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Hepatic lipid metabolism is affected by a daily 3-meal pattern with varying dietary crude protein with a pig model

Chunyan Xie [a, b], Xinyi Duan [a, c], Cimin Long [c], Xin Wu [b, c, *]

[a] College of Bioscience and Biotechnology, Hunan Agricultural University, Changsha 410128, China
[b] Institute of Biological Resources, Jiangxi Academy of Sciences, Nanchang 330096, China
[c] Key Laboratory of Agro-ecological Processes in Subtropical Region, Institute of Subtropical Agriculture, Chinese Academy of Sciences, National Engineering Laboratory for Pollution Control and Waste Utilization in Livestock and Poultry Production, Hunan Provincial Engineering Research Center for Healthy Livestock and Poultry Production, Changsha 410125, China

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ABSTRACT

The present study was conducted to evaluate the effects of 3 meals administered daily with varying dietary crude protein (CP) contents on hepatic lipid metabolism with a pig model. Pigs were divided into 3 groups according to the following feeding patterns: feeding a basal CP diet 3 times daily (3C); feeding a high CP diet for breakfast, the basal CP diet for lunch, and a low CP diet for dinner (HCL); and feeding the low CP diet for breakfast, the basal CP diet for lunch, and the high protein diet for dinner (LCH). Three groups took equivalent diet per meal ensuring that every pig was fed with similar dietary formulae daily. Results showed that HCL feeding pattern reduced the relative kidney weight ($P < 0.05$), and LCH feeding pattern increased the relative liver weight of pigs ($P < 0.05$) when compared with those in the 3C group. Plasma urea nitrogen ($P < 0.01$) and lipase ($P < 0.05$) decreased in the HCL group but increased in the LCH group. Both HCL and LCH feeding patterns reduced plasma triglycerides ($P < 0.01$), non-esterified fatty acids (NEFA) ($P < 0.01$), and hepatic crude fat ($0.05 < P < 0.10$) of pigs. Real-time quantitative PCR (RT-qPCR) results showed that dynamic feeding patterns down-regulated ($P < 0.05$) the mRNA level of lipid metabolism related genes, including adipose triglyceride lipase (ATGL), acetyl-CoA carboxylase (ACCα), liver X receptor (LXRα) in the liver, and negatively regulate elements of circadian clock, including period 1 (Per1), period 2 (Per2), cryptochrome (Cry2), which in turn, upregulated ($P < 0.05$) the protein expression of positive regulate element brain and muscle Arnt-like 1 (BMAL1) when compared with 3C group. Overall, our findings suggested that dynamic feeding patterns may affect hepatic lipid metabolism via regulation of the circadian clock.

1. Introduction

There is growing evidence on the interplay between feeding and the circadian system (Asher and Sassone-Corsi, 2015; Longo and Panda, 2016). It has been reported that time for food intake is a modifiable behavior that may influence energy regulation and consequently the risk of obesity. Several studies performed in experimental animals have demonstrated that animals become obese when they eat at the wrong time, whereas they apparently eat and expend the same amount of energy (Garaulet and Gomez-Abellan, 2014; Makwana et al., 2019). Mice fed a high fat diet during dark night (i.e., appropriate feeding time) weigh less than those fed during day time in light (feeding is reduced) (Arble et al., 2009). Wang et al. (2014) demonstrated that while energy intake in the morning was not associated with obesity, those who consumed more than 33% of daily energy intake in the evening were 2 times more likely to be obese than morning eaters (Wang et al., 2014). Our latest study also showed that feeding according to the circadian system could improve the growth performance of pigs or the production performance of sows (Wu et al., 2017, 2018). These findings

emphasized that time for food intake may play a significant role in weight control and obesity.

Liver has long been recognized to be one of essential metabolic organs. Studies have reported that increased carbohydrate metabolism and insulin actions in the liver following food consumption are responsible for the transcriptional activation of entire lipogenic pathways in mammals (Cuif et al., 1992), and the circadian clock generates approximately 24-h rhythms in feeding behavior, even under constant environmental conditions (Vollmers et al., 2009). Specifically for the liver, the rhythmic expression of clock genes has recently been reported in rats (Stokkan et al., 2001; Davidson et al., 2002), mice (Takata et al., 2002) and pigs (Xie et al., 2016). Studies on genome-wide circadian expression profiles in mice have uncovered potential connections between circadian clocks and metabolism, including energy generation (Davidson et al., 2002). Consequently, peripheral circadian clocks, especially those in the liver, can be strongly entrained by nutrients.

It has been shown that the efficient use of nutrients can be improved by adjusting the nutrient supply to the individual requirements of the animal (Ferket et al., 2002; Hauschild et al., 2012). Indeed, dietary different levels of protein affected the expression of peroxisome proliferator-activated receptor alpha (PPARα) in the liver (Weber et al., 2008). At present, it is fairly common to have excess protein in practical diets, particularly when the price of protein sources is low. Our previous work also showed that 3 meals administered daily with varying crude protein (CP) can regulate hormone secretion and growth performance (Xie et al., 2015). We have demonstrated that feeding high protein meal in the morning and a gradual reduction of the protein content in meals over the day could improve muscle quality characteristics, maximize performance, and reduce the pig feed cost (Wu et al., 2018). In this study, we further assessed the hepatic lipid metabolism affected by feeding pattern, which would supply a reference for animal or human nutrition.

2. Materials and methods

This study followed the guidelines for the treatment of animal subjects as approved by the Animal Care Committee of the Institute of Subtropical Agriculture, Chinese Academy of Science.

2.1. Animals and experimental treatments

Twenty one 60-day-old barrows (Duroc × Landrace × Large Yorkshire) with a similar initial weight (average body weight [BW] = 20.5 ± 1.5 kg) were fed 3 times a day at 08:00, 12:00 and 18:00. All pigs were randomly assigned to 1 of 3 groups (7 replicates/group) that varied in feeding sequence: feeding a basal protein diet 3 times daily (3C; control group); feeding a high CP diet for breakfast, the basal CP diet for lunch, and a low CP diet for dinner orderly (HCL); and feeding the low CP diet for breakfast, the basal CP diet for lunch, and the high protein diet for dinner orderly (LCH). Experiments were performed over two 6-week periods. During period 1, the control, high-protein, and low-protein diets were composed of 18.11%, 21.04%, and 15.29% CP, respectively. During period 2, the control, high-protein, and low protein diets were composed of 15.29%, 18.11%, and 12.53% CP, respectively. Pigs in the HCL and LCH groups were fed comparable amounts of their respective diets at 08:00 and 18:00 throughout the experimental periods ensuring that all pigs consumed the same formula of feed and the same amounts of calories a day. All pigs were housed individually in an environmentally controlled nursery and had free access to fresh water throughout the experiment.

In the present study, the nutrients comprising the feed of the control diets were considered adequate for pigs and met the National Research Council's (NRC) recommended requirements within the appropriate weight range. The compositions of the diets are listed in Table 1.

2.2. Sample collection

The 21 pigs were anesthetized, bled, and slaughtered at 144 d when the live weight was 60 to 70 kg. A 5-mL blood sample was collected from the jugular vein at 06:00 after 12 h fasting before being slaughtered at 08:00 orderly, and a plasma sample was obtained immediately by centrifugation at 3,000 × g for 10 min at 4 °C and stored at −80 °C. Visceral organs, including the heart, liver, kidney, and spleen, were excised and weighed. The relative weight of each visceral organ was calculated as the organ weight divided by the slaughter weight (%). In addition, a part of liver tissue was taken and immediately frozen in liquid nitrogen and stored at −80 °C for further analysis.

2.3. Determination of plasma biochemical parameters and non-esterified fatty acids (NEFA)

Plasma biochemical parameters, including alkaline phosphatase (ALP), alanine transaminase (ALT), aspartate aminotransferase (AST), glucose, ammonia (Amm), urea nitrogen (Urea), lipase, triglyceride (TG), high-density lipoprotein (HDL), low-density lipoprotein (LDL), and total cholesterol (CHO) were measured using a Biochemical Analytical Instrument (Beckman CX4, Beckman Coulter Inc., Brea, CA, USA) and commercial kits (Sino-German Beijing Leadman Biotech Ltd., Beijing, China).

In addition, the content of plasma free fatty acids was determined using a NEFA C test kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan) according to the manufacturer's instruction.

2.4. Determination of the crude fat proportion in the liver

The proportion of hepatic crude fat was determined according to the Soxhlet method. Freeze-dried powder of liver tissue was placed in a thimble measuring 22 mm × 28 mm (Foss North America, Eden Prairie, MN, USA), fitted with metal adaptors, and loaded into an automated SOXTHERM fat extraction system (Gerhardt, Germany). The resulting extract was then dried in an oven at 104 °C and cooled in a desiccator to determine the fat proportion gravimetrically.

2.5. Determination of the polyunsaturated fatty acid (PUFA) profile in the liver

Lipids from liver tissue were extracted with a mixture of chloroform and methanol according to the method described by Folch et al. and transmethylated with boron trifluoride (BF3) and methanolic KOH. The PUFA profile was then determined by gas chromatography (Agilent 6890, Boston, MA, USA). The results are expressed as a percentage of total fatty acids.

2.6. RNA extraction and cDNA synthesis

Approximately 100 mg of liver tissue was pulverized in liquid nitrogen. Total RNA was isolated from homogenate using the TRIzol reagent (100 mg liver tissue per 1 mL Trizol; Invitrogen, Carlsbad, CA, USA). The RNA integrity was checked by 1% agarose gel electrophoresis, stained with 10 μg/mL ethidium bromide. The quality and quantity of RNA were determined by ultraviolet spectroscopy using a NanoDrop ND-1000 (Thermo Fisher Scientific, DE, USA), and the RNA sample with A260:A280 ratio between 1.9 and 2.0 was selected. RNA (1,000 ng or 1 μg) was treated with DNase I according to the manufacturer's instructions before reverse transcription and

Table 1
Composition of the pig diets (%, as-fed basis).

Item	Feeds			
	21.04% CP	18.11% CP	15.29% CP	12.63% CP
Ingredients				
Corn (8% CP; < 13% H_2O)	54.60	63.50	72.40	81.3
Soybean expanded (43% CP)	29.00	21.27	13.54	5.81
Enzymatically decomposed Soybean meal (CP 48%)	8.35	7.50	6.65	5.80
L-lysine 98%	0.00	0.20	0.40	0.60
L-methionine	0.00	0.03	0.06	0.09
L-threonine	0.05	0.10	0.15	0.20
Soybean oil	4.00	3.40	2.80	2.20
Premix[1]	4.00	4.00	4.00	4.00
Total	100.00	100.00	100.00	100.00
Nutrient levels[2]				
CP	21.04	18.11	15.29	12.63
Lysine	1.06	1.02	0.99	0.95
Methionine	0.33	0.32	0.31	0.30
Methionine + Cystine	0.67	0.62	0.57	0.51
Threonine	0.88	0.79	0.70	0.61
Calcium	0.58	0.60	0.55	0.60
Total phosphorus	0.59	0.62	0.56	0.56
Available phosphorus	0.41	0.42	0.39	0.39
Digestible energy, MJ/kg	13.68	13.72	13.77	13.80

CP = crude protein.

[1] Providing the following in grams per kilogram diet: poly-mineral 25; carnitine 0.5; mixed vitamin 11.5; gourmet power 2.5; antioxidant 5; mildew preventive 12.5; $Fe_2(SO_3)_3$ 5; $MgSO_3$ 10; Cr 5; P–Ca 285; limestone 300; chaff 91.5; salt 75; feed carrier with zeolite powder.

[2] Nutrient levels are calculated.

polymerase chain reaction. Synthesis of the first strand cDNA was performed with Oligo (dT) 20 and Superscript II reverse-transcriptase and stored at −80 °C until use. All the reagents used in this process were purchased from Life Technologies, Tokyo, Japan.

2.7. Real-time quantitative PCR (RT-qPCR)

Primers were designed with Primer 5.0 using the pig gene sequence (http://www.ncbi.nlm.nih.gov/pubmed/) to produce an amplification product (Table 2). The RT-qPCR was performed on the ABI 7900HT Fast qPCR System (Applied Biosystems, CA) with a total volume of 10 μL containing 5 ng of cDNA, 5 μL SYBR Green mix, 0.2 μL ROX Reference Dye (50×), 0.6 μL primers (forward and reverse), and some purified water. Reactions were seeded in a 384-well plate, and the PCR cycles included initial pre-denaturation at 95 °C for 10 s and 40 cycles of denaturation at 95 °C for 5 s, annealing at 60 °C for 20 to 30 s. The relative level of mRNA expression was calculated using the $2^{-(\Delta\Delta Ct)}$ method after normalization with β-actin as a reference gene (Wu et al., 2012). Therefore, relative gene expressions of 3 groups were reported as a fold change of the mean of control value, and relative expression of target genes in 3C group was 1.0.

2.8. Protein extraction and Western blot analysis

Liver tissue was pulverized in liquid nitrogen and lysed in radioimmunoprecipitation assay (RIPA) buffer (Beyotime Biotechnology, China) mixed with 1 mmol/L phenylmethylsulfonyl fluoride (PMSF) and 1% phosphatase inhibitor. Following centrifugation at 12,000 × g for 10 min at 4 °C, the protein concentration in the supernatant was determined using a bicinchoninic acid assay (Beyotime Biotechnology, Shanghai, China). Western blots were performed using antibodies brain and muscle Arnt-like 1 (BMAL1, Abcam Inc., Cambridge, MA, USA), and β-actin (Santa Cruz Biotechnology Inc., Dallas, TX, USA). Briefly, equal amounts of protein were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 10% polyacrylamide gel), and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Western blot images were quantified by measuring the intensity of correctly sized bands using Alpha Imager 2200 software (Alpha Innotech Corporation, San Leandro, CA, USA), and normalized to β-actin (Duan et al., 2014).

2.9. Statistical analysis

Statistical analyses were carried out using one-way ANOVA within the SPSS statistics 17 (SPSS Institute, Inc.). All the results are expressed as means with their standard error means (SEM) (SPSS Institute, Inc.). A probability of $P < 0.05$ was considered to be statistically significant and $0.05 < P < 0.10$ was considered to present the tendency. Differences between individual means were determined by the New Duncan multiple comparison test.

3. Results

3.1. Weight of visceral organs

As shown in Table 3, nonsignificant difference was observed in the weights of the heart, kidney, and spleen among the 3 groups. However, the relative liver weight in the LCH group was significantly greater ($P < 0.05$) than that in other 2 groups, whereas the liver weight only presented the uptrend in the LCH group ($0.05 < P < 0.10$). In addition, the relative weights of the kidney and spleen were decreased ($P < 0.05$) by HCL feeding pattern when compared with those in the other 2 groups.

3.2. Plasma biochemical parameters

As shown in Table 4, changing the feeding sequence according to the CP induced a significant reduction in the plasma TG levels ($P < 0.01$), although nonsignificant changes occurred in the concentrations of total CHO, LDL, and HDL ($P > 0.05$). Interestingly, there was a significant decrease in the plasma Urea ($P < 0.01$) and lipase ($P < 0.05$) in the HCL group, but an increase in the LCH group compared with the 3C group. Consistent with TG level, plasma ALP

Table 2
Primers used for RT-qPCR.

Genes	GenBank accession no.	Nucleotide sequence of primers (5′-3′)	Size, bp
DGAT	NM_214051.1	F: CTGGCTCTGATGGTCTACGC	173
		R: TAGGTCAGGTTGTCGGGGTA	
PPARα	NM_001044526.1	F: GCTATCATTTGGTGCGGAGAC	139
		R: GGAGTTTGGGGAAGAGAAAGAC	
ATGL	NM_001098605.1	F: ATGGTGCCCTACACGCTG	111
		R: GCCTGTCTGCTCCTTTATCC	
ACCα	NM_001114269.1	F: TCCCAGTGCAAGCAGTATG	211
		R: TGCCAATCCACACGAAGAC	
FASN	NM_001099930.1	F: GTCCTGCTGAAGCCTAACTC	206
		R: TCCTTGGAACCGTCTGTG	
LXRα	NM_001101814.1	F: GTAGATGGCTGAGGCGTGAC	96
		R: TTCCCAACCCTTTGACTCTTT	
SREBP-1c	NM_214157.1	F: CCTCTGTCTCTCCTGCAACC	229
		R: GACCGGCTCTCCATAGACAA	
Per1	AJ277735.1	F: TGAGGTGCTGGATTGTTGTG	101
		R: GTGTGCCGTGTGGTGAAGAC	
Per2	XM_003483786.1	F: TCTCCCTGCCACCATTACTTAC	144
		R: CTTGACCCGTTTGGACTTCAG	
Cry1	XM_003126079.2	F: ATCTCCGATTTGGTTGTTTGTC	151
		R: GGATTATTTGTTGCTGCTGTGT	
Cry2	XM_003122835.1	F: ATCGTCAACCACGCTGAGA	127
		R: TGCTGAGGTCTTCCACACAG	
Rev-erba1	XM_003358340.2	F: TCTTCTCCTCCCTCTTCTGATTT	101
		R: TTTTCTCGCTGCTCCTGATTAT	
β-actin	XM_003357928.2	F: CGTTGGCTGGTTGAGAATC	132
		R: CGGCAAGACAGAAATGACAA	

DGAT = diacylglycerol acyltransferase; PPARα = peroxisome proliferator-activated receptor alpha; ATGL = adipose triglyceride lipase; ACCα = acetyl-CoA carboxylase; FASN = fatty acid synthase; LXRα = liver X receptor; SREBP-1c = sterol regulatory element binding protein-1c; Per1 = period 1; Per2 = period 2; Cry1 = cryptochrome 1 Cry2 = cryptochrome 2; Rev-erba1 = Rev-erb alpha1.

Table 3
The visceral organ weight and relative weight of visceral organ as affected by a daily 3-meal pattern with different dietary crude protein contents.[1]

Item	Feeding pattern[2]			SEM	P-value
	3C	HCL	LCH		
Visceral organ weight, g					
Heart	307	325	328	7.04	0.452
Liver	1,235[b]	1,305[ab]	1,364[a]	24.8	0.099
Kidney	132	123	130	3.08	0.529
Spleen	113	107	116	3.46	0.629
Relative weight of visceral organ, %					
Heart	0.414	0.417	0.451	0.012	0.374
Liver	1.67[b]	1.66[b]	1.87[a]	0.035	0.014
Kidney	0.177[a]	0.156[b]	0.185[a]	0.004	0.007
Spleen	0.152[a]	0.135[b]	0.147[ab]	0.003	0.105

a, b Within a row, means without a common superscript indicate significant difference (P < 0.05, one-way ANOVA method).
[1] Data are presented as means with total SEM, n = 7.
[2] 3C: feeding basal CP diet 3 times daily; HCL: feeding high CP diet for breakfast, basal CP diet for lunch, and low CP diet for dinner; LCH: feeding low CP diet for breakfast, basal CP diet for lunch, and high protein diet for dinner.

Table 4
The plasma biochemical parameters of pigs as affected by a daily 3-meal pattern with different dietary crude protein contents.[1]

Item	Feeding pattern[2]			SEM	P-value
	3C	HCL	LCH		
ALP, U/L	92.0[a]	80.2[ab]	66.0[b]	4.53	0.042
ALT, U/L	59.0	63.2	55.0	2.09	0.307
AST, U/L	39.5	51.8	43.6	3.80	0.409
Urea, mmol/L	6.55[b]	5.66[b]	7.66[a]	0.26	0.002
CHO, mmol/L	2.83	2.78	2.99	0.08	0.604
TG, mmol/L	0.54[a]	0.36[b]	0.35[b]	0.03	0.007
LDL, mmol/L	1.07	1.09	1.13	0.04	0.870
HDL, mmol/L	1.55	1.52	1.64	0.06	0.682
Lipase, U/L	14.4[ab]	12.5[b]	16.7[a]	0.66	0.016

ALP = alkaline phosphatase; ALT = alanine transaminase; AST = aspartate aminotransferase: Urea = urea nitrogen; CHO = total cholesterol; TG = triglyceride; LDL = low-density lipoprotein; HDL = high-density lipoprotein.
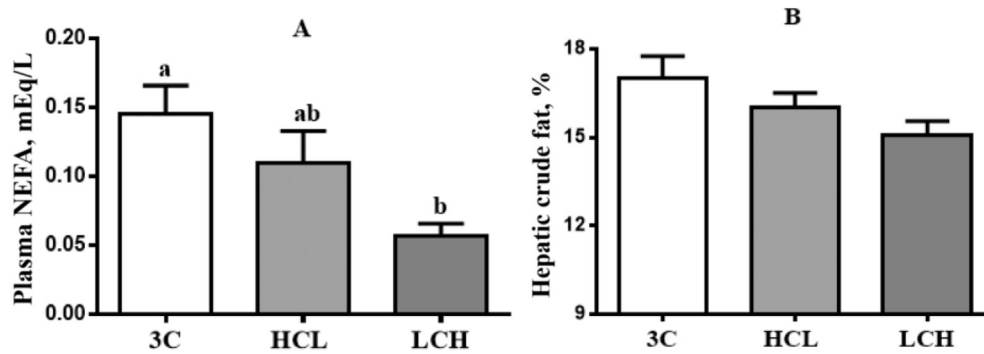a,b Within a row, means without a common superscript indicate significant difference (P < 0.05, one-way ANOVA method).
[1] Data are presented as means with total SEM, n = 7.
[2] 3C: feeding basal CP diet 3 times daily; HCL: feeding high CP diet for breakfast, basal CP diet for lunch, and low CP diet for dinner; LCH: feeding low CP diet for breakfast, basal CP diet for lunch, and high protein diet for dinner.

activity in LCH group (P < 0.05) also decreased whereas few differences were found in plasma ALT and AST among the 3 groups (P > 0.05).

3.3. Proportion of hepatic crude fat and plasma NEFA contents

Compared with 3C group, LCH feeding pattern reduced the plasma NEFA (P < 0.05) (Fig. 1A), which was modest with the result of the proportion of the hepatic crude fat to some extent (0.05 < P < 0.10) (Fig. 1B).

3.4. PUFA profile in the liver

Polyunsaturated fatty acid in the liver, expressed as a proportion of the total fatty acids, are presented in Table 5. However, unlike effects observed on hepatic crude fat, differences among 3 groups in the proportion of different PUFA were not significant (P > 0.05). In terms of PUFA, LCH feeding sequence reduced the polyunsaturated fatty acid c20:3, n-3 (P < 0.05), whereas c20:3, n-6 proportion was increased (P < 0.05) when compared with that in other 2 groups. No significant difference was observed in other PUFA among 3 groups (P > 0.05).

3.5. Relative mRNA expression of lipid metabolism-related genes

As shown in Fig. 2, the mRNA levels of ATGL (P < 0.01), ACCα (P < 0.01), and sterol regulatory element-binding protein 1c (SREBP1-c) (P < 0.05) in the LCH group were down-regulated, and LXRα also tended to be lower (0.05 < P < 0.10) when compared with those in the 3C group. However, we did not find significant

Fig. 1. Plasma non-esterified fatty acids (NEFA) content and hepatic crude fat as affected by a daily 3-meal pattern with different dietary crude protein contents. Values are means + SEM. [a, b] Means without a common superscript indicate a significant difference ($P < 0.05$, one-way ANOVA method). 3C: feeding basal CP diet 3 times daily; HCL: feeding high CP diet for breakfast, basal CP diet for lunch, and low CP diet for dinner; LCH: feeding low CP diet for breakfast, basal CP diet for lunch, and high protein diet for dinner.

Table 5
The proportion of polyunsaturated fatty acids in the liver affected by a daily 3-meal pattern with different dietary protein contents (%).[1]

Item	Feeding pattern[2]			SEM	P-value
	3C	HCL	LCH		
c18:2, n-6	18.01	17.83	19.2	0.610	0.622
c18:2, n-6	0.070	0.108	0.073	0.011	0.294
c18:3, n-3	0.044	0.096	0.046	0.016	0.304
c18:3, n-6	0.236	0.214	0.247	0.011	0.463
c20:2	0.550	0.605	0.639	0.020	0.193
c20:3, n-3	0.057[a]	0.052[ab]	0.048[b]	0.002	0.027
c20:3, n-6	0.710[ab]	0.598[b]	0.874[a]	0.046	0.032
c20:3	0.725[b]	0.642[b]	0.978[a]	0.050	0.004
c20:5	0.161	0.170	0.163	0.006	0.853
c22:6, n-3	0.430	0.496	0.575	0.040	0.381
PUFA	20.2	20.05	21.78	0.663	0.512

[a,b] Within a row, means without a common superscript indicate significant difference ($P < 0.05$, one-way ANOVA method).
[1] Data are presented as means with total SEM, $n = 7$.
[2] 3C: feeding basal CP diet 3 times daily; HCL: feeding high CP diet for breakfast, basal CP diet for lunch, and low CP diet for dinner; LCH: feeding low CP diet for breakfast, basal CP diet for lunch, and high protein diet for dinner.

differences in mRNA expression for diacylglycerol acyltransferase (DGAT), PPARα, or fatty acid synthase (FASN) among the 3 groups ($P > 0.05$).

3.6. Relative expression of circadian clock related genes

To investigate lipid metabolism in the liver, we also measured the relative mRNA expression of negative-regulated elements of circadian clock (Fig. 3) and positive-regulated protein BMAL1 (Fig. 4), which are involved in the regulation of lipid metabolism. Real-time quantitative PCR results showed that both 2 dynamic feeding patterns down-regulated the relative mRNA expression of Per1 ($P < 0.05$), Per2 ($P < 0.01$) and Cry2 ($P < 0.05$), which in turn, up-regulated the protein expression of BMAL1 ($P < 0.05$) when compared with the 3C group, which may indicate that changing the feeding sequence according to the CP could affect the liver lipid metabolism via regulating circadian clock related genes.

4. Discussion

It is well known that lifestyle interventions designed to prevent further weight gain in overweight individuals have a significant public health impact by preventing obesity-related chronic morbidities (Sayer et al., 2016). Incre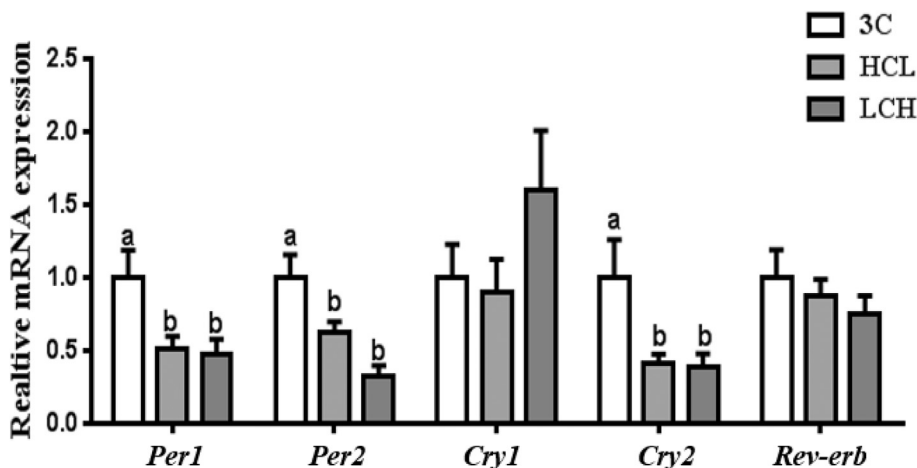asing the amount of dietary protein at breakfast may be desirable intervention have been previously shown to have greater effects on satiety compared to other meal-times (Leidy et al., 2009). Therefore, pig is expected to be an optimal species as applicable model in many research fields such as human nutrition, physiology and pathophysiology, and could be an important supplement to the many available rodent models (Fan and Lai, 2013). Moreover, pigs provide the greatest economic returns at intensive feeding systems because of their greater efficiency, reduced feeding costs, greater carcass weight and conformation, and greater lean yields. Accordingly, taking pig as animal model contributes to the acquisition of new knowledge to improve both animal and human health.

It has been reported that excessive intake of CP could affect energy metabolism (Koong et al., 1985; Yen, 1997; Nyachoti et al., 2000), by increasing energy expenditure due to increased nitrogen excretion (Noblet et al., 1987; Le Bellego et al., 2001), with a subsequent impact on organ size (Chen et al., 1999). The reduction in relative kidney weight due to ingesting a low-protein diet in the evening was expected in the HCL group, and reflected a lower workload on the kidneys in terms of the excretion of nitrogenous wastes (Lopez et al., 1994; Kerr and Easter, 1995; Chen et al., 1999). Correspondingly, opposite effect occurred in pigs fed with LCH sequence. No significant changes occurred in the liver, which in line with previous studies that no consistent change in liver weight in response to a low-protein diet (Lopez et al., 1994; Kerr and Easter, 1995; Knowles et al., 1998; Chen et al., 1999). Moreover, a high-protein diet may adversely affect metabolic acidosis and hepatic or renal health, and could lead to a considerable increase in liver and kidney weight (Aparicio et al., 2011). Consequently, it was inferred that the daily 3-meal pattern could affect the metabolism in the liver and kidney, especially the content of CP in the dinner, even though similar amounts of protein were consumed each day among 3 groups.

In addition to the organ size, plasma parameters also could indicate the metabolism in the liver or kidney. The index of liver function, such as ALP, was decreased by HCL and LCH feeding patterns, emphasizing that we could reduce the ALP level or blood fat of patients via changing the feeding pattern, such as feeding sequence according to dietary CP. A reduction in plasma urea in pigs is an indication of reduced energy needs for the deamination of excess AA (Lopez et al., 1994; Kerr and Easter, 1995), and greater plasma urea nitrogen and lipase may indicate impaired kidney function (Anwar et al., 2014). Consistent with the relative weight of the kidney, plasma urea nitrogen and lipase concentrations in the LCH group reached the greatest level among the 3 groups, which may be explained that LCH feeding pattern increased the workload of the kidney, then disrupted the AAs balance of the pigs.
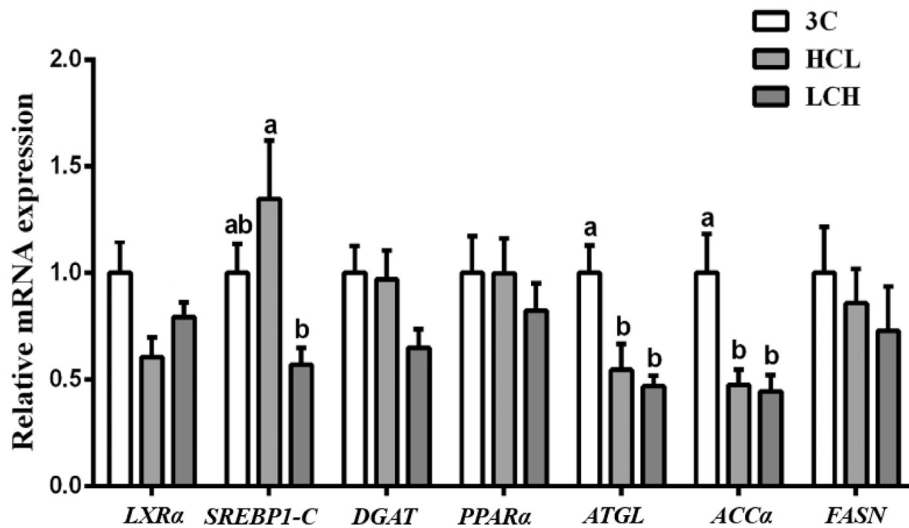
Fig. 2. Relative mRNA expression of genes involved in lipid metabolism in the liver of pigs as affected by a daily 3-meal pattern with different dietary crude protein contents. Values are means + SEM. [a,b] Means without a common superscript indicate a significant difference ($P < 0.05$, one-way ANOVA method). 3C: feeding basal CP diet 3 times daily; HCL: feeding high CP diet for breakfast, basal CP diet for lunch, and low CP diet for dinner; LCH: feeding low CP diet for breakfast, basal CP diet for lunch, and high protein diet for dinner. $LXR\alpha$ = liver X receptor; $SREBP-1c$ = sterol regulatory element binding protein-1c; $DGAT$ = diacylglycerol acyltransferase; $PPAR\alpha$ = peroxisome proliferator-activated receptor alpha; $ATGL$ = adipose triglyceride lipase; $ACC\alpha$ = acetyl-CoA carboxylase; $FASN$ = fatty acid synthase.
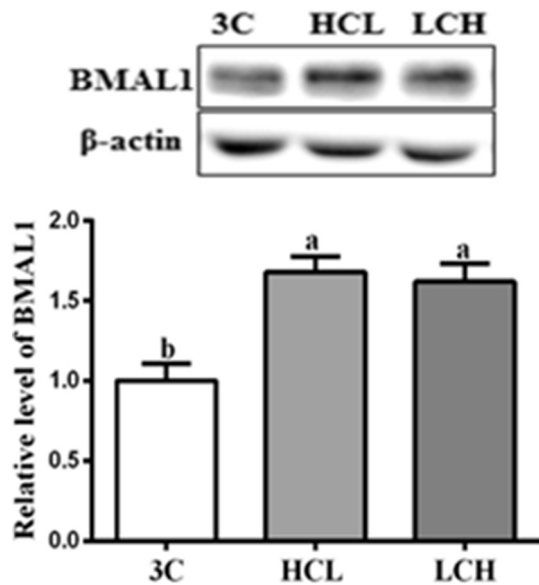

Fig. 3. Relative mRNA expression of negative-regulated element of circadian clock in the liver affected by a daily 3-meal pattern with different dietary protein contents. Values are means + SEM. [a,b] Means without a common superscript indicate a significant difference ($P < 0.05$, one-way ANOVA method). 3C: feeding basal CP diet 3 times daily; HCL: feeding high CP diet for breakfast, basal CP diet for lunch, and low CP diet for dinner; LCH: feeding low CP diet for breakfast, basal CP diet for lunch, and high protein diet for dinner. $Per1$ = period 1; $Per2$ = period 2; $Cry1$ = cryptochrome 1; $Cry2$ = cryptochrome 2; $Rev-erba1$ = Rev-erb alpha1.

Excess fat accumulation in the liver can result from increased fatty acid delivery, and fat stored in white adipose tissue that was transported to the liver by the way of the plasma NEFA pool, increased de novo fatty acid synthesis from glucose, reduced fatty acid oxidation or triglyceride export in the form of very low-density lipoproteins (VLDL) (Postic and Girard, 2008; Ferre and Foufelle, 2010). Liver takes up NEFA from the blood in proportion to their concentration (Nguyen et al., 2008). Therefore, the plasma TG level and hepatic crude fat in the HCL and LCH feeding patterns may be a result of the downregulation in the adipose lipolysis (VanNevel and Demeyer, 1996), or the decreased plasma NEFA pool caused by dynamic feeding pattern. It is well known that nutrient and hormonal could regulate lipid metabolism in the liver (Rui, 2014). There is also evidence that a high protein, lower carbohydrate diet can reduce plasma TG production (Parks et al., 1999), suggesting that a daily dynamic feeding pattern according to different CP may

have a similar effect on plasma TG, but this point needs to be verified.

As an essential peripheral tissue of the circadian clock, the liver contains self-sustaining circadian oscillators and plays a major role in regulating energy homeostasis in mammals (Liu et al., 2007; Rui, 2014). Hepatic lipid metabolism and cholesterol synthesis have long been known to be subject to circadian regulation (Guo et al., 2012; Betancor et al., 2014). A previous study found that BMAL1$^{-/-}$ mice developed obesity, and that the attenuation of BMAL1 function promoted adipogenesis, demonstrating that circadian disruption was associated with the development of obesity and BMAL1 suppressed adipogenesis (Guo et al., 2012). Consistent with the lower proportion of crude fat in the liver, protein expression of BMAL1 was stimulated in the HCL and LCH groups, indicating that feeding pattern may affect lipid metabolism by regulating the circadian clock gene expression in the liver.

Fig. 4. Relative protein expression of BMAL1 in the liver of pigs as affected by a daily 3-meal pattern with different dietary crude protein contents. Values are means + SEM. [a,b] Means without a common superscript indicate a significant difference ($P < 0.05$, one-way ANOVA method). 3C: feeding basal CP diet 3 times daily; HCL: feeding high CP diet for breakfast, basal CP diet for lunch, and low CP diet for dinner; LCH: feeding low CP diet for breakfast, basal CP diet for lunch, and high protein diet for dinner. _BMAL1_ = brain and muscle Arnt-like 1.

Hepatocellular TG storage does not appear to be directly hepatotoxic, but rather may be a marker of hepatocyte exposure to potentially toxic fatty acids (Jou et al., 2008). Liver-specific inhibition of diacylglycerol acyltransferase (_DGAT_), the enzyme responsible for the last step in hepatic TG synthesis, with antisense oligonucleotides improves hepatic steatosis in obese diabetic mice (Yamazaki et al., 2005; Yamaguchi et al., 2007). In this study, both HCL and LCH feeding patterns reduced hepatic crude fat, which was consistent with the result of the relative expression of _DGAT_, whereas the difference among 3 groups didn't reach the significant level.

Inhibition of the _ACCα_ expression in the liver could be expected because hepatic crude fat tended to be lower in the HCL and LCH groups, whereas no significant difference was observed in _FASN_ expression among the 3 groups. The liver has been shown to be a preferential site for PUFA synthesis (Scott and Bazan, 1989; Duran-Montge et al., 2009), and fatty acid composition in the liver reflects both the diet and endogenous synthesis (Ding et al., 2003). However, we did not find remarkable differences in _FASN_ mRNA level and the proportion of most PUFA. Many transcription factors have been identified as prospective targets for fatty acid regulation, including peroxisome proliferator-activated receptors _PPARα_ (Schoonjans et al., 1996), _SREBP-1c_ (Xu et al., 1999; Bommer and MacDougald, 2011), and _LXRα_ (Zelcer and Tontonoz, 2006). It has been reported that LXRα−/− mice wasn't vulnerable to be obesity when was fed with high-fat diet, reducing fatty acids synthesis and the TG content (Kalaany et al., 2005). In this study, both HCL and LCH feeding patterns inhibited the mRNA expression of _LXRα_ in the liver, which in turn suppressed the hepatic lipogenesis, and reduced the fat accumulation in the liver. However, further research is needed to elucidate the underlying mechanism.

5. Conclusions

In this study, the daily 3-meal pattern with different dietary CP contents influences, to some extent, lipid metabolism in the liver by regulating the relative mRNA expression of lipid metabolism-related genes and protein, although further studies are needed to clarify this point. Moreover, randomized trials are needed to test whether shifting other nutrition composition in one day would have similar effects in the liver. In summary, these findings reveal that we could regulate pig production by adjusting the feeding pattern, and suggest that the lifestyle-related diseases, such as obesity or metabolic syndrome, not only be related to the caloric energy intake, but also the timing of feeding.

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