Eur J Nutr (2012) 51:151–165
DOI 10.1007/s00394-011-0201-8

ORIGINAL CONTRIBUTION

Limited and excess protein intake of pregnant gilts differently affects body composition and cellularity of skeletal muscle and subcutaneous adipose tissue of newborn and weanling piglets

Charlotte Rehfeldt · Louis Lefaucheur · Jana Block · Bernd Stabenow · Ralf Pfuhl · Winfried Otten · Cornelia C. Metges · Claudia Kalbe

Received: 2 February 2011 / Accepted: 15 April 2011 / Published online: 11 May 2011

Abstract
Aim This study investigated whether dietary protein intake less (50%) or greater (250%) than requirements throughout gestation differently affects offspring body composition and cellular properties of skeletal muscle and subcutaneous adipose tissue (SCAT).
Methods Primiparous gilts were fed iso-energetic diets containing adequate (22 AP), high (21 HP), or low (19 LP) protein contents. Newborn ($n = 166$) and weanling piglets cross-fostered to sows fed a standard diet (day 28; $n = 83$) were examined by morphological, biochemical, histological, and molecular analyses of the body, SCAT, and *semitendinosus, longissimus, biceps femoris* muscles.
Results Lowered birth weight (BW) in response to the HP and LP diets ($p < 0.01$) resulted from decreases in all body constituents in LP, and mainly from reduced body fat in HP piglets ($p < 0.05$). In the light BW class within litters, HP piglets exhibited a greater percentage of muscle tissue ($p < 0.05$) than LP piglets. Less SCAT mass in HP and LP piglets resulted from reduced ($p < 0.05$) number, but not the size of adipocytes. The LP diet adversely affected myogenesis and muscular differentiation derived from less ($p < 0.01$) primary and secondary myofibers, lower creatine kinase activity ($p < 0.05$), less *IGF2* mRNA ($p < 0.10$), and greater expression of the embryonic myosin heavy chain isoform ($p < 0.01$). Catch-up growth of LP but not HP pigs until day 28 increased body fat ($p = 0.01$). Despite compensated muscle growth in LP piglets, the deficit in myofiber number remained.
Conclusion Poor intrauterine environment by limited and excess protein supply retards fetal growth, but only limited protein supply impairs myogenesis, persistently restricts muscle growth potential, and favors obesity at infancy.

Keywords Pregnancy protein intake · Pig · Fetal development · Body composition · Muscle fiber · Adipocyte

Electronic supplementary material The online version of this article (doi:10.1007/s00394-011-0201-8) contains supplementary material, which is available to authorized users.

C. Rehfeldt (✉) · J. Block · R. Pfuhl · C. Kalbe
Research Unit Muscle Biology and Growth,
Leibniz Institute for Farm Animal Biology (FBN),
Wilhelm-Stahl-Allee 2, 18196 Dummerstorf, Germany
e-mail: rehfeldt@fbn-dummerstorf.de

L. Lefaucheur
Institut National de la Recherche Agronomique (INRA),
Unité Mixte de Recherche sur les Systèmes d'Elevage,
la Nutrition Animale et Humaine, 35590 Saint-Gilles, France

B. Stabenow
Experimental Units, Leibniz Institute for Farm Animal Biology (FBN), Wilhelm-Stahl-Allee 2, 18196 Dummerstorf, Germany

W. Otten
Research Unit Behavioural Physiology, Leibniz Institute for Farm Animal Biology (FBN), Wilhelm-Stahl-Allee 2, 18196 Dummerstorf, Germany

C. C. Metges
Research Unit Nutritional Physiology, Leibniz Institute for Farm Animal Biology (FBN), Wilhelm-Stahl-Allee 2, 18196 Dummerstorf, Germany

Introduction

Inadequate nutrition in utero may retard fetal growth and permanently change physiology and metabolism of the organism. Intrauterine growth retardation (IUGR) is an important aspect of the developmental origin of health and disease (DOHaD) concept [1] based on Barker's "thrifty

phenotype hypothesis" [2] and the more advanced "immediately and predictive adaptive response hypothesis" in line with developmental plasticity [3]. On the whole, prediction of a poor nutrient supply during fetal development leads to obesity as well as altered insulin sensitivity, cardiovascular function, hypothalamic–pituitary–adrenal axis function and mood in postnatal life [4].

Low body weight at birth resulting from IUGR is also a significant problem in animal production [5]. This phenomenon is increasingly observed in the pig as a litter-bearing species. Low birth weight piglets show reduced survival [6], compromised postnatal growth and lower carcass quality compared with piglets of medium and/or heavy birth weight [7–9]. The fetal development of skeletal muscle is impaired in that lower numbers of myofibers are formed [8, 10], which cannot be compensated for during postnatal life and, hence, has long-lasting consequences in terms of decreased lean mass and increased fatness [8, 9].

Imbalanced maternal nutrition during pregnancy due to a deficit of energy and/or protein intake or other essential nutrients may also exert permanent effects on the developing fetus and result in IUGR. Consequences of maternal nutrient restriction during mouse and rat pregnancy, such as high blood pressure, glucose intolerance, insulin resistance, and a greater propensity to become obese, on offspring are well described [11]. Studies in pigs have shown that severe (0–0.5%) or moderate (8.5%) protein deficiency during gestation results in decreased placental and fetal growth and may permanently retard postnatal growth [12–14]. Some body compositional changes in response to virtually protein-free maternal diets have been reported [15–18]. However, information on the effects of a potentially more common moderate protein restriction is scarce and no data are available on changes in structural and functional properties of skeletal muscle and adipose tissue. The induction of persistent changes in these tissues as a consequence of embryonic/fetal environmental conditions is of particular interest because of their central role in body functions [19] with impact both on human health and sustainable farm animal production.

On the other hand, there is now a growing interest in the effects of nutrient excess, because this may reflect another developmental pathway to later obesity [4]. Some data in rodents suggest that a high-protein intake during gestation has negative effects on birth weight, albeit findings are inconsistent [20–22]. Epidemiological studies in women show that high-protein intakes during pregnancy also resulted in fetal growth retardation [23, 24]. Influences of longer term high-protein intake are not well described, and the effects on structural and functional properties of tissues are almost completely unknown.

The objective of this study using the pig as an animal model was to investigate the effects of a moderately reduced (6.5%) and an excess (30%) maternal protein intake throughout gestation on the newborns as the outcome of intrauterine development and on weanling piglets. A gestation diet of 30% protein was chosen to simulate a very high-protein intake observed in dieting women [25]. We tested the hypotheses that (1) the decreases in birth weight, which were similar in response to both diets [26], are associated with changes in body composition as well as structural and functional properties of skeletal muscle and adipose tissue and (2) that these changes differ in dependence on the maternal dietary intervention.

Materials and methods

Animals and experimental treatment

Newborn piglets ($n = 166$) born to forty-seven pure German Landrace gilts artificially inseminated with the semen of pure German Landrace boars were examined in this study. The gilts were fed iso-energetic corn-barley and soybean meal diets ($\sim$13.7 MJ ME/kg DM) containing a high (HP, 30%; $n = 16$), a low (LP, 6.5%; $n = 16$), or an adequate (AP, 12.1%; $n = 15$) protein level corresponding to a protein/carbohydrate ratio of 1:1.3, 1:10.4, and 1:5, respectively, throughout gestation. The experiment was conducted over eight temporally successive replicates with at least 6 gilts each (regularly 2 per diet). Diets were fed between 2.3 and 2.9 kg/day to achieve an average target energy intake of $\sim$34 MJ ME/day (AP: 34.4; HP: 35.8; LP: 33.3 [26]) during gestation following recommendations for primiparous sows [27]. Further experimental details are described previously [26]. Birth weight was recorded, and from each litter, regularly 4 piglets (the heaviest one, two of medium weight based on the median within litter, the lightest one, but >800 g body weight), in single cases only 2–3 piglets, were killed within 36 h by an *i.v.* injection of 1 mL T61 (200 mg embutramide, 50 mg mebezonium iodide, 5 mg tetracaine hydrochloride, Intervet, Unterschleissheim, Germany) and subjected to detailed analyses (AP, $n = 55$; HP, $n = 56$; LP, $n = 55$; sex almost equally distributed within diets). The remaining piglets were cross-fostered within 48 h after birth by 2–4 parity sows fed a standard diet during pregnancy (11 piglets per sow). The standard pregnancy diet (Provital RF R.324.0; Trede & Pein, Dammfleth, Germany) fed to foster sows at 2.4 and 3.0 kg/day during early and late gestation contained 11.4 MJ ME/kg, and 12.6% crude protein. After birth, foster sows were fed a single standard lactation diet (Provital LAC R.325.0; Trede & v. Pein) with 15.5% crude protein, 0.95% lysine, $\sim$13.0 MJ ME/kg at an increasing level of 3–5 kg/day within the first postnatal week. Male piglets were castrated at day 4 of age. On day 28 of age,

regularly 2 weanling piglets were randomly selected from each of 42 litters and killed (3 mL T61 *i.v.*) for detailed analyses as described for newborn piglets (AP, $n = 28$, HP, $n = 28$, LP, $n = 27$; sex almost equally distributed within diets).

All procedures including use and treatment of animals were in accordance with the German animal protection law and approved by the relevant authorities (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern, Germany; LVL M-V/TSD/7221.3-1.1-006/04; LALLF M-V/TSD/7221.3-1.2-05/06; LALLF M-V/TSD/7221.3-1.2-013/06).

Analysis of body composition

At slaughter, the weights of all internal organs, as well as of perirenal and omental fat, were recorded of the newborn and weanling piglets. For dissection-derived body composition, the left half carcass was dissected into cuts (e.g., loin, neck, ham, etc., according to the guidelines of the German Agricultural Society-DLG) that were further separated into muscle tissue, subcutaneous adipose tissue (SCAT), skin and bones after overnight cooling at 4 °C. Muscle tissue included the total amount of skeletal muscle and intermuscular fat, and the subcutaneous fat comprises the whole superficial fat layer. Loin muscle cross-sectional area (MCSA) of weanling piglets was measured manually by planimetry at the level of 13th/14th ribs. To determine the chemical composition of the whole piglet body (empty digestive tract), various body fractions were analyzed for moisture/dry matter, protein, fat, and ash. Subsequently, the chemical composition of the total body was calculated from the data of individual fractions. Water/dry matter, lipid, and ash were determined according to standard methods [28], and the protein was estimated by difference [29].

Tissue collection

All samples for histological, biochemical, and mRNA analyses were taken as soon as possible after death from the piglets' body (right half). If not otherwise stated, all tissue samples were immediately snap-frozen in liquid nitrogen and thereafter stored at −80 °C until analyses. Samples of skin together with SCAT for histological analyses were collected at the level of 13th/14th rib and mounted on cork-chucks. Samples of loin SCAT for biochemical and transcript analyses were taken from newborn piglets by scraping all remaining SCAT from the skin. The *semitendinosus* (ST) muscle was dissected from the right hind limb, and its weight, circumference, and length were recorded. In the case of newborn piglets, pieces of the ST muscle mid-belly were mounted on cork-chucks, frozen in isopentane. The isopentane was cooled in nitrogen. Remaining ST tissue and pieces dissected from *M. longissimus* (LM) and *M. biceps femoris* (BF) were placed into cryotubes. From weanling piglets (day 28), one sample each was collected from the dark (deep) and bright (superficial) portion of the ST mid-belly [30] and from the central LM muscle at the level of 13th/14th rib and mounted on cork-chucks. Additional ST and LM samples for biochemical analyses were placed into cryotubes.

Histo-morphological analyses of M. semitendinosus and subcutaneous adipose tissue

From whole ST muscle of newborn piglets, serial transverse sections of 12 and 10 µm were cut at −20 °C in a cryostat (Reichert-Jung, Leica, Nussloch, Germany) and stained with eosin [31] or with myosin ATPase (EC 3.6.1.32) after acid preincubation at pH 4.2 [32], respectively. MCSA was estimated from the circumference of the mid muscle by the circle formula. Estimations of total muscle fiber number, primary, and secondary fiber numbers were calculated after manual counting of randomly selected areas on table-projected microscopic images of the muscle cross-sections by a pen-counter. Between 8 and 12% of the whole muscle cross-section was included. Eosin-stained sections were used to count all fiber categories. Primary fiber number was estimated using ATPase-stained sections [33]. From muscle samples of weanling piglets (day 28), transverse sections of 10 µm were cut at −20 °C and stained with eosin [31]/alkaline phosphatase [34] to visualize capillaries. Another serial section was exposed to a combined reaction for NADH-tetrazolium reductase [35] and acid-preincubated ATPase at pH 4.2 [32], which enables to classify slow-twitch oxidative (STO), fast-twitch oxidative (FTO), and fast-twitch glycolytic (FTG) muscle fibers. Fiber type distribution, fiber cross-sectional area (FCSA), and capillary distribution were determined on 400–450 muscle fibers (50% in the dark, 50% in the bright region of ST) by image analysis (AMBA, IBSB, Berlin, Germany). The number of fibers per unit area was used to estimate the total fiber number by multiplication with the MCSA of ST and LM muscles.

Sections of the skin-SCAT samples were cut at 16 µm at −27 °C in a cryostat and stained with hematoxylin and eosin [31]. The thickness of the superficial (first) and the second SC fat layer (day 28 only), the cross-sectional area of 150 fat cells per fat layer, and the number of fat cells per unit area (day 1 only) were measured by image analysis (AMBA; TEMA v1.00, Scan Beam APS, Hadsund, Denmark).

Biochemical analyses and analysis of myosin heavy chains

As an equivalent of cell number, DNA was determined fluorometrically by the bisbenzimidazole dye Hoechst

33258 against a standard from calf thymus DNA (Sigma–Aldrich Chemie GmbH, Steinheim, Germany) A sample of 50 mg SCAT was homogenized in 2 mL of 50% ethanol (Carl Roth, Karlsruhe, Germany) using an Ultra Turrax tissue grinder on ice for 3 min. The homogenate was centrifuged at 500×*g* for 10 min at 4 °C. The fat supernatant was discarded, and the remaining sample was refilled to 2 mL and diluted 1:4 with 50% ethanol. Samples of 100 mg muscle tissue were homogenized in 2 mL of 0.01 mol/L potassium phosphate buffer (pH 6.9) using a Potter–Elvehjem tissue grinder and diluted 1:4. DNA was measured in triplicates using 100 µL of diluted sample and 100 µL of Hoechst 33258 solution [36]. RNA was quantified fluorometrically with SYBR®Green II (Molecular Probes, Eugene, OR, USA) against RNA from calf liver (Sigma–Aldrich) as a standard [37]. Fluorescence was measured in a 96-well quartz microtestplate (Hellma, Müllheim, Germany) using the Flx 800-I microplate reader (Bio-Tek instruments Inc., Bad Friedrichshall, Germany).

Creatine kinase activity (EC 2.7.3.2.) was measured at 37 °C in diluted supernatants (1:50) of muscle homogenates (in 2 mL 0.1 mol/L citrate-phosphate buffer; pH 6.7) using a commercial kit (Biomed, Oberschleissheim, Germany). Protein concentration was determined according to Peterson et al. [38]. Both assays were adapted to microplate, and optical densities were measured using a Spectramax 384 plate reader (Molecular Devices Corporation, Sunnyvale, CA, USA).

Myosin heavy chains (MyHC) of LM muscle samples from newborn piglets were separated using one-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) as described by Talmadge and Roy [39] after some modifications [40]. The relative proportions of the different MyHC isoforms were expressed as a percentage of the sum of all MyHC within each lane.

mRNA expression analyses

In a subset of 60 piglets belonging to large litters, the expression of selected genes was measured using quantitative polymerase chain reaction (qPCR). Total RNA was isolated from LM muscle with RNeasy fibrous Mini Kit and from SCAT with RNeasy lipid tissue Mini Kit (both Qiagen, Hilden, Germany). RNA was quantified in a NanoDrop instrument (PEQLAB, Erlangen, Germany), and the quality was monitored from randomly selected samples by denaturing agarose gel electrophoresis.

Reverse transcription (RT) was carried out with 1 µg of total RNA preparation from LM or SCAT, 2 µL random primer $p(dN)_6$ (600 µM, Roche, Mannheim, Germany), and Moloney mouse leukemia virus reverse transcriptase (M-MLV RT RNase H Minus Point Mutant, Promega, Mannheim, Germany) in 25 µL of the incubation buffer provided by the supplier, supplemented with deoxy-NTPs (Roche) and RNasin (Promega), for 60 min at 42 °C. The freshly synthesized cDNA samples were cleaned with the High Pure PCR Product Purification Kit (Roche) and eluted in 50 µL elution buffer. For qPCR, 1.25 µL of each purified cDNA sample (equivalent to an input of 25 ng total RNA) was amplified in duplicate with the LightCycler-FastStart DNA Master^{PLUS} SYBR Green I kit (Roche) in 10 µL total reaction volume as described previously [40]. Specific information for the investigated genes is given in Online Resource 1-Table S1. All primers were purchased from Sigma-Genosys (Steinheim, Germany). The relative quantification was performed with the LightCycler software version 4.5 using the quantification module: Relative Quantification—Monocolor as previously described [41]. Data for mRNA expression of genes are presented as relative expression ratio normalized to topoisomerase (DNA) II beta 180 kDa (*TOP2B*) as endogenous reference gene because its expression was unaffected by the fixed factors used in statistical analysis. This reference gene was formerly validated as potential housekeeping gene for normalization of qPCR data of porcine SCAT and LM [42].

Statistical analysis

For statistical analysis, data were subjected to ANOVA by the mixed procedure of SAS (version 9.2, SAS Inst. Inc., Cary, NC, USA) with diet, sex, litter size group (L1 $<$ 13 piglets; L2 $\geq$ 13 piglets; 13 was the median of litter size), birth weight class (BW; light, medium, heavy), and replicate as well as interactions of diet × sex and diet × L and diet × BW as fixed factors. BW class of day 28 piglets was determined according to the frequency distribution of birth weight within litters (25% light; 50% medium; 25% heavy); each BW class was represented within each dietary group. Diet × replicate interaction revealed no significance for all traits and was therefore not included. The gilt nested within diet × L × replicate was included as random factor. Data shown in tables and graphs are least squares means ± SE. Differences between least squares means were tested *post hoc* by the *Tukey* test. Differences were considered significant if $p \leq 0.05$ and were considered as tendencies if $0.05 < p \leq 0.10$.

Results

Body weight and body composition in newborn piglets

Both the maternal HP and the LP diet caused a reduction ($p < 0.05$) in offspring birth weight by 10% (Table 1), which is consistent with the results obtained for all piglets born [26]. Body weight and hot carcass weight at day 1 of

Table 1 Body weight and weights of tissues and organs of selected neonatal piglets (least squares means ± SE) born to gilts fed adequate (AP), high (HP), or low (LP) levels of protein throughout gestation

Item	AP	HP	LP	P^1			
				D[2]	Sex	L[3]	BW[4]
No. of piglets (sample)	55	56	55				
Birth weight (g)	1368 ± 40^{a}	1226 ± 37^{b}	1235 ± 37^{b}	0.02	0.14	0.02	–
Body weight day 1 (g)	1482 ± 60^{a}	$1327 \pm 51^{a,b}$	1268 ± 51^{b}	0.04	<0.01	<0.01	<0.0001
Hot carcass weight (g)	1050 ± 43^{a}	$943 \pm 37^{a,b}$	898 ± 36^{b}	0.05	<0.01	<0.01	<0.0001
Perirenal fat (g)	3.60 ± 0.20^{A}	2.99 ± 0.17^{B}	2.96 ± 0.17^{B}	0.05	0.84	0.001	<0.0001
Omental fat (g)	6.89 ± 0.40	5.99 ± 0.34	5.95 ± 0.34	0.18	0.04	0.53	<0.0001
Internal organs (g)	236.9 ± 8.24^{A}	$218.1 \pm 7.03^{A,B}$	210.5 ± 6.95^{B}	0.08	0.43	<0.01	<0.0001
Carcass tissues[5]							
Muscle tissue (g)[6]	223.3 ± 10.8^{A}	$202.9 \pm 9.2^{A,B}$	190.9 ± 9.1^{B}	0.10	0.001	<0.01	<0.0001
Subcutaneous fat (g)	$55.3 \pm 3.0^{a,A}$	$46.4 \pm 2.5^{a,b,B}$	$45.9 \pm 2.5^{b,B}$	0.05	0.63	0.01	<0.001
Bones (g)	172.2 ± 5.8^{a}	$157.4 \pm 5.0^{a,b}$	150.9 ± 4.9^{b}	0.04	0.01	<0.01	<0.0001
Skin (g)	59.4 ± 2.2	54.1 ± 1.9	53.0 ± 1.9	0.11	0.06	<0.01	<0.0001

[a,b] Least squares means not sharing a common superscript are significantly different ($p < 0.05$)

[A,B] Least squares means not sharing a common superscript tend to differ ($p < 0.10$)

[1] There were no D × Sex, D × L, and D × BW interactions

[2] D—diet

[3] L—litter size group

[4] BW—birth weight group within litter

[5] Dissection of left half carcass

[6] Includes intermuscular fat

age were significantly reduced ($p < 0.05$) in LP piglets, but not in HP compared with AP piglets. Both experimental maternal diets led to decreases ($p < 0.10$) in the mass of the perirenal fat depot in offspring by 17% compared with AP piglets, whereas omental fat was not affected ($p = 0.18$) (Table 1). The relative proportions of internal fat, however, remained unchanged in response to dietary treatments (Online Resource 1, Table S2). The sum of all internal organ weights tended to be reduced ($p < 0.10$) by 11% in response to the maternal LP, but not to the HP diet (Table 1). Considering individual organs, the weight of most organs, such as liver, tongue, kidneys, throat, stomach, colon, pancreas, diaphragm ($p < 0.05$), and spleen ($p < 0.10$), were lighter in LP, whereas only the weights of stomach ($p = 0.05$) and pancreas ($p < 0.10$) were reduced in HP compared with AP piglets (Online Resource 2, Fig. S1). The relative proportion of the sum of all internal organs weights was not affected by dietary treatments (Online Resource 1, Table S2). In addition, a significant interaction of diet by litter size group ($p < 0.01$) revealed that the proportion of kidneys was significantly reduced (0.86 ± 0.04 vs. $1.04 \pm 0.04\%$; $p < 0.05$) in LP piglets from large, but not from smaller litters compared with AP and HP piglets (Online Resource 2, Figure S2).

As obtained from manual dissection of the carcass, SCAT and bones were lighter ($p \leq 0.05$; by 17 and 12%, respectively) and the weight of muscle tissue tended to be reduced ($p = 0.10$; by 15%) in response to the LP diet (Table 1). In HP piglets, only the weight of SCAT tended to be reduced ($p < 0.10$) by 16%. On average, relative tissue proportions of the carcass remained unchanged in response to dietary treatments (Online Resource 1, Table S2). However, significant interactions of diet with BW for muscle tissue ($p = 0.04$) and SCAT percentages ($p = 0.01$) revealed that HP piglets exhibited a higher percentage of muscle tissue compared with LP piglets (44.0 ± 0.5 vs. $41.6 \pm 0.5\%$; $p < 0.05$) only in the category of light piglets within litters (Online Resource 2, Fig. S3). The respective difference in SCAT percentage was not significant (8.8 ± 0.4 vs. $9.8 \pm 0.4\%$; $p = 0.61$). The composition of the whole empty body in terms of protein, lipid, ash, and moisture obtained by chemical analysis was not significantly changed by dietary treatments (Online Resource 1; Table S2). However, the body lipid percentage tended to be lower in HP (1.67 ± 0.12 vs. $2.06 \pm 0.14\%$; $p < 0.10$) compared with AP piglets.

Subcutaneous fat cell size and numbers in newborn piglets

To elucidate the morphological basis of decreased SCAT deposition in HP and LP piglets, micro-structural and

biochemical features of loin SCAT that tended to weigh less (5.0 ± 0.3 vs. 5.9 ± 0.3 g; $p < 0.10$) in HP and LP piglets were examined. Adipocytes filled with lipid were clearly recognized in histological sections of SCAT in most animals (Online Resource 2; Fig. S4). Mostly, they were arranged in clusters surrounded by stromal cells. DNA concentration tended to be less ($p < 0.10$) in response to LP feeding, and the total DNA in loin SCAT was less ($p < 0.05$) in HP and LP piglets by 23 and 28% (Table 2), respectively, which is indicative of a lower number of cells. Likewise, the estimated total number of mature adipocytes in the superficial SCAT layer was decreased by 27% ($p = 0.01$) by maternal LP and HP feeding (Table 2). An interaction of diet with litter size ($p = 0.02$) revealed that adipocyte number was only reduced in HP ($p < 0.05$) and LP ($p < 0.01$) piglets of small litters (AP: 1591 × 10^6; HP: 1011 × 10^6 vs. AP: 904 × 10^6), which in tendency was also true for the weight of loin SCAT (data not shown). Thickness of the first fat layer that was the only one exactly measurable at this stage of age was not influenced by the diets (Online Resource 1, Table S3). Likewise, adipocyte cross-sectional area measured in both layers of loin SCAT was not different among dietary treatments (Table 2).

Histological and biochemical properties of skeletal muscle tissue in newborn piglets

To elucidate the effects of the maternal diets on myogenesis and its outcome at birth, histo-morphological and biochemical features of ST and/or LM and BF muscles were examined. Weight and cross-sectional area of ST muscle were reduced ($p < 0.05$) in LP by 17 and 16%, respectively, but not changed in HP ($p > 0.13$) compared with AP piglets (Table 2). ST length tended also to be reduced in LP piglets ($p < 0.10$). In LP muscle, this was associated with a clear decrease ($p < 0.01$) in the ST total fiber number by nearly 100 thousands fibers (−20%). Both the number of primary and secondary fibers was found to

Table 2 Structural and biochemical properties of subcutaneous adipose tissue (SCAT) and *semitendinosus* (ST) muscle of neonatal piglets (least squares means ± SE) born to gilts fed adequate (AP), high (HP), or low (LP) levels of protein throughout gestation

Item	AP	HP	LP	P^1				
				D[2]	Sex	L[3]	BW[4]	D × L
No. of piglets	55	56	55					
SCAT								
DNA (μg/g)	1290 ± 72^{A}	1136 ± 61^{A,B}	1075 ± 61^{B}	0.10	0.82	0.28	<0.01	0.08
DNA total (mg)	7.24 ± 0.54^{a}	5.54 ± 0.46^{b}	5.23 ± 0.45^{b}	0.02	0.29	0.40	<0.0001	0.61
Adipocyte number, total (×10^{-6})[5]	1257 ± 87^{a}	923 ± 73^{b}	920 ± 73^{b}	0.01	0.23	<0.01	<0.0001	0.02
Adipocyte area (μm^2)	345.4 ± 39.5	302.1 ± 33.8	320.0 ± 33.3	0.71	0.11	0.02	<0.01	0.86
ST muscle								
Weight (g)	3.23 ± 0.14^{a}	2.96 ± 0.12^{a,b}	2.68 ± 0.12^{b}	0.03	<0.01	0.001	<0.0001	0.87
Length (cm)	4.6 ± 0.10^{A}	4.4 ± 0.08^{AB}	4.3 ± 0.08^{B}	0.07	0.71	<0.01	<0.0001	0.76
MCSA (cm^2)[6]	1.24 ± 0.06^{a}	1.22 ± 0.05^{a,b}	1.04 ± 0.05^{b}	0.02	<0.01	0.02	<0.0001	0.98
Total fiber no. (×10^{-3})	486.4 ± 22.71^{a}	496.0 ± 19.25^{a}	388.8 ± 19.18^{b}	<0.001	0.03	0.25	<0.001	0.49
Primary fiber no. (×10^{-3})	21.48 ± 1.09^{a}	21.46 ± 0.92^{a}	16.93 ± 0.92^{b}	<0.01	0.56	0.61	<0.0001	0.81
Secondary fiber no. (×10^{-3})	464.0 ± 21.89^{a}	474.6 ± 18.66^{a}	369.8 ± 18.49^{b}	<0.001	0.02	0.27	<0.0001	0.48
Secondary: Primary	22.3 ± 0.92	22.7 ± 0.78	22.2 ± 0.78	0.90	0.09	0.38	0.18	0.81
DNA total (mg)	6.90 ± 0.27^{a}	6.55 ± 0.23^{a,b}	5.89 ± 0.23^{b}	0.03	<0.0001	<0.01	<0.0001	0.35
RNA total (mg)	6.05 ± 0.28^{a}	5.44 ± 0.24^{a,b}	5.06 ± 0.24^{b}	0.05	0.01	<0.01	<0.0001	0.42
Protein total (mg)	298.2 ± 13.5^{a}	275.6 ± 11.5^{a,b}	246.8 ± 11.4^{b}	0.03	<0.001	0.001	<0.0001	0.68
CK (IU/mg protein)[7]	6.33 ± 0.15^{a,b}	6.36 ± 0.13^{a}	5.91 ± 0.13^{b}	0.04	0.11	0.01	<0.0001	0.09

^{a,b} Least squares means not sharing a common superscript are significantly different ($p < 0.05$)

^{A,B} Least squares means not sharing a common superscript tend to differ ($p < 0.10$)

[1] There were no D × Sex and D × BW interactions

[2] D—diet

[3] L—litter size group

[4] BW—birth weight group within litter

[5] Estimated from adipocyte number/mm^3 and weight of loin SCAT

[6] MCSA—muscle cross-sectional area

[7] Average creatine kinase activity of ST—*M. semitendinosus*; LM—*M. longissimus*; BF—*M. biceps femoris*

be lower ($p \leq 0.01$) in LP compared with AP and HP muscles, whereas the ratio of secondary to primary fibers was not influenced by the diets (Table 2).

Biochemical analyses revealed decreases ($p < 0.05$) in total DNA, RNA, and protein amounts in ST muscle of LP versus AP piglets between 15 and 17%, but no changes in HP versus AP piglets (Table 2). DNA, RNA, and protein concentrations measured in all three muscles as well as resulting ratios (data not shown) of these constituents remained unchanged by dietary interventions. Creatine kinase (CK) activity as a marker of muscular differentiation and maturity was reduced ($p < 0.05$) in response to maternal LP diet in ST muscle, but not in LM and BF muscles (Online Resource 1, Table S4). As average of all three muscles, CK activity was lower ($p < 0.05$) in LP compared with HP piglets (Table 2). The maternal LP diet influenced the expression of the different MyHC isoforms analyzed in LM muscle (Online Resource 1, Table S4). The proportion of the embryonic isoform was greater in LP compared with AP and HP muscle (8.2 ± 0.3 vs. $7.0 \pm 0.3\%$, $p < 0.05$), whereas the proportion of the fast isoforms tended to be decreased in LP versus AP muscle (40.6 ± 0.7 vs. 44.2 ± 1.2; $p < 0.10$). Consequently, the ratios of fast to embryonic and fast to embryonic plus fetal MyHC tended to be decreased in LP piglets ($p < 0.10$). Fetal to embryonic MyHC ratio and the proportion of slow MyHC were not significantly different between LP and AP piglets (Online Resource 1, Table S4). With regard to the markers of muscular maturity, such as CK activity and MyHC, there were significant diet by sex interactions showing that in particular female piglets born to LP fed dams showed lower CK activity and higher proportions of the embryonic and fetal isoforms of MyHC appearing early in development. This in turn resulted in a decreased ratio of the later appearing adult fast isoforms to the sum of embryonic + fetal isoforms (Fig. 1a–c).

mRNA expression in SCAT and LM muscle tissue in newborn piglets

The transcript expression of selected genes of growth and development was measured in SCAT and LM tissue of a subset of 60 newborn piglets (Online Resource 1, Table S5). In SCAT, no differences in response to maternal dietary treatment could be detected in the mRNA expression of genes of the growth hormone/insulin-like growth factor (IGF) system, such as *IGF1*, *IGF2*, IGF-binding proteins (*IGFBP2*, *IGFBP3*, *IGFBP5*), and growth hormone receptor (*GHR*). Likewise, no dietary effects on the mRNA expression of follistatin (*FST*), leptin (*LEP*), delta-like 1 protein (*DLK1*, also known as *PREF1*), and CCAAT/enhancer-binding protein alpha (*CEBPA*) were detected. In skeletal muscle, *IGF2* mRNA expression tended to be affected ($p = 0.06$) by maternal dietary treatment in that lower levels ($p < 0.10$) were observed in LP compared with HP muscle with AP muscle lying in between

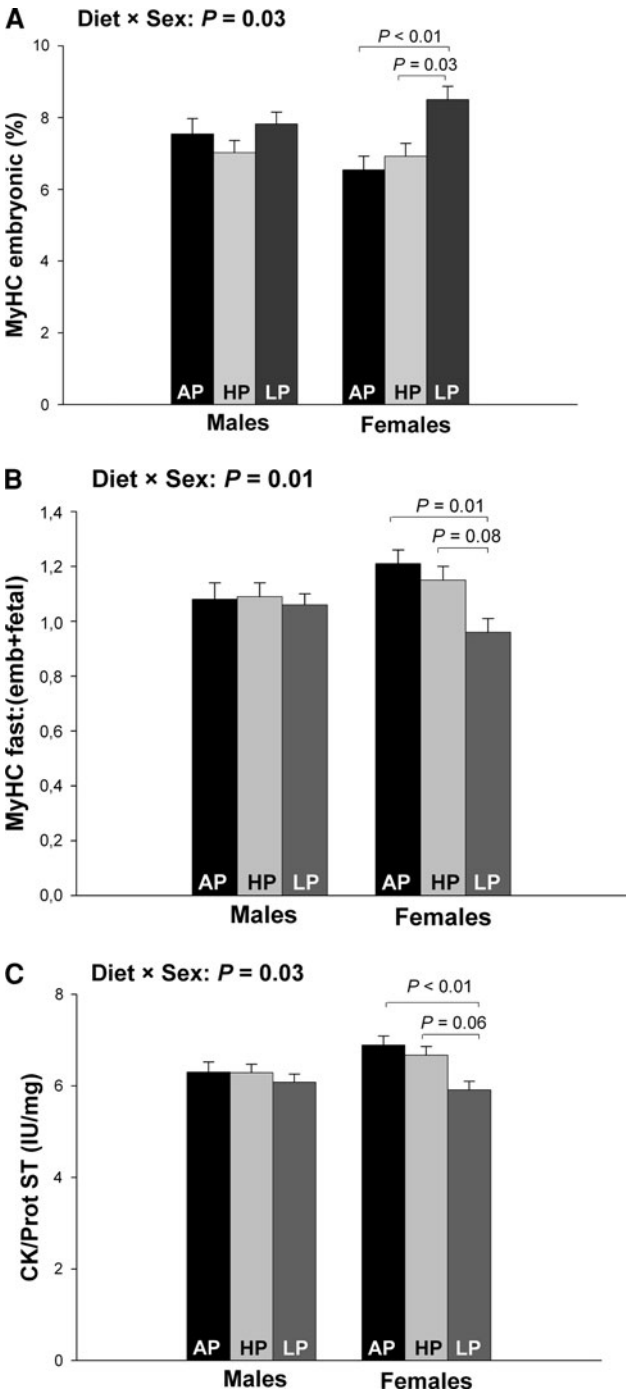

Fig. 1 Diet by sex interactions for markers of muscular differentiation and maturity in neonatal piglets born to gilts fed adequate (AP), high (HP), or low (LP) levels of protein throughout gestation. **a** Embryonic isoform of myosin heavy chain (MyHC) in longissimus muscle (LM). **b** (Embryonic + fetal) to fast MyHC isoform ratio in LM. **c** Creatine kinase activity in *semitendinosus* (ST) muscle. *Columns* and *errors bars* represent least squares means and SE

Table 3 Body composition of weanling piglets at day 28 of age (least squares means ± SE) born to gilts fed adequate (AP), high (HP), or low (LP) levels of protein throughout gestation

Item	AP	HP	LP	P^1				
				D[2]	Sex	L[3]	BW[4]	D × Sex
No. of pigs	28	28	27					
Body weight (kg)	7.70 ± 0.34	7.14 ± 0.38	7.60 ± 0.34	0.53	0.24	0.39	0.01	0.17
Hot carcass weight (kg)	5.77 ± 0.28	5.35 ± 0.31	5.73 ± 0.28	0.57	0.21	0.38	0.02	0.19
Perirenal fat (%)[5]	0.41 ± 0.02[a]	0.44 ± 0.02[a,b]	0.50 ± 0.02[b]	0.03	0.60	0.29	0.62	0.14
Omental fat (%)[5]	0.69 ± 0.03	0.71 ± 0.03	0.72 ± 0.03	0.88	0.21	0.35	0.65	0.84
Internal organs (%)[5]	15.3 ± 0.38	15.6 ± 0.42	15.2 ± 0.38	0.81	0.19	0.42	0.08	0.32
Carcass tissues[6]								
Muscle tissue (%)	57.0 ± 0.42	57.4 ± 0.50	57.3 ± 0.44	0.78	0.79	0.72	0.15	1.00
Subcutaneous fat (%)	12.0 ± 0.42[A]	13.3 ± 0.49[A,B]	13.4 ± 0.44[B]	0.04	0.50	0.07	0.43	0.27
Bones (%)	22.8 ± 0.43[A]	21.5 ± 0.52[A,B]	21.5 ± 0.46[B]	0.07	0.86	0.21	0.08	0.65
Skin (%)	8.2 ± 0.15	7.8 ± 0.18	7.8 ± 0.16	0.16	0.66	0.65	0.07	0.39
Analytical components[5]								
Protein (%)	16.6 ± 0.10	16.4 ± 0.12	16.5 ± 0.10	0.43	0.67	0.69	0.93	0.76
Fat (%)	11.6 ± 0.45[a]	12.9 ± 0.52[a,b]	13.6 ± 0.48[b]	0.02	0.29	0.14	0.24	0.08
Ash (%)	3.0 ± 0.05	2.9 ± 0.06	2.9 ± 0.05	0.12	0.72	0.73	0.57	0.59
Moisture (%)	68.7 ± 0.45[a]	67.9 ± 0.28[a,b]	67.0 ± 0.46[b]	0.03	0.36	0.10	0.27	0.08

[a,b] —least squares means not sharing a common superscript are significantly different ($p < 0.05$)

[A,B] —least squares means not sharing a common superscript tend to differ ($p < 0.10$)

[1] There were no D × L and D × BW interactions

[2] D—diet

[3] L—litter size group

[4] BW—birth weight group within litter

[5] Calculated from whole empty body

[6] Calculated from left half carcass

(1.43 ± 0.13, 2.01 ± 0.17, 1.80 ± 0.14 arbitrary units, respectively). No effects of diet or diet x sex interactions were observed for the expression of myogenic regulatory factors *MYF5*, *MYOD*, *MYOG*, and *MRF4*, as well as *IGF1*, *IGF1R*, *IGFBP2*, *IGFBP5*, *GHR*, and *FST*.

Body weight and body composition at day 28 of age

Body weight and weights of all body constituents were not significantly different at day 28 of age in response to limited or high maternal protein intake during gestation (Table 3 and Online Resource 1, Table S6). However, the piglets born to LP fed mothers showed an increased ($p < 0.05$) relative proportion of perirenal adipose tissue in the body, while omental fat percentage was not affected (Table 3). In addition, the carcass of LP pigs tended to contain a higher ($p < 0.10$) percentage of SCAT, mainly at the expense of bone ($p < 0.10$), and had an increased ($p = 0.01$) analytical lipid percentage in whole empty body (Table 3). A diet by sex interaction ($p = 0.08$) revealed that the increase in analytical lipid tended to be more pronounced in female than in castrated male LP piglets (13.8 vs. 10.6%; $p = 0.03$). Piglets exposed to maternal HP diet in utero did not exhibit significantly higher proportions of perirenal fat, SCAT, and body lipid ($p > 0.13$). Interestingly, piglets light at birth were still lighter than their littermates at weaning with 6.8 ± 0.3 kg compared with 7.5 ± 0.3 and 8.2 ± 0.4 kg in the medium and heavy BW class within litters, respectively ($p = 0.01$).

Subcutaneous fat cell size and numbers at day 28 of age

The mass and thickness of the loin SCAT was not different between the dietary groups of weanling piglets (Table 4; Online Resource 1, Table S7). However, the size of the adipocytes measured as cross-sectional area in histological sections of loin SCAT was significantly influenced by the maternal diet (Table 4). Piglets exposed to the LP diet in utero exhibited larger ($p < 0.01$) adipocytes in both SCAT layers compared with piglets from AP-fed gilts. Diet × BW interactions revealed that the adipocytes were only larger in LP pigs of medium birth weight ($p = 0.01$); however, there were only differences in the magnitude but not of the direction of the diet effect among BW groups.

Table 4 Structural and biochemical properties of subcutaneous adipose tissue (SCAT), *longissimus* (LM), and *semitendinosus* (ST) muscles of weanling piglets at day 28 of age (least squares means ± SE) born to gilts fed adequate (AP), high (HP), or low (LP) levels of protein throughout gestation

Item	AP	HP	LP	P[1]				
				D[2]	Sex	L[3]	BW[4]	D × BW
No. of pigs	28	28	27					
SCAT								
Thickness (mm)	4.30 ± 0.32	4.77 ± 0.36	5.17 ± 0.32	0.18	0.20	0.34	0.30	0.32
Adipocyte no. estimate[5]	102.4 ± 8.2	104.6 ± 9.0	116.0 ± 8.4	0.47	0.47	0.86	0.22	0.64
Adipocyte area (μm^2)	1683 ± 74[a]	1858 ± 83[a,b]	2070 ± 76[b]	<0.01	0.06	0.01	0.41	0.06
Skeletal muscles								
Weight ST (g)	28.5 ± 1.69	25.9 ± 1.85	26.8 ± 1.69	0.57	0.97	0.44	0.05	0.37
MCSA–ST (cm^2)[6]	6.27 ± 0.35	6.21 ± 0.38	6.27 ± 0.35	0.99	0.82	0.24	0.09	0.37
MSCA-LM (cm^2)[6]	5.03 ± 0.30	4.88 ± 0.34	5.14 ± 0.31	0.86	0.23	0.57	0.04	0.53
Fiber no./mm^2 ST	1492 ± 89	1400 ± 100	1392 ± 89	0.69	0.41	0.32	<0.01	0.05
Fiber no./mm^2 LM	2080 ± 115[a,b]	2275 ± 128[a]	1834 ± 115[b]	0.05	0.94	0.70	0.36	0.42
Total fiber no. ST ($\times 10^{-3}$)	878.9 ± 35.07	855.9 ± 38.77	836.9 ± 34.9	0.70	0.75	0.08	0.42	0.06
Total fiber no. LM ($\times 10^{-3}$)	1033.1 ± 44.52[A,B]	1061.7 ± 49.37[A]	906.06 ± 44.24[B]	0.06	0.29	0.60	0.02	0.42
FCSA-ST (μm^2)[7]	743.8 ± 34.4	735.1 ± 38.0	759.1 ± 34.2	0.90	0.80	0.80	0.03	0.05
FCSA-LM (μm^2)[7]	516.2 ± 25.3[a,b]	461.8 ± 27.9[a]	559.7 ± 25.2[b]	0.05	0.93	0.67	0.83	0.62

[a,b] —least squares means not sharing a common superscript are significantly different ($p < 0.05$)

[A,B] —least squares means not sharing a common superscript tend to differ ($p < 0.10$)

[1] There were no D × Sex and D × L interactions; D × BW interactions revealed no *post hoc* significance among diets

[2] D—diet

[3] L—litter size group

[4] BW—birth weight group within litter

[5] Layer thickness/adipocyte diameter * 1,000

[6] MCSA—muscle cross-sectional area

[7] FCSA—fiber cross-sectional area

Fat cell number index roughly calculated from adipocyte diameter and SCAT layer thickness was not significantly changed in response to maternal diets (Table 4).

Histological and biochemical properties of skeletal muscle tissue at day 28 of age

Weight, length, and cross-sectional area of ST muscle as well as LM area did not differ due to dietary treatments at day 28 of age (Table 4; Online Resource 1, Table S7). Myofiber number per mm^2 and total myofiber number tended to be influenced by the maternal diet in LM but not in ST muscle. Numbers were lower in LP compared with HP piglets ($p < 0.05$ and $p < 0.10$, respectively). Average fiber cross-sectional area was increased ($p < 0.05$) in LM but not in ST muscle from LP compared with HP pigs. No effects of dietary treatments on the frequency of fiber types STO, FTO, and FTG in ST and LM muscles were observed (data not shown). Likewise, there were no diet effects on the distribution of capillaries in terms of the number of capillaries per myofiber or fiber area per capillary (data not shown). DNA, RNA, and protein concentrations measured in ST, LM, and BF muscles as well as resulting ratios of these constituents as well as CK activity remained unchanged by maternal dietary treatments (Online Resource 1, Table S7).

Discussion

Offspring from primiparous sows fed diets with adequate, limited, or excess maternal protein levels during gestation have been examined in detail in this study. Recently, we have reported as first results of our experiment that both limited and excess protein intake retarded intrauterine growth resulting in lower body weight at birth [26]. The decreases in birth weight were consistent with previous findings in rodents showing that both low [43] and high [20] maternal protein feeding reduce birth weight. The major findings of the present study provide first insight into compositional and functional changes of adipose and skeletal muscle tissue in newborn and infant offspring born

to gilts receiving a diet containing excess (30%) or moderately reduced (6.5%) protein concentration throughout gestation. These novel findings demonstrate that the identical responses to these diets in terms of decreased birth weight are associated with similar changes in adipose tissue, but significantly differ at the level of structural and functional properties of skeletal muscle in neonates and weanling piglets.

Body composition

Body composition of the newborn piglets was differently affected in response to limited and excess maternal protein intake. Offspring of LP fed mothers showed a proportional decrease in the weight of most body constituents leading to nearly unchanged relative body composition. As a clear exception, the weights of the kidneys were over-proportionally reduced in piglets from large litters that were exposed to maternal protein restriction in utero. This suggests that renal development was adversely affected in LP piglets, when a high competition for nutrients per se due to litter size was additionally combined with an in utero exposure to low-protein supply. Consistently, relative kidney weights were reduced in fetuses 63 dpc in response to a severe maternal protein deprivation (0.7% protein) [17].

Excess protein in the maternal diet decreased the weights of adipose tissue depots like in the low-protein group, but not of other body constituents resulting in a slightly negative shift ($p < 0.10$) in analytical body fat in HP piglets. In the body of HP dams, the relative proportions of fat depots, such as perirenal, omental, and subcutaneous fat, were also significantly decreased [26]. Decreases in relative perirenal adipose tissue were also observed when piglets received a high-protein milk replacer from day 7 to day 28 [44], which supports the effectiveness of a high-protein diet in reducing adiposity. The high metabolic costs for irreversible nitrogen disposal, thermogenesis [45], and gluconeogenesis [46] may lead to a net energy deficit in the gravid maternal organism and partly explain why the HP fetuses gained less weight and deposited less adipose tissue. Analyses of fetal and maternal blood at day 93/94 of gestation in an experiment with the same dietary treatments as in this study suggest that IUGR in HP pigs mainly resulted from a glucose and energy deficit and in LP pigs from a deficit in insdispensible amino acids [47, 48, Metges et al., unpublished] and a dysfunction of the hypothalamic–pituitary–adrenal (HPA) axis [49]. Light piglets within litters from the HP group gained a higher percentage of muscle tissue compared with light LP piglets, suggesting that natural conditions in utero suboptimal for muscle accretion can be better tolerated at a high-protein/low-carbohydrate supply than vice versa.

Subcutaneous adipose tissue

During adipogenesis, mature adipocytes develop from mesodermal precursor cells via preadipocytes [50]. The structural basis of decreased SCAT deposition in response to both excess and low protein in the maternal diet has been identified to be a 23–28% lower total number of cells estimated by DNA amount and a 27% lower number of differentiated, lipid-filled adipocytes, whereas the degree of adipocyte differentiation in terms of cell size was not influenced. Conclusively, the reduced deposition of SCAT results mainly from decreased proliferation and/or increased apoptosis of precursor cells during fetal development, whereas adipogenic differentiation of existing precursor cells in newborn piglets seems not to be disturbed. This is in line with our finding that the mRNA expression of *CEBPA* as a marker of adipocyte differentiation [50] remained unchanged in SCAT. It is further in agreement with the observation that a moderate protein restriction during gestation in rats did not affect the capacity of preadipocytes to divide and to store fat [51]. The specific activity of the key lipogenic enzyme fatty acid synthase (FAS) was even greater in SCAT of a subset of piglets from our study exposed to low maternal protein [52]. Even though higher FAS activity was not reflected in increased adiposity at the neonatal stage, this may indicate a higher capacity for later lipid synthesis during subsequent growth.

Clear decreases in the mass of SCAT in response to both diets were not associated with changes in the mRNA expression of selected genes known to be involved in the regulation of adipogenesis. This suggests that at this stage of development regulation of adipogenesis with respect to these genes is not changed in response to limited or excess maternal protein. As the importance of the regulatory factors in adipogenesis are well established [53, 54], our results may indicate that regulatory events that contributed to the massive restriction of adipose tissue development occurred earlier during fetal development, and thereafter the system returned to normal by adaptation to the inadequate diets to maintain homeostasis. This hypothesis of an earlier adaptive response is also supported by a lack of differences in *IGF1, IGF2, IGF1R*, and leptin mRNA expression in SCAT between small and normal weight piglets at day 7 and 28 of age and in response to 3 weeks of high-protein feeding at day 28 [44]. Nevertheless, the proteome analysis of SCAT in a subset of piglets used in this study revealed a series of proteins (expressed from other genes than those chosen for mRNA analysis in this study) that were up- or downregulated in response to low or excess maternal protein during gestation [52]. A greater abundance of proteins involved in pathways related to glucose and fatty acid metabolism, lipid transport, and

regulation of apoptosis was observed in LP compared with AP piglets. The differences in regulated proteins in SCAT were less between HP and AP pigs, but the main changes concerned proteins putatively involved in amino acid metabolism.

Skeletal muscle

Myofiber formation in mammals undergoes at least two distinct phases. In the pig, primary fibers form from about 35 to 55 days and secondary fibers between about 50–55 and 90 days of gestation [55, 56]. Analysis of skeletal muscle revealed that the formation of myofibers during prenatal myogenesis was significantly impaired in response to the low-protein diet, but was not influenced by the high-protein diet. This is the first evidence that myofiber formation is adversely affected due to insufficient maternal protein intake. This result is of high relevance. Indeed, piglets of low birth weight due to natural IUGR have been shown to exhibit a lower number of myofibers compared with their heavier littermates and to develop decreased leanness and increased fatness during postnatal life [7, 8]. These piglets are not able to compensate for the lower number of myofibers and therefore the structural prerequisite for optimal lean growth is not achieved. Indeed, preliminary results reveal that pigs which were exposed to the low maternal protein diet do have more adipose and less lean tissue at market weight (day 188 of age; [57]). Because of the major role of skeletal muscle in regulating metabolic homeostasis [19, 58], this persisting reduction in myofiber number in response to low maternal protein supply may well have secondary consequences in terms of metabolic dysfunction and disease. The impairment of both primary and secondary fiber formation in response to the low-protein diet suggests that already early steps of myogenesis including stem cell commitment, proliferation, and apoptosis of myoblasts as well as differentiation and fusion of myoblasts to myotubes are adversely affected. Further studies that include various fetal stages of development are now needed to elucidate the processes involved in the impaired myogenesis in response to the low-protein supply.

Decreases in muscle mass and myofiber number in LP piglets were associated with reduced abundance of muscular DNA, RNA, and protein in almost equal terms. This result is in line with results obtained for *longissimus* muscle of newborn piglets in response to severely restricted protein feeding [17] and suggests that both myogenic cell proliferation and protein accretion were adversely affected. Definitely, our data obtained from newborn piglets reveal that muscle fiber maturation is delayed by low gestational protein supply. In pig muscle, muscle fiber-specific proteins, such as CK, and different isoforms of myofibrillar proteins are expressed with progressing differentiation [33, 54]. In developing muscle, the embryonic and fetal MyHCs are the predominant isoforms, which are gradually replaced by the adult isoforms, such as slow I, and fast IIa, IIx, and IIb MyHC [56, 59]. Hence, the lower CK activity and the expression of the embryonic and fast isoforms of MyHC at higher and lower level, respectively, indicate that skeletal muscle is less differentiated and mature at birth in response to the low-protein diet. However, in this respect, female fetuses showed a higher sensitivity than males. This sex-specific response may be related to the effects of the low-protein diet on the maternal and/or fetal HPA axis and to a blocking function of glucocorticoid action by testosterone. The feeding of a low-protein diet to rats during pregnancy resulted in reduced expression and activity of placental 11ß-hydroxysteroid dehydrogenase 2 (11ß-HSD2; [60]) that in the placenta converts active glucocorticoids, such as cortisol, to inactive forms [61]. Glucocorticoids are known for their catabolic action on muscle growth via decreasing protein synthesis and increasing protein degradation [62, 63]. Indeed, the calculated free cortisol index indicated increased concentrations of biologically active cortisol in day 93 fetuses from LP sows [49], which may be one of the reasons for restricted skeletal muscle growth. On the other hand, the male hormone testosterone, which is one of the major hormones found in the fetal pig [64], has anabolic actions on muscle. There is increasing evidence that testosterone blocks the adverse effects of glucocorticoids on muscle [65, 66], which may explain the sex-specific response indicating muscular immaturity in female LP piglets. As free cortisol in maternal plasma and saliva were only increased in late gestation (Otten et al., unpublished data; [67]), it is possible that negative effects on muscle protein accretion by glucocorticoids occurred only during late gestation. As fetal myofiber formation is finished by day 90 of gestation, this would also explain why markers of muscle differentiation but not fiber number showed a sex-specific response, although serum testosterone is biologically active early in pig development [64].

Together with the data on CK activity and MyHC isoforms, the lowered mRNA expression of *IGF2* in skeletal muscle of LP piglets suggests that the low-protein diet changed the program of myogenic differentiation. IGF-II plays a significant role in muscle differentiation [68], and the extent of autocrine expression of IGF-II in muscle has been shown to correlate with myogenic differentiation [69, 70]. In pig muscle, *IGF2* mRNA considerably decreases from day 90 of gestation to the day of birth [71, 72]. Gerrard et al. [73] have shown that there is a maximum of IGF-II expression in porcine skeletal muscle at day 59 of gestational age followed by a continuous decline until birth. It remains to be investigated, whether the differences in *IGF2* mRNA in our study occurred only temporarily

during the perinatal period or whether LP muscles exhibited a lower *IGF2* abundance throughout gestational treatment. The lack of changes in the mRNA expression of other genes of the GH-IGF system, of the myogenic regulatory factors, myostatin, and follistatin, all known to be involved into the regulation of myogenesis may indicate that regulatory events that contributed to restricted muscle development occurred earlier during fetal development.

Catch-up growth until weaning (day 28 of age)

From the results obtained for weanling piglets at day 28 of age, we conclude that piglets exposed to low (50%) and excess (250%) maternal dietary protein during gestation and thereafter cross-fostered to control sows showed catch-up growth with respect to all tissues and organs and thereby completely compensated the difference in body weight observed at birth. This is an important result, because piglets light at birth due to big litter size and/or intra-litter variation have also reduced body weight at weaning [9, 74, 75]. This suggests that the exposure to inadequate protein but adequate energy supply during prenatal life allows a compensating catch-up growth during suckling and may therefore be less impairing than overall nutrient restriction. It has to be considered, however, that in the present study, piglets were suckled by foster sows fed adequate control diets during pregnancy and lactation, suggesting that growth retardation due to a nutritionally inadequate situation during in utero life can be remedied preweaning by adequate nutrition. Notably, even under these conditions, piglets being light at birth irrespective of the maternal diet remained lightest at weaning.

The deposition of adipose tissue depots in terms of perirenal and SC fat was over-proportionally increased in piglets exposed to the LP diet exhibiting restricted adipose tissue development at birth, which is indicative of accelerated adipogenesis. In SCAT, we observed an enlargement of lipid-filled adipocytes. Our results are consistent with observations in rodents that a mismatch in early life nutrition, produced by forced catch-up growth after fetal protein restriction, has long-term consequences for the development of obesity [76, 77]. In contrast, piglets that were exposed to an excess maternal dietary protein in utero exhibited no clear signs of accelerated adipogenesis. Taken together, our results provide experimental evidence that the prediction of a poor environment given by limited supply with protein in utero may lead to a greater propensity to become obese, when the environment has changed to normal after birth [see 4, 11]. In contrast, signs of reduced obesity were apparent in weanling Meishan pigs, when maternal protein was restricted during gestation *and* lactation [78]. It remains to be investigated whether the changes toward obesity in our experiment will be maintained during further growth. Preliminary results reveal that only the offspring of LP dams, but not from HP dams, exhibit greater fat deposition at market weight [57].

Differences in muscle growth due to gestational low-protein treatment as observed at birth were no longer seen at day 28, suggesting that retardation of fetal muscle growth has been compensated during this 4-week postnatal period with adequate nutrient supply. This catch-up growth was at least in parts realized by accelerated myofiber hypertrophy as indicated by larger myofiber area in LM muscle. Accumulation of nuclei and protein accretion were similarly involved to redeem delayed muscle growth and differentiation observed in LP piglets at birth. However, the adverse effect of the LP diet on myofiber number was still apparent at day 28. Hence, the potential for lean growth may be limited in these pigs [7, 8]. Our observations further suggest that both the restricted and excess maternal protein diets did not influence the development of the capillary network as well as contractile and metabolic properties of skeletal muscle in weanling offspring. Reductions in the capillary network have been reported to occur in pancreatic islets [79] or within the cerebral cortex [80] of rats after fetal exposure to low maternal protein. We do not know, whether capillary density was adversely affected in our newborn pigs, but if this was the case, it was restored before weaning.

Conclusions

Feeding low or excess protein diets to gilts during gestation retards fetal growth, but has only marginal effects on relative body composition of the newborn offspring. In response to moderate protein restriction, all body constituents are nearly proportionally affected, whereas excess protein mainly impairs adipose tissue growth. Decreases in SCAT deposition in response to restricted or excess maternal protein intake are associated with lower cell numbers, but not with impaired adipocyte differentiation. The maternal low-protein diet leads to a clear remodeling of skeletal muscle tissue in terms of two major effects. First, less myofibers are formed during myogenesis. Second, muscular differentiation is delayed in female piglets only, presumably related to the blocking function of testosterone on the action of glucocorticoids during late gestation. Four weeks postnatal catch-up growth leads to full compensation in body mass. However, piglets exposed to low maternal protein supply develop increased adiposity associated with accelerated subcutaneous adipocyte differentiation. This suggests that a poor protein supply in utero may lead to a greater propensity to become obese in later life. Interestingly, this was not the case in HP piglets that also showed catch-up growth until weaning,

suggesting that catch-up growth is only leading to increased adiposity when myogenesis is adversely affected. The muscles of piglets exposed to a limited protein supply in utero continue to exhibit lower numbers of myofibers, which is indicative of restricted muscle growth potential and therefore may affect body composition of adult pigs. In contrast, differences in the degree of muscle differentiation are no longer apparent, and both excess and limited protein diets seem not to have consequences for metabolic and functional properties of skeletal muscle in infant pigs.

Acknowledgments This study was supported by a grant of the Deutsche Forschungsgemeinschaft (DFG), Bonn, to CK and CR (KA 1844/1-1; PAK24). The authors express their gratitude to H. Sievert and colleagues as well as Dr. vet. med. O. Bellmann for expert animal care, to Dr. U. Hennig for diet preparation, and M. Jugert-Lund, A. Steinborn, M. Günther, H. Brandt, G. Nehring, and I. Grünwald (FBN Dummerstorf) and P. Ecolan (INRA Saint-Gilles) for assistance with tissue sampling and laboratory analysis. Thanks also to Dr. S. Görs, I. S. Lang, S. Dwars, I. Brüning, and K. Karpati for tissue sampling.

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Gluckman PD, Hanson MA, Mitchell MD (2010) Developmental origins of health and disease: reducing the burden of chronic disease in the next generation. Genome Med 2:14
2. Hales CN, Barker DJ (1992) Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis. Diabetologia 35:595–601
3. Gluckman PD, Hanson MA, Spencer HG (2005) Predictive adaptive responses and human evolution. Trends Ecol Evol 20:527–533
4. Gluckman PD, Hanson MA, Cooper C, Thornburg KL (2008) Effect of in utero and early-life conditions on adult health and disease. N Engl J Med 359:61–73
5. Wu G, Bazer FW, Wallace JM, Spencer TE (2006) Board-invited review: intrauterine growth retardation: implications for the animal sciences. J Anim Sci 84:2316–2337
6. Litten JC, Drury PC, Corson AM, Lean IJ, Clarke L (2003) The influence of piglet birth weight on physical and behavioural development in early life. Biol Neonate 84:311–318
7. Gondret F, Lefaucheur L, Juin H, Louveau I, Lebret B (2006) Low birth weight is associated with enlarged muscle fiber area and impaired meat tenderness of the longissimus muscle in pigs. J Anim Sci 84:93–103
8. Rehfeldt C, Kuhn G (2006) Consequences of birth weight for postnatal growth performance and carcass quality in pigs as related to myogenesis. J Anim Sci 84(Suppl):E113–E123
9. Rehfeldt C, Tuchscherer A, Hartung M, Kuhn G (2008) A second look at the influence of birth weight on carcass and meat quality in pigs. Meat Sci 78:170–175
10. Dwyer CM, Stickland NC (1991) Sources of variation in myofiber number within and between litters of pigs. Anim Prod 52:527–533
11. Langley-Evans SC (2009) Nutritional programming of disease: unravelling the mechanism. J Anat 215:36–51
12. Mahan DC (1977) Effect of feeding various gestation and lactation dietary protein sequences on long-term reproductive performance in swine. J Anim Sci 45:1061–1072
13. Pond WG, Maurer RR, Mersmann HJ, Cummins S (1992) Response of fetal and newborn piglets to maternal protein restriction during early or late pregnancy. Growth Dev Aging 56:115–127
14. Schoknecht PA, Newton GR, Weise DE, Pond WG (1994) Protein restriction in early pregnancy alters fetal and placental growth and allantoic fluid proteins in swine. Theriogenology 42:217–226
15. Pond WG, Yen JT, Mersmann HJ (1987) Effect of severe dietary protein, nonprotein calories or feed restriction during gestation on postnatal growth of progeny in swine. Growth 51:355–371
16. Pond WG, Yen JT, Mersmann HJ, Maurer RR (1990) Reduced mature size in progeny of swine severely restricted in protein intake during pregnancy. Growth Dev Aging 54:77–84
17. Pond WG, Maurer RR, Klindt J (1991) Fetal organ response to maternal protein deprivation during pregnancy in swine. J Nutr 121:504–509
18. Schoknecht PA, Pond WG, Mersmann HJ, Maurer RR (1993) Protein restriction during pregnancy affects postnatal growth in swine progeny. J Nutr 123:1818–1825
19. Sayer AA, Stewart C, Patel H, Cooper C (2010) The developmental origins of sarcopenia: from epidemiological evidence to underlying mechanisms. JDOHaD 1:150–157
20. Daenzer M, Ortmann S, Klaus S, Metges CC (2002) Prenatal high protein exposure decreases energy expenditure and increases adiposity in young rats. J Nutr 132:142–144
21. Thone-Reineke C, Kalk P, Dorn M, Klaus S, Simon K, Pfab T, Godes M, Persson P, Unger T, Hocher B (2006) High-protein nutrition during pregnancy and lactation programs blood pressure, food efficiency, and body weight of the offspring in a sex-dependent manner. Am J Physiol Regul Integr Comp Physiol 291:R1025–R1030
22. Kucia M, Langhammer M, Görs S, Albrecht E, Hammon HM, Nürnberg G, Metges CC (2011) High protein diet during gestation and lactation affects mammary gland mRNA abundance, milk composition and pre-weaning litter growth in mice. Animal 5:268–277
23. Campbell-Brown M, Johnstone FD, Kerr-Grieve JF (1986) The effect on birthweight of a high-protein, low carbohydrate diet during pregnancy. Proc Nutr Soc 45:90A (Abstr.)
24. Andreasyan K, Ponsonby AL, Dwyer T, Morley R, Riley M, Dear K, Cochrane J (2007) Higher maternal dietary protein intake in late pregnancy is associated with a lower infant ponderal index at birth. Eur J Clin Nutr 61:498–508
25. Moran LJ, Noakes M, Clifton PM, Tomlinson L, Galletly C, Norman RJ (2003) Dietary composition in restoring reproductive and metabolic physiology in overweight women with polycystic ovary syndrome. J Clin Endocrinol Metab 88:812–819
26. Rehfeldt C, Lang IS, Görs S, Hennig U, Kalbe C, Stabenow B, Brüssow KP, Pfuhl R, Bellmann O, Nürnberg G, Otten W, Metges CC (2011) Low and excess dietary protein levels during gestation affect growth and compositional traits in gilts and impair offspring fetal growth. J Anim Sci 89:329–341
27. GfE (Gesellschaft für Ernährungsphysiologie) (2006) Empfehlungen zur Energie- und Nährstoffversorgung von Schweinen (Recommendations of energy and nutrient intake in pigs). DLG-Verlags-GmbH, Frankfurt am Main
28. AOAC (1990) Official methods of analysis, 15th edn. Assoc. Off. Anal. Chem, Washington, DC
29. Kuhn G, Ender K, Nürnberg K (1994) Effects of recombinant porcine somatotropin on the chemical composition of the whole edible body at pigs. Arch Anim Breed 37:621–623

30. Lösel D, Nürnberg G, Rehfeldt C (2011) Regional differences in micro-structural and biochemical characteristics of growth and metabolism in semitendinosus muscle of 28-day old piglets. Meat Sci 87:19–25
31. Romeis B (1989) Mikroskopische Technik. Urban & Schwarzenberg, Munich
32. Guth L, Samaha FJ (1970) Procedure for the histochemical demonstration of actomyosin ATPase. Exp Neurol 28:365–367
33. Rehfeldt C, Kuhn G, Vanselow J, Fürbass R, Fiedler I, Nürnberg G, Clelland AK, Stickland NC, Ender K (2001) Maternal treatment with somatotropin during early gestation affects basic events of myogenesis in pigs. Cell Tissue Res 306:429–440
34. Spannhof L (1967) Einführung in die Praxis der Histochemie. VEB Gustav-Fischer-Verlag, Jena
35. Novikoff AB, Shin WY, Drucker J (1961) Mitochondrial localization of oxidative enzymes: staining results with two tetrazolium salts. J Biophys Biochem Cytol 9:47–61
36. Rehfeldt C, Walther K (1997) A combined assay for DNA, protein, and incorporated [3H] label in cultured muscle cells. Anal Biochem 251:294–297
37. Oksbjerg N, Petersen JS, Sorensen IL, Henckel P, Vestergaard M, Ertbjerg P, Moller AJ, Bejerholm C, Stoier S (2000) Long-term changes in performance and meat quality of Danish Landrace pigs: a study on a current compared with an unimproved genotype. Anim Sci 71:81–92
38. Peterson GL (1977) A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal Biochem 83:346–356
39. Talmadge RJ, Roy RR (1993) Electrophoretic separation of rat skeletal muscle myosin heavy chain isoforms. J Appl Physiol 75:2337–2340
40. Lefaucheur L, Ecolan P, Lossec G, Gabillard JC, Butler-Browne GS, Herpin P (2001) Influence of early postnatal cold exposure on myofiber maturation in pig skeletal muscle. J Muscle Res Cell Motil 22:439–452
41. Kalbe C, Puppe B (2010) Long-term cognitive enrichment affects opioid receptor expression in the amygdala of domestic pigs. Genes Brain Behav 9:75–83
42. Erkens T, van Poucke M, Vandesompele J, Goossens K, van Zeveren A, Peelman LJ (2006) Development of a new set of reference genes for normalization of real-time RT-PCR data of porcine backfat and *longissimus dorsi* muscle, and evaluation with *PPARGCIA*. BMC Biotechnol 6:41
43. Langley-Evans SC, Gardner DS, Jackson AA (1996) Association of disproportionate growth of fetal rats in late gestation with raised systolic blood pressure in later life. J Reprod Fertil 106:307–312
44. Morise A, Seve B, Mace K, Magliola C, Le Huerou-luron I, Louveau I (2009) Impact of intrauterine growth retardation and early protein intake on growth, adipose tissue, and the insulin-like growth factor system in piglets. Pediatr Res 65:45–50
45. Halton TL, Hu FB (2004) The effects of high protein diets on thermogenesis, satiety and weight loss: a critical review. J Am Coll Nutr 23:373–385
46. Kuhla B, Kucia M, Gors S, Albrecht D, Langhammer M, Kuhla S, Metges CC (2010) Effect of a high-protein diet on food intake and liver metabolism during pregnancy, lactation and after weaning in mice. Proteomics 10:2573–2588
47. Görs S, Lang IS, Stabenow B, Hennig U, Brüssow K, Otten W, Rehfeldt C, Metges CC (2010) Foetal umbilical venous and arterial plasma amino acid concentrations are depending on the protein level of gestation diets fed to gilts. In: Matteo Corvetto G (ed) Energy and protein metabolism and nutrition. EAAP publication No. 127, 3rd EAAP ISEP, Parma, Italy, 6–10 Sept 2010, The Netherlands, pp 221–223
48. Görs S, Lang IS, Junghans P, Hennig U, Stabenow B, Schneider F, Otten W, Rehfeldt C, Metges CC (2009) Influence of maternal low and high protein diets during pregnancy in young sows on glucose metabolism of their offspring. Proc Soc Nutr Physiol 18 (Abstr.)
49. Kanitz E, Gräbner M, Tuchscherer M, Brüssow KP, Stabenow B, Rehfeldt C, Metges CC, Otten W (2010) An inadequate maternal protein diet during pregnancy in pigs alters the expression of corticosteroid receptors and 11ß-hydroxysteroid dehydrogenase isoforms in the placenta and fetal brain. Endocr Abstr 22:P261
50. Gregoire FM, Smas CM, Sul HS (1998) Understanding adipocyte differentiation. Physiol Rev 78:783–809
51. Bieswal F, Hay SM, McKinnon C, Reusens B, Cuignet M, Rees WD, Remacle C (2004) Prenatal protein restriction does not affect the proliferation and differentiation of rat preadipocytes. J Nutr 134:1493–1499
52. Sarr O, Louveau I, Kalbe C, Metges CC, Rehfeldt C, Gondret F (2010) Prenatal exposure to maternal low or high protein diets induces modest changes in the adipose tissue proteome of newborn piglets. J Anim Sci 88:1626–1641
53. Louveau I, Gondret F (2004) Regulation of development and metabolism of adipose tissue by growth hormone and the insulin-like growth factor system. Domest Anim Endocrinol 27:241–255
54. Kalbe C, Rehfeldt C (2005) Aspects of prenatal development of muscle and adipose tissue: principles, regulation, and influence of maternal nutrition. Berl Munch Tierarztl Wochenschr 118:509–520
55. Wigmore PM, Stickland NC (1983) Muscle development in large and small pig fetuses. J Anat 137:235–245
56. Lefaucheur L, Edom F, Ecolan P, Butlerbrowne GS (1995) Pattern of muscle-fiber type formation in the pig. Dev Dyn 203:27–41
57. Rehfeldt C, Kalbe C, Block J, Nürnberg G, Stabenow B, Metges CC (2008) Long-term effects of low and high protein feeding to pregnant sows on offspring at market weight. In: Proceedings of international congress of meat science & technology. 10–15 Aug 2008, Cape Town, South Africa, 7B.15
58. Bonen A (2010) Muscles as molecular and metabolic machines. Am J Physiol Endocrinol Metab 299:E143–E144
59. Lefaucheur L, Hoffman R, Okamura C, Gerrard D, Leger JJ, Rubinstein N, Kelly A (1997) Transitory expression of alpha cardiac myosin heavy chain in a subpopulation of secondary generation muscle fibers in the pig. Dev Dyn 210:106–116
60. Langley-Evans SC, Gardner DS, Jackson AA (1996) Maternal protein restriction influences the programming of the rat hypothalamic-pituitary-adrenal axis. J Nutr 126:1578–1585
61. Edwards CR, Benediktsson R, Lindsay RS, Seckl JR (1996) 11 beta-Hydroxysteroid dehydrogenases: key enzymes in determining tissue-specific glucocorticoid effects. Steroids 61:263–269
62. Kayali AG, Young VR, Goodman MN (1987) Sensitivity of myofibrillar proteins to glucocorticoid-induced muscle proteolysis. Am J Physiol 252:E621–E626
63. Millward DJ, Garlick PJ, Nnanyelugo DO, Waterlow JC (1976) The relative importance of muscle protein synthesis and breakdown in the regulation of muscle mass. Biochem J 156:185–188
64. Colenbrander B, de Jong FH, Wensing CJ (1978) Changes in serum testosterone concentrations in the male pig during development. J Reprod Fertil 53:377–380
65. Mayer M, Rosen F (1977) Interaction of glucocorticoids and androgens with skeletal muscle. Metabolism 26:937–962
66. Zhao W, Pan J, Zhao Z, Wu Y, Bauman WA, Cardozo CP (2008) Testosterone protects against dexamethasone-induced muscle atrophy, protein degradation and MAFbx upregulation. J Steroid Biochem Mol Biol 110:125–129
67. Graebner M, Kanitz E, Tuchscherer M, Stabenow B, Metges CC, Rehfeldt C, Otten W (2009) Maternal low and high protein diets

during pregnancy affect body weight and stress reactivity in the offspring of pigs. J Anim Sci 87 E-Suppl 2/J Dairy Sci 92 E-Suppl. 1:234 (Abstr.)
68. Florini JR, Magri KA, Ewton DZ, James PL, Grindstaff K, Rotwein PS (1991) "Spontaneous" differentiation of skeletal myoblasts is dependent upon autocrine secretion of insulin-like growth factor-II. J Biol Chem 266:15917–15923
69. Florini JR, Ewton DZ, Coolican SA (1996) Growth hormone and the insulin-like growth factor system in myogenesis. Endocr Rev 17:481–517
70. Kalbe C, Mau M, Rehfeldt C (2008) Developmental changes and the impact of isoflavones on mRNA expression of IGF-I receptor, EGF receptor and related growth factors in porcine skeletal muscle cell cultures. Growth Horm IGF Res 18:424–433
71. Lee CY, Chung CS, Simmen FA (1993) Ontogeny of the porcine insulin-like growth factor system. Mol Cell Endocrinol 93:71–80
72. Peng M, Pelletier G, Palin MF, Veronneau S, LeBel D, Abribat T (1998) Ontogeny of IGFs and IGFBPs mRNA levels and tissue concentrations in liver, kidney and skeletal muscle of pig. Growth Dev Aging 60:171–187
73. Gerrard DE, Okamura CS, Ranalletta MA, Grant AL (1998) Developmental expression and location of IGF-I and IGF-II mRNA and protein in skeletal muscle. J Anim Sci 76:1004–1011
74. Fix JS, Cassady JP, Herring WO, Holl JW, Culbertson MS, See MT (2010) Effect of piglet birth weight on body weight, growth, backfat, and longissimus muscle area of commercial market swine. Livest Sci 127:51–59
75. Beaulieu AD, Aalhus JL, Williams NH, Patience JF (2010) Impact of piglet birth weight, birth order, and litter size on subsequent growth performance, carcass quality, muscle composition, and eating quality of pork. J Anim Sci 88:2767–2778
76. Bieswal F, Ahn MT, Reusens B, Holvoet P, Raes M, Rees WD, Remacle C (2006) The importance of catch-up growth after early malnutrition for the programming of obesity in male rat. Obesity (Silver Spring) 14:1330–1343
77. Bol VV, Delattre AI, Reusens B, Raes M, Remacle C (2009) Forced catch-up growth after fetal protein restriction alters the adipose tissue gene expression program leading to obesity in adult mice. Am J Physiol Regul Integr Comp Physiol 297:R291–R299
78. Liang J, Zhang X, Zhao R, Maak S, Yang X (2010) Effect of maternal protein restriction on lipid metabolism in Meishan piglets at weaning. Livest Sci 136:157–163
79. Snoeck A, Remacle C, Reusens B, Hoet JJ (1990) Effect of a low protein diet during pregnancy on the fetal rat endocrine pancreas. Biol Neonate 57:107–118
80. Bennis-Taleb N, Remacle C, Hoet JJ, Reusens B (1999) A low-protein isocaloric diet during gestation affects brain development and alters permanently cerebral cortex blood vessels in rat offspring. J Nutr 129:1613–1619