Introduction

Intrauterine growth retardation (IUGR) is a frequent cause of perinatal morbidity which prevents the fetus from meeting its optimal growth potential [1]. IUGR is associated with impaired growth during childhood [2] as well as metabolic diseases in adulthood [3,4]. Overall, epidemiological studies show a convincing link between low body weight at birth and increased propensity for adult diseases [5–7]. From human studies, the most common definition of IUGR is a fetal weight below the 10th percentile for gestational age (birth weight <2.5 kg). Individuals born small for gestational age (SGA) show lower insulin sensitivity and higher abdominal fat mass, and more often disturbed lipoprotein levels and increased liver lipid content [8,9]. One study in male IUGR mice showed elevated triglyceride levels, which were attributed to increased hepatic fatty acid synthesis and decreased beta-oxidation [10].

It is well known that caloric restriction may prevent late-onset metabolic diseases such as hyperlipidemia and diabetes mellitus [11,12]. These effects were attributed to improvements of liver lipid metabolism and stress responses [13–15]. Based on these data and the fact that inadequate intrauterine conditions may alter hepatic energy and lipid storage state [9,16], liver tissue seems to be of interest for studying effects linked to fetal growth reduction. Because metabolic imprinting occurs during critical periods such as prenatal life [17], long-term alterations of gene expression may lead to persistent effects of IUGR on liver lipid homeostasis. Overall, there is growing evidence that IUGR alters the epigenetic state of the fetal genome and imprint gene expression [18,19]. Two mechanisms that underlie these
epigenetic effects are DNA methylation and histone modification [20,21].

Based on these previous observations, we investigated IUGR and normal birth weight female porcine offspring before and after postnatal feed restriction and a subsequent refeeding period in regard to hepatic molecular and physiological changes. The liver is the central metabolic organ, integrating nutrient intake and supply to the peripheral tissues. The pig is increasingly used as a biomedical animal model due to its similarity to human physiology [22]. Porcine offspring shows up to 20% of naturally occurring IUGR [23] leading to fetal metabolic aberrations, reduced postnatal growth and increased body fatness during puberty and young adulthood [24,25]. It has been also shown that early postnatal catch-up growth in low birth weight piglets was associated with insulin resistance in adult pigs [26]. In addition, in small porcine fetuses a delayed adipocyte differentiation as compared to normal weight fetuses and lower body fatness at birth has been observed [27].

In our study, the following questions were addressed at the transcriptional, epigenomic and metabolic level: 1) Are there differences in the hepatic transcriptional profile between IUGR and normal birth weight pigs? 2) Are these effects reflected on the metabolic level? 3) Could potential birth weight-dependent effects be modified through feed restriction intervention? 4) For how long do these effects persist after refeeding and, moreover, are there alterations in variables of lipid homeostasis? To our knowledge, there are no reports on effects of feed restriction and subsequent refeeding periods on liver lipid metabolism in IUGR vs. normal birth weight adolescent mammals. However, there is evidence from human studies that impaired fetal growth can induce long-term effects on ontogenetic development and disease state [28,29].

Materials and Methods

Animal selection and treatment

Procedures performed in this study were in accordance with the German animal protection law and approved by the Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei, Mecklenburg-Vorpommern, Germany (LALLF M-V/TSD/7221.3-1.1-049/09). Liver tissue studies were performed with a total of 42 female pigs which were born by multiparous German landrace sows with a mean litter size of 15 piglets. Littermates were selected for low (0.8–1.1 kg, U) or normal (1.4–1.6 kg, N) birth weight. From birth until weaning at d 28, U and N piglets were suckled by their dams with no creep feed provided. After weaning, piglets were housed individually and had free access to water and feed. Feed intake was determined daily and body weights were monitored at weekly intervals. Different diets were fed during the growing period to meet or exceed the nutrient intake recommendations according to the respective growing stage (GfE, Society of Nutritional Physiology, 2006). After weaning, piglets were fed a diet containing a mixture of 50% (w/w) commercial pig feed (22% crude protein; BabyCrisp EW, Bergophor-Futtermittelfabrik, Kulmbach, Germany), 30% rolled oats (12.5% crude protein; Holstenmühle, H. & J. Brüggen KG, Lübeck, Germany), and 20% sucrose (Nordzucker, Braunschweig, Germany) with a total energy content of 16.9 MJ ME/kg (16.2% crude protein, 98% dry matter). From d 78 until d 98 half of the U and N pigs were subjected to feed restriction (60% of ad libitum consumption) of a conventional pig diet (Vormast CAFO TO, Trede & von Pein Landhandel und Mischfutterwerk, Dammlenhof, Germany; 14.7 MJ ME/kg; 19.7% crude protein). Subsequently, both restricted (UR, NR) and control groups (UK, NK) were subjected to ad libitum feeding until d 131 (75% Vormast CAFO TOP, 15% rolled oats, and 10% sucrose; 15.2 MJ ME/kg; 16.5% crude protein). Overall, four pig groups (NK, NR, UK, UR) were included in our study to analyze effects related to birth weight (U vs. N) and/or feed restriction (R vs. K). Liver tissue was taken from animals after overnight fasting (18 h) at ages d 75 (before feed restriction, T1), d 98 (after 3 week feed restriction, T2), d 104 (after 5 d of refeeding, T2.1) and d 131 (after 5 weeks of refeeding, T3). Animals were killed by electrical stunning and exsanguination and livers were removed within 10 min after death. Liver studies were performed on three animals per group at each time point.

Whole genome expression profiling and bioinformatics analysis

Whole genome expression studies were performed on liver tissues of three randomly chosen animals per group at ages d 75 (T1), d 98 (T2) and d 131 (T3). Total RNA was isolated from the liver with the RNeasy Mini Kit (Qiagen, Hilden, Germany). After quantification, RNA aliquots of each animal were hybridized to porcine-specific Agilent 8 × 60 K multiplex arrays (Agilent, Santa Clara, USA). After quantile normalization of microarray data, statistical analysis for pairwise comparisons was performed with a paired t-test with unequal variance. Normalized intensity levels for each of the 19,864 transcripts were used to calculate mean expression values and fold changes for all compared groups. Pairwise comparison analysis resulted in increased, decreased, not changed or not detected gene expression levels. A gene list was selected applying the following criteria: thresholds for gene expression levels ≥1.3 with p-value≤0.05. For the classification of regulated genes according to their functional roles, and to identify common pathways between these genes, further bioinformatics analyses were conducted by using DAVID bioinformatics and Ingenuity pathway analysis (IPA). The complete microarray data sets and information about study design and methodology were submitted to Minimal Information about Microarray Experiments (MIAME) with the GEO accession number GSE43826 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=prephuscomeyvo&acc=GSE43826).

Oil red o staining and lipid droplet analysis

For histologic analysis, frozen liver tissue of UK, UR, NK, and NR pigs at three time points (T1, T2, T3) were cut into 10 μm sections and fixed with formal-calcium for 5 min. Thereafter, sections were rinsed in distilled water and neutral lipids were stained with Oil Red O for 15 min. Intracellular lipid droplets (LD) were detected as red spheres in liver tissue sections using computerized image analysis. The image analysis system was equipped with a Jenaval microscope (Carl Zeiss, Jena, Germany), an Altra20 color camera (OSIS, Munster, Germany), and CELL.D image analysis software (OSIS, Munster, Germany). The sequence of analysis steps was organized in a newly developed macro program. The sequence was as follows: At first, the color image was taken, the green channel was extracted and preprocessed to enhance the contrast and to improve the detectability of LD. A threshold was interactively determined for discrimination between background and objects (LD) to be measured. An interactive step was included to delete false detected objects like artifacts. The total number and area of LD in the selected region, area percentage occupied by LD, individual and mean LD size and distribution of LD as distance between neighbors were determined. The results presented are means ± SEM of three animals per experimental group with 16 observations per animal (yielding a total of 48 observations per experimental group).
Promoter DNA methylation analysis

Methylation studies were performed on liver tissue DNA of UKT1, UKT2, URT2, NK24, NRT2, UKT3, URT3, NK3, NRT3 pigs (n = 3 per group). Genes with changed expression profiles and publicly available sequence annotations were selected as candidate genes (PFRPS, PDX1, PPAR3E, SORT1, WNT5B, SFRP4, SAC, PDE9A, FGFR4, FABP5). Most promising target regions of highest CpG density (CpG islands) within the putative promoter region (up to 2000 base pairs upstream of the transcription start) were determined with EBI’s open access tool CpGplot (http://www.ebi.ac.uk/Tools/seqparts/emboss_cpgplot/). Except for FGFR4, the first transcribed exons contained increased CpG-density as well. Therefore, these regions were included in the analysis targets.

Quantitative methylation analysis was performed with the MassARRAY® system (Sequenom, Hamburg, Germany).

Assay design was aided by platform specific software EpiDesigner, accessible through Sequenom’s customer page www.mysequenom.com. The software divides the region of interest into suitable amplicons and delivers information on primer sequences and positions, amplicon size, and CpG-coverage. Where possible, assay design/analysis was performed for forward as well as reverse strand of the genomic DNA sequence to ensure best CpG-coverage and system-inherent confirmation with a second independent reaction. The design outcome is summarized in Table 1.

The MassCLEAVE™ biochemistry was applied after bisulfite treatment of DNA samples and MALDI-TOF mass spectrometry for analyte detection according to the standard protocols recommended by the supplier. Genomic DNA was extracted from pig liver with the DNeasy Kit (Qiagen, Hilden, Germany). One μg DNA was treated with sodium bisulfite bisulfite (DNA EZ Bisulfite Treatment Kit, Sequenom, Hamburg, Germany) according to supplier’s manual to convert Cytosin to Uracil at non-methylated CpG-sites whereas each 5-Methyl-Cytosin persists as Cytosin. Regions of interest were amplified by PCR from bisulfite treated DNA samples using methylation independent primers (Metabion International, Martinsried, Germany; Table 1). PCR products were then subject to simultaneous in vitro transcription and RNase A cleavage applying the T-reverse reaction following Sequenom’s recommended standard protocol. The generated fragments were displayed based on their molecular weight in the mass spectrum, which was acquired after sample conditioning with a MassARRAY® Analyzer Compact. The resulting methylation calls were analyzed with EpiTyper Software (Sequenom) to generate quantitative results for each CpG site.

Statistical analysis followed a boot-strap method where first, the methylation ratios differences between experimental and control group are determined. Then, samples were randomly reassigned to experimental and control group and the methylation difference calculated again. Finally, the data display statistical significance if the determined methylation differences persist in the true compared to the random groups.

Liver amino acid analysis

For amino acid analysis, liver tissue from 8 experimental groups (UKT2, URT2, NK2T2, NRT2, UKT3, URT3, NK3, NRT3) was included (n = 3 per group). In detail, 20 mg of each liver sample was homogenized in 60 μl lysis buffer containing 50 mM Tris (pH 7.8), 1 mM EDTA (GE Healthcare, Munich, Germany), 10 mM NaF (Fisher Scientific, Schwerte, Germany), 1% (v/v) Igepal GA-630 (Sigma-Aldrich, Taufkirchen, Germany), 0.1% (v/v) Triton X-100 (GE Healthcare, Munich, Germany), 0.5% (v/v) deoxycholic acid (DOC; Sigma-Aldrich), 0.1% (w/v) sodium dodecyl sulfate (SDS; USB Corporation, Cleveland, OH, USA) and Roche Phospho-Stop tablets (one tablet/10 ml buffer; Roche Diagnostics, Mannheim, Germany). Protein concentrations in solubilized homogenates were determined by Bradford assay (Sigma-Aldrich, Germany). Liver homogenates from three animals per group were diluted with water (1:20) and free amino acids were analyzed by HPLC equipped with a fluorescence detector (Series 1200, Agilent Technologies, Germany). The HPLC analysis method was adapted from the technique described by Kroemer et al. [30]. Briefly, amino acids were separated after automated pre-column derivatization with ortho-phthalaldehyde/3-mercaptopropionic acid and 9-fluorenylmethoxycarbonyl chloride after reaction with 3-mercaptopropionic acid as reducing agent and iodosacetic acid to block sulphonyl groups. Analyses were carried out at a flow rate of 0.8 ml/minute within 45 min on a 250 x 4 mm Hyperclone ODS (C18) 120 Å column protected by a 4 x 3 mm C18 pre-column (Phenomenex, Aschaffenburg, Germany) using a gradient with 40 mM phosphate buffer (pH 7.8) and acetonitrile/methanol/water (v:v:v: 45:45:10) ranging from 6–100%.

Statistics

Statistical evaluation was done with SigmaPlot 11.0 software (Systat Software GmbH, Erkrath, Germany). For four-group comparisons (UK, NK, UR, NR), Two-Way Analysis of Variance (ANOVA) with the factors birth weight and feed restriction was used. When data passed the normality test (p > 0.05), Bonferroni-test was used to detect group differences. Not-normally distributed data were log-transformed to obtain normality and to allow ANOVA analysis. For two-group comparisons of gene expression data, paired Student’s t-test was used. Results are depicted as means ± SEM, and were considered significantly different when p-values were p < 0.05. Trends were discussed when 0.05 < p < 0.1.

Results

Effects of feed restriction and three weeks refeeding on body weight development in low (U) and normal (N) birth weight pigs

As a basic requirement of the study, U and N grouped animals (21 animals each) used for liver tissue studies differed in birth weight (d 0) with 1.07 ± 0.02 and 1.56 ± 0.02 kg, respectively (p = 0.001) (Table 2, Figure 1). At later time points after birth (d 75, d 90, d 104 and d 131), no differences in body weight were found between groups (Table 2, Figure 2, n = 3). Accordingly, no correlation was observed between slaughter weight and birth weight of animals (data not shown; p > 0.1). However, after five weeks of refeeding (d 131) UK animals tended to have higher body weights as compared to UR, whereas we could not observe differences between NR and NK pigs (p < 0.1, Table 2, Figure 2).

Effects of low birth weight on hepatic gene expression profile and lipid droplet formation

Microarray-based whole genome expression profiles were analyzed in liver samples of low (U) and normal (N) birth weight pigs. Based on our selection criteria (fold change levels ±1.3, p ≤ 0.05), 194 genes (95 up- and 99 down-regulated) were identified to be differentially expressed in the liver of U vs. N pigs at the age of 75 d (prior to feed restriction). IPA (Figure 3) shows a key role for the regulated genes in processes of cell death, cellular growth and proliferation, and lipid metabolism. Since 20% of the strongest regulated genes (EIF2A, HPGDS, PLCG2, CYP7A1) were related to lipid metabolism (Table 3), effects on
| Selected regions | Start | End | Direction | Relative position to gene | CpGs | Left primer (5’-TAG) | Right primer (3’-TAG) |
|------------------|-------|-----|-----------|---------------------------|------|---------------------|---------------------|
| **PTPR5A** | F | 2 2675 | -2162 | 340 | 9 | 'aaggaagagagAGGGTTATAGGTGGAGG GTA' | 'cagtaatacgactcactatagggagaaggctACTTACCCACTCAAATCCAA' |
| **PTPR5A** | R | 2 2081 | 406 | 11 | | 'aaggaagagagAGGGTTATAGGTGGAGG GTA' | 'cagtaatacgactcactatagggagaaggctAATTTCCACCAAATCCAA' |
| **PTPR5A** | F | 2 1570 | -1099 | 472 | 20 | 'aaggaagagagGTTTGGTTTTGGTTAAAGTGGAAAT' | 'cagtaatacgactcactatagggagaaggctATTTAACCACCAAATCCAA' |
| **PTPR5A** | R | 2 1226 | 2 322 | 122 | 444 | 15 | 'aaggaagagagGTTTGGTTTTGGTTAAAGTGGAAAT' | 'cagtaatacgactcactatagggagaaggctAACCTACACCACAACTCAAATCAAT' |
| **PPP1R3B** | F | 7 135 | 135 | 11 | | 'aaggaagagagATGGGGAGTTATTTGTTAGAGGGTA' | 'cagtaatacgactcactatagggagaaggctCTTCAACTTCCAAAATCTTAAACCA' |
| **PPP1R3B** | R | 7 12 135 | 140 | 11 | | 'aaggaagagagATGGGGAGTTATTTGTTAGAGGGTA' | 'cagtaatacgactcactatagggagaaggctCTTCAACTTCCAAAATCTTAAACCA' |
| **SORT1** | F | 4 702 | 2 1735 | 1369 | 367 | | 'aaggaagagagTTTGGGATTAGAGATGGAGTAGAGA' | 'cagtaatacgactcactatagggagaaggctACTATCCTAAAAAACAAAAACCCCA' |
| **WNT5B** | R | 5 1180 | 2 821 | 360 | 11 | | 'aaggaagagagATTTTGGAGTAAGTTTTTGGAAAGG' | 'cagtaatacgactcactatagggagaaggctCACACACCTACCTATTAATACCCCC' |
| **WNT5B** | R | 5 487 | 166 | 322 | 8 | | 'aaggaagagagGGAGAGTTTGTTTTTTTAGGAAAGG' | 'cagtaatacgactcactatagggagaaggctCAACTTACCAATTACTTTCAACTCCA' |
| **PDE9A** | F | 13 1855 | 2 1392 | 464 | 11 | | 'aaggaagagagGGAGGGTTTAGGGAGATATTTGATA' | 'cagtaatacgactcactatagggagaaggctCAAATAAAAACAAAAAACCCCTCTT' |
| **PDE9A** | R | 13 250 63 | 293 | 14 | | | 'aaggaagagagTTATTGAAGGTAAAAGTAGGGTTGG' | 'cagtaatacgactcactatagggagaaggctAACCTACAACCCCATAATTCCTAAC' |
| **PDE9A** | F | 13 1907 | 1548 | 360 | 11 | | 'aaggaagagagTGTATTGGTTATAGAGTTGGGAGGA' | 'cagtaatacgactcactatagggagaaggctATCACAAACCAAACCAAACTCC' |
| **PDE9A** | R | 13 1572 | -1129 | 444 | 13 | | | 'aaggaagagagGTGGTTGGTTAGGGTTTTTTTTAAG' | 'cagtaatacgactcactatagggagaaggctTCCTCCCAACTCTATAACCAATACA' |
| **PDE9A** | F | 13 217 | 701 | 485 | 28 | | 'aaggaagagagGTGTTGGGTTTAGGGGTTAGGTAG' | 'cagtaatacgactcactatagggagaaggctAAAAACCTAATCCAAACTCTCTAAAAAA' |
| **PDE9A** | R | 13 1044 | 1495 | 373 | 4 | | | 'aaggaagagagTTAAGGATGAGTGATTTTTAGGTAGGA' | 'cagtaatacgactcactatagggagaaggctCAAAAAACCAACCTTAACCAACTAA' |
| **PDE9A** | R | 13 230 63 | 293 | 14 | | | | 'aaggaagagagTTTAGTTTAATTGGGGTTTTTGGAT' | 'cagtaatacgactcactatagggagaaggctTTACCTCCACCAAAATAACTACCCT' |
| **PDE9A** | R | 13 336 | 321 | 8 | | | | 'aaggaagagagATGTTTTTTTTGGGTTAGAGGATTT' | 'cagtaatacgactcactatagggagaaggctCTTCCTAAAACTTTTCCAACTACCC' |
| **FABP5** | F | 4 217 | 701 | 485 | 28 | | 'aaggaagagagGTGTTGGGTTTAGGGGTTAGGTAG' | 'cagtaatacgactcactatagggagaaggctAAAAACCTAATCCAAACTCTCTAAAAAA' |
| **FABP5** | R | 4 1044 | 1495 | 373 | 4 | | | 'aaggaagagagTTAAGGATGAGTGATTTTTAGGTAGGA' | 'cagtaatacgactcactatagggagaaggctCAAAAAACCAACCTTAACCAACTAA' |
| **FABP5** | R | 4 230 63 | 293 | 14 | | | | 'aaggaagagagTTTAGTTTAATTGGGGTTTTTGGAT' | 'cagtaatacgactcactatagggagaaggctTTACCTCCACCAAAATAACTACCCT' |
| **FABP5** | R | 4 336 | 321 | 8 | | | | 'aaggaagagagATGTTTTTTTTGGGTTAGAGGATTT' | 'cagtaatacgactcactatagggagaaggctCTTCCTAAAACTTTTCCAACTACCC' |

All sequences are given in 5’→3’ direction of the analytical PCR product.
lipid droplet (LD) count and formation were analyzed in liver tissues of U and N pigs. As shown in Figure 4A, the total mean area of LDs was 4.6-fold higher in U compared to N animals at the age of 75 d. Furthermore, a 3.7-fold higher LD count has been observed, which was also reflected by a reduced distance (1.85-fold) to nearest LD (μm) in U vs. N pigs (Figure 4B–C). In addition, the LD area (μm²) was 24.9% higher in U compared to N animals (Figure 4D). This observation was confirmed by larger LD diameter (μm), LD circumference (μm) and LD convex area (μm²) of 12.1%, 13.0% and 25.8%, respectively (Figure 4E–G). Assuming a typical round shape of lipid droplets, the volume of LD was calculated with 0.248 and 0.176 μm³ for U and N animals, respectively. Taking into account the LD count for equal sized areas, the volume per total LD area was 5.2-fold higher with 223.6 and 43.4 μm³ for U and N animals, respectively.

In summary, low birth weight induces distinct changes in the metabolic gene expression profile, which was reflected by higher hepatic lipid droplet count and size (Figure 4H–I).

Influence of feed restriction (R) on gene expression, lipid droplet count and size in the liver of U and N pigs

The influence of the 3-week feed restriction period was analyzed in regard to gene expression and lipid droplet formation in low (U) and normal (N) animals at the age of 75 d. Furthermore, a 3.7-fold higher LD count has been observed, which was also reflected by a reduced distance (1.85-fold) to nearest LD (μm) in U vs. N pigs (Figure 4B–C). In addition, the LD area (μm²) was 24.9% higher in U compared to N animals (Figure 4D). This observation was confirmed by larger LD diameter (μm), LD circumference (μm) and LD convex area (μm²) of 12.1%, 13.0% and 25.8%, respectively (Figure 4E–G). Assuming a typical round shape of lipid droplets, the volume of LD was calculated with 0.248 and 0.176 μm³ for U and N animals, respectively. Taking into account the LD count for equal sized areas, the volume per total LD area was 5.2-fold higher with 223.6 and 43.4 μm³ for U and N animals, respectively.

In summary, low birth weight induces distinct changes in the metabolic gene expression profile, which was reflected by higher hepatic lipid droplet count and size (Figure 4H–I).

 processes of ER stress-associated protein degradation and unfolded protein response (UPR) is also shown in Table 6. Additionally, the regulated genes seem to play a role in inflammatory processes (Figure 5B, Table 7). To analyze the transcriptional effects of feed restriction also on the metabolic level, amino acid concentrations were additionally determined. We could show that the free arginine concentration in the liver of UR animals was 17% higher than in NR (Figure 6). Indeed, no differences were found for other amino acids. Effects of feed restriction were also observed in regard to genes involved in lipid metabolism in N (e.g. ABCA9, FADS1, CROT, STAR2, STAD13, SC5DL, ACAD10) and U (e.g. CYP26A1, ATP8A2, DHCR24, ACOX3, SLC27A3, HNF4A) animals. Of note, these effects were accompanied by changes in

### Table 2. Effects of 3-week feed restriction (age d 98), 5-day (age d 104) and 5-weeks refeeding (age d 131) on body weight development in U and N pigs used for liver tissue studies.

| Body weight [kg; means ± SEM] | d 98 | d 98 | d 104 | d 131 |
|------------------------------|------|------|-------|-------|
| UK                           | 1.07 ± 0.02*** | 27.8 ± 2.9 | 31.3 ± 2.0 | 38.0 ± 2.4 | 71.3 ± 0.7 |
| UR                           | 27.0 ± 3.5 | 37.5 ± 3.2 | 63.4 ± 2.6 |
| NK                           | 1.56 ± 0.02 | 31.3 ± 1.5 | 29.3 ± 1.6 | 39.0 ± 6.2 | 64.9 ± 4.9 |
| NR                           | 33.5 ± 2.0 | 35.4 ± 1.3 | 68.0 ± 3.4 |

***P<0.001 between groups per time point,
^P<0.001 per group;
^n = 3 per group.
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lipid droplet (LD) count and formation. As summarized in Table 4, the total mean area of LD was reduced by 58.3% and 72.7% after feed restriction in U and N animals, respectively. Moreover, parameters of LD formation including mean droplet area (μm²), mean LD diameter (μm) and mean LD circumference (μm) were also reduced by 7.9%, 4.7% and 5.6%, respectively, in U animals after feed restriction (UR) (p≤0.05). Significant effects, although of lesser magnitude, were also found in N animals after 3-week feed restriction period (Table 4). However, when related to NK pigs, UK pigs showed higher levels of mean LD area, mean LD diameter and mean LD circumference of about 21.7%, 7.1% and 9.0%, respectively, but LD total mean area was not affected (Table 4).

In summary, feed restriction induced changes in the hepatic gene expression profile that was translated into increased free liver arginine levels in U animals. Moreover, LD numbers and formation were also reduced in U and N animals during feed restriction (Figure 7A–D).

Long-term effects of feed restriction on the molecular and physiological level in U and N pigs

To further analyze immediate effects of feed restriction that might persist after refeeding, previously feed-restricted (UR, NR) and age-matched non-restricted control animals (UK, NK) were fed ad libitum for another 5 weeks until the age of d 131. Subsequently, effects on gene expression and metabolic variables related to amino acid and lipid metabolism were analyzed. As shown in Table 8 and 9, 4 (2 up- and 2 down-regulated) and 22 genes (4 up- and 18 down-regulated) show persistent regulation in U and N animals after 5 weeks of refeeding, respectively. These genes are involved in processes of lipid (FABP5, WNT5B, ACSL5, FGFR4) and protein metabolism (PTPRS, HSPA8, FGFR4), glucose homeostasis (SORT1, PPP1R3E, FGFR4) and (post)trans-
scriptional modification (NFκB1,2, CC2D1A, NSUN2, CAND2, PRMT7). In view of the fact that long-term transcriptional changes may be correlated with CpG island variation [31], methylation analyses were conducted in putative promoter regions of persistently regulated genes. Finally, 7 relevant metabolic genes (PTPRS, WNT5B, FABP5, PPP1R3E, PDE9A, SORT1, FGFR4) were included in methylation analysis. As shown in Figure 8 A and B, effects were found for FGFR4 and PTPRS gene with regard to birth weight (U vs. N). Thus, in U pigs, regions with a tendency of increased methylation where identified for FGFR4 and PTPRS.

Table 3. “Top 10” regulated genes in the liver of 75 d old pigs with low (U) and normal (N) birth weight.

| Ensembl ID       | Gene symbol | Gene name                                           | Gene Ontology function or process associations                                      | Fold Change |
|------------------|-------------|-----------------------------------------------------|--------------------------------------------------------------------------------------|-------------|
| up-regulated     |             |                                                     |                                                                                     |             |
| ENSP00000351206  | TXLNB       | taxilin beta                                        | -                                                                                    | +3.24       |
| ENSP00000389272  | RP11-88G17.6| Novel protein similar to hemicentin                 | -                                                                                    | +3.06       |
| ENSP00000356849  | POBK        | pogo transposable element with KRAB domain          | DNA binding, multicellular organisational development                               | +2.68       |
| ENSP00000361580  | ZNF691      | zinc finger protein 691                             | DNA and metal ion binding                                                           | +2.41       |
| ENSP00000273435  | EIF2A       | eukaryotic translation initiation factor 2A, 65 kDa | mRNA binding, SREBP-mediated signaling pathway                                       | +2.31       |
| ENSP00000394670  | PPIA        | peptidylprolyl isomerase A (cyclophilin A)           | Unfolded protein binding, protein peptidyl-prolyl isomerization, blood coagulation  | +2.08       |
| ENSP00000315662  | IQSEC3      | IQ motif and Sec7 domain 3                          | ARF guanyl-nucleotide exchange factor activity, regulation of ARF protein signal transduction | +2.08       |
| ENSP00000295256  | HPGDS       | hematopoietic prostaglandin D synthase              | calcium ion binding, fatty acid biosynthetic process, prostaglandin metabolic process | +2.04       |
| ENSP00000388194  | RSP01       | R-spondin homolog (Xenopus laevis)                  | Protein binding, positive regulation of canonical Wnt receptor signaling pathway     | +2.02       |
| ENSP00000204961  | EFN81       | ephrin-B1                                           | Ephrin receptor and protein binding, cell differentiation, multicellular organisational development, positive regulation of T cell proliferation | +2.01       |
| down-regulated   |             |                                                     |                                                                                     |             |
| ENSP00000352336  | PLCG2       | phospholipase C, gamma 2 (phosphatidylinositol-specific) | Hydrolase activity, phosphatidylinositol, phospholipase C activity, Wnt receptor signaling pathway, B and T cell receptor signaling pathway, lipid and phospholipid catabolic process | −2.84       |
| ENSP00000301645  | CYP7A1      | cytochrome P450, family 7, subfamily A, polypeptide 1 | Cholesterol 7-alpha-monooxygenase activity, bile acid biosynthetic process, cellular lipid metabolic process, cellular response to cholesterol, cholesterol catabolic process, cholesterol homeostasis | −2.77       |
| ENSP00000401437  | LDB3        | LIM domain binding 3                                | Protein and metal ion binding                                                       | −2.69       |
| ENSP00000342518  | SLC35F4     | solute carrier family 35, member F4                 | -                                                                                    | −2.68       |
| ENSP00000326324  | LRRC37A     | leucine rich repeat containing 37A                  | -                                                                                    | −2.69       |
| ENSP00000410447  | IKZF2       | IKAROS family zinc finger 2 (Helios)                | DNA and metal ion binding, regulation of DNA-dependent transcription                | −2.44       |
| ENSP00000348019  | SLC17A5     | solute carrier family 17 (anion/sugar transporter), member 5 | Sialic acid transmembrane transporter activity, sugar:hydrogen symporter activity, anion transport | −2.43       |
| ENSP00000402584  | MDGA1       | MAM domain containing glycosylphosphatidylinositol anchor 1 | Multicellular organisational development, cell differentiation, brain development | −2.41       |
| ENSP00000316740  | TTC29       | tetratricopeptide repeat domain 29                 | Binding                                                                              | −2.35       |
| ENSP00000357844  | SLC16A10    | solute carrier family 16, member 10                | (aromatic) amino acid and transmembrane transport, ion transport                    | −2.33       |

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Birth Weight-Dependent Effects in Juvenile Pigs

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case of PTPRS CpG-sites with increased methylation ratio in “U”-pigs are located in regions 2186 to 2170 and 21033 to 2730 relative to mRNA/gene start. In case of FGFR4, regions 2675 to 2178 and 1392 to 893 display the methylation change. Of note, FGFR4 gene expression was also reduced (FC = 1.36, p = 0.018). No transcriptional effects have been found for PTPRS gene (FC = 1.44, p = 0.213). Further effects of refeeding were analyzed in regard to LD formation and free amino acid concentrations in the liver of U and N animals. As shown in Figure 9A, the 5-weeks refeeding period induced the recovery of total mean LD area (%) in previously feed-restricted U and N animals (UR, NR) when related to their age-matched non-restricted controls (UK, NK). The LD count was greater (+51.6%, p≤0.001) in UR compared to NR animals (Figure 9B), which was accompanied by a decreased distance to nearest LD (Figures 9C). However, the LD size (μm²) was reduced by 17.1% in UR vs. NR animals, and by 23.3% in UR vs. UK animals (Figure 9D). This finding was supported by decreases in LD diameter and circumference, and LD convex area of 11.5%, 14.0% and 27.8% in UR vs. UK animals, respectively (Figures 9E–G). Indeed, in N animals, the opposite effect was observed. Thus, in NR pigs, the LD size (μm²) was increased by 24.7% when compared to NK (Figure 9D). This finding was supported by increases in LD diameter, LD circumference and convex LD area of 11.5%, 13.7% and 28.4%, respectively (Figures 9E–G).

With regard to the observed effects of feed restriction on hepatic arginine levels (Figure 6), measurements were also done after a 5-week refeeding period. However, there were not observed any differences later on between UR and NR (data not shown). Thus, in terms of the range of free arginine concentrations it was similar to those observed before feed restriction (R = K).

**Discussion**

Studies in animals [32–34] and humans [35] show a relationship between IUGR and the risk for metabolic-related diseases in later life. Individuals born with SGA show higher adult abdominal fat mass, lower insulin sensitivity and disturbances of other physiological parameters related to e.g. hepatic lipid metabolism [8,36–38]. Moreover, there is also evidence in the literature that early postnatal dietary restriction may improve metabolic param-
In IUGR offspring [39,40]. Based on these observations, we used the swine model to determine responses to low birth weight (vs. normal birth weight) after a postnatal feed restriction period and subsequent refeeding on the liver of female juvenile pigs. The swine model was used, because the pig resembles the human physiology in more ways than any other non-primate mammalian species. This is primarily due to physiological and anatomical similarity of the digestive tract [22]. As a basic requirement of our study, birth weights differed significantly between low and normal birth weight animals (1.07 ± 0.02 vs. 1.56 ± 0.02 kg) used for liver tissue analysis. Liver was selected as the target organ due to its primary importance in carbohydrate, protein and lipid metabolism. IPA for the differentially regulated genes showed an involvement in processes of cell death, cellular growth and proliferation, and lipid metabolism. Hence, the two strongest down-regulated genes (PLCG2, CYP7A1) suggest an impact of low birth weight on the regulation of lipid and/or cholesterol metabolism processes [41–44]. So far, only few articles have been published for humans and rodents investigating the relationship between low birth weight (IUGR) and hepatic lipid metabolism [9,45–47]. The higher liver fat contents observed in e.g. guinea pigs [45] and rat fetuses [9] with IUGR were attributed to inflammatory responses [46,48,49]. In fact, there is strong evidence in the literature supporting a positive relationship between inflammatory stress conditions and lipid and/or cholesterol accumulation in the liver [50]. The higher inflammatory status observed in IUGR animals was accompanied by increased ER stress, finally leading to UPR [51,52]. In this process, the hydrophobic matrix of LDs has been shown to become a sequestering surface for misfolded proteins [53]. Zhang et al. described the excessive deposition of LDs in cell types such as hepatocytes and macrophages as a hallmark in ER-stress

**Table 4.** Effects of 3-weeks feed restriction (R, T2) on LD size in the liver of U and N pigs.

|                  | Total mean area of LD [%] | Mean droplet size [μm²] | LD diameter [μm] | LD circumference [μm] |
|------------------|--------------------------|------------------------|-----------------|-----------------------|
| vs.              | UKT2 | URT2 | NRT2 | UKT2 | URT2 | NRT2 | UKT2 | URT2 | NRT2 | UKT2 | URT2 | NRT2 |
| UKT2             | -58.3 | ns   | -7.9 | ns   | -4.7 | ns   | -    | 5.6 | ns   |
| URT2             | ns   | -    | ns   | ns   | -    | ns   | ns   | ns  | -    |
| NRT2             | ns   | -    | 72.7 | +21.7 | ns | ns   | +7.1 | ns   | -3.1 | +9.0 | ns   | -3.3 |

Reading example URT2 vs. UKT2: The total mean area of LD% in URT2 pigs was reduced by 58.3% as compared to UKT2. Values are expressed as % deviation from the age-matched control groups (i.e. UR vs. UK, UR vs. NR, and NR vs. NK) (p ≤ 0.05) (n = 3 per group).

Table 4. Effects of 3-weeks feed restriction (R, T2) on LD size in the liver of U and N pigs.

|                  | Total mean area of LD [%] | Mean droplet size [μm²] | LD diameter [μm] | LD circumference [μm] |
|------------------|--------------------------|------------------------|-----------------|-----------------------|
| vs.              | UKT2 | URT2 | NRT2 | UKT2 | URT2 | NRT2 | UKT2 | URT2 | NRT2 | UKT2 | URT2 | NRT2 |
| UKT2             | -58.3 | ns   | -7.9 | ns   | -4.7 | ns   | -    | 5.6 | ns   |
| URT2             | ns   | -    | ns   | ns   | -    | ns   | ns   | ns  | -    |
| NRT2             | ns   | -    | 72.7 | +21.7 | ns | ns   | +7.1 | ns   | -3.1 | +9.0 | ns   | -3.3 |

Figure 5. *In silico* analysis of regulated genes sensitive to feed restriction in N (A) and U pigs (B) via IPA analysis. In liver tissues of pigs with normal birth weight (N), IPA network tool identified regulated genes sensitive to feed restriction with a primary role in cell death, cellular growth and proliferation, and tissue development processes (A). In low birth weight pigs (U), a major role in processes of cell death, protein degradation and protein synthesis was identified (B).

Reading example URT2 vs. UKT2: The total mean area of LD% in URT2 pigs was reduced by 58.3% as compared to UKT2. Values are expressed as % deviation from the age-matched control groups (i.e. UR vs. UK, UR vs. NR, and NR vs. NK) (p ≤ 0.05) (n = 3 per group).

Non-significant differences among groups are indicated by “ns”.

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Figure 5. *In silico* analysis of regulated genes sensitive to feed restriction in N (A) and U pigs (B) via IPA analysis. In liver tissues of pigs with normal birth weight (N), IPA network tool identified regulated genes sensitive to feed restriction with a primary role in cell death, cellular growth and proliferation, and tissue development processes (A). In low birth weight pigs (U), a major role in processes of cell death, protein degradation and protein synthesis was identified (B).

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Table 5. Identification of differentially regulated genes involved in amino acid metabolism in the liver of N pigs after 3 weeks of feed restriction (age d 98).

| Ensembl ID | Gene symbol | Gene name                                             | Gene Ontology function or process associations                                                                 | Fold Change |
|------------|-------------|-------------------------------------------------------|---------------------------------------------------------------------------------------------------------------|-------------|
| up-regulated |             |                                                       |                                                                                                              |             |
| ENSP00000388036 | ASPHD1      | aspartate beta-hydroxylase domain containing 1         | peptidyl-amino acid modification                                                                             | +2.82       |
| ENSP00000217420 | SLC32A1     | solute carrier family 32 (GABA vesicular transporter), member 1 | gamma-aminobutyric acid-hydrogen symporter activity, glycine transmembrane transporter activity            | +2.40       |
| ENSP00000378854 | PSPH        | phosphoserine phosphatase                             | L-serine biosynthetic process, phosphoserine phosphatase activity                                           | +1.71       |
| ENSP00000333003 | PRSS55      | protease, serine, 55                                  | serine-type endopeptidase activity                                                                           | +1.67       |
| ENSP00000317836 | PRR15       | proline rich 15                                       | multicellular organismal development                                                                        | +1.54       |
| ENSP00000368135 | LRRN4       | leucine rich repeat neuronal 4                        |                                                                                                              | +1.52       |
| ENSP00000280527 | CRIM1       | cysteine rich transmembrane BMP regulator 1 (chordin-like) | regulation of cell growth, insulin-like growth factor binding, serine-type endopeptidase inhibitor activity | +1.49       |
| ENSP00000320324 | NPEPPS      | aminopeptidase puromycin sensitive                    | cytosol alanyl aminopeptidase activity,                                                                       | +1.49       |
| ENSP00000361470 | ASS1        | argininosuccinate synthase 1                          | Arginine biosynthesis, Amino-acid biosynthesis, urea cycle                                                  | +1.47       |
| ENSP00000413373 | TNK2        | tyrosine kinase, non-receptor, 2                       | protein autophosphorylation                                                                                 | +1.47       |
| ENSP00000329291 | TARS52      | threonyl-tRNA synthetase-like 2                       | threonyl-tRNA aminoacylation, threonine-tRNA ligase activity                                               | +1.36       |
| ENSP00000348043 | LRRC20      | leucine rich repeat containing 20                     |                                                                                                              | +1.36       |
| down-regulated |             |                                                       |                                                                                                              |             |
| ENSP00000317382 | SLC36A4     | solute carrier family 36 (proton/amino acid symporter), member 4 | L-alanine transport, proline transport, tryptophan transport                                               | −2.18       |
| ENSP00000319255 | CHORDC1     | cysteine and histidine-rich domain (CHORD)-containing 1 | Stress response                                                                                              | −2.02       |
| ENSP00000250237 | QTRT1       | queuine tRNA-ribosyltransferase 1                     | Queuosine biosynthesis, tRNA processing                                                                    | −1.93       |
| ENSP00000349932 | PTPRS       | protein tyrosine phosphatase, receptor type, 5        | peptidyl-tyrosine dephosphorylation                                                                        | −1.76       |
| ENSP00000344762 | SFRS3       | serine/arginine-rich splicing factor 3                 | insulin receptor signaling pathway, mRNA processing, tRNA splicing                                          | −1.62       |
| ENSP00000244020 | SFRS6       | serine/arginine-rich splicing factor 6                 | RNA binding, nucleotide binding, negative regulation of nuclear mRNA splicing, via spliceosome              | −1.59       |
| ENSP00000401121 | VARS        | valyl-tRNA synthetase                                 | valine-tRNA ligase activity, Aminoacyl-tRNA synthetase                                                     | −1.55       |
| ENSP00000413162 | VARS        | valyl-tRNA synthetase                                 | valine-tRNA ligase activity, Aminoacyl-tRNA synthetase                                                     | −1.54       |
| ENSP00000425634 | HARS        | histidyl-tRNA synthetase                              | histidine-tRNA ligase activity, histidyl-tRNA aminoaoylation                                              | −1.53       |
| ENSP00000355890 | EPRS        | glutamyl-prolyl-tRNA synthetase                       | proline-tRNA ligase activity, glutamate-tRNA ligase activity                                               | −1.50       |
| ENSP00000274562 | LARS        | leucyl-tRNA synthetase                                | leucine-tRNA ligase activity                                                                               | −1.49       |
| ENSP00000281828 | FARS5       | phenylalanyl-tRNA synthetase, beta subunit             | phenylalanine-tRNA ligase activity, phenylalanine-tRNA aminoaoylation                                    | −1.47       |
| ENSP00000361930 | YWHA8       | tyrosine 3-monooxygenase/tryptophan S-monooxygenase activation protein, beta polypeptide | monoxygenase activity, protein targeting                                                                  | −1.42       |
| ENSP00000367440 | TTRAP       | tyrosyl-DNA phosphodiesterase 2                       | 5’-tyrosyl-DNA phosphodiesterase activity, transcription corepressor activity, protein binding            | −1.42       |
| ENSP00000303754 | PPID        | peptidylprolyl isomerase D                            | peptidyl-prolyl cis-trans isomerase activity, protein folding                                              | −1.39       |
| ENSP00000409324 | PRMT7       | protein arginine methyltransferase 7                  | cell differentiation, DNA methylation involved in gamete generation, regulation of gene expression by genetic imprinting | −1.38       |
Table 5. Cont.

| Ensembl ID       | Gene symbol | Gene name                                    | Gene Ontology function or process associations                                                                 | Fold Change |
|------------------|-------------|----------------------------------------------|---------------------------------------------------------------------------------------------------------------|-------------|
| ENSP00000278723  | GRIK4       | glutamate receptor, ionotropic, kainate 4    | kainate selective glutamate receptor activity, synaptic transmission, extracellular-glutamate-gated ion channel activity | −1.34       |
| ENSP00000359557  | LDOC1       | leucine zipper, down-regulated in cancer 1   | -                                                                                                             | −1.33       |
| ENSP00000264094  | LOXL3       | lysyl oxidase-like 3                         | oxidoreductase activity, acting on the CH-NH2 group of donors, oxygen as acceptor, scavenger receptor activity | −1.31       |

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Table 6. Identification of differentially regulated genes involved in amino acid metabolism in the liver of U pigs after 3 weeks of feed restriction (age d 98).

| Ensembl ID       | Gene symbol | Gene name                                    | Gene Ontology function or process associations                                                                 | Fold Change |
|------------------|-------------|----------------------------------------------|---------------------------------------------------------------------------------------------------------------|-------------|
| **up-regulated** |             |                                              |                                                                                                               |             |
| ENSP00000217420  | SLC32A1     | Solute carrier family 32 (GABA vesicular transporter), member 1 | glycine transmembrane transporter activity                                                                  | +1.81       |
| ENSP00000409976  | ASPA        | Aspartoacyclase                               | hydrolyase activity, acting on carbon-nitrogen (but not peptide) bonds, in linear amides                     | +1.77       |
| ENSP0000013222   | INMT        | Indolethylamine N-methyltransferase          | methyltransferase activity                                                                                   | +1.48       |
| ENSP00000310447  | GLS2        | Glutaminase 2                                 | glutamine metabolic process, glutaminase activity                                                             | +1.41       |
| ENSP00000325589  | PTPRCAP     | protein tyrosine phosphatase, receptor type, C-associated protein | receptor activity                                                                                           | +1.33       |
| **down-regulated** |             |                                              |                                                                                                               |             |
| ENSP00000355632  | GALNT2      | UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminytransferase 2 | protein O-linked glycosylation via serine or threonine                                                       | −2.03       |
| ENSP00000356207  | PPFIA4      | protein tyrosine phosphatase, receptor type, f polypeptide (PTPFR), interacting protein (liprin), alpha 4 | Protein binding                                                                                             | −1.92       |
| ENSP00000296486  | AGXT2L1     | alanine-glyoxylate aminotransferase 2-like 1 | cellular amino acid metabolic process, alanine-glyoxylate transaminase activity                              | −1.89       |
| ENSP00000278360  | PAMR1       | peptidase domain containing associated with muscle regeneration 1 | serine-type endopeptidase activity                                                                           | −1.60       |
| ENSP00000332223  | CRELD2      | cysteine-rich with EGF-like domains 2         | Protein binding, calcium ion binding                                                                          | −1.60       |
| ENSP00000324173  | HSPAS       | heat shock 70 kDa protein 5 (glucose-regulated protein, 78 kDa) | ER overload response, ER-associated protein catabolic process, regulation of protein folding in ER            | −1.56       |
| ENSP00000379250  | AURKA       | aurora kinase A                              | protein serine/threonine/tyrosine kinase activity                                                             | −1.51       |
| ENSP00000391739  | PISD        | phosphatidylserine decarboxylase             | phosphatidylserine decarboxylase activity                                                                     | −1.49       |
| ENSP00000331368  | SERPINB8    | serpin peptidase inhibitor, clade B (ovalbumin), member 8 | serine-type endopeptidase inhibitor activity, protein binding                                                | −1.48       |
| ENSP00000384295  | XBP1        | X-box binding protein 1                      | activation of signaling protein activity involved in unfolded protein response, positive regulation of endoplasmic reticulum unfolded protein response, immune response | −1.43       |
| ENSP00000366156  | SRM         | spermidine synthase                          | polyamine metabolic process                                                                                   | −1.40       |
| ENSP00000341538  | SEC61G      | Sec61 gamma subunit                          | P-P-bond-hydrolysis-driven protein transmembrane transporter activity, SRP-dependent cotranslational protein targeting to membrane | −1.39       |
| ENSP00000387180  | PTHR2       | peptidyl-tRNA hydrolase 2                    | aminoacyl-tRNA hydrolase activity                                                                            | −1.38       |
| ENSP00000223641  | SEC61B      | Sec61 beta subunit                           | ER-associated protein catabolic process, retrograde protein transport, ER to cytosol                           | −1.38       |
| ENSP00000250237  | QRTT1       | queuine tRNA-ribosyltransferase 1            | queuine tRNA-ribosyltransferase activity                                                                      | −1.34       |

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associated metabolic diseases including fatty liver disease [52]. Additionally, Lee et al. showed that ER stress promotes hepatic lipogenesis and LD formation in vitro [51]. Lipid droplets are linked to many cellular functions, including lipid storage for energy generation and membrane synthesis, and protein degradation [54]. Additionally, LD biogenesis is considered a physiological defense mechanism of the liver. Thus, through esterification of free fatty acids and its conversion into triglycerides and LD storage, fatty acid-induced toxicity of cells is reduced [55]. However, to prevent uncontrolled LD expansion, lipolysis becomes activated under physiological conditions. Defects in the regulation of lipid accumulation induce liver steatosis [56]. Autophagy has been identified as the mechanism to regulate the control of hepatic LD growth under pathological conditions. This process is associated to the maintenance of blood glucose and amino acid levels [57]. Hence, the increased LD count and size observed in low birth weight animals (vs. N) in our study could be due, at least in part, to autophagy-related processes.

Energetic restriction is hypothesized to improve metabolic outcomes in IUGR offspring [40]. This assumption was tested in liver tissues of U and N animals after three weeks of feed restriction (d 98). Microarray results indicated 20 transcripts with different expression levels (FC $\geq$1.3, p $\leq$0.05) that have functions in inflammation (Table 6) and UPR response (XBP-1, SEC61B, SEC61G, GRP78 ( = HSPA5)). The X-box binding protein 1 (XBP-1) is required for the function of normal fatty acid synthesis in the liver, and is thus an important regulator of hepatic lipogenesis [58,59]. Moreover, a study in the liver of adult mice showed that 50% loss of glucose-regulated protein 78 (GRP78) caused an ER stress response, which was accompanied by the

| Ensembl ID     | Gene symbol | Gene name                                | Gene Ontology function or process associations                                      | Fold Change |
|----------------|-------------|------------------------------------------|--------------------------------------------------------------------------------------|-------------|
| ENSP00000225842| CCL1        | chemokine (C-C motif) ligand 1           | Chemokine activity, immune response                                                  | +2.37       |
| ENSP00000369485| TNFRSF11A   | tumor necrosis factor receptor superfamily, member 11a, NFKB activator | Adaptive immune response, response to cytokine stimulus, positive regulation of NF-kappaB transcription factor activity | +2.25       |
| ENSP00000386239| NFkB1L2     | nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor-like 2   | histone binding, protein binding, transcription corepressor activity                  | +1.62       |
| ENSP00000399105| IL2RB       | interleukin 2 receptor, beta             | cytokine-mediated signaling pathway, interleukin-2 receptor activity, interleukin-2 binding | +1.40       |
| ENSP00000398379| STAT5B      | signal transducer and activator of transcription 5B                                 | T cell homeostasis, cytokine-mediated signaling pathway, isoleucine metabolic process, liver development | +1.40       |
| ENSP00000241052| CAT         | catalase                                 | NADP binding, catalase activity, oxidoreductase activity, aminoacylase activity        | +1.39       |
| ENSP00000420269| ADH7        | alcohol dehydrogenase 7                 | oxidoreductase activity                                                              | +1.38       |
| ENSP00000366729| TNFRSF9     | tumor necrosis factor receptor superfamily, member 9                                | induction of apoptosis, receptor activity                                             | −3.82       |
| ENSP00000247668| TRAF2       | TNF receptor-associated factor 2         | activation of NF-kappaB-induced kinase activity, positive regulation of T cell activation, innate immune response, tumor necrosis factor-mediated signaling pathway | −2.31       |
| ENSP00000329418| SOCS1       | suppressor of cytokine signaling 1       | type I interferon-mediated signaling pathway, interferon-gamma-mediated signaling pathway | −2.00       |
| ENSP00000394473| DOCK8       | dedicator of cytokinesis 8               | blood coagulation                                                                    | −1.87       |
| ENSP00000371676| IFNAR2      | interferon (alpha, beta and omega) receptor 2                                      | type I interferon binding, type I interferon receptor activity                        | −1.78       |
| ENSP00000355046| MT-ND2      | mitochondrially encoded NADH dehydrogenase 2                                         | NADH dehydrogenase (ubiquinone) activity, mitochondrial electron transport, NADH to ubiquinone | −1.74       |
| ENSP00000368698| HIVEP1      | human immunodeficiency virus type 1 enhancer binding protein 1                      | zinc ion binding                                                                     | −1.65       |
| ENSP00000416956| Pdia5       | protein disulfide isomerase family A, member 5                                       | protein disulfide oxidoreductase activity, electron carrier activity                  | −1.49       |
| ENSP00000376500| TRAF3       | TNF receptor-associated factor 3         | innate immune response, toll-like receptor signaling pathway, tumor necrosis factor-mediated signaling pathway | −1.41       |
| ENSP00000376092| CASP8       | caspase 8, apoptosis-related cysteine peptidase                                      | response to tumor necrosis factor, innate immune response, positive regulation of I kappaB kinase/NF-kappaB cascade | −1.37       |
| ENSP00000337127| SOD2        | superoxide dismutase 2, mitochondrial   | removal of superoxide radicals, age-dependent response to reactive oxygen species, oxygen homeostasis | −1.35       |
| ENSP00000355190| NFE2L1      | nuclear factor (erythroid-derived 2)-like 1                                          | inflammatory response, heme biosynthetic process                                      | −1.35       |
| ENSP00000262053| ATF1        | activating transcription factor 1         | innate immune response, Toll signaling pathway, stress-activated MAPK cascade         | −1.33       |
onset of apoptosis [60]. Furthermore, these mice exhibited increased fat accumulation in the liver. In agreement with our text mining analysis, the regulated genes in pig liver were attributed to processes of cell death, protein degradation and protein synthesis. Of interest is that these effects were not found in NR pigs. In N animals, the effects were mainly seen in a reduction of mRNA levels of aminoacyl tRNA synthetases and their processing (TARSL2, QTRT1, VARS, HARS, EPRS, LARS, FARSb). Since the presence of aminoacyl tRNA synthetases is a precondition for translation, this finding likely reflects the reduced protein synthesis during feed restriction [61–64]. With regard to the observed transcriptional effects of feed restriction on processes related to protein degradation and synthesis, amino acids were additionally determined in liver tissues of U and N animals. We could show that a 3-week feed restriction period induced an increase of free arginine levels in the liver of U pigs as compared to feed-restricted N pigs. Arginine, the nitrogenous precursor of nitric oxide [65] has been shown to regulate multiple metabolic pathways involved in the metabolism of fatty acids, glucose, amino acids, and proteins through cell signaling and gene expression [66]. Of note, arginine is converted to nitric oxide (NO) by nitric oxide synthase (NOS) in almost all mammalian cells [65,67]. Secondly, NO increases the phosphorylation of hormone-sensitive lipase and perilipins, finally leading to the translocation of the lipase to the neutral LDs, and, hence, the stimulation of lipolysis [68–71]. With regard to the results from gene expression and amino acid analysis, diet-induced effects on hepatic LD content were also hypothesized. A 3-week feed restriction period induced a significant decrease in the total mean area of LDs of 58.32 and 72.67% in U and N animals, respectively, when related to their age-matched controls. In general, feed restriction effects were more pronounced on LD count than on LD size in both U and N animals. The observed strong decrease in LD count was also found in liver tissue of caloric-restricted rats when related to ad libitum fed animals [72]. Moreover, a recent study in rats showed that early postnatal caloric restriction protected adult male IUGR offspring from obesity [73]. However, there is a lack of studies determining effects of feed restriction and subsequent refeeding in view of birth weight [74–76]. The first evidence of how deficient nutrient supply in utero affects birth weight, and the subsequent risk for health disorders in the offspring came from large epidemiological studies such as The Dutch Hunger Winter Families study in 1944–1945 [77] [78,79]. It was thus assumed that environmental conditions in early life can change epigenetic settings which remain throughout life [80,81].

The second aim of our pig study was to determine long-term effects of feed restriction on molecular features of hepatic lipid metabolism in relation to birth weight. First of all, significantly regulated genes sensitive to feed restriction, and moreover, with...
Persisting expression levels after five weeks of refeeding, were selected. Overall, the 26 identified genes have functions in DNA-dependent transcription processes, cell differentiation and metabolic homeostasis. Of note, the up-regulated IGLV3-1 gene (in U animals) encodes an antibody of the innate immune system that has been shown to induce tumor-specific cell death via intracellular lipid accumulation, a process that is named lipoptosis [82]. Long-term regulated gene expression is thought to be mediated by epigenetic mechanisms [83]. Epigenetic changes are changes in gene function that occur without changes in gene sequence [84]. Epigenetic regulations can occur at the level of DNA methylation, histone modification or microRNA regulation. DNA methylation of CpG dinucleotides is accompanied by the inability of transcription factors to bind to specific DNA regulatory sequences, and is thus closely linked to silencing of gene transcription. With regard to our study, persistently regulated genes were further selected for methylation analysis. Hence, only genes with detectable CpG island regions in the promoter region, and with publicly available sequence annotations were further considered.

With regard to the selected genes (PTPRS, WNT5B, FABP5, PPP1R3E, PDE9A, SORT1, FGFR4), detectable effects were found for FGFR4 and PTPRS genes in relation to birth weight (U vs. N). Thus, U animals showed increased methylation status in each two regions of fibroblast growth factor receptor 4 (FGFR4) and protein tyrosine phosphatase, receptor type, S (PTPRS) gene. In concordance to common opinion, the increased methylation ratio in the FGFR4 gene in U vs. N animals corresponds with a decreased expression rate. A study in FGFR4-deficient mice on a normal diet exhibited features of the metabolic syndrome, including increased mass of white adipose tissue, hyperlipidemia, hypercholesterolemia and insulin resistance [85]. Moreover, restoration of FGFR4 in hepatocytes of FGFR4-deficient mice restored fatty liver with a simultaneous decrease of plasma lipids. Thus, FGFR4 seems to play a role in hepatic lipid metabolism and might explain, at least in part, the increased LD count and size observed in low birth weight animals (U vs. N) in our study. To get an idea about the persistence of metabolic effects related to lipid metabolism in low birth weight animals, previously feed-restricted

Table 8. Identification of long-term regulated genes in the liver of U pigs responsive to 3-week feed restriction (T2, age d 98) and after 5 weeks of refeeding (T3, age d 131).

| Ensembl ID       | Gene symbol | Gene name                                  | Gene Ontology function or process associations                          | Fold Change URT2 vs. UKT2 | Fold Change URT3 vs. UKT3 |
|------------------|-------------|--------------------------------------------|------------------------------------------------------------------------|---------------------------|---------------------------|
| up-regulated     |             |                                            |                                                                        |                           |                           |
| ENSP00000374854  | IGLV3-1     | immunoglobulin lambda variable 3-1         | -                                                                     | +1.82                     | +2.07                     |
| ENSP00000386239  | NFKBI2      | Inhibitor of kappa B-related protein       | histone binding, transcription corepressor activity                    | +1.62                     | +1.42                     |
| down-regulated   |             |                                            |                                                                        |                           |                           |
| ENSP00000281924  | TMEM163     | transmembrane protein 163                  | cation transmembrane transporter activity                              | −1.89                     | −2.01                     |
| ENSP00000355046  | MT-ND2      | NADH-ubiquinone oxidoreductase chain 2     | NADH dehydrogenase activity, small molecule metabolic process          | −1.74                     | −1.33                     |

Figure 8. Promoter methylation analysis of FGFR4 and PTPRS gene. As shown in Figure A, low birth weight (U) induced increased methylation status of FGFR4 gene in amplicons 04 (#1 and #2 = −2635, −263 and −2625, −2624 and #9 = −2453, −2452) and amplicon 44 (#4 = −1046, −1045 and #5 = −1109, −1108) when related to normal birth weight animals (N). This was supported by significant decreases in gene expression (FC = −1.36, p = 0.018, data not shown). An increased methylation status with regard to birth weight was also indicated in PTPRS gene promoter (B) (amp41 #3 = −477, −476, #4 and #5 = −492, −491 and −498, −497, #6 = −520, −519; amp09 #5 = −1818, −1817), however, with no changes in gene expression (FC = −1.44, p = 0.213, data not shown).

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Table 8. Identification of long-term regulated genes in the liver of U pigs responsive to 3-week feed restriction (T2, age d 98) and after 5 weeks of refeeding (T3, age d 131).
pigs were also analyzed after five weeks of refeeding. Of specific interest is that the mean LD size increased about 24.7% in NR animals when related to NK animals. Indeed, in U animals the opposite effect was observed after 5 weeks of refeeding. Thus, LD size was 23.3% lower in UR vs. UK animals. This finding was supported by further reductions of parameters related to mean LD size.

### Table 9. Identification of long-term regulated genes in the liver of N pigs responsive to 3-week feed restriction (T2, age d 98) and after 5 weeks of refeeding (T3, age d 131).

| Ensembl ID       | Gene symbol | Gene name                                      | Gene Ontology function or process associations                                                                 | Fold Change  |
|------------------|-------------|-----------------------------------------------|---------------------------------------------------------------------------------------------------------------|---------------|
|                  |             |                                               | NRT2 vs. NKT2                                                                                                 | NRT3 vs. NKT3* |
| up-regulated     |             |                                               |                                                                                                              |               |
| ENSP00000363369  | MIA         | melanoma inhibitory activity                 | growth factor activity                                                                                      | +1.63         | +3.99         |
| ENSP00000313601  | CC2D1A      | coiled-coil and C2 domain containing 1A       | regulation of transcription, DNA-dependent, signal transducer activity                                       | +1.58         | +1.79         |
| ENSP00000354481  | FAM5B       | family with sequence similarity 5, member B   | -                                                                                                             | +1.42         | +1.85         |
| ENSP00000418356  | MRAS        | muscle RAS oncogene homolog                   | GTP binding, GTPase activity                                                                                 | +1.48         | +1.97         |
| down-regulated   |             |                                               |                                                                                                              |               |
| ENSP00000256637  | SORT1       | sortilin 1                                    | glucose import, response to insulin stimulus, negative regulation of apoptotic process                        | -1.82         | -1.94         |
| ENSP00000230050  | RPS12       | ribosomal protein 512                          |                                                                                                              | -1.38         | -1.60         |
| ENSP00000264670  | NSUN2       | NOP2/Sun domain family, member 2              | tiRNA binding, tiRNA (cytosine-5-) methyltransferase activity                                               | -1.53         | -1.37         |
| ENSP00000297258  | FABP5       | fatty acid binding protein 5                  | fatty acid binding, protein binding, transporter activity                                                   | -1.32         | -1.70         |
| ENSP00000308887  | WNT5B       | wingless-type MMTV integration site family, member 58 | Positive regulation of fat cell differentiation and cell migration, cellular response to retinoic acid          | -1.62         | -1.92         |
| ENSP00000322061  | C7          | complement component 7                        | Complement activation, cytosis                                                                               | -1.48         | -1.74         |
| ENSP00000320040  | RPL3        | small nuclear RNA, C/D box 43                 | translation                                                                                                  | -1.33         | -1.35         |
| ENSP00000349932  | PTPRS       | protein tyrosine phosphatase, receptor type, 5 | transmembrane receptor protein tyrosine phosphatase activity, protein binding                                | -1.76         | -1.64         |
| ENSP00000354717  | PPP1R3E     | protein phosphatase 1, regulatory (inhibitor) subunit 3E | glycogen metabolic process                                                                                     | -1.45         | -1.66         |
| ENSP00000355688  | GUK1        | guanylate kinase 1                            | purine nucleotide metabolic process, nucleobase-containing small molecule interconversion                      | -1.44         | -1.35         |
| ENSP00000362524  | ANGPTL2     | angiotensin-like 2                            | multicellular organisal development, signal transduction                                                    | -1.35         | -1.46         |
| ENSP00000364240  | UBK10       | UBX domain protein 10                         |                                                                                                              | -1.51         | -1.61         |
| ENSP00000376526  | HSPA8       | heat shock 70 kDa protein 8                   | protein folding and binding, response to unfolded protein, negative regulation of transcription, DNA-dependent | -1.89         | -1.94         |
| ENSP00000376796  | ACSL5       | acyl-CoA synthetase long-chain family member 5| long-chain fatty acid-CoA ligase activity, ATP binding, fatty acid transport, response to cholesterol and glucose stimulus | -1.36         | -1.44         |
| ENSP00000381280  | PDE9A       | phosphodiesterase 9A                          | 3',5'-cyclic-GMP phosphodiesterase activity, blood coagulation, metal ion and protein binding                 | -1.52         | -1.52         |
| ENSP00000387641  | CAND2       | cullin-associated and neddylation-dissociated 2 (putative) | Positive regulation of transcription, DNA-dependent                                                          | -1.60         | -1.35         |
| ENSP00000409324  | PRMT7       | protein arginine methyltransferase 7          | DNA methylation involved in gamete generation, regulation of gene expression by genetic imprinting, cell differentiation, regulation of protein binding | -1.38         | -1.42         |
| ENSP00000366412  | FGFR4       | fibroblast growth factor receptor 4           | glucose homeostasis, peptidyl-tyrosine phosphorylation, regulation of cholesterol homeostasis, positive regulation of proteolysis | -1.41*        | -1.52         |

*p≤0.05 or the highest absolute signal in one group is equal or lower than the lowest value in the other group and vice versa.

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size such as diameter, circumference and convex area. In summary, it seems that the 3-week feed-restriction period sensitized U animals for an increased catabolic rate of LDs in the liver. This is due to the fact that the smaller LD droplets observed in the liver of previously feed-restricted U animals are possibly more accessible for surface lipases and thus lipid oxidation.

Figure 9. Effect of refeeding period on lipid droplet (LD) count and formation in previously feed restricted (R) and non-restricted (K) N and U pigs. As shown in Figure A, 5-week refeeding period induced the recovery of total mean LD area in previously feed-restricted animals (UR, NR) when compared to their non-restricted age matched controls (UK, NK). Moreover, LD count was increased by 51.6% in UR vs. NR animals (B), which was accompanied by a decreased mean distance to nearest LD (C). In contrast, the mean lipid droplet size (μm²) was reduced by 23.3% in UR vs. UK animals (D). This was supported by decreases of mean LD diameter (E), mean LD circumference (F), and mean convex area (G) of 11.5%, 14.0% and 27.8%, respectively. Of note, in N animals, the opposite effect was observed. Here, the mean LD size (μm²) was increased by 24.7% when compared to controls (NK) (D). This finding was supported by increases of LDs in mean diameter (E), mean circumference (F) and mean convex area (G) of 11.5%, 13.7% and 28.4%, respectively. These results are illustrated by representative microscopic observations of oil-red stained liver sections of UK (H), UR (I), NK (K) and NR (L) pigs.

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The expression was markedly reduced (FC = 2.03, p = 0.03) in UR compared to NR animals after five weeks of refeeding. Serum PON1 is an HDL-associated lipolactonase, which is synthesized and secreted by the liver [99]. PON1 enzyme’s capability to prevent oxidation processes in HDL [101] was reduced about 2.1-fold (p = 0.05) in UR compared to NR animals when related to their age-matched controls after five weeks of refeeding. Thus, when related to NK animals, FADS2 expression was markedly reduced (FC = -1.66, p = 0.067) in NR animals. FADS2 belongs to the group of fatty acid desaturase (FADS) enzymes of the omega 6 family that regulate the unsaturation of fatty acids through the introduction of double bonds [94]. In turn, dietary polyunsaturated fatty acids (PUFA) of the omega 3 and omega 6 family suppress the expression of lipogenic genes while concomitantly inducing the expression of genes related to fatty acid oxidation [95–97]. Of note, these mice were resistant to diet-induced as well as genetic obesity. Overall, based on our data, we suggest that the 3-week feed-restriction period sensitized UR animals for a higher lipolytic activity in later life. This assumption was supported by the increased expression levels of fatty acid desaturase 2 (FADS2, FC = 2.03, p = 0.03) in UR compared to NR animals after five weeks of refueling. Thus, when related to NK animals, FADS2 expression was markedly reduced (FC = -1.66, p = 0.067) in NR animals. FADS2 belongs to the group of fatty acid desaturase (FADS) enzymes of the omega 6 family that regulate the unsaturation of fatty acids through the introduction of double bonds [94]. In turn, dietary polyunsaturated fatty acids (PUFA) of the omega 3 and omega 6 family suppress the expression of lipogenic genes while concomitantly inducing the expression of genes related to fatty acid oxidation [95–97].

Furthermore, are the data from FADS2 knockout mice, which show an increased macrophage cholesterol biosynthesis and decreased cellular paraoxonase 2 (PON2) expression [98]. In our study, PON1 and PON3 have been found to be the strongest down-regulated genes (FC = -11.13 and -5.73, respectively, p≤0.05) in the liver of NR animals when related to their age-matched controls after five weeks of refeeding. Serum PON1 is an HDL-associated lipolactonase, which is synthesized and secreted by the liver [99]. PON1 has antioxidant properties [100], which are associated with the enzyme’s capability to prevent oxidation processes in HDL [101] and LDL [102], to decrease the oxidative status in macrophages [103] and atherosclerotic lesions [104], and to stimulate cholesteryl efflux from macrophages [105]. Thus, based on PON1 and PON3 expression levels, oxidative stress conditions seem to be increased in previously feed-restricted N animals after five weeks of refeeding. This assumption is supported by data of a mouse model of moderate caloric restriction, in which a 10–15% weight loss that is comparable to human dieting induced increases of the level of the stress hormone plasma corticosterone [106]. Of note, in this study the observed effects on gene expression and promoter methylation of corticotropin-releasing factor were not normalized after refueling. Moreover, studies in adult rats show that catch-up growth after feed restriction resulted in increased intramuscular and intrahepatic lipid content, visceral fat deposition, and dyslipidemia [75,76]. Thus, a link between inflammatory response and hepatic lipid accumulation is suggested. Another study showed that treatment of microglia with pro-inflammatory lipopolysaccharide induced not only accumulation of LDs but also increased their size [107]. This stress-associated response may partly explain the observed increased LD size in previously feed-restricted N animals after refueling. On the basis of our observation period we conclude that early-life feed restriction may program juvenile female pigs with low birth weight for an increased rate of hepatic lipolysis in later life. Finally, the observed differences in the metabolic responses between low and normal birth weight animals might be due to metabolic imprinting during critical periods of life [17,108], inter alia mediated by epigenetic mechanisms such as DNA methylation. However, other epigenetic mechanisms such as histone modification and microRNA (miR) regulation may also explain the observed long-term effects [109]. These investigations are planned to be addressed in future research.

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