Effects of breed, postnatal development, and nutrition on mRNA expression of the *FTO* gene in porcine muscle and its relationship with intramuscular fat deposition

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ABSTRACT: The effects of breed, development, and nutrition on mRNA expression of the fat mass and obesity-associated gene (*FTO*) and its relationship with intramuscular fat (IMF) content in porcine muscle (*m. longissimus dorsi*; *m.l.d.*) were estimated. Purebred Jinhua, Zhongbai, Yorkshire, Duroc, Duroc × Zhongbai (DZ), and Duroc × Yorkshire × Landrace (DYL) pigs were used to investigate the effect of breed. Pigs weighing 2.5, 10, 20, 40, 60, and 100 kg were selected to study the effects of different stages of development. To study the effect of nutrition, four diets were selected: corn-soybean (CS), CS with 1.2% conjugated linoleic acid (CLA) or 0.05% creatine monohydrate (CMH), and barley-soybean (BS). All eighty animals were slaughtered, and *m.l.d.* samples were collected to examine *FTO* mRNA expression and IMF content. Results showed that breed significantly affected *FTO* mRNA expression and IMF content. *FTO* mRNA expression in the studied pigs was in the order: Zhongbai and Yorkshire > Duroc and DZ > Jinhua and DYL. The IMF content ordered by breed was Duroc > DZ > DYL > Jinhua > Zhongbai > Yorkshire. Both *FTO* mRNA expression and IMF content increased with age of the pigs, with the greatest difference seen between 100 kg pigs and all other weights. In the study, none of the four diets had a significant effect ($P > 0.05$) on *FTO* mRNA expression or IMF content. The study demonstrated that *FTO* mRNA expression increased with increasing body weight and was significantly affected by the breed of pigs. The results showed that *FTO* mRNA expression had an inconsistent correlation with IMF content between breeds and developmental ages.

Keywords: pig breeds; development ages; *FTO* mRNA expression; IMF; pig

The fat mass and obesity-associated gene (*FTO*), also known as Fatso (*Fto*), was originally described in the fused-toed (Ft) mouse (van der Hoeven., 1994). The *FTO* gene encodes a 2-oxoglutarate-dependent nucleic acid demethylase, which is functionally involved in energy homeostasis by controlling energy expenditure (Fischer et al., 2009). The *FTO* gene is located on porcine chromosome 6, where many fat quantitative trait loci (QTLs) have been identified. Studies on pig *FTO* have reported associations of several single nucleotide polymorphisms with some fat-related traits (Fan et al., 2009; Fontanesi et al., 2009; 2010; Zhang et al., 2009; Dvořáková et al., 2012). A polymorphism in the 5' regulatory region of *FTO* has been shown to be associated with IMF content in a Jinhua × Pietrain F_2 reference population (Zhang et al., 2009). Fan et al. (2009) also identified two DNA markers in the *FTO* gene that were associated with total lipid percentage in muscle of a Berkshire × Yorkshire F_2 population. Moreover, it was reported that different genotypes of *FTO* affected intermuscular fat deposition in Italian Duroc pigs and feed conversion rate in Italian Large White pigs (Fontanesi et al., 2009).

Although previous studies have investigated *FTO* mRNA expression in the brain and adipose tissue, few papers have reported *FTO* expression characteristics in porcine muscle (Huang et al., 2010; Madsen

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et al., 2010). In humans, *FTO* mRNA expression in skeletal muscle has been demonstrated to be regulated by both age and sex, and is negatively correlated with fat deposition (Klöting et al., 2008; Grunnet et al., 2009). In pigs, the transcriptional expression of additional known candidate genes that affect IMF deposition has been shown to be significantly affected not only by age and breed, but also by IMF content. For example, a significant relationship was found between the fatty acid binding protein (*FABP*) mRNA expression, but not protein expression level, in the muscle and IMF content of pigs (Gerbens et al., 2001; Wang et al., 2009).

More recently, diacylgycerol acyltransferase (*DGAT*) mRNA expression has been demonstrated to be breed-dependent, and correlated to IMF content in Laiwu, Lulai Black, and Large White pigs (Cui et al., 2011). The highly positive correlation between the low density lipoprotein receptor (*LDLR*) and *FABP3* expression and IMF content was also confirmed in porcine muscle in three genetic groups (Serão et al., 2011). In addition to growth and genetics, nutritional manipulation also affected gene expression and IMF deposition (Gao et al., 2009). The mRNA expression of several genes related to lipid metabolism and IMF deposition in animals (*PPARγ*, *H-FABP*, and *C/EBPα*) was found to be influenced by the following factors: dietary protein, lysine, energy level, or functional additives (Witte et al., 2000; Gondret et al., 2002; da Costa et al., 2004; Saez et al., 2009; Guo et al., 2011; Kyoya et al., 2011). However, no studies have reported the effects of nutritional status on *FTO* expression in animals.

Therefore, the objective of this study was to explore the characteristics of *FTO* mRNA expression in porcine muscle (*m. longissimus dorsi*; *m.l.d.*), its relationship with IMF deposition, and the influence of breed, development, and nutritional status.

MATERIAL AND METHODS

All animal studies were conducted in accordance with the principles and procedures outlined by the Zhejiang Farm Animal Welfare Council of China.

Animals and feeding

The effect of breed and crossbred pigs. Thirty-six market-weight barrows were sampled. Six groups (n = 6 per group) were selected: Jinhua pigs (70 ± 2.0 kg, 200 days), Zhongbai pigs (95 ± 2.5 kg, 180 days), Yorkshire pigs (100 ± 2.5 kg, 180 days), Duroc pigs (100 ± 2.5 kg, 180 days), Duroc × Zhongbai crossbred pigs (DZ, 100 ± 2.5 kg, 180 days), and Duroc × Yorkshire × Landrace crossbred pigs (DYL, 100 ± 2.5 kg, 180 days). All pigs were fed with the same corn-meal diet for 30 days in Lvjia Yuan Livestock Industry Co., Ltd., Zhejiang Province, China.

The effect of age group. Twenty-four male DYL pigs weighing 2.5 ± 0.2 kg (7 days), 10 ± 0.25 kg (30 days), 20 ± 0.5 kg (50 days), 40 ± 0.5 kg (70 days), 60 ± 1.5 kg (100 days), 100 ± 2.5 kg (180 days) live weight (n = 4 per developmental age) were approved by the Lvjia Yuan Livestock Industry Co., Ltd. With the exception of the 2.5 kg and 10 kg pigs, all were castrated boars.

The effect of nutritional treatments. Sixty castrated male DYL pigs with an average initial body weight of 70 ± 2.5 kg were randomly divided into four groups (n = 15 per group). The first group was fed a corn-soybean basal diet (CS), the second and third groups were fed a corn-soybean basal diet with 1.2% conjugated linoleic acid (CLA) and 0.05% creatine monohydrate (CMH) added, and the last group was fed a barley-soybean basal diet (BS). Compositions of the basal diet and nutrient levels for the growing and finishing phases are listed in Table 1. Each group consisted of five pens (three pigs per pen), feed and water were supplied *ad libitum*. The feeding experiment lasted for 30 days after 7 days of adaptation. The feed intake per pen was recorded for the experimental period, and each pig was weighed at the beginning and the end of the experiment to determine average daily gain (ADG), average daily feed intake (ADFI), and the gain : feed (G : F) ratio. One pig per pen was selected for slaughter.

Slaughtering and sampling

All pigs were transferred to the same abattoir by ordinary commercial trucks, kept off feed, and given free access to water for 16 h, and then electrically stunned, exsanguinated, scalded, and rinsed. About a 200 mg sample was obtained from the *m.l.d.* adjacent to the last rib immediately after exsanguination, and rapidly frozen in liquid nitrogen for the *FTO* mRNA expression analysis. An additional *m.l.d.* sample of about 5 g was collected for the IMF content analysis.

Table 1. Composition of experimental diets[1]

Ingredients (%)	Diet	
	corn-soybean	barley-soybean
Corn	60.5	25.15
Barley	–	50
Wheat bran	16	–
Soybean meal	20.6	22
Monocalcium phosphate	0.5	0.5
Limestone	0.95	0.95
Salt (NaCl)	0.35	0.35
Lysine	0.1	0.05
Premix[2]	1	1
Total	100	100
Calculated analysis[3]		
CP (%)	16.2	17
ME (Mcal/kg)	3040	2975
Ca (%)	0.52	0.52
Total P (%)	0.54	0.45

CP = crude protein, ME = metabolizable energy

[1]basal diets in the nutritional experiment

[2]per 1 kg complete diet: vitamin A 3950 IU, vitamin D_3 595 IU, vitamin E 23 IU, vitamin B_2 5.5 mg, vitamin B_{12} 0.03 mg, biotin 0.15 mg, nicotinic acid 18 mg, Cu ($CuSO_4 \times 5H_2O$) 200 mg, Fe ($FeSO_4 \times 7H_2O$) 55 mg, Zn ($ZnSO_4 \times 7H_2O$) 120 mg, Mn ($MnSO_4 \times H_2O$) 60 mg, Se ($NaSe_2O_3$) 0.15 mg

[3]based on nutrient contents of ingredients listed in National Research Council (1998)

Real-time PCR

Total RNA was extracted from the *m.l.d.* samples using an E.Z.N.A.® HP Total RNA Isolation Kit (Omega Bio-Tek, Norcross, USA) according to the manufacturer's instructions. Total RNA concentration was determined using an ND1000 spectrophotometer (Thermo, Wilmington, USA), and the integrity of the RNA was verified by 1.4% agarose-formaldehyde electrophoresis. About 1 µg of total RNA was reverse transcribed in a 20 µl mixture using a ReverTra Ace® qPCR RT Kit (Toyobo Co., Ltd., Osaka, Japan). Reactions were incubated at 65°C for 5 min, 37°C for 15 min, and then 98°C for 5 min to inactivate the reverse transcriptase. Prior to use in qPCR, all cDNA samples were diluted 1 : 10 with H_2O.

The qPCR primers were synthesized and gave an expected amplification of 240 bp (Fan et al., 2009), and the *β-actin* gene was used as the reference (Nygard et al., 2007). The parameters of the primers are presented in Table 2. Fast SYBR® Green PCR Master Mix, 0.5mM of each primer and 2 µl of 10 × diluted cDNA were mixed and used for PCR amplification in duplicate on an ABI StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, USA). The baseline adjustment method of the StepOne Software v2.1 was used to determine the *Ct* in each reaction. Three replicates were run for each sample. Standard curve analysis of the primers showed a high linearity ($R^2 > 0.998$) and efficiency of amplification (Eff%, 90.62–91.98%), and a reasonable range of *Ct* (15–32). Relative quantification (RQ) was calculated using the $2^{-\Delta\Delta Ct}$ formula (Yuan et al., 2006).

$$2^{-\Delta\Delta Ct} = 2^{\Delta Ct_{FTO} - \Delta Ct_{\beta\text{-}actin}}$$

where: $\Delta Ct_{FTO} = Ct_{\text{control}} - Ct_{\text{treatment}}$

$\Delta Ct_{\beta\text{-}actin} = Ct_{\text{control}} - Ct_{\text{treatment}}$

Determination of intramuscular fat content

To assess IMF content, muscle samples were carefully trimmed of all external fat and epimysium, avoiding the intermuscular fat deposits surrounding the muscle. IMF content was determined in triplicate for each sample using Soxhlet petroleum-ether extraction, and expressed as g/100 g of wet muscle tissue. IMF contents were not measured in the 2.5 kg and 10 kg pigs due to insufficient samples.

Statistical analyses

The data were statistically analyzed by the One-Way ANOVA program using the SPSS (Version 17.0,

Table 2. Parameters of gene-specific primers for *FTO* and *β-actin* genes

Gene symbol	Nucleotide sequence (5' to 3')	GenBank Accession No.	T_a (°C)	Size (bp)
FTO	TGCAGATTGAGACCATCCAG TCTTCCCCATGCCAAAGTAG	GU138673	60	240
β-actin	CACGCCATCCTGCGTCTGGA AGCACCGTGTTGGCGTAGAG	DQ845171	60	100

FTO = fat mass and obesity-associated gene, T_a = annealing temperature

2009) statistical software package. The results were presented as means ± SE. The *P*-value for significance was set at 0.05.

RESULTS

The effect of breed

***FTO* mRNA expression.** The levels of *FTO* mRNA expression in *m.l.d.* were significantly affected by breed (Figure 1). The RQ values of *FTO* mRNA expression in the studied pigs were in the order: Zhongbai and Yorkshire > Duroc and DZ > Jinhua and DYL. There were significant differences ($P < 0.05$) among the above three pairs.

Intramuscular fat content. The statistical results are presented in Figure 2. In summary, the variation tendency of IMF contents was inconsistent with the RQ values of *FTO* mRNA expression in different breeds. The IMF contents were the lowest ($P < 0.05$) in Yorkshire and the highest in Duroc pigs. The IMF content ordered by breed was Duroc > DZ > DYL > Jinhua > Zhongbai > Yorkshire.

The effect of developmental age

***FTO* mRNA expression.** The levels of *FTO* mRNA expression in *m.l.d.* were greatly influenced by age (Figure 3), and increased with weight gain. The highest RQ value was found in 100 kg pigs, being very significantly higher ($P < 0.05$) than in the other weights. RQ values were significantly higher ($P < 0.05$) in 60 kg pigs than in 2.5 kg animals. No significant differences ($P > 0.05$) were found between 2.5 kg and 40 kg pigs.

Intramuscular fat content. IMF content showed a similar trend to *FTO* mRNA levels (Figure 4). The data of IMF content in *m.l.d.* were also increased with increasing body weight. The highest IMF contents were observed in 100 kg pigs, being very significantly higher ($P < 0.05$) than in the 20 kg pigs and also significantly higher ($P < 0.05$) than in the 40 kg animals. No significant differences ($P > 0.05$) were found between 20 kg and 40 kg pigs, 40 kg and 60 kg pigs, or 60 kg and 100 kg pigs. IMF contents in the 2.5 kg and 10 kg pigs were not analyzed because of insufficient samples.

Experiment of nutritional regulations

Growth performances. The results of the feeding trial showed no significant difference ($P > 0.05$) between the four treatments on any of the growth performance indicators, including ADG, ADFI, and G : F ratio (Table 3). Although the growth performance of CLA group showed a small increase, this finding did not reach the level of statistical significance.

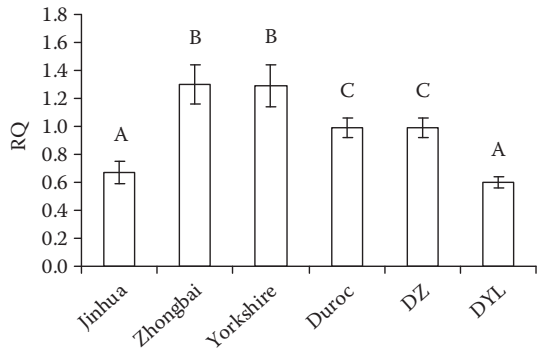

Figure 1. *FTO* mRNA expression in *m. longissimus dorsi* of different breeds

FTO = fat mass and obesity-associated gene, RQ = relative quantities, DZ = Duroc × Zhongbai crossbred pigs, DYL = Duroc × Yorkshire × Landrace crossbred pigs

RQ of *FTO* mRNA expressions were calculated using the $2^{-\Delta\Delta Ct}$ formula

six pigs were analyzed for each breed/crossbred

$^{A–C}P < 0.05$, error bars represent the standard error

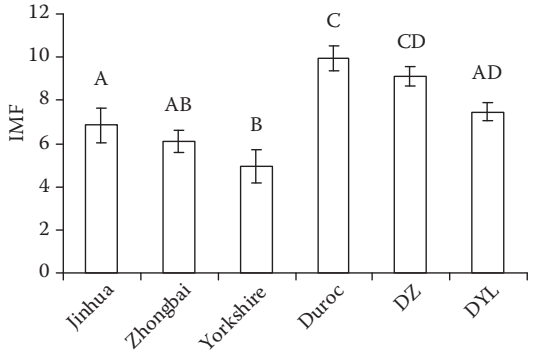

Figure 2. Intramuscular fat (IMF) contents in *m. longissimus dorsi* of different breeds

DZ = Duroc × Zhongbai crossbred pigs, DYL = Duroc × Yorkshire × Landrace crossbred pigs

IMF values are g/100 g of wet *m. longissimus dorsi* samples

six pigs were analyzed for each breed/crossbred

$^{A–D}P < 0.05$, error bars represent the standard error

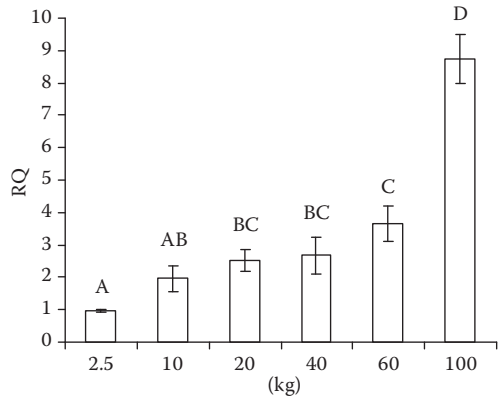

Figure 3. *FTO* mRNA expressions in *m. longissimus dorsi* of DYL of different weight categories

FTO = fat mass and obesity-associated gene, RQ = relative quantities, DYL = Duroc × Yorkshire × Landrace crossbred pigs
RQ of *FTO* mRNA expressions were calculated using the $2^{-\Delta\Delta Ct}$ formula
four pigs were analyzed for each weight category
$^{A–D}P < 0.05$, error bars represent the standard error

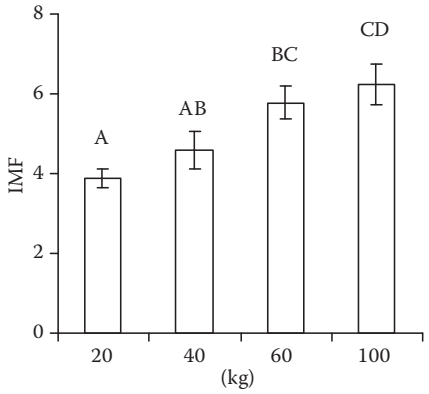

Figure 4. Intramuscular fat (IMF) contents in *m. longissimus dorsi* of DYL of different weight categories

DYL = Duroc × Yorkshire × Landrace crossbred pigs
IMF values are g/100 g of wet *m. longissimus dorsi* samples
four pigs were analyzed for each weight category
$^{A–D}P < 0.05$, error bars represent the standard error

***FTO* expression and IMF content.** Neither *FTO* mRNA expression nor IMF content showed a significant difference ($P > 0.05$) between the four nutritional treatments (Table 4).

DISCUSSION

Previous studies (Huang et al., 2010; Madsen et al., 2010) showed the *FTO* mRNA could be expressed in most pig tissues, including muscle. In our study, different breeds showed different patterns of *FTO* mRNA expression in *longissimus dorsi* muscle tissue. And *FTO* mRNA expression had higher levels in those breeds whose muscle tissue contained lower IMF content. This may be due to the fact that there were different genotypes of *FTO* gene in different breeds, and accordingly affecting the levels of *FTO* mRNA expression. However, we did not investigate the genotypes of *FTO*, because the low number of pigs from each treatment would result in the data of no statistical significance. The research of Dvořáková et al. (2012) indicated allele *C* was significantly associated with back fat depth and allele *G* in exon 3 of the *FTO* with muscling traits in commercial pigs. Moreover, different genotypes, such as *H-FABP* (Zhao et al., 2009), leptin receptor (Tyra et al., 2011), and sterol regulatory element binding transcription factor 1 gene (Chen et al., 2008) also

Table 3. Pig growth performances of different nutritional treatments[1]

Item	Nutritional treatment			
	CS	CLA	CMH	BS
Initial weight (kg)	70.75 ± 3.44	70.82 ± 3.20	69.77 ± 3.72	70.11 ± 3.27
Final weight (kg)	95.89 ± 4.19	96.79 ± 4.24	94.55 ± 4.62	96.69 ± 3.90
ADG (g)	842.78 ± 57.43	874.17 ± 70.04	811.11 ± 18.60	901.94 ± 41.09
ADFI (kg)	2.70 ± 0.05	2.65 ± 0.06	2.65 ± 0.06	2.78 ± 0.05
Gain : feed (g/kg)	311.30 ± 18.09	328.30 ± 20.80	311.76 ± 5.35	324.89 ± 13.98

CS = corn-soybean basal diet, CLA = conjugated linoleic acid, CMH = creatine monohydrate, BS = barley-soybean basal diet, ADG = average daily gain, ADFI = average daily feed intake
[1]five pens ($n = 3$) were analyzed for each group, values are expressed as means ± SE, values in individual rows do not differ significantly ($P > 0.05$)

Table 4. *FTO* mRNA expression and IMF content of nutritional treatments[1]

Item	Nutritional treatment			
	CS	CLA	CMH	BS
RQ[2]	0.73 ± 0.10	0.62 ± 0.09	0.77 ± 0.14	0.73 ± 0.09
IMF[3]	8.90 ± 0.81	7.11 ± 0.42	7.14 ± 1.40	7.25 ± 0.67

CS = corn-soybean basal diet, CLA = conjugated linoleic acid, CMH = creatine monohydrate, BS = barley-soybean basal diet, RQ = relative quantities, IMF = intramuscular fat, *FTO* = fat mass and obesity-associated gene

[1]five pigs were analyzed for each group; values are expressed as means ± SE, values in the same row do not differ significantly at $P > 0.05$

[2]RQ of *FTO* mRNA expressions were calculated using the $2^{-\Delta\Delta Ct}$ formula

[3]IMF values are g/100 g of wet *m. longissimus dorsi* samples

appear to affect mRNA expression. On the other hand, expressions of mRNA have relation to the alternative isoforms of the gene in different breeds. Huang et al. (2010) have identified three novel splice variants were in Large White and indigenous Chinese Tibetan pigs.

It is well known that the order of adipose deposition is initially subcutaneous, followed by intermuscular and finally at intramuscular adipose sites (Mourot and Kouba, 1999). In other words, more IMF is deposited in the later stages of animal development. Interestingly, in our study, *FTO* mRNA expression appeared to follow the same rule. It has been suggested that *FTO* mRNA expression is involved in the regulation of IMF deposition during the growth of animals. As no further evidence was found for the involvement of the *FTO* gene in IMF content, this relationship is most likely to be a consequence of higher lipid metabolism as *FTO* is upregulated in tissue containing a higher concentration of IMF content.

However, in the current study, the inconsistent results were found, possibly due to both the different *FTO* genotypes and splice variants of *FTO* expression. Firstly, it has been demonstrated that alleles of *FTO* gene affected IMF deposition in pig breeds (Fontanesi et al., 2009). Secondly, splice variants of *FTO* expression were found to be breed- and tissue- specific; for example, three splice variants have been found in the fat tissue of Large White and Chinese Tibetan pigs (Huang et al., 2010).

Our results showed that the nutritional factors, CLA and CMH, had no significant effects on either *FTO* mRNA expression or IMF content of pigs. However, the existing papers showed that feeding status had indeed affected *FTO* mRNA expression. Hypothalamic *FTO* mRNA levels have been reported to be higher (Fredriksson et al., 2008; Olszewski et al., 2009; Rask-Andersen et al., 2011), decreased (Poritsanos et al., 2011) or without difference (McTaggart et al., 2011) in fasted mice. Fasting was also found to change hepatic *FTO* mRNA expression, but had no effect on *FTO* mRNA expression in muscle and adipose tissues both in mice and broiler chickens (Poritsanos et al., 2010; McTaggart et al., 2011; Tiwari et al., 2012). In the present study, all animals had been fasting for 16 h before slaughter, which eliminated the effect of metabolic state and should have no effect on *FTO* mRNA expression in muscle of pigs in any case.

Studies have reported that adding certain doses of CLA, CMH, linseed, and other additives increased the IMF content of pigs (Stahl et al., 2001; Meadus et al., 2002; Huang et al., 2008; Martin et al., 2008; Luo et al., 2009; Cordero et al., 2010; Zhong et al., 2011). However, our results showed that adding CLA or CMH to the diet had no significant effect on either IMF content or *FTO* mRNA expression of pigs. This may be related to additive dosage and feeding time of the additives.

In conclusion, our results showed that *FTO* mRNA expression in muscle was significantly affected by both breed and developmental stage in pigs. However, an inconsistent relationship was found between *FTO* mRNA expression and IMF content. The results indicated that *FTO* is involved in the genetic variation of intramuscular fat content at different developmental stages and breeds of pigs through a distinct genetic mechanism. It might be that the *FTO* protein differs with different phenotypes and splice variants, which could be more related to intramuscular fat content in different breeds.

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