Mol Biol Rep (2015) 42:559–565
DOI 10.1007/s11033-014-3801-6

Expression of genes related to mitochondrial function in Nellore cattle divergently ranked on residual feed intake

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Received: 24 October 2013 / Accepted: 18 October 2014 / Published online: 14 January 2015

Abstract Several measures have been proposed to investigate and improve feed efficiency in cattle. One of the most commonly used measure of feed efficiency is residual feed intake (RFI), which is estimated as the difference between actual feed intake and expected feed intake based on the animal's average live weight. This measure permits to identify and select the most efficient animals without selecting for higher mature weight. Mitochondrial function has been indicated as a major factor that influences RFI. The analysis of genes involved in mitochondrial function is therefore an alternative to identify molecular markers associated with higher feed efficiency. This study analyzed the expression of PGC1α, TFAM, UCP2 and UCP3 genes by quantitative real-time PCR in liver and muscle tissues of two groups of Nellore cattle divergently ranked on RFI values in order to evaluate the relationship of these genes with RFI. In liver tissue, higher expression of TFAM and UCP2 genes was observed in the negative RFI group. Expression of PGC1α gene did not differ significantly between the two groups, whereas UCP3 gene was not expressed in liver tissue. In muscle tissue, higher expression of TFAM gene was observed in the positive RFI group. Expression of PGC1α, UCP2 and UCP3 genes did not differ significantly between the two groups. These results suggest the use of TFAM and UCP2 as possible candidate gene markers in breeding programs designed to increase the feed efficiency of Nellore cattle.

Keywords Quantitative real-time PCR · Liver tissue · Muscle tissue · PGC1α · TFAM · UCP2 · UCP3

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Introduction

The production of more efficient animals increases the profitability of cattle farming. According to Oliveira [1], feed is the largest cost item in animal production, accounting for approximately 60 % of total costs. This value can reach 70 % in the case of feedlot cattle. One strategy to increase the profitability of the production system is to select high feed efficiency animals without compromising growth, reproduction or meat quality. Furthermore, improvement of feed efficiency minimizes

environmental impacts since it reduces the pasture area necessary for cattle feeding, as well as the production of environmental pollutants such as manure and methane, increasing sustainability of the system [2].

More than 40 measures have been proposed to improve feed efficiency [3]. The most commonly used measure is feed conversion (kg feed intake/kg gain). However, selection to improve feed conversion leads to an increase of mature size, compromising especially reproductive functions under conditions of limited nutrients [4]. As an alternative, in 2000, Canada and Australia started to study residual feed intake (RFI), a technique developed in the 1960s in the United States [5].

RFI is defined as the difference between actual feed intake and expected feed intake based on the weight gain and average live weight of the animal. Negative RFI animals are more efficient since observed feed intake is lower than expected intake. In contrast, positive RFI animals are less efficient, i.e., they consume more than predicted. Therefore, RFI is a measure that permits to identify and select more efficient animals without concomitantly selecting for higher weight gain and higher mature weight [6]. RFI is a trait of moderate heritability. In a meta-analysis, Del Claro et al. [7] reported a mean heritability for this trait of 0.29 and concluded that genetic variability exists wich could be explored for the identification and selection of genetically superior animals for feed efficiency.

Mitochondrial function has been indicated as a major factor that influences RFI [8]. Bottje et al. [9] demonstrated a greater loss of electrons in mitochondria of low feed efficient broiler chickens compared to high feed efficient animals. In another study, Bottje et al. [10] showed that broiler chickens with low feed efficiency (positive RFI) present a greater loss of electrons during transport and consequently release more heat, produce more reactive oxygen species and less ATP, thus consuming more feed. Similar results have been reported by Kolath et al. [11] for cattle, suggesting a relationship between mitochondrial respiration and feed efficiency. In this respect, the use of markers of mitochondrial function to identify animals with higher feed efficiency may be a suitable selection approach, since the analysis of RFI in each animal requires infrastructure and is time consuming, thus increasing the cost of the process. Possible candidate genes include PGC1, TFAM, UCP2 and UCP3, which play a role in mitochondrial function and biogenesis.

The objective of the present study was to evaluate the differential expression of genes involved in mitochondrial function in muscle and liver tissue of Nellore animals (*Bos taurus indicus*) divergently ranked on RFI, using quantitative real-time PCR (qRT-PCR).

Materials and methods

Animals and tissue collection

The animals used in this study belong to a stabilized Nellore herd of 160 dams and eight sires, which have been selected within herd (about 8 % of males and 60 % of females) since 1978 based on individual yearling weight performance. Animals of the entire contemporary group ($n = 60$) had been submitted to weight gain testing between May 4 and October 19, 2010. The animals were weighed without prior fasting three times per week on consecutive days. RFI values were estimated according to the model proposed by Koch et al. [6]. After the weight gain test, 24 animals were selected based on RFI values. These animals were slaughtered and samples of facial muscle tissue (masseter muscle) and liver tissue were collected immediately and stored in an RNAholder (BioAgency, São Paulo, Brazil) at −80 °C until RNA extraction.

Total RNA extraction and quantitative real-time PCR

Total RNA was extracted using the RNeasy Lipid Tissue Mini Kit (Qiagen, Valencia, CA, USA) according to manufacturer instructions. The extracted RNA was then treated with the RNase-Free DNase Set (Qiagen) for removal of genomic DNA. The concentration and purity of RNA were evaluated in a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Santa Clara, CA, USA, 2007) by measuring absorbance at 260, 280 and 230 nm. The reference values are ratios of 1.8 to 2 and ≥2, respectively. Total RNA quality was tested in an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA, 2009) using the Agilent RNA 6000 Nano Kit (Agilent). Values of RNA Integrity Number (RIN) equal or higher than 7.0 were classified as satisfactory. In addition, the absence of contamination of the samples with genomic DNA was confirmed in a Qubit® 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA, 2010) using the Qubit dsDNA Assay Kit (Invitrogen).

The first complementary DNA (cDNA) strand was synthesized from 1 μg total RNA using the SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen) according to the manufacturer's instructions.

The nucleotide sequences used to design the primers were obtained from mRNA sequences present in the GenBank database (http://www.ncbi.nlm.nih.gov) to prevent annealing to intron regions. Four primer pairs were designed for the target genes (PGC1α, TFAM, UCP2, and UCP3). In addition, two other genes (beta-actin and GAPDH) were used as reference genes. The Primer Express 3.0 software (Applied Biosystems, 2004) was used

Table 1 Sequence of the forward (F) and reverse (R) primers used for quantitative real-time PCR

Gene	Accession number (NCBI)	Sequence (5′–3′)	TM (°C)[a]	Amplicon size (bp)	Amplification efficiency in liver tissue (%)	Amplification efficiency in muscle tissue (%)
GAPDH F	NM_001034034	CCGTTCGACAGATAGCCGTAA	83	69	90.39	90.60
GAPDH R		CGACCTTCACCATCTTGTCTCA				
Beta-actin F	NM_173979	CAGCAAGCAGGAGTACGATGAG	83	85	106.34	90.22
Beta-actin R		AAGGGTGTAACGCAGCTAACAGT				
PGC1α F	NM_177945	TGTGCGCGTCACGTTCA	84	81	93.85	90.64
PGC1α R		TGAGCCTTTCGTGCTGGTACT				
UCP2 F	NM_001033611	CTGCCTATACCCGCCTGTTC	80	73	103.93	90.55
UCP2 R		TGGTTGGTAGACGAAATATCTAATGG				
UCP3 F	NM_174210	ACCACCTGCTCACCGACAA	84	64	–	91.33
UCP3 R		GCACAGAAGCCAGCTCCAA				
TFAM F	NM_001034016	TAGCCGGGTTGCAGTTTCC	85	63	96.95	91.32
TFAM R		GGTGGGCGGGACTACGA				

[a] Denaturation temperature of the amplicon

to design the primers (Table 1). Next, the primers selected were aligned in the appropriate databases using the NCBI BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST) in order to analyse them for specificity. The expression stability of the references genes was tested using the Genorm program (http://medgen.ugent.be/~jvdesomp/genorm) and Expression Suite Software v1.0 (Applied Biosystems, Foster, CA, USA, 2012).

In the present study, all qRT-PCR reactions were performed in a 7500 Real-Time PCR apparatus (Applied Biosystems, 2009) using the SYBR Green Master Mix (Applied Biosystems). This kit contains all components (except primers, samples, and RNA free water) necessary for PCR: 2X PCR buffer, dNTPs, $MgCl_2$, SYBR Green I Dye, AmpliTaq Gold® DNA polymerase, and ROX as a passive reference. The reaction mixture contained 1X SYBR Green Master Mix, 100 ng cDNA, and forward and reverse primers in a final volume of 12.5 µL. The following primer concentrations were determined by titration: 100 nM forward and reverse primers (100/100) for beta-actin, PGC1α and UCP2; 600 nM forward and reverse primers (600/600) for TFAM, and 300 nM forward and reverse primers (300/300) for UCP3. The qRT-PCR efficiency was calculated for each gene from a standard curve constructed with serial dilutions of cDNA (1:5). Only PCR primers showing an efficiency of 90–110 % were used [12].

The amplification conditions were 40 cycles at 50 °C for 2 min, 95 °C for 10 min, and 60 °C for 1 min. A dissociation analysis step was added after each reaction to monitor the specificity of the reactions. All reactions were performed in triplicate and cDNA of the 24 animals was quantified relative to the four target genes, two reference genes, and two tissues. In addition, a negative control for each gene (target and reference) and a positive control were included in all reactions since the samples had been tested previously. At the end of qRT-PCR, threshold cycle (*Ct*) values were calculated and analyzed with the Expression Suite Software v1.0 (Applied Biosystems, 2012).

Statistical analysis of the data

The *Ct* values calculated in triplicate for each animal in muscle and liver tissue were analyzed using the Statistical Analysis System program (SAS Institute, Cary, NC, USA, 2002). First, the geometric means of *Ct* values generated for the beta-actin and GAPDH reference genes, for each sample, for each tissue, and for each target gene were calculated as suggested by Vandesompele et al. [13]. For the analysis of the target genes (PGC1α, TFAM, UCP2 and UCP3) expression and geometric means of the reference genes (beta-actin and GAPDH), in each tissue, a mixed linear model was fitted using the mixed procedure of the SAS program (SAS Institute, 2002) as proposed by Steibel et al. [14]:

$$Y_{gikr} = T_{ig} + G_k + D_{ik} + e_{gikr},$$

where y_{gikr} is the *Ct* obtained from the thermocycler software for gene *g* (geometric mean of the reference genes and target gene) in the *r*th well of the plate (referring to the technical replicate) in a sample obtained from animal *k* of treatment *i* (positive or negative RFI group). T_{ig} is the group of animals effect *i* (RFI positive or negative) on the expression of gene *g*; G_{k} is the effect of slaughter groups

Table 2 Expression of the PGC1α, TFAM and UCP2 genes in liver tissue of Nellore cattle with positive and negative residual feed intake

Gene	RFI	Mean *Ct*	Standard error	*P* value (GxR)[a]
PGC1α	Negative	27.88	0.17	0.6448 [ns]
	Positive	27.80	0.17	
TFAM	Negative	32.30	0.15	<0.0001*
	Positive	33.23	0.16	
UCP2	Negative	25.74	0.17	<0.0001*
	Positive	26.55	0.17	

RFI residual feed intake, *Ct* threshold cycle, [a]*GxR* gene x RFI interaction

* Significant at the 5 % level, *ns* not significant

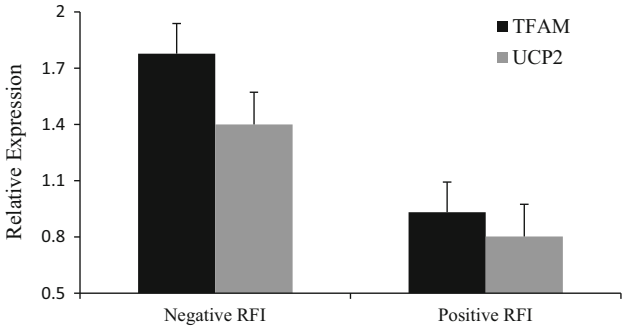

Fig. 1 Relative expression of the TFAM and UCP2 genes in liver tissue of Nellore cattle with positive and negative residual feed intake (RFI)

for animal k; D_{ik} is a random sampling specific effect which captures differences between samples shared by both genes, particularly those affecting RNA concentration such as different extraction and amplification efficiency, and e_{gikr} is a residual effect.

Results

In liver tissue, the expression levels of the TFAM and UCP2 genes differed significantly between the positive and negative RFI groups (Table 2). Analysis of the relative expression of the genes in liver tissue (Fig. 1) showed that expression of the TFAM gene was 1.9 times higher in negative RFI animals (more efficient) compared to positive RFI animals (less efficient). The expression of the UCP2 gene was 1.74 times higher in negative RFI animals compared to the positive RFI group.

In muscle tissue, only the expression of the TFAM gene differed significant between the positive and negative RFI groups (Table 3). The relative expression of the TFAM gene in muscle tissue was 1.72 times higher in positive RFI animals compared to the negative RFI group (Fig. 2).

Table 3 Expression of the PGC1α, TFAM, UCP2 and UCP3 genes in muscle tissue of Nellore cattle with positive and negative residual feed intake

Gene	RFI	Mean *Ct*	Standard error	P-value (GxR)[a]
PGC1α	Negative	24.08	0.26	0.2846 [ns]
	Positive	24.35	0.26	
TFAM	Negative	32.44	0.21	0.0018 *
	Positive	31.66	0.21	
UCP2	Negative	25.41	0.14	0.4124 [ns]
	Positive	25.54	0.14	
UCP3	Negative	25.93	0.35	0.1170 [ns]
	Positive	25.32	0.35	

RFI residual feed intake, *Ct* threshold cycle, [a]*GxR* gene x RFI interaction

* Significant at the 5 % level, *ns* not significant

Discussion

UCPs are proteins found in the inner mitochondrial membrane which are involved in different processes such as the control of ATP synthesis, production of reactive oxygen species, and regulation of fatty acid metabolism [15, 16]. The function of UCP2 is to prevent the formation of reactive oxygen species. This protein is also activated during the fever response to infection and is therefore related to immune function [17]. According to Ricquier et al. [18], the physiological function of UCP2 is associated with the regulation of metabolism, with the protein playing a role in diet-induced thermogenesis and weight loss. UCP3 is involved in body weight control. This protein is regulated by dietary energy availability and its expression increases in the presence of glucose and lipids [19], increasing energy expenditure [20].

In the present study, no significant differences were observed in the expression of the UCP2 or UCP3 gene in muscle tissue between positive and negative RFI animals. These results agree with the findings of Kolath [21] who found no difference in the expression of UCP2 or UCP3 in *longissimus dorsi* muscle of Angus cattle with positive and negative RFI values. Similarly, Kelly et al. [22], studying *longissimus dorsi* muscle in Limousin x Friesian heifers, observed no effect of RFI on the expression of UCP2. However, in that study, expression of the UCP3 gene tended to be higher in positive RFI animals (inefficient). In broiler chickens, Ojano-Dirain et al. [23] also observed a tendency towards higher expression of the avUCP gene (avian uncoupling protein, which shows 70 and 71 % of

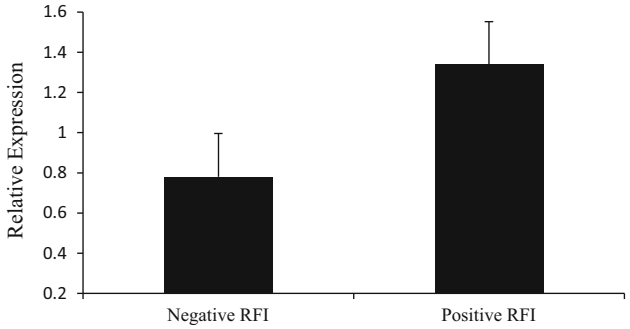

Fig. 2 Relative expression of the TFAM gene in muscle tissue of Nellore cattle with positive and negative residual feed intake (RFI)

identity with UCP2 and UCP3, respectively) in breast muscle tissue of animals with low feed efficiency.

In liver tissue, higher expression of the UCP2 gene was observed in the negative RFI group (more efficient). One of the functions of UCP2 is to reduce ATP production, an event increasing energy requirements and heat generation [24, 25]. The present finding is therefore unexpected since, according to Basarab et al. [26], more efficient animals consume less metabolizable energy, retain less energy, and generate less heat than less efficient animals. However, these authors evaluated the performance of beef cattle, but did not specifically analyze liver tissue. We found no studies in the literature investigating the expression of this gene in the liver.

The exact function of TFAM in mammals is unclear. Some evidence indicates that mitochondrial DNA is packaged with TFAM, with one DNA molecule being coated by 900 TFAM molecules [27–29]. A study using rat embryos has shown that TFAM regulates mitochondrial DNA copy number in mammals, since this number is directly proportional to total TFAM levels [30]. In addition, TFAM stimulates the transcription of mitochondrial DNA [31]. This protein acts in combination with secondary factors, TFBM1 and TFBM2, in the process of mitochondrial transcription. These proteins heterodimerize with mitochondrial RNA polymerase and, like histones, bind to mitochondrial DNA [32]. Proteins TFBM1 and TFBM2 also exert RNA methyltransferase activity. Taken together, these findings suggest that TFAM is a major regulator of mitochondrial biogenesis in mammals [33] and is a regulator of mitochondrial transcription and is activated in an attempt to overcome problems with oxidative phosphorylation through the stimulation of the co-activator PGC1, which controls the transactivators NRF1 and NRF2. The latter, in turn, regulate the expression of TFAM proteins [34]. In the present study, the expression of TFAM in muscle tissue was significantly higher in positive RFI animals (less efficient), whereas in liver tissue higher expression of this gene was observed in negative RFI animals (more efficient). These results disagree with those reported by Kelly et al. [22], who observed no significant differences in the expression of this gene in *longissimus dorsi* muscle of Limousin x Friesian heifers with high and low RFI.

The production of energy by oxidative phosphorylation requires the coordinated action of the genes involved in this process. One of the main regulators is co-activator PGC1α, which is essential to guarantee that the oxidative capacity is adjusted according to the energy needs of the cells [35, 36].

Messenger RNA of the PGC1 gene is found in tissues with high energy demand that are rich in mitochondria, such as the heart, skeletal muscle, brown fat, kidney and liver. Consistent with the profile of expression and induction, ectopic expression of PGC1 induces a cellular response that is related to the physiology and energy metabolism of mitochondria. Overexpression of this gene in white adipose tissue, muscle and heart stimulates mitochondrial biogenesis [37].

In adipocytes, PGC1α and β induce the production of the inner mitochondrial membrane uncoupling protein, UCP1, which uncouples fuel oxidation from ATP production, dissipating energy as heat. In muscle, PGC1 induces uncoupling protein UCP2, generating heat. These results suggest that PGC1 acts as a mitochondrial trigger to meet the specific metabolic requirements of tissues and plays a crucial role in the control of mitochondrial function [37].

In the present study, no significant differences in the expression levels of PGC1α in muscle or liver tissue were observed between positive and negative RFI animals. Similar results have been reported by Ojano-Dirain et al. [23] who studied the expression of this gene in duodenum and breast muscle of broiler chickens and found no differences between animals with high and low feed efficiency. Similarly, Bottje and Carstens [36], studying the expression of protein PGC1α by Western blotting, observed no differential expression of this protein in muscle tissue of high and low feed efficient broiler chickens. However, the authors detected a higher amount of this protein in the liver of low feed efficient animals.

The result of PGC1α expression in muscle tissue was consistent with that obtained for UCPs, i.e., PGC1α regulates the expression of these proteins, and there was not differential expression in both groups of animals in these genes. However, there were significant differences in UCP2 gene expression levels in liver tissue. This inconsistency was also observed for TFAM. PGC1α is a regulator of TFAM and expression of this gene differed significantly between positive and negative RFI animals. Inconsistencies in the expression of these genes have also been reported by Kelly et al. [22] for cattle of European

origin. Although these authors observed significant differences in the expression of the PGC1α gene in *longissimus dorsi* muscle between high and low RFI animals, they found no significant differences in the expression of UCP2 and TFAM between the same groups of animals.

In conclusion, this study showed differences in the expression levels of the UCP2 (liver tissue) and TFAM (liver and muscle tissue) genes in Nellore cattle divergently ranked on RFI. These results suggest that TFAM and UCP2 are possible candidate gene markers in breeding programs designed to increase the feed efficiency of Nellore cattle.

Acknowledgments This study was supported by the state funding agency São Paulo Research Foundation FAPESP (grants 2009/16118-5 and 2010/13502-6). We thank the Instituto de Zootecnia for providing the tissue samples and database used in this study. We are also grateful to Departamento de Tecnologia, Laboratório de Bioquímica e Biologia Molecular and Programa de Pós-graduação em Genética e Melhoramento Animal, Faculdade de Ciências Agrárias e Veterinárias for their cooperation.

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