British Journal of Nutrition (2016), **115**, 937–950
doi:10.1017/S0007114515005218
© The Authors 2016

Influence of betaine and arginine supplementation of reduced protein diets on fatty acid composition and gene expression in the muscle and subcutaneous adipose tissue of cross-bred pigs

Marta S. Madeira[1], Eva A. Rolo[1], Cristina M. Alfaia[1], Virgínia R. Pires[1], Richard Luxton[2], Olena Doran[2], Rui J. B. Bessa[1,3] and José A. M. Prates[1]*

[1]*Centro de Investigação Interdisciplinar em Sanidade Animal (CIISA), Faculdade de Medicina Veterinária, Universidade de Lisboa, Avenida da Universidade Técnica, Alto da Ajuda, 1300-477 Lisboa, Portugal*

[2]*Centre for Research in Biosciences, Faculty of Health and Life Sciences, University of the West of England, Coldharbour Lane, Bristol BS16 1QY, UK*

[3]*Unidade de Investigação em Produção Animal (UEISPA), Instituto Nacional de Investigação Agrária e Veterinária, Fonte Boa, 2005-048 Vale de Santarém, Portugal*

(Submitted 2 July 2015 – Final revision received 19 November 2015 – Accepted 7 December 2015 – First published online 28 January 2016)

Abstract

The isolated or combined effects of betaine and arginine supplementation of reduced protein diets (RPD) on fat content, fatty acid composition and mRNA levels of genes controlling lipid metabolism in pig *m. longissimus lumborum* and subcutaneous adipose tissue (SAT) were assessed. The experiment was performed on forty intact male pigs (Duroc × Large White × Landrace cross-breed) with initial and final live weights of 60 and 93 kg, respectively. Pigs were randomly assigned to one of the following five diets (*n* 8): 16·0 % of crude protein (control), 13·0 % of crude protein (RPD), RPD supplemented with 0·33 % of betaine, RPD supplemented with 1·5 % of arginine and RPD supplemented with 0·33 % of betaine and 1·5 % of arginine. Data confirmed that RPD increase intramuscular fat (IMF) content and total fat content in SAT. The increased total fat content in SAT was accompanied by higher GLUT type 4, lipoprotein lipase and stearoyl-CoA desaturase mRNA expression levels. In addition, the supplementation of RPD with betaine and/or arginine did not affect either IMF or total fat in SAT. However, dietary betaine supplementation slightly affected fatty acid composition in both muscle and SAT. This effect was associated with an increase of carnitine *O*-acetyltransferase mRNA levels in SAT but not in muscle, which suggests that betaine might be involved in the differential regulation of some key genes of lipid metabolism in pig muscle and SAT. Although the arginine-supplemented diet decreased the mRNA expression level of *PPARG* in muscle and SAT, it did not influence fat content or fatty acid composition in any of these pig tissues.

Key words: Betaine: Arginine: Reduced protein diets: Lipid metabolism: Intramuscular fat: Fatty acid composition: Pigs

Pork is the most consumed meat in European Union countries, with 22 358 000 tonnes of pig carcass produced in 2014[1]. Owing to the genetic selection towards reduced subcutaneous fat, the amount of intramuscular fat (IMF) in commercial cross-bred pigs has also been decreased[2]. It was proposed that acceptable pork eating quality requires a minimum IMF of 2·5 %[3]. However, according to Daszkiewicz *et al.*[4] about 84 % of the carcass from commercial pigs have a IMF content below the level required for acceptable eating quality. In addition, fatty acid composition plays an important role in the eating quality and nutritional value of meat. Thus, one of the main goals of the meat industry is to improve fat partitioning – namely, the production of pork with higher amounts of IMF and a balanced fatty acid composition – without an increase in subcutaneous fat.

In pigs, fat partitioning can be improved by using different feeding strategies. These strategies are mainly based on the manipulation of dietary amino acid supplementation and reduction of the dietary protein content (reduced protein diets (RPD))[5,6]. Betaine, or trimethylglycine, is a metabolic product present in plant and animal tissues. Its acts as an organic osmoprotectant or as a methyl donor, which may partially reduce the requirements for other methyl donors during lipid metabolism[7].

Abbreviations: *ACACA*, acetyl-CoA carboxylase α; cDNA, complementary DNA; *CPT-1B*, carnitine palmitoyltransferase 1B; *CRAT*, carnitine *O*-acetyltransferase; *FABP4*, fatty acid binding protein 4, adipocyte; *FADS1*, fatty acid desaturase 1; *FADS2*, fatty acid desaturase 2; FAME, fatty acid methyl esters; *FASN*, fatty acid synthase; *GLUT4*, solute carrier family 2, facilitated GLUT member 4; IMF, intramuscular fat; *LPL*, lipoprotein lipase; *MLXIPL*, MLX interacting protein-like; RPA, reduced protein diet with arginine; RPB, reduced protein diet with betaine; RPBA, reduction protein diet with betaine and arginine; RPD, reduced protein diets; RPLP0, ribosomal protein large P0; SAT, subcutaneous adipose tissue; *SCD*, stearoyl-CoA desaturase.

* **Corresponding author:** J. A. M. Prates, fax +351 213 652 895, email japrates@fmv.ulisboa.pt

British Journal of Nutrition

https://doi.org/10.1017/S0007114515005218 Published online by Cambridge University Press

In fact, dietary betaine supplementation may decrease the requirements for other methyl donors such as methionine and choline[8]. It has been demonstrated that dietary betaine supplementation in pigs may repress overall fat deposition[9]. As reported by Huang *et al.*[9,10], the addition of betaine to the diet of growing–finishing pigs results in decreased carcass fat deposition by increasing the rate of lipolysis and/or decreasing the rate of lipogenesis. Moreover, arginine is a semi-essential amino acid that, in addition to playing multiple physiological functions in animals, enhances lipolysis through the expression of key genes responsible for the activation of fatty acid oxidation in a tissue-specific manner[11,12]. Previous studies have suggested that dietary arginine supplementation to growing–finishing pigs increased the IMF content, and thus improved fat partitioning[12,13]. However, we have recently showed that dietary arginine supplementation, either alone or in combination with RPD, does not increase IMF content or change the fatty acid composition in pigs[14]. In addition to the use of dietary amino acid supplementation, the use of RPD for increasing IMF content in pigs with less effect on subcutaneous fat deposition has also been reported[5,15]. Although the mechanisms of the tissue-specific effects of RPD are not clear[5], one possibility might be a dietary-stimulated increase in stearoyl-CoA desaturase (SCD) activity in pig muscle but not in subcutaneous adipose tissue (SAT)[5]. Moreover, recent results from our research group[15] showed the existence of breed-specific effects of fat deposition promoted by RPD in pigs, with increased IMF content in lean pig genotypes but not in fat ones.

The main site for *de novo* fatty acid biosynthesis and lipogenesis in pig is the white adipose tissue[16]. In contrast, muscle is one of the tissues playing the main role in the metabolism of glucose and degradation of lipids[17]. The mechanisms that regulate adipogenesis and lipogenesis are controlled by a range of key transcription factors including sterol regulatory element binding protein 1 (SREBP1), CCAAT/enhancer binding protein α (CEBPA) and PPARG[18]. In addition, MLX interacting protein-like (MLXIPL) is a critical glucose-responsive transcription factor that regulates lipogenic and glycolytic genes, highly controlled by the insulin-regulated solute carrier family 2, facilitated GLUT member 4 (GLUT4) in adipose tissue[19]. Furthermore, the MLXIPL also regulates various enzymes involved in glycolysis and lipogenesis, such as acetyl-CoA carboxylase (ACACA) and fatty acid synthase (FASN)[20]. ACACA[21] and FASN[22] are the lipogenic enzymes controlling the rates of SFA biosynthesis, and SCD catalyses the rate-limiting step of MUFA biosynthesis. Fatty acid desaturase 1 (*FADS1*) and desaturase 2 (*FADS2*) genes encode for $\Delta 5$ and $\Delta 6$ desaturases, respectively, which are membrane-bound enzymes that catalyse the synthesis of PUFA[23]. Moreover, carnitine palmitoyltransferase 1B (CPT-1B) and carnitine *O*-acetyltransferase (CRAT) are the rate-limiting enzymes of lipid catabolism and are responsible for the transport of fatty acid esters from the cytosol to the mitochondria for β-oxidation[18]. PPARA is involved in fatty acid oxidation by up-regulating the expressions of acyl-CoA oxidase and carnitine palmitoyltransferase enzymes[24]. Lipoprotein lipase (LPL) is the rate-limiting enzyme in the conversion of chylomicrons and VLDL into chylomicron remnants and LDL in tissues. Therefore, LPL controls TAG partitioning between adipose tissue and muscle, thereby increasing fattening or providing energy in the form of fatty acids for muscle growth[25]. Finally, fatty acid binding protein 4 (FABP4) is responsible for fatty acid transport in the adipocytes[26]. It remains unclear whether and how these processes contribute to the mechanisms controlling dietary regulation of fat partitioning in pigs.

We have recently shown that adipogenesis and lipogenesis are regulated differently in the muscle and SAT of commercial cross-bred pigs[14]. In addition, it was suggested that increased IMF promoted by RPD is due to lysine restriction, and it is mediated by the up-regulation of both the adipogenic transcription factor *PPARG* and the lipogenic enzyme *SCD*. Moreover, the supplementation of RPD with leucine seems to be interesting to increase MUFA content in pork[14]. Thus, in order to assess the influence of new feeding strategies, we tested the following hypotheses: (1) RPD supplemented with betaine, arginine or both improve fat partitioning and fatty acid composition in commercial cross-bred pigs; and (2) the tissue-specific effect of betaine and/or arginine supplementation of RPD is mediated via the expressions of key genes controlling lipid metabolism. The general aim of this study was, therefore, to assess whether the increased IMF content induced by RPD in the growing–finishing phase of commercial cross-bred pigs could be modulated by dietary supplementation of betaine and arginine, or both (to assess additive/interactive effects), without major undesirable increases in SAT.

Methods

Animals and diets

The trial was conducted at the facilities of Unidade de Investigação em Produção Animal (Instituto Nacional de Investigação Agrária e Veterinária (UEISPA-INIAV)), and all the experimental procedures involving animals were reviewed by the Ethics Commission of the Centro de Investigação Interdisciplinar em Sanidade Animal/Faculdade de Medicina Veterinária and approved by the Animal Care Committee of the National Veterinary Authority (Direcção-Geral de Alimentação e Veterinária), following the appropriated European Union guidelines (2010/63/EU Directive). All the staff members involved in animal trials had licence for conducting experiments on live animals from the Portuguese Veterinary Services.

In all, forty commercial cross-bred (50 % Duroc, 25 % Large White and 25 % Landrace) entire male pigs with an initial body weight of 59·9 (SD 1·65) kg were used. Animals were fed a standard commercial concentrate diet from weaning until the beginning of the experiment. The forty animals were randomly allocated to ten pens and the five dietary treatments were randomly allocated to each animal, according to an incomplete balanced block design. The five diets were isoenergetically formulated (13·3 MJ metabolisable energy/kg) and differed in crude protein, betaine and arginine contents as follows: 16·0 % of crude protein (normal protein diet, control); 13·0 % of crude protein (reduced protein diet, RP); 13·0 % of crude protein with 0·33 % betaine supplementation (reduced protein diet with betaine, RPB), 13·0 % of crude protein with 1·5 % arginine supplementation (reduced protein diet with arginine, RPA) and

British Journal of Nutrition

https://doi.org/10.1017/S0007114515005218 Published online by Cambridge University Press

13·0 % of crude protein with 0·33 % betaine and 1·5 % arginine supplementation (reduction protein diet with betaine and arginine, RPBA). The amino acids were obtained from Fh Diedrichs & Ludwig Post. The ingredients, chemical composition and fatty acid profile of the experimental diets are shown in Table 1. During the experiment, the animals were fed individually twice a day and had access to water *ad libitum*. Feed offered and refusals were recorded daily in order to calculate feed intake. Pigs were weighed weekly, just before feeding, throughout the experiment.

Slaughter and sampling

Feed was removed 17–19 h before the animals were slaughtered. Pigs were slaughtered at an average live body weight of 92·7 (SD 2·54) kg, with no significant differences ($P > 0·05$) between animal groups, at the UEISPA Experimental Abattoir. Immediately after electrical stunning and exsanguination, samples of *m. longissimus lumborum* and SAT were collected from the right side of the carcass at the first lumbar vertebra level for gene expression analysis. The samples were rinsed with sterile RNAse-free cold saline solution, cut into small pieces (approximately 0·3-cm thick), stabilised in RNA Later solution (Qiagen) and stored at −80°C until analysed. For analysis of IMF and fatty acid composition, *m. longissimus lumborum* and SAT samples were collected after slaughter from the right carcass side between the third and fifth lumbar vertebrae. Muscle samples were collected and trimmed of visible connective and adipose tissues before being blended in a food processor. The samples of muscle and SAT were vacuum packed and stored at −20°C until analysed. Backfat thickness was measured in left carcass side, at the P_2 (last rib position) location, using a Vernier calliper (Bochem Lab Supply).

Feed analysis

Feed samples, collected five times during the trial (the first collection was in the beginning of the trial, followed by regular collections with a 3-week interval until the slaughter) were analysed for DM by drying a sample at 100°C to a constant weight. N content was determined by the Kjeldahl method[27], and crude protein was calculated as 6·25 × N. Crude fibre was determined by the procedure described by the Association of Official Analytical Chemists (AOAC)[27]. The samples were extracted with petroleum diethyl ether, using an automatic Soxhlet extractor (Gerhardt Analytical Systems), and crude fat was determined. Analysis of ash and starch contents was carried out according to the procedures described by the AOAC[27] and Clegg[28], respectively. Gross energy in the feed was determined by adiabatic bomb calorimetry (Parr 1261; Parr Instrument Company). Fatty acid methyl esters (FAME) of the feed samples were analysed by one-step extraction and transesterification, using heptadecanoic acid (17 : 0) as the internal standard[29]. Total amino acids were extracted from feed according to the method described by the AOAC[30]. The extract was analysed by HPLC (Agilent 1100; Agilent Technologies) to quantify total amino acids in the feed, following the procedure described by Henderson *et al.*[31].

Intramuscular fat and fatty acid composition

The *m. longissimus lumborum* and SAT samples were lyophilised (−60°C and 2·0 hPa) to constant weight using a lyophilisator (Edwards High Vacuum International), maintained dry at −20°C

Table 1. Ingredients and chemical, amino acid and fatty acid compositions of the experimental diets

	Control	RP	RPB	RPA	RPBA
Ingredients (%)					
Maize	55·0	55·0	55·0	55·0	55·0
Soyabean meal	19·0	10·7	11·4	3·90	4·05
Barley	10·0	15·9	16·0	25·5	24·9
Wheat	6·92	10·0	10·0	10·0	10·0
Sunflower meal	5·27	4·79	3·65	–	–
Soyabean oil	0·95	0·94	0·94	1·01	1·16
Calcium carbonate	0·89	0·89	0·89	0·90	0·90
Bicalcium phosphate	0·49	0·58	0·58	0·70	0·70
Salt	0·40	0·40	0·35	0·39	0·41
Vitamin–trace mineral premix	0·40	0·40	0·40	0·40	0·40
Mould inhibitor mixture	0·10	0·10	0·10	0·10	0·10
Fermentation products	0·10	0·10	0·10	0·10	0·10
Phytase mixture	0·10	0·10	0·10	0·10	0·10
Acid mixture	0·05	0·05	0·05	0·05	0·05
Antioxidant mixture	0·01	0·01	0·01	0·01	0·01
Sodium bicarbonate	–	–	0·08	0·02	–
L-Lys	0·25	0·07	0·06	0·27	0·27
L-Thr	0·04	–	–	0·05	0·05
DL-Met	0·02	–	–	–	–
L-Trp	–	–	–	0·01	0·01
Betaine HCl	–	–	0·33	–	0·33
L-Arg	–	–	–	1·50	1·50
Chemical composition (% diet)					
DM	88·5	88·5	88·6	88·8	88·7
Crude protein	16·1	13·1	13·0	12·8	13·0
Starch	45·0	49·3	48·4	47·6	49·9
Crude fat	3·17	3·35	3·35	3·85	3·97
Crude fibre	4·20	3·93	4·12	2·60	2·77
Ash	4·21	3·85	3·64	3·60	3·64
Ca	0·77	0·61	0·70	0·73	0·67
P	0·42	0·40	0·41	0·41	0·39
ME (MJ ME/kg)	13·3	13·6	13·3	13·2	13·2
Amino acid composition (% diet)					
Ala	0·39	0·36	0·35	0·28	0·28
Arg	0·53	0·39	0·44	1·05	1·15
Asp	0·68	0·43	0·52	0·35	0·34
Glu	1·17	0·89	1·01	0·81	0·79
Gly	0·32	0·37	0·27	0·20	0·20
His	0·23	0·15	0·21	0·16	0·17
Ile	0·26	0·17	0·24	0·17	0·17
Leu	0·64	0·56	0·56	0·45	0·45
Lys	0·51	0·35	0·35	0·30	0·33
Met	0·04	0·01	0·02	0·03	0·02
Phe	0·33	0·23	0·28	0·21	0·21
Pro	0·65	0·61	0·59	0·55	0·53
Ser	0·36	0·32	0·28	0·21	0·21
Thr	0·22	0·14	0·17	0·15	0·15
Tyr	0·24	0·15	0·19	0·15	0·14
Val	0·26	0·18	0·25	0·20	0·21
Fatty acid composition (% total fatty acids)					
16 : 0	20·4	16·1	15·5	14·2	14·1
18 : 0	4·52	3·27	3·22	2·77	2·79
18 : 1*c*9	32·4	28·5	27·9	26·2	26·1
18 : 1*c*11	1·21	1·03	0·99	0·91	0·92
18 : 2*n*-6	39·4	48·3	49·4	52·7	52·7
18 : 3*n*-3	2·04	2·75	2·88	3·24	3·27

Control, normal protein diet; RP, reduced protein diet; RPB, reduced protein diet with betaine addition; RPA, reduced protein diet with arginine addition; RPBA, reduced protein diet with betaine and arginine addition; ME, metabolisable energy.

British Journal of Nutrition

https://doi.org/10.1017/S0007114515005218 Published online by Cambridge University Press

and analysed within 2 weeks. The total fat content of the muscle samples (IMF) and SAT was determined using fresh samples by hydrolysis with 4 M-HCl, followed by Soxhlet extraction for 6 h with petroleum diethyl ether[27]. For fatty acid analysis of *m. longissimus lumborum* and SAT samples, FAME were extracted from the lyophilised samples (approximately 250 and 50 mg, respectively), according to the method described by Folch *et al.*[32], using dichloromethane–methanol (2:1, v/v) instead of chloroform–methanol (2:1, v/v), as described by Carlson[33]. All the extraction solvents contained 0·01 % butylated hydroxytoluene as an antioxidant. Fatty acids were converted to methyl esters by a combined transesterification procedure with NaOH in anhydrous methanol (0·5 M), followed by HCl–methanol (1:1, v/v), at 50°C for 30 and 10 min, respectively, as described by Raes *et al.*[34]

Quantification of FAME in muscle and SAT was performed using a GC HP7890A (Hewlett-Packard), equipped with a flame ionisation detector and a Supelcowax™ 10 capillary column (30 m × 0·20 mm i.d., 0·20-μm film thickness; Supelco). The column temperature of 150°C was held for 11 min, then increased to 210°C at a rate of 3°C/min and maintained for 30 min. He was used as the carrier gas at a flow rate of 1·3 ml/min, the split ratio was 1:20 and 1 μl of the sample was injected. The injector and detector temperatures were 250 and 280°C, respectively. The quantification of total FAME was carried out using nonadecanoic acid (19 : 0) as the internal standard. Results for each fatty acid were expressed as a percentage of the sum of detected fatty acids (% total fatty acids).

RNA isolation and complementary DNA synthesis

Total RNA from *m. longissimus lumborum* and SAT samples was isolated using QIAzol® Lysis Reagent (Qiagen) and purified with RNeasy® Lipid Mini Kit (Qiagen). All the procedures were performed in accordance with the manufacturer's protocols, and all RNA were subjected to an on-column DNAse I (Qiagen) treatment to remove any contamination with genomic DNA. RNA concentration was determined by analysis of absorbance at 260 nm using a NanoDrop ND-2000c spectrophotometer (Nanodrop; Thermo Fisher Scientific). The A260/280 ratios were between 1·9 and 2·1, and RNA integrity was evaluated by electrophoresis using 1·5 % agarose gel and ethidium bromide staining (1·25 ng/μl; Sigma-Aldrich); 750 ng of total RNA was reversed-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems), based on the use of both oligodT and random hexamers as primers, following the manufacturer's protocol as was previously described by Madeira *et al.*[14]. Control reactions were carried out in the absence of RT in order to check for DNA contamination. Complementary DNA (cDNA) quality was tested by end-point PCR, amplifying all the housekeeping and target genes used in this study. The obtained cDNA was divided into aliquots and stored at −20°C until further analysis.

Real-time quantitative PCR

Genes used in the present study were selected based on their role in the transcriptional control of adipogenesis regulation/differentiation (*MLXIPL*, *PPARG*, *SREBP1*, *CEBPA*), regulation of lipogenesis (*ACACA*, *FASN*, *FADS1*, *FADS2*, *SCD*), glucose uptake (*GLUT4*), fatty acid uptake (*LPL*) and lipid oxidation (*CRAT*, *CPT-1B*, *PPARA*) (Table 2). Gene-specific intron-spanning primers were designed with the aid of Primer3 (http://frodo/wi.mit.edu/primer3/) and Primer Express® 2.0 software (Applied Biosystems), based on *Sus scrofa* sequences (www.ncbi.nlm.nih.gov), to generate amplicons ranging in size from 71 to 145 bp. Sequences of primers, GenBank accession numbers, amplicon length and span exons for PCR products are provided in Table 2. Primers were synthesised commercially by NZYTech. Sequence homology searches against the database of GenBank showed that these primers were specific to the sequence to which they were designed. In order to test the primers and verify the amplified products, a conventional PCR was carried out for all the genes investigated in this study before performing the real-time quantitative PCR experiments. In brief, genes were amplified by conventional qualitative PCR (using 1 μl of cDNA) with the same primers that were designed for real-time PCR. PCR products were extracted from gels using QIAquick® Gel Extraction Kits (Qiagen). The fragments were then cloned into the pGEM®-Teasy cloning vector (Promega), transformed into pMOS Blue *Escherichia coli* and selected on Luria-Bertani (LB) agar plates containing ampicillin (50 μg/ml). Plasmids containing inserts of the right size were sequenced by Stab Vida, and homology searches were performed using Blast (www.ncbi.nlm.nih.gov/blast) to confirm the identity of the amplified fragments. The PCR efficiency was calculated for each amplicon, in triplicate, using StepOnePlus™ PCR System software (Applied Biosystems), by amplifying 5-fold serial dilutions of pooled cDNA. All primer sets exhibited an efficiency ranging between 90 and 110 %, and the correlation coefficients were higher than 0·99.

The gene expression profiles of the five candidate references genes (glyceraldehyde-3-phosphate dehydrogenase, 60S ribosomal protein L27 (*RPL27*), ornithine decarboxylase antizyme 1, ribosomal protein large P0 (*RPLP0*) and 40S ribosomal protein S29 (*RPS29*)) were analysed in twenty-four randomly selected different samples (four pigs from each group). The geNorm algorithm[35] and NormFinder algorithm[36] were used to evaluate their stability in all the samples. *RPLP0* and *RPS29* were identified as the most stable pair of endogenous control genes for normalisation of results in the *m. longissimus lumborum*, whereas *RPLP0* and *RPL27* genes were identified as the most stable pair for SAT. Quantitative real-time PCR reactions were carried out using MicroAmp® Optical 96-well plates (Applied Biosystems) in a StepOnePlus™ thermocycler (Applied Biosystems) in standard cycling conditions. Measurements of each sample for each gene were conducted in duplicate; 12·5 μl of PCR reaction mixtures contained 6·25 μl of 2 × Power SYBR® Green PCR Master Mix (Applied Biosystems), 160 nM of gene-specific forward and reverse primers and 1·5 μl of diluted cDNA as a template. Controls included no template cDNA to monitor contamination and primer dimer formation and a minus RT sample to check for genomic DNA contamination. A melting curve analysis was performed after the final cycle to ensure specificity of primer and absence of primer dimer formation. The relative amount of each target gene was

British Journal of Nutrition

https://doi.org/10.1017/S0007114515005218 Published online by Cambridge University Press

British Journal of Nutrition

Table 2. Characterisation of the selected genes used in the real-time quantitative PCR assay

	Full gene name	GenBank accession number	Forward primer	Reverse primer	Product size (bp)	Spanned coding exons
Gene symbols						
ACACA	Acetyl-coenzyme A carboxylase α	NM_001114269	ggccatcaaggacttcaacc	acgatgtaagcgccgaactt	120	46–47
CEBPA	CCAAT/enhancer binding protein α (C/EBP)	XM_003127015	ggccagcacacacacattaga	cccccaaagaagagaaccaag	71	1
CPT-1B	CPT-1B carnitine palmitoyltransferase 1B	NM_001007191.1	cagatggagcggatgttcaa	gagccctcgtagagccacac	133	8–9
CRAT	Carnitine acetyltransferase	NM_001113047	ggcccaccgagcctacac	atggcgatggcgtaggag	138	12–13
FABP4	Fatty acid binding protein 4, adipocyte	NM_001002817	gggccaggaatttgatgaag	ctttccatcccacttctgcac	103	2–3
FASN	Fatty acid synthase	NM_001099930	acaccttcgtgctggcctac	atgtcggtgaactgctgcac	112	40–41
FADS1	Fatty acid desaturase 1	NM_001113041.1	ccactgttggggctgaagg	gatgtgcatggggatgtggt	108	8–9
FADS2	Fatty acid desaturase 2	NM_001171750.1	gccttacaaccaccagcatga	aggccaagtccacccagtc	122	6–7
GLUT4	SLC2A4 solute carrier family 2, member 4	NM_001128433	gctgcctcctacgagatgct	tggccagctggttgagtgt	145	4–5
LPL	Lipoprotein lipase	NM_214286	atctgcgggatacaccaagc	ccaaggctgtatcccaggag	110	3–4
MLXIPL	MLX interacting protein-like	XM_003481002	tgacatgatccagcctgacc	gggggctcagagaagtttga	126	7–8
PPARA	PPAR α	NM_001044526	tttccctctttgtggctgct	ggggtggttggtctgcaag	128	5–6
PPARG	PPAR γ	NM_214379	gagggcgatcttgacaggaa	gccacctctttgctctgctc	124	6–7
SCD	Stearoyl-CoA desaturase (Δ-9-desaturase)	NM_213781	agccgagaagctggtgatgt	gaagaaaggtggcgacgaac	140	5–6
SREBP1	Sterol regulatory element binding protein factor 1	NM_214157	gtgctggcggaggtctatgt	aggaagaagcgggtcagaaag	96	11–12
Housekeeping genes						
RPLP0	Ribosomal phosphoprotein large, P0 subunit	NM_001098598	tccaggctttaggcatcacc	ggctcccactttgtctccag	95	4–5
RSP29	Ribosomal protein S29	NM_001001633	ggtcagggttctcgctcttg	cactggcggcacatattgag	120	1–2
RPL27	Ribosomal protein L27	NM_001097479.1	gtactccgtggatatcccttg	aacttgaccttggcctctcga	102	1

calculated using the geometric mean of *RPLP0/RPS29* and *RPLP0/RPL27* as a normaliser for muscle and SAT, respectively. The relative gene expression levels were calculated using the Livak method[37], corrected for variation in amplification efficiency, as described by Fleige *et al.*[38]

Statistical analysis

All the data were checked for normal distribution (Shapiro–Wilk test) and variance homogeneity (χ^2 test). As variance heterogeneity was detected for most fatty acids and genes, these data were analysed using Proc MIXED of SAS software package[39] (version 9.2; SAS Institute). The model included the effect of dietary protein reduction (PR), betaine and Arg. The contrast among diet types was performed as follows: PR = control *v.* (RP, RPB, RPA, RPBA)/4; Bet = RP *v.* RPB; Arg = RP *v.* RPA; Bet + Arg = RP *v.* RPBA; Bet × Arg = RPBA *v.* (RPB + RPA)/2. The contrast Bet × Arg enables to assess additive or interactive effects between dietary betaine and arginine supplementation of RP. Pearson's correlation matrices were computed using the PROC CORR of SAS.

Results

This study presents and discusses the results of a trial in commercial pigs aiming to investigate the dietary modulation of fat content and fatty acid composition in the *m. longissimus lumborum* and SAT. Furthermore, the possible molecular mechanisms underlying fat deposition in muscle and SAT were elucidated through the assessment of mRNA expression levels of genes encoding key lipogenic transcription factors and enzymes. This animal trial also generated results on pigs' performance, carcass traits and sensory quality of meat that are presented elsewhere[40]. In brief, the results confirmed that dietary PR enhances pork eating quality but negatively affects pigs' growth performance. Moreover, it was suggested that betaine and/or arginine supplementation of RPD does not further increase IMF content but improves some pork sensory traits, including overall acceptability.

Intramuscular fat and fatty acid composition of muscle

The results of IMF content, fatty acid composition and partial sums of fatty acids in the *m. longissimus lumborum* of crossbred pigs are presented in Table 3. The IMF content was increased by 25 % for reduced protein diets (RP, RPA, RPB and RPBA groups; $P = 0{\cdot}041$) relative to the normal protein diet (control group). However, IMF content was not affected by the supplementation of reduced protein diet (RP) by betaine (RPB; $P = 0{\cdot}730$), arginine (RPA; $P = 0{\cdot}344$) or both (RPBA; $P = 0{\cdot}610$).

The predominant fatty acids in IMF were 18 : 1*cis*-9 (33–35 % of total FAME), 16 : 0 (22–23 %), 18 : 0 (12–14 %) and 18 : 2*n*-6 (11–12 %) for all the experimental groups. The term 'others' in Table 3 refers to unidentified minor fatty acids and to the 16 : 0, 18 : 0 and 18 : 1 plasmalogen-derived dimethyl acetals. Dietary PR (RP, RPA, RPB and RPBA) resulted only in a decrease of the percentage of 16 : 1*cis*-7 ($P < 0{\cdot}001$), when compared with the control diet, out of the twenty-four fatty acids identified in

https://doi.org/10.1017/S0007114515005218 Published online by Cambridge University Press

Table 3. Effect of dietary protein reduction (PR), betaine (Bet) and arginine (Arg) on intramuscular fat (IMF; % muscle), fatty acid composition (% total fatty acids), partial sums of fatty acids and related nutritional ratios in the *m. longissimus lumborum* of pigs
(Mean values with their standard errors)

	Control		RP		RPB		RPA		RPBA		*P*				
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	PR*	Bet†	Arg‡	Bet + Arg§	Bet × Arg‖
IMF	1·63	0·158	2·03	0·158	1·95	0·158	2·25	0·158	1·92	0·158	0·041	0·730	0·344	0·610	0·362
Fatty acid composition															
12 : 0	0·07	0·003	0·07	0·002	0·07	0·003	0·09	0·017	0·07	0·002	0·568	0·789	0·462	0·498	0·374
14 : 0	1·12	0·028	1·14	0·028	1·13	0·067	1·06	0·067	1·14	0·028	0·913	0·842	0·316		0·426
15 : 0	0·13	0·020	0·12	0·020	0·13	0·020	0·12	0·020	0·13	0·020	0·907	0·552	0·986	0·548	0·732
16 : 0	22·6	0·34	22·9	0·27	23·1	0·34	22·7	0·56	23·1	0·34	0·388	0·646	0·769	0·742	0·772
16 : 1*cis*-7	0·31	0·008	0·27	0·008	0·26	0·008	0·25	0·008	0·27	0·008	<0·001	0·857	0·279	0·959	0·501
16 : 1*cis*-9	2·29	0·118	2·54	0·118	1·98	0·118	2·29	0·118	2·22	0·118	0·777	0·003	0·160	0·071	0·565
17 : 0	0·55	0·037	0·52	0·037	0·56	0·037	0·52	0·037	0·49	0·037	0·512	0·414	0·997	0·545	0·245
17 : 1*cis*-9	0·26	0·020	0·26	0·017	0·24	0·017	0·23	0·018	0·23	0·017	0·462	0·198	0·385	0·163	0·703
18 : 0	13·0	0·51	12·7	0·51	14·1	0·51	13·2	0·51	13·7	0·51	0·473	0·071	0·478	0·190	0·959
18 : 1	0·12	0·007	0·12	0·007	0·11	0·007	0·12	0·007	0·11	0·007	0·728	0·107	0·889	0·599	0·781
18 : 1*cis*-9	33·5	1·14	35·6	1·14	33·0	1·14	34·6	1·14	34·6	1·14	0·454	0·127	0·539	0·551	0·572
18 : 1*cis*-11	3·25	0·105	3·45	0·105	2·99	0·105	3·32	0·105	3·19	0·105	0·938	0·005	0·404	0·099	0·763
18 : 2*n*-6	13·4	0·82	11·8	0·82	12·7	0·82	12·1	0·82	12·1	0·82	0·184	0·413	0·757	0·804	0·713
18 : 3*n*-3	0·43	0·016	0·39	0·016	0·34	0·045	0·37	0·016	0·37	0·016	0·021	0·260	0·387	0·387	0·462
20 : 0	0·15	0·010	0·15	0·009	0·16	0·009	0·16	0·010	0·17	0·010	0·831	0·546	0·822	0·207	0·323
20 : 1*cis*-11	0·54	0·028	0·57	0·028	0·52	0·028	0·54	0·028	0·56	0·028	0·762	0·280	0·475	0·790	0·463
20 : 2*n*-6	0·39	0·015	0·37	0·015	0·37	0·015	0·34	0·015	0·33	0·015	0·058	0·880	0·309	0·172	0·277
20 : 3*n*-3	0·37	0·037	0·32	0·037	0·39	0·037	0·37	0·037	0·33	0·037	0·722	0·248	0·346	0·955	0·253
20 : 3*n*-6	2·82	0·324	2·54	0·324	2·97	0·324	2·79	0·324	2·57	0·324	0·778	0·356	0·592	0·943	0·447
20 : 4*n*-6	0·085	0·0084	0·080	0·0084	0·081	0·0084	0·063	0·0084	0·075	0·0084	0·301	0·978	0·153	0·660	0·751
20 : 5*n*-3	0·035	0·0137	0·052	0·0137	0·069	0·0137	0·064	0·0137	0·050	0·0137	0·142	0·400	0·565	0·892	0·332
22 : 4*n*-6	0·43	0·046	0·39	0·046	0·43	0·046	0·42	0·046	0·36	0·046	0·549	0·567	0·668	0·686	0·299
22 : 5*n*-3	0·29	0·042	0·27	0·042	0·28	0·042	0·25	0·042	0·22	0·042	0·506	0·926	0·755	0·405	0·403
22 : 6*n*-3	0·092	0·0288	0·041	0·0288	0·059	0·0288	0·067	0·0288	0·052	0·0288	0·258	0·659	0·532	0·791	0·756
Others	3·79	0·418	3·33	0·418	3·98	0·418	3·88	0·418	3·55	0·418	0·830	0·290	0·367	0·716	0·472
Fatty acid partial sums															
SFA¶	37·6	0·83	37·6	0·83	39·2	0·83	37·9	0·83	38·8	0·83	0·406	0·202	0·820	0·328	0·794
MUFA**	40·2	1·25	42·8	1·25	39·1	1·25	41·3	1·25	41·2	1·25	0·537	0·048	0·421	0·373	0·539
PUFA††	18·4	1·27	16·2	1·27	17·7	1·27	16·9	1·27	16·4	1·27	0·288	0·418	0·730	0·907	0·594
n-6 PUFA‡‡	17·2	1·18	15·2	1·18	16·6	1·18	15·8	1·18	15·4	1·18	0·289	0·398	0·723	0·879	0·604
n-3 PUFA§§	1·22	0·098	1·08	0·050	1·11	0·098	1·10	0·159	1·05	0·098	0·236	0·779	0·875	0·799	0·673
Fatty acid ratios															
PUFA:SFA	0·49	0·041	0·43	0·041	0·46	0·041	0·45	0·041	0·42	0·041	0·293	0·658	0·720	0·862	0·509
n-6:*n*-3	14·3	0·75	14·2	0·75	15·3	0·75	14·7	0·75	15·0	0·75	0·588	0·282	0·620	0·451	0·969

Control, normal protein diet; RP, reduced protein diet; RPB, reduced protein diet with BET addition; RPA, reduced protein diet with Arg addition; RPBA, reduced protein diet with betaine and Arg addition.
* Contrast for PR = control *v.* (RP + RPB + RPA + RPBA)/4.
† Contrast for Bet = RP *v.* RPB.
‡ Contrast for Arg = RP *v.* RPA.
§ Contrast for Bet + Arg = RP *v.* RPBA.
‖ Contrast for Bet × Arg = (RPBA) *v.* (RPB + RPA)/2.
¶ SFA = 12 : 0 + 14 : 0 + 15 : 0 + 16 : 0 + 17 : 0 + 18 : 0 + 20 : 0.
** MUFA = 16 : 1*cis*-7 + 16 : 1*cis*-9 + 17 : 1*cis*-9 + 18 : 1 + 18 : 1*cis*-9 + 18 : 1*cis*-11 + 20 : 1*cis*-11.
†† PUFA = 18 : 2*n*-6 + 18 : 3*n*-3 + 20 : 2*n*-6 + 20 : 3*n*-3 + 20 : 3*n*-6 + 20 : 4*n*-6 + 20 : 5*n*-3 + 22 : 4*n*-6 + 22 : 5*n*-3 + 22 : 6*n*-3.
‡‡ *n*-6 PUFA = 18 : 2*n*-6 + 20 : 2*n*-6 + 20 : 3*n*-6 + 20 : 4*n*-6 + 22 : 4*n*-6.
§§ *n*-3 PUFA = 18 : 3*n*-3 + 20 : 3*n*-3 + 20 : 5*n*-3 + 22 : 5*n*-3 + 22 : 6*n*-3.

https://doi.org/10.1017/S0007114515005218 Published online by Cambridge University Press

British Journal of Nutrition

https://doi.org/10.1017/S0007114515005218 Published online by Cambridge University Press

the muscle. Pigs fed the RPB had lower proportions of 16 : 1*cis*-9 ($P = 0.003$) and 18 : 1*cis*-11 ($P = 0.005$) than those fed the reduced protein diet (RP). Arginine supplementation of the reduced protein diet (RPA) had no effect on fatty acid profile relative to the reduced protein diet.

Concerning the partial sums of fatty acids (Table 3), only betaine supplementation of the reduced protein diet decreased the percentage of MUFA ($P = 0.048$), when compared with the reduced protein diet without amino acid supplementation (RP). Finally, PUFA:SFA and *n*-6:*n*-3 ratios, which are commonly used indices to assess the nutritional value of fatty acids, were not affected by any of the experimental treatments.

Fatty acid content and composition of subcutaneous adipose tissue

The results of backfat thickness at the P_2 site, total fat and fatty acid composition for SAT are presented in Table 4. Dietary betaine, arginine and betaine plus arginine supplementation did not affect backfat thickness at the P_2 site or total fat content. However, a 7 % increase in total fat content was observed for pigs fed the reduced protein diets ($P = 0.046$) when compared with those fed the control diet.

The major fatty acids in SAT were 18 : 1*cis*-9 (34–35 % of total FAME), 16 : 0 (23–24 %), 18 : 0 (16–18 %) and 18 : 2*n*-6 (14–16 %) across experimental groups. A reduction in the level of dietary protein (RP) resulted in a decrease of the percentages of 16 : 1*cis*-7 ($P = 0.001$) and 18 : 1*cis*-11 ($P = 0.035$) when compared with the control diet. Betaine supplementation of the reduced protein diet decreased the percentages of 16 : 1*cis*-9 ($P = 0.020$) and 18 : 1*cis*-11 ($P = 0.013$). Neither arginine nor the combination of betaine and arginine affected the fatty acid profile in SAT.

The partial sums of fatty acids (Table 4) were not affected by any experimental treatment. However, PUFA:SFA ratio was decreased under the reduced protein diet ($P = 0.043$) when compared with the control diet. In contrast, pigs fed the reduced protein diet supplemented with betaine had a higher *n*-6:*n*-3 ratio ($P = 0.048$) when compared with those fed the reduced protein diet.

Gene expression levels in muscle and subcutaneous adipose tissue

Expression analysis of genes controlling lipid metabolism was carried out in order to elucidate whether the tissue-specific effects of dietary protein level, betaine and arginine are associated with differential modulation of gene expression. Fig. 1 and 2 show the expression levels of fourteen key genes associated with lipid metabolism in the *m. longissimus lumborum* and SAT of pigs, respectively.

In the *m. longissimus lumborum*, the relative expression levels of two out of the fourteen genes analysed were affected by, at least, one dietary treatment. The expression level of the *PPARA* gene was lower ($P = 0.027$) in pigs fed the reduced protein diet (RP) when compared with that fed the normal protein diet (control diet). In addition, both arginine ($P = 0.022$) and betaine plus arginine ($P = 0.025$) supplementation of the reduced protein diet decreased the mRNA level of the *PPARG* gene.

In SAT, the mRNA levels of six out of the fourteen genes analysed were affected by one dietary treatment. The expression levels of *GLUT4* ($P = 0.026$), *LPL* ($P = 0.015$) and *SCD* ($P = 0.023$) genes were up-regulated in pigs fed the reduced protein diet when compared with those fed the control diet. Dietary betaine supplementation of the reduced protein diet decreased the *CRAT* mRNA level ($P = 0.004$), whereas dietary arginine supplementation decreased the *PPARG* expression level ($P = 0.045$). Finally, dietary betaine plus arginine supplementation increased the *FADS2* expression level ($P = 0.028$) when compared with the diets with either betaine or arginine supplementation.

Correlation between fatty acid composition and gene expression levels

The correlation coefficients (r) between fatty acid composition and gene expression levels for the *m. longissimus lumborum* and for SAT are shown in Table 5. In the *m. longissimus lumborum*, 16 : 1*cis*-9 was positively and moderately correlated ($0.7 \geq r \geq 0.3$) with *FABP4* ($P < 0.01$), *SCD* ($P < 0.01$), *LPL* ($P < 0.05$) and *PPARG* ($P < 0.05$) and was negatively correlated with *PPARA* ($P < 0.05$). Furthermore, 18 : 1*cis*-9 was positively and moderately correlated with *FABP4* ($P < 0.01$), *PPARG* ($P < 0.01$), *SCD* ($P < 0.01$) and *CPT-1B* ($P < 0.05$) and was negatively correlated with *FADS1* ($P < 0.05$). A moderate and positive correlation was found between 18 : 1*cis*-11 and *FABP4* and *SCD* expression levels ($P < 0.05$). The fatty acid 18 : 2*n*-6 was negatively and moderately correlated with *CPT-1B* ($P < 0.05$), *PPARG* ($P < 0.05$) and *SCD* ($P < 0.05$) and was positively correlated with *FADS1* ($P < 0.05$). MUFA were positively and moderately correlated with *FABP4* ($P < 0.01$), *PPARG* ($P < 0.01$), *SCD* ($P < 0.01$) and *CPT-1B* ($P < 0.05$) and were negatively correlated with *FADS1* ($P < 0.05$). PUFA were negatively and moderately correlated with *PPARG* ($P < 0.01$) and *SCD* ($P < 0.01$) and were positively associated with *FADS1* ($P < 0.05$).

In SAT, 16 : 0 and 18 : 0 fatty acid percentages were positively and moderately correlated with *ACACA* ($P < 0.05$), *FADS2* ($P < 0.05$) and *GLUT4* ($P < 0.05$) expression levels. Furthermore, 16 : 0 was positively correlated with *LPL* ($P < 0.01$), *MLXIPL* ($P < 0.01$), *FASN* ($P < 0.05$) and *PPARG* ($P < 0.05$). Similar to 16 : 0, SFA was positively correlated with *FADS2* ($P < 0.001$), *ACACA* ($P < 0.01$), *GLUT4* ($P < 0.01$), *FASN* ($P < 0.05$), *LPL* ($P < 0.05$) and *MLXIPL* ($P < 0.05$). MUFA and 18 : 1*cis*-9 were negatively and moderately correlated with *FADS2* ($P < 0.001$), and MUFA were also negatively correlated with *FADS1* ($P < 0.05$), *GLUT4* ($P < 0.05$) and *LPL* ($P < 0.05$). A moderate negative correlation was found between 18 : 1*cis*-11 content and *FADS2* ($P < 0.05$) and *GLUT4* ($P < 0.05$) expression levels. A positive correlation was established between 18 : 1*cis*-11 content and *CRAT* ($P < 0.05$). PUFA and 18 : 2*n*-6 percentages were negatively correlated with *ACACA* ($P < 0.05$) and *FASN* ($P < 0.05$) expression levels.

Table 4. Effect of dietary protein reduction (PR), betaine (Bet) and arginine (Arg) on backfat thickness P_2 (mm), total fat (% fat), fatty acid composition (% total fatty acids), partial sums of fatty acids and related nutritional ratios in subcutaneous adipose tissue of pigs
(Mean values with their standard errors)

	Control		RP		RPB		RPA		RPBA		*P*				
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	PR*	Bet†	Arg‡	Bet + Arg§	Bet × Arg‖
P_2 backfat thickness	19·6	1·42	20·9	1·42	21·2	1·42	20·0	1·42	19·1	1·42	0·659	0·659	0·663	0·391	0·398
Total fat	76·6	2·12	81·6	2·12	77·6	2·12	83·4	2·12	84·2	2·12	0·046	0·195	0·574	0·412	0·181
Fatty acid composition															
12 : 0	0·07	0·003	0·07	0·003	0·06	0·003	0·06	0·003	0·07	0·003	0·548	0·710	0·750	0·951	0·638
14 : 0	1·15	0·036	1·14	0·036	1·11	0·036	1·15	0·036	1·16	0·036	0·712	0·495	0·956	0·692	0·415
16 : 0	23·4	0·37	23·8	0·37	23·6	0·37	24·0	0·37	24·2	0·37	0·243	0·638	0·716	0·504	0·406
16 : 1*cis*-7	0·35	0·013	0·30	0·013	0·33	0·013	0·28	0·013	0·28	0·013	0·001	0·188	0·184	0·194	0·138
16 : 1*cis*-9	1·36	0·084	1·35	0·084	1·06	0·084	1·32	0·084	1·18	0·084	0·168	0·020	0·757	0·157	0·943
17 : 0	0·55	0·036	0·55	0·036	0·54	0·036	0·53	0·036	0·49	0·036	0·519	0·843	0·707	0·205	0·254
17 : 1*cis*-9	0·33	0·025	0·33	0·025	0·27	0·025	0·30	0·025	0·27	0·025	0·213	0·120	0·469	0·101	0·547
18 : 0	16·5	0·65	16·9	0·65	18·5	0·65	17·6	0·65	18·5	0·65	0·057	0·091	0·453	0·090	0·566
18 : 1	0·07	0·004	0·06	0·004	0·05	0·004	0·06	0·004	0·06	0·004	0·078	0·073	0·965	0·239	0·777
18 : 1*cis*-9	34·5	0·52	34·7	0·52	33·9	0·52	35·4	0·52	34·4	0·52	0·898	0·306	0·344	0·734	0·730
18 : 1*cis*-11	1·96	0·061	1·91	0·061	1·67	0·061	1·89	0·061	1·76	0·061	0·035	0·013	0·764	0·090	0·756
18 : 2*n*-6	16·3	1·08	15·3	0·53	14·3	0·53	14·2	0·53	14·3	0·53	0·152	0·174	0·148	0·174	0·919
18 : 3*n*-3	0·99	0·046	0·93	0·046	0·91	0·046	0·84	0·046	0·87	0·046	0·-057	0·714	0·167	0·313	0·878
20 : 0	0·24	0·010	0·33	0·093	0·25	0·010	0·24	0·010	0·26	0·023	0·293	0·430	0·382	0·521	0·466
20 : 1*cis*-11	0·67	0·036	0·69	0·036	0·68	0·036	0·71	0·036	0·71	0·036	0·608	0·947	0·715	0·686	0·768
20 : 2*n*-6	0·68	0·026	0·66	0·026	0·69	0·026	0·64	0·026	0·64	0·026	0·345	0·352	0·608	0·621	0·416
20 : 3*n*-3	0·16	0·006	0·15	0·006	0·14	0·006	0·14	0·006	0·15	0·006	0·119	0·451	0·473	0·495	0·950
20 : 3*n*-6	0·09	0·004	0·09	0·004	0·09	0·004	0·08	0·004	0·08	0·004	0·257	0·696	0·270	0·066	0·194
20 : 4*n*-6	0·29	0·022	0·-30	0·022	0·29	0·022	0·27	0·022	0·27	0·022	0·633	0·731	0·342	0·455	0·909
Others	0·28	0·021	0·25	0·021	0·22	0·021	0·26	0·021	0·23	0·021	0·136	0·490	0·689	0·619	0·685
Fatty acid partial sums															
SFA¶	41·9	0·886	42·9	0·886	44·1	0·886	43·6	0·886	44·7	0·886	0·063	0·335	0·538	0·147	0·431
MUFA**	39·3	0·58	39·3	0·58	38·0	0·58	39·9	0·58	38·7	0·58	0·666	0·115	0·463	0·436	0·691
PUFA††	18·5	0·71	17·5	0·71	17·6	0·71	16·1	0·71	16·3	0·71	0·054	0·889	0·175	0·239	0·510
n-6 PUFA‡‡	17·4	1·13	16·4	0·56	15·2	0·56	15·2	0·56	15·2	0·53	0·154	0·179	0·157	0·179	0·935
n-3 PUFA§§	1·15	0·08	1·08	0·04	1·05	0·01	1·01	0·04	1·00	0·04	0·238	0·517	0·271	0·222	0·573
Fatty acid ratios															
PUFA:SFA	0·45	0·025	0·41	0·025	0·40	0·025	0·37	0·025	0·37	0·025	0·043	0·804	0·263	0·227	0·538
n-6:*n*-3	15·1	0·22	15·0	0·15	15·7	0·26	15·3	0·15	15·1	0·15	0·461	0·048	0·175	0·750	0·062

Control, normal protein diet; RP, reduced protein diet; RPB, reduced protein diet with Bet addition; RPA, reduced protein diet with Arg addition; RPBA, reduced protein diet with betaine and Arg addition.
* Contrast for PR = Control *v.* (RP + RPB + RPA + RPBA)/4.
† Contrast for Bet = RP *v.* RPB.
‡ Contrast for Arg = RP *v.* RPA.
§ Contrast for Bet + Arg = RP *v.* RPBA.
‖ Contrast for Bet × Arg = (RPBA) *v.* (RPB + RPA)/2.
¶ SFA = 12 : 0 + 14 : 0 + 16 : 0 + 17 : 0 + 18 : 0 + 20 : 0.
** MUFA = 16 : 1*cis*-7 + 16 : 1*cis*-9 + 17 : 1*cis*-9 + 18 : 1 + 18 : 1*cis*-9 + 18 : 1*cis*-11 + 20 : 1*cis*-11.
†† PUFA = 18 : 2*n*-6 + 18 : 3*n*-3 + 20 : 2*n*-6 + 20 : 3*n*-3 + 20 : 3*n*-6 + 20 : 4*n*-6.
‡‡ *n*-6 PUFA = 18 : 2*n*-6 + 20 : 2*n*-6 + 20 : 3*n*-6 + 20 : 4*n*-6.
§§ *n*-3 PUFA = 18 : 3*n*-3 + 20 : 3*n*-3.

https://doi.org/10.1017/S0007114515005218 Published online by Cambridge University Press

British Journal of Nutrition

https://doi.org/10.1017/S0007114515005218 Published online by Cambridge University Press

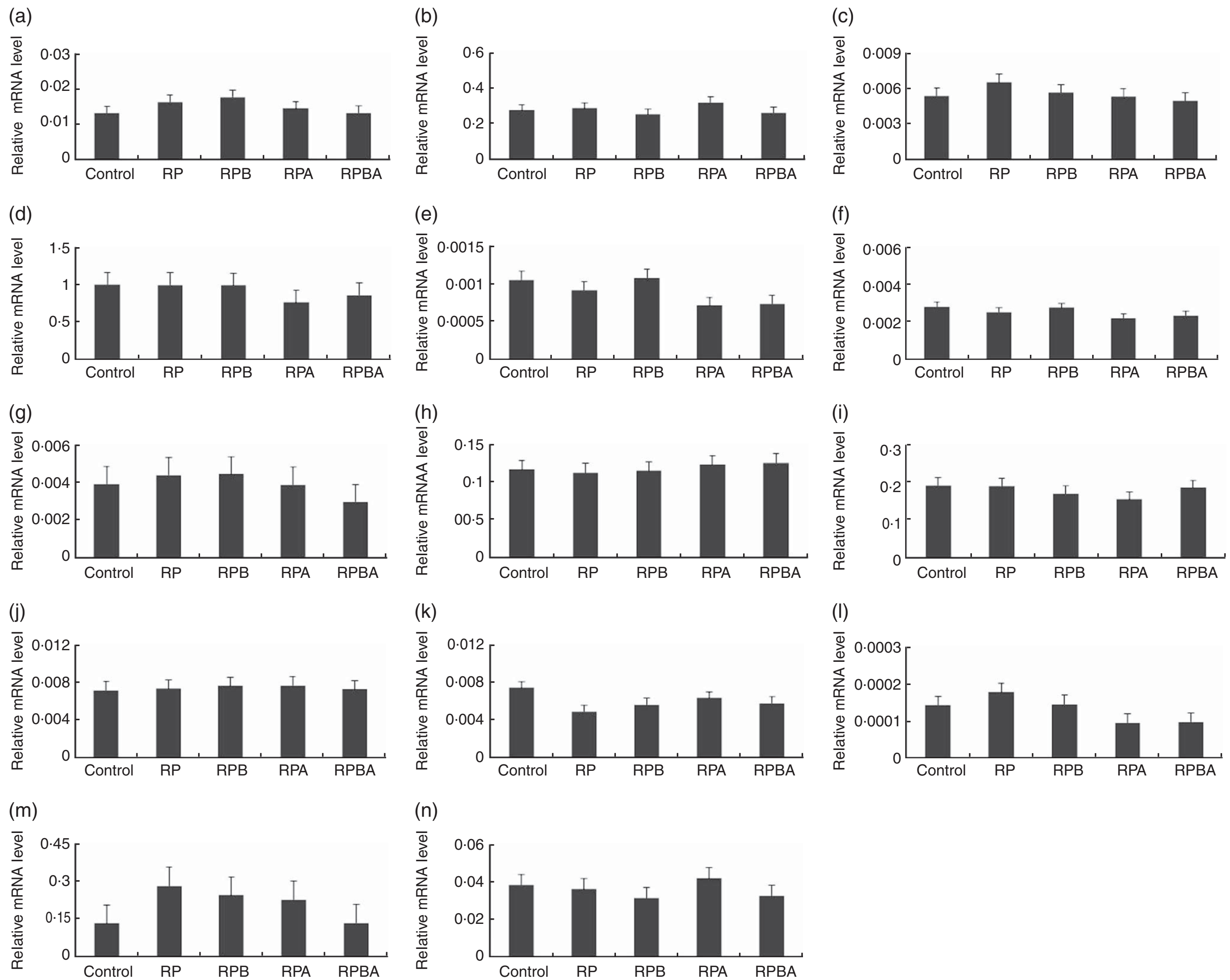

Fig. 1. Effect of dietary protein reduction, betaine and arginine on gene expression in the *m. longissimus lumborum* of pigs: (a) acetyl-CoA carboxylase; (b) carnitine *O*-acetyltransferase; (c) carnitine palmitoyltransferase 1; (d) fatty acid binding protein 4; (e) fatty acid desaturase 1; (f) fatty acid desaturase 2; (g) fatty acid synthase; (h) GLUT type 4; (i) lipoprotein lipase; (j) MLX interacting protein-like; (k) PPARA (protein reduction, $P = 0·027$ (control diet (control) *v.* reduced protein diet (RP), reduced protein diet with betaine addition (RPB), reduced protein diet with arginine addition (RPA), reduced protein diet with betaine and arginine addition (RPBA)); (l) PPARG (arginine, $P = 0·022$ (RP *v.* RPA)); betaine + arginine, $P = 0·025$ (RP *v.* RPBA)); (m) stearoyl-CoA desaturase, (n) sterol regulatory element binding protein 1. Values are means, with their standard errors represented by vertical bars.

Discussion

In the present study, a 19 % reduction of dietary protein (16 *v.* 13 % of crude protein) during the growing–finishing phase of commercial cross-bred pigs resulted in an increased IMF of 25 %. These results agree with previous studies, which showed that a range of dietary protein concentrations (e.g. 21 *v.* 18 %[5], 17 *v.* 15 %[41] and 16 *v.* 13 %[14]) increases IMF content in commercial cross-bred pigs. It was previously suggested that an increase in IMF is likely due to a dietary lysine restriction, and it might be mediated by up-regulation of the lipogenic enzyme SCD[15], which is responsible for the regulation of MUFA biosynthesis. However, the present study did not confirm the up-regulation of *SCD* mRNA expression by RPD in pig muscle, although a significant correlation between *SCD* expression level and MUFA proportion was observed. This is likely explained by the different pig genotypes and dietary lysine restriction percentages used in the different experiments. In fact, in the study by Madeira *et al.*[14], the cross-bred pig used was 25 % Duroc × 25 % Pietrain × 25 % Large White × 25 % Landrace, whereas in this study the cross-bred pig used was 50 % Duroc × 25 % Large White × 25 % Landrace. In addition, although the protein level was the same in both studies (16 *v.* 13 %), lysine level was lower in the present study (0·8 *v.* 0·5[14]; 0·5 *v.* 0·3). In line with this, we have previously shown that the relationship between SCD protein expression and IMF content is breed specific[42]. Finally, the mRNA expression level of *PPARA*, a key transcription factor involved in the promotion of fatty acid oxidation[24], decreased with dietary PR, which has not yet been previously described.

Our hypothesis was that tissue-specific responses of IMF content, fatty acid composition and backfat thickness promoted

British Journal of Nutrition

https://doi.org/10.1017/S0007114515005218 Published online by Cambridge University Press

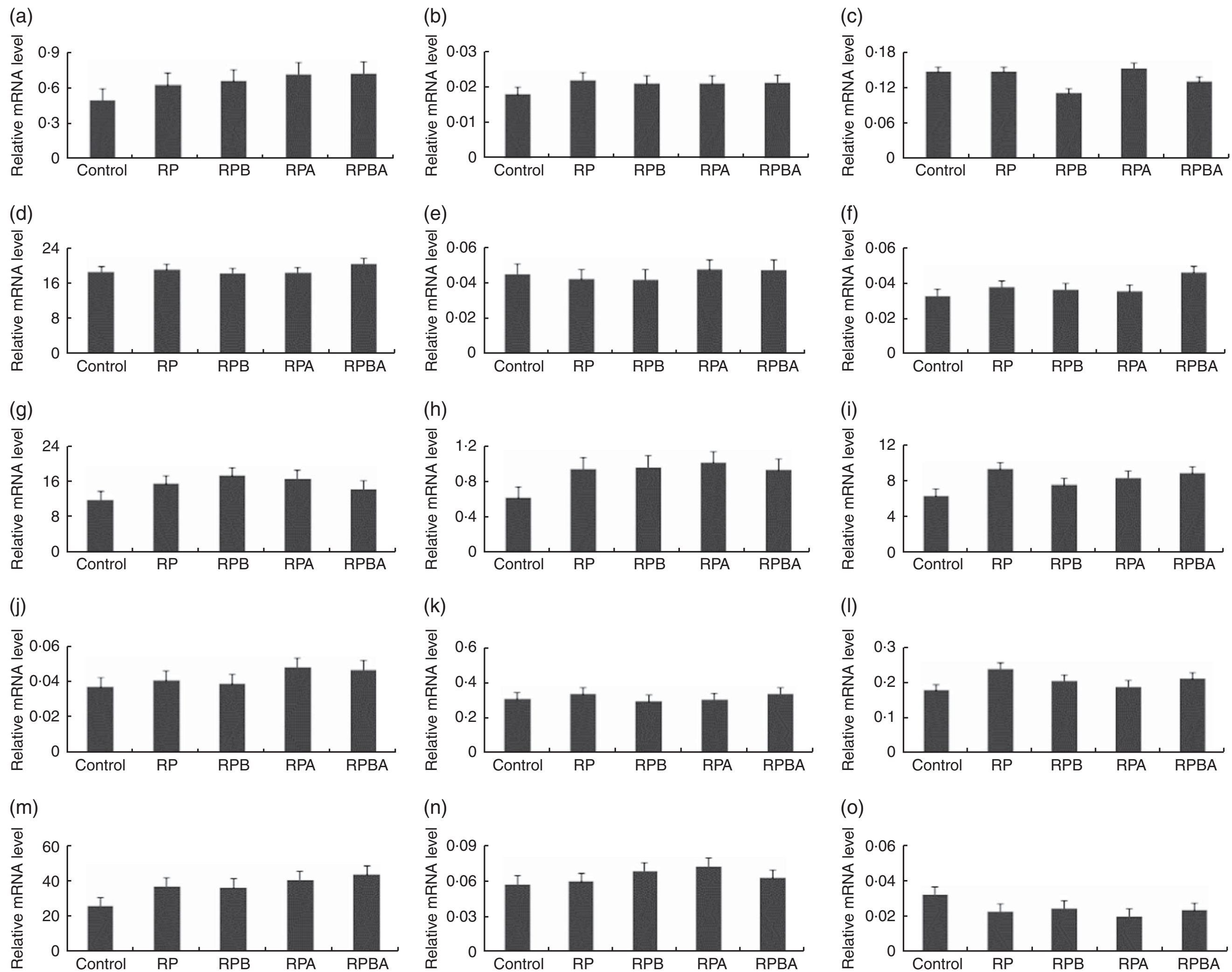

Fig. 2. Effect of dietary protein reduction, betaine and arginine on gene expression in the subcutaneous adipose tissue of pigs: (a) acetyl-CoA carboxylase; (b) CCAAT/enhancer binding protein *α*; (c) carnitine *O*-acetyltransferase (betaine, $P=0.004$ (reduced protein diet (RPD) *v.* reduced protein diet with betaine addition (RPB)); (d) fatty acid binding protein 4, (e) fatty acid desaturase 1, (f) fatty acid desaturase 2 (betaine × arginine, $P=0.028$ (reduced protein diet with betaine and arginine addition (RPBA) *v.* RPB and reduced protein diet with arginine addition (RPA)) (g) fatty acid synthase, (h) GLUT type 4 (protein reduction, $P=0.026$ (control diet (control) *v.* reduced protein diet (RP), RPB, RPA, RPBA)), (i) lipoprotein lipase (protein reduction, $P=0.015$ (control *v.* RP, RPB, RPA, RPBA)), (j) interacting protein-like, (k) PPARA, (l) PPARG (arginine, $P=0.045$ (RP *v.* RPA)), (m) stearoyl-CoA desaturase) (protein reduction, $P=0.023$ (control *v.* RP, RPB, RPA, RPBA)), (n) sterol regulatory element binding protein 1, (o) LPL muscle/subcutaneous adipose tissue. Values are means, with their standard errors represented by vertical bars.

by RPD in cross-bred pigs could be improved by the dietary supplementation of betaine and/or arginine. It was also our goal to elucidate the molecular mechanisms controlling lipid metabolism by these dietary supplementations in muscle and SAT. This hypothesis was based on some studies indicating that dietary betaine supplementation can increase IMF content[43,44], decrease carcass fat deposition and increase carcass leanness in pig[45,46]. In addition, the increase in IMF content by dietary arginine supplementation has also been previously reported[13,47].

The results of the present study regarding the effect of RPD supplemented by betaine are in agreement with the data of Rojas-Cano *et al.*[48], who suggested that 0·5 % dietary betaine supplementation of a normal protein diet does not increase IMF content in Iberian pigs from 20 to 50 kg of live body weight. In contrast, Feng[43] and Ma *et al.*[49] reported that betaine supplementation of pig diets with 0·10–0·18 % increases IMF content in the *m. longissimus*. Martins *et al.*[44] also found that 0·1 % betaine supplementation in Alentejano pigs increases total intramuscular lipids. The explanation for this discrepancy might be the use of distinct pig genotype, age and degree of maturity, as well as different dietary protein/lysine levels (normal protein diet *v.* RPD) in the different studies. Our recent study[15] showed that the increase in IMF content under RPD can be observed in genetically lean pigs such as cross-bred pigs with Pietrain, Large White and Landrace genetics but not in fatty breeds such as Alentejano breed.

Betaine is involved in lipid metabolism through its role in both regulation of phosphatidylcholine synthesis and fatty acid oxidation as a methyl donor during carnitine synthesis[50]. Dietary carnitine supplementation has been shown to decrease carcass fat

Table 5. Pearson's correlation coefficients among fatty acid composition (% total fatty acids), partial sums of fatty acids and gene expression levels (relative mRNA level) in the *m. longissimus lumborum* and subcutaneous adipose tissue of pigs

	16:0	16:1 *cis*-9	18:0	18:1 *cis*-9	18:1 *cis*-11	18:2 *n*-6	SFA	MUFA	PUFA	*SREBP1*	*SCD*	*PPARG*	*PPARA*	*MLXIPL*	*LPL*	*GLUT4*	*FASN*	*FADS2*	*FADS1*	*FABP4*	*CRAT*	*CPT-1B/CEBPA*	*ACACA*
m. longissmus lumborum																							
ACACA	0·13	0·23	−0·07	0·21	0·18	−0·20	0·00	0·22	−0·20	−0·08	0·86***	0·47**	−0·05	0·43**	0·05	0·22	0·80***	0·20	0·04	0·53***	−0·10	0·03	
CPT−1B	−0·08	−0·05	0·09	0·39*	0·15	−0·33*	0·00	0·37*	−0·30	0·35*	−0·00	0·31	0·21	0·13	0·02	0·20	0·03	0·01	−0·27	0·01	0·54***		
CRAT	−0·07	−0·11	0·08	0·25	−0·01	−0·19	0·00	0·22	−0·19	0·22	−0·04	0·00	0·24	−0·04	0·25	0·11	0·12	−0·44**	−0·34*	−0·26			
FABP4	0·14	0·46**	−0·21	0·41**	0·34*	−0·30	−0·09	0·44**	−0·31	−0·18	0·70***	0·71***	−0·15	0·25	0·43**	−0·13	0·55***	0·25	0·01				
FADS1	−0·13	−0·11	−0·01	−0·39*	−0·19	0·34*	−0·06	−0·38*	0·34*	0·05	−0·03	0·03	0·06	0·05	0·02	0·14	0·11	0·56***					
FADS2	−0·09	−0·12	0·00	−0·10	−0·09	0·11	−0·04	−0·11	0·12	0·35*	0·08	0·29	0·24	0·15	−0·20	0·22	0·21						
FASN	0·20	0·25	−0·01	0·26	0·11	−0·25	0·07	0·27	−0·25	0·11	0·82***	0·63***	0·11	0·33*	0·30	−0·08							
GLUT4	−0·06	−0·17	0·15	0·02	−0·07	−0·03	0·06	−0·00	−0·03	0·28	0·02	−0·09	0·27	0·25	−0·22								
LPL	0·30	0·36*	−0·07	0·28	0·17	−0·28	0·09	0·29	−0·28	−0·04	0·25	0·32*	−0·08	0·04									
MLXIPL	0·09	−0·04	0·17	−0·08	−0·12	−0·04	0·14	−0·10	0·02	−0·03	0·31	0·21	−0·10										
PPARA	−0·07	−0·32*	0·23	0·07	−0·25	−0·03	0·11	0·01	−0·06	0·44**	−0·05	0·05											
PPARG	0·26	0·33*	−0·02	0·41**	0·17	−0·37*	0·09	0·42**	−0·38*	0·09	0·62***												
SCD	0·16	0·43**	−0·19	0·43**	0·32*	−0·36*	−0·07	0·45**	−0·35*	−0·12													
SREBP1	0·07	−0·10	0·21	0·11	−0·06	−0·16	0·17	0·08	−0·15														
Subcutaneous adipose tissue																							
ACACA	0·38*	−0·06	0·37*	−0·22	−0·27	−0·33*	0·45**	−0·26	−0·33*	0·52***	0·76***	0·27	0·60***	0·53***	0·59***	0·88***	0·76***	0·41**	0·24	0·17	0·28	0·03	
CEBPA	0·07	−0·11	0·07	−0·08	−0·15	−0·04	0·10	−0·10	−0·03	−0·04	0·05	0·49**	0·05	−0·02	0·23	0·07	0·09	0·41**	0·22	0·29	0·00		
CRAT	0·04	0·33*	−0·25	0·13	0·34*	0·01	−0·16	0·21	0·00	0·00	0·20	0·20	0·14	0·20	0·14	0·23	0·06	−0·02	−0·07	0·18			
FABP4	0·10	−0·13	0·14	−0·14	−0·16	−0·04	0·15	−0·17	−0·04	−0·15	0·27	0·26	−0·07	0·07	0·28	0·24	−0·01	0·15	−0·08				
FADS1	0·10	−0·08	0·19	−0·38	−0·12	0·08	0·20	−0·37*	0·08	0·27	0·17	0·22	0·39*	0·17	0·16	0·22	0·02	0·70***					
FADS2	0·36*	−0·22	0·47**	−0·62***	−0·38*	−0·08	0·53***	−0·64***	−0·08	0·01	0·21	0·55***	0·36*	0·33*	0·49**	0·39*	0·17						
FASN	0·45**	0·11	0·20	−0·09	−0·10	−0·36*	0·36*	−0·09	−0·37*	0·49**	0·64***	0·13	0·28	0·32*	0·34*	0·64***							
GLUT4	0·33*	−0·14	0·39*	−0·27	−0·33*	−0·26	0·44**	−0·32*	−0·26	0·47**	0·68***	0·35*	0·50**	0·46**	0·63***								
LPL	0·43**	0·07	0·24	−0·39*	−0·13	−0·13	0·38*	−0·38*	−0·13	−0·00	0·29	0·66***	0·29	0·54***									
MLXIPL	0·47**	0·19	0·18	−0·33*	−0·07	−0·15	0·35*	−0·30	−0·16	0·08	0·22	0·16	0·37*										
PPARA	0·05	−0·09	0·18	−0·14	−0·18	−0·04	0·16	−0·18	−0·04	0·35*	0·53***	0·19											
PPARG	0·34*	0·18	0·03	−0·36*	0·04	0·05	0·18	−0·30	0·05	−0·15	0·13												
SCD	0·10	−0·07	0·14	0·14	−0·13	−0·28	0·15	0·08	−0·28	0·65***													
SREBP1	−0·10	−0·00	−0·04	0·26	0·04	−0·14	−0·07	0·24	−0·14														

ACACA, acetyl-CoA carboxylase; *CPT-1B*, carnitine palmitoyltransferase 1B; *CRAT*, carnitine *O*-acetyltransferase; *FABP4*, fatty acid binding protein 4; *FADS1*, fatty acid desaturase 1; *FADS2*, fatty acid desaturase 2; *FASN*, fatty acid synthase; *GLUT4*, GLUT type 4; *LPL*, lipoprotein lipase; *MLXIPL*, MLX interacting protein-like; *SCD*, stearoyl-CoA desaturase; *SREBP1*, sterol regulatory element binding protein 1; *CEBPA*, CCAAT/enhancer binding protein α.

* $P<0·05$, ** $P<0·01$, *** $P<0·001$.

https://doi.org/10.1017/S0007114515005218 Published online by Cambridge University Press

content in pigs[51]. Betaine has been reported to increase carnitine content in pig liver and muscles[43], which indicates an effect of betaine on the reduction of carcass pig fat content in pigs. Huang *et al.*[50] reported that supplementation of pig diet with betaine leads to a decrease in the activity of CPT1 – an enzyme that plays a key role in the regulation of lipid metabolism. The present study did not find any significant effect of dietary betaine supplementation on *CPT-1B* gene expression. This fact can be explained by the use of distinct pig genotypes (Duroc × (Seghers × Seghers) cross-breed *v.* Duroc × Large White × Landrace cross-breed in the study by Huang *et al.*[50] and the present study, respectively). In addition, the concentrations of dietary betaine and protein levels used in this study (RPD with 13·0 % of protein and 0·33 % of betaine) were different from that used by Huang *et al.*[50] (normal protein diet with 14·9 % of protein and 0·12 % of betaine).

The effect of supplementing RPD with 1·5 % of arginine described in this study is in agreement with a report by Go *et al.*[52], who found that 0·82 % dietary arginine supplementation does not increase IMF content in pigs. In contrast, the studies by Tan *et al.*[13] and Ma *et al.*[47] reported an increase in IMF content in dietary trials that used 1 % dietary arginine supplementation, which is a value lower than that used in our study. The discrepancy between our findings and the report by Ma *et al.*[47] might be explained by the use of pigs with a different genetic background (Du × (Chang × Da)) and distinct slaughter weights (93 *v.* 110 kg[47], approximately). In addition, it is important to note that the feeding strategies reported in this study were based on RPD supplemented by betaine and/or arginine, whereas the studies mentioned above used betaine and arginine supplementation of diets with the recommended protein levels for those pig genetic lines. Taken together, the present study did not find any additional effect of dietary betaine and/or arginine supplementation to the increased IMF promoted by RPD in this lean commercial pig cross-breed.

The feeding strategies used in this study had a slight effect on the fatty acid compositions in muscle and SAT. The animals fed the diet supplemented with betaine had a lower percentage of beneficial MUFA in muscle, which was mainly due to a decrease in 16 : 1*cis*-9 and 18 : 1*cis*-11 proportions. Although *SCD* mRNA expression level was not affected in muscle by dietary betaine supplementation, a positive correlation was found between *SCD* mRNA and 16 : 1*cis*-9, 18 : 1*cis*-9, 18 : 1*cis*-11 and MUFA proportions. The increased *n-6*:*n-3* PUFA ratio in SAT of betaine-supplemented pigs indicates the lower nutritional value of the fat from these animals. Our results are in agreement with those of Martins *et al.*[44], who observed a slight effect of dietary betaine on fatty acid composition in the *m. longissimus lumborum* of Alentejano pig. In contrast to betaine, our study did not find any effect of dietary arginine supplementation on fatty acid composition in muscle. However, dietary supplementation with both arginine and betaine resulted in a decrease of *PPARG* mRNA level.

In the present study, the RPD increased the total fat content in SAT but did not affect backfat thickness. The increased fat content in SAT was accompanied by increased *GLUT4*, *LPL* and *SCD* mRNA levels. In addition, strong significant correlations among *GLUT4*, *LPL* and *SCD* mRNA levels were observed. This is consistent with our previous study[14], in which the increased total fat content in SAT was associated with the *LPL* and *SCD* mRNA levels promoted by the RPD.

Betaine supplementation of RPD did not affect total fat content in SAT or backfat thickness, but decreased 16 : 1*cis*-9 and 18 : 1*cis*-11 proportions. In addition, a down-regulation of *CRAT* mRNA expression under the betaine-supplemented diet was obtained. *CRAT* is a rate-limiting enzyme of lipid catabolism responsible for the transport of fatty acids from the cytosol to the mitochondria for β-oxidation[18].

The present study established that dietary arginine supplementation did not affect total fat content or fatty acid composition in SAT or backfat thickness at the P_2 site. However, arginine induced the down-regulation of *PPARG* transcription factor in muscle and SAT. It is well known that the transcription factor *PPARG* is involved in fat deposition through the expression regulation of some lipogenic enzymes[53].

In the present study, SAT had, in general, higher mRNA expression levels of genes controlling lipid metabolism when compared with the muscle. In addition, the number of correlations between major fatty acids and expression levels of key lipogenic enzymes and transcription factors was also higher in SAT than in muscle. This is likely explained by the fact that SAT is the main site for *de novo* fatty acid biosynthesis and lipogenesis, whereas muscles play a major role in the metabolism of glucose and degradation of lipids[17]. Therefore, SAT is more sensitive than IMF to changes in feeding strategies, mainly in those that affect lipid metabolism.

Conclusions

The present study confirms that RPD with restricted lysine levels increase IMF content and total fat content of SAT in pigs, but do not change backfat thickness at the P_2 site. The increased total fat content in SAT seems to be mediated by the up-regulation of *GLUT4*, *LPL* and *SCD* mRNA levels. These data indicate that the supplementation of RPD with betaine and/or arginine does not affect IMF content, total fat content in SAT or backfat thickness at the P_2 site. However, dietary betaine supplementation slightly affects fatty acid composition in both muscle and SAT. Betaine-supplemented diet decreased the expression of *CRAT* in SAT but not in muscle. Therefore, betaine might be involved in the differential regulation of some key genes of lipid metabolism in pig muscle and SAT. In spite of the lack of effect of arginine supplementation on fat content and fatty acid composition in muscle and SAT, the arginine-supplemented diet decreased the expression of *PPARG* transcription factor in both tissues.

Taken together, our data indicate that, under our experimental conditions, dietary betaine and/or arginine supplementation of RPD does not seem to be useful to further increase IMF content or to improve the nutritional value of meat fatty acid composition in pigs. The results of this research reinforce current evidence that adipogenesis and lipogenesis are differently regulated in pig muscle and SAT. These data contribute to understand the mechanisms of dietary regulation of fat partitioning in pigs, and therefore could help improve pig feeding strategies to address industry needs and consumer demands.

British Journal of Nutrition

https://doi.org/10.1017/S0007114515005218 Published online by Cambridge University Press

https://doi.org/10.1017/S0007114515005218 Published online by Cambridge University Press

Acknowledgements

The authors are grateful to J. Santos Silva and António Sequeira, from the Unidade de Investigação em Produção Animal (INIAV), for technical assistance.

The authors acknowledge the financial support from Fundação para a Ciência e a Tecnologia (FCT) grant (PTDC/CVT/2008/99210), CIISA project (UID/CVT/00276/2013) and individual fellowships to M. S. M. (SFRH/BPD/97432/2013). V. M. R. P. is an assistant researcher supported through an IF-FCT contract (2013 FCT investigator).

M. S. M. and J. A. M. P. performed the animal experiments. M. S. M., E. A. R., V. R. P., C. M. A. and J. A. M. P. performed the tissue sampling, laboratory work and prepared the manuscript. M. S. M., R. L., O. D., R. J. B. B. and J. A. M. P. were responsible for interpretation of the results and preparation of the manuscript. R. J. B. B. and J. A. M. P. were responsible for design of the study. All authors read and approved the findings of the study.

The authors declare that there are no conflicts of interest.

References

1. Eurostat (2015) Production of meat. http://epp.eurostat.ec.europa.eu/ (accessed March 2015).
2. Wood JD, Nute GR, Richardson RI, *et al.* (2004) Effects of breed, diet and muscle on fat deposition and eating quality in pigs. *Meat Sci* **67**, 651–667.
3. De Vol DL, McKeith FK, Bechtel PJ, *et al.* (1988) Variations in composition and palatability traits and relationships between muscle: characteristics and palatability in a random sample of pork carcasses. *J Anim Sci* **66**, 385–395.
4. Daszkiewicz T, Bak T & Denaburski J (2005) Quality of pork with a different intramuscular fat (IMF) content. *Pol J Food Nutr Sci* **14**, 31–35.
5. Doran O, Moule SK, Teye GA, *et al.* (2006) A reduced protein diet induces stearoyl-CoA desaturase protein expression in pig muscle but not in subcutaneous adipose tissue: relationship with intramuscular lipid formation. *Br J Nutr* **95**, 609–617.
6. Hyun Y, Kim JD, Ellis M, *et al.* (2007) Effect of dietary leucine and lysine levels on intramuscular fat content in finishing pigs. *Can J Anim Sci* **87**, 303–306.
7. Simon J (1999) Choline, betaine and methionine interactions in chickenm pigs and fish (including crustaceans). *World Poultry Sci* **J55**, 353–374.
8. Eklund M, Bauer E, Wamatu J, *et al.* (2005) Potential nutritional and physiological functions of betaine in livestock. *Nutr Res Rev* **18**, 31–48.
9. Huang QC, Xu ZR, Han XY, *et al.* (2008) Effect of dietary betaine supplementation on lipogenic enzyme activities and fatty acid synthase mRNA expression in finishing pigs. *Anim Feed Sci Tchnol* **140**, 365–375.
10. Huang QC, Xu ZR, Han XY, *et al.* (2006) Changes in hormones, growth factor and lipid metabolism in finishing pigs fed betaine. *Livest Sci* **105**, 78–85.
11. Jobgen WS, Fried SK, Fu WJ, *et al.* (2006) Regulatory role for the arginine-nitric oxide pathway in metabolism of energy substrates. *J Nutr Biochem* **17**, 571–588.
12. Tan B, Yin Y, Liu Z, *et al.* (2011) Dietary L-arginine supplementation differentially regulates expression of lipid-metabolic genes in porcine adipose tissue and skeletal muscle. *J Nutr Biochem* **22**, 441–445.
13. Tan BE, Yin YL, Liu ZQ, *et al.* (2009) Dietary L-arginine supplementation increases muscle gain and reduced body fat mass in growing-finishing pigs. *Amino Acids* **37**, 169–175.
14. Madeira MS, Pires VM, Alfaia CM, *et al.* (2014) Combined effects of dietary arginine, leucine and protein levels on fatty acid composition and gene expression in the muscle and subcutaneous adipose tissue of crossbred pigs. *Br J Nutr* **111**, 1521–1535.
15. Madeira MS, Pires VMR, Alfaia CM, *et al.* (2013) Differential effects of reduced protein diets on fatty acid composition and gene expression in muscle and subcutaneous adipose tissue of alentejana purebred and large white × landrace × pietrain crossbred pigs. *Br J Nutr* **110**, 216–229.
16. Dodson MV, Hausman GJ, Guan L, *et al.* (2010) Lipid metabolism, adipocyte depot physiology and utilization of meat animals as experimental models for metabolic research. *Int J Biol Sci* **6**, 691–699.
17. Bergen WG & Mersmann HJ (2005) Comparative aspects of lipid metabolism: impact on contemporary research and use of animal models. *J Nutr* **135**, 2499–2502.
18. Zhao S, Wang J., Song X, *et al.* (2010) Impact of dietary protein on lipid metabolism-related gene expression in porcine adipose tissue. *Nutr Metab (Lond)* **7**, 6.
19. Herman MA, Peroni OD, Villoria J, *et al.* (2012) A novel ChREBP isoform in adipose tissue regulates systemic glucose metabolism. *Nature* **484**, 333–338.
20. Dentin R, Pégorier JP, Benhamed F, *et al.* (2004) Hepatic glucokinase is required for the synergistic action of ChREBP and SREBP1-1c on glycolytic and lipogenic gene expression. *J Biol Chem* **279**, 20314–20326.
21. Liu CY, Grant AL, Kim KH, *et al.* (1994) Porcine somatotropin decreases acetyl-CoA carboxylase gene expression in porcine adipose tissue. *Domest Anim Endocrinol* **11**, 125–132.
22. Clarke SD (1993) Regulation of fatty acid synthase gene expression: an approach for reducing fat accumulation. *J Anim Sci* **71**, 1957–1965.
23. Nakamura MT & Nara TY (2004) Structure, function, and dietary regulation of delta6, delta5, and delta9 desaturases. *Annu Rev Nutr* **24**, 345–376.
24. Torra IP, Gervois P & Steals B (1999) Peroxisome proliferator-activated receptor alfa in metabolic disease, inflammation, atherosclerosis and aging. *Curr Opin Lipidol* **10**, 151–159.
25. Hocquette JF, Graulet B & Olivecrona T (1998) Lipoprotein lipase activity and mRNA levels in bovine tissues. *Comp Biochem Physiol B Biochem Mol Biol* **121**, 201–212.
26. Hocquette JF, Gondret F, Baéza E, *et al.* (2010) Intramuscular fat content in meat-producing animals: development, genetic and nutritional control, and identification of putative markers. *Animal* **4**, 303–319.
27. Association of Official Analytical Chemists (2000) *Official Methods of Analysis*, 17th ed. Arlington, VA: AOAC.
28. Clegg KM (1956) The application of the anthrone reagent to the estimation of starch in cereals. *J Sci Food Agric* **70**, 40–44.
29. Sukhija PS & Palmquist DL (1988) Rapid method for determination of total fatty acid content and composition of feedstuffs and feces. *J Agric Food Chem* **36**, 1202–1206.
30. Association of Official Analytical Chemists (2005) Amino acid analysis using Zorbax Eclipse-AAA columns and the Agilent 1100 HPLC. In *Official Methods of Analysis of the Association of Official Analytical Chemists International*, 18th ed. p. 473 [GW Latimer and W Horwitz, editors]. Gaithersburg, MD: AOAC International.
31. Henderson JW, Ricker RD, Bidlingmeyer BA, *et al.* (2000) *Rapid, Accurate, Sensitive and Reproducible Analysis of Amino Acids*. Palo Alto, CA: Agilent Technologies (Agilent Publication No. 5980-1193EN).

32. Folch J, Lees M & Stanley GHS (1957) A simple method for the isolation and purification of total lipids from animal tissues. *J Biol Chem* **226**, 497–509.
33. Carlson LA (1985) Extraction of lipids from human whole serum and lipoproteins and from rat liver tissue with methylene chloride-methanol: a comparison with extraction chloroform-methanol. *Clin Chim Acta* **149**, 89–93.
34. Raes K, De Smet SD & Demeyer D (2001) Effect of double-muscling in Belgian Blue young bulls on the intramuscular fatty acid composition with emphasis on conjugated linoleic acid and polyunsaturated fatty acids. *Anim Sci* **73**, 253–260.
35. Vandesompele J, De Preter K, Pattyn F, *et al.* (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* **3**, 7.
36. Andersen CL, Jensen JL & Orntoft TF (2004) Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res* **64**, 5245–5250.
37. Livak KJ & Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta C(T)) method. *Methods* **25**, 402–408.
38. Fleige S, Walf V, Huch S, *et al.* (2006) Comparison of relative mRNA quantification models and the impact of RNA integrity in quantitative real-time RT-PCR. *Biotechnol Lett* **28**, 1601–1613.
39. SAS Institute Inc. (2009) *SAS/STAT 9.2 User's Guide*, 2nd ed. Cary, NC: SAS Institute Inc.
40. Madeira MS, Alfaia CM, Costa P, *et al.* (2015) Effect of betaine and arginine in lysine-deficient diets on growth, carcass traits, and pork quality. *J Anim Sci* **93**, 4721–4733.
41. Alonso V, Campo MDM, Provincal L, *et al.* (2010) Effect of protein level in commercial diets on pork meat quality. *Meat Sci* **85**, 7–14.
42. Bessa RJB, Hughes RA, Jeronimo E, *et al.* (2013) Effect of pig breed and dietary protein level on selected fatty acids and stearoyl-coenzyme A desaturase protein expression in longissimus muscle and subcutaneous fat. *J Anim Sci* **91**, 4540–4546.
43. Feng J (1996) Effects of betaine on growth performance, carcass characteristics and meat quality and approach to mechanism of the effects in finishing swine. Master Thesis, Zhejiang Agriculture University, Hangzhou.
44. Martins JM, Neves JA, Freitas A, *et al.* (2012) Effect of long-term betaine supplementation on chemical and physical characteristics of three muscles from the Alentejano pig. *J Sci Food Agric* **92**, 2122–2127.
45. Matthews JO, Southern LL, Higbie AD, *et al.* (2001) Effects of betaine on growth, carcass characteristics, pork quality, and plasma metabolites of finishing pigs. *J Anim Sci* **79**, 722–728.
46. Yan XC (2001) Effects of betaine on growth hormone releasing factor (GRF) and approach to the mechanism in the hypothalamus of finishing pig. MD Dissertation, Zhejiang University, Hangzhou.
47. Ma X, Lin Y, Jiang Z, *et al.* (2010) Dietary arginine supplementation enchances antioxidative capacity and improves meat quality of finishing pigs. *Amino Acids* **38**, 95–102.
48. Rojas-Cano ML, Lara L, Lachica M, *et al.* (2011) Influence of betaine and conjugated linoleic acid on development of carcass cuts of Iberian pigs growing form 20 to 50 kg body weight. *Meat Sci* **88**, 525–530.
49. Ma YL, Wang L, Yong JH, *et al.* (2000) Effects of betaine on carcass composition and meat quality I finishing pigs. *J Ningxia Univ (Natural Science Edition)* **21**, 349–351.
50. Huang QC, Han XY, Xu ZR, *et al.* (2009) Betaine suppresses carnitine palmitoyltransferase I in skeletal muscle but not in liver of finishing pigs. *Lives Sci* **126**, 130–135.
51. Owen KQ, Nelssen JL, Goodband RD, *et al.* (1996) Effect of L-carnitine and soybean oil on growth performance and body composition of early-weaned pigs. *J Anim Sci* **74**, 1612–1619.
52. Go G, Wu G, Silvey DT, *et al.* (2012) Lipid metabolism in pigs fed supplemented conjugated linoleic acid and/or dietary arginine. *Amino Acids* **43**, 1713–1726.
53. Guo X, Tang R, Wang W, *et al.* (2011) Effects of dietary protein/carbohydrate ratio on fat deposition and gene expression of peroxisome proliferator activated receptor γ and heart fatty acid-binding protein of finishing pigs. *Lives Sci* **140**, 111–116.

British Journal of Nutrition

https://doi.org/10.1017/S0007114515005218 Published online by Cambridge University Press