Hepatic mitochondrial function in Hereford steers with divergent residual feed intake phenotypes¹

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ABSTRACT: Variations in phenotypic expression of feed efficiency could be associated with differences or inefficiencies in mitochondria function due to its impact on energy expenditure. The aim of this study was to determine hepatic mitochondrial density and function in terms of respiration, gene and protein expression, and enzyme activity of mitochondrial respiratory complex proteins, in steers of divergent residual feed intake (RFI) phenotypes. Hereford steers ($n = 111$ and $n = 122$ for year 1 and 2, respectively) were evaluated in postweaning 70 d standard test for RFI. Forty-six steers exhibiting the greatest ($n = 9$ and 16 for year 1 and 2; high-RFI) and the lowest ($n = 9$ and 12 for year 1 and 2; low-RFI) RFI values were selected for this study. After the test, steers were managed together until slaughter under grazing conditions until they reached the slaughter body weight. At slaughter, hepatic samples (biopsies) were obtained. Tissue respiration was evaluated using high-resolution respirometry methods. Data were analyzed using a mixed model that included RFI group as fixed effect and slaughter date and year as a random effect using PROC MIXED of SAS. RFI and dry matter intake were different ($P < 0.001$) between low

and high-RFI groups of year 1 and year 2. Basal respiration and maximum respiratory rate were greater ($P \leq 0.04$) for low than high-RFI steers when complex II substrates (succinate) were supplied. However, when Complex I substrates (glutamate/malate) were used maximum respiratory capacity tended to be greater ($P < 0.09$) for low vs. high-RFI steers. Low-RFI steers presented greater mitochondria density markers (greater ($P < 0.05$) citrate synthase (CS) activity and tended ($P \leq 0.08$) to have greater CS mRNA and mtDNA:nDNA ratio) than high-RFI steers. Hepatic expression *SDHA*, *UQCRC1*, and *CYC1* mRNA was greater ($P \leq 0.02$) and expression of *NDUFA4*, *NDUFA13*, *SDHD*, *UQCRH*, and *ATP5E* mRNA tended ($P \leq 0.10$) to be greater in low than high-RFI steers. Hepatic SDHA protein expression tended ($P < 0.08$) to be greater while succinate dehydrogenase activity was greater ($P = 0.04$) and NADH dehydrogenase activity was greater ($P = 0.03$) for low than high-RFI steers. High-efficiency steers (low-RFI) probably had greater efficiency in hepatic nutrient metabolism, which was strongly associated with greater hepatic mitochondrial density and functioning, mainly of mitochondrial complex II.

Key words: beef cattle, feed efficiency, liver, mitochondria, oxygen consumption

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INTRODUCTION

Residual feed intake (RFI), the difference between an animal's actual dry matter intake (DMI) and its expected DMI based on the requirements for maintenance and growth over a specified period is a useful measure of feed efficiency (Koch et al., 1963). Variations in phenotypic expression of feed efficiency could be associated to differences or inefficiencies in mitochondria function (Harper et al., 2002) due to its impact on energy expenditure (Rolfe and Brand, 1997).

Studies on mitochondrial function and phenotypic expression of feed efficiency in beef cattle are scarce. Reduced activity but increased expression of some proteins of respiratory complexes was reported in muscle and lymphocytes of high-efficiency steers when compared with low-efficiency steers (Sandelin et al., 2005; Ramos and Kerley, 2013). In addition, research showed greater muscle or hepatic mitochondrial respiration rates in low than high RFI steers, without differing in mitochondrial function or protein leak kinetics (Kolath et al., 2006; Lancaster et al., 2014). However, previous research has focused on isolated mitochondria in which several functional properties can be lost (Kuznetsov et al., 2008). Indeed, high-resolution respirometry studies in biopsies have been indicated as the most physiologically relevant approach to study intracellular energy metabolism (Gneiger et al., 2008). Particularly, mitochondria function in the liver, a central organ of metabolism that represents ~25% of whole body oxygen consumption (Baldwin et al., 2004), could be key for identifying pathways affecting cattle feed efficiency.

Thus, our hypothesis was that efficiency of hepatic mitochondrial function would be improved in low RFI when compared to high RFI animals. Our objective was to determine hepatic mitochondrial density and function in terms of respiration, gene and protein expression, and enzyme activity of mitochondrial respiratory complex proteins, in steers with divergent RFI phenotypes.

MATERIALS AND METHODS

Animals, RFI Test, and Tissue Sampling

The RFI tests were conducted at the Kiyú Test Station (San José, Uruguay; 32°S, 56°W)

of the Uruguayan Hereford Breed Association. Animal procedures were approved by the Animal Experimentation Committees of Universidad de la República (Uruguay). Animals used in this study were part of a larger project with the aim of building a training population of 1,000 animals for genomic selection of RFI in the Uruguayan Hereford breed (Navajas et al., 2014).

Hereford steers ($n = 111$ and $n = 122$ for year 1 and 2, respectively) were evaluated in postweaning tests for RFI. Steers came from 10 breeders and were sired by 24 pedigree bulls, confirmed by SNP paternity test before the RFI evaluation (data not shown). Steers entered the test with 290 ± 10 d of age, weighed 226 ± 33 kg, and had electronic tags for measuring individual feed intake with the GrowSafe system (Model 6000 GrowSafe Systems Ltd., Airdrie AB, Canada). The 70-d test started after 21 d of acclimatization to the feeding system and diet by the animals. All steers had ad libitum access to a total mixed ration (Table 1) and water. During the tests, animals were weighed fortnightly. Ultrasound measurements of subcutaneous back fat were taken by certified technicians. The RFI value for each animal was calculated for the test period as the difference (or residual) between the actual feed intake and the expected feed intake based on its body weight (BW) and average daily gain (Koch et al., 1963). Forty-six steers exhibiting the greatest ($n = 9$ and 16 for year 1 and 2; high-RFI) and the lowest ($n = 9$ and 12 for year 1 and 2; low-RFI) RFI values were selected for this study.

After the test, steers were managed together until slaughter in grazing conditions until they reached the slaughter BW (326 or 400 d; 500 ± 15 kg). During the summer, steers grazed on a sorghum pasture (*Sorghum vulgare*; herbage mass and allowance of 4,782 kg dry matter (DM)/ha and 9.5 ± 2.6 kg DM/d for year 1 and 5,294 kg DM/ha and 15.0 ± 2.5 kg DM/d for year 2; Table 1) without concentrate supplementation or supplemented with sorghum silage when pasture availability was limiting. During the fall-winter period, steers grazed on an oat pasture (*Avena sativa*; herbage mass and allowance of 1,735 kg DM/ha and 4.5 ± 2.3 kg DM/d for year 1 and 2,099 kg DM/ha and 7.5 ± 2.1 kg DM/d for year 2; Table 1) and were supplemented with 5.4 kg DM/animal of corn grain (Table 1). During the period that the animals consumed the forage diet, initial (final RFI test

weight), final (slaughter), and average daily gain did not differ between low and high RFI steers and their weight was not significantly different either (Table 2).

Steers were slaughtered in a commercial abattoir (Breeders and Packers Uruguay S.A; Durazno, Uruguay). Prior to transport, steer BW were recorded. Steers were stunned with a captive bolt

gun, exsanguinated, and carcass was weighed (Table 2). The liver was dissected, separated from surrounding connective tissue and liver samples from the caudal lobe were obtained ~20 min after the animal was killed. For respiration measurements, liver samples (~25 mg) were obtained by duplicate using a 14-gauge biopsy needle (Tru-Core-II Automatic Biopsy Instrument; Angiotech,

Table 1. Ingredients and chemical composition of total mixed ration, pastures and corn grain provided to steers

Component	TMR ¹		Sorghum grass		Oats grass		Corn grain	
	Year 1	Year 2	Year 1	Year 2	Year 1	Year 2	Year 1	Year 2
<i>Ingredients, % DM</i>								
Sorghum silage	55	38	...	...	...	...	...	...
Barley silage	21	35	...	...	...	...	...	...
Corn grain	21	20	...	...	...	...	...	...
Vitamin and mineral mix	3	7	...	...	...	...	...	...
<i>Chemical composition²</i>								
DM, %	47.5	54.2	23.2	16.1	18.1	18.3	89.6	89.3
CP, % DM	12.3	13.5	8.1	9.8	22.5	22.5	9.1	9.8
NDF, %DM	39.8	42.8	65.2	62.3	50.5	48.3	10.3	10.9
ADF, %DM	26.3	27.8	40.7	41.5	30.5	30.2	3.2	3.6
ME (Mcal/kgDM) ³	2.96	2.48	1.98	1.96	2.26	2.25	2.51	2.63

ADF = acid detergent fiber; CP = crude protein; DM = dry matter; NDF = neutral detergent fiber.

¹Total Mixed Ration offered during the RFI test.

²Estimated by AOAC (2000).

³ME = Metabolizable energy of pastures estimated: ME = $[-0.027 + 0.0428 \times (88.9 - 0.779 \times \text{acid detergent fiber})] \times 0.82$ according to (Reid et al., 1991).

Table 2. Steer performances according to RFI phenotype

Item	Year 1				Year 2				Pooled data			
	Low-RFI (n = 9)	High-RFI (n = 9)	SE	P-value	Low-RFI (n = 12)	High-RFI (n = 16)	SE	P-value	Low-RFI (n = 21)	High-RFI (n = 25)	SE	P-value
RFI, kg DM/d	-0.69	0.90	0.62	<0.01	-0.79	0.62	0.70	<0.01	-0.75	0.72	0.66	<0.01
Feed intake, kg DM/d	9.2	10.6	0.53	<0.01	9.0	10.5	0.33	<0.01	9.0	10.4	0.31	<0.01
RFI test ADG, kg/d ¹	1.61	1.52	0.84	ns	1.41	1.46	0.65	ns	1.51	1.48	0.53	Ns
RFI test initial weight, kg	226.2	218.7	16.62	ns	243.8	246.5	21.61	ns	225.8	224.2	16.11	Ns
RFI test final weight, kg	364.7	354.4	19.89	ns	342.9	349.3	25.13	ns	351.4	343.4	14.72	Ns
Sorghum ADG, kg/d	0.92	0.93	0.51	ns	0.95	0.96	0.49	ns	0.91	0.94	0.48	Ns
Oat ADG, kg/d	0.84	0.81	0.48	ns	0.82	0.83	0.51	ns	0.83	0.82	0.53	Ns
Slaughter weight, kg	520.6	508.7	11.45	ns	505.1	506.1	14.01	ns	510.4	508.7	7.43	Ns
Carcass weight, kg	277.5	274.1	5.11	ns	260.7	263.4	4.12	ns	267.9	267.1	3.44	Ns
Liver weight, kg	5.31	5.79	0.25	0.06	5.62	5.81	0.22	0.10	5.56	5.74	0.18	0.09
Liver relative weight, g/kg EBW ²	11.5	11.8	0.60	ns	13.08	13.21	0.28	ns	12.29	12.65	0.45	Ns

DM = dry matter; RFI = residual feed intake.

¹ADG = average daily gain.

²EBW = empty body weight calculated according to NRC, 2000.

Lausanne, Switzerland), immersed immediately in 1 mL of modified University of Wisconsin medium [20 mM histidine, 20 mM succinate, 3 mM glutathione, 1 μ M leupeptin, 2 mM glutamate, 2 mM malate, 2 mM ATP, 0.5 mM ethylene glycol tetraacetic acid (EGTA), 3 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 60 mM 3-(N-morpholino)propanesulfonic acid (MOPS), 20 mM taurine, 10 mM KH_2PO_4 , 20 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES), 110 mM sucrose, 1 g/L BSA, and 10% dimethylsulfoxide, pH 7.4] in cryotubes and frozen in a stepwise freezing procedure (Garcia-Roche et al., 2018). For gene, protein expression and enzymes activity analyses, a liver sample (10 g) was snap-frozen in liquid nitrogen. Both sets of samples were stored at -80°C until analyses.

High-resolution respirometry assays were performed in all samples ($n = 46$, steers of year 1 and 2) while hepatic DNA, RNA and protein contents, quantitative real-time qPCR, western blot, and enzyme activity assays were only determined in year 1 animals ($n = 18$) when differences between high and low-RFI steers were the greatest.

Oxygen Consumption Rate Measurements

The evaluation of mitochondrial function was analyzed by studying the oxygen consumption in liver frozen samples (Garcia-Roche et al., 2018). Briefly, samples were measured in 2.4 mL chambers using high-resolution respirometry in an Oroboros Oxygraph 2-k (Oroboros Instruments, Innsbruck, Austria) at 37°C . Chambers were previously calibrated with MIR05 mitochondrial respiration medium (0.5 mM EGTA, 3 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 60 mM MOPS, 20 mM taurine, 10 mM KH_2PO_4 , 20 mM HEPES, 110 mM sucrose, 1g/L BSA, pH 7.1) with a calculated saturated oxygen concentration of 190 nmol O_2 per mL at 100 kPa barometric pressure. Weight-specific oxygen flux ($\text{pmol O}_2 \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ wet weight) was calculated using the DatLab 4 analysis software (Oroboros Instruments). A substrate-inhibitor titration protocol was utilized (Fig. 1). The assay was initiated with the addition cytochrome *c* titration (1 to 3.5 μM ; controlling outer mitochondrial membrane integrity), before to addition of complex specific substrates: 10 mM glutamate and 5 mM malate for complex I or 20 mM succinate for complex II, followed by ADP (1 to 4 mM). Oligomycin (2 μM) was added to inhibit ATPase activity and carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP; 1 to 4 μM ; controlling outer mitochondrial membrane integrity) was added to uncouple oxidative

Figure 1. Representative high-resolution respirometry experiment and titration protocol. Respiration rates were measured at 37°C and substrate-inhibitor titration protocol was utilized. Oxygen consumption rates were measured after the sequential addition of Cytochrome *c* (1 to 3.5 μM), addition of complex specific substrates (10 mM Glutamate and 5 mM Malate for complex I or 20 mM Succinate for complex II), ADP (1 to 4 mM), Oligomycin (2 μM) and 1 mM of Rotenone to inhibit complex I or Antimycin A 1 mM to inhibit complex II. Respiratory parameters and indices: Non-mitochondrial respiration (*rate e*) is substrate from other values: State 4 respiration (*rate a*); State 3 respiration after the addition of ADP that resembles the basal respiration of the tissue at saturating concentrations of substrates and ADP (*rate b*); Oligomycin-resistant respiration (*rate c*); Maximum respiratory rate (*rate d*). ATP-linked respiration (*rate b-rate c*). Spare respiratory capacity (*rate d-rate b*); Coupling efficiency ($((\text{rate } b - \text{rate } c) / \text{rate } b)$) [adapted to Garcia-Roche et al., 2018; Brand and Nicholls, 2011).

phosphorylation. Finally, 1 mM rotenone was added to inhibit complex I or antimycin A 1 mM to inhibit complex III and consequently complex II. Titration curves were done for ADP and FCCP in order to reach saturating concentrations for each reagent. Different respiratory parameters and indices were obtained from oxygen consumption rate measurements using the DatLab 4 analysis software (Oroboros Instruments).

Hepatic DNA, RNA, and Protein Concentrations

The high-salt protocol procedure was used to determine genomic DNA concentrations (Sunnucks and Hales, 1996) and total RNA was isolated using TRIzol (Invitrogen, Life Technologies, Carlsbad, CA, USA) starting from 50 mg of tissue for each analysis. Concentrations of genomic DNA and total RNA were determined by measuring absorbance at 260 nm (NanoDrop ND-1000 Spectrophotometer; Nanodrop Technologies Inc., Wilmington, DE, USA), and purity of all DNA and RNA isolates were assessed from 260/280 nm and 260/230 nm absorbance ratios. The Coomassie brilliant blue G (Bradford) assay was used to determine protein concentration of liver homogenates (300 mg of tissue; Polytron; Brinkmann, Westbury,

NY) in 10 volumes of modified MRM buffer (5 mM KH_2PO_4 , 1 mM EGTA, 5 mM MOPS, 300 mM sucrose, pH 7.4) Hepatic DNA, RNA, and protein contents were calculated by multiplying liver weight by DNA, RNA, and protein concentrations, respectively. Tissue concentrations of protein and nucleic acids were used to estimate cellularity indices according to Casal et al. (2014), relative cell number (hyperplasia; DNA concentration), cell size (hypertrophy; protein:DNA), or protein synthesis capacity (RNA concentration and RNA:protein ratio). Isolated DNA and RNA were used for real time qPCR analyses and, protein homogenates for mitochondrial extraction and enzymes activity analyses.

Quantitative Real-time qPCR

Isolated RNA was precipitated with lithium chloride, DNase-treated with a DNA-Free kit (Applied Biosystems Ambion, Austin, TX, USA). Integrity was assessed by electrophoresis in 1% agarose gel. Isolated RNA was stored at -80°C until analyzed. The SuperScript III Transcriptase (Invitrogen), with random hexamers and 2 μg of total RNA as a template, was used to conduct the reverse transcription. The cDNA was stored at -20°C until its use. Primers (Supplementary Table S1) to specifically amplify cDNA of 12 target genes of the mitochondrial respiratory complex and citrate synthase and 3 internal control genes: β -actin (*ACTB*), hypoxanthine phosphoribosyl-transferase (*HPRT1*) and ribosomal protein L19 (*RPL19*), were obtained from literature or specifically designed using the Primer3 website (<http://bioinfo.ut.ee/primer3-0.4.0/>) based on bovine nucleotide sequences available from NCBI (<http://www.ncbi.nlm.nih.gov/>). Before use, primer product size (as estimated by 1% agarose gel separation) and sequence (Macrogen Inc., Seoul, Korea) were determined to ensure that primers produced the desired amplicons (data not shown). The genes selected for this study were selected based on previous results from a microarray analysis (Laporta et al., 2014). Mitochondrial respiratory complex candidate genes selected were: NADH:ubiquinone oxidoreductase subunit C1 (*NDUFC1*, complex I); NADH:ubiquinone oxidoreductase subunit A4 (*NDUFA4*, complex I); NADH:ubiquinone oxidoreductase subunit A13 (*NDUFA13*, complex I); succinate dehydrogenase complex, subunit A, flavoprotein (Fp) (*SDHA*, complex II); succinate dehydrogenase complex, subunit D (*SDHD*, complex II); ubiquinol-cytochrome c reductase core

protein I (*UQCRC1*, complex III); ubiquinol-cytochrome c reductase hinge protein (*UQCRH*, complex III); cytochrome c oxidase assembly factor (*COX19*, complex IV); cytochrome c-1 (*CYCI*, complex IV); ATP synthase, H^+ transporting, mitochondrial F1 complex, epsilon subunit (*ATP5E*, complex V); ATP synthase, H^+ transporting, mitochondrial F1 complex, O subunit (*ATP5O*, complex V); and citrate synthase (*CS*).

Real-time qPCR reactions were performed according to Casal et al. (2014) in a total volume of 15 μL using KAPA SYBR FAST Universal 2X qPCR Master Mix (Kapa Biosystems, Inc. Woburn, MA, USA) using the following standard amplification conditions: 3 min at 95°C and 40 cycles of 3 s at 95°C , 40 s at 60°C , and 10 s at 72°C in a Rotor-Gene 6000 (Corbett Life Sciences, Sidney, Australia). Dissociation curves were run on all samples to detect primer dimers, contamination, or presence of other amplicons. Each run included a pool of total RNA from bovine liver samples analyzed in triplicate to be used as the basis for the comparative expression results (exogenous control) and duplicate tubes of water (nontemplate control). Gene expression was measured by relative quantification (Pfaffl, 2009) to the endogenous control and normalized to the geometric mean expression of internal control genes (*ACTB*, *HPRT1*, and *RPL19*). Expression stability of 3 selected house-keeping genes was evaluated using the MS-Excel add-in Normfinder (MDL, Aarhus, Denmark). The stability values obtained with Normfinder they were 0.343, 0.379, and 0.285 for *ACTB*, *HPRT1*, and *RPL19*, respectively. Amplification efficiencies of target and endogenous control genes were estimated by linear regression of a dilution cDNA curve ($n = 5$ dilutions, from 100 to 6.25 ng/tube (Supplementary Table S1). The intra and interassay CV for all genes were less than 1.4% and 4.2%, respectively.

In addition, mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) ratio (mtDNA:nDNA ratio) was used to estimate tissue mitochondrial density (Guo et al., 2009). The real-time qPCR was carried out in separate tubes for mtDNA and nDNA amplification using PCR primers (Supplementary Table S1) for detecting the mitochondrial encoded cytochrome c oxidase I (*mt-COI*, mitochondrial gene) and NADH:ubiquinone oxidoreductase core subunit V1 (*NDUFV1*, nuclear gene), respectively and real-time qPCR reactions were conducted as described above. The cycle number (Ct) at which the fluorescent signal of a given reaction crossed the threshold value was used as basis for quantification

of mtDNA and nDNA copy numbers. The difference between cycle numbers measured at the threshold (designated β) was used to calculate the relative ratio of mtDNA:nDNA using the equation: 2^{β} .

Western Blot Analysis

Total protein extracts of hepatic samples were obtained from homogenates 10% (wt/vol) in ice-cold RIPA buffer (with protease inhibitors) [50 mM Tris, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1% Triton X-100 and 0.1% Sodium dodecyl sulfate (SDS)] and stored at -80°C until its use. Protein samples (30 to 40 μg) were separated on a 12% SDS-polyacrylamide gel and transferred to nitrocellulose membrane overnight at 20 mV and 4°C . The immunochemical detection of ATP synthase subunit alpha (ATP5A), succinate dehydrogenase subunit A (SDHA) or β -actin (ACTB) proteins were performed using specific mouse monoclonal anti-ATP5A, anti-SDHA, or anti-ACT antibodies (Abcam, Cambridge, UK). The membranes were blocked for 1 h at room temperature in TBS buffer (50 mM Tris-HCL, 150 mM NaCl, pH 7.4) with 0.6% (vol/vol) of Tween-20 and 5% (wt/vol) of milk. Next, membranes were incubated with the mouse monoclonal anti-ATP5A and anti-SDHA antibodies (1:2,000) and mouse monoclonal anti-ACTB (1:3,000) in the same blocking buffer (without milk) for 1 h at room temperature with gentle agitation. Subsequently, membranes were washed twice for 10 min and 3 times for 5 min in TBS plus 0.6% Tween-20. Then, membranes were incubated with anti-mouse IR Dye 680CW (Li-COR, Lincoln, NE, USA) at 1:15,000 for 1 h in TBS plus 0.6% Tween-20. The membranes were washed four times for 15 min, and signals were detected using the ODYSSEY CLx Infrared Imaging System (Li-COR) and analyzed using ODYSSEY CLx and ImageJ-software. Density of the area of each lane was measured. The relative levels of ATP5A and SDHA were normalized with ACTB density and normalized values for high-RFI were expressed as a percentage relative to the low-RFI group.

Enzyme Activity

Enzyme activity assays were adapted from Spinazzi et al. (2012) and Janssen et al. (2007). Assays were performed in duplicate at 37°C using a final volume of 500 μL in a UV-2401 PC spectrophotometer (Shimadzu Corporation, Tokyo, Japan). Mitochondria from liver protein homogenates were isolated by differential centrifugation

according to Cassina and Radi (1996). Enzyme activities were expressed as mU per mg of mitochondrial or homogenate protein.

Citrate synthase activity: Since citrate synthase is present only in mitochondria, it is commonly used as an indirect marker of tissue mitochondrial density. The assay was performed at 412 nm following the reduction of 0.1 mM 5,5'-dithiobis(2-nitrobenzoic acid) in the presence of 30 to 80 μg of liver homogenate protein, 0.2 mM acetyl-CoA in a medium with 100 mM Tris-HCl, pH 7.5, and 0.1% Triton X-100. The reaction was incubated 5 min at 37°C , and the absorbance change determined 5 min before and after the addition of 0.25 mM oxalacetic acid and an extinction coefficient of $13.6\text{ mM}^{-1}\text{cm}^{-1}$ was used to quantify enzyme activity.

Nicotinamide adenine dinucleotide (NADH) ubiquinone oxidoreductase activity: The assay was performed at 600 nm following the reduction of 2,6-dichlorophenolindophenol (DCPIP) by decylubiquinone in the presence of 80 to 120 μg of mitochondrial protein, 60 μM DCPIP, 10 μM antimycin, 3.5 mg/mL bovine serum albumin and 70 μM decylubiquinone in a 25 mM phosphate buffer, pH 7.2. The reaction was initiated by the addition of 200 μM NADH. The decrease in the optical density at 600 nm measured 5 min before and after the addition of 2 μM rotenone and the extinction coefficient of $21\text{ mM}^{-1}\text{cm}^{-1}$ were used to quantify enzyme activity.

Succinate dehydrogenase (SDH) activity: The assay was performed at 600 nm following the reduction of DCPIP by decylubiquinone in the presence of 80 to 120 μg of mitochondrial protein, 50 μM DCPIP, 1 mM potassium cyanide, 5 μM rotenone and 50 μM decylubiquinone in a 25 mM phosphate buffer, pH 7.2. The absorbance change determined 5 min before and after addition of 10 mM succinate and an extinction coefficient of $21\text{ mM}^{-1}\text{cm}^{-1}$ was used to quantify the enzyme activity.

Statistical Analyses

Data were analyzed using the SAS System program (SAS Institute Inc., Cary, NC, USA). The UNIVARIATE analyses were performed on all variables to identify outliers and inconsistencies and to verify normality of residuals. Respiratory data were analyzed using the MIXED procedure with a model that included RFI group as fixed effect and slaughter date and year as a random effect. Liver weight (fixed) and breeders and sires data (random) were included in the model and when covariance parameter estimates were zero or close to zero it was

removed from the model. All the other data were analyzed using the MIXED procedure with a model that included RFI group as fixed effect and slaughter date as a random effect. Correlation coefficients were estimated using the CORR procedure. Mean separation was performed using the Tukey test, and differences were considered significant at $P \leq 0.05$ and a trend when $0.05 < P \leq 0.10$. Data are presented as least square means $\pm$ pooled standard errors.

RESULTS

RFI Test

RFI and DMI were different ($P < 0.001$) between low and high-RFI groups of year 1, year 2 and when pooled data were analyzed together (Table 2). However, RFI groups neither differed in the initial and final weights of the RFI test, average daily gain, nor in preslaughter and carcass weights (Table 2).

Hepatic Oxygen Consumption Rate

A representative oxygen consumption assay is shown in Fig. 1. Basal respiration (state 3) was greater ($P = 0.03$) for low than high-RFI steers when complex II substrates were supplied but not when complex I substrates were added (Table 3). Maximum respiratory rate was greater ($P = 0.01$) with complex II substrates and tended ($P = 0.09$)

to be greater with complex I substrates for low than high-RFI steers (Table 3). The ATP-linked respiratory, spare respiratory capacity, and coupling efficiency did not differ between RFI groups (Table 3). RFI values showed a moderate and negative correlation with maximum respiratory rate of complex II ($r = -0.33$, $P = 0.05$, $n = 17$).

Liver Mass, Cellularity Indices, and Mitochondrial Density

High-RFI steers tended ($P < 0.06$) to have greater absolute liver weight than low-RFI steers. Hepatic protein concentration tended ($P < 0.08$) to be greater for low than high-RFI steers but DNA and RNA concentrations as well as protein:DNA and RNA:protein ratios did not differ between RFI groups. Low-RFI steers presented greater ($P < 0.05$) CS activity and tended ($P \leq 0.08$) to have greater CS mRNA and mtDNA:nDNA ratio than high-RFI steers (Table 4).

Hepatic Gene Expression of Mitochondrial Respiratory Chain Proteins

Hepatic expression *SDHA*, *UQCRC1*, and *CYCI* mRNA was greater ($P \leq 0.02$) and expression of *NDUFA4*, *NDUFA13*, *SDHD*, *UQCRH*, and *ATP5E* mRNA tended ($P \leq 0.10$) to be greater in low than high-RFI steers (Table 5). However, *NDUFC1*, *COX19*, and *ATP5O* mRNA did not differ between RFI groups (Table 5). RFI values showed a moderate to high and negative correlation

Table 3. Hepatic respiratory parameters (pmol O₂ min⁻¹·mg⁻¹) and indices of steers differing in RFI phenotype

Item	RFI group		SE	P-value
	Low (n = 21)	High (n = 25)		
<i>Complex I^l</i>				
Respiratory parameters and indices ²				
Basal respiration (State 3)	6.23	6.32	0.57	ns
Maximum respiratory rate	8.08	6.34	0.71	0.09
ATP-linked respiration	3.42	3.56	0.73	ns
Spare respiratory capacity	1.06	0.95	0.19	ns
Coupling efficiency	0.56	0.47	0.05	ns
<i>Complex II^l</i>				
Respiratory parameters and indices ²				
Basal respiration (State 3)	31.46	23.71	3.58	0.03
Maximum respiratory rate	38.66	28.70	3.71	0.01
ATP-linked respiration	5.68	5.72	0.65	ns
Spare respiratory capacity	4.75	6.23	1.61	ns
Coupling efficiency	0.22	0.21	0.06	ns

¹Mitochondrial Complex I NADH ubiquinone oxidoreductase or Complex II Succinate dehydrogenase.

²Respiratory parameters and indices according to Figure 1.

Table 4. Hepatic tissue characterization according to RFI phenotype

Item	RFI group		SE	P-value
	Low (n = 9)	High (n = 9)		
<i>Tissue cellularity indices</i>				
Protein, mg/g tissue	203.6	184.4	7.2	0.08
RNA, µg/mg tissue	1.43	1.30	0.12	ns
DNA, µg/mg tissue	1.66	1.36	0.26	ns
Protein:DNA ratio	122.5	135.5	28.2	ns
RNA:Protein ratio	0.007	0.007	0.001	ns
<i>Mitochondrial density indices</i>				
Citrate synthase activity, mU/mg protein	48	43	2	0.05
mRNA Citrate synthase, AU	1.57	0.96	0.55	0.08
mtDNA/nDNA ratio, AU ¹	148.1	89.7	29.8	0.08

RFI = residual feed intake.

¹mtDNA/nDNA ratio = mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) ratio

coefficients with hepatic *CS*, *SDHA*, and *CYC1* mRNA ($r = -0.59$, -0.51 , and -0.50 , respectively, $P \leq 0.04$, $n = 17$).

Hepatic Mitochondrial Respiratory Chain Protein Expression and Enzyme Activity

Hepatic SDHA protein expression tended ($P < 0.08$) to be greater while SDH activity was greater ($P = 0.04$) for low than high-RFI steers (Figs. 2 and 3). RFI values and SDHA protein expression were higher and negatively correlated ($r = -0.77$, $P < 0.01$, $n = 17$). In addition, NADH dehydrogenase activity was also greater ($P = 0.03$) for low than high-RFI steers (Fig. 3). Hepatic ATP5A protein expression did not differ between RFI groups (data not shown).

DISCUSSION

This study determined hepatic mitochondrial density and function in steers with divergent RFI phenotypes. Results showed that high-feed efficiency steers (low-RFI) would have a better efficiency of nutrient utilization associated with greater hepatic mitochondrial density and better mitochondrial functioning (greater gene and protein expression and enzyme activity) than low-feed efficient steers (high-RFI). Herein, indicators of liver mitochondrial function are discussed in the context of beef cattle feed efficiency.

In agreement with previous reports, low-RFI steers achieved similar body and carcass weight and had a similar growth rate with a reduced DMI than high-RFI steers (Kolath et al., 2006; Castro Bulle et al., 2007; Lancaster et al., 2014). Individual

Table 5. Hepatic gene expression of mitochondrial respiratory complex (Cx) proteins of steers differing in RFI phenotype

Gene ²	Complex	RFI group		SE	P-value
		Low ($n = 9$)	High ($n = 9$)		
<i>NDUFC1</i>	CxI	0.85	0.91	0.35	ns
<i>NDUFA4</i>	CxI	0.93	0.69	0.20	0.06
<i>NDUFA13</i>	CxI	1.18	0.91	0.14	0.09
<i>SDHA</i>	CxII	1.26	0.84	0.19	0.02
<i>SDHD</i>	CxII	1.01	0.62	0.19	0.06
<i>UQCRC1</i>	CxIII	1.28	0.96	0.23	0.07
<i>UQCRC1</i>	CxIII	1.02	0.59	0.30	0.02
<i>CYC1</i>	CxIV	1.05	0.83	0.17	<0.01
<i>COX19</i>	CxIV	0.91	0.99	0.10	ns
<i>ATP5E</i>	CxV	1.06	0.85	0.33	0.10
<i>ATP5O</i>	CxV	0.98	1.03	0.27	ns

RFI = residual feed intake.

¹Genes: NADH:ubiquinone oxidoreductase subunit C1 (*NDUFC1*), NADH:ubiquinone oxidoreductase subunit A4 (*NDUFA4*), NADH:ubiquinone oxidoreductase subunit A13 (*NDUFA13*), succinate dehydrogenase complex, subunit A, flavoprotein (Fp) (*SDHA*), succinate dehydrogenase complex, subunit D (*SDHD*), ubiquinol-cytochrome c reductase core protein I (*UQCRC1*), ubiquinol-cytochrome c reductase hinge protein (*UQCRC1*), cytochrome c oxidase assembly factor (*COX19*); cytochrome c-1 (*CYC1*), ATP synthase, H⁺ transporting, mitochondrial F1 complex, epsilon subunit (*ATP5E*); ATP synthase, H⁺ transporting, mitochondrial F1 complex, O subunit (*ATP5O*).

Figure 2. Western blot analysis with monoclonal anti-SDHA antibody in liver (A). Means $\pm$ SE of pixels intensity expressed with proportion of low-RFI animals (B) according to steer RFI phenotype ($n = 9$ and 9 for low and high-RFI steers, respectively).

Figure 3. Hepatic enzyme activity (mU/mg of mitochondrial protein) of NADH dehydrogenase (■) and Succinate dehydrogenase (□) of steers differing in RFI phenotypes. Each bar represents the mean $\pm$ SE of 18 observations ($n = 9$ and 9 for low and high-RFI steers, respectively).

variations in feed efficiency resulted from differences in feed intake and, consequently, would impact on the metabolic workload of visceral organs, especially the liver (Montanholi et al., 2017). Indeed, high-RFI steers tended to present greater liver mass compared to low-RFI steers. However, this trend disappeared when liver mass was expressed as relative to empty body weight (g/kg EBW) which would indicate that the increase in liver mass associated with DMI, was a compensatory mechanism for metabolic nutrient processing.

Lancaster et al. (2014) and Montanholi et al. (2017) observed no differences in liver biometrics when efficient and inefficient steers were compared, suggesting gravimetric assessments of organ function do not capture structural and functional changes such as those due to variation in feed efficiency. However, in agreement with the trend for a greater liver protein content for low vs. high-RFI steers, these latter authors (Montanholi et al., 2017) reported larger hepatocytes and hepatocyte nuclei in efficient than inefficient steers. These results would suggest an increased hepatic metabolic rate with improved feed efficiency.

The present study, to our knowledge, is the first work that compared mitochondrial respiration of low vs. high RFI steers using high-resolution respirometry in cryopreserved hepatic biopsies. Low-RFI steers exhibited a greater basal respiration (state 3 respirations after addition of ADP; maximum ADP-stimulated respiration) and

maximum respiratory rate when provided succinate (complex II) as respiratory substrates with respect to high-RFI steers. The greater oxygen consumption recorded in low vs. high-RFI steers at the same level of substrates reflected a greater capacity to obtain ATP per unit of substrate. Similar to our results, greater basal respiration was also reported in muscle and hepatic isolated mitochondria for low than high-RFI steers and heifers (Kolath et al., 2006; Lancaster et al., 2014).

The rate of energy use by an organism—the metabolic rate—is directly proportional to the rate of oxygen consumption. Therefore, rate of oxygen consumption can be measured and used to infer metabolic rate indirectly (Brand and Nicholls, 2011). Maximum respiratory rate is induced by the uncoupler (FCCP) and represents maximum activity of electron transport and substrate oxidation achievable by the cells (Brand and Nicholls, 2011), being, when decreased, a strong indicator of potential mitochondrial dysfunction (Hill et al., 2012). The greater feed efficiency of low than high-RFI steers would be related to greater availability of respiratory chains to cope with their metabolic demands in the former ones and could be considered as an indirect indicator of mitochondrial density. Indeed, a greater amount of available respiratory chains activity in low than high-RFI steers may be the result of a greater number of mitochondria per unit of hepatic weight in the former ones as indicated by differences in mitochondrial content biomarkers (CS activity and mRNA, mtDNA:nDNA ratio; Larsen et al., 2012). These differences between low and high-RFI steers could be explained by a greater proton or electron leak, uncoupling proteins or protein modifications (oxidation, nitration, acetylation; Bottje and Carstens, 2009; Cimen et al., 2010). Thus, low-RFI steers would have a greater reserve capacity to protect cells, among other stresses, from energy/substrate insufficiency and oxidative stress than high-RFI steers.

In addition, in the present study, stronger evidence on differences on maximum respiratory rate between low and high-RFI steers was observed when substrates for complex II than for the complex I was supplied. Complex II is the only enzyme complex component of the electron transport chain that not only catalyzes a tricarboxylic acid (TCA) cycle reaction (oxidation of succinate to fumarate with the reduction of ubiquinone to ubiquinol by coupling the two reactions together) but it is also a key step in the use of ruminal propionate for neoglucogenesis. These characteristics confer this enzyme complex an unique position in the metabolism to serve as a

regulated source of the maximum and spare respiratory capacity that would remain unused under normal conditions, but becomes manifest when oxygen consumption is uncoupled from ATP production, or if there is an increase in energy demand (Pfleger et al., 2015). Moreover, it has been suggested (Guaras et al., 2016; Scialò et al., 2017) that the increase in the activity of complex II in low-RFI animals, could lead to an increase in the flow of electrons from the oxidation of FADH_2 . This could cause reverse electron transport that fosters separation of Complex I and III supercomplex and a greater passage of the electrons coming from the FADH_2 to the rest of the respiratory chain. Thus, low-RFI could optimize nutrient utilization when compared to high-RFI steers.

Contrary to our results, Lancaster et al. (2014) did not find differences in maximum respiratory rate between low and high-RFI steers fed high concentrate (trial 1) or a more fibrous diet (trial 2) when hepatic mitochondria were provided with substrates for any of the two complexes. Similarly, Acetoze et al. (2015) did not report differences in hepatic respiration rates or proton leak of steers descent of bulls of divergent lines of RFI. Moment of tissue collection related to RFI measure could explain differences between results from this vs. previous reported studies. Phenotypic correlations between RFI measured during growing and finishing periods were positive and moderate to high. (0.50 and 0.85 using high- or medium-concentrate diets; Arthur et al., 2001; Kelly et al., 2010; McGee et al., 2014) and others (Durunna et al., 2011; Russell et al. 2016) reported relative classification of a steer as highly or lowly feed efficient was repeatable between growing and finishing phases even if steers changed of diet. Moreover, positive and moderate correlations between RFI values derived from forage- and grain-based diets in heifers were reported (Trujillo et al., 2013; Cassady et al. 2016). Thus, it could be expected that more efficient young animals when fed one diet type were also more efficient when the diet is changed, however, reranking may occur and could contribute to explain some of the differences between our and other studies findings. Methodological differences could also explain, at least partially, differences between studies, as in our study measurements were performed in a tissue sample vs. isolated mitochondria and in a high-resolution equipment vs. Clark-type oxygen electrode. The isolation process could decrease mitochondrial integrity and mitochondria isolated from cells and tissues are essentially stripped from their intracellular locales (Salabei et al., 2014). The use of high-resolution equipment allowed us

a more precise titration of the FCCP, critical to determine uncontrolled respiration (Brand and Nicholls, 2011). Thus, greater basal respiration and maximum respiratory rate associated with greater mitochondria content would indicate an improved bioenergetics capacity in feed efficient animals as they are important quantitative indicators of oxidative capacity (Lee and Wei, 2005) and metabolic activity (Weikard and Kühn, 2016).

The greater estimated mitochondrial respiration for low than high-RFI steers was associated with greater expression of various genes encoding for respiratory chain protein complexes in the liver of low-RFI steers (*NDUFA4* and *NDUFA13*) for complex I, *SDHA* and *SDHD* for complex II, *UQCRH* and *UQCRC1* for complex III, *CYCI* for complex IV and *ATP5E* for complex V). Similar to our results, Iqbal et al. (2005) reported that only a few protein respiratory chain subunits were differentially expressed between broilers with low and high feed efficiency in liver mitochondria, being most of the subunits expressed at similar levels between groups. Mitochondrial complexes are not separate entities, but assembled multiprotein subunits, which have a great structural and functional dependence of the individual subunits as the alteration of one of them can cause partial or global disturbance of the whole complex functionality. Connor et al. (2010) studied differential hepatic gene expression in feed restricted steers using microarrays and reported up regulation of nearly 30 genes participating in mitochondrial electron transport, including some of the ones evaluated in the present study (complex II: *SDHA*, complex IV: *CYCI*, complex V: *ATP5O*) in steers exhibiting high vs. low feed efficiency during the re-alimentation period. In contrast, other hepatic transcriptome studies—using total RNA sequencing or microarray—did not report differently expressed genes related to electron transport chain or oxidative phosphorylation when high vs. low feed efficiency cattle were compared (Chen et al., 2011; Alexandre et al., 2015; Tizioto et al., 2015; Zarek et al., 2017). Differences between methodologies (qRT-PCR vs. RNA-seq or microarrays) or breeds (*Bos Taurus* vs. *Bos indicus*) may explain contradictory results. Interestingly, these latter studies reported, differences in expression of genes related to mitochondrial biogenesis, oxidative stress, transport of fatty acids to the mitochondrion for β -oxidation, antioxidant enzymes and uncoupling proteins (Chen et al., 2011; Alexandre et al., 2015; Tizioto et al., 2015; Zarek et al., 2017). In addition, although Kelly et al. (2011) reported no differences between

low and high RFI-steers in muscle gene expression of complex I, II, or III proteins, in agreement with our results, they observed a negative correlation between RFI values and *COXII* (complex IV) gene expression. Results suggest that the differences in respiration parameters between steers of low or high-RFI would be associated with changes at the transcription level, by affecting rates of mRNA synthesis and/or degradation, of some proteins of the mitochondrial respiratory chain complexes.

In agreement with the greater *SDHA* mRNA expression, we detected an increased protein expression of SDHA in low than high-RFI steers. As already mentioned, SDH is an enzyme complex (complex II) that participates in both the TCA cycle and the respiratory chain and constitutes one of the fundamental steps in the utilization of propionate (generated at ruminal level) for hepatic gluconeogenesis. Therefore, this could result in an increase in the performance of hepatic gluconeogenesis in low-RFI steers. In addition, in agreement with Sharifabadi et al. (2012), hepatic activity of both, NADH dehydrogenase (complex I) and SDH (complex II) was between 15% and 20% less in high than low-RFI steers, which would indicate complex I and complex II of respiratory chain complex activities may be compromised in low-efficiency animal phenotypes. Electrons enter the respiratory chain from NADH or FADH₂ at complex I and complex II respectively, transferred through coenzyme Q to complex III, and shuttled by cytochrome *c* from complex III to complex IV. We postulated that a greater activity of complex I and complex II would result in a greater proton gradient—that can finally generate ATP—generated per unit of time.

CONCLUSIONS

In summary, high-feed efficiency steers (low-RFI) probably had greater efficiency in hepatic nutrient metabolism, which was strongly associated with greater hepatic mitochondrial density and function, mainly of mitochondrial complex II. Further research is needed to identify the mechanism involved in these different physiological processes between animals with low and high efficiency.

SUPPLEMENTARY DATA

Supplementary data are available at *Journal of Animal Science* online.

LITERATURE CITED

- Acetoze, G., K. L. Weber, J. J. Ramsey, and H. A. Rossow. 2015. Relationship between liver mitochondrial respiration and proton leak in low and high RFI steers from two lineages of RFI Angus bulls. *Int. Sch. Res. Notices*. 2015:194014. doi:10.1155/2015/194014
- Alexandre, P. A., L. J. Kogelman, M. H. Santana, D. Passarelli, L. H. Pulz, P. Fantinato-Neto, P. L. Silva, P. R. Leme, R. F. Strefezzi, L. L. Coutinho, et al. 2015. Liver transcriptomic networks reveal main biological processes associated with feed efficiency in beef cattle. *BMC Genomics*. 16:1073. doi:10.1186/s12864-015-2292-8
- AOAC. 2000. Official methods of analysis. 17th ed. Association of Official Analytical Chemists, Gaithersburg, MD.
- Arthur, P. F., G. Renand, and D. Krauss. 2001. Genetic and phenotypic relationships among different measures of growth and feed efficiency in young Charolais bulls. *Livest. Prod. Sci.* 68:131–139. doi:10.1016/S0301-6226(00)00243-8
- Baldwin, R. L., K. R. McLeod, and A. V. Capuco. 2004. Visceral tissue growth and proliferation during the bovine lactation cycle. *J. Dairy Sci.* 87:2977–2986. doi:10.3168/jds.S0022-0302(04)73429-3
- Bottje, W. G., and G. E. Carstens. 2009. Association of mitochondrial function and feed efficiency in poultry and livestock species. *J. Anim. Sci.* 87(14 Suppl):E48–E63. doi:10.2527/jas.2008-1379
- Brand, M. D., and D. G. Nicholls. 2011. Assessing mitochondrial dysfunction in cells. *Biochem. J.* 435:297–312. doi:10.1042/BJ20110162
- Casal, A., M. Veyga, A. L. Astessiano, A. C. Espasandin, A. I. Trujillo, P. Soca, and M. Carriquiry. 2014. Visceral organ mass, cellularity indexes and expression of genes encoding for mitochondrial respiratory chain proteins in pure and crossbred mature beef cows grazing different forage allowances of native pastures. *Livest. Sci.* 167:195–205. doi:10.1016/j.livsci.2014.06.024
- Cassady, C. J., T. L. Felix, J. E. Beever, and D. W. Shike. 2016. Effects of timing and duration of test period and diet type on intake and feed efficiency of Charolais-sired cattle. *J. Anim. Sci.* 94:4748–4758. doi:10.2527/jas.2016-0633
- Cassina, A., and R. Radi. 1996. Differential inhibitory action of nitric oxide and peroxynitrite on mitochondrial electron transport. *Arch. Biochem. Biophys.* 328:309–316. doi:10.1006/abbi.1996.0178
- Castro Bulle, F. C., P. V. Paulino, A. C. Sanches, and R. D. Sainz. 2007. Growth, carcass quality, and protein and energy metabolism in beef cattle with different growth potentials and residual feed intakes. *J. Anim. Sci.* 85:928–936. doi:10.2527/jas.2006-373
- Chen, Y., C. Gondro, K. Quinn, R. M. Herd, P. F. Parnell, and B. Vanselow. 2011. Global gene expression profiling reveals genes expressed differentially in cattle with high and low residual feed intake. *Anim. Genet.* 42:475–490. doi:10.1111/j.1365-2052.2011.02182.x
- Cimen, H., M. J. Han, Y. Yang, Q. Tong, H. Koc, and E. C. Koc. 2010. Regulation of succinate dehydrogenase activity by SIRT3 in mammalian mitochondria. *Biochemistry*. 49:304–311. doi:10.1021/bi901627u
- Connor, E. E., S. Kahl, T. H. Elsasser, J. S. Parker, R. W. Li, C. P. Van Tassell, R. L. Baldwin, 6th, and S. M. Barao. 2010. Enhanced mitochondrial complex gene function and reduced liver size may mediate improved feed efficiency of beef cattle during compensatory growth. *Funct. Integr. Genomics*. 10:39–51. doi:10.1007/s10142-009-0138-7
- Durunna, O. N., F. D. Mujibi, L. Goonewardene, E. K. Okine, J. A. Basarab, Z. Wang, and S. S. Moore. 2011. Feed efficiency differences and reranking in beef steers fed grower

- and finisher diets. *J. Anim. Sci.* 89:158–167. doi:10.2527/jas.2009-2514
- García-Roche, M., A. Casal, M. Carriquiry, R. Radi, C. Quijano, and A. Cassina. 2018. Respiratory analysis of coupled mitochondria in cryopreserved liver biopsies. *Redox Biol.* 17:207–212. doi:10.1016/j.redox.2018.03.008
- Gnaiger E. 2008. Polarographic oxygen sensors, the oxygraph and high-resolution respirometry to assess mitochondrial function. In: J. A. Dykens and Y. Will, editors. *Mitochondrial dysfunction in drug-induced toxicity*. John Wiley & Sons, Inc., Hoboken, NJ. p. 327–352.
- Guarás, A., E. Perales-Clemente, E. Calvo, R. Acín-Pérez, M. Loureiro-Lopez, C. Pujol, I. Martínez-Carrascoso, E. Nuñez, F. García-Marqués, M. A. Rodríguez-Hernández, et al. 2016. The coqh2/coq ratio serves as a sensor of respiratory chain efficiency. *Cell Rep.* 15:197–209. doi:10.1016/j.celrep.2016.03.009
- Guo, W., L. Jiang, S. Bhasin, S. M. Khan, and R. H. Swerdlow. 2009. DNA extraction procedures meaningfully influence qPCR-based mtDNA copy number determination. *Mitochondrion.* 9:261–265. doi:10.1016/j.mito.2009.03.003
- Harper, M. E., A. Antoniou, L. Bevilacqua, V. Bezaire, and S. Monemdjou. 2002. Cellular energy expenditure and the importance of uncoupling. *J. Anim. Sci.* 80(E-Suppl_2):E90–E97. doi:10.2527/animalsci2002.80E-Suppl_2E90x
- Hill, B. G., G. A. Benavides, J. R. Lancaster, Jr, S. Ballinger, L. Dell'Italia, Z. Jianhua, and V. M. Darley-Usmar. 2012. Integration of cellular bioenergetics with mitochondrial quality control and autophagy. *Biol. Chem.* 393:1485–1512. doi:10.1515/hsz-2012-0198
- Iqbal, M., N. R. Pumford, Z. X. Tang, K. Lassiter, C. Ojano-Dirain, T. Wing, M. Cooper, and W. Bottje. 2005. Compromised liver mitochondrial function and complex activity in low feed efficient broilers are associated with higher oxidative stress and differential protein expression. *Poult. Sci.* 84:933–941. doi:10.1093/ps/84.6.933
- Janssen, A. J., F. J. Trijbels, R. C. Sengers, J. A. Smeitink, L. P. van den Heuvel, L. T. Wintjes, B. J. Stoltenborg-Hogenkamp, and R. J. Rodenburg. 2007. Spectrophotometric assay for complex I of the respiratory chain in tissue samples and cultured fibroblasts. *Clin. Chem.* 53:729–734. doi:10.1373/clinchem.2006.078873
- Kelly, A. K., M. McGee, D. H. Crews, Jr, T. Sweeney, T. M. Boland, and D. A. Kenny. 2010. Repeatability of feed efficiency, carcass ultrasound, feeding behavior, and blood metabolic variables in finishing heifers divergently selected for residual feed intake. *J. Anim. Sci.* 88:3214–3225. doi:10.2527/jas.2009-2700
- Kelly, A. K., S. M. Waters, M. McGee, R. G. Fonseca, C. Carberry, and D. A. Kenny. 2011. mRNA expression of genes regulating oxidative phosphorylation in the muscle of beef cattle divergently ranked on residual feed intake. *Physiol. Genomics.* 43:12–23. doi:10.1152/physiolgenomics.00213.2009
- Koch, R. M., L. A. Swiger, D. Chambers, and K. E. Gregory. 1963. Efficiency of feed use in beef cattle. *J. Anim. Sci.* 22:486–494. doi:10.2527/jas1963.222486x
- Kolath, W. H., M. S. Kerley, J. W. Golden, and D. H. Keisler. 2006. The relationship between mitochondrial function and residual feed intake in Angus steers. *J. Anim. Sci.* 84:861–865. doi:10.2527/2006.844861x
- Kuznetsov, A. V., V. Veksler, F. N. Gellerich, V. Saks, R. Margreiter, and W. S. Kunz. 2008. Analysis of mitochondrial function in situ in permeabilized muscle fibers, tissues and cells. *Nat. Protoc.* 3:965–976. doi:10.1038/nprot.2008.61
- Lancaster, P. A., G. E. Carstens, J. J. Michal, K. M. Brennan, K. A. Johnson, and M. E. Davis. 2014. Relationships between residual feed intake and hepatic mitochondrial function in growing beef cattle. *J. Anim. Sci.* 92:3134–3141. doi:10.2527/jas.2013-7409
- Laporta, J., G. J. Rosa, H. Naya, and M. Carriquiry. 2014. Liver functional genomics in beef cows on grazing systems: Novel genes and pathways revealed. *Physiol. Genomics* 46:138–147. doi:10.1152/physiolgenomics.00120.2013
- Larsen, S., J. Nielsen, C. N. Hansen, L. B. Nielsen, F. Wibrand, N. Stride, H. D. Schroder, R. Boushel, J. W. Helge, F. Dela, et al. 2012. Biomarkers of mitochondrial content in skeletal muscle of healthy young human subjects. *J. Physiol.* 590:3349–3360. doi:10.1113/jphysiol.2012.230185
- Lee, H. C., and Y. H. Wei. 2005. Mitochondrial biogenesis and mitochondrial DNA maintenance of mammalian cells under oxidative stress. *Int. J. Biochem. Cell Biol.* 37:822–834. doi:10.1016/j.biocel.2004.09.010
- McGee, M., C. M. Welch, J. A. Ramirez, G. E. Carstens, W. J. Price, J. B. Hall, and R. A. Hill. 2014. Relationships of feeding behaviors with average daily gain, dry matter intake, and residual feed intake in red angus-sired cattle. *J. Anim. Sci.* 92:5214–5221. doi:10.2527/jas.2014-8036
- Montanholi, Y. R., L. S. Haas, K. C. Swanson, B. L. Coomber, S. Yamashiro, and S. P. Miller. 2017. Liver morphometrics and metabolic blood profile across divergent phenotypes for feed efficiency in the bovine. *Acta Vet. Scand.* 59:24–35. doi:10.1186/s13028-017-0292-1
- Navajas, E. A., M. I. Pravia, M. Lema, J. Clariget, I. Aguilar, O. Ravagnolo, G. Brito, P. Peraza, M. Dalla Rizza, and F. Montossi. 2014. Genetic improvement of feed efficiency and carcass and meat quality of Hereford cattle by genomics. 60th International Congress of Meat Science and Technology, Maldonado, Uruguay, Arch. Latinoam. Prod. Anim. 22:13–16. http://www.alpa.org.ve/ojs/index.php/ojs_files/article/viewFile/2457/835 (accessed 15 May 2017).
- NRC. 2000. Nutrient requirements of beef cattle. 7th rev. ed. Natl. Acad. Press, Washington, DC.
- Pflaffl, M. W. 2009. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 29:e45. doi:10.1093/nar/29.9.e45
- Pfleger, J., M. He, and M. Abdellatif. 2015. Mitochondrial complex II is a source of the reserve respiratory capacity that is regulated by metabolic sensors and promotes cell survival. *Cell Death Dis.* 6:e1835. doi:10.1038/cddis.2015.202
- Ramos, M. H., and M. S. Kerley. 2013. Mitochondrial complex I protein differs among residual feed intake phenotype in beef cattle. *J. Anim. Sci.* 91:3299–3304. doi:10.2527/jas.2012-5589
- Reid, C. R., C. M. Bailey, and M. B. Judkins. 1991. Metabolizable energy for maintenance of beef-type bos taurus and bos indicus x bos taurus cows in a dry, temperate climate. *J. Anim. Sci.* 69:2779–2786. doi:10.2527/1991.6972779x
- Rolfé, D. F., and M. D. Brand. 1997. The physiological significance of mitochondrial proton leak in animal cells and tissues. *Biosci. Rep.* 17:9–16. doi:10.1023/A:1027327015957
- Russell, J. R., W. J. Sexten, and M. S. Kerley. 2016. Effect of corn inclusion on soybean hull-based diet digestibility and growth

- performance in continuous culture fermenters and beef cattle. *J. Anim. Sci.* 94:2919–2926. doi:10.2527/jas.2015-0180
- Salabei, J. K., A. A. Gibb, and B. G. Hill. 2014. Comprehensive measurement of respiratory activity in permeabilized cells using extracellular flux analysis. *Nat. Protoc.* 9:421–438. doi:10.1038/nprot.2014.018
- Sandelin, B. A., A. H. Brown Jr, M. A. Brown, C. Ojano-Dirain, and R. T. Baublits. 2005. Association of mitochondrial function with feed efficiency in Angus cattle. Arkansas Animal Science Department Report. 535:118–121. <http://arkansasagnews.uark.edu/535-30.pdf> (accessed 20 January 2016).
- Scialò, F., D. J. Fernández-Ayala, and A. Sanz. 2017. Role of mitochondrial reverse electron transport in ROS signaling: Potential roles in health and disease. *Front. Physiol.* 8:1–7. doi:10.3389/fphys.2017.00428
- Sharifabadi, H. R., M. J. Zamiri, E. Rowghani, and W. G. Bottje. 2012. Relationship between the activity of mitochondrial respiratory chain complexes and feed efficiency in fat-tailed Ghezel lambs. *J. Anim. Sci.* 90:1807–1815. doi:10.2527/jas.2011-4791
- Spinazzi, M., A. Casarin, V. Pertegato, L. Salviati, and C. Angelini. 2012. Assessment of mitochondrial respiratory chain enzymatic activities on tissues and cultured cells. *Nat. Protoc.* 7:1235–1246. doi:10.1038/nprot.2012.058
- Sunnucks, P., and D. F. Hales. 1996. Numerous transposed sequences of mitochondrial cytochrome oxidase I-II in aphids of the genus *Sitobion* (Hemiptera: Aphididae). *Mol. Biol. Evol.* 13:510–524. doi:10.1093/oxfordjournals.molbev.a025612
- Tizioto, P. C., L. L. Coutinho, J. E. Decker, R. D. Schnabel, K. O. Rosa, P. S. Oliveira, M. M. Souza, G. B. Mourão, R. R. Tullio, A. S. Chaves, et al. 2015. Global liver gene expression differences in Nelore steers with divergent residual feed intake phenotypes. *BMC Genomics.* 16:242. doi:10.1186/s12864-015-1464-x
- Trujillo, A. I., A. Casal, F. Peñagaricano, M. Carriquiry, and P. Chilibroste. 2013. Association of SNP of neuropeptide Y, leptin, and IGF-1 genes with residual feed intake in confinement and under grazing condition in Angus cattle. *J. Anim. Sci.* 91:4235–4244. doi:10.2527/jas.2013-6254
- Weikard, R., and C. Kuhn. 2016. Mitochondrial biogenesis and DNA content in metabolically tissues of lactating cows with divergent milk production. *J. Anim. Sci.* 94(supplement 5):522–523 (Abstr.). doi:10.2527/jam2016-1089
- Zarek, C. M., A. K. Lindholm-Perry, L. A. Kuehn, and H. C. Freetly. 2017. Differential expression of genes related to gain and intake in the liver of beef cattle. *BMC Res. Notes.* 10:1. doi:10.1186/s13104-016-2345-3