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Expression patterns of *GHRL, GHSR, LEP, LEPR, SST* and *CCK* genes in the gastrointestinal tissues of Tibetan and Yorkshire pigs

*Wen-Kui Sun[1,2], Chi Cheng[3], Rui Liu[4], Yi-Hui Chen[1], Kai Zeng[4], Xiao-Hui Chen[4], Yi-Ren Gu[4], Jiang-Ling Li[4], Xue-Bin Lv[4], Rong Gao[1]**

[1]*Key Laboratory for Bio-Resource and Eco-Environment of Education Ministry, Key Laboratory for Animal Disease Prevention and Food Safety of Sichuan Province, College of Life Science, Sichuan University, Chengdu, P.R. China*

[2]*Chengdu Medical College, School of Laboratory Medicine, Chengdu, P.R. China*

[3]*College of Chemistry and Life Science, Chengdu Normal University, Chengdu, P.R. China*

[4]*Sichuan Academy of Animal Science, Chengdu, P.R. China*

**Corresponding author: gaorong96@163.com*

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Abstract: The aim was to characterize the expression patterns of several genes in the gastrointestinal tracts of Tibetan pigs (TP) and Yorkshire pigs (YP) and to explore their correlation with digestion and growth difference of the two breeds. The body weights and growth of YP and TP were studied at 6, 12 and 24 weeks of age, and their plasma levels of ghrelin (GHRL), leptin (LEP), somatostatin (SST) and cholecystokinin (CCK) were determined by enzyme linked immunosorbent assay (ELISA). Blood and gastrointestinal sections (stomach, duodenum, jejunum, ileum, caecum and colon) were collected and assayed for mRNA expression of the six genes (*GHRL*, ghrelin receptor (*GHSR*), *LEP*, leptin receptor (*LEPR*), *SST* and *CCK*) by reverse transcription-qPCR (RT-qPCR). TP generally had higher mRNA expressions of *GHSR*, *LEP, LEPR, SST* and *CCK* genes compared to YP, and expressed lower levels of the *GHRL* gene in most tissues of the digestive tract. In both breeds, plasma levels of the expressed proteins were more closely correlated with the feed intake and growth than with mRNA levels of the target genes. Our data indicate that TP possess special gene expression patterns in the gastrointestinal tract compared to YP, which is consistent with its unique feed intake and adaptation to harsh environment.

Keywords: gene transcript; hormone level; growth performance; digestive traits

Different porcine breeds have different gene expression patterns that are correlated with their different phenotypes (Zhang et al. 2013; Shen et al. 2014). The ingestion of nutrients triggers numerous changes in gastrointestinal (GI) peptide hormone secretions that affect appetite and eating, especially in the stomach (Steinert et al. 2013). The development of the GI tract is closely related to GI peptides that affect feed intake, energy and glucose homeostasis, as well as immune functions (Monteiro and Batterham 2017).

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W.-K. Sun and C. Cheng contributed equally to this work.

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The Tibetan pig (TP) is an indigenous pig of the Qinghai-Tibet plateau of China and is a rare and valuable genetic resource. In addition to strong resistance against disease compared with other pig breeds, it shows good adaptation to poor quality feed and has excellent meat quality and taste (Li et al. 2012). However, little is known about the molecular regulation of its unique digestion traits.

Ghrelin (GHRL), leptin (LEP), somatostatin (SST) and cholecystokinin (CCK) are important gastrointestinal hormones, with many studies suggesting that they have multiple physiological functions, stimulating a wide array of nutrition-related processes such as regulation of ingestion, digestion, and absorption of nutrients (Krejs 1986; Barb et al. 2001; Little et al. 2005; Dong et al. 2009). These hormones have been found to be associated with growth and carcass traits by affecting food intake and energy balance in many animals of economic importance. The active proteins are found in both endocrine cells and neurons of the peripheral nervous system or the central nervous system. However, previous studies have focused on the protein levels in main secretory cells, and little is known about the expression profiles of these genes in the GI tract of the TP.

Therefore, for the sake of employing the TP as a genetic breeding resource for the development of hybrid pigs with excellent meat quality and strong disease resistance, the growth, digestive and immunity characteristics of this animal are worth of further intensive investigation. The present work mainly evaluated the transcriptional expression of *GHRL*, *LEP*, ghrelin receptor (*GHSR*), leptin receptor (*LEPR*), *SST* and *CCK*, in the gastrointestinal tissues of Tibetan pigs (TP) and Yorkshire pigs (YP). We also conducted an analysis of the growth characteristics of both breeds and their plasma levels of GHRL, LEP, SST and CCK hormones.

MATERIAL AND METHODS

Animals and samples. Thirty-six healthy purebred pigs, eighteen half-sib Tibetan and eighteen half-sib Yorkshire, were provided by the Chengdu Research Base of Sichuan Academy of Animal and Husbandry Science. The parents of the test TP were second generation following introduction from the Qinghai-Tibet plateau. Each breed consisted of three age groups: 6-week-old weanlings, 12-week-old young pigs and 24-week-old adult pigs, with three males and three females in each group. They were maintained under the same environmental and feeding conditions, i.e., with the same housing, feedstuff and water supplies (Table 1).

Experimental procedure. At specific times, the animals were weighed (Tables 2 and 3) and then euthanized by electric shock. Blood samples were collected in EDTA tubes, plasma was separated by centrifugation and stored at –20°C and blood cells were retained for RNA isolation. Small tissue specimens were excised immediately from identical positions of the following organs: stomach (pyloric region mucosa), duodenum, jejunum and ileum, caecum and ascending colon. After washing in PBS, the samples were individually homogenized and snap-frozen in liquid nitrogen until required for total RNA determination.

Maintenance of the animals and euthanizations were performed according to Chinese animal welfare laws and regulations and approved by the Institutional Animal Care and Use Committee at Sichuan University under permission No. SCUBC20160603.

Quantification of plasma hormone levels. Plasma GHRL, LEP, SST and CCK were determined using a commercial porcine-specific ELISA kit (Senbeijia, China) according to the manufacturer's instruc-

Table 1. Diet formulation and nutrition content

Feed stuffs	(%)	Main nutrient indexes	Nutrition content
Corn	65.00	digestible energy (MJ/kg)	13.38
Soybean meal	12.50	crude protein (%)	16.50
Wheat bran	12.40	calcium (%)	0.80
Grass meal	3.00	phosphorus (%)	0.62
Fish meal	5.00	lysine (%)	0.60
Calcium carbonate	1.10	crude fibre (%)	5.22
Calcium hydrogen phosphate	0.55		
Salt	0.30		
Additive	0.15		

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Table 2. Weights of Yorkshire pigs (YP) and Tibetan pigs (TP) at different ages

Breed	Born (n = 18; 18)	6 weeks (n = 18; 18)	12 weeks (n = 12; 12)	24 weeks (n = 6; 6)
YP (kg)	1.22 ± 0.14	8.41 ± 2.23	24.45 ± 4.22	58.14 ± 6.02
TP (kg)	0.71 ± 0.16	4.56 ± 1.33	11.32 ± 2.68	24.92 ± 5.16
P-value	< 0.0001	< 0.0001	< 0.0001	< 0.0001

values are expressed as means ± SD; *P*-values were calculated between Tibetan and Yorkshire pigs at the same time point

Table 3. Growth performance of Yorkshire pigs (YP) and Tibetan pigs (TP) at different ages

Trait	Weeks 0–6 (n = 18; 18)			Weeks 7–12 (n = 12; 12)			Weeks 13–24 (n = 6; 6)		
	YP	TP	*P*-value	YP	TP	*P*-value	YP	TP	*P*-value
ADFI (g/day)	–	–	–	964.68 ± 86.52	609.99 ± 58.72	< 0.0001	1248.54 ± 135.67	593.48 ± 86.43	< 0.0001
ADG (g/day)	211.42 ± 62.54	124.52 ± 22.36	0.0015	380.95 ± 78.84	257.38 ± 54.52	0.0012	476.19 ± 102.43	179.52 ± 50.18	< 0.0001
F : G	–	–	–	3.10 ± 0.58	2.37 ± 0.66	0.0341	2.75 ± 0.49	2.96 ± 0.71	0.3162

ADFI = average daily feed intake, ADG = average daily gain, F = feed, G = gain, (–) = no results for 6-week-old weanlings
values are expressed as means ± SD

tions. The R value of the correlation coefficient between the linear regression and the expected concentration was above 0.990. The intra- and inter-assay coefficients of variation were 9% and 11%, respectively.

Total RNA extraction and reverse transcription. Total RNA from pig tissues and whole blood cells collected by centrifugation were extracted using RNAiso Plus Reagent (TaKaRa, China) according to the manufacturer's instructions under sterile conditions, and purity was measured by A_{260}/A_{280} ratio to be 1.8–2.0 using a NanoDrop ND-8000 spectrophotometer (Thermo Fisher Scientific, USA). The integrity of the RNA was verified to be qualified for RT-PCR by denaturing agarose gel electrophoresis (18s and 28s RNA bands were clear). RNA preparations were diluted to 500 ng/µl and rapidly reverse-transcribed using TransScript® One-Step gDNA Removal and cDNA Synthesis SuperMix kit (TransGen, China) according to the manufacturer's instructions.

Gene expression by qPCR. Nine pairs of primers were designed (Table 4) to be optimal using Primer Premier 5.0 software and NCBI Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and were commercially synthesized (Invitrogen, USA). Where possible, primers were designed over introns. A specific validation of primer pairs was conducted by melting curve analysis and by PCR products sequencing. SYBR Green PCR assays were performed on an iQ5 iCycler iQ™ Real-Time PCR Detection System (Bio-Rad, USA). For each 15 µl of SYBR Green PCR reaction, 1.0 µl cDNA, 0.75 µl sense primer (100 µM), 0.75 µl anti-sense primer (100 µM), 7.5 µl SYBR Green PCR Master Mix (Bio-Rad) and 5 µl PCR-grade water were mixed. The parameters for real-time PCR were as follows: a pre-run at 95°C for 3 min, 40 cycles with a 5 s denaturation step at 95°C, followed by an optimal annealing temperature (Table 4) step for 10 s and a 72°C extension step for 10 s. Fluorescence was measured immediately after the end of each extension step.

Gene expression relative to reference genes (*RPL4*, *PPIA* and *YWHAZ*) was performed in order to correct for the variance of RNA input in the reactions in porcine tissues of different ages (Uddin et al. 2011). The amplification efficiency of each set of primers (listed in Table 4) was determined by running a log dilution series of purified conventional PCR products and approximating to 100%. Each sample was amplified in triplicate. A no-template control (NTC) was also included in each assay.

Statistical analysis. Relative gene expression compared to reference genes was calculated by the $2^{-\Delta\Delta Ct}$ method as described previously (Livak and Schmittgen 2001). Statistical analyses

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Table 4. Primer sequences for qPCR

Gene	GenBank Accession No.	Primer sequence (5'-3')	Product length (bp)	Annealing temperature (°C)	Amplification efficiency (%)	R^2
GHRL	NM_213807	F: GTTGGGATCAAGTTGTCAGG R: TGGGAGAACAGAGGTGGC	144	55.5	98.9	0.983
GHSR	NM_214180	F: TCTTCTGCCTCACTGTGCTCTAT R: CACGGTTTGTTTGTGGTTCTG	113	56.5	98.0	0.957
LEP	GQ268936	F: TGCCGATTCCTGTGGCTTTG R: GTGACAATCGTCTTGATGAGGG	102	55.5	97.1	0.998
LEPR	GQ268934	F: GCAGTCGCTCAGTGCTTATCC R: GAGGAAGGGATTCTGAGCCAT	150	51.5	98.3	0.993
SST	NM_001009583	F: TGGCTCTGGGCGGTGTCACT R: CACCCAAGGGAGGAGGGGCA	172	59.5	98.7	0.991
CCK	NM_214237	F: AACCACGCACCTCAACAA R: AATAGCATAGGGACACTTTCA	89	48.5	99.1	0.997
RPL4[1]	XM_003121741	F: CCACCATGGCGTGTGCTCGT R: ATGTCTGGTCGAATGGGAGC	112	55.0	99.52	0.989
PPIA[1]	NM_214353	F: AGACAGCAGAAAACTTCCGTG R: ACTTGCCACCAGTGCCATTA	138	53.5	95.57	0.985
YWHAZ[1]	XM_001927228	F: GATTGGAGGAAACCCCGTGT R: GGATGTTCTGTGTCCGGAGTG	145	58.5	90.45	0.996

F = forward primer, R = reverse primer, R^2 = correlation coefficient, *PPIA* = peptidylprolyl isomerase A (cyclophilin A), *RPL4* = ribosomal protein L4, *YWHAZ* = tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta polypeptide

[1]genes used as a reference for normalization

were carried out using Graph Pad Prism 6 for Student's *t*-test between the two breeds. Differences between means were considered significant at $P < 0.05$.

RESULTS

Growth performance. The average daily feed intake and average daily weight gain were significantly higher in YP than in TP at three time points (Tables 2 and 3). However, TP had significantly better feed/gain rate than YP before 12 weeks of age, and the average daily feed intake and daily gain of TP decreased after 12 weeks of age.

Plasma hormone levels. Plasma hormone levels such as GHRL in the 2 breeds, LEP and SST in YP, and CCK in TP tended to increase with age (Table 5). Notably, there was a downward trend of LEP and SST levels in TP at 24 weeks and of CCK in YP at 12 weeks.

Compared with YP, the LEP plasma levels in TP were significantly higher at all 3 time points,

Table 5. Hormone concentrations of plasma in Yorkshire pigs (YP) and Tibetan pigs (TP) at different weeks of age

Hormone (ng/ml)	6 weeks (n = 6; 6)			12 weeks (n = 6; 6)			24 weeks (n = 6; 6)		
	YP	TP	*P*-value	YP	TP	*P*-value	YP	TP	*P*-value
GHRL	24.67 ± 3.02	29.89 ± 2.14	0.0384	35.25 ± 3.35	31.54 ± 2.27	0.0619	39.23 ± 4.07	33.16 ± 2.64	0.0159
LEP	0.62 ± 0.03	1.46 ± 0.15	< 0.0001	0.85 ± 0.06	2.48 ± 0.35	0.0002	0.88 ± 0.04	2.16 ± 0.21	< 0.0001
SST	231.12 ± 9.40	237.23 ± 11.45	0.2082	253.61 ± 15.35	436.27 ± 21.36	< 0.0001	279.68 ± 15.62	382.16 ± 16.74	0.0001
CCK	20.24 ± 2.37	20.59 ± 1.51	0.6512	17.36 ± 2.04	24.34 ± 1.57	0.0004	38.45 ± 3.62	31.21 ± 2.56	0.0016

values are expressed as mean ± SD

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however, SST was borderline higher at 12 and 24 weeks, as was CCK at 12 weeks. TP had higher levels of GHRL than YP at 6 weeks, while this was reversed at the later ages.

GHRL and GHSR expression. The mRNA of *GHRL* was highly expressed in the blood cells, stomach, duodenum, jejunum, and in the lower colon and caecum in both breeds (Figure 1A) . In

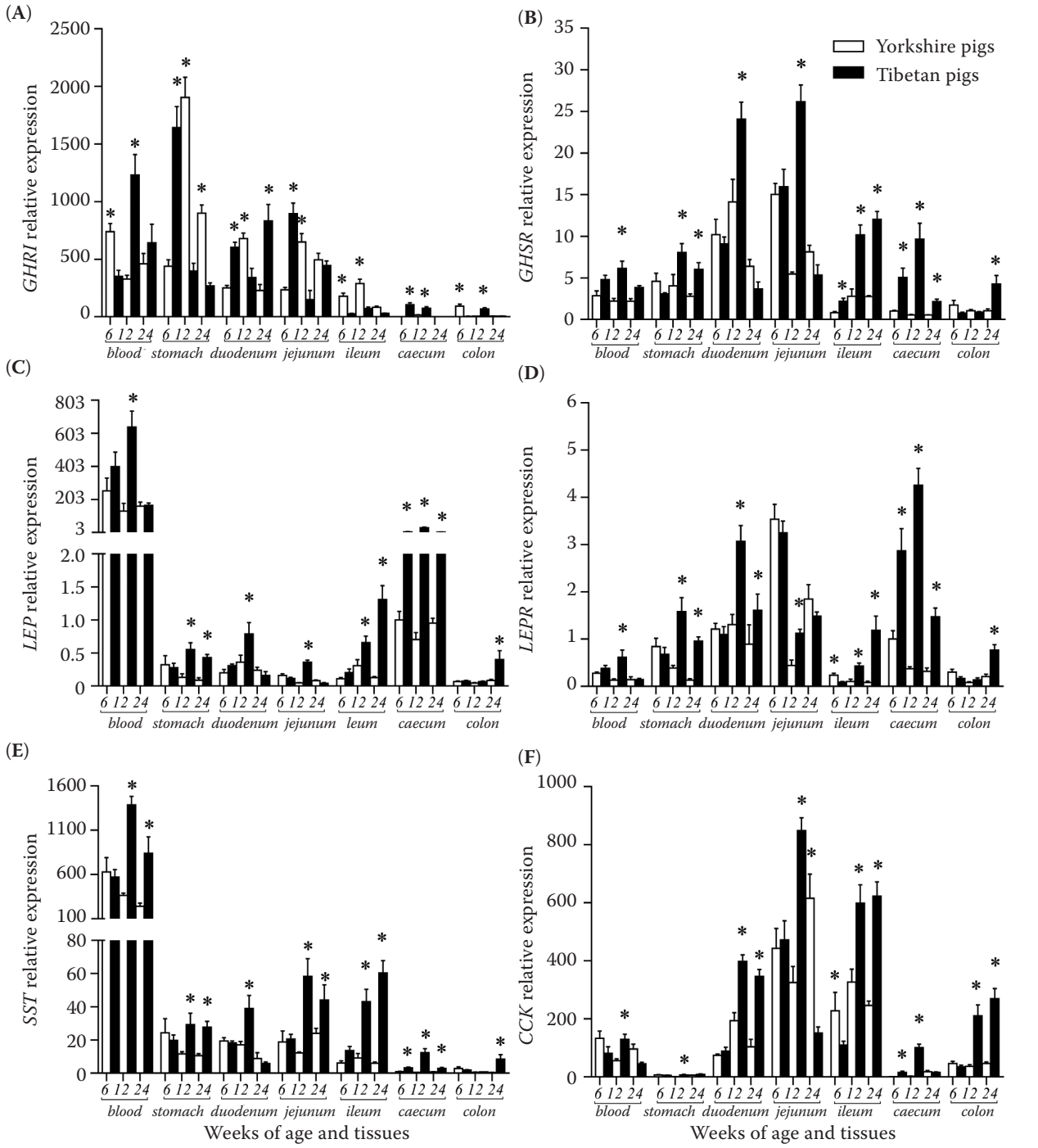

Figure 1. Relative mRNA level of *GHRL* (**A**), *GHSR* (**B**), *LEP* (**C**), *LEPR* (**D**), *SST* (**E**) and *CCK* (**F**) genes in the tissues of Yorkshire and Tibetan pigs at different ages. The mRNA level was normalized against a set of three internal control genes (*RPL4*, *PPIA* and *YWHAZ*), and the relative index was determined against the transcript level in the caecum of Yorkshire pigs at 6 weeks of age

blood = blood cells; data are presented as means ± SEM (*$P < 0.05$)

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6-week-old weanling pigs, *GHRL* mRNA levels in TP were significantly higher than in YP in stomach, duodenum, jejunum and caecum. Compared to YP, only the blood cells, caecum and colon levels were significantly higher in the 12-week-old Tibetan piglets. In 24-week-old adults, only the duodenum levels were significantly higher than in YP. Overall, however, *GHRL* expression in the digestive tissues was significantly higher at 6 weeks in TP than in YP, but this situation was reversed in the older animals.

GHSR mRNA was expressed more highly in the duodenum and jejunum than in the other samples (Figure 1B). Expression of the *GHSR* mRNA in TP was significantly higher than in YP in most tissues, except at 6 weeks. Overall, the relative expression of the *GHSR* in the two breeds was found to vary with age.

LEP and LEPR expression. *LEP* expression levels in blood cells were much higher than in other tissues of the two breeds (Figure 1C). The *LEP* expression levels in TP were significantly higher than in YP at 12 and 24 weeks, especially in the caecum. Overall, the *LEP* gene expression levels followed a pattern similar to *GHSR* when comparing the two breeds at the different ages.

Compared to the other tissues, it is noteworthy that the highest levels of *LEPR* mRNA were mainly in the small intestine and caecum of the two breeds, and a near lowest mRNA abundance of *LEPR* was found in their blood cells (Figure 1D).

SST and CCK expression. *SST* was expressed at the highest levels in the blood cells and the lowest in the large intestine of the two breeds (Figure 1E). The *SST* expression levels were significantly higher in most tissues of TP than in YP, except at 6 weeks of age.

A noticeably higher *CCK* expression level was detected in the small intestine than in the other tissues in both breeds, and the lowest expression level was detected in the stomach (Figure 1F). In 6-week-old animals, the mRNA expression of *CCK* in YP was significantly higher than in TP in the ileum. However, this trend was reversed in most tissues of the older animals, except in the jejunum at 24 weeks.

DISCUSSION

In our case, we were only able to obtain 18 half-sib but not all-sib TP for analysis, since the reproductive rate of TP is limited, the average number of Tibetan piglets per litter being only 5~8. So we chose to use six Tibetan piglets per cote. They did, however, have the same male parent. We used a set of three stably expressed reference genes previously determined to confirm the final quantitative PCR results reliably. The same method was used in our previous study for the normalization of mRNA expression in samples collected from the various tissues of the two breeds (Cheng et al. 2015). Therefore, we are confident that our results are reliable.

The average daily feed intake and average daily gain of TP had decreased significantly by 24 weeks – the age of sexual maturity (Gong et al. 2009). At the same time, there was a correlation between plasma levels of GHRL, LEP and CCK. GHRL is important for growth performance and, with LEP, regulates the sex hormone levels (Shintani et al. 2001; Yadav and Deo 2013). Furthermore, the lower CCK at 24 weeks is also an important reason for the lower growth performance by affecting feed intake (Matson et al. 2015). Plasma hormone levels of both breeds correlated more strongly with the growth performance of the two breeds than the transcript expression pattern. The former is systemically affected by many factors, while the latter is mainly regulated within the local gastrointestinal tract.

Our experimental results have shown that the transcriptional abundance of *GHRL*, *LEP* and their receptor genes, as well as *SST* and *CCK*, was influenced by age and tissue type, and the expression levels varied considerably between TP and YP. Previous research on β-defensins and Toll-like receptors 1–10 revealed similar expression patterns in different tissues of pigs (Qi et al. 2009; Uddin et al. 2013; Jiao et al. 2017).

Transcripts of *GHRL* were most abundant in the stomach, less present in the small intestine and least in the large intestine. This result is consistent with the pattern previously described in pigs at different ages (Vitari et al. 2012). In addition to stimulating the GH secretion, GHRL has various physiological functions: it strongly stimulates feeding and increase in body weight while blocking LEP-induced feeding reduction (Shintani et al. 2001). Our results indicate that the *GHRL* expression level in 6-week-old TP is higher than in YP except in blood cells; however, at 12 and 24 weeks, this ratio in TP and YP is reversed. A possible reason for this is that

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TP may have stronger appetite than YP during the weaning period; hence, the growth rate of TP is higher during weaning but lower later. At the ages of 12 and 24 weeks, the *GHRL* expression levels in the YP stomachs were significantly higher than in TP, which may be associated with the important physiological effect of GHRL in the gastrointestinal tract on gastric acid secretion and gastrointestinal motility, resulting in more efficient digestion and higher body weight. These results suggest that attention should be given to the manipulation of GHRL during the later weaning period if we wish to improve on the economically valuable traits in TP. *GHRL* and the *GHSR* are highly conserved across all vertebrate species examined, which indicates that the two genes have important physiological functions and are indispensable (Dong et al. 2009; Kaiya et al. 2013). *GHRL* had a higher expression pattern in YP; however, its receptor level was higher in most TP samples. GHRL has a widespread distribution in various tissues to stimulate GH release by GHSR, but the different expression patterns in tissues suggest that it may exert other regulatory activities via different receptors (Fujimiya et al. 2011; Kitazawa et al. 2011).

The expression pattern of *LEP* in most tissues of the two breeds is almost the reverse of that of *GHRL* but with the same general trend as *GHSR*. The *LEP* expression levels in TP were higher than in YP in blood cells at 6 weeks. However, this trend was reversed in most tissues of the two breeds at older ages. These results are consistent with the opposing effects of GHRL and LEP in gastrointestinal emptying and food intake (Shintani et al. 2001; Vitari et al. 2010). They may explain why TP have lower appetite-inducing *GHRL* and higher *LEP* levels in most tissues than YP, thereby helping the former to maintain stronger viability in a harsh feed environment. In both young and adult animals, the lower *GHRL* levels of TP possibly mirror their slow growth rate due to low feed intake and metabolism. The higher *LEP* levels may have a relationship with the role of LEP in immunity and female reproduction (Yadav and Deo 2013; Perez-Perez et al. 2015). Our results show that the lowest mRNA abundance of *LEPR*, but the highest of *LEP*, appeared in the blood cells samples of the two breeds. A possible reason for this discrepancy may be because *LEPR*, a member of six class I cytokine receptor super-family isoforms, is primarily found in the hypothalamus and is involved in satiety response (Perez-Montarelo et al. 2013). Another possible reason is that *LEP*, acting as an endocrine and paracrine regulating factor, is involved in the peripheral short-term regulation of food intake (Zieba et al. 2008).

The data reported here show that the highest *SST* expression occurs in the blood cells of both breeds. The gene expression of *SST* in tissues at different ages exhibited a trend similar to those of the *GHSR* and *LEP*. *SST* exerts a powerful suppressive effect on gastric emptying, gallbladder contractility and propulsive activity of the small and large intestines (Den Bosch et al. 2009). The expression patterns are consistent with the habits and growth characteristics of the two breeds, and may explain why TP have a rapid growth rate before weaning and a relatively slow growth rate thereafter. TP have a higher expression level of *SST* than YP except in the stomach, jejunum and colon at 6 weeks, which would result in a greater inhibition of gastric acid secretion, intestinal motility and digestive enzyme secretion (Corleto 2010).

CCK is a gut hormone and neuropeptide the function of which is considered to be an antagonist of *GHRL* in its effect on appetite and metabolism (Matson et al. 2000; Little et al. 2005). The current data show that *CCK* expression levels in small and large intestinal tissues were higher in TP than in YP. This indicates that TP have higher stimulation of the exocrine pancreas and exhibit stronger gallbladder contractions to promote the secretion of pancreatic enzymes and bile acids as well as to control gut motility and gastric emptying for digestion. This may be why TP have an excellent ability to digest harsh food within a hostile plateau environment. Additionally, as a satiety factor, TP will benefit from higher *CCK* levels in their adaptation to a cold environment and more limited nutrients.

CONCLUSION

Our experiment has compared the hormone levels of GHRL, LEP, SST and CCK in plasma and the transcript expression patterns of *GHRL*, *GHSR*, *LEP*, *LEPR*, *SST* and *CCK* in the gastrointestinal tracts of each eighteen half-sib purebred YP and TP. The plasma hormone levels had a greater positive correlation with the growth performance of the two breeds than with the transcript expression patterns. Transcript expression levels of *GHSR*,

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LEP, *LEPR*, *SST* and *CCK* were higher in all samples of TP than in YP at older ages, with lower levels of *GHRL* transcript in the main tissues. Our results indicate that, compared to YP, TP have different expression patterns of these genes in the gastrointestinal tract, as well as different plasma levels of these hormone proteins. These observations contribute to an understanding of the genetic basis of the unique digestion of the TP with a view of developing hybrid pigs with a better growth performance while retaining the excellent quality of TP meat and the adaptation of this breed to limited feed. Nevertheless, our studies of gene expression patterns in the two breeds are limited and a more definitive analysis will require more animals and further research.

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