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Intrauterine crowding impairs formation and growth of secondary myofibers in pigs

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There are indications that intrauterine crowding may cause intrauterine growth retardation with the possibility of an impaired myofiber hyperplasia. The aim of the study was to confirm this by generating large differences in uterine space using sows that were unilaterally hysterectomized-ovariectomized (HO; crowded) or unilaterally oviduct ligated (OL; non-crowded). In the study, seven HO and seven OL Swiss Large White third parity sows were used. At farrowing, litter size and litter birth weight were determined. Subsequently, within each litter two male and two female progenies each with the respectively lowest (L) and highest (H) birth weight were sacrificed. Internal organs and brain were weighed, and longissimus (LM) and semitendinosus muscle (SM) samples were collected. Histological analyses were performed in both muscles using mATPase staining after preincubation at pH 4.3 and 10.2. Myosin heavy chain (MyHC) polymorphism was determined in the LM by means of SDS-PAGE. The number of piglets born alive was similar in both sow groups, but litter size expressed per uterine horn was lower ($P < 0.05$) in OL than HO sows. Consequently, OL progeny were markedly heavier ($P < 0.01$). Regardless of gender, the organs, the brain and the SM were heavier ($P < 0.001$) in OL and H compared with HO and L offspring, respectively. Compared with HO pigs, the SM of OL offspring tended ($P < 0.1$) to have more myofibers, which were of larger ($P < 0.05$) size. However, myofiber density appeared to be lower ($P < 0.1$) in the SM of OL than HO pigs. The impact of birth weight on myofiber characteristics was limited to the lower ($P < 0.05$) myofiber density in the SM and the larger ($P < 0.01$) myofiber size in the light portion of the SM of H than L offspring, whereas myofiber hyperplasia did not differ between birth weight categories. The SM, but not the LM, of male offspring had a greater ($P < 0.05$) myofiber density. This did not affect total SM myofiber number. The relative abundance of fetal and type I MyHC in the LM was lower ($P < 0.05$) and that of type II MyHC was greater ($P < 0.001$) in OL than HO pigs. The current data suggest that regardless of birth weight and gender, in the LM and SM of individuals born from a crowded environment, not only hyperplasia but also hypertrophy of myofibers is impaired and their maturity seems delayed.

Keywords: birth weight, intrauterine crowding, myogenesis, pig

Implications

In sows, selection for increasing litter size has led to a decrease in litter birth weight as a likely result of elevated intrauterine crowding. It is important to better understand how uterine space availability affects fetal development. This study validated the connection between the level of intrauterine crowding and the degree of fetal growth retardation. This includes reduced myofiber size and number and ultimately impaired pre- and postnatal muscle development. These results will help to convey the litter size where negative effects of intrauterine crowding are not counterbalancing the economic advantage given by extra offspring.

Introduction

Intrauterine growth retardation in newborn pigs has been hypothesized to be linked to intrauterine crowding. This gets apparent in lower overall fetal growth, markedly lower birth weight and impaired myofiber hyperplasia especially of that of the secondary myofibers in the *semitendinosus* (SM) and *psoas major* muscle (Bérard *et al.*, 2010). Primiparous sows of the common Large White breed kept in Switzerland were shown to have a very high ovulation rate of on average 31.3 (Bérard and Bee, 2010). Thus, naturally occurring intrauterine crowding cannot be excluded. Individual birth weight may be of importance for the actual effects of intrauterine crowding because of its influence on postnatal growth. There seems to be a close relationship between birth weight and low meat quality, resulting in part from low myofiber numbers and an

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accelerated fiber hypertrophy in piglets with low birth weights (Bee, 2004; Gondret *et al.*, 2006). Some studies have even proposed that a birth weight \times gender interaction exists regarding postnatal development, suggesting that the negative effects of low birth weight exhibit an influence in adult life more in female than in male pigs (Poore and Fowden, 2004; Rehfeldt *et al.*, 2008).

The effects of differences in available uterine space on fetal development and maternal metabolism have been previously studied with different experimental models such as unilateral ovary-hysterectomy (HO) (Huang *et al.*, 1987; Bérard *et al.*, 2010) or unilateral oviduct ligation (OL; Town *et al.*, 2004) or both (Père *et al.*, 1997). However, only Town *et al.* (2004) and Bérard *et al.* (2010) studied the effects of intrauterine crowding on muscle development using these experimental approaches. Compared with OL sows, HO sows have only half the uterine space, whereas the overall ovulation rate is similar for both experimental models (Christenson *et al.*, 1987). Therefore, combining the HO and OL experimental model makes uterine conditions regarding space and nutrient availability per fetus even more divergent and allows to better determine the effects of intrauterine crowding on fetal development and myogenesis. In addition, the use of OL also prevents the possibility of intrauterine crowding, which might happen in intact sows with a high ovulation rate as observed by Bérard and Bee (2010).

The aim of the present study was to confirm or disprove effects of intrauterine crowding on organ and muscle development by generating large and unbiased differences in uterine space with the combined use of both, HO and OL, models. For that purpose, progeny were selected at birth in a way that birth weight extremes and both genders were included. This approach would help identifying which type of piglet will be most adversely affected.

Material and methods

Animals and treatments

All procedures imposed on the animals were approved by the Committee for Animal Care and Use of the Canton Fribourg, Switzerland.

In all, 14 third parity Swiss Large White gilts were used in the present study (seven pairs of siblings). The HO sows originated from a previous experiment (Bérard *et al.*, 2010). In their intact sisters, unilateral OL surgery of the left oviduct was performed two days after weaning of the second parity. Before the surgery, sows were sedated with a mix of 25 mg romidifin (Sedivet, Boehringer Ingelheim, Basel, Switzerland) and 30 mg butorphanol (Morphasol-10, Dr. E. Gräub AG, Bern, Switzerland). Subsequently, ligation of the oviduct was carried out under general anesthesia induced with 15 mg/kg BW of ketamine (Ketasol-100, Dr. E. Gräub AG) and maintained with isoflurane at a saturation of 2% to 3.5% (Attane, Provet AG, Lyssach, Switzerland), via a face mask, with 2 l/min of oxygen. Post-operative analgesia was provided by administration of 400 mg carprofen (Rimadyl Rind, Pfizer GmbH Tiergesundheits,

Karlsruhe, Germany). No postsurgical problems occurred and the wounds healed completely.

For estrus stimulation, 5 ml/day of Regumate Porcine (Intervet International B.V., Boxmeer, the Netherlands) was injected subcutaneously during 18 days. At day 18, 5 ml of Folligon (Intervet International B.V.) and, 4 days later, 1.5 ml of Chorulon 1500 (Intervet International B.V.) were injected subcutaneously. During estrus, the 14 gilts were artificially inseminated three times with unfrozen semen from Swiss Large White boars (Suisag, Sempach, Switzerland).

From mating to farrowing, gestating sows were reared with other multiparous gilts in group pens equipped with an automatic feeder (Compident, Model 2000, Schauer, Prambachkirchen, Austria). Sows were offered 2.5 kg/day of a gestation diet, which was formulated according to the Swiss nutrient recommendations for pigs (Agroscope Liebefeld Posieux Research Station (ALP), 2012). The ingredient composition (g/kg as fed) was: barley, 242; oat, 200; dried whole maize plant, 200; wheat bran, 100; soybean meal, 100; rapeseed meal, 50; molasses, 50; 1 : 1 lard–tallow mixture, 30.2; potato protein, 10; NaCl, 4.8; calcium carbonate, 11.7; dicalcium phosphate, 0.8; lysine HCl, 0.9; L-threonine, 0.6; vitamin–mineral premix, 4.0; binder (Pellan, Mikro-Technik GmbH & Co. KG, Bürgstadt, Germany), 4.0. The premix supplied per kg of diet: all-trans retinol, 1.2 mg; cholecalciferol, 0.006 mg; vitamin E, 9.9 mg; riboflavin, 2.8 mg; vitamin B-6, 1.3 mg; vitamin B-12, 0.015 mg; vitamin K3, 0.2 mg; pantothenic acid, 102 mg; niacin, 10 mg; folic acid, 0.48 mg; Fe as FeSO₄, 84 mg; I as Ca(IO₃)₂, 0.56 mg; Se as Na₂Se, 0.2 mg; Cu as CuSO₄, 9.2 mg; Zn as ZnO₂, 81 mg; Mn as MnO₂, 2.5 mg; choline, 196 g; biotin, 0.99 mg. The estimated composition per kg dry matter was: digestible energy, 12.1 MJ; total ash, 62.2 g; ether extract, 68.4 g; CP, 133 g; crude fiber, 94.0 g. The animals had free access to water.

Data and tissue sample collection at farrowing

At farrowing, one HO sow gave birth to only two piglets and was excluded from the experiment for that reason. At the end of farrowing, the number of piglets born alive and stillborn and their individual BWs were recorded. Then, where possible, the male and female piglets with the highest (H) and the male and female piglets with the lowest (L) birth weight of each litter were sacrificed. Pigs with a birth weight of <800 g were considered runts and were not used for sample collection. One OL sow had only male progeny, one OL sow had no low birth weight male (L) and two OL sows did not give birth to either a H or a L female, respectively. Consequently, only 47 instead of the 56 piglets intended to be collected from the 14 sows were selected. The selected newborn pigs were anesthetized using isoflurane at a 4% saturation in oxygen with a flow rate of 2 l/min, for at least 5 min and euthanized by exsanguination. Subsequently, kidney, spleen, liver, lung, heart and brain were dissected and weighed. From the left side of the carcass caudal to the 10th rib, a 3 cm long section of the *longissimus dorsi* muscle (LM) was collected. In addition, the entire SM consisting of a light (SM_{light}) and dark (SM_{dark}) portion was excised and weighed.

These muscles and sections were selected because in slaughter pigs they differ in their metabolic and contractile properties where the LM and SM_{light} will be predominately composed of fast-twitch glycolytic myofibers and the SM_{dark} of slow-twitch oxido-glycolytic myofibers (Bee, 2004). The two muscles were then snap frozen by submersion in 2-methylbutane cooled in liquid nitrogen and stored at -80°C for subsequent histochemical analysis.

Histochemical analysis

The number of primary and secondary myofibers, the secondary : primary myofiber ratio and the cross-sectional muscle area were determined histochemically as described previously by Bérard *et al.* (2010). Briefly, 10 μm thick cross-sections of the muscles were prepared and stained for the determination of myofibrillar ATPase activity after alkaline (pH 10.3) or acid (pH 4.5) preincubation. In the SM_{dark} and the LM, the primary myofibers stain light and the secondary myofibers dark using the basic preincubation condition, whereas the opposite occurs after acid preincubation. In addition, primary myofibers can be differentiated by size from the secondary myofibers. This was possible in the LM and the SM_{dark}, whereas, in accordance to observations of Lefaucheur *et al.* (1995), primary and secondary myofibers were not distinguishable in the SM_{light} of newborn pigs using this mATPase histochemistry assay. To determine the cross-sectional area of the total SM, SM_{dark} and SM_{light}, cross-sections of the muscles were stained using anti-slow myosin heavy chain (MyHC) monoclonal antibodies (Novocastra lyophilized mouse monoclonal antibody myosin heavy chain (NCL-MHCs) diluted 1 : 20 in ultrapure water; Novocastra, Newcastle, UK). The number of primary myofibers was determined in the mATPase sections after acid preincubation, where ~ 750 primary myofibers were counted in an area of 0.89 mm^2 . The number of primary myofibers, counted in the selected area, and the cross-sectional area of the SM_{dark} was used to estimate the total number of primary myofibers. The number of secondary myofibers was determined in mATPase sections after alkaline preincubation. Four images containing at least 33 primary myofibers were selected, and all the surrounding secondary myofibers were counted (~ 1000 secondary myofibers). This permitted estimating the total secondary myofiber number as well as calculating the secondary to primary myofiber ratio. Total number of myofibers in the SM_{dark} was calculated from the respective estimated total number of primary and secondary myofibers. In the SM_{light}, the number of myofibers was determined in mATPase sections after acid preincubation, where ~ 3000 myofibers were counted in an area of 1.47 mm^2 . The number of counted myofibers, the according measurement area (1.47 mm^2) and the cross-sectional area of the SM_{light} were used to estimate the total number of myofibers. Different from the SM, myofiber alignment in the LM is not perpendicular to the muscle axis, which makes it difficult to estimate the total myofiber number. As an indicator of myofiber density, the number of primary and secondary myofibers was expressed per mm^2 in both muscles. The diameter of at least 60 primary and 180 secondary myofibers

was determined in each LM and SM_{dark}. In the SM_{light} the diameter of at least 300 myofibers was measured.

MyHC isoform distribution in the LM

The MyHC isoform distribution was determined in the LM of 47 newborn pigs using the SDS-PAGE. The samples (500 mg) were dissolved in a solution of 5 ml of homogenization buffer (g/l: NaH_2PO_4 0.54, Na_2HPO_4 1.08, SDS 20, pH 7.0), $5\ \mu\text{l}$ of phenylmethylsulfonyl fluoride 10 mM and $50\ \mu\text{l}$ of cysteine protease inhibitor E64 1 mM (Merck KGaA, Calbiochem, Darmstadt, Germany), and centrifuged at $1500 \times g$ for 15 min. From the supernatant, the protein concentration was determined using the bicinchoninic acid assay (Micro BCA, Thermo Scientific, Rockford, IL, USA). To obtain the final protein concentration of 1 mg/ml, samples were diluted using the homogenization buffer and $400\ \mu\text{l}$ of Lämmli buffer (126 mM Tris-HCl, pH 6.8, 126 g/l glycerol, 50 g/l SDS, 0.02 g/l bromophenol blue and 50 ml/l 2-mercaptoethanol). Denaturation of the protein was carried out at 95°C for 5 min. From each sample, $1\ \mu\text{g}$ of protein was used for loading onto a 7% polyacrylamide separating gel. Gel preparation, running conditions and staining were performed as previously described by Bérard *et al.* (2010). The gels were scanned using a densitometer (GS-710 BioRad Laboratories, Reinach, Switzerland) and the proportion of the fetal, type I and type II MyHC isoforms, identified according to Lefaucheur *et al.* (1995), were quantified using Quantity One software (BioRad Laboratories). The relative proportion of fetal, type I and type II MyHC was expressed as a percentage of the sum of all MyHC isoforms detected.

Statistical analyses

The data were analyzed as an incomplete block design, considering the sow sisters as block, using the MIXED procedure of SAS (version 9.1; SAS Institute Inc., Cary, NC, USA). The statistical model used for analyzing the reproductive performance data from the sows included the surgical treatment (OL and HO) as fixed effects and the sire as random effect. Data from the piglets were analyzed by considering surgical treatment, birth weight category, gender and the two- and three-way interactions as fixed and sire as a random effect. However, none of the interactions were statistically significant. Therefore, they were removed from the final model. The piglet was the experimental unit. Least squares means were compared using the PDIF option with probability levels of $P < 0.05$ being considered significant, and levels of $P < 0.1$ were referred to as tendencies. The results are presented in the tables as least squares means together with the pooled standard error. Pearson correlation coefficients were calculated between MyHC isoforms and myofiber density using the CORR procedure of SAS.

Results

Sow performance

The total number of piglets born per sow was non-significantly lower in OL than HO sows (Table 1). However, as

Table 1 Reproductive characteristics of unilaterally OL and unilaterally HO sows at farrowing

Trait	Surgical treatment		s.e.	P-value [†]
	OL	HO		
Sows (n)	7	6		
Total piglets born (n)	7.2	9.7	1.0	ns
Total piglets born alive (n)	7.1	7.6	1.0	ns
Males (n)	4.4	4.1	0.9	ns
Females (n)	2.8	4.1	0.8	ns
Male proportion of total live borns	0.60	0.56	0.06	ns
Average birth weight of total borns (kg)	1.89	1.14	0.06	**
Average birth weight of born alive (kg)	1.89	1.20	0.07	**
Males (kg)	1.96	1.20	0.10	*
Females (kg)	1.79	1.22	0.05	**

OL = oviduct ligated; HO = hysterectomized-ovariectomized.
[†]**P < 0.05; ***P < 0.01; ns, not significant.

Table 2 Birth weight, body organs, semitendinosus muscle weight and brain to liver weight ratio of selected male and female littermates with the respectively highest and lowest birth weight born to unilaterally OL and unilaterally HO sows

Trait	Surgical treatment		Birth weight category		Gender		s.e.	P-value [†]		
	OL	HO	High	Low	Male	Female		Trt	BtW	Gender
Observations (n)	23	24	24	23	25	22				
Birth weight (kg)	1.86	1.23	1.71	1.38	1.59	1.50	0.03	***	***	**
Heart (g)	12.6	8.1	11.5	9.3	10.6	10.1	0.3	***	***	ns
Liver (g)	49.2	32.5	45.6	36.1	41.2	40.5	2.5	***	***	ns
Kidney (g)	14.9	10.3	13.7	11.5	12.6	12.5	0.4	***	***	ns
Lung (g)	25.8	16.8	23.9	18.7	21.9	20.7	0.7	***	***	ns
Spleen (g)	1.6	1.2	1.6	1.3	1.5	1.4	0.1	***	***	ns
Brain (g)	34.2	31.8	34.0	32.1	33.1	33.0	0.4	***	***	ns
Semitendinosus muscle (g)	4.1	2.5	3.6	3.0	3.3	3.3	0.3	***	***	ns
Brain to liver weight ratio	0.75	1.16	0.86	1.06	0.94	0.97	0.05	***	***	ns

OL = oviduct ligated; HO = hysterectomized-ovariectomized; Trt = surgical treatment; BtW = birth weight category.
[†]Probability values for Trt, BtW and gender. **P < 0.01; ***P < 0.001; ns, not significant.

the number of stillborn in OL sows also tended to be lower (0.1 v. 2.1; *P* < 0.1), the total number of piglets born alive were almost the same in the two sow groups. When litter size was expressed per uterine horn, OL sows gave birth to less than half the number of piglets than HO sows (3.6 v. 9.7; *P* < 0.05). Per litter, the average OL progeny were 750 g heavier (*P* < 0.01) at birth compared with the HO progeny. Compared with average male and female HO offspring born alive, OL progeny were heavier by 760 and 570 g (*P* < 0.05 for each), respectively. Furthermore, the number of female but not of male offspring tended to be lower (*P* < 0.1) in OL compared with HO sows.

Birth weight and morphometric measurements of the piglets selected at birth

As expected, birth weight was greater (*P* < 0.001) in OL and H than HO and L offspring, respectively (Table 2). Regardless of surgical treatment or birth weight category, the selected females tended to be lighter (*P* < 0.1) than their male littermates. Absolute weights of heart, liver, kidney, lung,

spleen, brain and SM were greater (*P* < 0.01) in piglets from OL sows and the H birth weight category compared with piglets from HO sows and the L birth weight category, respectively. Gender had no effect on absolute organ and muscle weights. However, except for the brain and SM, relative organ weights, expressed as percentage of the birth weight, were similar in offspring from OL and HO sows and the H and L birth weight category (data not shown), indicating that the observed treatment differences found in absolute organ weights paralleled those in birth weights. By contrast, the relative muscle weights were slightly but significantly greater (*P* < 0.05) in OL compared with HO offspring (0.22% v. 0.21%) and greater in female than male piglets (0.22% v. 0.20%). Relative weights of the brain were greater (*P* < 0.001) in piglets from HO sows and the L birth weight category compared with piglets from OL sows (2.80% v. 1.89%) and the H birth weight category (2.55% v. 2.13%). Brain:liver ratio was lower in OL and H progeny, whereas no difference was observed between male and female piglets.

Muscle characteristics of the piglets selected at birth

Because of the lower ($P < 0.01$) density of primary but not of secondary myofibers, the secondary : primary myofiber ratio of the LM was greater ($P < 0.05$) in OL than HO progeny (Table 3). Furthermore, the secondary myofibers of OL offspring were larger ($P < 0.1$) and the primary tended also to be larger in size ($P < 0.1$) than those of HO offspring. Regardless of the sow group, in H progeny primary but not secondary myofibers tended ($P < 0.1$) to be smaller than in L progeny, whereas density of primary and secondary myofibers as well as secondary : primary ratio were not different between the two birth weight categories.

Overall myofiber density tended ($P < 0.1$) to be lower in the SM of OL compared with HO progeny (Table 4). This difference resulted mainly from a lower ($P < 0.01$) primary and secondary myofiber density in the SM_{dark} of OL offspring. Conversely to the LM, the secondary : primary ratio did not differ. In the SM_{dark} the secondary myofibers were larger ($P < 0.001$), but not the primary, and in the SM_{light} overall the myofibers were larger ($P < 0.05$) in the OL than the HO offspring. Compared with the HO piglets, the SM cross-sectional area of OL piglets was greater ($P < 0.001$) because of the greater ($P < 0.05$) cross-sectional area of both, the SM_{light} and SM_{dark}. In addition, OL progeny tended

Table 3 Muscle-fiber related traits of the longissimus muscle of selected male and female littermates with the respectively highest and lowest birth weight born to unilaterally OL and unilaterally HO sows

Trait	Surgical treatment		Birth weight category		Gender		s.e.	P-value [†]		
	OL	HO	High	Low	Male	Female		Trt	BtW	Gender
Observations (n)	23	24	24	23	25	22				
Prim density	212	307	243	276	262	257	50	**	ns	ns
Sec density	6087	7382	6574	6896	6679	6790	1135	ns	ns	ns
Sec : Prim ratio	28.8	24.9	27.7	26.0	26.3	27.4	1.4	*	ns	ns
Prim diameter (μm)	15.7	13.8	13.9	15.6	14.7	14.8	0.8	ns	ns	ns
Sec diameter (μm)	9.2	7.4	8.2	8.4	8.2	8.4	0.9	***	ns	ns

OL = oviduct ligated; HO = hysterectomized-ovariectomized; Trt = surgical treatment; BtW = birth weight category; Prim = primary myofiber; Sec = secondary myofiber; Sec:Prim ratio = ratio of primary and secondary myofibers; Density = density of myofibers was expressed as number of myofibers per mm² of the longissimus muscle cross-sectional area.

[†]Probability values for Trt, BtW and gender. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns, not significant.

Table 4 Muscle-fibre related traits of the semitendinosus muscle of selected male and female littermates with the respectively highest and lowest birth weight born to unilaterally OL and unilaterally HO sows

Trait	Surgical treatment		Birth weight category		Gender		s.e.	P-value [†]		
	OL	HO	High	Low	Male	Female		Trt	BtW	Gender
Observations (n)	23	24	24	23	25	22				
Dark portion										
CSA (mm ²)	39.8	32.3	36.2	35.9	36.2	35.8	1.9	*	ns	ns
Prim density	193	236	201	228	227	202	10	**	*	*
Sec density	7042	8337	7519	7858	8127	7252	375	**	ns	*
Sec : Prim ratio	37.2	36.4	38.3	35.3	37.2	36.4	1.6	ns	ns	ns
Prim diameter (μm)	19.4	18.9	19.6	18.7	19.2	19.1	0.5	ns	ns	ns
Sec diameter (μm)	10.8	9.1	10.2	9.8	9.9	10.1	0.6	***	ns	ns
Light portion										
CSA (mm ²)	55.1	45.9	52.8	48.2	49.4	51.6	2.7	*	ns	ns
Myofiber diameter (μm)	9.1	8.4	9.1	8.3	8.7	8.8	0.2	*	**	ns
Myofiber density	9410	10007	9120	10298	10096	9321	630	ns	**	ns
Total muscle										
CSA (mm ²)	95.1	77.7	89.1	83.7	85.4	87.4	3.0	***	ns	ns
Myofiber density	8452	9286	8457	9282	9261	8478	564	ns	*	*
Myofiber number (× 10 ⁵)	7.913	7.125	7.467	7.571	7.701	7.336	0.302	ns	ns	ns

OL = oviduct ligated; HO = hysterectomized-ovariectomized; Trt = surgical treatment; BtW = birth weight category; CSA = cross-sectional area; Prim = primary myofiber; Sec = secondary myofiber; Sec:Prim ratio = ratio of primary and secondary myofibers; Density = density of myofibers was expressed as number of myofibers per mm² of the dark, light and total semitendinosus muscle cross-sectional area; Myofiber number = total myofiber number in the semitendinosus muscle was calculated from the estimated total number of Prim and Sec myofibers in the dark portion and in the light portion by estimating the total number of myofibers in mATPase sections after acid preincubation.

[†]Probability values for Trt, BtW and gender. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns, not significant.

Table 5 Myosin heavy-chain isoform distribution in the longissimus muscle of selected male and female littermates with the respectively highest and lowest birth weight born to unilaterally OL and unilaterally HO sows

Trait [†]	Surgical treatment		Birth weight category		Gender		s.e.	P-value [‡]		
	OL	HO	High	Low	Male	Female		Trt	BtW	Gender
Observations (n)	23	24	24	23	25	22				
Myosin heavy-chain isoforms (%)										
Fetal	37.8	43.0	41.4	39.4	40.5	40.2	2.8	*	ns	ns
Type I	12.1	15.4	12.4	15.1	13.9	13.6	2.6	*	*	ns
Type II	50.0	40.8	45.8	45.1	45.1	45.7	5.2	***	ns	ns

OL = oviduct ligated; HO = hysterectomized-ovariectomized; Trt = surgical treatment; BtW = birth weight category.

[‡]Probability values for Trt, BtW and gender. * $P < 0.05$; *** $P < 0.001$; ns, not significant.

[†]The abundance of the individual isoforms was expressed as the percentage of the individual isoform abundance to the total abundance of the three isoforms.

($P < 0.1$) to have a greater total myofiber number than HO progeny. Regardless of the surgical treatment, H offspring had a lower ($P < 0.05$) primary but similar secondary myofiber density in the SM_{dark}. However, the secondary myofibers tended ($P < 0.1$) to be larger. As a result of the greater ($P < 0.01$) myofiber diameter in the SM_{light} of the H pigs, the myofiber density was lower ($P < 0.01$) than in the L offspring. Compared with males, females had fewer ($P < 0.05$) primary and secondary myofibers per mm² in the SM_{dark} and myofiber number per mm² tended ($P < 0.1$) to be lower in the SM_{light}.

MyHC polymorphism in the LM of the piglets selected at birth

The MyHC isoform distribution in the LM was affected by surgical treatment and birth weight but not gender (Table 5). The LM of OL offspring had a lower ($P < 0.05$) relative abundance of the fetal and type I MyHC isoforms and a greater ($P < 0.001$) abundance of the type II MyHC isoform compared with the OL progeny. In H piglets, relative abundance of the type I MyHC was lower ($P < 0.05$) than in L piglets. The number of primary myofibers was positively correlated ($r = 0.51$; $P < 0.001$) with the abundance of the type I and negatively correlated ($r = -0.43$; $P < 0.001$) with the abundance of the type II MyHC isoforms.

Discussion

Combining the OL and HO sow model, the effects of different degrees of intrauterine crowding on fetal development were studied. The present results demonstrated the suitability of this approach to generate the expected effects on litter size and litter birth weight. When expressed per uterine horn, HO sows gave birth to 1.7 times more piglets and, although average birth weight was lower by 750 g, total litter birth weight was 0.6 times greater compared with OL sows. Regardless of gender and birth weight category, the greater intrauterine crowding markedly hampered the development of internal organs and myogenesis.

Effects of intrauterine crowding on litter characteristics

It is well established that uterine capacity is a limiting factor for fetal survival and development, thereby affecting litter

size, number of born alive and of stillborn as well as litter birth weight (Dziuk, 1968; Fenton *et al.*, 1972, Ford *et al.*, 2002). By determining the estrone sulfate concentration, which is a good indicator of the numbers of viable embryos (Gaustad-Aas *et al.*, 2002), in the plasma of HO and intact sows at day 24 of gestation, Bérard *et al.* (2010) suggested that implantation rate was not impaired by different extents of intrauterine crowding in primiparous Swiss Large White sows. However, at birth they observed a greater number of mummies in the HO sows compared with the intact sows suggesting that uterine capacity limited prenatal survival rate in late gestation in these gilts. In the current study, neither smaller nor larger mummies were found. Smaller mummies (length: < 1 cm) would represent fetal death at about days 35 to 40 of gestation and larger mummified fetuses (length: > 10 cm) represent fetal losses from day 55 of gestation to parturition (van der Lende and van Rens, 2003). However, it cannot be excluded that smaller and very small mummified fetuses may have gone unnoticed at farrowing in the present study as experienced by van der Lende and van Rens (2003). In addition, the uterus is able to reabsorb non-calcified bone tissues, thus additionally hampering the detection at birth of fetal death occurring from implantation to around day 40 of gestation. However, in the current study a greater number of stillborns occurred in HO compared with OL sows. The relatively high average birth weight of the stillborn (0.86 kg) compared with the littermates born alive suggests that they perished just before or during farrowing. Under these conditions where the uterine horn was occupied by 9.7 piglets during the whole gestational period, not only uterine space but also competition for maternal nutrient resources might have restricted the normal development of the entire litter. When the number of embryos/fetuses exceeds 14 (seven per uterine horn), normal offspring development is considered to be limited by the uterine space and placental development and efficiency (Town *et al.*, 2004; Foxcroft *et al.*, 2007). Therefore, with each additional fetus a linear decrease in the average litter birth weight can be expected (Foxcroft *et al.*, 2007). In HO sows the aforementioned threshold in fetus number was exceeded by on average 2.6 piglets, which clearly explains the observed low average birth weight of the HO offspring.

Furthermore, Leenhouders *et al.* (1999) found a positive relationship between the number of stillbirths and pre-weaning mortality of live borns, indicating an overall lower viability of litters in which stillbirths occur. Altogether, these findings allow concluding that increased intrauterine crowding will negatively affect birth weight as well as pre- and perinatal survival rate. In the case of the OL offspring, intrauterine competition was minimal, which permitted an adequate placental development and, consequently, an improved nutrient exchange between fetuses and dam. These intrauterine conditions allowed the fetuses to reach maximal prenatal growth, which is reflected in the markedly higher birth weight compared with OH offspring.

Effects of intrauterine crowding on organ and muscle development

Regardless of the birth weight and gender, the selected HO progeny were lighter by 34% than the OL offspring. Hearts, livers, kidneys and lungs of HO offspring were lighter to a similar extent (range between 30% and 36%), indicating that at around birth allometric growth paralleled that of the whole body. However, it has to be pointed out that these results do not allow to exclude that allometric growth of these organs was constant during gestation (Vallet and Freking, 2006). Surprisingly, at farrowing, the reduction of the SM weight relative to birth weight was, with 39%, slightly larger than for the other organs in HO compared with OL offspring. Similarly, Powell and Aberle (1981) as well as Wigmore and Stickland (1983) found that growth retarded piglets with a low birth weight had relatively smaller muscles than their larger littermates. One possible explanation for this greater growth restriction is that secondary myofiber hyperplasia, which is predominantly responsible for prenatal muscle growth (Handel and Stickland, 1987), occurs at a time in the intrauterine development when nutrient requirements for overall fetal growth dramatically increase. Because of the aforementioned great intrauterine crowding in HO sows, muscle development might therefore have been more than proportionately impaired. By contrast, and in agreement with other studies (Bauer *et al.*, 2003; Town *et al.*, 2004; Bérard *et al.*, 2010), brain growth was partly maintained through compensatory mechanisms as the difference in absolute brain weight between HO and OL offspring amounted only to 7%. This phenomenon, also known as brain sparing effect, illustrates that the maintenance of brain weight is of primary importance for growth restricted piglets (McMillen *et al.*, 2001). Similarly, brain weight was only 6% lower in L than H offspring, whereas weights of the SM and those of the other organs were lower to a similar extent (19%) as birth weight.

Effects of intrauterine crowding on myofiber characteristics and MyHC polymorphism

Increased intrauterine crowding, as induced by HO, resulted in impaired myofiber hyperplasia and, as a consequence, a smaller muscle cross-sectional area of the SM. These findings confirm results reported by Town *et al.* (2004)

showing lower secondary myofiber number and smaller cross-sectional area of the SM in 90 day old fetuses subjected to modest intrauterine crowding. However, in that study, like as in the present study, a confounding effect between intrauterine crowding and birth weight exists, because offspring born from a crowded compared with an un-crowded intrauterine environment were also of lower birth weight. However, Bérard *et al.* (2010) demonstrated that independent of birth weight, intrauterine crowding negatively affects myofiber hyperplasia (shown in the *psaos major* muscle and SM_{dark}); this probably through malnutrition of the fetuses (Père and Etienne, 2000). Unexpectedly, in the present study, myofiber hyperplasia in the SM of HO offspring was impaired even though there was a greater myofiber density. This discrepancy might be explained by the differences observed in myofiber diameter. Thus, intrauterine crowding not only seems to reduce hyperplasia but also impair hypertrophy of myofibers.

In the LM, the muscle cross-sectional area was not determined but, as reported earlier (Bee, 2004), a similar positive relationship between the cross-sectional area of the LM and birth weight can be expected as that found with the SM in the present study. Because the secondary myofiber density was similar, the secondary : primary ratio was lower and primary and secondary myofiber sizes were smaller in the HO offspring; it is likely that, as in the SM, the LM had fewer myofibers than that of the OL offspring as well. Furthermore, in the LM, the relative abundance of fetal and type I MyHC was greater in HO offspring compared with OL offspring. Lefaucheur *et al.* (1997) showed that embryonic and fetal MyHC isoforms are transitional MyHC isoforms, which within 2 weeks after birth mature to become the adult type II isoforms. Therefore, this result indicates a delayed muscle development in HO offspring.

Effects of intrauterine crowding on piglets with different birth weight and gender

The complete lack of surgical treatment \times birth weight interactions suggests that contrasting levels of intrauterine crowding similarly affected muscle development of H and L offspring. However, the effect of birth weight on myofiber characteristics was very limited in the present study. Only in the SM, myofiber density was greater and myofiber size was smaller, whereas total myofiber number did not differ between L and H offspring. On a first glance, this was unexpected considering numerous previous evidence that birth weight has a distinct effect on myogenesis (Wigmore and Stickland, 1983; Rehfeldt and Kuhn, 2006; Tristan *et al.*, 2009). However, in the present study the average birth weight even of the L pigs was rather high with 1.38 kg and the weight difference to the H pigs amounted to only 320 g. On the basis of earlier results where similar birth weight ranges were compared, no differences in myofiber hyperplasia were observed as well (Rehfeldt and Kuhn, 2006; Bérard *et al.*, 2010).

In a similar way, the average birth weight of both genders was high with an average of 1.54 kg and the weight

difference between them amounted only to 80 g. As there was also no significant interaction between surgical treatment and gender, it can be assumed that intrauterine crowding has a similar effect on these high average weight piglets. Although myofiber densities in the SM were greater in male compared with female piglets, these differences were not sufficiently large to affect total myofiber number of the muscle. Because myofiber diameter and cross-sectional area were similar in both genders, myofibers were more densely packed within the muscle of the males. The lack of a general gender effect on myofiber number is in agreement with previous results obtained in the SM of slaughter pigs (Bee, 2004). The author observed a heavier muscle in gilts than barrows, related to a greater myofiber hypertrophy but not hyperplasia. By contrast, Bérard *et al.* (2010) reported greater myofiber hyperplasia in the SM_{dark} and *rhomboideus* muscle, the latter being also larger in size, of male compared with female newborns. This discrepancy of results might be related to differences in birth weight of the newborn pigs between studies (on average 130 g lighter in the study by Bérard *et al.*, 2010).

Conclusion

The present results revealed that HO offspring carried all negative phenotypic characteristics that are indicative of intrauterine growth retardation such as lower birth weight, reduced organ and muscle weights as well as brain sparing effects. Furthermore, elevated intrauterine crowding impaired hyperplasia and growth of myofibers along with a delayed myofiber maturation. On the basis of the current knowledge about the relationship between prenatal muscle development and postnatal growth, carcass characteristics and meat quality, an impaired myogenesis induced by intrauterine crowding can be expected in common genotypes with important adverse effect on economically relevant traits of pig production.

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