed a strong negative correlation to mean fetal weight ($r = -0.82$, $P < 0.0001$; Fig. 5a). In contrast, mean relative liver weight was only weakly correlated with average fetal weight ($r = 0.42$; $P > 0.05$; Fig. 5b), and no correlations were evident ($P \geq 0.1$) with mean relative heart, lung, spleen and semitendinosus muscle weights and semitendinosus CSA. The mean brain:liver weight ratio was negatively correlated with mean fetal weight ($r = -0.76$; $P < 0.0001$; Fig. 6a) and mean placental weight ($r = -0.74$; $P < 0.0001$; Fig. 6b), but positively correlated to the number of viable fetuses ($r = 0.73$; $P < 0.0001$; Fig. 7).

Immunohistochemical analysis

Myosin isoforms

Immunohistochemistry results are shown in Fig. 8. For the day 90 fetal semitendinosus tissue, positive specific staining for MHCIβ (NOQ7.5.4D and BA-D5), and MHCIIa (SC-71) was observed. MY-32 (all fast MHC) seemed to give a positive reaction with primary fibres in addition
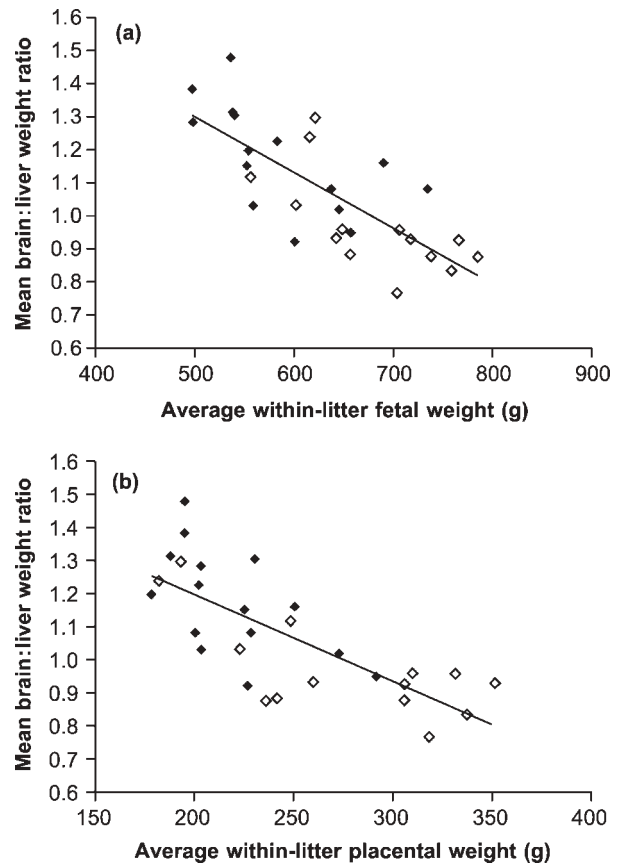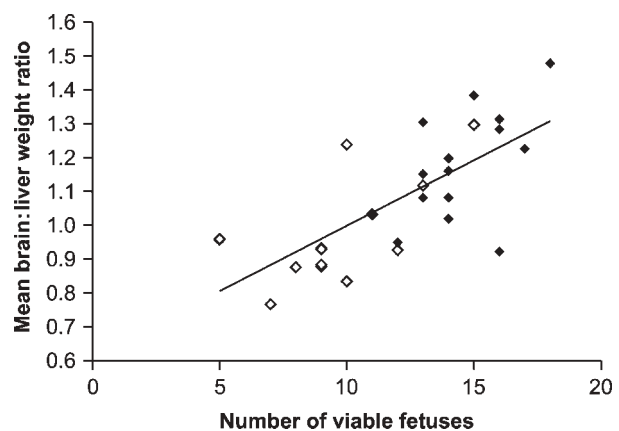
to positively staining secondary muscle fibres in day 90 tissue. BF-35 (recognizing all MHC isoforms except for MHCIId(x)) resulted in staining of all fibres present, probably indicating that no IId(x) fibres are present at day 90. No staining was observed for F88.12F8 (specific to
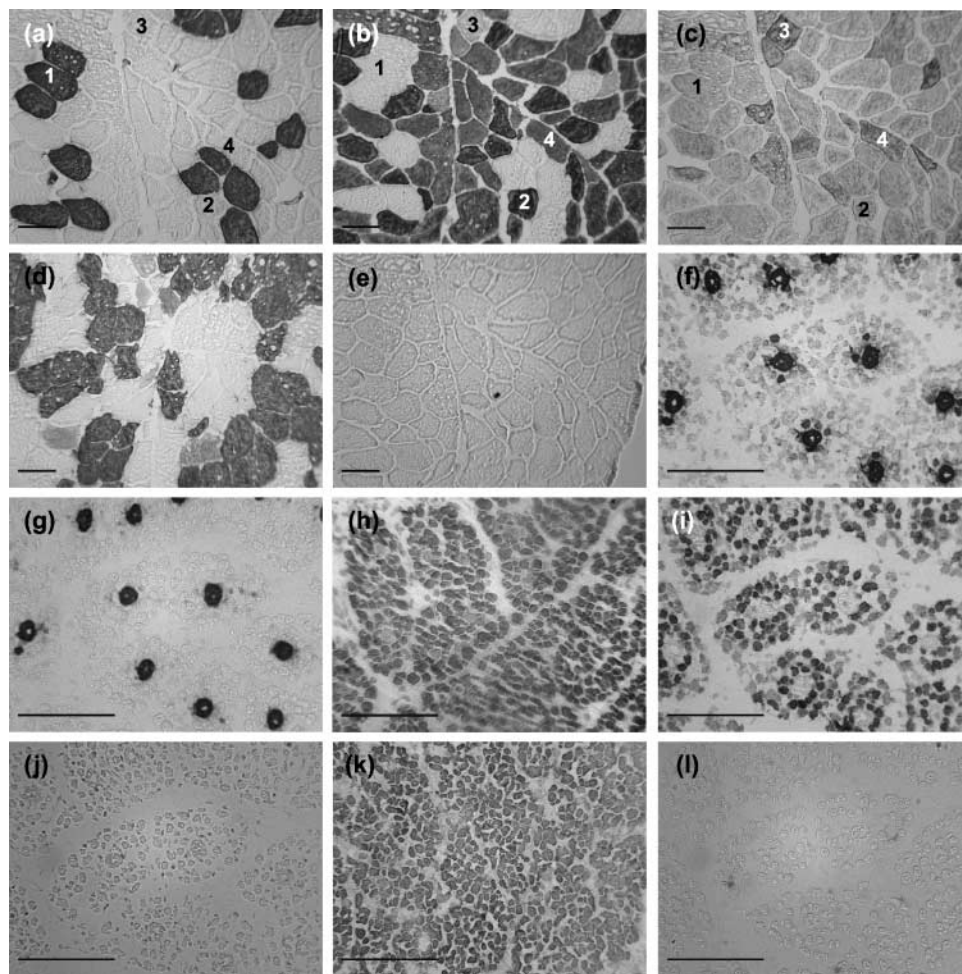
Figure 8 Immunohistochemical staining of the triceps brachii muscle of an adult pig (a-e) and the semitendinosus muscle of a day 90 fetus (f-l). (a) BA-D5 (anti-MHCIβ), (b) SC-71 (anti-MHCIIa), (c) BF-F3 (anti-MHCIIb), (d) BF-35 (anti-MHC except IIx/d), (e) IgG control. Numbers denote the same fibres across serial sections. (f) NOQ7.5.4D (anti-MHCIβ), (g) BA-D5 (anti-MHCIβ), (h) MY-32 (anti-MHCII all), (i) SC-71 (anti-MHCIIa), (J) BF-F3 (anti-MHCIIb), (k) BF-35, (l) IgG control. Scale bars represent 50 μm.

MHCIα; data not shown) or BF-F3 (specific to MHCIIb). Surprisingly, a negative result was also obtained for the developmental antibodies NCL-d (anti-MHC embryonic), NCL-n (anti-MHC neonatal) and BF-45 (anti-MHC embryonic). In adult triceps brachii tissue, which was used to confirm reactivity of the antibodies used with pig tissue, positive staining was observed using NOQ7.5.4D, BA-D5, and SC-71 and, in contrast to the day 90 semitendinosus tissue, BF-35 did not stain all fibres, indicating the presence of pure IId(x) fibres. Additionally, positive staining was observed for BF-F3, indicating that MHCIIb fibres are also present in adult tissue. F88.12F8 and MY-32 have previously been shown to react with swine MHCIα (Lefaucheur *et al.* 1995, 1997) and all fast MHC respectively (Fazarinc *et al.* 1995).

Muscle fibre count data

Muscle fibre development data obtained from the muscle sections stained using the antibody specific to MHCIβ (NOQ7.5.4D) are shown in Table 4. Average fibre number per mm^2 was not different between groups for either primary or secondary fibres, nor was average fibre CSA. However, muscle weight and muscle CSA were greater in fetuses from LIG sows. As a consequence, the total number of secondary fibres across the whole muscle was also greater in the fetuses of LIG sows. There was no difference between the LIG and the CTR fetuses in the number of primary fibres, or the secondary:primary fibre ratio. Fetal weight was positively correlated with the mean absolute number of secondary fibres ($r = 0.63$; $P = 0.0004$); however there was no correlation between fetal weight and the relative number of secondary fibres.

Standard SDS-PAGE

The MHC complement of whole muscle extracts of day 90 fetal tissue was analysed by SDS-PAGE and measured densitometrically. The electrophoretic separation of four different MHC isoforms is shown in Fig. 9. A comparison of their mobilities with bands obtained from rat muscle, adult pig muscle and neonatal pig muscle (Fig. 9) was carried out, and the distribution of MHC isoforms is shown in Table 5. The fetal MHC isoform was most abundant, followed by embryonic, type IIa and type Iβ. There were no differences in relative isoform distribution between fetuses from LIG and CTR sows (Table 5).

Table 4 Muscle fibre development data (means± S.E.M.) for day 90 fetuses from control (CTR) and unilaterally oviduct ligated (LIG) sows ($n = 28$).

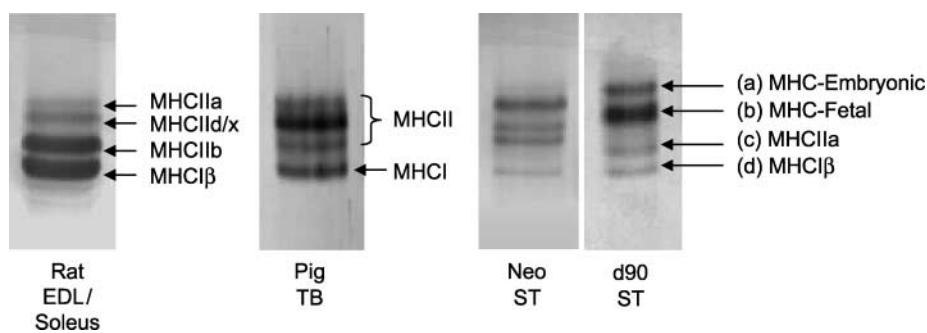|	Treatment group		
Parameter	CTR ($n = 14$) 'Relatively crowded'	LIG ($n = 14$) 'Non-crowded'	**P**
Primary fibre no./mm^2	29.5 ± 1.5	25.8 ± 1.3	0.073
Primary fibre CSA (μm^2)	123.5 ± 5.6	130.4 ± 4.0	0.33
Secondary fibre no./mm^2	678.7 ± 16.5	673.3 ± 18.6	0.83
Secondary fibre CSA (μm^2)	23.1 ± 1.5	20.2 ± 0.5	0.072
ST muscle weight (g)	1.25 ± 0.06	1.47 ± 0.09	0.014
ST muscle CSA (mm^2)	47.71 ± 2.85	58.78 ± 2.65	0.009
Number total primary fibres	1394 ± 81	1480 ± 57	0.39
Number total secondary fibres	32,691 ± 2098	39,628 ± 2074	0.027
Secondary:primary fibre ratio	24.01 ± 1.49	26.80 ± 0.06	0.13

ST, semitendinosus.

Figure 9 Electrophoretic separation of the MHC isoforms in rat exterior digitorum longus/soleus muscle, adult pig triceps brachii muscle, neonatal semitendinosus muscle and day 90 fetal semitendinosus muscle tissue.

Table 5 Myosin heavy chain (MHC) isoform distribution (mean± S.E.M.) in day 90 fetal semitendinosus muscle from control (CTR) and unilaterally oviduct ligated (LIG) sows ($n = 28$).

	Treatment group		
Parameter	CTR ($n = 14$) 'Relatively crowded'	LIG ($n = 14$) 'Non-crowded'	**P**
Embryonic MHC (%)	23.30 ± 2.37	25.05 ± 1.18	0.52
Fetal MHC (%)	58.61 ± 2.46	55.46 ± 1.72	0.30
Type IIa MHC (%)	13.26 ± 2.34	12.66 ± 0.82	0.81
Type Iβ MHC (%)	6.45 ± 1.12	6.84 ± 1.14	0.81

In-Gel immunodetection

In-Gel immunodetection with the MF-20 antibody that recognizes all MHC isoforms, confirmed that the four bands observed on the protein gels were all isoforms of MHC. Various antibodies were used to determine the identification of the four myosin bands. In the day 90 tissue, the band with the greatest mobility (band d) was identified as MHCIβ based on reactivity with NOQ7.5.4D. The band with the second greatest mobility (band c) was identified as MHCIIa based on reactivity with SC-71. Positive bands were not observed using the developmental antibodies NCL-d, NCL-n and BF-45 due to the apparent lack of reactivity of these antibodies with porcine muscle. However, the two upper bands observed in the day 90 samples were tentatively identified as fetal (band b) and embryonic (band a), based on the gel patterns established by Lefaucheur *et al.* (2001). As expected, these fetal and embryonic bands were not observed in adult tissue.

Discussion

Based on existing literature, the central hypothesis tested in this study was that increased uterine crowding in early gestation will have important consequences for fetal muscle fibre development of all surviving fetuses.

An ovulation rate of 19.90 ± 0.36 for this third parity sow population allowed the possibility of substantially increased uterine crowding compared with the gilts studied by van der Lende *et al.* (1990). Although this ovulation rate was considerably lower than that reported in other multiparous dam-line sows (Orzechowski 1998, Vonnahme *et al.* 2002, Town 2004), the number of viable embryos in CTR sows at day 30 (15.1) was similar to these earlier studies. In contrast, unilateral oviduct ligation resulted in a major reduction in the number of embryos at day 30 (9.3) and fetuses at day 90 (9.4), allowing us to test our central hypothesis over a range of uterine crowding that is known to exist in commercial herds. As the proportion of embryos surviving to day 30, and fetuses surviving to day 90, was higher in the LIG sows, it appears that mechanisms that drive selective reduction in the number of pre-implantation embryos, and match the number of conceptuses in the post-implantation period to functional uterine capacity, were both operative in the CTR sows.

Consistent with earlier studies (Almeida *et al.* 2000, Vonnahme *et al.* 2002), average placental weight was lighter in conceptuses of CTR sows at day 30 and was negatively correlated with the number of viable embryos; however, embryo weight was not different between groups. At day 90 of gestation, both placental weight and fetal weight were lighter in CTR sows, and placental weight was positively correlated with fetal weight. These relationships are consistent with the positive correlation between average placental weight at term and average birth weight seen in our previous studies (Town *et al.* 2002, $r^2 = 0.76$, $P < 0.001$), and reported by Biensen *et al.* (1999) and Wilson and Ford (2000). Also, both fetal and placental weight were inversely related to the number of viable fetuses at day 90, in agreement with data on fetal weight reported by Bauer *et al.* (1998). Collectively, we interpret these data as indicating that day 30 embryos were less sensitive to nutrient limitations than fetuses in later gestation. However, the early limitation in placental size in CTR sows at day 30 ultimately limited fetal development later in gestation, as was borne out by the day 90 data.

Generally, IUGR in animal studies has been defined in terms of fetuses or neonates weighing less than two standard deviations below the mean body weight for gestational age, or more frequently, below the mean body weight of the relevant study population. Whilst statistical definitions of IUGR are useful to classify extremely compromised and 'runted' individuals, the effects of IUGR are likely more complex and probably exist as a gradient of effects of growth restriction within a litter. As part of the central hypothesis tested in the present study, it is important to recognise that the fetuses and neonates chosen for measurement deliberately excluded the extremes of IUGR. Clearly, individuals within the relatively crowded CTR litters would be expected to show even greater IUGR. Indeed, using the classical definition of IUGR as those fetuses falling two standard deviations below the mean body weight of the litter, runt fetuses were present in twelve of the litters from the CTR, compared with only two of the litters from the LIG sows (data not shown).

Based on an analysis of relative organ weights used in previous studies (McMillen *et al.* 2001), relative heart, lung and spleen weights were unrelated to fetal weight, and relative liver weight was only weakly correlated with fetal weight, whereas relative brain weight showed a strong negative correlation to fetal weight (Fig. 5a). Together with strong negative correlations between brain:liver weight ratio and both fetal weight (Fig. 6a) and placental weight (Fig. 6b), the higher brain:liver weight ratio in fetuses from CTR litters, and the strong positive correlation between brain:liver weight ratio and the number of viable fetuses (Fig. 7), the data indicate detrimental effects of decreased placental size on prenatal development in the present study. Although not as large as the increase in brain:liver weight ratio in IUGR fetuses reported by Bauer *et al.* (1998, 2002), the increase in the day 90 brain:liver

weight ratio from 0.97 ± 0.04 in LIG fetuses to 1.17 ± 0.04 in CTR fetuses was, nevertheless, associated with a significant decrease in the number of secondary fibres in CTR fetuses.

The existence of limited periods or 'critical windows' of time for cell multiplication and differentiation in different organs and tissues is an essential driver of detrimental long term effects of IUGR. It is generally understood that a growth-retarding stress during the phase of cell proliferation in an organ will cause permanent restriction of that cell population, while a similar stress during the later growth phase may cause a reversible impairment in cell size. This is particularly relevant in the context of muscle fibre development. All available evidence indicates that differentiation of primary and secondary muscle fibres will be completed by day 90 in the pig (Wigmore & Stickland 1983). Therefore, even if the effects of uterine crowding on muscle fibre numbers are associated with decreased fetal weight at day 90, subsequent fetal growth will not correct the problem of limited numbers of muscle fibres. Evidence that the brain:semitendinosus muscle weight ratio, and the brain:total secondary muscle fibre ratio, were also higher in the CTR sows, is therefore of considerable economic, as well as physiological, significance.

Although the scope of the present experiment did not allow analysis of postnatal growth effects, earlier studies (Handel & Stickland 1987, Dwyer *et al.* 1993, 1994) have clearly demonstrated reduced postnatal growth potential due to quantitative effects on fetal muscle development through maternal undernutrition. The results of the present study extend the findings of previous work in the pig in which both naturally occurring IUGR within a litter (Aberle 1984) and nutritional manipulation during gestation (Dwyer *et al.* 1994) preferentially affected secondary muscle fibre development.

A clearer understanding of the origin and extent of effects of uterine crowding on muscle fibre development is clearly important and appropriate immunohistochemical and gel electrophoresis technologies for identifying crowding effects on muscle fibre development were established as part of the present study. In an extension of the methodology used by Lefaucheur *et al.* (2002), In-Gel immunodetection was used in combination with immuno-histochemistry to further elucidate the MHC isoforms present in porcine fetal muscle tissue. In-Gel immunodetection (data not shown) with the MF-20 antibody that recognizes all MHC isoforms confirmed that the bands observed by silver staining after gel electrophoresis were all isoforms of MHC. Consistent with the findings of Lefaucheur *et al.* (2001), the isoform that displayed the greatest electrophoretic mobility was identified as MHCIβ. Using immunohistochemistry, positive reactivity was also observed using BAD-5 (specific staining for MHCIβ) for both adult triceps brachii tissue and fetal day 90 semitendinosus tissue, and NOQ7.5.4D also showed specific staining for MHCIβ in day 90 semitendinosus tissue (Fig. 8). As reported by Lefaucheur *et al.* (2001) using

newborn piglet tissue, none of the primary fibres reacted with the antibody F88.12F8 (specific to MHCIα) in day 90 semitendinosus tissue, confirming that MHCIα is not present in the semitendinosus muscle until after birth in the pig.

Using immunohistochemistry, positive staining was observed for MHCIIa (using SC-71) for both day 90 and adult tissue and expression of this isoform was confirmed using In-Gel immunodetection. In contrast to Lefaucheur *et al.* (2001) who reported that all three adult MHCII isoforms (i.e. IIa, IIx and IIb) co-migrated using standard SDS-PAGE techniques, three fast MHC isoforms were observed in adult tissue in the present study and a total of four bands in these adult tissue samples is in agreement with the immunohistochemical evidence for the Iβ, IIa, IIb and IIx isoforms. In the case of day 90 fetal tissue, it is likely that band c (Fig. 9) is solely MHCIIa, as there was no evidence for the presence of IIb or IIx fibres based on the immunohistochemical results.

Based on the relative electrophoretic mobilities of the two upper bands observed in the day 90 samples by gel electrophoresis (Fig. 9), they were tentatively identified as fetal (band b) and embryonic (band a), described by Lefaucheur *et al.* (2001). Based on this definition, the fetal MHC isoform was most abundant, followed by embryonic, -IIa and -Iβ. Interestingly, developmental antibodies NCL-d, NCL-n and BF-45 that have been used in studies of tissue from other species (NCL-d, Putman *et al.* 2000; NCL-n, Ecob-Prince *et al.* 1989; BF-45, Schiaffino *et al.* 1988, Putman *et al.* 2003) do not appear to recognise the corresponding porcine isoforms by either immunohistochemical or In-Gel immunodetection methods. From the perspective of effects of crowding *in utero* and IUGR on myogenesis, there were no qualitative differences in the patterns of isoform distribution between fetuses from LIG and CTR sows, confirming previous results from our group (Town 2004). From a commercial perspective, this suggests that the size rather than the quality of the developing muscle is most affected in IUGR fetuses.

In summary, the present results are consistent with the central hypothesis tested, that even moderate crowding of the uterus at day 30 of gestation compromises subsequent fetal development and, specifically, the number of fetal secondary muscle fibres. In prolific sow genotypes, increased uterine crowding will exert even more severe effects on fetal muscle development and consequently on postnatal growth potential. These data provide important insights into the biological basis of variability in postnatal growth performance that has become an important economic concern for the swine industry.

Acknowledgements

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