Responses to maternal GH or ractopamine during early–mid pregnancy are similar in primiparous and multiparous pregnant pigs

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Abstract

Fetal growth is restricted in primiparous pigs (gilts) compared with dams who have had previous pregnancies (sows), as in other species. In gilts, daily maternal porcine GH (pGH) injections from day 25 to 50 of pregnancy (term ~115 day) increase fetal growth and progeny muscularity, and responses in sows are unknown. Whether feeding the β_2 -adrenergic agonist ractopamine during this period increases progeny growth rates in either parity and fetal responses in gilts, have not been investigated. We hypothesised that fetal and placental growth and fetal muscle development would be increased more by maternal pGH and/or ractopamine during early–mid pregnancy in gilts than sows, since fetal growth is restricted in gilts causing lower birth weights. Large White $\times$ Landrace gilts and sows were injected daily with water (controls) or pGH (~15 μ g/kg per day), or were fed 20 ppm

ractopamine, between day 25 and 50 of pregnancy. Maternal pGH increased litter average fetal weight (11%, $P=0.007$) and length (3%, $P=0.022$), but not placental weight, at day 50 of pregnancy, irrespective of parity, and had the greatest effects in the heaviest fetuses of each litter. Maternal ractopamine increased average fetal weight (9%, $P=0.018$), but not length. Muscle fiber diameter was increased by pGH in heavy littermates and by ractopamine in median littermates. Similar fetal growth responses to pGH and ractopamine in gilts and sows suggest that these hormones increase fetal nutrient availability similarly in both parities. We therefore predict that sustained pGH treatment will increase progeny birth weight, postnatal growth and survival, in both sows and gilts.

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Introduction

Primiparity and adolescent pregnancies each restrict fetal growth in humans and other species, including pigs (Ritter *et al.* 1984, Rasmussen & Fischbeck 1987, Bryan & Hindmarsh 2006). These may both contribute to the reduced birth weight and poorer subsequent performance of progeny of primiparous pigs (first pregnancy, gilts) compared with multiparous pigs (sows), since fetal growth and birth weight predict neonatal survival and postnatal growth and metabolism in pigs, as in other species (Winters *et al.* 1947, Fahmy & Bernard 1971, Campbell & Dunkin 1982, Wigmore & Stickland 1983, Dwyer *et al.* 1993). Multiple factors can restrict fetal growth and birth weight in pigs; competing maternal demands in growing adolescent animals, large and variable litter size, and restricted maternal nutrition used in commercial pig production systems during pregnancy. Interventions to increase fetal growth may

therefore be more effective in the gilt than the multiparous sow, where fetal growth and development are less constrained.

Manipulation of nutrition in the pig shows that early–mid pregnancy is a critical period for fetal growth and development, when increasing fetal growth leads to improved postnatal growth and muscle gain (Dwyer *et al.* 1994). This corresponds to the period of most rapid placental growth and differentiation in the pig (Knight *et al.* 1977). We and others have shown that daily maternal injections of gilts with porcine GH (pGH) during early–mid pregnancy increases fetal growth (Kelley *et al.* 1995, Gatford *et al.* 2000), numbers of muscle fibers in fetuses or progeny at birth (Rehfeldt *et al.* 1993, 2001b) and increases growth rates and muscle size of their postnatal progeny (Kelley *et al.* 1995, Gatford *et al.* 2003). Some studies have reported that the greatest responses to pGH are greatest in the smallest piglets within each litter (Sterle *et al.* 1995, Rehfeldt *et al.* 2001a, Rehfeldt & Kuhn 2006),

suggesting that the effects of pGH are greatest in the most restricted fetuses. Limited evidence from pigs and sheep also suggests that GH may act at least in part by increasing placental growth and/or function (Sterle *et al.* 1995, Harding *et al.* 1997, Rehfeldt *et al.* 2001a, Wallace *et al.* 2004). Whether pGH promotes fetal growth in sows with lower competing nutrient demands for maternal growth is not known.

Feeding pregnant pigs with β_2 -adrenergic agonists has been suggested as an alternate endocrine strategy to increase fetal and progeny growth and development. Progeny growth rates and carcass weight, but not birth weight, muscle size nor muscle fiber numbers, were increased in sows fed with the β_2 -adrenergic agonist ractopamine from day 25 to 50 of pregnancy (Hoshi *et al.* 2005a,b). Feeding sows the β_2 -adrenergic agonist salbutamol in the first third of pregnancy increased muscle size and altered muscle fiber types of progeny (Kim *et al.* 1994). Whether these changes in progeny muscle development are preceded by increased fetal and placental growth is not known, and parity differences in responses to β -adrenergic agonists have also not been explored.

We therefore tested the hypotheses that daily pGH injections or feeding ractopamine during early-mid pregnancy (from day 25 to 50) would promote fetal and placental growth and fetal muscle development to a greater extent in gilts than in sows, and that these effects would be greatest in the smallest fetuses in each litter.

Materials and Methods

Animals

The study was designed in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (National Health and Medical Research Council of Australia 2004) and approved by the University of Adelaide Animal Ethics Committee. The *in vivo* studies were conducted at the University of Adelaide Roseworthy Piggery. Totally 24 Large White $\times$ Landrace gilts were mated at 23 weeks of age, and 24 mature Large White $\times$ Landrace, third to fifth parity sows were mated at the first post-weaning estrus. Pigs were mated twice by artificial insemination using semen from Landrace or Large White boars, either in the morning and afternoon of the same day or on the afternoon and morning of consecutive days. The day of second insemination was taken as day 0 of pregnancy. All animals were individually housed in stalls throughout the study.

Nutrition and treatments

Gilts and sows were fed ~ 1 kg of a dry sow diet (13.0 MJ DE/kg, 15.2% total protein, 0.65% total lysine) on the day of mating (day 0) and day 1. From day 2 until the end of the

study, gilts were fed 2.2 kg/day and sows were fed 2.5 kg/day of the same diet. Pregnancy was confirmed by ultrasound scanning at day 23 of pregnancy, and dams were randomly allocated within parity groups to control, pGH-injected or ractopamine-fed treatment groups ($n=8$ per treatment and parity). Control dams were injected i.m. daily with 1 ml sterile water from day 25 to 50 of pregnancy. pGH-injected gilts and sows were injected i.m. daily with 1 ml sterile water containing 2.0 or 3.5 mg pGH respectively (recombinant pGH, Reporcin, OzBiopharm Pty Ltd, Knoxfield, Vic., Australia), which was calculated to provide a dose of ~ 15 μ g pGH/kg per day in both parity groups, based on previous live weight data from the herd. Ractopamine-fed dams were fed ractopamine at 20 ppm in the diet (added to the daily ration as Paylean, 2.2 g/day in gilts and 2.5 g/day in sows, Elanco Animal Health, Greenfield, IN, USA). One gilt (pGH-injected) returned to estrus in the first week of the treatment period and was removed from the study. Pregnant dams were weighed, and backfat depth were measured by ultrasound at the P2 site (110 mm from the midline over the 13th rib, using B-mode live ultrasound), at the start of treatments and on the day of post-mortem (Gatford *et al.* 2000).

Post-mortem

Maternal blood was collected by jugular venepuncture into EDTA tubes on the morning of post-mortem (day 50 of gestation) and placed on ice. Dams were then humanely killed and a hysterectomy performed. Number of fetuses and visible resorption sites were counted, and individual fetuses and their intact placentae were dissected from the uterus. Placentae were cut lengthways, laid out flat and digitally photographed on a board marked with a 1 cm grid for later measurement of placental area, made by tracing the perimeter of the placenta in VideoPro software (Leading Edge software, Adelaide, Australia). Fetal blood was collected into EDTA tubes from the umbilical cord immediately after removal of each fetus and placed on ice. Blood was centrifuged and plasma collected and stored frozen at -20 °C for later analyses. Weights, crown-rump length, abdominal circumference, head width, and sex of each fetus were measured and recorded. The fetal liver was dissected and weighed, and the left fetal hindlimb was removed, and a mid-femur cross-section was taken through the upper hindlimb. Fetal muscle sections were fixed in 4% paraformaldehyde in PBS for 24 h, then washed in PBS and embedded in paraffin wax. The maternal ovaries were removed and number of corpora lutea counted as a measure of ovulation rate. The proportion of fetuses plus visible implantations was calculated as the number of fetuses plus resorption sites divided by the number of corpora lutea. Fetal survival was calculated as the number of fetuses divided by the number of corpora lutea. Runt piglets were defined as fetuses with weight more than 2 s.d. below the mean weight calculated using the mean and s.d. of weight for their litter.

Hormone and metabolite analyses

GH concentrations in maternal plasma were measured by RIA. GH purified from porcine pituitaries (Lot# AFP10888C) was obtained from the National Hormone and Peptide Program (NHPP; Harbor–UCLA Medical Center, Torrance, CA, USA), NIDDK and Dr Parlow, and used as standard and as tracer for iodination. Rabbit anti-pGH (Lot# AFP422801), also obtained from the NHPP, was used as the primary antibody. All maternal samples were analysed for pGH in a single RIA with an intra-assay coefficient of variation (CV) of 9.9%. Insulin-like growth factors (IGFs) were extracted from plasma by size exclusion HPLC at pH 2.5, using a modification of the original procedure (Scott & Baxter 1986), as previously described (Owens *et al.* 1990). IGF-I concentrations were determined by RIA of neutralised chromatography fractions devoid of IGF binding proteins, using the Conlon rabbit polyclonal antibody to human IGF-I (Francis *et al.* 1989). All maternal plasma samples were extracted in a single HPLC run with a recovery of ^{125}I -IGF-I of 87.5% and assayed in a single RIA with an intra-assay CV of 13.7% for a HPLC eluate pool containing 18 ng/ml IGF-I. Covariance for extraction and assay of a maternal porcine plasma quality control pool containing 174 ng/ml IGF-I and included at the start, middle and end of the HPLC run of maternal samples was 7.2% ($n=1$ HPLC runs, 3 measurements). Insulin in maternal plasma was measured in a single assay using a commercially available RIA kit (Human insulin-specific RIA kit, HI-14K, Linco Research Inc., St Charles, MO, USA) with an intra-assay CV of 6.4 and 100% cross-reactivity with porcine insulin. Glucose and urea in maternal and fetal plasma were measured by colorimetric enzymatic analysis on a Hitachi 912 automated metabolic analyser using Roche/Hitachi Glucose/HK and UREA/BUN kits respectively (Roche Diagnostics GmbH).

Fetal muscle histology

Fetal muscle fiber density and size were measured on the lightest (non-runt), median weight, and heaviest fetus of each litter. Cross-sections (7 μm) of the fetal muscle were cut from embedded blocks and mounted on electrostatically charged slides (Superfrost Plus, Menzel GmbH & Co. KG, Braunschweig, Germany). Sections were stained with hemotoxylin–eosin, and images digitally captured using a 40 $\times$ lens and the NanoZoomer slide capture system (Hitachi). *M. semitendinosus* muscle cross-sectional area, muscle fiber density and muscle fiber diameters were measured on all sections where the *M. semitendinosus* was complete and fibers approximately perpendicular to the section. Cross-sectional area was measured by tracing the *M. semitendinosus* perimeter in the NanoZoomer viewing software (NDP View, Hitachi). For each section, 12 fields were captured at 20 $\times$ resolution for later counting of muscle fibers using random-systematic sampling, starting in the upper left-hand quadrant and capturing fields with constant horizontal and vertical spacing. All fibers (primary and secondary

combined) were counted in an area of 0.1055 mm² per field using VideoPro image analysis software (Leading Edge). Fiber diameter of primary fibers was measured at $\times 40$ resolution using NDP View software for 20 fibers per field and 12 fields per *M. semitendinosus* (total 240 fibers per fetus), with diameter measured on primary fibers falling closest to points on a sampling grid.

Statistical analyses

One gilt returned to estrus within 1 week of commencing treatment and was excluded from the study. The effects of maternal parity, treatment and their interaction on maternal and litter outcomes were analysed using a two-way ANOVA model, with litter size (total number of fetuses) included as a covariate. Specific contrasts were performed to test the *a priori* hypotheses that maternal pGH treatment or ractopamine treatment would increase fetal growth and survival. Hormone and metabolite data were excluded for two pGH-treated sows that were not injected on the morning of post-mortem. Mixed model procedures were used to further evaluate weights of individual fetuses and placentae and fetal:placental weight ratios, to allow analysis of effects of fetal sex and also to compare responses to maternal factors in littermates of varying weight within each litter. All fetuses were ranked and assigned to a quartile of weight within their litter. Sire breed did not affect outcomes and was excluded as a factor in the models. These mixed models included maternal parity, maternal treatment, fetal sex, fetal weight quartile, and their interactions, and litter size was included as a covariate. Measures on each fetus within a litter were treated as repeated measures on the dam, and multiple comparisons between treatment groups for repeated measures models used the Bonferroni's correction method. Where effects of parity or treatment varied with fetal weight quartile, effects of sex, maternal treatment, and maternal parity were evaluated separately for fetuses of each weight quartile. Fetal *M. semitendinosus* characteristics were similarly evaluated, using a repeated measures model including maternal parity, maternal treatment, fetal size group (lightest, median, or heaviest of litter), and their interactions. Fetal sex and litter size did not affect *M. semitendinosus* characteristics and were excluded from these models. Data were log transformed before analysis where necessary to achieve equal variances and normality. All tests were carried out using SPSS v17.0 for Windows (SPSS Inc., Chicago, IL, USA) and are presented as mean $\pm$ S.E.M. for reproductive data and estimated mean $\pm$ S.E.M. where litter size was included in the model, unless otherwise indicated.

Results

Maternal weight and backfat

Prior to the start of treatments, maternal weight and maternal P2 backfat depth were greater in sows than in gilts ($P<0.001$ for each), but did not differ between treatment groups (Fig. 1).

Figure 1 Effects of maternal treatments and parity on maternal weight (A and B) and P2 backfat depth (C and D). Data are estimated means $\pm$ S.E.M., corrected for the average litter size of 9.87 fetuses. *Indicates $P < 0.05$, #indicates $P < 0.1$.

After treatment, maternal weight was higher in sows than in gilts ($P < 0.001$) and not different between treatments ($P = 0.541$, Fig. 1). Maternal P2 backfat depth after treatment remained higher in sows than gilts ($P < 0.001$) and differed between treatments ($P = 0.078$), being lower in pGH-treated dams ($P = 0.029$) but not in ractopamine-treated dams ($P = 0.120$) than in controls (Fig. 1). The effect of treatment on change in maternal weight during the treatment period differed between parities ($P = 0.010$). In sows, pGH-treated dams gained more weight than control dams ($P = 0.013$, controls: 14.4 ± 1.1 kg, pGH-treated: 19.7 ± 1.2 kg), and ractopamine-treated dams tended to gain less weight than controls ($P = 0.070$ ractopamine-treated: 9.9 ± 1.2 kg). In gilts, weight change across the treatment period did not

differ between groups ($P = 0.362$). Neither maternal treatment ($P = 0.792$) nor parity ($P = 0.448$) affected the change in P2 backfat depth during the treatment period (mean 0.23 ± 0.53 mm).

Ovulation rate and litter size

Total ovary weight was greater in sows than in gilts at day 50 of pregnancy ($P < 0.001$; multiparous: 20.1 ± 0.7 g; primiparous: 14.6 ± 0.7 g). Ovulation rate (Table 1) was higher in sows than in gilts ($P < 0.001$) and did not differ between animals allocated to each treatment group after mating ($P = 0.299$). The number of resorption sites ($P = 0.065$) and the total number of fetuses ($P = 0.086$) tended to be greater in sows than in gilts, but were not different between treatments (Table 1). Fetal survival at day 50 of gestation was higher in gilts than in sows ($P = 0.010$; sows: $53 \pm 4\%$, gilts: $69 \pm 4\%$) and did not differ between treatments (Table 1). Numbers of fetuses plus visible implantation sites as a proportion of ovulation rate was also higher in gilts than sows ($P = 0.049$) and did not differ between treatments overall or within either parity ($P > 0.1$ for all, Table 1). Litter size (total number of fetuses, Table 1) tended to be higher in sows than gilts ($P = 0.086$) and did not vary with treatment overall ($P = 0.761$) or within either parity (gilts: $P = 0.134$, sows: $P = 0.266$).

Fetal and placental size

Litter average fetal weight was increased by maternal pGH treatment (11%, $P = 0.007$) and by maternal ractopamine treatment (9%, $P = 0.018$) compared with controls, and did not differ between gilts and sows or with litter size (Table 2). The sum of fetal weights for each litter increased with increasing litter size ($P < 0.001$) was increased by maternal pGH-treatment ($P = 0.014$), tended to be increased by maternal ractopamine treatment ($P = 0.071$), and did not differ between parities ($P = 0.181$). Litter average fetal crown-rump length was increased in pGH-treated dams

Table 1 Effects of maternal treatments and parity on reproductive performance^a

	Gilts			Sows			Significance		
	Control	pGH	Ractopamine	Control	pGH	Ractopamine	Treatment	Parity	T $\times$ P
Outcome									
Number of dams	8	7	8	8	8	8			
Ovulation rate	12.8 ± 1.1	12.9 ± 0.7	14.3 ± 1.4	21.4 ± 1.5	18.9 ± 0.7	21.3 ± 1.4	NS	< 0.001	NS
No. of fetuses	7.4 ± 1.2	10.4 ± 0.5	8.9 ± 1.1	12.4 ± 1.1	9.8 ± 1.7	9.6 ± 1.1	NS	0.086	0.053^b
No. of resorptions	0.6 ± 0.4	0.7 ± 0.4	1.5 ± 0.7	3.1 ± 1.1	1.5 ± 0.8	1.9 ± 1.0	NS	0.065	NS
Fetal survival (%)	62 ± 10	82 ± 5	63 ± 7	59 ± 6	51 ± 8	48 ± 7	NS	0.010	NS
Fetuses plus visible resorptions (%)	66 ± 9	88 ± 5	71 ± 7	73 ± 4	58 ± 10	56 ± 8	NS	0.049	0.053^c

^aMaternal data are mean $\pm$ S.E.M. NS indicates $P > 0.1$.

^bLitter size was not altered by treatment in either gilts ($P = 0.134$) or sows ($P = 0.266$).

^cThe number of fetuses plus visible resorptions as a proportion of corpora lutea number was not altered by treatment in either gilts ($P = 0.111$) or sows ($P = 0.251$).

Table 2 Effects of maternal treatments and parity on litter average fetal and placental size^a

	Gilts			Sows			Significance		
	Control	pGH	Ractopamine	Control	pGH	Ractopamine	Litter size	Treatment	T×P
Outcome									
Number of dams	8	7	8	8	8	8			
Number of fetuses	59	73	71	99	78	77			
Fetal weight (g)	37.1 ± 1.5	41.7 ± 1.5	40.6 ± 1.4	36.0 ± 1.5	39.6 ± 1.4	39.4 ± 1.4	NS	0.013 ^b	NS
Fetal crown-rump length (mm)	117 ± 2	121 ± 2	119 ± 1	114 ± 2	118 ± 1	116 ± 1	NS	0.068 ^c	0.019
Fetal abdominal circumference (mm)	76.4 ± 1.1	79.9 ± 1.1	78.4 ± 1.0	77.5 ± 1.1	79.5 ± 1.0	78.8 ± 1.0	NS	0.037 ^d	NS
Fetal head width (mm)	15.9 ± 0.2	16.8 ± 0.2	16.6 ± 0.2	15.8 ± 0.2	16.3 ± 0.2	16.2 ± 0.2	NS	0.012 ^e	NS
Fetal liver weight (g)	2.74 ± 0.14	3.20 ± 0.15	2.94 ± 0.14	2.86 ± 0.14	3.28 ± 0.14	2.95 ± 0.14	0.050	0.010 ^f	NS
Fetal liver weight (% of fetal weight)	7.40 ± 0.26	7.65 ± 0.26	7.19 ± 0.25	7.88 ± 0.26	8.29 ± 0.25	7.45 ± 0.25	0.001	0.046 ^g	0.034
Placental weight (g)	97 ± 8	116 ± 8	108 ± 8	105 ± 8	115 ± 8	110 ± 8	0.013	NS	NS
Placental area (cm ²)	880 ± 80	875 ± 74	853 ± 70	901 ± 73	994 ± 69	1014 ± 69	0.035	NS	0.100
Fetal:placental weight	0.40 ± 0.03	0.40 ± 0.03	0.41 ± 0.03	0.38 ± 0.03	0.40 ± 0.03	0.43 ± 0.03	0.002	NS	NS

^aFetal data are estimated mean ± s.e.m. of within-litter average, corrected to an average litter size of 9.72. NS indicates $P > 0.1$.^bAverage fetal weight was higher in pGH-treated ($P = 0.007$) and in ractopamine-treated than in control dams ($P = 0.018$).^cAverage fetal crown-rump length was higher in pGH-treated than control dams ($P = 0.022$), and did not differ between ractopamine-treated and control dams ($P > 0.3$).^dAverage fetal abdominal circumference was higher in pGH-treated than control dams ($P = 0.011$), and did not differ between ractopamine-treated and control dams ($P > 0.1$).^eAverage fetal head width was higher in pGH-treated ($P = 0.004$) and in ractopamine-treated than in control dams ($P = 0.029$).^fAverage fetal liver weight (g) was higher in pGH-treated than control dams ($P = 0.003$), and did not differ between ractopamine-treated and control dams ($P > 0.1$).^gAverage fetal liver weight (as a % of fetal body weight) did not differ between pGH-treated and control dams ($P = 0.195$), or between ractopamine-treated and control dams ($P = 0.203$).

(3%, $P = 0.022$), not altered in ractopamine-treated dams ($P > 0.3$), and was higher in gilts than in sows (3%, $P = 0.019$, Table 2). Similarly, litter average fetal abdominal circumference was increased in pGH-treated dams (4%, $P = 0.011$) but not in ractopamine-treated dams ($P > 0.1$), and was not altered by maternal parity (Table 2). Litter average fetal head width was increased by maternal treatment with pGH (4%, $P = 0.004$) or ractopamine (3%, $P = 0.029$) and not altered by litter size or parity (Table 2). Litter average fetal liver weight was increased by maternal pGH treatment (16%, $P = 0.003$), not altered by maternal ractopamine ($P > 0.1$), and negatively related to litter size ($P = 0.050$). Relative to fetal body weight, fetal liver weight did not differ in pGH- or ractopamine-treated dams compared with controls ($P > 0.1$ for each), was strongly and negatively related to litter size ($P = 0.001$), and was higher in sows than in gilts (6%, $P = 0.034$). Placental weight and area were negatively related to litter size, not altered by maternal treatment, and the latter tended to be greater in sows than in gilts (12%, $P = 0.100$). Fetal:placental weight ratios were positively related to litter size and not altered by maternal treatment or parity (Table 2).

Effects of treatment and fetal sex varied between fetuses of varying weight quartiles within their litters (treatment × quartile interaction: $P = 0.005$, fetal sex × quartile interaction: $P < 0.001$, Fig. 2), effect of parity differed between male and female fetuses ($P = 0.042$), and a three-way interaction between treatment, parity and fetal sex was also evident ($P = 0.048$). Effects of fetal sex, maternal treatment and maternal parity were therefore tested separately for fetuses within each fetal weight quartile, including litter size as a covariate. For fetuses within the first quartile for fetal weight (all fetuses within the lowest 1/4 by rank of weight for their litters), fetal weight tended to be negatively related to litter size ($P = 0.089$), was greater in males than females ($P = 0.001$), and fetal weight did not vary with treatment ($P = 0.217$) or parity ($P = 0.345$). For fetuses within the second quartile of fetal weight, fetal weight was not affected by sex ($P = 0.599$) or maternal parity ($P = 0.353$), and tended to be altered by treatment ($P = 0.094$), but did not differ between any pairs of treatment groups ($P > 0.1$ for each). For fetuses within the third quartile of fetal weight, fetal weight was not affected by litter size ($P = 0.334$), sex ($P = 0.376$), or maternal parity ($P = 0.998$), but was altered by maternal treatment ($P = 0.027$), with fetal weight increased in pGH-treated dams ($P = 0.008$) and ractopamine-treated dams ($P = 0.020$) compared with controls. For fetuses within the fourth quartile of fetal weight (heaviest within litters), fetal weight was not affected by litter size ($P = 0.310$), sex ($P = 0.107$), or parity ($P = 0.285$), and was altered by maternal treatment ($P = 0.036$), with fetal weight tending to be increased in pGH-treated dams ($P = 0.082$) and in ractopamine-treated dams ($P = 0.070$) compared with controls.

Effects of litter size, maternal parity and treatment, fetal sex and fetal weight quartile were similarly tested for placental weight and fetal:placental weight ratio, treating measures of individual placentae and fetal-placental pairs as repeated

Figure 2 Effects of (a) maternal treatments and (b) fetal sex on fetal weight within each quartile of fetal weight. Data are estimated means $\pm$ S.E.M. Panel (a): data for control dams are shown in the lightest bars, for pGH-treated dams in mid-gray bars, and for ractopamine-treated dams in dark gray bars. Panel (b): data for female fetuses are shown in the light gray bars and for male fetuses in dark gray bars.

measures on the dam and including litter size as a covariate. Placental weight was negatively related to litter size ($P=0.011$). Placental weight varied with fetal weight quartile ($P<0.001$), being heaviest in placentae from the heaviest fetuses and differing between each quartile of fetal weight ($P<0.01$ for each). Placentae from female fetuses were heavier than those from males overall ($P<0.001$, males:

Figure 3 Effects of maternal parity on placental weight within each quartile of fetal weight. Data are estimated means $\pm$ S.E.M. Data for gilts are shown in the lightest bars and for sows in dark gray bars.

99 ± 4 g, females: 108 ± 4 g). Effects of maternal treatment varied with parity and fetal weight quartile ($P=0.032$, three-way interaction), while effects of maternal parity varied with fetal weight quartile ($P<0.001$, Fig. 3). For fetuses in the lightest quartile of weight for their litter, placental weight was negatively related to litter size ($P=0.001$), and did not differ between maternal treatments ($P=0.413$), parity ($P=0.431$) or fetal sex ($P=0.708$). For fetuses in the second quartile of weight for their litter, placental weight was also negatively related to litter size ($P=0.004$), was higher in female than male fetuses ($P<0.001$), and not altered by maternal parity or treatment. For fetuses in the third quartile of weight for their litter, placental weight was also negatively related to litter size ($P=0.012$), was greater in female than male fetuses ($P=0.037$), and higher in gilts than in sows ($P=0.045$), but not altered by maternal treatment ($P>0.5$). For the heaviest littermates in the fourth quartile of fetal weight, placental weight did not differ with litter size, maternal treatment or parity or fetal sex ($P>0.1$ for each).

Fetal:placental weight ratio was positively related to litter size ($P=0.003$), decreased with increasing fetal weight quartile ($P<0.001$), was higher for male than female fetuses ($P<0.001$), and not affected by maternal treatment ($P=0.405$) overall. Effects of maternal parity varied between quartiles of fetal weight ($P=0.002$), with a similar trend was observed for a treatment $\times$ quartile interaction ($P=0.054$), and interactions between parity $\times$ treatment $\times$ quartile and parity $\times$ treatment $\times$ fetal sex were also observed ($P=0.004$ and $P=0.011$ respectively). Fetal:placental weight ratio was positively related to litter size in each quartile of fetal weight ($P<0.1$ for each quartile) and not affected by maternal treatment or parity in any quartile.

Maternal and fetal hormones and metabolites

Maternal plasma GH and IGF-I were each altered by treatment (Table 3). Maternal plasma GH and IGF-I were each increased in GH-treated dams (~ 9 -fold, $P<0.001$ and ~ 2.7 -fold, $P<0.001$ respectively), and maternal plasma IGF-I was negatively affected by litter size ($P=0.004$). Maternal plasma insulin did not differ between parities or with litter size and was altered by treatment, tending to be decreased in ractopamine-fed dams (-33% , $P=0.053$), but not altered by maternal pGH-treatment ($P=0.513$, Table 3). Maternal plasma glucose was unaltered by litter size, treatment or parity (Table 3). Litter average fetal plasma glucose, however, decreased with increasing litter size ($P=0.026$), was unaffected by maternal parity ($P>0.1$), and tended to be decreased in ractopamine-fed dams compared with controls (-23% , $P=0.051$). Fetal:maternal plasma glucose ratio was negatively related to litter size ($P=0.013$), and tended to vary with treatment ($P=0.067$), tending to be decreased in pGH-treated dams (-24% , $P=0.059$) and being decreased in ractopamine-fed dams (-26% , $P=0.034$, Table 3). Maternal plasma urea was decreased by pGH-treatment (-28% , $P=0.001$) and

Table 3 Effects of maternal treatments and parity on maternal and fetal metabolites^a

	Gilts			Sows			Significance		
	Control	pGH	Ractopamine	Control	pGH	Ractopamine	Litter size	Treatment	Parity
Outcome									
Number of dams	8	7	8	8	6	8			T×P
Maternal plasma GH (ng/ml)	1.4±3.7	25.5±4.1	1.3±3.5	3.3±3.8	14.7±3.8	1.4±3.8	0.311	<0.001	0.183
Maternal plasma IGF-I (ng/ml)	118±15	261±16	95±14	104±15	337±15	94±15	0.004	<0.001	NS
Maternal plasma insulin (mU/l)	41.1±6.0	45.4±8.1	24.3±5.7	30.6±6.1	34.8±6.1	23.6±6.6	NS	0.045 ^c	NS
Maternal plasma glucose (mmol/l)	3.26±0.20	3.37±0.22	3.23±0.19	3.10±0.22	3.50±0.20	3.06±0.19	NS	NS	NS
Fetal plasma glucose (mmol/l)	1.48±0.17	1.26±0.19	1.19±0.16	1.44±0.18	1.26±0.17	1.07±0.16	0.026	NS	NS
Fetal:maternal plasma glucose	0.47±0.06	0.38±0.06	0.37±0.06	0.50±0.07	0.36±0.06	0.35±0.06	0.013	0.067	NS
Maternal plasma urea (mmol/l)	2.61±0.16	2.00±0.18	2.71±0.15	2.59±0.16	1.73±0.16	2.58±0.15	NS	<0.001 ^d	NS
Fetal plasma urea (mmol/l)	2.88±0.18	2.26±0.20	2.85±0.17	2.97±0.18	2.35±0.18	2.69±0.17	NS	0.005 ^e	NS
Fetal:maternal plasma urea	1.11±0.08	1.15±0.09	1.06±0.08	1.16±0.08	1.39±0.08	1.06±0.08	NS	0.042 ^f	NS

^aData from two dams not treated on the day of post-mortem were excluded from this analysis. Maternal data are estimated mean $\pm$ S.E.M., and fetal data are estimated mean $\pm$ S.E.M. of within-litter averages, each corrected to the average litter size of 9.56 in these dams. NS indicates $P>0.1$.

^bIn sows, maternal plasma IGF-I was higher in pGH-treated dams than in controls ($P<0.001$) and not altered by ractopamine treatment ($P>0.9$). In gilts, maternal plasma IGF-I was increased in pGH-treated dams ($P<0.001$) and decreased in ractopamine-treated dams ($P=0.046$).

^cMaternal plasma insulin did not differ between controls and pGH-treated dams ($P>0.5$) and tended to be decreased in ractopamine-treated dams ($P=0.053$ for each).

^dMaternal plasma urea was lower in pGH-treated dams than in control dams ($P<0.001$) and not altered in ractopamine-treated dams ($P<0.7$).

^eFetal plasma urea was lower in pGH-treated dams than in control dams ($P=0.001$) and not altered in ractopamine-treated dams ($P>0.3$).

^fFetal:maternal plasma urea was not different in pGH-treated dams ($P=0.111$) or ractopamine-treated dams ($P>0.3$) than in control dams.

unaltered by ractopamine-treatment ($P>0.7$), parity or litter size (Table 3). Fetal plasma urea was similarly decreased in pGH-treated dams compared with control dams (-21% , $P=0.005$), but was unaltered in fetuses from ractopamine-treated dams ($P>0.3$), and not altered by parity or litter size (Table 3). Fetal:maternal plasma urea ratios were not altered by litter size or maternal parity, were altered by treatment ($P=0.042$), but did not differ in pGH- or ractopamine-treated dams compared with controls ($P>0.1$ for each, Table 3).

Fetal muscle development

Infrequent secondary fibers ($<5\%$ of all fibers) were present in 91% of analysed sections (Fig. 4), and the proportion of sections with secondary fibers was similar in fetuses of varying size (smallest non-runs 25/27; median-weight 20/23; largest 22/24). All fibers in a section were counted in order to obtain fiber density, while diameter was measured only for primary fibers, since effects on primary muscle fiber size may underlie consequences for later secondary fiber development (Wigmore & Stickland 1983).

The cross-sectional area of the *M. semitendinosus* tended to differ between fetal size groups and was not affected by maternal treatment ($P=0.109$), while the effect of maternal parity differed between fetal size groups ($P=0.038$). Within each fetal size group, maternal parity did not alter *M. semitendinosus* cross-sectional area ($P>0.1$ for each group), while maternal ractopamine tended to increase *M. semitendinosus* cross-sectional area in the heaviest ($P=0.078$) and lightest ($P=0.056$) fetuses, but not in median-weight fetuses ($P>0.7$). Overall, muscle fiber density varied significantly with fetal size group ($P<0.001$), and tended to differ between parities (gilts greater than sows, $P=0.057$) and with maternal treatments ($P=0.090$). This mainly reflected responses in the heaviest fetuses, where muscle fiber density was higher in fetuses from gilts than those from sows ($P=0.015$), and was decreased by pGH treatment ($P=0.018$). The total number of muscle fibers in the *M. semitendinosus*, calculated as the product of cross-sectional area and fiber density, did not differ between fetal size groups ($P=0.979$) or maternal treatments ($P=0.219$). Effects of parity on total fiber number again differed between fetal size groups ($P=0.001$). In the heaviest fetuses, total fiber number was greater in gilt than sow fetuses ($P=0.017$), and ractopamine tended to increase total fiber number ($P=0.053$). In median and lightest fetuses, total fiber number was unaffected by maternal parity or treatment.

Fetal average *M. semitendinosus* primary fiber diameter (Fig. 5) varied between fetal size groups ($P<0.001$), and was not altered by maternal parity ($P=0.134$), while the effects of maternal treatment differed between fetal size groups ($P=0.025$). In the largest littermates, fiber diameter tended to be increased by maternal pGH ($P=0.064$), in median-weight littermates, fiber diameter was increased by maternal

Figure 4 Cross-section of day 50 fetal pig muscle, stained with haematoxylin-eosin and showing visible secondary fibers (arrows). Full colour version of this figure available via <http://dx.doi.org/10.1677/JOE-09-0131>.

ractopamine ($P=0.025$), and in the lightest littermates, fiber diameter was unaffected by maternal treatment ($P=0.895$). Across all fetuses, fetal weight correlated positively with *M. semitendinosus* cross-sectional area ($r=0.327$, $P=0.004$, $n=74$) and primary muscle fiber diameter ($r=0.285$, $P=0.014$, $n=74$), and negatively with fiber density ($r=-0.483$, $P<0.001$, $n=74$). Fiber density correlated negatively with primary muscle fiber diameter across all fetuses ($R=-0.624$, $P<0.001$, $n=74$), as well as within each size group ($P<0.011$ for each).

Figure 5 Effects of maternal treatments on fetal *M. semitendinosus* primary fiber diameter within each quartile of fetal weight. Data for control dams are shown in the lightest bars, for pGH-treated dams in mid-gray bars, and for ractopamine-treated dams in dark gray bars.

Discussion

We have shown for the first time that treating the mother with pGH in early-mid pregnancy increases fetal growth in mature multiparous sows as well as in gilts in their first pregnancy. Maternal treatment with ractopamine also increased fetal weight, and this response did not differ between dams of different parities, contrary to our initial hypothesis. This suggests that these maternal treatments increase nutrient availability to the fetus to a similar extent in both parities and that the faster relative growth rates of gilts, and hence likely nutrient demand for maternal growth, do not limit the actions of these maternal treatments. The lack of difference in fetal weight between control gilts and sows at day 50 of gestation also suggests that primiparity and adolescent pregnancy may not restrict fetal growth until later in pregnancy in the pig, resulting in the lower birth weight of gilt compared with sow progeny (Ritter *et al.* 1984). Fetal growth was increased by 14 and 11% respectively in pGH-treated gilts and sows, compared with the control dams in each parity, with no significant parity $\times$ treatment interactions. Increased fetal growth in pGH-treated dams in the present study is consistent with reports from previous studies of pGH in gilts, in which both placental and fetal growth increases have been reported at the end of treatment (Kelley *et al.* 1995, Sterle *et al.* 1995, Gatford *et al.* 2000). Although litter average progeny birth weights or near term fetal weights are not increased when maternal pGH treatment stops around mid-pregnancy (Rehfeldt *et al.* 1993, 2001b, Kelley *et al.* 1995, Okere *et al.* 1997, Gatford *et al.* 2004), fetal and subsequent progeny development is altered and progeny from pGH-treated dams have larger muscles after weaning (Kelley *et al.* 1995, Gatford *et al.* 2003). In contrast with previous reports of greater progeny muscle development and placental length responses to pGH in small than in large littermates (Sterle *et al.* 1995, Rehfeldt *et al.* 2001a,b, Rehfeldt & Kuhn 2006), we found greater fetal growth responses to pGH in heavier littermates than in lighter littermates. Maternal pGH did not reduce fetal weight variability or act selectively to improve growth in the smallest fetuses in the present study. This difference in relative responses may reflect a dose effect (2 mg/day in our gilts cf. 5–6 mg/day in previous studies), or greater maternal constraint of large fetuses in the present study due to more restricted maternal nutrition, reflecting current commercial practice, such that large fetuses had a greater potential to respond to increased nutrient availability than small fetuses. Importantly, these results together suggest that hormonal treatments to increase fetal growth and subsequent progeny growth and performance will be similarly effective in young growing gilts and mature sows.

GH does not cross the placenta in rats (Fohlenhag *et al.* 1994), and the absence of circulating GH in decapitated fetal pigs suggests that this is also true in pigs (Martin *et al.* 1984). Increased fetal growth in pGH-treated dams must therefore be due to changes in maternal metabolism and/or placental

growth and function. In the present study, maternal pGH did not alter placental weight or size at the end of treatment, although placental weight was negatively related to litter size and greater in heavier fetuses, consistent with placental nutrient delivery as a major determinant of fetal growth, even in early gestation. Recent research also suggests that the fetus may signal the placenta to in part drive nutrient supply (Fowden *et al.* 2009), so that rapidly growing fetuses may partially determine the growth and function of their own placenta as well as vice-versa. Consistent with this hypothesis, studies of placentae from small and large littermates also suggest that placental structural adaptation throughout gestation is accelerated in fetal pigs with small placentae, which may help to maintain fetal nutrient supply (Vallet & Freking 2007). In contrast to the lack of effect of maternal pGH on placental weight in the present study, Sterle *et al.* (1995) reported that 14 day maternal pGH treatment increased placental weight and increased fetal weight:placental length ratio in gilts at day 44 of gestation, implying increased placental function. The different effects of pGH on placental weight in these two studies may reflect the approximately twofold higher doses of pGH used by the latter, and/or the different timing of treatments. In sheep, twice daily bGH injections in late pregnancy increased placental nutrient transport capacity during treatment although not placental size, consistent with effects on placental function (Harding *et al.* 1997). Maternal pGH may also alter placental differentiation, either due to direct actions of GH at the placenta or indirect actions through endocrine actions of increased maternal circulating IGF-I. Porcine placenta expresses GH as well as type 1 and type 2 IGF receptors (Chastant *et al.* 1994, Sterle *et al.* 1998). Altered placental differentiation may cause changes in the capacity of the placenta to supply nutrients to the fetus, which continue after treatment and may contribute to changes in fetal and postnatal development following maternal pGH treatment in early-mid pregnancy (Kelley *et al.* 1995, Rehfeldt *et al.* 2001a, Gatford *et al.* 2003). Persistent effects on placental function occur in guinea pigs following maternal infusion with IGF-I from day 20 to 37 of pregnancy (term ~67 day), with increased fetal and placental weight at the end of treatment and increased fetal weight, active and passive nutrient transport to the fetus and altered placental gene expression near term (Sferruzzi-Perri *et al.* 2006, 2007a,b, Roberts *et al.* 2008). Expression of IGF axis components were altered in maternal uterine and placental tissues over a month after pGH treatment of early pregnant gilts (days 10–27), although few changes in the axis were observed in placentae collected immediately after treatment (Sterle *et al.* 1998, Freese *et al.* 2005). Similarly, Rehfeldt *et al.* (2001a) reported that increased placental protein content and trends to increased placental total and dry matter weights at the end of gestation, but not earlier, in gilts that were treated with 6 mg pGH/day from day 10 to 27 of gestation. This suggests that although maternal pGH did not alter placental weight and size at day 50

of gestation in the present study, it may nevertheless affect subsequent placental growth, differentiation, and function.

Maternal pGH treatment may also affect fetal nutrient supply during the treatment period through its effects on maternal nutrient partitioning. In pregnant sheep, as well as pigs, maternal GH increases maternal circulating levels of other anabolic hormones, including IGF-I and insulin, which may act directly on the placenta as well as to modify maternal metabolism, and sustained GH treatment alters nutrient partitioning away from maternal and towards uteroplacental growth (Sterle *et al.* 1995, 1998, Harding *et al.* 1997, Okere *et al.* 1997, Gatford *et al.* 2000, Rehfeldt *et al.* 2001a, 2004, Schneider *et al.* 2002, Wallace *et al.* 2004). The ~170% increase in maternal plasma IGF-I in pGH-treated dams may therefore contribute to the increases in fetal growth seen in the present study. We did not observe increases in maternal or fetal plasma insulin or glucose in the present study, consistent with our other study in fetuses of nutrient-restricted dams (Gatford *et al.* 2000), and smaller and less sustained effects of maternal GH on maternal plasma insulin in ewes fed at moderate levels compared with those fed at high levels (Wallace *et al.* 2004). Plasma urea concentrations were reduced in both dams and fetuses in the present study, suggestive of decreased protein catabolism in the mother as well as fetus, which may increase the amino acid supply for fetal growth, as well as improving maternal organ function.

Effects of maternal treatments and parity on fetal muscle development in fetal pigs differed between small, median and large littermates in the present study. Maternal treatments did not affect total fiber number at day 50 gestation (predominantly primary fibers in the present study), with the exception of a trend to increase in large fetuses from ractopamine-treated dams. This suggests that changes in secondary fiber numbers account for the majority of increases in progeny muscle fiber numbers seen at birth in response to maternal treatments including increased nutrition and pGH treatment (Rehfeldt *et al.* 1993, Dwyer *et al.* 1994), although one study reported increases in numbers of primary as well as secondary fibers (Rehfeldt *et al.* 2001b). Interestingly, secondary fibers were apparent, although comprising <5% of all fibers, in the majority of sections in the present study and the proportion of sections in which secondary fibers could be distinguished was consistent between fetal size groups. Previous studies had suggested that secondary fiber hyperplasia did not begin until day 50 gestation in the pig (Wigmore & Stickland 1983), and that maternal pGH or nutritional treatment between days 25 and 50 of pregnancy acted on primary fetal muscle fibers, which begin to develop in fetal pigs by day 35 of gestation (Ashmore *et al.* 1973). Increases in secondary fiber numbers at birth were proposed to occur via maternal treatments increasing the size and hence surface area of primary fibers to form the scaffold for later secondary fiber development (Wigmore & Stickland 1983), and/or by increasing proliferation of myoblasts that subsequently fuse to form secondary muscle fibers (Rehfeldt *et al.* 2001b). The presence of secondary fibers in fetal muscle sections at day 50 of gestation

suggest that maternal treatments between days 25 and 50 of pregnancy may also act directly on secondary fibers during their early development. In the present study, maternal pGH increased *M. semitendinosus* fiber diameter in the largest littermates, suggesting that this mechanism may contribute to the increased muscle fiber numbers in progeny of pGH-treated dams.

In the present study, maternal treatment with the β_2 -adrenoreceptor agonist ractopamine in early-mid pregnancy increased fetal growth at the end of treatment but was less effective than maternal pGH. Although maternal pGH and ractopamine increased fetal weight, only pGH increased fetal length, abdominal circumference or liver weight, suggesting that the two hormones may differentially affect growth of particular fetal tissues. Nevertheless, a 9% increase in average fetal weight in response to maternal ractopamine may have practical significance if maintained to term. This is the first report of fetal growth responses to maternal β_2 -agonists in the pig. Similar to effects of maternal pGH, previous studies of maternal β_2 -adrenergic agonist administration in early-mid pregnancy in the pig have reported changes in progeny postnatal development without changes in birth weight (Kim *et al.* 1994, Hoshi *et al.* 2005a,b). *In utero* exposure to the β_2 -adrenergic agonist L_{644,969} from day 25 to 95 of pregnancy in sheep (~ 0.16 – 0.63 gestation, term ~ 150 days) did not increase weight or alter body composition of neonatal twin lambs, with the exception of increased heart weight (Shackelford *et al.* 1995). Similarly in rats, fetal weight in late pregnancy and weight of neonatal pups were not changed by feeding pregnant dams the β_2 -adrenergic agonist clenbuterol from day 4 after mating and throughout pregnancy, although neonatal cardiac hypertrophy was also reported in this species (Maltin *et al.* 1990, Downie *et al.* 2008). Limited studies in women with suspected impaired fetal growth have also found no change in birth weight in response to short-term treatment with the β -adrenergic agonist ritodine in late pregnancy (Say *et al.* 2001). Taken together, the smaller fetal weight responses to maternal β_2 -adrenergic agonist in early-mid pregnancy compared with maternal pGH in the present study in pigs, and lack of increase in fetal or neonatal weights in previous studies of sustained fetal exposure in rat and sheep, imply that β_2 -adrenergic agonists do not induce large and sustained changes in fetal growth.

In the present study, maternal ractopamine increased *M. semitendinosus* fiber diameter in median-weight littermates, although not in the light or heavy fetuses. Mixed effects of *in utero* exposure to β -agonists have been reported previously in the pig, with no change in total fiber numbers or muscle cross-sectional areas reported in progeny of sows treated with ractopamine (Hoshi *et al.* 2005a), but increased muscle size and the proportion of type 1 muscle fibers in progeny of sows treated with salbutamol (Kim *et al.* 1994). In lambs, *in utero* exposure to the β_2 -adrenergic agonist L_{644,969} from day 25 to 95 of pregnancy also did not alter fiber type proportion or size in young adult offspring

(Shackelford *et al.* 1995). In contrast, feeding rats the β_2 -adrenergic agonist clenbuterol throughout most of pregnancy and through lactation increased muscle fiber size but decreased muscle weights and secondary:primary fiber ratios in fetuses (Maltin *et al.* 1990, Downie *et al.* 2008). This decreased muscle weight persisted postnatally, with a decrease in total fiber numbers (Maltin *et al.* 1990). Effects of β_2 -adrenergic agonists on fetal muscle development may thus differ between species, with the specific β_2 -adrenergic agonists used, and with time and length of fetal exposure. Our results do, however, suggest that previously reported improved progeny performance and muscle size in pigs treated with β_2 -adrenergic agonists in early-mid pregnancy reflects increased fetal muscle development during this period.

Fetal responses to maternal dietary β_2 -adrenergic agonists may reflect direct actions of these drugs on fetal muscle development, as described for the rat above, and possibly effects via the placenta, uterine blood flow, and maternal metabolism. The pig placenta expresses β -adrenergic receptors, and β_1 - and β_2 -adrenergic agonists and antagonists regulate Na⁺ transfer in pig placenta at least *in vitro*, implying that it can respond directly to β_2 -agonists (Sibley *et al.* 1986). β_2 -Adrenergic agonists may also increase blood flow to the fetus, by acting on receptors in smooth muscle cells to elevate cAMP and cause vasodilation, as shown in human umbilical arteries *in vitro* (Karadas *et al.* 2007). Finally, β_2 -adrenergic agonists alter metabolism, increasing skeletal muscle deposition in growing pigs (Dunshea *et al.* 1993), and thus may increase nutrient availability for fetal growth, although we did not see changes in maternal or fetal plasma glucose or urea concentrations in response to maternal ractopamine in the present study.

Our results suggest that maternal treatments with hormones that repartition nutrients in the dam and increase fetal growth in gilts will also increase fetal growth in sows, despite the lower growth rates and hence nutrient demands for maternal growth in the latter. Fetal growth increases were larger in response to maternal pGH than ractopamine, suggesting that it is likely to have greater effects on fetal development and lead to greater improvements in postnatal performance than ractopamine. Our results suggest that maternal pGH treatment is likely to improve progeny performance in sows as well as gilts, and we are presently investigating postnatal growth and muscle development responses to maternal pGH in gilt and sow progeny.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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