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Dietary vitamin A restriction affects adipocyte differentiation and fatty acid composition of intramuscular fat in Iberian pigs

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ABSTRACT

The aim of this study was to investigate whether dietary vitamin A level is associated with differences in adipocyte differentiation or lipid accumulation in Iberian pigs at early growing (35.8 kg live weight) and at finishing (158 kg live weight). Iberian pigs of 16.3 kg live weight were allocated to two feeding groups, one group received 10,000 IU of vitamin A/kg diet (control); the other group received a diet with 0 IU of vitamin A (var) for the whole experimental period. The dietary vitamin A level had no effect on growth performance and carcass traits. The early suppression of vitamin A increased the preadipocyte number in *Longissimus thoracis* (LT) muscle in the early growth period ($P < 0.001$) and the neutral lipid content and composition (higher MUFA and lower SFA content) at the end of the finishing period ($P < 0.05$). Vitamin A restriction in young pigs increases their lipogenic potential without affecting carcass traits.

1. Introduction

Adipocyte differentiation is an important factor for fat accumulation in the body. Adipocytes are derived from fibroblast-like preadipocytes and grow in size by accumulation of lipids in the cytoplasm in association with terminal differentiation (Hausman, Campion, & Martin, 1980). In the early stage of adipocyte differentiation, many adipocyte characteristic genes are sequentially activated and play established roles in promoting the differentiation process (Ntambi & Kim, 2000).

Adipocyte differentiation is regulated by various kinds of hormones (Boone, Gregoire, & Remacle, 2000; Gregoire, Smas, & Sul, 1998). Furthermore, it is well known that fat-soluble vitamins, especially metabolites of vitamin A and D, modulate adipocyte differentiation in cultured cells in mammals (Kawada et al., 1990). All-*trans* retinoic acid (RA, the active metabolite of vitamin A) and 1,25-dihydroxyvitamin D3 (1,25(OH)2D3) inhibit adipocyte differentiation in cultured cells at a supraphysiological concentration (Kawada et al., 1990; Sato & Hiragun, 1988; Suryawan & Hu, 1997). However, very low concentration (1 pM — 10 nM) of RA stimulates adipocyte differentiation (Safonova et al., 1994).

Due to its role in reproduction, growth, development and immune response, commercial pig diets in the European Union contain vitamin A concentrations approximately six- to ten-fold higher than NRC recommendation (1317 IU/kg diet) (Fraga and Villamide, 2000). However, studies in vivo showed that a dietary level of 1300 IU of vitamin A for 11 weeks was associated with a higher intramuscular fat (IMF) content in *Longissimus thoracis* (LT) muscle than a diet with 13,000 IU in pigs (Olivares, Reya, Daza, & Lopez-Bote, 2011). However, in another experiment, Olivares, Daza, Rey, and Lopez-Bote (2009a) found no effect of dietary vitamin A on IMF content in pigs. Thus, the effect of vitamin A on body fat accumulation in swine remains unclear. Also, previous studies have found that dietary vitamin A concentration alters fatty acid composition of adipose tissue in sheep (Daniel, Salter, & Buttery, 2004), beef (Siebert et al., 2006) and pigs (Olivares, Daza, Rey, & Lopez-Bote, 2009b; Olivares et al., 2009a, 2011) but no effect was found on the fatty acid composition of IMF in pigs (Olivares et al., 2011). These experiments have been performed with different animals (ruminant vs. non-ruminant), genotypes (Duroc vs. lean pigs) and different times of supplementation or restriction of vitamin A. Both, IMF content and fatty acid composition are determinant factors affecting meat quality (Wood et al., 2008) and they are of special interest in high quality meat products, such as those obtained from Iberian pigs. Moreover, the effects of dietary vitamin A level have never been

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assessed in this breed. On the other hand, Iberian pigs have a high adipogenic and lipogenic potential, which could modify the effects of vitamin A restriction on fatness and thus, the use of dietary vitamin A restriction as a strategy to increase IMF should be tested in Iberian pigs.

Several authors have established that vitamin A exerts its effects on adipose tissue via regulation of expression of genes involved in adipogenesis and lipid metabolism (Bonet, Ribot, Felipe, & Palou, 2003; Daniel et al., 2004; Fernandez et al., 2011; Schwarz, Reginato, Shao, Krakow, & Lazar, 1997). Indeed, vitamin A, and specifically its metabolite, RA, is well known as a potent transcriptional regulator. Balmer and Blomhoff (2002) established that more than 500 genes are regulated by RA. Many genes are involved in the adipogenic process, from the very beginning, when *C/EBP* and *PPAR* (mainly *PPARG*) families are induced, to the final differentiation process, when mature adipocytes express genes involved in lipid metabolism such as ATP citrate lyase, malic enzyme, acetyl-CoA carboxylase, fatty acid synthase and others (Gregoire et al., 1998; Rosen, Walkey, Puigserver, & Spiegelman, 2000). The expression of these genes is considered a signal of the mature adipocyte phenotype. However, most of the available data in the literature are obtained from adipocyte culture studies. Results coming from studies in vivo are scarce and to our knowledge, there is no information about the effect of a restriction of dietary vitamin A on gene expression in pigs.

The objective of this study was to investigate how dietary vitamin A restriction affects gene expression in young pigs (35.8 kg) and impacts adipocyte differentiation and lipid accumulation in Iberian pigs at early growth (35.8 kg) and finishing (158 kg) periods.

2. Materials and methods

2.1. Animals and diets

Animal manipulations were done in compliance with the regulations of the Spanish policy for animal protection RD1201/05, which meets the European Union directive 86/609 about the protection of animals used in experimentation. The experiment was specifically assessed and approved (report CEEA 2010/003) by the Spanish National Institute for Agricultural and Food Research and Technology (INIA) Committee of Ethics in Animal Research. The trial was conducted at CIA Dehesón del Encinar (Oropesa, Toledo, Spain).

Thirty-eight castrated male (Torbiscal Pure Iberian) were randomly selected from a population. They were weaned at four weeks of age at a live weight (LW) of 11.7 ± 2.2 kg and were housed in pens until 2 months of age (average weight of 16.3 ± 2.5 kg) when piglets were randomly assigned to the two treatment groups, housed individually and given the experimental diets. One group was fed a vitamin A-enriched starter diet (10,000 IU vitamin A/kg diet) (control) and the other group received a starter diet formulated with no vitamin A (var, the same content in all periods) added in the premix (Table 1) from 16.3 ± 2.5 kg LW to 32.2 ± 4.5 kg LW. Diets were adjusted to meet requirements depending on the growing period. The pigs were changed to the corresponding control (10,000 IU vitamin A/kg diet) and vitamin A-restricted growing (from 32.2 ± 4.5 kg LW to 101 ± 4.1 kg LW) and finishing diets (from 101 ± 4.1 kg LW to 158 ± 7 kg LW). The pigs were fed 3.5% LW restriction until four months of age, 3% LW restriction until eight months and 2.5% LW restriction from this age until slaughter. The pigs had ad libitum access to water.

Ingredients, chemical composition and main fatty acids of experimental diets are shown in Table 1. Diets were formulated according to general guidelines proposed by De Blas, Gasa, and Mateos (2013) for Iberian pigs.

Table 1
Ingredient composition, calculated analysis (g/kg, as-fed basis unless stated otherwise) and fatty acid composition of the experimental diets.

	Starter		Growth		Finishing	
	Control[a]	Var[b]	Control	Var	Control	Var
Ingredient						
Barley	280.0	280.0	500.0	500.0	453.2	453.2
Soybean meal (440 g CP/kg)	155.1	155.1	169.4	169.4	75.9	75.9
Wheat	250.0	250.0	290.3	290.3	300.0	300.0
Soybean protein concentrate (650 g CP/kg)	25.0	25.0				
Corn	194.9	194.9				
Whey powder, sweet (cattle)	25.0	25.0				
Full fat soybean toasted	20.0	20.0				
High oleic sunflower seed					120.0	120.0
Lard	17.0	17.0	10.0	10.0	20.0	20.0
Calcium carbonate	5.4	5.4	8.2	8.2	8.2	8.2
Dicalcium phosphate	13.6	13.6	12.0	12.0	12.0	12.0
Control—mineral and vitamin premix[c]	4.0	0	4.0	0	4.0	0
Var—mineral and vitamin premix[d]	0	4.0	0	4.0	0	4.0
Salt	4.0	4.0	4.5	4.5	4.0	4.0
L-Lysine (500 g/kg)	4.0	4.0	1.6	1.6	2.2	2.2
Methionine-OH	1.4	1.4				
L-Threonine	0.6	0.6				
Calculated analysis[e]						
Net energy (MJ/kg)	10.0	10.0	9.5	9.5	10.4	10.4
Crude protein	178.2	178.2	171.9	171.9	147.0	147.0
Crude fat	41.9	41.9	29.0	29.0	85.0	85.0
Crude fiber	35.7	35.7	40.9	40.9	55.2	55.2
Crude Ash	48.0	48.0	49.2	49.2	49.2	49.2
Fatty acid composition (g/100 g total fatty acids)						
C12:0	1.8	1.6	7.2	8.5	1.0	1.0
C14:0	2.0	2.0	3.6	3.5	1.1	1.2
C16:0	18.8	19.2	18.0	17.8	11.2	11.8
C16:1 n−9	0.1	0.8	0.8	0.9	0.6	0.7
C16:1 n−7	1.0	0.4	0.3	0.3	0.2	0.2
C17:0	0.5	0.2	0.0	0.0	0.1	0.1
C17:1	0.2	0.6	0.0	0.0	0.2	0.2
C18:0	6.4	5.8	3.9	4.2	3.9	4.2
C18:1 n−9	26.6	26.3	21.1	20.5	57.0	57.5
C18:1 n−7	1.7	1.4	1.3	1.3	0.2	0.2
C18:2 n−6	36.2	36.8	39.0	37.8	21.4	20.1
C18:3 n−3	3.1	3.3	3.6	3.9	1.5	1.5
C20:0	0.3	0.3	0.3	0.3	0.4	0.3
C20:1 n−9	0.6	0.6	0.6	0.6	0.6	0.6
C20:3 n−6	0.2	0.2	0.2	0.2	0.3	0.2
C20:4 n−6	0.5	0.4	0.2	0.2	0.4	0.2
SFA[f]	29.8	29.1	32.9	34.3	17.6	18.7
MUFA[g]	30.2	30.1	24.1	23.6	58.8	59.4
PUFA[h]	40.0	40.7	43.0	42.1	23.6	22.0

[a] Control = control diet. 10,000 IU vitamin A/kg feed supplementation.
[b] Var = vitamin A restricted diet. 0 IU vitamin A/kg feed supplementation.
[c] Control—mineral and vitamin premix provided per kg of feed: vitamin A, 10,000 IU; vitamin D3, 2000 IU; vitamin E, 26.7 mg; vitamin B1, 1.3 mg; vitamin B2, 4.0 mg; vitamin B12, 0.020 mg; vitamin B6, 1.3 mg; calcium pantothenate, 13.3 mg; nicotinic acid, 20 mg; biotin, 0.1 mg; folic acid, 0.1 mg; vitamin K3, 2 mg; Fe, 133.3 mg; Cu, 26.7 mg; Co, 0.30 mg; Zn, 133.3 mg; Mn, 76.7 mg; I, 1.3 mg; Se, 0.30 mg; ethoxyquin, 150 mg.
[d] Var—mineral and vitamin premix provided per kg of feed: vitamin A, 0 IU; vitamin D3, 2000 IU; vitamin E, 26.7 mg; vitamin B1, 1.3 mg; vitamin B2, 4.0 mg; vitamin B12, 0.020 mg; vitamin B6, 1.3 mg; Calcium pantothenate, 13.3 mg; Nicotinic acid, 20 mg; Biotin, 0.1 mg; Folic acid, 0.1 mg; vitamin K3, 2 mg; Fe, 133.3 mg; Cu, 26.7 mg; Co, 0.30 mg; Zn, 133.3 mg; Mn, 76.7 mg; I, 1.3 mg; Se, 0.30 mg; Ethoxyquin, 150 mg.
[e] According to Fundación Española Desarrollo Nutrición Animal (2010) (supplied per kg of diet).
[f] SFA (C12:0 + C14:0 + C16:0 + C17:0 + C18:0 + C20:0); sum of saturated fatty acids.
[g] MUFA (C16:1n−9 + C16:1n−7 + C17:1 + C18:1n−9 + C18:1n−7 + C20:1n−9); sum of monounsaturated fatty acids.
[h] PUFA (C18:2n−6 + C18:3n−3 + C20:3n−9 + C20:4n−6); sum of polyunsaturated fatty acids.

2.2. Sample collection

Nine pigs per treatment were slaughtered at 4 months of age (early growing) and the remaining (n = 10) at 11 months of age (finishing) (Industrias Cárnicas Alonso, S.L., Toledo, Spain) when pigs reached the averaged weights of 35.8 ± 3.1 and 158 ± 7 kg LW, respectively. In the slaughterhouse, carcass length from the posterior edge of the

Symphysis pubica to the anterior edge of the first rib, ham length from the anterior edge of the *Symphysis pubica* to the *Articulatio tarsi*, and ham circumference at its widest point were measured on the left side of each carcass were determined with a tape measure. Backfat thickness at the 10th rib on the midline of the carcass (skin included) was also measured with a ruler. Samples from loin (LT) at the level of the last rib were taken, weighed, vacuum-packed in low-oxygen permeable film and kept frozen at −20 °C until fatty acid composition analysis. Prior to fatty acid analysis, muscle samples were freeze dried for two days in a lyophilizer (Lyoquest, Telstar, Tarrasa, Spain) and grounded in a Mixer Mill MM400 (Retsch Technology, Haan, Germany) until muscle was completely powdered. Loin samples for gene expression study were immediately frozen in liquid nitrogen and kept at −80 °C until analysis.

2.3. Laboratory analysis

2.3.1. Fatty acid composition of diets

Fatty acids of diets were extracted and quantified by the one-step procedure of Sukhija and Palmquist (1988) from lyophilized samples. Fatty acid methyl esters (FAMEs) were analyzed by a gas chromatograph (Hewlett Packard HP-6890, Avondale, PA, USA) with a flame ionization detector and a capillary column (HP-Innowax, Agilent Technologies Gmbh, Germany) as previously described (Lopez-Bote, Rey, Isabel, & Sanz, 1997). A temperature program of 170 to 245 °C was used. The injector and detector were maintained at 250 °C. The carrier gas (helium) flow rate was 2 ml/min. Results were expressed as gram per 100 g of detected FAMEs.

2.3.2. Neutral and polar lipid content and fatty acid composition of samples

Longissimus thoracis muscle lipids were extracted as proposed by Segura and Lopez-Bote (2014) and separated into neutral lipids (NLs) and polar lipids (PLs) (main fractions of IMF) using aminopropyl minicolumns, following the method used by Ruiz, Antequera, Andres, Petron, and Muriel (2004). Lipid fraction extracts were methylated in the presence of sulphuric acid and analyzed by gas chromatography as described by Lopez-Bote et al. (1997).

The activity of stearoyl-CoA desaturase (SCD, 1.14.19.1) enzyme was estimated as C18:1/C18:0 and MUFA/SFA ratios (Hulver et al., 2005).

2.3.3. Tissue handling for immunohistochemistry

The muscle tissue (LT) was removed from the carcass at the level of the last rib. The tissue was divided into 2 to 4 cm sections cut perpendicular to the long axis of the body. The sections were fixed in Bouin's fluid (saturated picric acid, buffered formalin stabilized with methanol to pH = 7.0 and glacial acetic acid) for up to two days. Large specimens were fixed for up to three days. After the fixing period the samples were transferred to 70% alcohol. If the tissue was still yellow in the paraffin block, the hydrated sections were placed in alkaline solution to remove residual picric acid, and then rinsed with water before staining. Then the specimen was embedded in paraffin, cut at 4 μm and routinely stained with hematoxylin and eosin (Pathology Service, Veterinary Teaching Hospital of Complutense University, Madrid, Spain) for routine examination (García del Moral, 1993).

2.3.4. Immunohistochemistry

Preadipocyte factor-1, pref-1, is a transmembrane protein that is part of the family of epidermal growth factor-like repeat-containing proteins that are involved in cell fate determination. Pref-1, which is coded by an adipocyte differentiation inhibitor gene, is highly expressed in preadipocytes, but its expression is completely abolished during differentiation into a mature adipocyte (Gondret, Perruchot, Tacher, Berard, & Bee, 2011; Huff, Lozeman, Weselake, & Wegner, 2005). Several authors have proposed the immunohistochemical localization of pref-1 or its expression to quantify the amount of preadipocytes in different tissues (Deiuliis, Li, Lyvers-Peffer, Moeller, & Lee, 2006; Gondret et al., 2011; Huff et al., 2005). Thus, immunohistochemistry was used to investigate preadipocyte presence, marked with delta-like homolog (DLK1) also known as preadipocyte factor 1 (DLF/Pref-1 Polyclonal Antibody — Proteintech, Manchester, United Kingdom) (Huff et al., 2005).

Preadipocyte cell detection was investigated using the StreptAvidin–biotin complex method as described by (Carrasco et al., 2011).

Sections were deparaffinized, rehydrated and further placed in a steel pressure cooker containing 2 l of 10 mM sodium citrate buffer (pH 6.0) and heated for 3 min after the maximum pressure had started, as the antigen unmasking protocol. The slides were cooled at room temperature in the same buffer for 20 min and washed in distilled water and Tris-buffered saline (TBS) (0.1 M Tris base, 0.9% NaCl, pH 7.4). After this procedure, the samples were incubated for 15 min with 1% H_2O_2 to block endogenous peroxidase activity, followed by a 5-min wash in distilled water and another 5-min wash in TBS. Previously, to add the primary antibody, the sections were incubated for 30 min with normal serum block (normal goat serum code no. X0901, Dako Cytomation, Glostrup, Denmark) at 1:30 (v/v) dilution. Then, the slides were incubated overnight at 4 °C with the primary antibody (polyclonal rabbit anti — DLF/Pref-1) at dilution of 1:100 (v/v). The slides were incubated with goat anti-rabbit biotinylated secondary antibody (1:400; v/v), 30 min at room temperature (E 0353, DakoCytomation, Glostrup, Denmark). Next, all the slides were incubated with StreptAvidin–biotin–peroxidase complex (1:400; v/v), 30 min at room temperature (P50242, Zymed, San Francisco, CA). All washes (5 min, two times between each incubation step) and dilutions were made in TBS buffer. Immunoreactivity was observed with 3,3′-diaminobenzidine tetrachloride (D5050, Sigma Chemical Co., St. Louis, MO) and H_2O_2 (0.01%) in distilled water. After washing in tap water for 10 min, slides were counterstained for 3 min with Carrazzi's hematoxylin, washed in tap water, dehydrated, and mounted. Negative control slides were included within each batch of slides, which were prepared in all cases by omitting the primary antibody and incubating tissue sections with TBS.

For evaluation of cells, an optical light microscope (Olympus BX50, Hamburg, Germany) was used with 10× magnification. Positive DLF/Pref-1 stained sections were counted in five random fields of each slide. Five photographs (Olympus DP50, Hamburg, Germany) in different fields of the histological section stained with the polyclonal rabbit anti-DLF/Pref-1 antibody were taken and analyzed with a computer image analyzer (software Viewfinder Lite® version 1.0). After obtaining the photographs, the preadipocytes were identified and counted. Cells expressing DLF/Pref-1 showed nuclear brown staining.

2.3.5. Quantitative gene expression

A quantitative gene expression analysis was performed in LT muscle from early growing animals in a panel of seven candidate genes (Table 2). We selected a panel of 7 candidate genes, which are involved in RA signaling and transcriptional control of adipogenesis mediated by RA (*RARA*, *RXRG* and *CRABPII*) adipocyte differentiation (*CEBP*, *PPARG* and SREBP*1C*) and fatty acids metabolism (*SCD*). Retinoic acid is considered an inhibitor of adipocyte differentiation by inhibiting the *CEBPB* signaling pathway and thus, blocking the expression of *PPARG* (Schwarz et al., 1997), which is considered the master regulator of adipogenesis.

RNA was extracted from 50 to 100 mg frozen muscle tissue samples using the Ribopure kit according to the manufacturer's instructions (Ambion, Austin, TX). The RNA concentration was quantified using a Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, USA). RNA quality was evaluated by an Agilent Bioanalyzer 2100 device (Agilent Technologies, Palo Alto, USA). The RNA Integrity Number values ranged from 7.7 to 8.4. First-strand cDNA was synthesized using 1 μg of total RNA as template treated with Superscript II (Invitrogen, Life Technologies, Paisley, UK) and random hexamers in a total volume of 20 μl.

Table 2
Primer design for qPCR, gene details and PCR efficiencies (%) in *Longissimus thoracis* muscle (Eff).

Gene name	Gene symbol	GenBank Acc. number	Forward primer sequence	Reverse primer sequence	Size (bp)	Eff
Adipocyte differentiation						
CCAAT/enhancer binding protein β	CEBPB	AB569088.1	GTGGCGCCGGCAAAACTT	GAGGGGGCAGGAGGAGAGGCAGAG	203	96.8
Peroxisome proliferator-activated receptor γ	PPARG	DQ437884	GGCGAGGGCGATCTTGACAG	GATGCGAATGGCCACCTCTTT	148	99.9
Sterol regulatory binding transcription factor 1	SREBP-1c	AY307771	TTGCGCAAGGCCATCGACTACATC	GTCTACCACCTCCGGCTTCACACC	180	94.7
Retinoic acid signaling and transcriptional control of adipogenesis						
Retinoic acid receptor α	RARA	XM_003131474	TCCGCCGAAGCATCCAGAAGAAC	ACCTCCGGCGTCAGCGTGTAGC	217	92.9
Retinoid X receptor γ	RXRG	NM_001130213	GGGGTTGGCTCCATCTTTGA	ACCTGCCCGGCTGTTCTG	223	95.6
Cellular retinoic acid binding protein 2	CRABP2	NM_001164509	GTACCACGGAGATCAACTTCAA	TGCCGTCATGGTCAGGA	200	91.8
Fatty acids metabolism						
Stearoyl-CoA desaturase	SCD	JN613287	TCCCGACGTGGCTTTTTCTTCTC	CTTCACCCCAGCAATACCAG	205	88.9

Primer pairs used for quantification were designed using Primer Select software (DNASTAR, Wisconsin, USA) from the available GenBank and/or ENSEMBL pig sequences, covering different exons in order to assure the amplification of the cDNA. Sequence of primers and amplicon lengths are indicated in Table 2.

Standard PCRs (polymerase chain reactions) on cDNA were carried out to verify amplicon sizes. Transcript quantification was performed using SYBR Green mix (Roche, Basel, Switzerland) with a LightCycler480 (Roche, Basel, Switzerland) in 384-well reaction plates (Roche, Basel, Switzerland). The qPCR (quantitative polymerase chain reaction) reactions were prepared in a total volume of 20 μl containing 2.5 μl of cDNA (1/20 dilution), 10 μl of SYBR Green mix and 0.15 μM of both forward and reverse primers. As negative controls, mixes without cDNA were used. Cycling conditions were 95 °C for 10 min, followed by 45 cycles of 95 °C (15 s) and 60 °C (1 min) where the fluorescence was acquired. Finally, a dissociation curve to test PCR specificity was generated by one cycle at 95 °C (15 s) followed by 60 °C (20 s) and ramp up to 95 °C with acquired fluorescence during the ramp to 0.01 °C/s. Data were analyzed with LyghtCycler480 SW1.5 software (Roche, Basel, Switzerland). All samples were run in triplicate as technical replicates and dissociation curves were carried out for each individual replicate. Single peaks in the dissociation curves confirmed the specific amplification of the genes. PCR efficiency was estimated by standard curve calculation using four concentrations of cDNA five-fold dilutions from a pool of samples and calculated from the following: $E = 10^{-1/\text{slope}}$. Values of PCR efficiency are indicated in Table 2. Average crossing points (*Cp*) values were employed for the statistical analyses of differential expression. Four commonly used housekeeping genes (*ACTB*, *B2M*, *GAPDH* and *TBP*) were tested with geNorm software (Vandesompele et al., 2002) to evaluate their stability based on the "M" value. *GAPDH* and *ACTB* were selected.

Table 3
Carcass characteristics according to dietary vitamin A treatment and productive phase (early growth or finishing).

		Treatment		RMSE[a]	P-value
		Control[b]	Var[c]		
Carcass weight	Growth[d]	28.24	27.57	2.61	0.5941
	Finishing[e]	122.75	124.41	5.38	0.1555
Ham weight	Growth	3.61	3.50	0.35	0.5032
	Finishing	14.60	13.79	0.85	0.4653
Shoulder weight	Growth	2.39	2.41	0.28	0.8881
	Finishing	8.70	8.72	0.43	0.3979
Backfat thickness (mm)	Growth	16.30	16.00	0.87	0.8340
	Finishing	63.50	62.50	4.46	0.7163
Carcass length (cm)	Finishing	81.30	80.55	1.05	0.4486
Ham length (cm)	Finishing	44.65	43.85	0.75	0.2285
Ham perimeter (cm)	Finishing	77.15	75.60	1.43	0.1759

[a] RMSE = root-mean-square error.
[b] Control = control diet. 10,000 IU vitamin A/kg feed supplementation.
[c] Var = vitamin A restricted diet. 0 IU vitamin A/kg feed supplementation.
[d] Growth = early growth phase; carcass traits measured at 35.8 kg LW.
[e] Finishing = finishing phase; carcass traits measured at 158 kg LW.

2.4. Statistical analysis

Phenotype data were analyzed as a completely randomized design using the general linear model (GLM) procedure contained in the SAS version 9.2 (2010). Dietary treatment was considered as systematic effect, and residual effects as random. The animal was the experimental unit for all data analysis. Live weight and performance data are presented as means ± SD. For statistical analysis of performance parameters, initial weight was used as covariate when it was significant and removed from the model when not.

Statistical analysis of gene expression data was carried out following the method proposed by Steibel, Poletto, Coussens, and Rosa (2009),

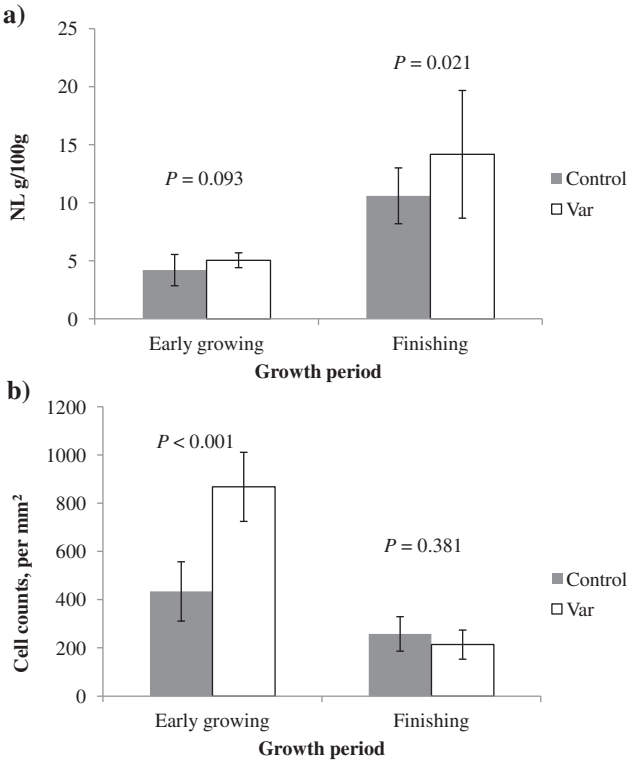

Fig. 1. Effect of vitamin A restriction on a) neutral lipids from *Longissimus thoracis* muscle (g/100 g total fatty acids) and b) preadipocyte number of Iberian pigs (cell counts/mm^2) slaughtered at early growing and finishing. Black bars = control group; pigs received a 10,000 IU vitamin A supplementation/kg diet. Open bars = var group; pigs received a 0 IU vitamin A supplementation/kg diet.

which consists of the analysis of cycles to threshold values (Cp), for the targets and endogenous genes using a linear mixed model. The following model was used for analyzing the joint expression of the target and housekeeping genes in muscle:

$$y_{gijkr} = TG_{gi} + L_{gj} + B_{gjk} + D_{ijk} + e_{gijkr}$$

where $y_{gijkr} = -\log_2\left(E_g^{-Cp_{gijkr}}\right)$, E_g is the efficiency of the PCR of gth gene, Cp_{gijkr} is the value obtained from the thermocycler software for the gth gene from the rth replicate in a sample collected from the kth animal of the jth litter fed with the ith dietary treatment, TG_{gi} is the specific effect of the ith dietary treatment on the expression of gene gth, L_{gj} and B_{gjk} are specific random effects of the jth full-sib family and the kth pig on the expression of gene gth, D_{ijk} is a random sample-specific effect common to all the genes, and e_{gijkr} is a residual effect.

To test differences in the expression rate of genes of interest ($diff_{TG}$) between classes normalized by the housekeeping genes, different comparisons were performed between the respective estimates of TG levels. Significance of $diff_{TG}$ estimates was determined with the t statistic. To obtain fold change (FC) values from the estimated $diff_{TG}$ values, the following equation was applied: $FC = 2^{-diff_{TG}}$. Asymmetric 95% confidence intervals (CI) were calculated for each FC value by using the standard error (SE) of the estimated difference: 95% CI from $2^{[-\mathrm{diff_{TG}}+(1.96\times SE)]}$ to $2^{[-\mathrm{diff_{TG}}-1.96\times \mathrm{SE}]}$.

3. Results

3.1. Carcass traits and performance parameters

Dietary vitamin A restriction had no effect ($P > 0.05$) on the studied carcass traits (carcass weight, carcass length, shoulder and ham weight, ham length and backfat thickness) at early growing or finishing periods, as shown in Table 3. Performance parameters were also evaluated on the overall period; there were no differences on average daily gain (0.52 ± 0.01 and 0.52 ± 0.02 kg/d in control and var animals, respectively; $P = 0.75$), average daily feed intake (2.09 ± 0.9 and 2.08 ± 0.08 kg feed/d in control and var animals, respectively; $P = 0.99$) and feed conversion ratio (4.02 ± 0.18 and 4.01 ± 0.13 kg gain/kg in control and var animals, respectively; $P = 0.99$).

3.2. Neutral lipids and preadipocyte content of intramuscular fat

Neutral lipid content in the IMF did not show any difference at early growing (4.2 ± 1.3 vs 5.1 ± 0.6 g/100 g tissue for control and var groups, respectively; $P = 0.09$). The Iberian pigs from var group showed a higher content in NL than those from the control group at finishing (Fig. 1a) (10.6 ± 2.4 vs 14.2 ± 3.4 g/100 g tissue for control and var groups, respectively; $P = 0.02$).

In an effort to identify changes in the IMF induced by dietary vitamin A level, we conducted immunohistochemical analysis to identify and quantify preadipose cells. The number of preadipocytes showed significant differences between diets at early growing but we did not find

Table 4
Fatty acid composition of *Longissimus thoracis* muscle (g/100 g total fatty acids) at the early growth phase.

	Neutral lipids				Polar lipids			
	Feeding treatments		RMSE[a]	P-value	Feeding treatments		RMSE	P-value
	Control[b]	Var[c]			Control	Var		
C14:1	1.41	1.40	0.09	0.938	2.36	2.31	0.18	0.602
C16:0	24.94	24.25	1.50	0.344	23.34	22.72	0.79	0.117
C16:1 n−9	0.32	0.30	0.06	0.402	0.33	0.33	0.05	0.727
C16:1 n−7	3.59	3.61	0.35	0.914	1.03	0.87	0.14	0.026
C17:0	0.35	0.33	0.04	0.496	0.75	0.72	0.10	0.479
C17:1	0.48	0.48	0.07	0.990	0.88	0.99	0.23	0.332
C18:0	14.16	13.68	0.64	0.131	11.12	10.23	0.87	0.045
C18:1 n−9	40.39	40.80	1.59	0.587	16.67	14.18	2.10	0.023
C18:1 n−7	2.92	2.82	0.31	0.483	2.79	2.78	0.18	0.924
C18:2 n−6	7.65	8.35	1.38	0.296	25.13	27.29	2.17	0.050
C18:3 n−3	0.43	0.48	0.07	0.116	0.89	0.86	0.12	0.688
C18:4 n−3	0.17	0.16	0.02	0.408	0.11	0.11	0.03	0.804
C20:0	0.17	0.18	0.03	0.592	0.65	0.33	0.23	0.008
C20:1 n−9	0.83	0.82	0.07	0.911	0.35	0.27	0.07	0.026
C20:2 n−6	0.40	0.41	0.05	0.691	0.45	0.54	0.11	0.109
C20:3 n−6	0.16	0.17	0.04	0.527	0.93	1.01	0.12	0.196
C20:4 n−6	0.93	1.04	0.31	0.486	7.65	8.79	0.73	0.004
C20:5 n−3	0.05	0.06	0.02	0.079	0.34	0.39	0.04	0.009
C22:1 n−9	0.09	0.07	0.03	0.260	0.10	0.06	0.05	0.085
C22:4 n−6	0.23	0.23	0.05	0.857	1.50	1.68	0.18	0.049
C22:5 n−3	0.14	0.16	0.03	0.190	1.07	1.92	1.08	0.112
C22:6 n−3	0.07	0.05	0.03	0.136	0.48	0.52	0.13	0.558
SFA[d]	39.62	38.45	1.62	0.141	36.55	34.46	1.51	0.010
MUFA[e]	50.02	50.30	1.91	0.761	24.56	21.90	2.03	0.013
PUFA[f]	10.35	11.25	1.81	0.308	38.89	43.63	2.93	0.003
Σn3	0.94	1.00	0.11	0.229	2.94	4.01	1.05	0.046
Σn6	9.42	10.25	1.71	0.317	35.95	39.63	2.67	0.010
Σn6/Σn3	9.96	10.23	0.97	0.565	12.31	10.78	2.30	0.178
C18:1/C18:0	3.06	3.20	0.17	0.115	1.75	1.66	0.15	0.220
MUFA/SFA	1.26	1.31	0.10	0.275	0.67	0.64	0.06	0.165

[a] RMSE = root-mean-square error.
[b] Control = control diet. 10,000 IU vitamin A/kg feed supplementation.
[c] Var = vitamin A restricted diet. 0 IU vitamin A/kg feed supplementation.
[d] SFA (C12:0 + C14:0 + C16:0 + C17:0 + C18:0 + C20:0); sum of saturated fatty acids.
[e] MUFA (C16:1n−9 + C16:1n−7 + C17:1 + C18:1n−9 + C18:1n−7 + C20:1n−9); sum of monounsaturated fatty acids.
[f] PUFA (C18:2n−6 + C18:3n−3 + C20:3n−9 + C20:4n−6); sum of polyunsaturated fatty acids.

Table 5
Fatty acid composition of *Longissimus thoracis* muscle (g/100 g total fatty acids) at finishing.

	Neutral lipids				Polar lipids			
	Feeding treatments		RMSE[a]	P-value	Feeding treatments		RMSE	P-value
	Control[b]	Var[c]			Control	Var		
C14:1	1.57	1.31	0.21	0.014	2.66	2.80	0.49	0.529
C16:0	26.06	24.06	1.10	0.001	19.55	18.67	1.76	0.275
C16:1 n−9	0.17	0.17	0.03	0.752	0.12	0.10	0.04	0.291
C16:1 n−7	4.11	3.98	0.53	0.600	0.82	0.82	0.26	0.950
C17:0	0.14	0.16	0.04	0.465	0.42	0.43	0.05	0.764
C17:1	0.19	0.21	0.04	0.141	1.93	2.24	0.60	0.272
C18:0	11.58	10.61	0.75	0.011	8.92	7.78	2.02	0.222
C18:1 n−9	48.12	51.29	1.34	<0.0001	16.79	16.07	3.44	0.644
C18:1 n−7	3.35	3.41	0.36	0.757	3.27	2.96	0.49	0.175
C18:2 n−6	2.85	3.02	0.53	0.499	27.16	29.57	4.05	0.199
C18:3 n−3	0.17	0.19	0.04	0.294	0.36	0.46	0.04	<0.0001
C18:4 n−3	0.10	0.12	0.01	0.043	0.06	0.06	0.02	0.612
C20:0	0.17	0.15	0.02	0.063	0.40	0.27	0.28	0.318
C20:1 n−9	0.89	0.85	0.11	0.422	0.38	0.34	0.26	0.756
C20:2 n−6	0.19	0.21	0.06	0.497	0.50	0.51	0.06	0.810
C20:3 n−6	0.05	0.04	0.01	0.094	1.05	1.11	0.15	0.407
C20:4 n−6	0.13	0.12	0.02	0.145	11.20	10.93	1.71	0.735
C20:5 n−3	ND[d]	ND	ND	ND	0.35	0.43	0.10	0.109
C22:1 n−9	0.02	0.03	0.01	0.485	0.43	0.40	0.24	0.792
C22:4 n−6	0.05	0.04	0.01	0.107	1.36	1.38	0.23	0.853
C22:5 n−3	0.07	0.06	0.06	0.816	1.15	1.23	0.20	0.413
C22:6 n−3	ND	ND	ND	ND	0.42	0.48	0.18	0.454
SFA[e]	37.96	34.97	1.02	<0.0001	29.72	27.54	3.86	0.223
MUFA[f]	58.44	61.24	1.00	<0.0001	26.16	25.55	2.96	0.650
PUFA[g]	3.60	3.79	0.64	0.537	44.12	46.91	5.98	0.311
Σn3	0.34	0.36	0.07	0.471	2.34	2.66	0.35	0.055
Σn6	3.27	3.43	0.59	0.567	41.27	43.50	5.67	0.390
Σn6/Σn3	9.99	9.50	1.40	0.460	17.72	16.54	2.32	0.270
C18:1/C18:0	4.46	5.18	0.32	<0.0001	2.33	2.48	0.38	0.379
MUFA/SFA	1.54	1.75	0.07	<0.0001	0.89	0.93	0.10	0.339

[a] RMSE = root-mean-square error.
[b] Control = control diet. 10,000 IU vitamin A/kg feed supplementation.
[c] Var = vitamin A restricted diet. 0 IU vitamin A/kg feed supplementation.
[d] ND = not detectable.
[e] SFA (C12:0 + C14:0 + C16:0 + C17:0 + C18:0 + C20:0); sum of saturated fatty acids.
[f] MUFA (C16:1n−9 + C16:1n−7 + C17:1 + C18:1n−9 + C18:1n−7 + C20:1n−9); sum of monounsaturated fatty acids.
[g] PUFA (C18:2n−6 + C18:3n−3 + C20:3n−9 + C20:4n−6); sum of polyunsaturated fatty acids.

any difference at finishing. In young animals the number of preadipose cells was higher in the var group than in the control group (868 ± 143 cells/mm^2 vs 502 ± 211 cells/mm^2, P < 0.001) (Fig. 1b).

3.3. Fatty acids composition of Longissimus thoracis muscle

Fatty acid profiles were analyzed in LT muscle samples at early growing and finishing (Tables 4 and 5, respectively). At the end of the early growth period, no effect of dietary treatment was observed in NL, but PL in control group showed higher C16:1 n−7, C18:0, C18:1 n−9, C20:0, C20:1 n−9, SFA and MUFA, and a lower C18:2 n−6, C20:4 n−6, C20:5 n−3, C22:4 n−6, PUFA, ∑n−6 and ∑n−3 concentrations than those fed the var diet.

Regarding the fatty acid composition in the finishing period, main differences were observed in the NL fraction. The var animals showed lower SFA and higher MUFA concentrations, mainly due to lower C16:0 and C18:0 acids and higher C18:1 n−9 content, respectively. Pigs fed the var diet showed also higher C18:4 n−3 and lower C14:1. In the PL fraction, only a higher C18:3 n−3 content and a trend (P = 0.06) for higher n−3 fatty acids were observed in the var group.

No effects of vitamin A restriction were observed on desaturase index (C18:1/C18:0 and MUFA/SFA) at the end of the early growth period in the NL or PL fractions. However we found higher desaturation index in the NL of IMF at finishing in Iberian pigs fed the var diet (P < 0.0001).

3.4. Expression of candidate genes

Fig. 2 shows relative gene expression values of both dietary treatments in LT muscle. *CRABPII* gene expression tended to be higher in control than in the var group (P = 0.099), with 1.85-fold increase.

However, no differences for *PPARG*, *RARA*, *RXRG*, *SCD*, *SREBP* or *CEBPB* genes expression were observed.

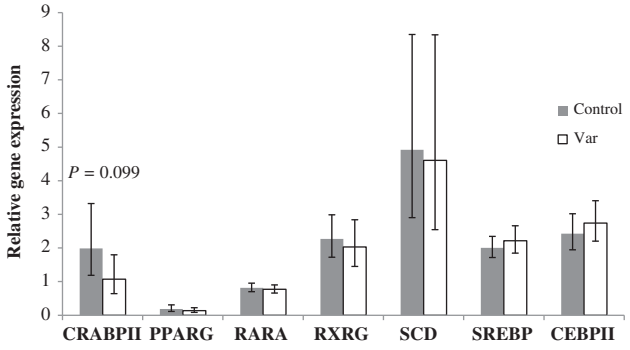

Fig. 2. Relative expression values of candidate genes of control and var groups in *Longissimus thoracis* muscle at early growing. CRABP2: Cellular retinoic acid binding protein 2. PPARG: Peroxisome proliferator-activated receptor γ. RARA: Retinoic acid receptor α. RXRG: Retinoid X receptor γ. SCD: Stearoyl-CoA desaturase. SREBP-1c: Sterol regulatory binding transcription factor 1. CEBPB: CCAAT/enhancerbinding protein β.

4. Discussion

4.1. Carcass traits and performance parameters

Vitamin A restriction did not affect carcass characteristics at either early growing or finishing periods, in agreement with Olivares et al. (2009a,b) and D'Souza, Pethick, Dunshea, Pluske, and Mullan (2003). It is noteworthy that vitamin A restriction applied from so early at growth stages did not affect growth and body development, whereas in the study of Olivares et al. (2009a), vitamin A was restricted only during the last 5 weeks of life, in the fattening phase.

4.2. Preadipocyte and Neutral lipid content of intramuscular fat

To our knowledge, this is the first study reporting the preadipocyte number in LT muscle; a large number of preadipocytes were observed in muscle tissue at growing, and their number decreased in both treatments over time. Adipocyte differentiation is an extremely complex process and meat-animal-derived preadipocytes and adipocytes studies have shown that conversion and differentiation processes are not necessarily the same as those observed in rodent systems or in cell lines (Hausman, Basu, Du, Fernyhough-Culver, & Dodson, 2014).

Lipid accumulation is the result of both hyperplasia and hypertrophy. Hyperplasia leads to an increase in adipose cell number during growth stages that may be related to higher IMF content in adult animals (Hausman et al., 2014). An effect of dietary vitamin A level on hyperplasia ability was observed in this study in Iberian pigs at early growing; samples from LT muscle showed significantly more preadipocytes in the var group ($P < 0.001$). However, hypertrophy is a posterior event and no effect on NL content was observed at that early stage, although we found higher NL in var group at finishing. Neutral lipid fraction represents 60–70% of total fatty acids in the IMF (Tous et al., 2013) and is mainly composed by reserve lipids and thus, it can be used as an estimator of IMF. The increase in NL content at 158 ± 7 kg LW may be a consequence of a higher potential for lipid deposition in the var group (Fig. 1a). The presence of a higher number of preadipocytes in early growing pigs can indicate greater potential to differentiate and accumulate lipids over the experimental period, since IMF accumulation is dependent on the increase in the number of adipocyte differentiations from preadipocytes. This fact is an important regulatory step in the deposition of marbling; thus, higher number of preadipose cell counts (associated with higher potential for lipid deposition) in muscle tissue during growth stages may be related to higher IMF content in adult animals, as reported by Hausman et al. (2014). This is consistent with the higher NL content observed in de var pigs at finishing.

In pigs, the research from Quiniou, Richard, Mourot, and Etienne (2008) showed that preadipocyte and adipocyte cells may change depending on dietary fat in the sow diets at an early age (10.2 kg LW) in subcutaneous adipose tissue and in the IMF without affecting slaughter weight or carcass lean meat content.

It has been reported that vitamin A restriction may influence specifically the IMF cell number (the cellularity of the IMF) without influencing the subcutaneous depot in steers (Gorocica-Buenfil, Fluharty, Reynolds, & Loerch, 2007). In agreement with these authors, backfat thickness was not affected by long-term vitamin A restriction in our experiment. On the other hand, the effect of vitamin A level on IMF content is still controversial. In accordance to our results, Olivares et al. (2011), in a study in lean pigs from 55.8 kg LW to 125 kg LW (approx. 11 weeks of restriction) found that pigs fed low dietary vitamin A levels (1300 IU) had a higher IMF content in LT muscle when compared with a control group fed 13,000 IU, 10-fold higher than the standard recommended level (NRC, 1998). However the same authors in a previous study with high dietary vitamin A supplementation (100,000 IU vs. 0 IU) found different responses to vitamin A supplementation depending on the genotype (Olivares et al., 2009b). In Duroc pigs, these authors found a 20% increase in the IMF content of LT muscle in those animals fed the 100,000 IU vitamin A enriched diet, while no effect was observed in Landrace × Large White for a restriction time of 8 weeks and 114.5 kg LW at slaughter. In our study the restriction time for Iberian pigs was two months at 35.8 ± 3.1 kg LW (early growing) and nine months for the pigs slaughtered at 158 ± 7 kg LW (finishing). According to these results, the effect of dietary vitamin A on IMF content might depend on restriction or supplementation duration, age at the beginning of the restriction or supplementation treatment and pig genotype. Thus, further studies are needed to determine the effect of different dietary vitamin A restriction levels and times on pig breeds of interest. Similarly, the results obtained in ruminant animals are not consistent (Gorocica-Buenfil et al., 2007; Siebert et al., 2006).

4.3. Fatty acids concentration of Longissimus thoracis muscle

The higher PUFA content observed at early growing could be related to a preferential mitochondrial transport and beta-oxidation for polyunsaturated rather than for saturated fatty acids in Iberian pigs fed the vitamin A supplemented diet. There is a lack of information on the influence of the dietary vitamin A inclusion level on enzyme activities. Sanz, Lopez-Bote, Menoyo, and Bautista (2000) and Shimomura, Tamura, and Suzuki (1990) observed that dietary saturated fat decreases beta-oxidation but the effect of other nutrients on beta-oxidation remains unclear.

Siebert et al. (2006) found that the desaturation index was inversely related to plasma vitamin A levels in Angus steers. Jeyakumar, Vajreswari, and Giridharan (2008) found that feeding a high level of vitamin A led to an increase of SCD activity in lean rats, but this effect was not observed in obese rats. Olivares et al. (2011) reported a decrease in the MUFA/SFA ratio in backfat but not in the IMF in pigs fed a diet with 1300 IU vitamin A for eleven weeks prior to slaughter. These results are not in agreement with those obtained in the present experiment since a higher desaturation index (MUFA/SFA) was observed in the NL of IMF from pigs fed the var diet at finishing. However it should be noticed that in the present experiment the animals were subjected to vitamin A restriction for a total of nine months, and they were of an obese genotype, whereas in the experiment of Olivares et al. (2011), vitamin A was restricted for just 11 weeks and the animals were of a lean genetic line (Large White × Landrace). This suggests that the effect of vitamin A on fatty acids profile might be dependent on vitamin A inclusion levels, genotype and length of experimental period, in accordance to the results obtained in LN fraction content.

4.4. Expression of candidate genes

To understand the role of vitamin A in preadipocyte differentiation, a gene expression analysis was carried out. Retinoic acid is well known as a potent transcription regulator, and some genes regulated by RA are involved in adipocyte differentiation and RA signaling, like nuclear receptors or binding proteins (Bonet et al., 2003; Noy, 2013). We investigated gene expression in young (35.8 kg) pigs, when genes related to adipocyte differentiation are expected to be more active and thus may have a greater response potential. However, we found no difference in gene expression in this stage. A possible reason of this lack of changes in gene expression after two months of treatment may be because the effects of dietary vitamin A restriction need a bit longer treatment to be detectable. Vitamin A is a liposoluble vitamin that accumulates in body tissues and thus body depots depletion is needed prior to changes in retinol homeostasis. A trend was observed for greater expression of the *CRABPII* gene in the control group. This gene codes for an intracellular protein that binds RA in the cytoplasm and transports it into the nucleus, thus, increasing its binding into nuclear receptors (Dong, Ruuska, Levinthal, & Noy, 1999). Previous studies have shown the influence of this protein in adipocyte development. Berry, Soltanian, and Noy (2010) confirmed that downregulation of *CRABPII* is a critical component in the differentiation process and that this protein markedly sensitizes preadipocytes to RA-induced inhibition of adipogenesis. Thus,

our finding may suggest that adipogenesis is a more active process in the var group; this fact together with an increased number of preadipose cells in this group may lead to a greater development of the fat compartment in adult animals, as shown in the vitamin A restricted animals at finishing.

5. Conclusion and implications

Decreasing added vitamin A in feed from two to four months of age caused an increase in the number of preadipocytes and suggestive downregulates the *CRABPII* gene. In addition, vitamin A restriction for 9 months caused a pronounced increase of total NL, monounsaturated fatty acids and a decrease of saturated fatty acids in NL fraction of IMF. This change in fatty acids profile has a positive effect on costumer's health.

This approach is an easy and no cost-increasing strategy to increase the lipid content in muscle of Iberian pigs with no detrimental effects on carcass traits. The greater lipid content would lead to an increase in meat quality, which is highly appreciated in meat products obtained from this breed. However, further studies on different added dietary vitamin A level (0, NRC recommended levels, commercial levels) would improve the knowledge about this topic.

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