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# Diet induced the change of mtDNA copy number and metabolism in Angus cattle

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## Abstract

**Background:** Grass-fed and grain-fed Angus cattle differ in the diet regimes. However, the intricate mechanisms of different beef quality and other phenotypes induced by diet differences are still unclear. Diet affects mitochondrial function and dynamic behavior in response to changes in energy demand and supply. In this study, we examined the mtDNA copy number, mitochondria-related genes expression, and metabolic biomarkers in grass-fed and grain-fed Angus cattle.

**Results:** We found that the grass-fed group had a higher mtDNA copy number than the grain-fed group. Among different tissues, the mtDNA copy number was the highest in the liver than muscle, rumen, and spleen. Based on the transcriptome of the four tissues, a lower expression of mtDNA-encoded genes in the grass-fed group compared to the grain-fed group was discovered. For the mitochondria-related nuclear genes, however, most of them were significantly down-regulated in the muscle of the grass-fed group and up-regulated in the other three tissues. In which, *COX6A2*, *POLG2*, *PPIF*, *DCN*, and *NDUFA12*, involving in ATP synthesis, mitochondrial replication, transcription, and maintenance, might contribute to the alterations of mtDNA copy number and gene expression. Meanwhile, 40 and 23 metabolic biomarkers were identified in the blood and muscle of the grain-fed group compared to a grass-fed group, respectively. Integrated analysis of the altered metabolites and gene expression revealed the high expression level of *MDH1* in the grain-fed group might contribute to the mitochondrial NADH oxidation and spermidine metabolism for adapting the deletion mtDNA copy number.

**Conclusions:** Overall, the study may provide further deep insight into the adaptive and regulatory modulations of the mitochondrial function in response to different feeding systems in Angus cattle.

**Keywords:** Beef quality, Different diet, Gene expression, Metabolism, MtDNA

## Background

Recently, grass-fed beef production is booming in the United States, which is considered more beneficial for human health [1]. The main difference between grass-fed and grain-fed beef cattle is attributable to the diet regimes. As we know, mitochondria play an essential role in the cellular response to environmental stressors, such as air, water, temperature, and food [2]. Mitochondria are dynamic organelles that are present in almost

eukaryotic cells and play a crucial role in several cellular pathways, producing most of the cellular ATP employing oxidative phosphorylation (OXPHOS). Different tissues have specialized mitochondrial features due to differences in their metabolic profiles and energy demands [3–6]. The difference is not only restricted to the OXPHOS function [6] and mitochondria protein compositions [7] but also gene expressions, mitochondrial DNA maintenance, and replications [8, 9].

Mitochondria contain their genomes (mtDNA), encoding a total of 13 proteins, together with 22 tRNAs and two rRNAs, necessary for translations of the respiratory subunit mRNAs within the mitochondrial matrix. Alterations

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of mtDNA mean inactivating genetic mutations or depletion of mtDNA copy numbers [10]. The copies of mtDNA serve as the initial template for mtDNA replication and maintenance, allowing cells to acquire the appropriate numbers of mtDNA copy as they differentiate into mature cell types [11–13]. Mitochondria replication, maintenance as well as gene expression are controlled by nuclear-encoded factors, such as nuclear respiratory factor 1 and 2 (*NRF-1*, *NRF-2*), mitochondrial transcription factor A (*TFAM*), and peroxisome proliferator-activated receptor-gamma coactivator-1a (*PGC-1a*) [14, 15]. The mitochondrial and nuclear genomes coordinate and co-evolve in eukaryotes to adapt to environmental changes. Variation in the mitochondrial genome is capable of affecting the expression of genes on the nuclear genome [16]. However, how the diet regime influences the mitochondria dynamics, further associated with beef quality, has yet to know.

Provisioning of dietary macronutrients to mitochondria is influenced by genetic variations that affect the activities of the electron transport system, retrograde organelle signaling to the nuclear genome, and anterograde signaling to the mitochondrion [17, 18]. In diet-induced obesity, impaired mitochondrial function and decreased mitochondria contents were found in liver and skeletal muscle [19, 20]. Dietary supplementation with plasminogen increased mitochondrial copy number and improved mitochondrial function in mice [19–21]. A reduction of the mtDNA copy number and the expression of genes involved in mitochondrial biogenesis in the liver of rats fed a high-fructose diet [22]. When feeding flies with a high carbohydrate diet, the mitochondria-related gene expression, and mtDNA were changed [23]. RNA-seq analysis revealed that genes associated with mitochondrial function were differentially expressed between low-protein fed heifers on high- or low- energy diets [24].

Meanwhile, diet by mitochondrial DNA haplotype interactions drives metabolic flexibility and organismal fitness [23]. Metabolomic profiling provides an additional layer of knowledge for the complete representation of the phenotype of the animal, revealing the combined contributions of gene expression, enzyme activity, and environmental context [23, 25]. What a cow eats can have a significant effect on the metabolic and nutrient composition of the beef via modulating metabolism and energy expenditure [1]. Grass-fed beef may contain less total fat than grain-fed beef, but a lot more omega-3 fatty acids and conjugated linoleic acid [26–28]. In our previous studies, the alterations in divergences in free fatty acids, lipid levels, and gene expression patterns have been observed between grass-fed and grain-fed Angus cattle [29–31]. However, information on the possible mechanism on mitochondrial function and metabolism between the grass-fed and grain-fed is lacking. Here, we propose using trio measurements of mtDNA

copy number, mRNA transcribed from mtDNA, and mRNA transcribed from nuclear DNA- related to mitochondria, to explore mitochondrial roles responded to different diets in Angus cattle. In parallel, we combined these data with metabolomics to assess the correlation between mitochondria-related genes and metabolites levels. These findings additionally provide insights into patterns of transcriptional coordination between the mitochondrial and nuclear genomes.

## Methods

### Animals and ethics statement

The grass-fed and grain-fed Angus cattle were raised at the Wye Angus farm, which has been closed more than 70 years, have similar genetics. The grain-fed group received a conventional diet comprised of shelled corn, corn silage, soybean, and trace minerals. The grass-fed steers usually consumed grazed alfalfa. The diet composes, and feeding regiments for grass-fed and grain-fed cattle are described in Additional file 1: Table S1. The longissimus dorsi muscle, liver, spleen, and rumen tissues were collected from steers with grass-fed and grain-fed, respectively, when they reached the market weight. The grain-fed animals reached the market weight around the age of 14 months, while grass-fed steers needed approximately 200 additional days to reach a similar weight value. Samples were taken immediately after euthanasia, frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$  until used for extracting RNA and DNA. Blood collection for both groups was performed before slaughtering. The metabolomics profiling analysis were performed in blood and muscle samples from eight individuals of the grass-fed group and the grain-fed group, respectively. Then, the four tissues from two randomly selected individuals in each group were used for deep sequencing. MtDNA copy number was detected in 24 samples (4 tissues  $\times$  3 individuals  $\times$  2 groups).

All animal experiments were conducted according to the NIH guidelines for housing and care of laboratory animals and following the regulations of the University of Maryland at College Park (UMCP). The UMCP Institutional Animal Care and Use Committee (IACUC) reviewed and approved the protocols (permit number R-08-62).

### mtDNA copy number analysis

DNA was prepared, and mtDNA copy number analysis was performed according to the previous report [32, 33]. Briefly, total DNA was extracted from longissimus dorsi muscle, liver, spleen, and rumen tissues using Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA), respectively. The DNA concentration was detected using Nanodrop-2000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE) and adjusted to 50 ng/ $\mu\text{L}$ .

Primers were designed for four mitochondrial genes: the *ND2* (NADH dehydrogenase subunit 2), *ND5* (NADH dehydrogenase subunit 5), *CYTB* (cytochrome B), *12sRNA*, and *COX3* (cytochrome oxidase subunit III). The *ACTB* (actin B) gene was used as a nuclear control gene. For the analysis of mtDNA copy number, quantitative real-time PCR (qPCR) amplification of genomic DNA was performed on a C1000 Touch thermal cycler (BioRad, Hercules, CA, USA). The reactions for each gene were performed with 50 ng genomic DNA in triplicates in a final volume of 10  $\mu$ L using 300 nmol/L of the specific primers and the 2  $\times$  SYBR Green PCR mix (Biorad, Hercules, CA, USA). The PCR program was 95 °C for 5 min, 40 cycles of 95 °C for 15 s, 60 °C for the 30 s, and 72 °C for 30 s, with a melting curve analysis (65 °C–95 °C) in the last cycle to evaluate amplification specificity. For each run, a standard curve was generated from 10-fold serial dilutions ( $10^{-1}$  to  $10^{-8}$ ). Relative mtDNA copy numbers were calculated following equation [34]: MtDNA copy number =  $2^{1 + (Ct_{n\_gene} - Ct_{mt\_gene})}$ , where Ct represents the average cycle threshold. The mtDNA copy number data from the four tissues were analyzed separately.

#### RNA sequencing and analysis

The RNA extraction, cDNA synthesis, library preparation, transcriptome sequencing, and raw data were carried out from our reported protocols [29–31]. We employed FastQC v.0.11.2 (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) to check the sequence data quality and Trim Galore v 0.4.0 ([https://www.bioinformatics.babraham.ac.uk/projects/trim\\_galore/](https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/)) to clean the data. The cleaned data were aligned to the reference genome downloaded from Ensembl (*Bos taurus*.ARS-UCD 1.2) using Hisat2 (<https://ccb.jhu.edu/software/hisat2/>) [35]. Differentially expressed genes were computed by using Cuffdiff (cole-trapnell-lab.github.io/cufflinks/cuffdiff). A gene was considered to be differentially expressed when fold changes  $\geq 2$  and *FDR* < 0.15. Gene Ontology (GO) enrichment analysis and Kyoto Encyclopedia of Gene and Genomes (KEGG) pathway analysis were implemented with STRING tools (<http://string-db.org/>) and KOBAS 3.0 (<http://kobas.cbi.pku.edu.cn/>).

#### Metabolomics profiling

Metabolomics profiling analysis was performed by the Metabolon Platform, as previously described [29]. The whole blood and muscle tissues were assigned a unique identifier by the Metabolon Laboratory Information Management System (LIMS). Samples were prepared using the automated MicroLab STAR system from Hamilton Company. A recovery standard was added before the first step in the extraction process for quality control (QC) purposes. Sample preparation was conducted using aqueous

methanol extraction process to remove the protein fraction while allowing for maximum recovery of small molecules. The resulting extract was divided into four fractions: one for analysis by liquid chromatography/mass spectroscopy (LC/MS) (positive mode), one for LC/MS (negative mode), one for gas chromatography/mass spectroscopy (GC/MS), and one for backup. Samples were placed briefly on a TurboVap (Zymark) to remove the organic solvent. Each sample was then frozen and dried under a vacuum. Samples were then prepared for the appropriate instrument. The progress of LC/MS, GC/MS, QA/QC, data extraction, and compound identification were detail described in our lab previous publication. The MUVR package in R was used to find the metabolic biomarkers [36].

#### Metabolomic enrichment and pathway characterization

The pathway analysis was performed by MetaboAnalyst 4.0 [37]. The common pathway databases in the MetaboAnalyst 4.0 were used to explore metabolic impact pathways. The pathway with a *P*-value less than 0.05, as well as an impact value greater than 0.1, was defined as a significant impact pathway. Degree Centrality was selected for the node importance, and the pathway impact value calculation in the topological analysis. The hypergeometric distribution in the overrepresentation analysis was applied to assess the significance of each pathway based on its overlap with pathway lists in the joint databases. Statistical significance was defined at the joint *P* < 0.05.

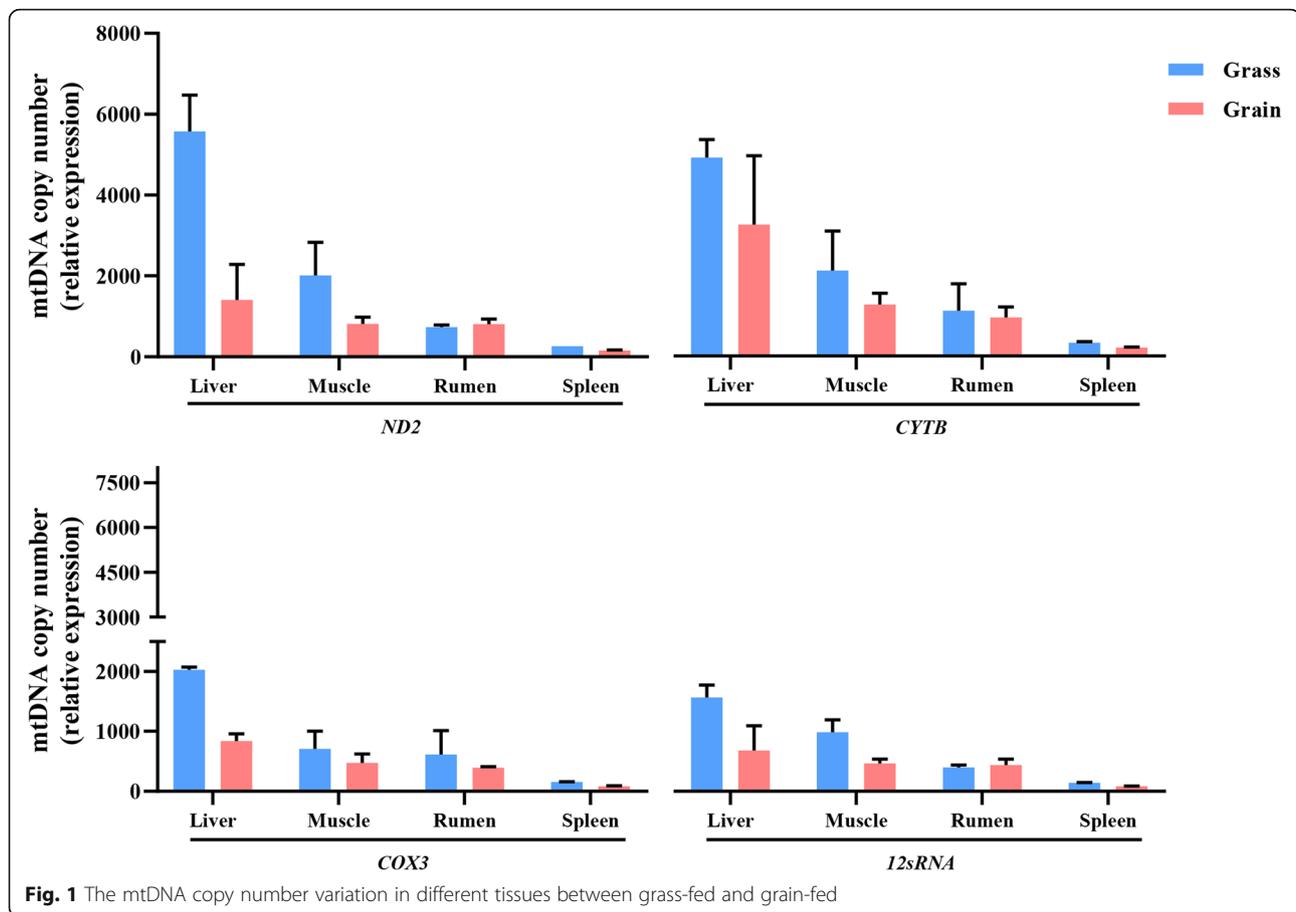
## Results

#### MtDNA copy number variation in different tissues

MtDNA copy number, assayed with independent primer sets, showed the difference in grass-fed and grain-fed groups. Overall, grass-fed steers had higher mtDNA copy number than grain-fed steers. Among tissues, mtDNA copy number was higher in the liver than the other three tissues, regardless of grass-fed and grain-fed ones. The following was muscle. In regards to the *ND2/ACTB* primer set, diet significantly affected copy number. In liver and muscle, the mtDNA copy number showed significant differences between grass-fed and grain-fed (*P* < 0.05). But there was little influence on the copy number in rumen and spleen. Copy number with regards to *CYTB/ACTB*, *COX3/ACTB*, or *12SRNA/ACTB* showed a similar result (Fig. 1).

#### The expression of mitochondria DNA-encoded genes

To identify whether the mitochondrial gene expression was altered in grass-fed and grain-fed cattle, the 13 mtDNA-encoded genes were examined using RNA sequencing data (Fig. 2). In muscle, seven of the 13 genes, *ND1*, *ND2*, *ND3*, *ND4*, *ND5*, *COX2*, and *CYTB*, were significantly up-regulated in the grain-fed group



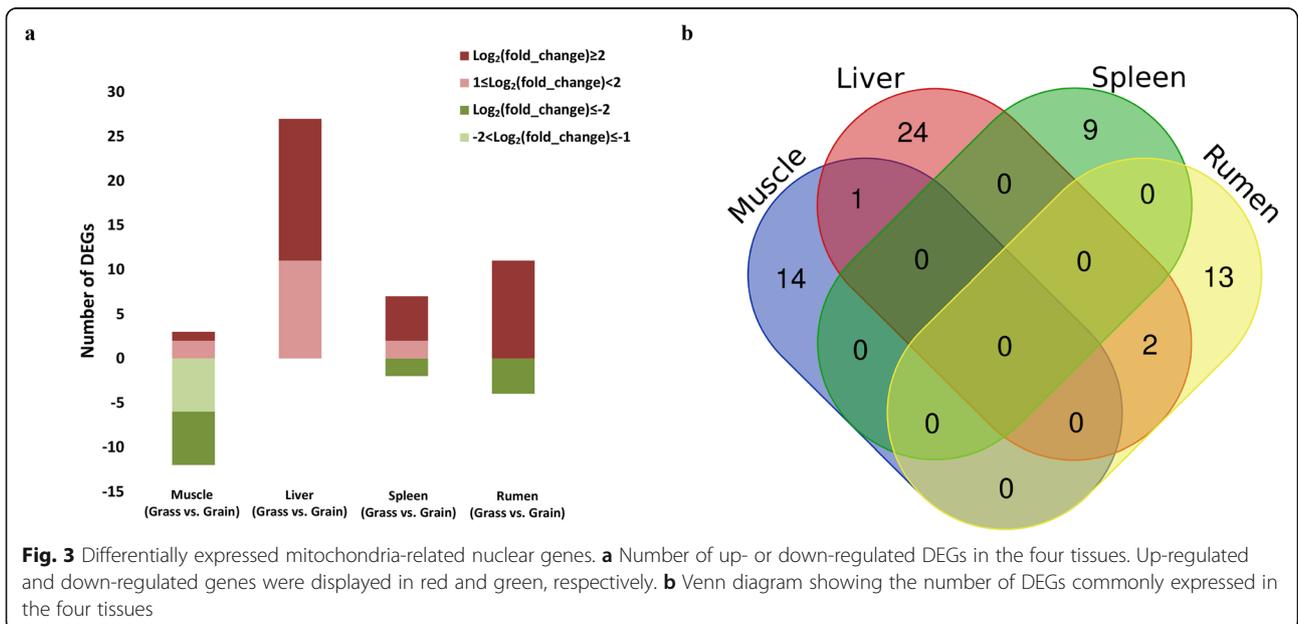
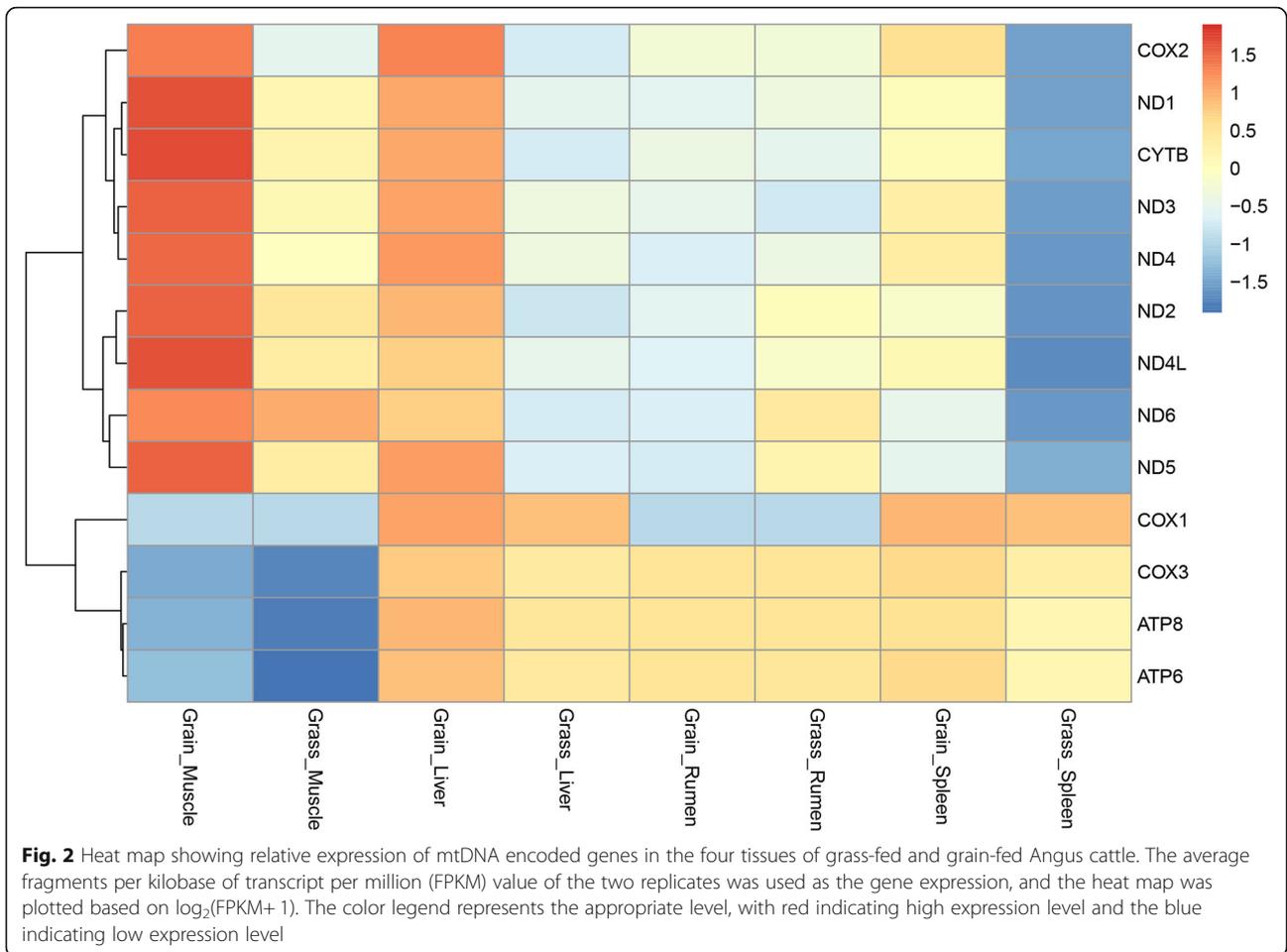
compared to the grass-fed group. In the liver, *ND1*, *ND2*, *ND5*, *COX2*, and *CYTB* had lower expression levels in the grass-fed group. In the spleen, only one mtDNA-encoded gene, *ND3*, was showed significantly lower in the grass-fed group. In the rumen, there was no mtDNA-encoded gene was significantly expressed between the two diets. Interestingly, in the four tissues, no differentially expressed mtDNA-encoded genes were shown up-regulation in grass-fed Angus cattle.

#### Differentially expressed mitochondria-related nuclear genes

The human mitochondria-related genes were downloaded from the MitoProteome Human Mitochondrial Protein Database [38]. Gene names were directly compared with those in cattle using the BioMart data-mining tool (<http://useast.ensembl.org/biomart/martview>). Meanwhile, the genes reported to be involved in mitochondrial function was generated by searching the “cattle mitochondrial” into the NCBI Gene database (National Center for Biotechnology Information, U.S. National Library of Medicine, Gene, <http://www.ncbi.nlm.nih.gov/gene>). Finally, 1283 genes were matched, including 13 mtDNA encoded protein genes as well as

1270 mitochondria-related nuclear genes (Additional file 1: Table S2).

The expression levels of 1270 mitochondria-related nuclear genes were also investigated based on the RNA sequencing data, and differentially expressed gene (DEG) analyses were performed between the two diets across different tissues. The numbers of the differentially expressed that mitochondria-related nuclear genes were shown in Fig. 3 and Additional file 1: Table S3-S7. Compared to the grain-fed group, most of the DEGs were down-regulated in the muscle of the grass-fed group, but more DEGs were up-regulated in the other three tissues (Fig. 3a). In muscle, 15 mitochondria-related nuclear genes were differentially expressed in a diet-dependent manner. *CKMT2* (creatine kinase, mitochondrial 2), *UBB* (ubiquitin B), and *MDH1* (malate dehydrogenase 1) were up-regulated in the grass-fed group, while other nine genes had the opposite expression pattern. In the liver, the effect of diet change showed that 27 mitochondria-related nuclear genes were differentially expressed, all of which were up-regulated in the grass-fed group. Among the nine DEGs observed in spleen, seven genes were up-regulated, and two genes, *LYRM7* (LYR motif containing 7) and *CYP11A1*



(cytochrome P450, family 11, subfamily A, polypeptide 1), were down-regulated in the grass-fed group. In the rumen, 15 mitochondria-related nuclear genes were differentially expressed (fold changes  $\geq 2$ ,  $FDR < 0.15$ ), of which 11 genes were up-regulated, and four genes were down-regulated in the grass-fed compared to the grain-fed ones. There were no DEGs in common among the four tissues of the two different diets. *UBB* was commonly differentially expressed in muscle and liver. While there were two DEGs, *DHCR24* (24-dehydrocholesterol reductase) and *SHMT1* (serine hydroxymethyltransferase 1), commonly expressed in liver and rumen (Fig. 3b).

### Bioinformatics functions analysis

To further define the potential biological function of differentially expressed mitochondrial-related genes, including mtDNA encoded protein genes, as well as mitochondria-related nuclear genes, GO analysis and KEGG pathway analysis were performed. The enriched GO terms of biological processes, molecular functions and cellular components were shown in Table 1 and Additional file 1: Table S8 ( $FDR < 0.05$ ). For the KEGG pathway analysis, most of the differentially expressed pathways ( $P < 0.05$ ) were associated with metabolic pathways in the four tissues (Additional file 2: Fig. S1). In muscle, the metabolic pathways included carbon metabolism, glyoxylate and dicarboxylate metabolism, and butanoate metabolism. In liver, the metabolism were involved in pyruvate metabolism, amino acid metabolism, glyoxylate, and dicarboxylate metabolism, carbon metabolism, and glycerolipid metabolism. Meanwhile, The FoxO signaling pathway was also differentially expressed, which was associated with development.

### Prediction potential metabolic biomarkers and significant impact metabolic pathways

LC/MS and GC/MS data detected 326 and 353 compounds in blood and muscle [29] (Additional file 3). Then, the compounds were analyzed using MUVR package in R to find the metabolic biomarkers. There were 40 and 23 metabolic biomarkers were identified in blood and muscle of the grain-fed group compared to a grass-fed group, respectively (Fig. 4). The involved metabolites belonged to the amino acid, carbohydrate, cofactors and vitamins, lipid, nucleotide, peptide, and xenobiotics. For the 40 metabolic biomarkers in blood, 14 metabolites had higher relative abundance in blood of the grain-fed group than the grass-fed group ( $P < 0.01$ ). Another 26 metabolites significantly decreased in the grain-fed group. In muscle, 14 metabolic biomarkers were increased in a grain-fed group compared to the grass-fed group, and the other nine were decreased ( $P < 0.01$ ). Among these metabolites, four lipids (1-eicosapentaenoyl glycerophosphoethanolamine, 2-eicosapentaenoyl

glycerophosphoethanolamine, 1-linolenoyl glycerophosphocholine (18:3n-3), and 3-dehydrocarnitine), one amino acid (N-methyl proline), and one xenobiotic (homostachydrine) were proposed as conventional metabolic biomarkers for the two tissues, which had same abundance trend between the two different diet groups.

Impact pathway analysis revealed that eight pathways were significant impact pathways based on the 23 metabolic biomarkers in muscle (Fig. 5a): glycerophospholipid metabolism ( $P = 0.0018$ , impact value = 0.6), arginine and proline metabolism ( $P = 0.0021$ , impact value = 0.2162), alpha-linolenic acid metabolism ( $P = 0.0036$ , impact value = 0.25), purine metabolism ( $P = 0.0102$ , impact value = 0.1077), glutathione metabolism ( $P = 0.0164$ , impact value = 0.2222), linoleic acid metabolism ( $P = 0.0359$ , impact value = 0.25), D-glutamine and D-glutamate metabolism ( $P = 0.0359$ , impact value = 0.5), nitrogen metabolism ( $P = 0.0429$ , impact value = 0.2). Meanwhile, there were four significant impact pathways based the 40 metabolic biomarkers in blood (Fig. 5b): neomycin, kanamycin and gentamicin biosynthesis ( $P = 0.0171$ , impact value = 1), glycine, serine and threonine metabolism ( $P = 0.0328$ , impact value = 0.1515), glycerophospholipid metabolism ( $P = 0.0364$ , impact value = 0.4857), linoleic acid metabolism ( $P = 0.0423$ , impact value = 0.25).

### Integrated analysis of metabolites and mitochondrial-related genes

The over-representation analysis and pathway topology analysis were conducted between the diet-different metabolic biomarkers and differentially expressed mitochondrial-related genes in the muscle of grass-fed and grain-fed groups (analysis I). The nutrients digested in the rumen are absorbed by the rumen epithelial wall and are then transported to the mammary to the liver for glycogenesis, followed by transportation to other tissues through the bloodstream [39, 40]. Therefore, we integrated the differentially expressed mitochondria-related nuclear genes in the liver and rumen together to ascertain the metabolic biomarkers in the blood, and did the over-representation analysis and pathway topology analysis (analysis II). Topology analysis uses the structure of a given pathway to evaluate the relative importance of the genes/metabolites based on their relative locations. In the analysis I, 27 pathways were identified, and 20 of them were displayed in Fig. 6a. In analysis II, 46 pathways were identified, the top 20 of which were shown in Fig. 6b. Six and nine pathways were significantly enriched in the analysis I and analysis II, respectively. They include Arginine and proline, glyoxylate and dicarboxylate, alpha-Linolenic acid, butanoate, glycerophospholipid, and beta-Alanine metabolisms. Other metabolisms also include pyruvate, glycine, serine and threonine, lysine degradation, glyoxylate and

**Table 1** Top five gene ontology terms in the four tissues

Tissue	GO terms	FDR	
Muscle	Biological Process		
	Cellular respiration	1.51E-11	
	Oxidation-reduction process	4.18E-11	
	ATP synthesis coupled electron transport	8.73E-10	
	Nucleobase-containing small molecule metabolic process	5.99E-09	
	ATP metabolic process	5.28E-08	
	Cellular Component		
	Mitochondrion	8.58E-14	
	Mitochondrial respiratory	6.93E-13	
	Respiratory chain complex	6.93E-13	
	An inner mitochondrial membrane protein complex	4.88E-12	
	Mitochondrial part	1.80E-11	
	Molecular Function		
	Oxidoreductase activity	2.07E-12	
	NADH dehydrogenase (ubiquinone) activity	6.48E-09	
	Catalytic activity	6.57E-09	
	Electron transfer activity	0.00029	
	Cytochrome-c oxidase activity	0.0025	
	Liver	Biological Process	
		Oxidation-reduction process	3.08E-17
Small-molecule metabolic process		1.32E-13	
Metabolic process		9.41E-11	
Cellular metabolic process		9.41E-11	
Cellular process		1.04E-08	
Cellular Component			
Mitochondrion		8.72E-21	
Mitochondrial part		6.00E-19	
Cytoplasmic part		1.95E-13	
Cytoplasm		4.00E-13	
Mitochondrial membrane		1.82E-11	
Molecular Function			
Oxidoreductase activity		4.54E-16	
Catalytic activity		1.61E-12	
Cofactor binding		2.96E-06	
Aldehyde dehydrogenase (NAD) activity		2.75E-05	
Coenzyme binding		5.48E-05	
Spleen		Biological Process	
		Oxidation-reduction process	0.0117
	Cellular Component		
	Mitochondrial part	7.57E-10	
	Mitochondrial membrane	6.12E-09	
	Mitochondrial inner membrane	4.01E-08	
	Inner mitochondrial membrane protein complex	0.00012	

**Table 1** Top five gene ontology terms in the four tissues (Continued)

Tissue	GO terms	FDR
	Molecular Function	
	Oxidoreductase activity	0.0014
	NADH dehydrogenase (ubiquinone) activity	0.003
	Catalytic activity	0.014
Rumen	Biological Process	
	Positive regulation of mitochondrion organization	0.0356
	Cellular metabolic process	0.0356
	Organonitrogen compound metabolic process	0.0356
	Cellular modified amino acid metabolic process	0.0419
	Phosphorylation	0.0419
	Cellular Component	
	Mitochondrial part	0.00044
	Mitochondrial envelope	0.0016
	Cytoplasm	0.0028
	Mitochondrial membrane	0.0098
	Cytoplasmic part	0.0126
	Molecular Function	
	Drug binding	0.00042
	Kinase activity	0.0046
Catalytic activity	0.0076	
Transferase activity	0.0076	
Carbohydrate derivative binding	0.0076	

dicarboxylate, and tryptophan metabolisms. The gene-metabolite interaction networks were also shown in Fig. 6.

## Discussion

### MtDNA copy number variation

According to the nutritional analysis of grass-diet and grain-diet, the contents of non-fiber carbohydrates and starch were much higher in the grain-diet, providing more total-digestible nutrients and available energy as well. Contrarily, proteins, lignin, fiber matter, and ash were more abundant in the grass- than grain-diet (Additional file 1: Table S1). It has been reported that when the flies were fed a high protein diet, the mtDNA copy number was increased compared to the high carbohydrate diet [23]. In our results, grass-fed steers had higher mtDNA copy number than grain-fed steers, which might be that the administration of insulin and amino acids enhances mitochondrial biogenesis and ATP production [41]. Meanwhile, the mtDNA copy number was higher in the liver than muscle, rumen, and spleen, regardless of grass- or grain-fed group. According to the ND2/ACTB primer set, the mtDNA copy number was 3.96-fold higher

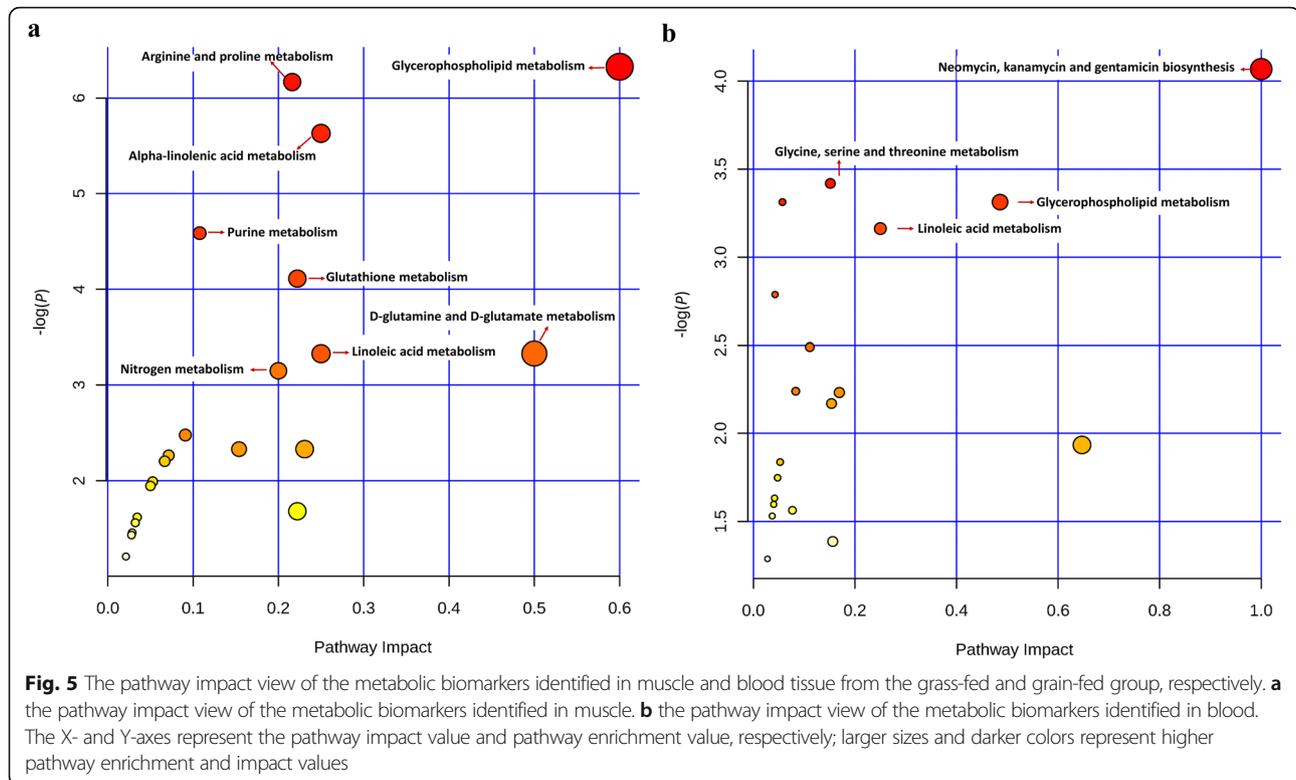
Pathway	Sub-pathway	Color Scale		Biochemical Name	RF*
		Blood	Muscle		
Amino Acid	Alanine and Aspartate Metabolism	Red	Red	alanine	B
	Glutamate Metabolism	Red	Red	glutamate	M
	Glycine, Serine and Threonine Metabolism	Red	Red	sarcosine (N-Methylglycine)	B
	Guanidino and Acetamido Metabolism	Red	Red	4-guanidinobutanoate	M
	Lysine Metabolism	Red	Red	lysine	B
	Methionine, Cysteine, SAM and Taurine Metabolism	Red	Red	cystathionine	B
	Phenylalanine and Tyrosine Metabolism	Red	Red	N-acetylmethionine	M
	Polyamine Metabolism	Red	Red	3-(4-hydroxyphenyl)lactate	M
Carbohydrate	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	Red	Red	spermidine	M
		Red	Red	N-methyl proline	B M
		Red	Red	glucose	B
		Red	Red	isobar	B
Cofactors and Vitamins	Pentose Metabolism	Red	Red	lactate	B
	Nicotinate and Nicotinamide Metabolism	Red	Red	ribose	B
Lipid	Fatty Acid Metabolism(Acyl Carnitine)	Red	Red	nicotinamide adenine dinucleotide reduced (NADH)	B
		Red	Red	3-dehydrocarnitine*	B M
	Fatty Acid, Branched	Red	Red	oleoylcarnitine	B
		Red	Red	13-methylmyristic acid	B
	Fatty Acid, Dicarboxylate	Red	Red	azelate (nonanedioate)	M
		Red	Red	docosadioate	B
		Red	Red	hexadecanedioate	B
	Fatty Acid, Methyl Ester	Red	Red	palmitate, methyl ester	B
		Red	Red	stearate, methyl ester	B
	Long Chain Fatty Acid	Red	Red	arachidate (20:0)	B
		Red	Red	nonadecanoate (19:0)	B
		Red	Red	pentadecanoate (15:0)	B
		Red	Red	1-docosapentaenoylglycerophosphocholine (22:5n3)*	B
		Red	Red	1-eicosapentaenoylglycerophosphocholine (20:5n3)*	B
	Lysolipid	Red	Red	1-eicosapentaenoylglycerophosphoethanolamine*	B M
		Red	Red	1-linolenoylglycerophosphocholine (18:3n3)*	B M
		Red	Red	1-stearoylglycerophosphoserine*	B
		Red	Red	2-eicosapentaenoylglycerophosphoethanolamine*	B M
Red		Red	2-linolenoylglycerophosphocholine(18:3n3)*	M	
Red		Red	2-linolenoylglycerophosphoethanolamine (18:3n3)*	M	
Phospholipid Metabolism	Red	Red	glycerophosphoethanolamine	M	
	Red	Red	docosapentaenoate (n6 DPA; 22:5n6)	B	
	Red	Red	eicosapentaenoate (EPA; 20:5n3)	B	
Polyunsaturated Fatty Acid (n3 and n6)	Red	Red	linolenate [alpha or gamma; (18:3n3 or 6)]	M	
	Red	Red	inosine	M	
Nucleotide	Purine Metabolism, (Hypo)Xanthine/Inosine containing	Red	Red	inosine 5'-monophosphate (IMP)	B
		Red	Red	urate	B
		Red	Red	xanthosine	M
Peptide	Purine Metabolism, Guanine containing	Red	Red	guanosine	M
		Red	Red	serylisoleucine*	M
		Red	Red	tyrosylglutamate	M
		Red	Red	valylaspartate	M
Xenobiotics	Benzoate Metabolism	Red	Red	3-hydroxyhippurate	B
		Red	Red	4-vinylphenol sulfate	B
	Chemical	Red	Red	hippurate	M
		Red	Red	iminodiacetate (IDA)	B
	Food Component/Plant	Red	Red	cinnamoylglycine	B
		Red	Red	equol glucuronide	B
		Red	Red	equol sulfate	B
		Red	Red	erythritol	B
Red	Red	homostachydrine*	B M		
Red	Red	phytate	B		
Red	Red	stachydrine	B		

**Fig. 4** Potential metabolic biomarkers and metabolic pathways in blood and muscle of grain-fed group compared to the grass-fed group. B, Blood; M, Muscle; Color highlighting indicates the common six metabolites in the two tissues

in the liver of the grass-fed group than the grain-fed group and was 2.46-fold higher in the muscle, which might be due to the different mechanism of mtDNA replication and maintenance in different tissues [9, 42]. The liver and kidney cells replicate their mtDNA using the asynchronous mechanism, skeletal muscle and brown fat with high OXPHOS activity employ a strand-coupled replication mode, combined with increased levels of recombination [9].

### The expression of mitochondria-related genes

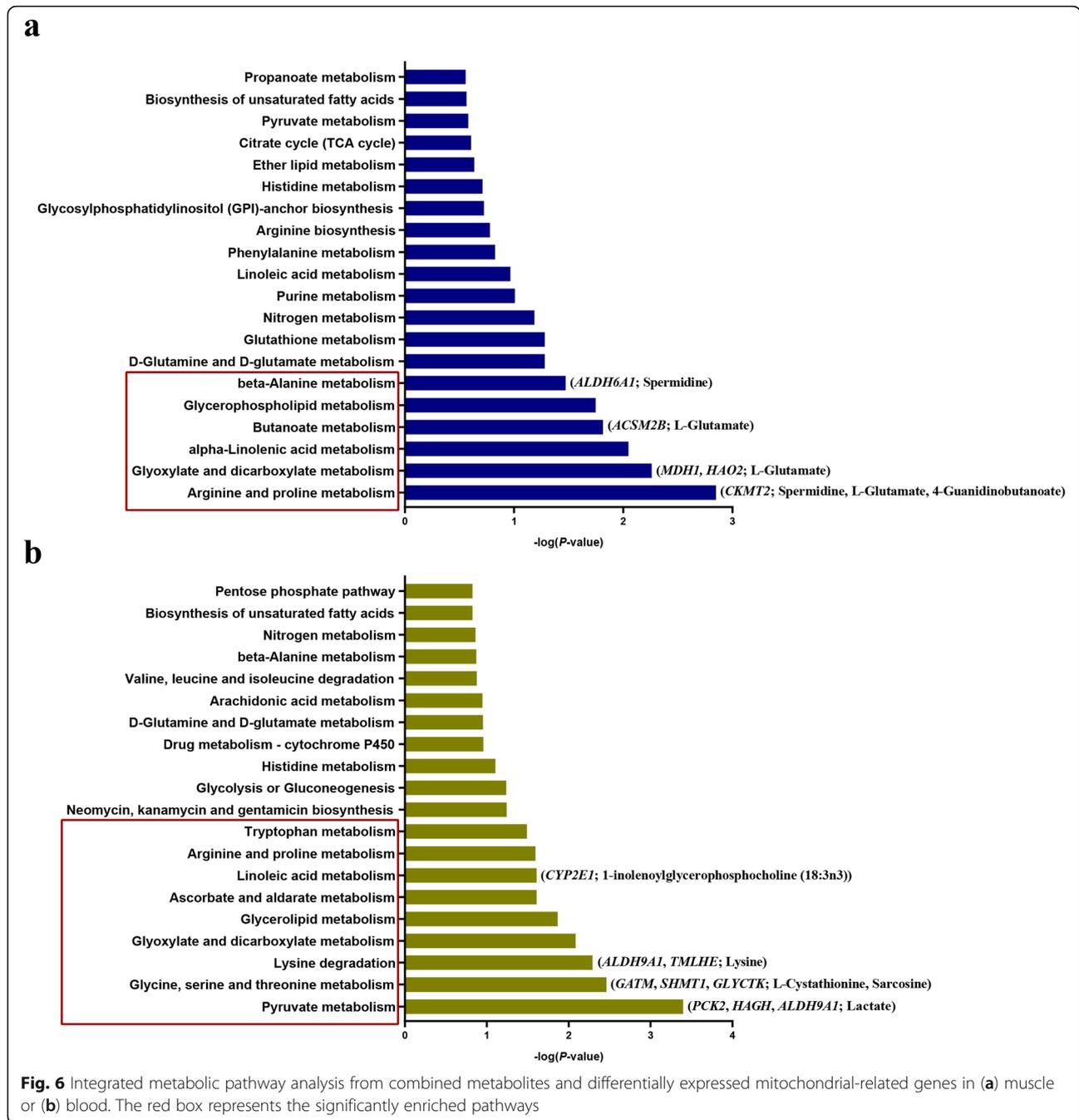
By RNA-seq profiling, 13 mtDNA-encoded genes were diet-dependent and could be differently regulated at the level of transcription. Concerning differential expression, most of the genes encoding subunits of complex I (*ND1*, *ND2*, *ND3*, *ND4*, *ND5*), complex III (*CYTB*) and complex IV (*COX2*) were down-regulated in the grass-fed group, suggesting that the high protein diet might have



an adverse effect on the mtDNA-encoded genes [10]. Mitochondrial Complexes I, III and IV are considered the energy-conserving core of the electron transport chain because they pump protons across the mitochondrial inner membrane, which is responsible for ATP production, and affecting mitochondrial function directly [43, 44]. Future studies should assay the ATP levels in the different tissues of the grass-fed group and grain-fed group. However, the higher expression levels of mtDNA-encoded genes were along with lower mtDNA copy number, implicating that compensation for differentially expressed mtDNA-encoded genes resulting from the different diet might be via transcriptional mechanisms, rather than changes to mtDNA ploidy. Several factors, including but not limited to mtDNA copy number, ultimately determine the steady-state abundance of mtRNAs and derived proteins in a cell [32, 33, 45], most of which were nuclear-encoded factors [16, 46].

To see if the mitochondrial related nuclear genome experience analogous changes in gene expression due to different diets, we examined the expression of mitochondria-related nuclear genes in different tissues of the grass-fed and grain-fed group. In muscle, an inspection of differentially expressed genes showed evidence for up-regulation of ATP synthesis coupled electron transport (*COX6A2*) in grain-fed steers. The *COX6A2* protein is one subunit of the respiratory chain complex IV, of which expression is restricted to striated

muscles [47, 48]. *COX6A2* has an important role in thermogenesis and whole-body energy metabolism and maybe a potential new target for therapy against high-fat diet-induced obesity or insulin resistance [49, 50]. In the liver, all of the differentially expressed genes were up-regulated in the grass-fed group. According to the GO term analysis, the *POLG2* gene was involved in the respiration electron transport chain. *POLG2* encodes the accessory subunits of DNA polymerase gamma, which is the only DNA replicative polymerase involved in the human mitochondria and is crucial for the replication and repair of mtDNA [51, 52]. *POLG2* could enhance interactions with the DNA template and increases both the catalytic activity. Mutations in *POLG2* have a dominant-negative effect and lead to multiple mtDNA deletions [53]. The higher level of *POLG2* might contribute to the larger amount mtDNA copy number in the liver of grass-fed steers compared to the grain-fed. In the rumen, *PPIF* was up-regulated, and *DCN* was down-regulated in the grass-fed group compared to the grain-fed group. These two genes were involved in the GO term of “positive regulation of mitochondrion organization”. *PPIF* is previously known as *cypD*, which is an important mitochondrial chaperone protein and well known for regulating mitochondrial function and coupling of the electron transport chain and ATP synthesis by controlling the mitochondrial permeability transition pore [54, 55].



Overexpression of *cypD* in HEK293 cell mitochondria increased respiratory activity, particularly the activity of complex III. This led to the increasing assembly of supercomplexes containing complexes I, III, and IV. At the same time, *cypD* binds to complex III and supercomplexes containing complex III and faster incorporation of complex III into these supercomplexes [56]. *DCN* is involved in multiple cellular functions such as proliferation, migration, and invasion and acts as a structural molecule, as well as a ligand for receptors [57, 58].

It has been reported that *DCN* potentially attenuated mitochondrial respiratory complexes and mtDNA [59]. In the spleen, *NDUFA12* was involved in the GO term of “oxidation-reduction process”, which was up-regulated in the grass-fed group compared to the grain-fed group. *NDUFA12* was required for the formation of the extra membrane arm of mitochondrial complex I [60] and had the function of the stability of complex I [61]. Based on these results, it suggested that different mitochondria-related nuclear factors, involved in ATP synthesis,

mitochondrial replication, transcription, and maintenance, might contribute to the mtDNA copy variation and changing of mtDNA expression in different tissues of grass-fed and grain-fed steers.

### Key metabolic pathways after single and integrated analysis

According to the previous study, alterations in glucose metabolism and divergences in free fatty acids were found between the grass-fed and grain-fed Angus steers [29]. In the present study, 40 and 23 metabolic biomarkers were identified in the blood and muscle of the grain-fed group compared to a grass-fed group, respectively. Based on the single metabolic pathway analysis, the most significantly impacted pathway in the muscle of grass-fed and grain-fed group was glycerophospholipid metabolism, which was also significantly different in the blood of the two different diet group. Changes in lipid abundance may also be indicative of altered membrane metabolism. Phosphoethanolamine, glycerophosphocholine, and glycerophosphoethanolamine were elevated in grain-fed muscle and may suggest increased membrane turnover, which may support enhanced tissue growth [62]. In blood, the most significantly impacted pathway was Neomycin, kanamycin, and gentamicin biosynthesis, which was also the top pointed in the combined level (enrichment and biological meaning). The involved metabolite was NADH, which was important for the TCA cycle. In contrast, NADH was elevated in the blood, but diminished elevated in the muscle of grass-fed compared to grain-fed cattle and may highlight differential energy regulation by the muscle versus other tissues in response to these diets. The related gene *MDH1* was down-regulated in the muscle of the grain-fed group compared to grass-fed, while up-regulated in the liver and rumen. MDH1 is a NAD(H)-dependent enzyme and a part of the malate–aspartate shuttle (MAS). The synthesis of cytosolic malate was via MDH1. MDH1 generates NAD<sup>+</sup> upon the reduction of oxaloacetate to malate. MAS is important for intracellular NAD(H) redox homeostasis as it transfers, reducing equivalents across the mitochondrial membrane [63].

Meanwhile, the most critical pathway was arginine and proline metabolism based on the integrated analysis in muscle. Three metabolites (spermidine, *L*-glutamate, 4-guanidinobutanoate), and one gene (*CKMT2*) were involved in this pathway. *CKMT2* is a mitochondrial creatine kinase responsible for the transfer of high energy phosphate from the mitochondria to the cytosolic compartment, and at the same time for returning ADP to the mitochondrial respiratory system, thereby stimulating oxidative phosphorylation, which was negatively associated with spermidine, *L*-glutamate, and 4-guanidinobutanoate in our results. Spermidine,

*L*-glutamate, and 4-guanidinobutanoate were elevated levels in grain-finished muscle and may reflect higher synthesis and availability for tissue growth. Spermidine displays pleiotropic effects that include anti-inflammatory properties, antioxidant functions, enhancement of mitochondrial metabolic function, and respiration [64]. ATP, as polyanion, is capable of suppressing the polycationic effect of spermidine [65]. However, the relationship between the *CKMT2* and spermidine needed to be further studied. Meanwhile, spermidine is a polyamine compound, and the higher level in grain-finished muscle might occur due to the maintenance of mitochondrial NADH oxidation that responded to lower mtDNA copy number [66].

### Conclusions

In summary, these data suggest that the grass and grain diets in cattle would cause the differences in the mtDNA copy number, mtDNA expression, and mRNA expression of selected nuclear genes involved in mitochondrial function. Meanwhile, the changes of mitochondria-related gene expression might contribute to the metabolites level variations. However, further research is required to better understand the relationships between mitochondria function, metabolic and molecular mechanisms, and beef quality.

### Supplementary information

**Supplementary information** accompanies this paper at <https://doi.org/10.1186/s40104-020-00482-x>.

**Additional file 1: Table S1.** Feed components analysis. **Table S2.** The 1283 mitochondria-related genes. **Table S3.** Differentially expressed mitochondria-related gene in the four tissues. **Table S4.** The differentially expressed genes in muscle of grass-fed and grain-fed Angus cattle. **Table S5.** The differentially expressed genes in liver of grass-fed and grain-fed Angus cattle. **Table S6.** The differentially expressed genes in rumen of grass-fed and grain-fed Angus cattle. **Table S7.** The differentially expressed genes in spleen of grass-fed and grain-fed Angus cattle. **Table S8.** Gene ontology terms in the four tissues.

**Additional file 2: Figure S1.** KEGG pathways differentially expressed between grass-fed and grain-fed Angus cattle. (A) muscle, (B) liver, (C) spleen, (D) rumen.

**Additional file 3: Table S1.** The 326 compounds were detected in blood. **Table S2.** The 353 compounds were detected in muscle.

### Abbreviations

OXPHOS: Oxidative phosphorylation; mtDNA: Mitochondrial DNA; NRF: Nuclear respiratory factor; TFAM: Mitochondrial transcription factor A; PGC-1 $\alpha$ : Peroxisome proliferator-activated receptor- $\gamma$  coactivator-1 $\alpha$ ; ND2: NADH dehydrogenase subunit 2; ND5: NADH dehydrogenase subunit 5; CYTB: Cytochrome B; COX3: Cytochrome oxidase subunit III; ACTB: Actin B; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Gene and Genomes; QC: Quality Control; LC/MS: Liquid Chromatography/Mass Spectroscopy; GC/MS: Gas Chromatography/Mass Spectroscopy; DEG: Differentially expressed gene; CKMT2: Creatine kinase, mitochondrial 2; UBB: Ubiquitin B; MDH1: Malate dehydrogenase 1; LYRM7: LYR motif containing 7; CYP11A1: Cytochrome P450, family 11, subfamily A, polypeptide 1; DHCR24: 24-dehydrocholesterol reductase; SHMT1: Serine hydroxymethyltransferase 1

**Acknowledgements**

Not applicable.

**Authors' contributions**

JS designed the study. JAC and YL collected samples. YL, JAC and YB generated the data. YB, JAC and YH analyzed the data. YB contributed to the writing of the manuscript. YB and JAC constructed the Figures. All authors have revised and approved the final manuscript.

**Funding**

This work was supported by Maryland Agricultural Experiment Station (MAES), Jorgensen Endowment Funds.

**Availability of data and materials**

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request. The datasets analysed during the current study are available in the GEO DataSets repository. The web links to datasets were <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE70248>, <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE67018>, <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE63550>, <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE145375>

**Ethics approval**

All animal experiments were conducted according to the NIH guidelines for housing and care of laboratory animals and following the regulations of the University of Maryland at College Park (UMCP). The UMCP Institutional Animal Care and Use Committee (IACUC) reviewed and approved the protocols (permit number R-08-62).

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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Received: 20 February 2020 Accepted: 9 June 2020

Published online: 21 July 2020

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