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Full Length Article

Effect of Weaning Age on Plasma Biochemistry and Muscular GHR, IGF-1 and IGF-1R Gene Expression in Piglets

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Abstract

The effects of weaning age on plasma biochemistry parameters and expression of growth-related genes in piglets were investigated. Twenty-four piglets were randomly divided into 4 weaning groups (14 d, 21 d, 28 d and 35 d) and slaughtered at the age of 42 days. Blood, longissimus dorsi muscle (LD), and semitendinosus tissue samples were collected. Plasma biochemistry parameters and GHR, IGF-1, and IGF-1R mRNA and protein were measured. Weaning piglets at 14 days resulted in significantly lower plasma TP and GLU compared to weaning at 28 and 35 days ($p < 0.05$). Weaning at 28 and 35 days resulted in lower plasma CK activity compared to weaning at 14 and 21 days ($p < 0.05$). Weaning piglets at 35 days resulted in lower plasma LDH activity compared to weaning at 14 days ($p < 0.05$). Weaning at 21 days after birth resulted in significantly higher expression of GHR mRNA and protein in LD and semitendinosus muscles compared to weaning at 14 days ($p < 0.05$). IGF-1 protein in LD and IGF-1 mRNA in LD and semitendinosus muscles were significantly elevated in the 21 days weaning group compared to weaning at 14 days ($p < 0.05$). Weaning at 21 days resulted in significantly elevated mRNA and protein levels for IGF-1R in LD and semitendinosus muscles compared to weaning on any other day ($p < 0.05$). © 2018 Friends Science Publishers

Keywords: Dorsi muscle; Growth related gene; Longissimus plasma biochemistry index; Piglet; Semitendinosus; Weaning age

Introduction

The effects of weaning age on productivity and safety in pork production have garnished substantial attention (Do, 2012). The chosen weaning age of piglets in many countries has changed often in recent years. Weaning ages as young as 7 and 12 days have been reported. However, the current EU recommendations for minimum weaning age of swine are 28 days, in accordance with animal welfare concerns. In the UK, the recommended weaning age for organic pork production is 42 days (Worobec *et al*., 1999; Li *et al*., 2016).

Mucosal barrier dysfunction in piglets may be due to the effects of early weaning (7–14 days) on immunoglobulin A (IgA) synthesis and Th17 pathways. In contrast, stimulation of IgA synthesis by endogenous milk factors has a protective effect on piglets when weaning is delayed (Levast *et al*., 2010). In a recent study, pigs weaned at 21 days showed higher fecal *E. coli* counts and higher mortality rates compared to piglets weaned at 28 days, which suggests that negative health effects are associated with earlier weaning (Leliveld *et al.,* 2013). Weaning age of piglets may influence economic traits throughout the lifetime of the pig (Collins *et al.*, 2013).

Growth hormone (GH) is an ancestral hormone in the pituitary glands of primitive vertebrates that promotes cell division, regeneration, and growth (Møller and Jørgensen, 2009; Brooks and Waters, 2016). The actions of GH are mediated by activation of the GH receptor (GHR). The cytokine receptor superfamily includes GH, prolactin, erythropoietin, leptin, and interleukin receptors (Birzniece *et al.,* 2009). GH elicits insulin-like growth factor 1 (IGF-1) production, and IGF-1 subsequently binds to type 1 IGF-1 receptor (IGF-1R) in target tissues (Gan *et al.,* 2014). IGF binding proteins (IGFBPs, termed IGFBP-1 through -6) bind IGF in the circulation, and regulate the biological activity and accessibility of IGF-1 in blood (Gow *et al.,* 2010).

To date, investigations into the effects of weaning age have focused on gastrointestinal growth (Smith *et al.,* 2010; Bomba *et al.,* 2014; Eckert *et al.,* 2015; Tsukahara *et al.,* 2016), immunity (Annamalai *et al.,* 2015; García *et al.,* 2016), and other traits. The effects of weaning age on expression of growth-related genes and proteins in piglet muscle tissue has not been previously reported. The current study aimed to determine the effects of piglet weaning age on growth performance, along with changes in plasma

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biochemistry parameters and expression levels of growth-related genes and proteins in muscle tissue, including GHR, IGF-1, and IGF-1R.

Materials and Methods

Animals and Treatments

All experiments were conducted according to the regional Animal Ethics Committee guidelines. Healthy Duroc × Landrace × Yorkshire piglets from eight litters with similar body weights were randomized into 4 treatment groups (n=6/group). Piglets were weaned at 14, 21, 28, and 35 days (labeled W14, W21, W28, and W35, respectively).

Feeding Management

Piglets were vaccinated according to standard protocols and supplied with two-stage feed beginning 8 days after birth. Piglets had access to water, ad libitum, and free access to sow milk and creep feed, before and after weaning, respectively. Piglet health was monitored and recorded for the entire experimental period.

Sample Collection

Piglets were fasted for 12 h, then sacrificed by exsanguination at 42 days. Blood samples were collected into evacuated tubes and allowed to clot before centrifugation. After centrifugation to separate the plasma (1300 × g, 25 min; 4°C), samples were stored at -20°C. Samples of longissimus dorsi (LD) and semitendinosus muscle tissue were stored immediately at -80°C after collection.

Assessments of the Plasma Biochemistry Parameters of Piglets

A fully automatic biochemical analyzer and commercially available kits were used to measure plasma TP content, GLU content, TG content, CHO content, CK activity, LDH activity, ALT activity, AST activity and ALP activity in duplicate, following the manufacturer's guidelines (Nanjing Research Institute of Biotechnology, Beijing, China).

Reverse Transcription and Polymerase Chain Reaction

All procedures were performed as described previously (Monteiro *et al*., 2016). TRIzol reagent was used to extract total RNA from the LD and semitendinosus muscles (Invitrogen, USA). RNA integrity was determined by visualizing intact 18S and 28S ribosomal RNA bands after electrophoresis (1.2% TAE agarose gel). Total RNA concentration was determined by spectrophotometry at a wavelength of 260 nm (Astra Gene II). The purity of RNA samples was determined by calculating the A260/A280 ratio (1.8–2). RNA samples were stored at -80°C.

Synthesis of cDNA was achieved with the following reverse transcription (RT) reaction: 2 μg of RNA, 10 m*M* deoxynucleotide triphosphates (dNTPs), 10 μM random hexamer primers, 200 U Moloney murine leukemia virus reverse transcriptase, 40 U RNase inhibitor, and 5 × RT buffer. After denaturation at 70°C, samples were incubated with random hexamer primers and dNTPs prior to the RT reaction. The RT reaction was performed as follows: 1 h at 37°C, 95°C for 5 min, cooling to 4°C. The resulting cDNAs were stored at -20°C.

The polymerase chain reaction (PCR) mix (25 μL) contained the following: 2 μL cDNA, 1.5 U Taq DNA polymerase, 10 mmol/L dNTPs, 10× PCR Buffer (20 mmol/L Tris-HCl pH 8.0, 100 mmol/L KCl, 0.1 mmol/L EDTA, 1.0 mmol/L DTT, 50% glycerol), and 1.0–30.0 μmol/L each of the forward and reverse primers. Primer Premier 5.0 was used for primer design. Design of primers for GHR, IGF-1, IGF-1R and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers was based on porcine gene sequences from GenBank (Table 1). In both the RT and PCR reactions, controls were used to detect environmental or genomic DNA contamination. A pooled sample consisting of equal amounts of RNA from each sample was created for optimization of PCR conditions and for intra-assay normalization. The number of cycles was optimized to assure that the PCR reaction was in the linear range. All samples were analyzed simultaneously and at least 3 repetitions were performed. RT and PCR were executed using the Gene Amp PCR System 9600 (Perkin Elmer, USA). PCR products (9 μL) were separated by agarose gel electrophoresis (2%) and band intensities were determined using Image Lab software and a Bio-Rad Analysis System 120 (Bio-Rad Co. Ltd., USA). Abundance of mRNA transcripts was determined using the target gene to GAPDH ratio.

Western Blotting

All procedures were performed as described previously (Li *et al*., 2016). LD and semitendinosus muscle samples were extracted with radioimmunoprecipitation assay (RIPA) lysis buffer, then centrifuged (5000 × g for 10 min). Protein extracts (36 μL) were combined with 4× loading buffer, boiled (5 min), and subjected to SDS-PAGE (10%). The total protein concentration of each sample was measured by spectrophotometry (595 nm). Protein samples were stored at -80°C.

Separated proteins were transferred to polyvinylidene fluoride (PVDF) membranes. After blocking for 2 h (25°C) with fat-free dry milk (5%) dissolved in Tween-Tris-buffer saline (TTBS), membranes were washed with TTBS. Membranes were then incubated at 4°C, overnight, with specific primary monoclonal antibodies at the following dilutions: GHR (Abcam, Cambridge, UK, dilution 1:5000), IGF-1 (Abcam, Cambridge, UK, dilution 1:5000), or IGF-1R (Abcam, Cambridge, UK, dilution 1:500).

Table 1: Primer sequences used for amplification of pig genes

Target gene	Product length (bp)	Primer sequence (F, forward; R, reverse)	Reference
GAPDH	285	F: 5′-TACATGGTCTACATGTTCCAGTATG-3′ R: 5′-CAGGAGGCATTGCTGACAATCTTG-3′	AF017079
GHR	344	F: 5′-GATGAGTTGAGTCAGTTCCA-3′ R: 5′-CTCGATATTGATGACCCCTGA-3′	X54429
IGF-1	222	F: 5′-GGAGCTGTGATCTGAGGA-3′ R: 5′-ACAGTAACCTCGTGCAGA-3′	M31175
IGF-1R	362	F: 5′-TCCTCACTGTAGTAGAAGGA-3′ R: 5′-CGAGAGACATCTATGAGACA-3′	AB003362

Table 2: Effect of weaning age on the serum biochemistry parameters of piglets

Items	Weaned at 14 d	Weaned at 21 d	Weaned at 28 d	Weaned at 35 d
TP (g/L)	$65.77^{c} \pm 5.25$	$70.73^{bc} \pm 6.57$	$78.14^{ab} \pm 9.29$	$84.68^{a} \pm 5.89$
GLU (mmol/L)	$5.30^{b} \pm 0.99$	$6.23^{ab} \pm 1.67$	$7.02^{a} \pm 0.65$	$7.64^{a} \pm 0.91$
CHO (mmol/L)	1.82 ± 0.16	1.86 ± 0.22	1.83 ± 0.41	1.80 ± 0.20
TG (mmol/L)	0.43 ± 0.05	0.41 ± 0.05	0.42 ± 0.12	0.40 ± 0.07
CK (U/L)	$389.01^{a} \pm 96.25$	$384.03^{a} \pm 77.19$	$307.07^{b} \pm 63.79$	$289.68^{b} \pm 72.33$
LDH (U/L)	$1074.43^{a} \pm 99.33$	$1040.45^{ab} \pm 178.32$	$896.44^{ab} \pm 72.04$	$825.23^{b} \pm 76.94$
ALT (U/L)	51.33 ± 3.91	48.38 ± 6.15	49.42 ± 6.89	46.94 ± 1.98
AST (U/L)	162.94 ± 11.09	166.90 ± 12.82	153.69 ± 23.48	156.34 ± 31.29
ALP (U/L)	419.03 ± 16.73	411.79 ± 22.74	417.12 ± 44.08	396.51 ± 35.99

Note: Values represent the mean ± SEM of 6 samples from each group. Means in the same column without common superscript letters differ significantly ($p < 0.05$)

After incubation with primary antibodies, membranes were washed in TTBS (1 × for 15 min, 4 × for 5 min) to remove unbound antibodies followed by incubation with secondary antibody- horseradish peroxidase conjugates (Anti-rabbit or Anti-rat IgG) (Abcam, Cambridge, UK, dilution 1:5000). Finally, each membrane was washed 5 times (TTBS, 1 × for 15 min, 4 × for 5 min). Chemiluminescence was used to detect secondary antibody binding (Pierce Super Signal West Pico Trial Kit). Western blot analysis was performed using Image Lab software and a Bio-Rad Analysis System 120 (Bio-Rad Co. Ltd., USA).

Statistical Analysis

Groups were compared using one-way ANOVA. Data are presented as means ± standard error of the mean. The threshold for significance was $p < 0.05$.

Results

Plasma Biochemistry Parameters

As shown in Table 2, weaning piglets at 14 days resulted in significantly lower plasma TP and GLU levels compared to weaning at 28 and 35 days ($p < 0.05$), and weaning at 21 days significantly lowered the plasma TP content of piglets compared to weaning at 35 days ($p < 0.05$). Weaning at 14 and 21 days resulted in significantly elevated plasma CK activity levels compared to 28 and 35 days ($p < 0.05$). The plasma LDH activity level was significantly greater at 14 days weaning compared to 35 days ($p < 0.05$). The plasma CHO content, TG content, ALT activity level, AST activity level, and ALP activity levels did not vary significantly between the weaning groups ($p > 0.05$).

Longissimus Dorsi Muscle GHR, IGF-1, and IGF-1R mRNA

As shown in Fig. 1, the LD GHR and IGF-1R mRNA levels were significantly greater at 21 days of weaning compared to weaning at 14, 28, and 35 days ($p < 0.05$) (Fig. 1B and D). Weaning at 14 days resulted in significantly elevated LD IGF-1 mRNA when compared to weaning at 21 days ($p < 0.05$) (Fig. 1C).

Semitendinosus GHR, IGF-1, and IGF-1R mRNA Expression

As shown in Fig. 2, semitendinosus GHR and IGF-1R mRNA levels in piglets weaned at 21 days were significantly greater compared to piglets weaned at 14, 28, and 35 days ($p < 0.05$) (Fig. 2B and D). IGF-1 mRNA levels in semitendinosus were significantly greater in piglets weaned at 14 days compared to 21, 28 and 35 days ($p < 0.05$) (Fig. 2C).

Longissimus Dorsi muscle Content of GHR, IGF-1 and IGF-1R Protein

As shown in Fig. 3, immunoreactive bands for GHR at 72 kDa, IGF-1 at 17 kDa, and IGF-1R at 95 kDa were detected in the LD muscle (Fig. 3A). Weaning at 21 days resulted in significantly higher LD GHR and IGF-1R protein levels compared to weaning at 14, 28, and 35 days ($p < 0.05$) (Fig. 3B and D). Weaning at 14 days resulted in significantly higher LD IGF-1 protein levels compared to weaning at 21 days ($p < 0.05$) (Fig. 3C).

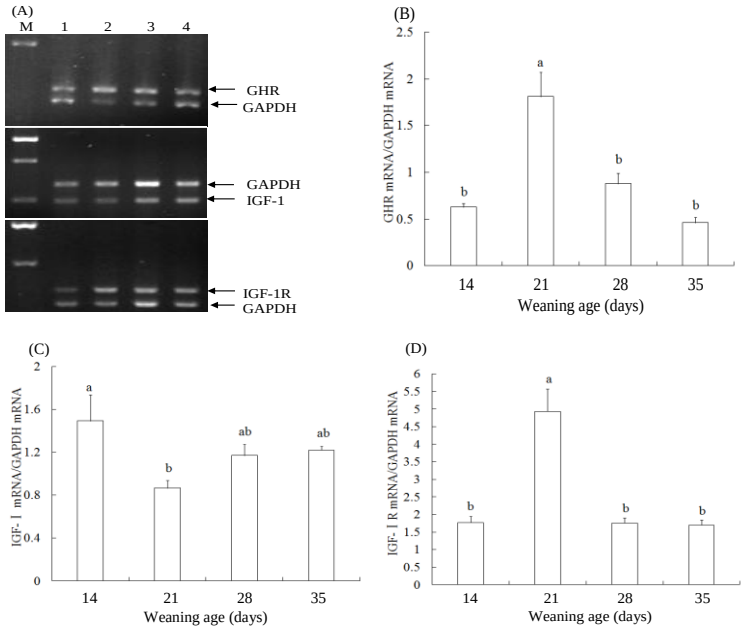

Fig. 1: Longissimus dorsi muscle GHR, IGF-1, and IGF-1R mRNA in piglets weaned at different ages. (A) Representative electrophoresis images. M: DNA ladder (DL2000); 1-4: electrophoresis images of piglets weaned at 14, 21, 28 and 35 days; (B-D) mRNA expression levels for GHR, IGF-1, and IGF-1R in semitendinosus muscle. The values are mean ± SEM and n=6/group. The average abundance of each target gene from 14 days weaning was considered as 100%. Mean values without a common superscript letter differed significantly between different weaning groups ($p < 0.05$)

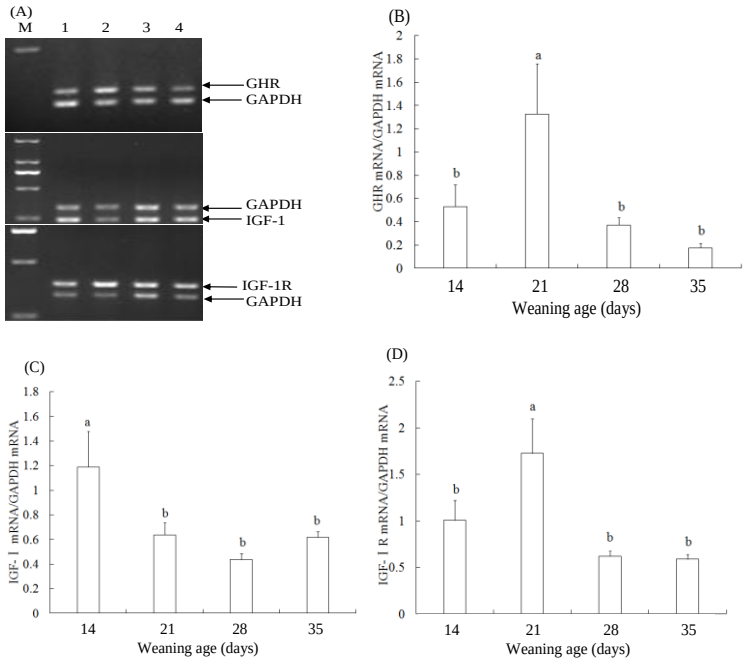

Fig. 2: Semitendinosus GHR, IGF-1, and IGF-1R mRNA in piglets weaned at different ages. (A) Representative electrophoresis images. M: DNA ladder (DL2000); 1-4: electrophoresis images from 14, 21, 28, and 35 days piglet weaning groups, respectively; (B-D) Relative expression levels of semitendinosus GHR, IGF-1, and IGF-1R mRNA, respectively. The values are mean ± SEM and n=6/group. The average abundance of each target gene in piglets weaned at 14 days was considered as 100%. Mean values without a common superscript letter differed significantly between different weaning groups ($p < 0.05$)

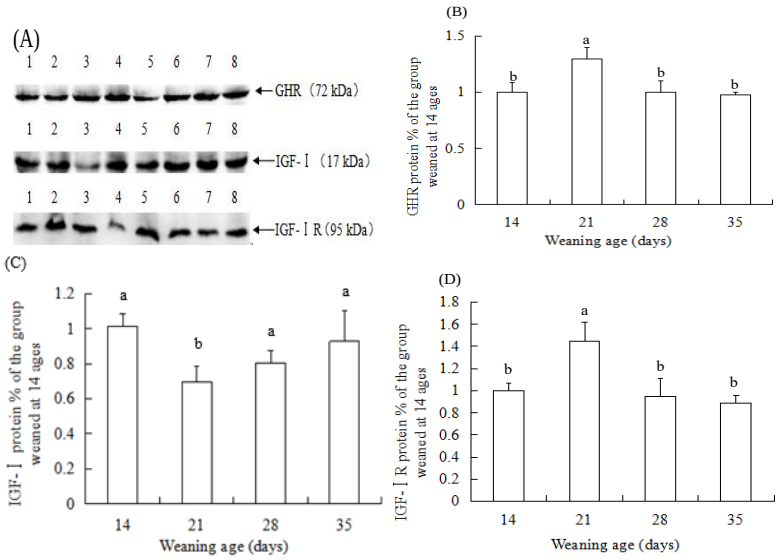

Fig. 3: GHR, IGF-1, and IGF-1R protein levels in longissimus dorsi muscle of piglets weaned at different ages. (A) Immunoreactive bands were detected using western blotting. 1-4 and 5-8: western blots of protein samples from piglets weaned at 14, 21, 28, and 35 days, respectively; (B-D) Densitometric data for GHR, IGF-1, and IGF-1R protein, respectively. The values are mean ± SEM and n=6/group. The average abundance of each target gene in piglets weaned at 14 days was considered as 100%. Mean values without a common superscript letter differed significantly between different weaning groups ($p < 0.05$)

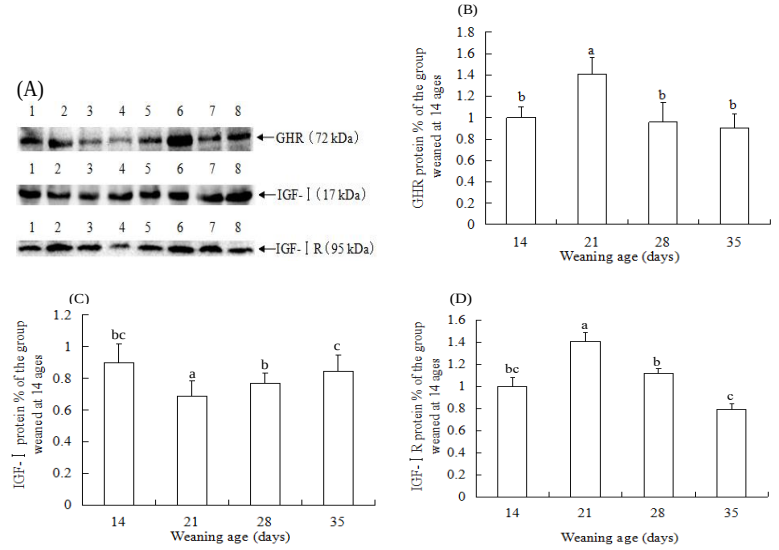

Fig. 4: GHR, IGF-1, and IGF-1R protein levels in semitendinosus muscle in piglets weaned at different ages. (A) Immunoreactive bands were detected using western blotting. 1-4 and 5-8: western blots of protein samples from piglets weaned at 14, 21, 28, and 35 days, respectively; (B-D) Densitometric data for GHR, IGF-1, and IGF-1R protein, respectively. The values are mean ± SEM and n=6/group. The average abundance of each target gene in piglets weaned at 14 days was considered as 100%. Mean values without a common superscript letter differed significantly between different weaning groups ($p < 0.05$)

Semitendinosus Muscle Content of GHR, IGF-1 and IGF-1R Protein

Fig. 4 shows western blotting analysis for GHR, IGF-1, and IGF-1R proteins in semitendinosus muscle (Fig. 4A). Weaning at 21 days resulted in significantly greater GHR and IGF-1R protein in the semitendinosus muscle compared to weaning at 14, 28, and 35 days ($p < 0.05$) (Fig. 4B and D). IGF-1R protein levels in semitendinosus muscle were significantly greater in piglets weaned at

28 days compared to 35 days ($p < 0.05$) (Fig. 4D). IGF-1 protein levels in semitendinosus muscles of piglets at different weaning ages did not differ significantly ($p > 0.05$) (Fig. 4C).

Discussion

Basic information about serum biochemistry and hematology are essential for understanding experimental data acquired using animal models (Yeom *et al*., 2012). Homeostatic regulation of blood glucose is essential for organisms to avoid adverse clinical outcomes (Arum *et al*., 2014). In the present study, weaning at 14 days resulted in significantly lower plasma GLU compared to weaning at 28 and 35 days, but the CHO and TG did not differ significantly in these groups. These results suggest that more glucose was incorporated into lipids when animals were weaned at 28 or 35 days compared to those weaned at 14 days. These findings agree with the published results of Mersmann (Mersmann *et al*., 1973). In this study, plasma ALT and AST levels in piglets weaned at different ages did not differ significantly. These data are in concordance with studies by Dritz and Fenton (Fenton *et al*., 1985; Dritz *et al*., 1996), in which liver and lipogenesis were not affected by piglet weaning age. In the present study, the plasma TP and CK in piglets differed depending on weaning age ($p < 0.05$), whereas only small differences in these parameters were found in a recent study of lambs weaned at different days (Abdel-Fattah *et al*., 2013). Lactic acid is the end-product of anaerobic glycolysis in muscle tissue (Holbrook *et al*., 1975). Therefore, the significant increase in LDH content when pigs were weaned at 14 days indicates that they had consumed more GLU than those weaned at 35 days. Indeed, when piglets were weaned at 14 days, GLU content was lower compared to piglets weaned at 35 days.

In the present study, piglets weaned at 21 days had significantly higher IGF-1R mRNA levels in the LD and semitendinosus muscles compared to piglets weaned at 14 days, but the former group had significantly lower levels of IGF-1 mRNA ($p < 0.05$). Götz (Götz *et al*., 2001) describes both inhibitory and stimulatory control of IGF function to balance IGF-I and IGF1R growth promoting activities, which may explain the variations in IGF-1R and IGF-1 mRNA levels in LD and semitendinosus muscle when weaning is delayed. Weaning at 14 days resulted in significantly greater expression of IGF-1 mRNA in the LD and semitendinosus muscles compared to weaning at 21 days. In contrast, weaning at 14 days resulted in significantly lower IGF-1R mRNA levels in the LD and semitendinosus muscles compared to weaning at 21 days. This expression pattern was similar to the findings of Tang (Tang *et al.*, 2002).

In this study, IGF-1 mRNA expression in LD and semitendinosus muscles when piglets were weaned at 14 days was significantly different from piglets weaned at 21 days ($p < 0.05$), IGF-1 protein also differed in LD muscles. In contrast, Li found no significant differences in body weights when piglets were weaned between 14 and 21 days (Li *et al.*, 2016). This conclusion was similar to findings of Sjögren showing that IGF-1 is not required for postnatal body growth (Sjögren *et al*., 1999). Several studies have shown that the function of IGF-1 is modulated by binding to IGFBPs (Hwa *et al*., 1999). Therefore, future studies should assess the potential effect of weaning age on IGFBP expression in piglets, to determine the mechanism of changes in IGF-1 mRNA and protein in piglets weaned at different ages.

Conclusion

The age at which piglets are weaned significantly affects the mRNA and protein levels of growth-related genes and proteins in the longissimus dorsi and semitendinosus muscles.

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