University of São Paulo
"Luiz de Queiroz" College of Agriculture

The biochemistry of feed efficiency, energy metabolism, and mitochondrial function, an animal and molecular approach

Welder Angelo Baldassini

Thesis presented to obtain the degree of Doctor in Science. Area: Animal Science and Pastures

Piracicaba
2017

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Animal Scientist

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versão revisada de acordo com a resolução CoPGr 6018 de 2011

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DEDICATION

To my parents,

Angelo Baldassini Neto and **Clarinda dos Anjos Baldassini**

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“I believe that science is the engine of prosperity, that if you look around at the wealth of civilization today, it's the wealth that comes from science.”

Dr. Michio Kaku

CONTENTS

RESUMO

Bioquímica da eficiência alimentar, metabolismo energético e função mitocondrial, uma abordagem animal e molecular

A eficiência energética é importante para a saúde humana (gênese da obesidade), sistemas de produção de carne (custo dos alimentos para produzir proteínas de alta qualidade) e para o meio ambiente (uso de recursos naturais e mitigação de gases de efeito estufa). As mitocôndrias são organelas que desempenham papel central no metabolismo e homeostase relacionada a utilização da energia. Nas células, diversas proteínas são importantes para melhorar a eficiência energética. Como exemplos, as proteínas de sinalização *Shc* são fundamentais na oxidação de substratos e metabolismo energético e, nas mitocôndrias, existem as proteínas desacopladoras (UCPs), que participam do gasto energético e produção de calor. Entretanto, os mecanismos que controlam o gasto energético nos animais ainda é bastante desconhecido. Assim, para estudar o metabolismo energético e a função das mitocôndrias foram conduzidos dois estudos utilizando-se estratégias nutricionais, bioquímicas e moleculares com camundongos (1) e bovinos (2). Objetivou-se, no estudo 1, determinar se as proteínas *Shc* influenciam a resposta metabólica à alimentação contendo dieta rica em gordura (HFD) por 7 dias. Enzimas da via glicolítica, ciclo de Krebs, cadeia transportadora de elétron (CTE) e β-oxidação foram analisadas no fígado e músculo de camundongos com baixa expressão de *Shc* (*knockout* ou ShcKO) e comuns (*wild-type* ou WT) submetidos à uma dieta controle ou à HFD. O gasto energético foi medido por câmara calorimétrica de respiração nos animais. O genótipo ShcKO apresentou maior gasto energético ($P < 0.05$) ajustado para o peso corporal total ou massa magra. Essa mudança poderia explicar o menor ganho de peso observado no genótipo ShcKO comparado ao WT quando consumindo a HFD. Esses resultados sugerem que as proteínas *Shc* podem contribuir no desenvolvimento de estratégias para mitigar o ganho de peso. Embora a redução dos níveis de *Shc* (ShcKO) tenha modificado a atividade de enzimas da β-oxidação em resposta a HFD, tal condição não produziu mudanças semelhantes na via glicolítica, ciclo de Krebs ou CTE. Por isso, mais estudos são necessários para compreender a significância fisiológica dessas alterações. No experimento 2, objetivou-se estudar a associação entre produção de calor, variáveis sanguíneas e número de cópias de DNA mitocondrial (mtDNA) em bovinos Nelore agrupados pelo consumo alimentar residual (CAR). O CAR foi obtido por regressão do consumo de matéria seca em relação ao ganho de peso diário e peso metabólico do teste de desempenho (fase de crescimento). Assim, 18 bovinos (9 alto CAR *versus* 9 baixo CAR) foram confinados em baias individuais por 98 dias (fase de terminação). Os batimentos cardíacos (BC) dos bovinos foram monitorados por quatro dias consecutivos e, então, utilizados para o cálculo da produção de calor estimada (PC_e). O consumo e pulso de oxigênio (O_2) foram obtidos por meio de analisador de gás conectado à

uma máscara facial, com medição simultânea dos BC por 15 minutos. A PC_e diária foi calculada por multiplicação do pulso de O_2 pela média dos BC, assumindo-se a constante 4.89 kcal/L de O_2. Foram analisadas variáveis sanguíneas como hematócrito, hemoglobina e glicose (alto vs. baixo CAR). Imediatamente após o abate dos animais, amostras de fígado, músculo e tecido adiposo foram coletadas para determinação do mtDNA por PCR em tempo real. Adicionalmente, o proteoma do tecido hepático e os níveis de UCPs nos tecidos foram também investigados. Não houve diferença para PC_e e consumo de O_2 ($P > 0.05$) entre os grupos experimentais, entretanto, os animais baixo CAR (mais eficientes em conversão alimentar) demonstraram menor BC, concentração de hemoglobina e percentagem de hematócrito ($P < 0.05$), confirmando resultados previamente observados em nossos estudos. No fígado, 71 spots proteicos foram diferentes ($P < 0.05$) entre os grupos alto e baixo CAR, mas nenhuma diferença foi observada para os níveis de UCPs no músculo, fígado ou tecido adiposo. Por fim, não existiu diferença ($P > 0.05$) entre o número de cópias do mtDNA por célula entre os fenótipos estudados, sugerindo que o número de mitocôndrias e possivelmente a fosforilação oxidativa foi semelhante entre os grupos de animais eficientes e ineficientes. Contudo, são necessários estudos adicionais para confirmar essa hipótese.

Palavras-chave: Bioenergética; Bioquímica; Metabolismo; Mitocôndrias; DNA mitocondrial

ABSTRACT

The biochemistry of feed efficiency, energy metabolism, and mitochondrial function, an animal and molecular approach

Energetic efficiency is important for health (e.g. genesis of obesity in humans), socio-economically important for meat production systems (e.g. feed cost to produce high quality protein) and important for the environment (e.g. use of natural resources and production of green house gases for meat production). Mitochondria are organelles that play an essential role in cellular metabolism and homeostasis related to energy utilization. These processes involve several proteins to ensure continuous availability of energy to the cells. The Shc proteins play a key role in substrate oxidation and energy metabolism. Additionally, the mitochondrial uncoupling proteins (UCPs) participate in physiological processes that may account for variation in energy expenditures in tissues. However, the mechanisms behind energy expenditure in animals are largely unknown. Thus, in order to study the energy metabolism and mitochondria function, studies using a nutritional, biochemical and molecular approaches were conducted with mice and cattle. The purpose of the first study was to determine if Shc proteins influence the metabolic response to acute (5-7 days) feeding of a high fat diet (HFD). To this end, whole animal energy expenditure and substrate oxidation were measured in the Shc knockout (ShcKO) and wild-type (WT) male mice consuming either a control or HFD diet. The activities of enzymes of glycolysis, the citric acid cycle, electron transport chain (ETC), and β-oxidation were investigated in liver and skeletal muscle. The study showed that ShcKO increases ($P < 0.05$) energy expenditure (EE) adjusted for either total body weight or lean mass. This change in EE could explain the decrease in weight gain observed in ShcKO versus WT mice fed an HFD. Thus, our results indicate that Shc proteins should be considered as potential targets for developing interventions to mitigate weight gain on HFD by stimulating EE. Although decreased levels of Shc proteins influenced the activity of some enzymes in response to high fat feeding, such as increasing the activity of acyl-CoA dehydrogenase, it did not produce concerted changes in enzymes of glycolysis, citric acid cycle or the ETC. However, the physiological significance of these changes in enzyme activities remains to be determined. The purpose of experiment 2 was to study the association among heat production, blood parameters and mitochondrial DNA (mtDNA) copy number in Nellore bulls with high and low residual feed intake (RFI). The RFI values were obtained by regression of dry mater intake (DMI) in relation to average daily gain and mid-test metabolic body weight. Thus, 18 animals (9 in each group) were individually fed in a feedlot for 98 days. The heart rate (HR) of bulls was monitored for 4 consecutive days and used to calculate the estimated heat production (EHP). Electrodes were fitted to bulls with stretch belts and oxygen consumption was obtained using a facemask connected to the gas analyzer and HR was simultaneously measured for 15 minutes period.

Daily EHP was calculated multiplying oxygen pulse (O2P) by the average HR, assuming 4.89 kcal/L of O_2. Blood parameters such as hematocrit, hemoglobin, and glucose were assayed between 45 and 90 days. Immediately after slaughter, liver, muscle and adipose tissues (subcutaneous and visceral fat) were collected and, subsequently, mtDNA copy number per cell was quantified in tissues by quantitative real-time PCR. The proteome of hepatic tissue and levels of mitochondrial UCPs were also investigated. We found similar EHP and O_2 consumption between RFI groups, while low RFI bulls (more efficient in feed conversion) shown lower HR, hemoglobin and hematocrit percentage ($P < 0.05$), confirming previous data from our group. In addition, 71 protein spots in liver were differentially expressed ($P < 0.05$) and no differences were detected for UCPs levels between RFI groups. Finally, there was no association between amounts of mtDNA and the RFI phenotypes, suggesting that mitochondrial abundance in liver, muscle, and adipose tissue was similar between efficient and inefficient groups. However, additional studies to confirm this hypothesis are needed.

Keywords: Bioenergetics; Cell biochemistry; Energy metabolism; Mitochondrial content; Mitochondrial DNA

1. INTRODUCTION

Energy expenditure and the efficiency of energy conversion into body weight gain is of enormous importance. First, it is related to obesity in humans, which has extraordinary health and economic implications. Secondly, in animal agriculture feed efficiency (FE) is related to cost and profitability of meat production. Thirdly, feed efficiency is of paramount importance in determining the environmental impact of animal agriculture. Animal feed conversion efficiency accounts for most of the variation in the emission of pollutants, green house gases, and in the utilization of many natural resources for meat production.

In animal cells, Shc proteins act as adaptor molecules which specifically bind to phosphorylated tyrosine residues on the cytoplasmic motif of growth factor receptors, thereby serving as mitogenic signaling molecules. These proteins have an important influence on energy metabolism and previous reports have implicated their role in energy expenditure, whereby animals with low levels of Shc proteins (Shc knockout or ShcKO) have lower body weight than wild-type (WT) at the same level of energy intake [1]. In addition, Shc proteins appear to influence the fuels used for energy metabolism in the mitochondria [2]. Thus, a decrease in Shc levels may help animals adapt to periods of consumption of high fat diet (HFD). However, the overall impact of Shc proteins on energy metabolism is not entirely clear when ShcKO animals are fed a HFD.

In parallel, the mitochondrial uncoupling proteins (UCPs) are involved in different processes such as control of ATP synthesis and mediating the production of reactive oxygen species (ROS) [3]. Additionally, the UCPs may increase energy expenditure (by producing heat instead of ATP), which aroused interest in the studies regarding FE traits in animal agriculture. The association between mitochondrial function (and UCPs) and FE relates to the fact that cells of less efficient animals may have higher requirements for the production of the same amount of ATP [4-6]. Additionally, in mitochondria from these animals there may be a greater protein oxidation due to greater basal mitochondrial ROS production [5]. This increased ROS production could oxidize proteins, as well as mitochondrial DNA (mtDNA) causing impaired protein synthesis and oxidative phosphorylation [3, 7-9]. However, more

studies are needed to understand the role of mtDNA and UCPs on the phenotypic expression of FE.

In the context of energy metabolism and mitochondrial function, this thesis has two main objectives: I) To determine if Shc proteins influence the metabolic response of animals to acute feeding of an HFD; II) To determine the possible association between heat production, blood physiological indicators, mitochondrial content (mtDNA copy number), hepatic protein profile and levels of UCPs in liver, muscle, and adipose tissue from animals classified according to FE (efficient versus inefficient).

To this end, in chapter 2 we discuss the results of published studies regarding the role of Shc proteins on growth, enzyme activities of energy metabolism**,** mitochondrial function, and oxidative stress. We also review the importance of UCPs on energy metabolism, as well as how the mtDNA and the abundance of mitochondria are regulated in animals under different physiological conditions.

In chapter 3, we provide a study with ShcKO and WT mice fed a control (chow) or HFD. In addition, in this study we performed a whole animal energy expenditure and substrate oxidation study, where we measured the activities of enzymes of glycolysis, the citric acid cycle, electron transport chain, and β-oxidation in liver and skeletal muscle. We report higher energy expenditure in ShcKO mice and these results could be due to a better ability to alter substrate oxidation in response to a shift in diet composition and/or energy metabolism. Although decreased levels of Shc proteins influenced the activity of a central enzyme of β-oxidation in response to high fat feeding, it did not produce concerted changes in enzymes of glycolysis, citric acid cycle or the electron transport chain.

In chapter 4 we performed a study with Nellore bulls which were classified according to residual feed intake, an index commonly used to measure FE. Residual feed intake (RFI) is the difference between observed feed intake and that predicted based on a regression of average body weight and growth rate in animals fed the same diet in the same group environment. Efficient animals (low RFI) eat less that what is predicted based on their weights and rate of growth. On the other hand, animals that eat more than predicted are considered inefficient (high RFI).

Our hypothesis was that estimated heat production (EHP), blood parameters, liver protein profile, UCPs levels, and mtDNA copy number in liver, muscle, and adipose tissue would differ among RFI phenotypes (high versus low RFI). Briefly, we

describe that EHP and mtDNA copy number per cell were similar between RFI groups, while heart rate, hemoglobin, and hematocrit were lower in the efficient phenotype (low RFI). We also found that liver protein profile measured by two-dimensional electrophoresis is different between RFI groups. Also, we investigated the levels of UCP in the liver, muscle, and adipose tissue from these animals and discuss these results as related to FE. Finally, in chapter 5, we present the general conclusions of this thesis.

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2. KEY PROTEINS AT THE INTERFACE OF BIOENERGETICS AND MITOCHONDRIAL FUNCTION

Abstract

The Shc and mitochondrial uncoupling proteins (UCP) have been proposed to play an important role in energy expenditure (EE) and cellular metabolism. Thus, our objective was to discuss the influence of Shc proteins and UCP on energy metabolism of mice and beef cattle. Additionally, we review the possible association between mitochondrial DNA (mtDNA copy number is an indicator of cell mitochondrial content) and levels of UCP in tissues from animals classified according to feed efficiency (FE) values. Several studies in mice, humans, and cattle have been conducted to investigate the role of Shc proteins play in aging and control of reactive oxygen species (ROS) production. Some studies have investigated the impact of low Shc levels (ShcKO) in preventing oxidative stress, apoptosis, and hyperglycemia. In general, ShcKO is associated with changes in mitochondrial function and EE and protection of tissues against oxidative stress. However, little is known about the role of Shc proteins on energy metabolism in animals consuming a high fat diet. In parallel, UCP activity in mitochondria provides adaptive thermogenesis, carbon flux maintenance and also protection of cell membranes against oxidative stress caused by ROS. In mitochondrial metabolism, UCP activity (uncoupling) may represent a cellular inefficiency but also a reduction in oxidative stress by attenuating mitochondrial ROS production. Thus, studies suggest that mitochondria from less FE animals have greater protein oxidation due to greater basal mitochondrial ROS production. This increased ROS production could oxidize proteins, as well as mtDNA and nuclear DNA causing impaired protein synthesis. Additionally, studies have shown that mtDNA content reflects the abundance of mitochondria within a cell and may vary according to the energy demands of tissues. However, additional studies are needed to understand the physiological significance of these changes in mitochondrial function and energy metabolism.

Keywords: Energy expenditure; Feed efficiency; Metabolism; Oxidative stress; Shc protein; Uncoupling

2.1. Introduction

Signaling proteins are essential for regulating the cellular processes that contribute to whole energy expenditure and metabolism. In particular, Shc proteins have an important influence on energy metabolism, and studies have shown that mouse with low levels of Shc proteins (ShcKO) have lower body weight than wild-

type (WT) at the same level of energy intake (Berniakovich et al., 2008). In addition, Shc proteins appear to influence the fuels used for energy but they do not produce a net change in overall capacity for energy metabolism in the mitochondria (Hagopian et al., 2016). Thus, a decrease in Shc levels may help animals adapt to periods of caloric restriction or consumption of a high fat diet (HFD). However, little is known about Shc proteins on energy metabolism in animals fed a HFD.

In parallel, others key proteins for bioenergetics and metabolism are the mitochondrial uncoupling proteins (UCPs). In these organelles, the UCPs are localized in the inner membrane and are involved in different processes such as control of ATP synthesis and control of reactive oxygen species (ROS) (Azzu et al., 2010). Additionally, the UCPs may increase energy expenditure (by producing heat instead of ATP), which aroused interest in studies investigating the impact of these proteins on feed efficiency (FE) (Bottje and Carstens, 2009).

Moreover, oxidative damage to mitochondrial proteins may be increased in animals with less active UCPs due to greater mitochondrial ROS production (Bottje and Carstens, 2009). This increased ROS production could oxidize proteins, as well as mitochondrial DNA (mtDNA) causing impaired protein synthesis and oxidative phosphorylation (Azzu and Brand, 2010; Azzu et al., 2010; Baughman and Mootha, 2006; Laubenthal et al., 2016).

This review has two main objectives: I) To describe the influence of Shc proteins on energy metabolism; II) To discuss the role of UCPs and mtDNA copy number in metabolic tissues from animals classified according to FE (efficient versus inefficient).

2.2. Shc proteins

There are three isoforms at the mammalian Shc locus, the highly expressed isoforms p46Shc and p52Shc, and the minor p66Shc. The Shc molecule is frequently described as a longevity protein, although it has recently been shown that lifespan is not increased in mice with low levels of Shc proteins (Ramsey et al., 2014). Thus, Shc proteins do not alter lifespan, but they do influence stress response (Migliaccio et al., 1999). However, cells derived from these mice exhibit lower levels of ROS (Nemoto et al., 2006).

Although several studies considered the depletion of p66Shc isoform as the main driver for changes in metabolism (Camici et al., 2007; Natalicchio et al., 2009), the p52Shc and p46Shc depletion in cells from ShcKO mice are the more likely candidates for alterations in insulin sensitivity and mitochondrial activation. Thus, decreases in the major Shc isoforms p52/p46 appear to drive both animal adiposity and insulin sensitivity phenotypes, and also alterations in mitochondrial function (Tomilov et al., 2014).

Higher ROS generation may occur in tissues from wild-type (WT) compared to animals with low Shc protein levels. During mitochondrial respiration, electrons are extracted from substrates and transferred to molecular oxygen through successive redox reactions that are catalyzed by enzymatic complexes (termed Complexes I–IV). In the final step of this electron transfer chain (ETC), cytochrome c oxidase (Complex IV) ensures the reduction of molecular oxygen to water, without the formation of oxygen radicals. However, partial reduction of oxygen with the generation of ROS can occur in sites other than Complex IV with the mitochondrial ETC (Orsini et al., 2006).

In mitochondria, ROS originated from the respiration process are inducers of oxidative damage and tissue dysfunction. Thus, when produced in excess, ROS affect many cellular processes such as energetic metabolism, signal transduction, gene expression, cell cycle and apoptosis (Orsini et al., 2006). It is important to note that ROS may be produced in a controlled way through specialized enzymes, and take part in regulating cellular processes (Bartosz, 2009). Several studies in mice, humans, and cattle have been conducted to investigate the role Shc proteins play in cellular metabolism and oxidative damage (Table 1). In particular, these studies evaluated the impact of decreased Shc protein levels on preventing oxidative stress, apoptosis, and hyperglycemia.

Table 1. Studies with different animal species regarding the role of Shc proteins on celullar metabolism and regulation of reactive oxygen species (ROS).

Reference	Species	Hypothesis	Results
(Camici et al., 2007)	Mice	Shc proteins are involved with hyperglycemia and diabetes mellitus	Deletion of p66Shc protects against hyperglycemia-induced, and ROS-dependent endothelial dysfunction
(Natalicchio et al., 2009) ¥	Mice	Shc proteins are involved with glucose transport in skeletal muscle	p66Shc regulate the glucose transport system in skeletal muscle by controlling, via MAP kinase, the integrity of the actin cytoskeleton and by modulating cellular expression of GLUT1 and GLUT3 transporter proteins
(Betts et al., 2014)	Cattle	Shc proteins regulates the oxidative stress response in bovine early embryo development	p66Shc knockdown (ShcKO) embryos exhibited reduced intracellular ROS and DNA damage (were stress resistant), exhibiting reduced oxidative stress and apoptosis
(Perrini et al., 2015)	Human	Shc proteins mediates oxidative stress-related injury in liver cells and alcoholic steatohepatitis	Increased hepatocyte p66Shc protein levels may enhance susceptibility to DNA damage by oxidative stress by promoting ROS synthesis and repressing antioxidant pathways

¥ MAP kinase = mitogen activated protein kinase; GLUT1 = glucose transporter 1; GLUT3 = glucose transporter 3

It has been known that Shc proteins play a role in insulin signaling (Ravichandran, 2001; Sasaoka and Kobayashi, 2000), but relatively little has been known about the overall impact of these proteins on energy metabolism. The first evidence that Shc proteins may have an important influence on energy metabolism came from the observation that body weight and composition are altered in Shc knockout (ShcKO) mice. It was shown that body weight is decreased in ShcKO mice, despite the fact that their energy intake is not different than WT animals (Berniakovich et al., 2008). This decrease in body weight is due to the fact that the weights of all fat pads are lower in ShcKO compared to WT mice (Berniakovich et al., 2008; Tomilov et al., 2011).

Similarly, it has been reported that ShcKO mice are resistant to weight gain on a HFD (Berniakovich et al., 2008) and ShcKO in leptin-deficient ob/ob mice decreases weight gain in these obese animals (Ranieri et al., 2010). Insulin sensitivity and glucose tolerance were increased in ShcKO mice (Ranieri et al., 2010; Tomilov et al., 2011), and this change in insulin sensitivity would be expected to influence energy metabolism in the fed state. However, results indicate that there are substantial changes in energy metabolism in ShcKO mice in the fasted state (Hagopian et al., 2015). In particular, enzyme activity measurements in skeletal muscle and liver indicate a shift in metabolism towards increased capacity for fatty acid oxidation, ketogenesis, ketone body catabolism, gluconeogenesis, and amino acid catabolism, while capacity for glycolysis is decreased in ShcKO compared to WT mice under fasting conditions (Hagopian et al., 2016). These changes in enzyme activities mirror those observed in animals under caloric restriction (CR) conditions (Hagopian et al., 2003; Stern et al., 2012a).

Additional studies with mouse models (ShcKO versus WT mice) were reported in the literature to investigate the role of Shc proteins in mitochondrial function and energy metabolism (Table 2). In general, the approaches associate decreased Shc levels with changes in the mitochondrial generation of ATP, regulation of mtDNA replication, energy expenditure and protection against oxidative stress in main body tissues following consumption of a HFD.

Table 2. Studies regarding the influence of Shc proteins on mitochondrial function and energy metabolism of mice.

Reference	Hypothesis	Results
(Nemoto et al., 2006)	Shc proteins are localized in mitochondria and regulate energy expenditure	The p66Shc is localized within the mitochondria, and functions as a regulator of mitochondrial metabolism, as well as regulate the partition of ATP generation in the cell
(Orsini et al., 2006)	Shc proteins are regulated by transcription factors, protein stabilization, and post-translational modifications	p66Shc activity is finely regulated both by cytosolic signals and by an intrinsic mitochondrial control, whereby changes in energetic status result in altered release of p66Shc
(Trinei et al., 2006)	Shc proteins are involved in the regulation of mitochondrial DNA (mtDNA) copy number	p66Shc is part of the signaling pathway that regulates mtDNA replication both *in vitro* and *in vivo*
(Hagopian, K. et al., 2012)	Shc proteins influence enzymes involved in β-oxidation	Shc knockout mice showed increased liver and muscle β-oxidation enzyme activities in response to fasting and induce chronic increases in the activity of liver ketogenic enzymes
(Stern et al., 2012b)	Shc proteins regulate whole body energy metabolism at 22˚C and at 12˚C (acute cold exposure).	Shc knockout mice demonstrate a slightly lighter total body mass and fat-free mass and, therefore, energy expenditure was decreased in Shc knockout compared to wild-type animals (kJ/mouse or adjusted for body weight)
(Bellisario et al., 2014)	Shc knockout mice are protected from the metabolic stressful induced by high fat diet (HFD)	Shc knockout mice showed greater resistance toward glucose challenge (by HFD) compared to wild-type, and this Shc deletion results in insulin desensitization, acting as a protective factor

It is important to note that the p66Shc knockout mice used by investigators have recently been shown to have low levels of all Shc isoforms in many tissues (Hagopian et al., 2016; Tomilov et al., 2011). Thus, it is difficult to determine if observed changes are truly due to p66Shc or due to one of the other Shc isoforms.

2.3. Shc proteins and body composition

Studies have shown no differences in energy expenditure (adjusted for body weight or lean body mass) or respiratory quotient (RQ) between ShcKO and WT when allowed *ad libitum* access to food (Stern et al., 2012a, b). Similarly, there were no difference in energy expenditure and RQ between *ad libitum* fed groups of mice when exposed to an ambient temperature of 12°C for 24 hours (Stern et al., 2012b). However, energy expenditure was decreased ($P < 0.05$) in the ShcKO versus WT mice immediately following the initiation of CR. Mice were sacrificed following completion of the calorimetry measurements and body composition was determined, including organ and fat pad weights. Very clear decreases in all fat pad weights were noticed in one-year-old ShcKO mice, and it was also noticed that small, but significant ($P < 0.05$), decreases in epididymal, perirenal and subcutaneous fat pads were already present at 3 months of age (Figure 1) (Hagopian et al., 2012).

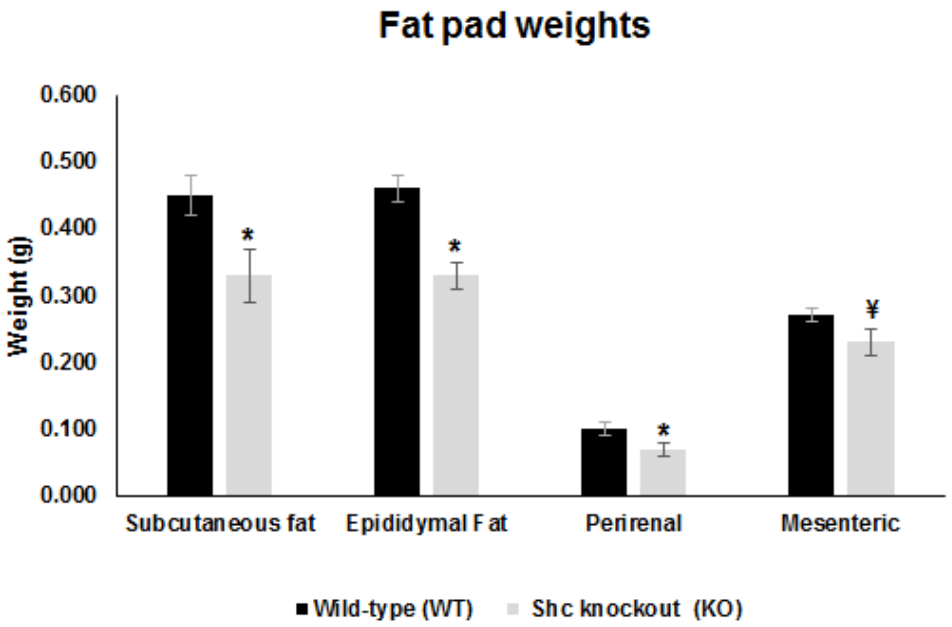

Figure 1. Fat pad weights in knockout (KO) and wild-type (WT) mice at 3 months of age and *ad libitum* fed on chow diet. *$P < 0.05$ and ¥ $P < 0.10$ between KO and WT mice. Adapted from (Hagopian et al., 2012a).

2.4. Shc proteins and enzymes activities of energy metabolism

Significant differences ($P < 0.05$) were observed between the two groups of mice (WT vs. ShcKO) for enzyme activities in the major pathways of intermediary metabolism as follow: *i*) Glycolytic metabolites were decreased in ShcKO versus WT mice ($P < 0.05$) corresponding with a decrease in glycolytic enzyme activity; *ii*) Ketone body levels were increased in ShcKO versus WT mice, corresponding with increased ketone body synthesis. Metabolite levels are consistent with a shift in substrate metabolism towards increased fatty acid and decreased glucose oxidation during fasting in ShcKO mice (Hagopian et al., 2012; Tomilov et al., 2011; Tomilov et al., 2016).

Levels of lactate and pyruvate were also measured and the ratio of lactate to pyruvate was taken to indicate the redox state of the cytosol (Hagopian et al., 2015). These results indicate that cytosolic redox state was significantly altered in fasted ShcKO versus fed WT mice (Figure 2).

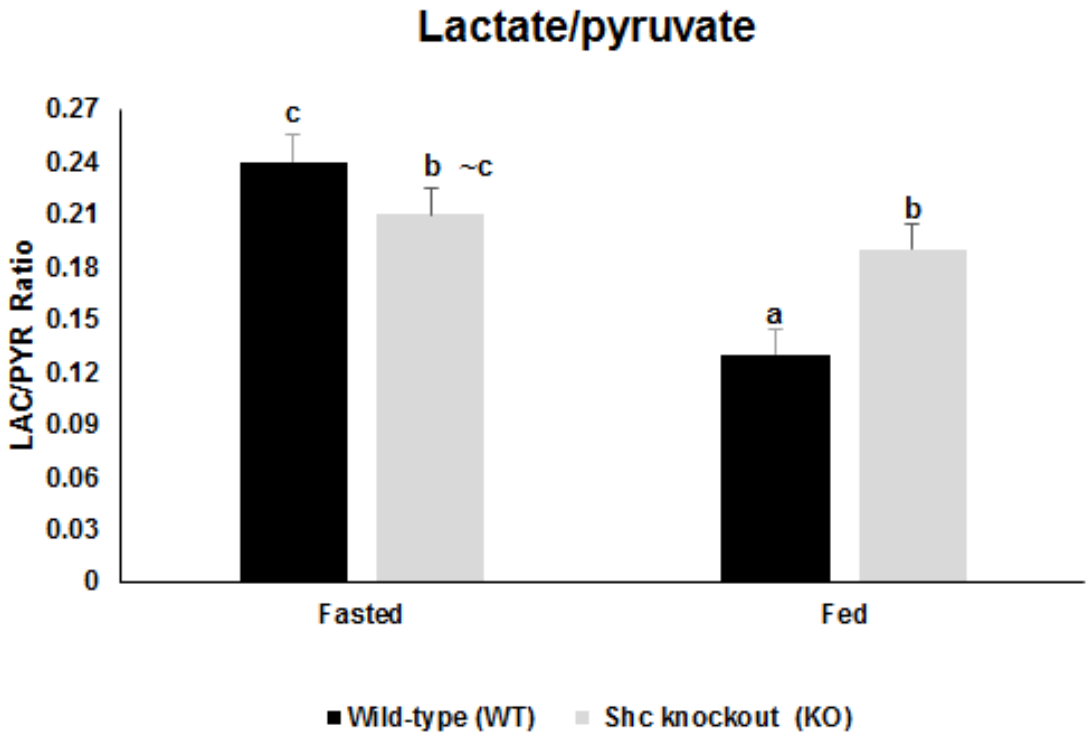

Figure 2. Ratios of muscle lactate/pyruvate as indicators of redox state in the cytosol in Shc knockout (KO) and wild-type (WT) mice. Animals were compared within a genotype, fasted versus fed, across genotype, fasted versus fasted and fed versus fed. Adapted from (Hagopian et al., 2015).

2.5. β-oxidation and ketone body metabolism

The activities of the β-oxidation enzymes acyl-CoA dehydrogenase, hydroxyacyl-CoA dehydrogenase, and ketoacyl-CoA thiolase were measured in skeletal muscle from fed and fasted ShcKO and WT mice (Hagopian et al., 2012). In the fed state, there were no differences ($P > 0.05$) in the activities of any of these enzymes between the groups of mice. However, the activities of all of the β-oxidation enzymes were increased ($P < 0.05$) in ShcKO versus WT mice following an overnight (16 hours) fast (Figure 3).

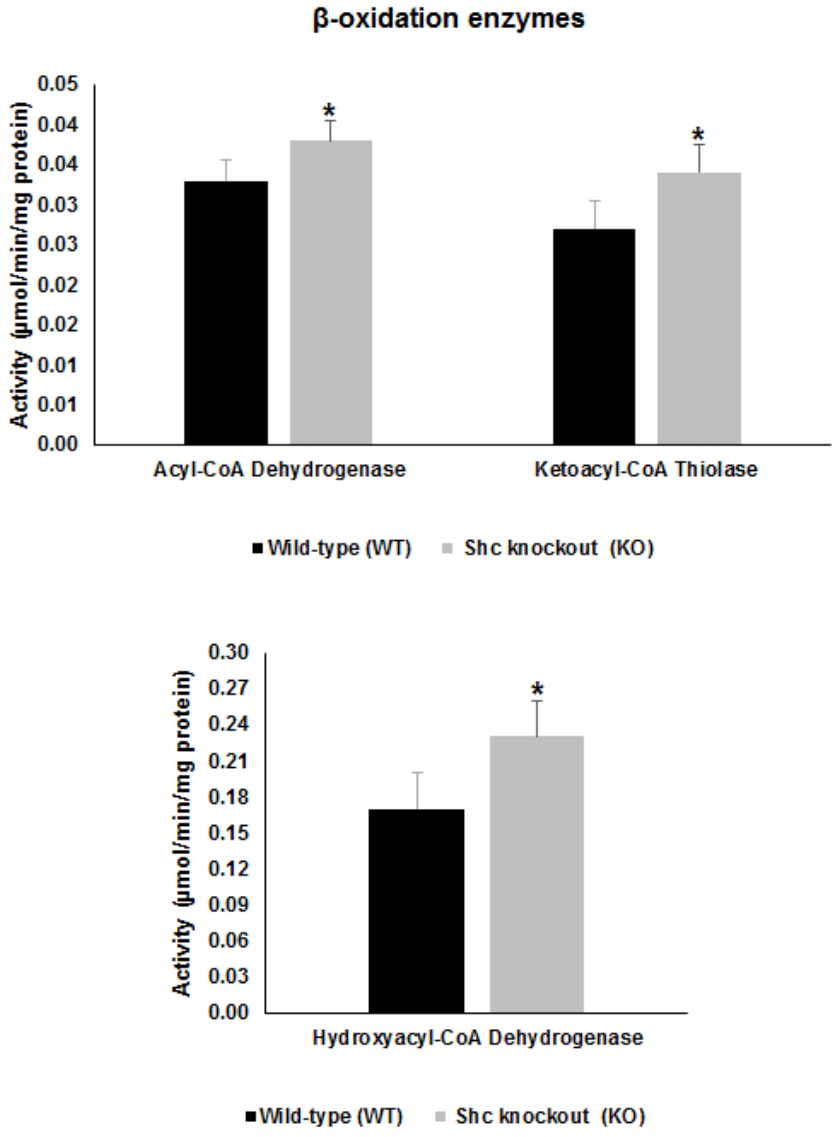

Figure 3. Activities of β-oxidation enzymes in skeletal muscles of Shc knockout (KO) and wild-type (WT) fasted mice. *$P < 0.05$ between KO and WT mice. Adapted from (Hagopian et al., 2012a).

The activities of enzymes involved in ketone body synthesis and catabolism were also measured in skeletal muscle and liver (Hagopian et al., 2012). In both liver and muscle, the activities of β-hydroxybutyrate dehydrogenase and acetoacetyl-CoA thiolase were increased ($P < 0.05$) in both the fed and fasted states in the ShcKO compared to WT mice. Overall, these results indicate that skeletal muscle from the ShcKO animals has an increased capacity for oxidizing both fatty acids and ketone bodies (Hagopian et al., 2012; Tomilov et al., 2011; Tomilov et al., 2016).

2.6. Gluconeogenesis and Glycolysis

The activities of the regulatory enzymes of gluconeogenesis (glucose-6-phos-phatase, fructose-1,6-bisphos-phatase, pyruvate carboxylase and phosphoenolpyruvate carboxykinase) were measured in liver from fed and fasted ShcKO and WT mice (Hagopian et al., 2016). There were no differences in enzyme activities between the groups in the fed state, but in the fasted stated, the activities of phosphoenolpyruvate carboxylase and glucose-6-phosphatase were increased ($P < 0.05$) in the ShcKO versus WT mice. These results are consistent with an increased capacity for gluconeogenesis in the ShcKO mice under fasting conditions (Hagopian et al., 2015).

In the same study, the activities of key regulatory enzymes of glycolysis (hexokinase, phosphofructokinase-1, and pyruvate kinase) were measured in skeletal muscle from fed and fasted mice (Hagopian et al., 2015). The activities of all three enzymes were decreased ($P < 0.05$) in the ShcKO compared to WT animals under both fasting and fed conditions, although the magnitude of these differences were greater in the fasted mice (Figure 4).

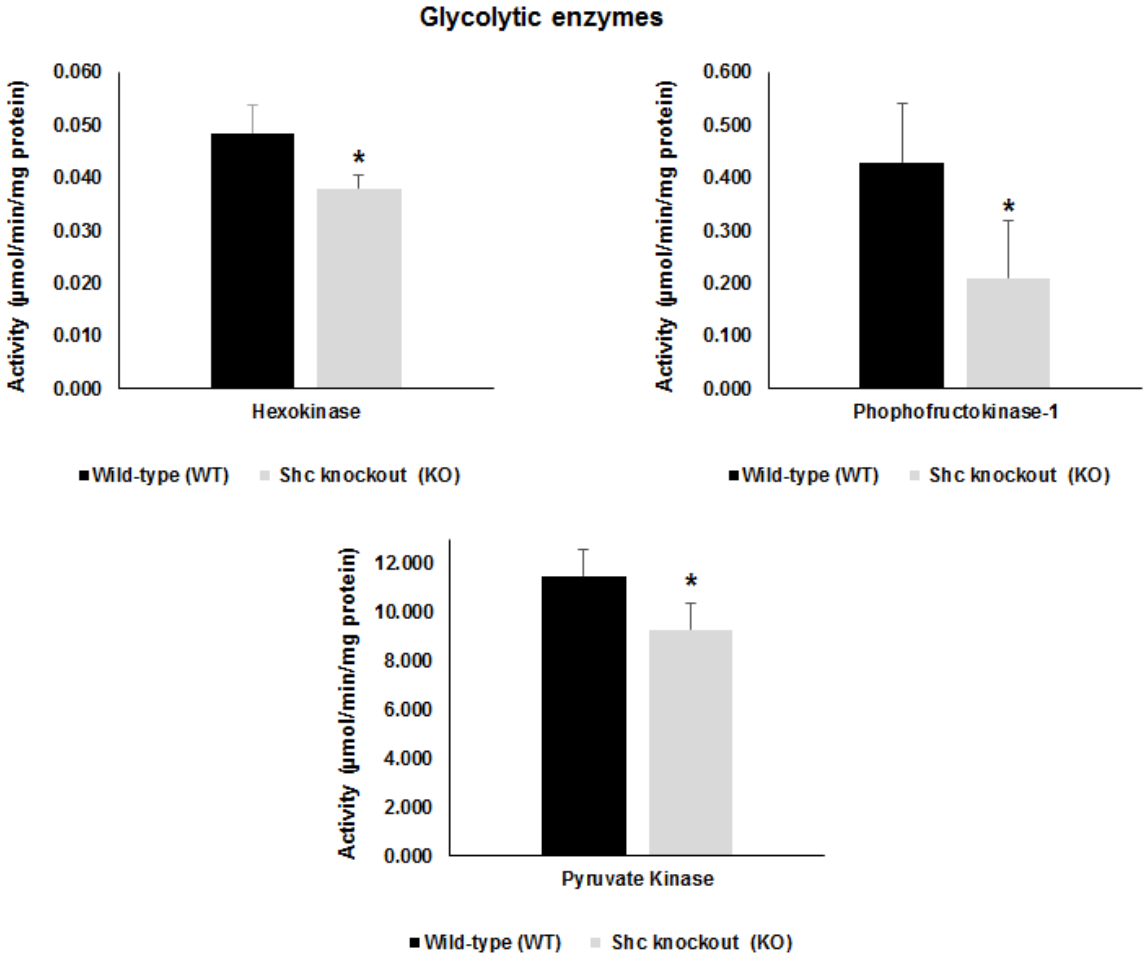

Figure 4. Activities of glycolytic enzymes in skeletal muscles of fasted Shc knockout (KO) and wild-type (WT) mice. *P < 0.05 between KO and WT mice. Adapted from (Hagopian et al., 2015).

2.7. The citric acid cycle

Measures of enzyme activity indicated no overall changes in the citric acid cycle between ShcKO and WT mice under fasted or fed conditions (Hagopian et al., 2012). These results indicate that Shc proteins may influence the fuels used for energy but they do not produce a net change in overall capacity for energy metabolism in the mitochondria. Shc proteins may play an important role in energy metabolism and, particularly, a decrease in Shc levels leads to an increased capacity for β-oxidation, ketone body metabolism, amino acid catabolism and gluconeogenesis under fasting conditions (Hagopian et al., 2012; Tomilov et al., 2016). Decreases in Shc proteins may play an important role in transitioning the animal from a fed to a fasted state. Thus, a decrease in Shc levels may help animals

adapt to periods of CR or chronic consumption of low carbohydrate or HFD. However, little is known about the role of Shc proteins in energy metabolism in animals fed a HFD. More studies are needed to determine if Shc proteins play any role in changes in energy expenditure or enzymes activities of major metabolic pathways in response to a HFD.

2.8. The uncoupling proteins

The UCPs are localized in the inner membrane (Figure 5) of mitochondria. UCPs are involved in different processes such as control of ATP synthesis, modulating ROS production and regulation of fatty acid metabolism (Echtay, 2007). These proteins may increase energy expenditure in tissues, generating great interest in the potential role they play in obesity, diabetes, and energy metabolism (Bouillaud et al., 2016).

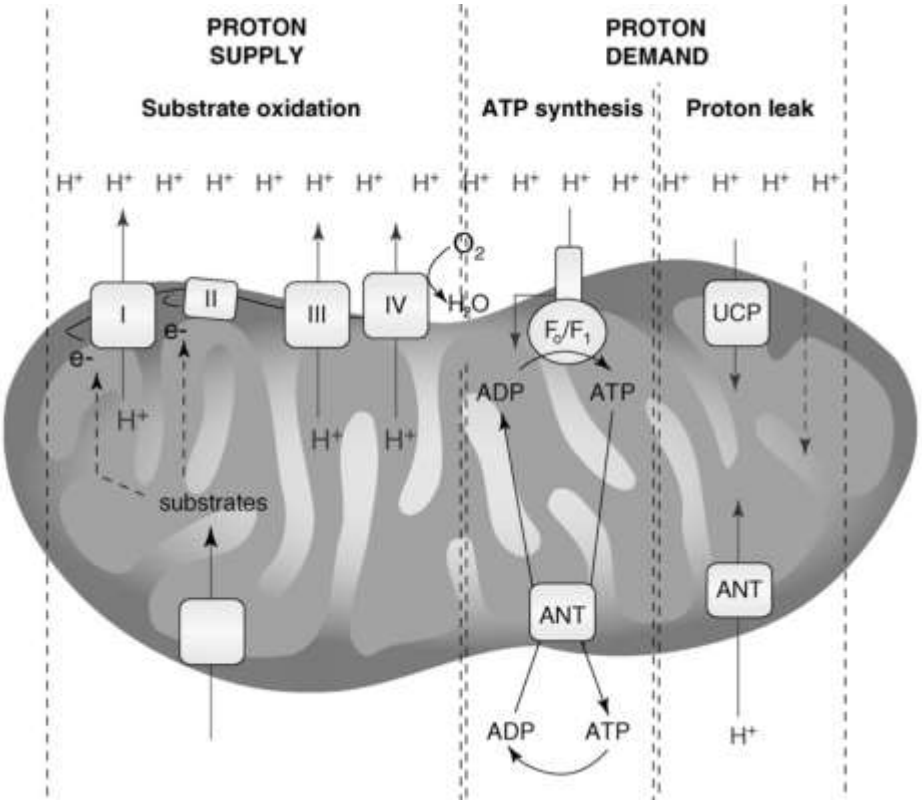

Figure 5. Oxidative phosphorylation and proton leak pathways in mitochondria. Proton leak pathways can be mediated by uncoupling protein (UCP) or by adenine de nucleotide translocase (ANT). Adapted from (Azzu and Brand, 2010).

Respiratory substrates are oxidized at mitochondrial respiratory complexes I–IV, leading to the ejection of protons (H+) into the intermembrane space. This proton electrochemical gradient is consumed by the F_0/F_1 ATP synthase to produce ATP or by proton leak pathways, which stimulate heat production without ATP synthesis (Azzu and Brand, 2010). This proton leak is the movement of protons through the inner membrane of mitochondria mediated by UCPs (main pathway), adenine nucleotide translocase (ANT) and other carriers and porosities (Azzu and Brand, 2010; Azzu et al., 2010). Proton leak represents an inefficiency that occurs because there is an incomplete coupling of oxidative phosphorylation. On the other hand, proton leak mediated by UCPs provide adaptive thermogenesis, carbon flux and protection of cell membranes against oxidative stress (Azzu et al., 2010; Divakaruni and Brand, 2011).

The role of UCPs in these physiological processes in the liver, muscle, and adipose tissue may account for inter-animal variation in energy expenditures and heat production. In addition, because mitochondria are responsible for producing approximately 90% of the energy for the cell, how efficiently this process is conducted has implications in animal growth and development (Bottje and Carstens, 2012). In this context, several studies focus on the association between mitochondrial function, UCP activity, and FE of beef cattle (Kolath et al., 2006a; Kolath et al., 2006b; Fonseca et al., 2015), poultry and livestock species (Bottje and Carstens, 2009; Bottje and Carstens, 2012; Grubbs et al., 2013).

2.9. The UCP family

The first member of the UCP family, brown adipose tissue (BAT) uncoupling protein 1 (UCP1), was identified in 1976. Twenty years later, two closely related proteins, UCP2 and UCP3, were described in mammals (Bouillaud et al., 2016)

In adipose tissue, adipocytes can be divided into white and brown cells. While white cells are specialized in storing chemical energy, brown adipocytes produce heat through a large amount of mitochondria-rich in UCP1 (Wu et al., 2013). UCP1 is known for its role in adaptive and noshivering thermogenesis (neonates and hibernation in animals). Studies have also investigated the role of UCP1 in the regulation of body weight (Azzu et al., 2010; Divakaruni and Brand, 2011), and UCP1

appears to regulate the body weight of animals by activating the thermogenic program in adipose tissue, producing heat and burning off energy from oxidized substrates.

The UCP2 gene is widely present in tissues, and in contrast to UCP1 it is expressed not only in BAT but also in white adipose tissue (WAT), skeletal muscle, heart, kidney, lung, spleen and others (Erlanson-Albertsson, 2003). Whereas UCP2 is widely expressed in tissues, UCP3 is expressed almost exclusively in skeletal muscle and brown adipose tissue and to a lesser extent in the heart (Mailloux and Harper, 2011).

In humans, tremendous interest has been generated in targeting energy expenditure in order to provide treatments to obesity. This could be possible by increasing activation of brown adipocyte progenitors to induce BAT differentiation and increasing UCP1-mediated uncoupling. However, there is a need for an explicit description of UCP1 mechanism (Divakaruni and Brand, 2011) and only few studies with mice have tried to describe this process. On the other hand, a study describe that increasing the uncoupling particularly in skeletal muscle is an effective obesity treatment, since overexpression of UCP3 in mice causes fat-specific weight loss (Clapham et al., 2000). Additionally, a study demonstrated that deranged expression of UCP confers resistance to diet induced obesity (Li et al., 2000).

2.10. UCPs, mitochondrial function, and feed efficiency

Transcription factors and other genes encoding mitochondrial proteins may be critical determinants of cellular function associated with the phenotypic expression of FE (Kolath et al., 2006b; Sherman et al., 2008). A study regarding molecular markers of FE evaluated the differential expression (mRNA levels) of genes involved with mitochondrial function (peroxisome proliferator-activated receptor-gamma coactivator 1-α [PGC-1α], mitochondrial transcription factor A [TFAM], and UCPs) in Nellore cattle divergently ranked by residual feed intake (RFI) (Fonseca et al., 2015), one of the most common indexes to measure FE. This study showed differences (high versus low RFI) in the expression levels of the UCP2 (liver) and TFAM (liver and muscle), suggesting that UCP2 and TFAM are possible candidate gene markers in breeding programs designed to increase the FE of Nellore cattle.

However, these results should be taken with great caution since RFI is based on the conversion of amount of feed (feed weight) to body weight. Animals ranked for RFI have been shown to have different compositions of body weight growth. More efficient animals (lower RFI) have leaner carcasses (Nascimento et al., 2016) and less internal fat (Basarab et al., 2003; Gomes et al., 2012). Thus, changes in metabolism and intake may not only be related to changes in energy expenditure, but also to changes in body tissue composition. Taken together these results suggest that RFI ranking could introduce a bias as it deviates from energetic efficiency rankings.

The role of UCPs instruct several studies in order to investigate the condition that cells from less efficient animals could decouple the proton gradient and thus sharply increase their energy requirements for the production of the same amount of ATP. Additionally, the increase of ROS may also alter gene expression for different reasons. Thus, expression of other genes and transcription factors encoding mitochondrial proteins may be changed. These adjustments may be critical determinants of the modulation of cellular functions associated with the phenotypic expression of FE (Bottje and Carstens, 2009).

Furthermore, there are many other proteins (e.g., structural, scaffolding, and transport proteins) that could impact mitochondrial function. Using proteomic approach (*i.e*., two-dimensional gel electrophoresis in combination with mass spectrometry) differentially expressed proteins (both mitochondrial and extra-mitochondrial) were identified (Bottje and Carstens, 2009;c Ramos and Kerley, 2013). These studies indicated that a large number of proteins are differentially expressed in FE phenotypes. The causal relationships among these different genes and proteins remain to be established.

2.11. Mitochondrial DNA

Mammalian mtDNA is a high-copy-number, maternally inherited genome that codes for a small number of essential proteins involved in oxidative phosphorylation (OXPHOS) (Trinei et al., 2006). For these reasons, damages in mtDNA can cause inefficiencies of mitochondrial activity (and consequently on OXPHOS). This may contribute to the phenotypic expression of the less-efficient animal (Bottje and

Carstens, 2009; Bottje and Carstens, 2012). Additionally, the mtDNA reflects the abundance of mitochondria within a cell (Trinei et al., 2006) and may vary according to the energy demands of the cell. In particular, multiple metabolic processes such as glucose, lipid, and protein metabolism can increase energy demands in the liver, muscle and adipose tissue (Laubenthal et al., 2016).

However, it remains unclear how the number of copies of mtDNA and the abundance of mitochondria are regulated under different physiological and developmental conditions (Trinei et al., 2006). Thus, it is impossible to assess its association with differences observed among animals in energetic efficiency, even that the association of mitochondria abundance and maintenance requirements have not been evaluated. Only a few studies have investigated the variation in mtDNA copy number on main metabolic tissues of livestock species and beef cattle. It is possible that not only protein synthesis, but cell division and apoptosis could be affected. Thus, not only the number of mitochondria per cell can be changed, but the number of cells in tissues such as liver and muscle could vary. Future studies are needed to understand the complex nature of the interaction of nuclear and mtDNA-encoded proteins that comprise both the fully functional organelle and the fully functional organ.

The discussion presented above highlight the enormous challenge to develop markers or a system to identify efficient or inefficient animals based solely on mitochondrial-related genes and/or proteins.

2.12. Mitochondrial function on biological efficiency of energy metabolism

A study (Herd et al., 2004) suggested that approximately 67% of variation among animals that are efficient and those that are inefficient relate to basal metabolic rate, cellular maintenance requirements, and energy lost as heat (Figure 6). In their study, for animals classified according to RFI, one-third of the biological variation of growing cattle could be explained by interanimal differences in digestion, heat increment, the composition of growth, and activity and posited that the remaining two-thirds was linked to interanimal variation in energy expenditure, including the mitochondrial efficiency and metabolism. However, these results should be taken with caution since it is not clear how these values were estimated by the authors in order to explain the variation in FE and RFI. Moreover, data from Nellore

cattle studies (Gomes et al., 2012; Chaves et al., 2015; Nascimento et al., 2016), whereby animals were ranked for RFI, did not find similar results as those reported by Herd et al. (2004).

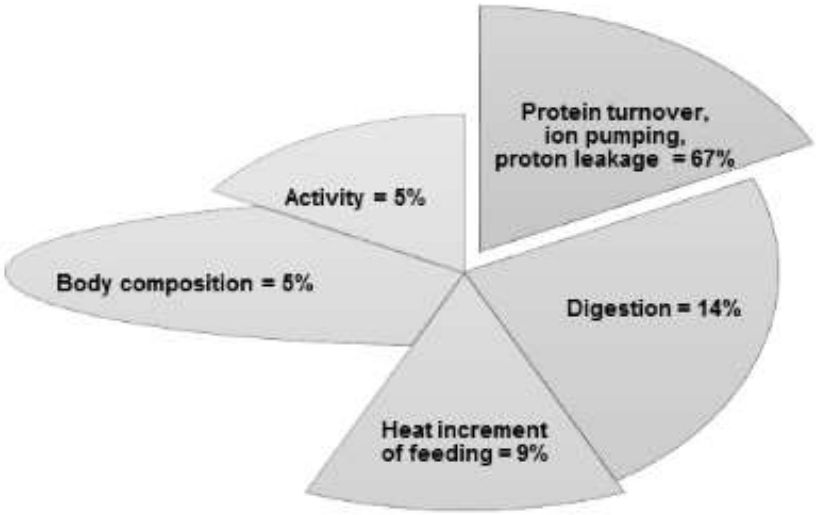

Figure 6. Estimates of percentage contribution of different mechanisms to variation in residual feed intake in beef cattle. Adapted from (Herd et al., 2004)

In the physiological processes described in Figure 6, it has been estimated that mitochondrial proton leak, Na+/K+ ATPase, and protein turnover each contribute approximately 20% to the total interanimal variation in basal energy expenditures (Bottje and Carstens, 2009). Reaction in which energy expenditure is not directly controlled through hydrolysis of high-energy phosphatic bounds (proton leakage, protein turnover) could contribute significantly to variation in efficiency (Herd and Arthur, 2009). However, these reactions remain difficult to quantify even in experimental conditions.

The proton motive force that develops from proton pumping across the inner mitochondrial membrane is used to drive ATP synthesis (as previously showed in Figure 5). However, protons may also flow back into the mitochondria at sites other than the ATP synthase (mainly by UCP), dissipating proton motive force and effectively short-circuit the coupling of ATP synthesis (proton leak process). An study estimate these process as responsible as much as 25% of the total basal metabolic rate of an animal and are possibly involved in the phenotypic expression of FE (Bottje and Carstens, 2012). On the other hand, the physiological benefit of proton leak for obesity, diabetes, and age-related disease was extensively reviewed (Azzu et al.,

2010; Divakaruni and Brand, 2011). The incompletely coupled oxidative phosphorylation permits adjustments in energy metabolism to regulate metabolic homeostasis and maintain body function (Divakaruni and Brand, 2011).

The paradigm in mitochondrial metabolism is that uncoupling by UCP may represents a cellular inefficiency but also reduces oxidative stress by attenuating mitochondrial ROS production. A study reported that proton leak in isolated muscle mitochondria obtained from superior FE phenotype broilers treated with chemical inhibitors of cellular respiration process was consistently less than that observed in mitochondria isolated from broilers with inferior FE (Bottje and Carstens, 2009). Thus, these results suggest that there are differences in membrane characteristics that affect proton conductance in broiler muscle mitochondria, which could contribute to higher mitochondrial ROS associated with the phenotypic expression of inferior FE (Bottje and Carstens, 2009; Bottje and Carstens, 2012). Furthermore, a study showed that cattle with low RFI had a greater coupling of oxidative phosphorylation in skeletal muscle mitochondria than cattle with high RFI (Kolath et al., 2006a). However, the physiological significance of these association including mitochondrial function, proton leak, UCPs activity (uncoupling), mtDNA copy number, and FE remains to be determined. Few studies have been able to confirm these results across species.

2.13. Conclusion

Taken together, in the context of energy metabolism and mitochondrial function, Shc proteins should be considered as potential molecular markers in genetic selection due to its association with weight gain and body composition of animals. It could be proposed to be used as a means to manipulate gain, body composition and efficiency.

Although proton leak and the total abundance of mitochondria are key to any estimate of feed efficiency traits, it remains very difficult to determine these in organs or animals. The variation in efficiency in farming animals is extremely low (2 to 5% being of great economic importance). Studies conducted to observe differences of these magnitudes in proton leak, mitochondrial abundance, energy coupling or gene expression are not common.

Initial studies have pointed out that in mitochondria from inferior FE animals, there may be greater protein oxidation due to greater ROS production. This increased ROS levels could oxidize proteins, as well as mtDNA. Thus, ETC and OXPHOS appear to be different in the liver, muscle, and adipose tissue among FE phenotypes. However, additional studies measuring protein amount or enzyme activity would be needed to confirm this hypothesis.

Acknowledgments

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3. THE INFLUENCE OF SHC PROTEINS AND HIGH FAT DIET ON ENERGY METABOLISM OF MICE

Abstract

The objective of this study was to determine if Shc proteins influence the metabolic response to acute (7 days) feeding of a high fat diet (HFD). To this end, whole animal energy expenditure (EE) and substrate oxidation were measured in the Shc knockout (ShcKO) and wild-type (WT) mice fed a control or HFD. The activities of enzymes of glycolysis, the citric acid cycle, electron transport chain (ETC), and β-oxidation were also investigated in liver and skeletal muscle of ShcKO and WT animals. The study showed that ShcKO increases (P < 0.05) EE adjusted for either total body weight or lean mass. This change in EE could explain the decrease in weight gain in ShcKO versus WT mice fed a HFD. Thus, our results indicate that Shc proteins should be considered as potential targets for developing interventions to mitigate weight gain on HFD by stimulating EE. Although decreased levels of Shc proteins influenced the activity of some enzymes in response to high fat feeding, such as increasing the activity of acyl-CoA dehydrogenase, it did not produce concerted changes in enzymes of glycolysis, citric acid cycle or the ETC. However, the physiological significance of these changes in enzyme activities remains to be determined.

Keywords: Beta-oxidation; Bioenergetics; Glycolysis; Mitochondria; Metabolism

3.1. Introduction

The mammalian Shc locus encodes the p46Shc, p52Shc and p66Shc proteins (1). These adaptor proteins were originally shown to play a role in growth factor signaling (1), although subsequent studies have also shown that these proteins participate in signaling from a wide range of cell surface receptors, including the insulin receptor (2, 3). This indicates that Shc proteins may be involved in energy metabolism. The p66Shc knockout mouse has been used to investigate the possible roles p66Shc plays in insulin signaling and metabolism. While this mouse model was intended to provide a way to investigate p66Shc depletion alone, it has been shown that these animals also have decreased levels of p46Shc and p52Shc in liver and

muscle (4). In these tissues, the p66Shc knockout mice (referred to as ShcKO hereafter) provides a model for studying the overall decreases in Shc proteins.

It was shown that body weight is decreased in ShcKO mice, despite the fact that their energy intake is not different than wild-type (WT) animals (5). This decrease in body weight is due to the fact that the weights of all fat pads are lower in ShcKO compared to WT mice (5, 6). Similarly, it has been reported that ShcKO mice show less weight gain on a high fat diet than WT animals (5) and knockout of ShcKO in leptin-deficient ob/ob mice decreases weight gain in these obese animals (7). The results of these mouse studies suggest that Shc proteins play a role in regulating whole animal energy metabolism.

In addition to the whole animal measures of metabolism in the ShcKO mice, it has also been shown that Shc proteins influence the activities of key regulatory enzymes of intermediary metabolism in liver and skeletal muscle (8-10). The activities of regulatory enzymes of glycolysis (gluckinase/hexokinase, phosphofructokinase-1 and pyruvate kinase) were significantly decreased in liver and skeletal muscle of ShcKO compared to WT mice with fasting and 3 hours following feeding (9, 10). ShcKO mice also showed a significant increase in the activities of mitochondrial β-oxidation enzymes, although this change in enzyme activities only occurred under fasting conditions (8). The results of these studies suggest that Shc proteins may play a role in fuel selection. Thus, it is not known if ShcKO mice are better able to alter the activities of enzymes of energy metabolism than WT animals in response to metabolic challenges, such as sustained consumption of a high fat diet.

The purpose of this study was to determine if Shc proteins influence the metabolic response to acute (5-7 days) feeding of a high fat diet (HFD). To this end, the activities of enzymes of glycolysis, the citric acid cycle, electron transport chain, and β-oxidation were investigated in liver and skeletal muscle of ShcKO and WT mice fed a control or HFD. Whole animal energy expenditure and substrate oxidation (respiratory exchange ratio) were also measured in the ShcKO and WT animals consuming either a control or HFD diet.

3.2. Material and Methods

3.2.1. Animals and diet

All animal care and use protocols were approved by the UC Davis Institutional Animal Care and Use Committee (IACUC) and are in accordance with the National Institutes of Health (NIH) guidelines for the Care and Use of Laboratory Animals. ShcKO and WT mice came from a breeding colony at UC Davis that was established from mice provided by Dr. Pier Giuseppe Pelicci (Department of Experimental Oncology, European Institute of Oncology, Milan, Italy). The breeding stocks were backcrossed onto C57BL/6J mice to full congenic status, and the homozygous ShcKO and WT lines were generated by mating heterozygous ShcKO mice.

Male WT and ShcKO mice used for this study were 2 months old and were fed a chow diet (Lab diet 5001) prior to feeding the HFD. At 2 months of age, the mice of both genotypes (WT and ShcKO) were randomly divided and fed either the chow or HFD for an additional 5 (indirect respiration calorimetry study) or 7 days (enzyme study). A total of 8 WT and 8 ShcKO mice were used for the indirect respiration calorimetry study. Separate groups of mice were used for the enzyme study. For the enzyme study, a group of mice were fed the chow diet (10 WT and 8 ShcKO) and another group of mice were fed the HFD (11 WT and 8 ShcKO).

All animals had free access to food and water. The mice were housed at 22–24 °C and 40–60% humidity, with a 12 h light: dark cycle. The nutrient compositions of the diets were as follows: chow lab diet 5001 (% of Kcal; protein = 30%, carbohydrates = 57%, fat = 13%) and HFD (% kcal; protein = 18.3%, carbohydrates = 21.4%, fat = 60.3%). The HFD uses the same macronutrients and vitamin and mineral mixes as the semi-purified AIN93G diet, with the increase in percent of total energy from fat achieved by adding lard and removing carbohydrates from the AIN93G diet.

3.2.2. Indirect respiration calorimetry

Total daily energy expenditure (EE) and respiratory exchange ratio (RER) were measured using whole-body indirect respiration calorimetry through the UC

Davis Mouse Metabolic Phenotyping Center. The Oxymax/CLAMS calorimetry system (Columbus Instruments, Columbus, OH) was housed in a room maintained on a 12 hour light/12 hour dark cycle at 22°C. The mice were placed in acclimation cages (calorimetry chambers not connected to the calorimeter) and housed in the Oxymax/CLAMS room for 24 hours. The mice were then transferred to calorimetry chambers contained in a 22°C incubator and calorimetry measurements were then completed over a 7 day period. Mice were fed the control diet for the first two days in the calorimetry chamber, and then all mice were switched to the HFD for the remaining 5 days in the calorimetry chambers.

Calorimetry measurements were completed on the mice on days 1 and 2 (chow diet) and day 7 (HFD). Room air was drawn through the calorimetry chambers at 500 ml/min. Samples of dried room and chamber air were analyzed for oxygen and carbon dioxide content using the Oxymax system. Calorimeter calibration was performed daily prior to the beginning of each 24 hour measurement. A 0.50% CO_2 and 20.50% O_2 (balance nitrogen) calibration gas (Airgas, Sacramento, CA) and dry room air were used to calibrate the analyzers. At the start and end of the experiments, the performance of the entire calorimetry system was validated by bleeding a 20% CO_2 (balance nitrogen) standard (Airgas, Sacramento, CA) into each calorimetry chamber at a regulated flow rate using an OxyVal gas infusion system (Columbus Instruments, Columbus, OH) and measuring recovery of CO_2 and O_2 dilution in the chamber exhaust. Energy expenditure was calculated from oxygen consumption and carbon dioxide production using the Oxymax system and RER was determined as the ratio of the volume of CO_2 produced to the volume of O_2 produced.

The calorimetry chambers were also fitted with an infrared photocell system (Columbus Instruments, Columbus, OH) which measured activity as “counts” each time an infrared beam break occurred. Activity was measured in the horizontal (x) and vertical (z) plane. Immediately following calorimetry measurements, the mice were anesthetized with 2-4% isoflurane and body composition (lean and fat mass) were measured using a dual energy X-ray absoptiometry (DEXA) machine (PixiMus, Fitchburg, WI). Each mouse was scanned in less than 5 minutes, and body composition was determined using the PixiMus software.

3.2.3. Chemicals

The chemicals and reagents were purchased from MilliporeSigma (St. Louis, MO), except bovine serum albumin (BSA, MP Biochemicals, Santa Ana, CA), Bio-Rad protein assay dye (BioRad, Hercules, CA) and NAD, NADH and ATP (Roche Diagnostics, Indianapolis, IN). Auxiliary enzymes used in the assays were from Roche Diagnostics (Indianapolis, IN), MilliporeSigma (St Louis, MO) and Megazyme (Bray, Ireland).

3.2.4. Sample preparation

For the enzyme study, the food was removed from the cage at 8 AM and mice were sacrificed by cervical dislocation between 9–10 am. Body weight (BW) was record and liver and hindlimb skeletal muscles were rapidly removed, weighed immediately and frozen in liquid nitrogen. The visible fat and connective tissue were trimmed from the muscle samples prior to freezing. In addition to BW, organs (heart, kidneys, and spleen) and epididymal fat pad weights were also recorded. Frozen liver and muscles were then powdered in a porcelain mortar and pestle maintained under liquid nitrogen. All tissue powders were stored under liquid nitrogen until used in enzyme assays. The powders were homogenized at a 1:10 ratio (w/v) in buffer containing 10 mM HEPES, 250 mM sucrose, 0.5 mM EDTA, 100 mM KCl and 10% glycerol (pH = 7.4 at 4˚ C). The homogenates were centrifuged at 600 G for 10 minutes at 4˚ C and the supernatants carefully collected and stored on ice.

3.2.5. Enzyme assays

All assays were performed in a 1 ml final volume using a Perkin Elmer Lambda 25 UV/Vis spectrophotometer equipped with a Peltier heating control system and 9 cell changer (Perkin Elmer, Shelton, CT). Enzyme activities were expressed as μmol/min/mg protein using the respective extinction coefficient (ε), and presented as mean ± SEM determined from at least eight animals per group.

3.2.6. Glycolytic enzymes

The activities of the glycolytic enzymes glucokinase/hexokinase (GK/HK, EC 2.7.1.1), phosphofructokinase-1 (PFK-1, EC 2.7.1.11) and pyruvate kinase (PK, EC 2.7.1.40) were measured in both liver and muscle samples of WT and ShcKO animals, at 340nm (ε = 6.22 $mM^{-1}.cm^{-1}$), as previously described (11). GK activity was determined in the presence of 0.5 and 100 mM glucose and taken as the activity at 100 mM glucose minus the activity at 0.5 mM glucose. HK activity in muscle samples was determined using 10 mM glucose. Lactate dehydrogenase activity (LDH, EC 1.1.1.27) was also measured in both liver and muscle samples, at 340 nm (ε = 6.22 $mM^{-1}.cm^{-1}$) as previously described (8)

3.2.7. Citric acid cycle and electron transport chain (ETC) enzymes

The activities of citrate synthase (CS, EC 2.3.3.1) and aconitase (AC, EC 4.2.1.3) were measured in both liver and muscle samples of WT and ShcKO animals. CS and AC were determined as previously described (12), at 412 nm (ε = 13.6 $mM^{-1}.cm^{-1}$) and 240nm (ε = 3.6 $mM^{-1}.cm^{-1}$), respectively. For the ETC enzymes, complex I (NADH: Ubiquinone oxidoreductase, EC 1.6.5.3) and complex IV (Cytochrome C oxidase, EC 1.9.3.1) activities were measured at 340nm (ε = 6.22 $mM^{-1}.cm^{-1}$) and 550nm (ε = 28 $mM^{-1}.cm^{-1}$), respectively, as previously described (13).

3.2.8. β-oxidation and ketone body metabolism enzymes

Activities of acyl-CoA dehydrogenase [EC 1.3.8.8] was measured in skeletal muscle and liver as previously described (8). Acyl-CoA dehydrogenase activity was measured at 600nm (ε =21 $mM^{-1}cm^{-1}$), using n-palmitoyl-CoA as substrate. For ketone body synthesis, the activity of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA synthase, EC 2.3.3.10), was determined at 303 nm (ε =12.2 $mM^{-1}cm^{-1}$), as previously reported (14).

3.2.9. Protein assays

Protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA), with BSA as the standard.

3.2.10. Statistical analysis

Mean of body weights of WT and ShcKO mice in each diet group were compared using two-sample *t*-tests. A two-way ANOVA was used considering the mains effects of diets (chow or HFD) and genotypes (WT or ShcKO). Both factors and their interaction (diet*genotype) were evaluated. All analyses were conducted with SAS software (version 9.3) and for interaction effect ($P < 0.05$), Tukey's multiple comparison procedure was used to identify which diet*genotype groups differed significantly. To control for multiple testing, a Bonferroni adjustment or false discovery rating (FDR) methods were used to maintain the family-wise error rate at 0.05 for the pairwise comparisons. For the variables not normally distributed, log transformation was applied and then used as log-transformed data for ANOVA tests. When this procedure failed, Friedman's two-way nonparametric ANOVA was applied. For energy expenditure data, analysis of covariance (ANCOVA) was used with either BW or lean mass as a covariate in the model.

3.3. Results

3.3.1. Body weights, epididymal fat and organs weights

Final body weights, weight change, and organ weights are summarized in Table 1. No weight change was observed in ShcKO mice on chow ($P = 0.370$), while a significant increase ($P < 0.05$) in body weight was observed in WT animals after consuming the HFD for one week. Changes in body weight following consumption of the HFD also reach statistical significance ($P < 0.01$) in the ShcKO mice after consuming the HFD for 7 days. However, changes in body weight trended ($P = 0.075$) to be lower in ShcKO compared to WT mice on HFD. There were no differences in final body weight ($P = 0.446$) between the WT or ShcKO mice on HFD, reflecting the fact that the two genotypes had similar body weights on chow ($P =$

0.370) and any body weight changes with the HFD over the one week period were relatively small. In both genotypes, the HFD increases ($P < 0.01$) the amounts of epididymal fat compared to their counterparts on chow diet. Also, WT and ShcKO mice fed a HFD showed similar results to organs weights such as heart, kidneys, spleen and epididymal fat.

Table 1. Effects of 7 days on chow (CH) or high fat diet (HFD) on body, epididymal fat and organs weights of wild-type and ShcKO (deletion of Shc) male mice.

Variable	Wild-type (WT)		ShcKO		*P* value			
	CH	HFD	CH	HFD	Diet effect		Genotype effect	
	n=10	n=11	n=8	n=8	WT	ShcKO	CH	HFD
	Mean ± SEM				CH vs HFD	CH vs. HFD	WT vs. ShcKO	WT vs. ShcKO
Final BW[1]	22.501 ± 0.570	26.356 ± 0.371	23.770 ± 0.322	26.476 ± 0.779	<0.01	<0.01	0.370	0.446
Change in BW (%)	2.063 ± 0.604	10.916 ± 1.720	1.645 ± 1.166	7.560 ± 1.084	<0.01	<0.01	0.370	0.075
Liver	1.104 ± 0.049	1.131 ± 0.030	1.001 ± 0.021	1.021 ± 0.047	0.972	0.703	0.038	0.027
Skeletal Muscle	1.049 ± 0.043	1.130 ± 0.029	1.078 ± 0.044	1.200 ± 0.039	0.131	0.057	0.327	0.080
Heart	0.113 ± 0.003	0.119 ± 0.003	0.114 ± 0.005	0.121 ± 0.005	0.181	0.329	0.449	0.355
Kidneys	0.310 ± 0.008	0.337 ± 0.008	0.329 ± 0.010	0.359 ± 0.019	0.029	0.179	0.078	0.158
Spleen	0.069 ± 0.003	0.074 ± 0.005	0.074 ± 0.005	0.076 ± 0.003	0.626	0.680	0.196	0.335
Ep.fat[2]	0.356 ± 0.018	0.742 ± 0.045	0.324 ± 0.014	0.740 ± 0.057	<0.01	<0.01	0.096	0.490

[1] BW = body weight; [2] Ep.fat = epididymal fat. All weights in g.

3.3.2. Indirect respiration calorimetry

No differences between initial BW (P = 0.914) and final BW (P = 0.678) of ShcKO and WT mice were detected during calorimetry measurements. The initial BW of the ShcKO and WT mice when they were placed in the calorimetry chamber (CLAMS) was 24.477 ± 0.665 g and 24.570 ± 0.518 g, respectively. In addition, the final BW of the ShcKO and WT when they were removed from CLAMS was 25.904 ± 0.565 and 26.269 ± 0.643 g, respectively. The lean body mass (LBM), measured following completion of the calorimetry study in the CLAMS, was also similar (P = 0.572) between ShcKO and WT mice, and LBM mean were 19.584 ± 0.586 and 19.221 ± 0.216 g, respectively.

Whole animal energy expenditure (EE) data of WT and ShcKO mice are summarized in Table 2. No interaction between genotype and diet was observed (P > 0.05) for EE expressed as kJ/min or normalized for BW or LBM using ANCOVA (EE_{BW} and EE_{LBM}, respectively). However, there is an effect (P < 0.05) of genotype, whereby ShcKO mice showed increased (P < 0.05) in 24h EE compared to WT animals fed on chow or HFD. No effects of genotype (P > 0.05) were observed for daily respiratory exchange ratio (RER). As expected, there is an overall effect of HFD (P < 0.01) increasing EE and decreasing RER of WT and ShcKO mice (Figure 1).

The interaction between BW and genotype was significant (P < 0.05) for a few comparisons. Whenever this occurred the Johnson-Neyman technique (15, 16) was used to investigate possible differences between experimental groups. This technique provides cut-offs which show which weights showed differences between groups (see Table 2). Thus, the HFD comparison between the WT and ShcKO mice showed a significant (P < 0.05) interaction between BW and genotype, whereby there is a significant increase in EE_{BW} 24h in ShcKO mice at BW ≤ 18.12 or ≥ 23.69 g. Additionally, on chow diet comparison, there is a significant (P < 0.05) increase in EE_{BW} in dark cycle of ShcKO animals at BW ≥ 24.44 g.

Table 2. Energy expenditure (EE) and respiratory exchange ratio (RER) in the wild-type (WT) and Shc knockout (ShcKO) animals consuming either a control chow (CH) or high fat diet (HFD).

Variables[1]	Wild-type (WT)		Shc knockout (ShcKO)		*P* value			
					Diet effect		Genotype effect	
	CH	HFD	CH	HFD	WT	ShcKO	CH	HFD
	Mean ± SEM (n = 7)				CH vs. HFD	CH vs. HFD	WT vs. ShcKO	WT vs. ShcKO
24h								
EE (kcal/hr)	0.364 ± 0.008	0.443 ± 0.008	0.389 ± 0.019	0.487 ± 0.013	<0.001	<0.001	0.261	0.015
EE_{BW}	0.360 ± 0.009	0.440 ± 0.009*	0.390 ± 0.009	0.490 ± 0.009 *	<0.001	<0.001	0.098	0.001
EE_{LBM}	0.370 ± 0.008	0.450 ± 0.008	0.380 ± 0.009	0.480 ± 0.009	<0.001	<0.001	0.304	0.003
RER	0.909 ± 0.026	0.808 ± 0.009	0.898 ± 0.021	0.802 ± 0.007	<0.001	<0.001	0.189	0.189
Light								
EE (kcal/hr)	0.326 ± 0.013	0.399 ± 0.015	0.347 ± 0.018	0.453 ± 0.008	<0.001	<0.001	0.012	0.012
EE_{BW}	0.330 ± 0.014	0.400 ± 0.014	0.350 ± 0.010	0.450 ± 0.010	0.003	<0.001	0.207	0.006
EE_{LBM}	0.340 ± 0.014	0.410 ± 0.014	0.340 ± 0.010	0.440 ± 0.010	0.004	<0.001	0.484	0.009
RER	0.886 ± 0.016	0.814 ± 0.005	0.864 ± 0.009	0.806 ± 0.005	<0.001	<0.001	0.138	0.138
Dark								
EE (kcal/hr)	0.403 ± 0.005	0.488 ± 0.009	0.431 ± 0.021	0.522 ± 0.020	<0.001	<0.001	0.053	0.053
EE_{BW}	0.400 ± 0.008 ¥	0.490 ± 0.008	0.430 ± 0.009 ¥	0.520 ± 0.009	<0.001	<0.001	0.038	0.015
EE_{LBM}	0.410 ± 0.014	0.500 ± 0.014	0.420 ± 0.009	0.510 ± 0.009	<0.001	<0.001	0.155	0.079
RER	0.932 ± 0.005	0.803 ± 0.004	0.932 ± 0.008	0.798 ± 0.004	<0.001	<0.001	0.62	0.62

[1] EE_{BW} = energy expenditure (kcal/hr) normalized for body weight; EE_{LBM} = energy expenditure (kcal/hr) normalized for lean body mass.

* Interaction between BW and genotype was significant ($P < 0.05$) and Johnson-Neyman technique was used to compare the experimental groups (WT HFD vs. ShcKO HFD). Johnson-Neyman Cutoffs: 18.12 ≤ BW ≥ 23.69 g.

¥ Interaction between BW and genotype was significant ($P < 0.05$) and Johnson-Neyman technique was used to compare the experimental groups (WT CH vs. ShcKO CH). Johnson-Neyman Cutoffs: 8.108 ≤ BW ≥ 24.44 g

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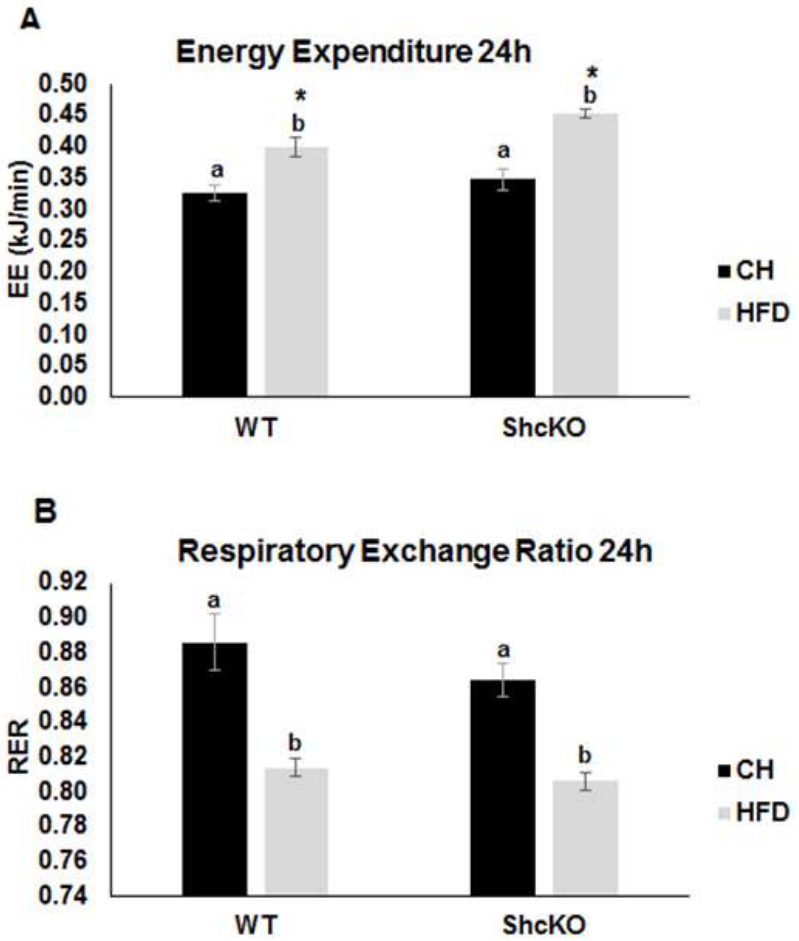

Figure 1. Energy expenditure (**A**) and respiratory exchange ratio (**B**) of wild-type (WT) and Shc knockout (ShcKO) male mice over 24h fed on chow (CH) or high fat diet (HFD). All values are mean ± SEM (n = 8). Different letters are used to indicate a diet effect ($P < 0.05$) within a genotype. The symbol (*) indicated the difference between diets across genotypes ($P < 0.05$).

3.3.3. Activities of glycolytic enzymes

The activities of the enzymes GK, PFK-1, PK and LDH from hepatic tissues of WT and ShcKO mice fed chow or an HFD are summarized in Figure 2. Interactions between diet and genotype ($P < 0.05$) were observed for PK and GK. In contrast to the WT animals, ShcKO mice fed a HFD showed an increase in GK acitivity ($P < 0.05$) compared to the chow diet. Similarly, ShcKO mice fed a HFD showed higher ($P < 0.05$) PK activity when compared to chow diet while diet did not significantly alter PK activity in the WT animals. PK, PFK-1 and LDH activities decreased ($P < 0.05$) in ShcKO compared to WT mice on the chow diet while no differences between the genotypes were observed for these enzymes with the HFD. Additionally, LDH activity was increased ($P < 0.05$) in both genotypes with the HFD.

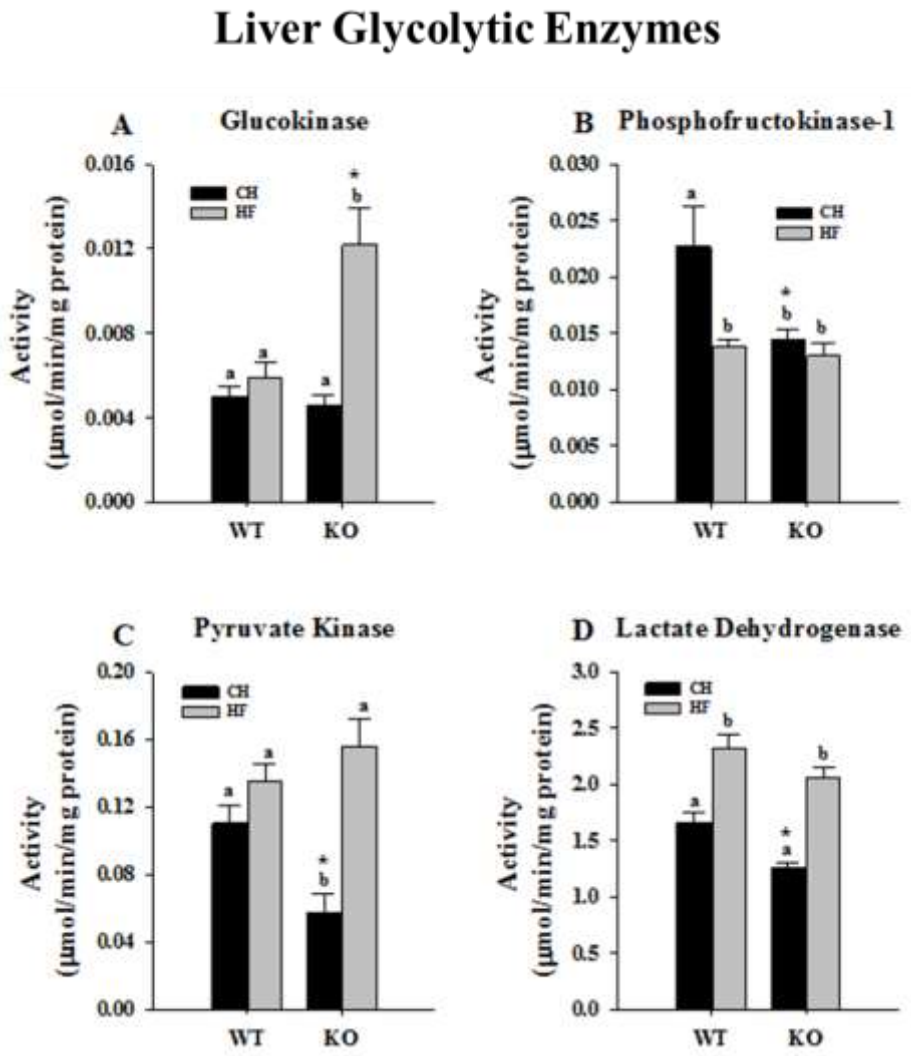

Figure 2. Activities of glucokinase (**A**), phosphofructokinase-1 (**B**), pyruvate kinase (**C**), and lactate dehydrogenase (**D**) in hepatic tissue of wild-type (WT) and Shc knockout (KO) male mice fed on chow (CH) or high fat diet (HFD). All values are mean ± SEM (n = 8 -- 11). Different letters are used to indicate a diet effect ($P < 0.05$) within a genotype. The symbol (*) indicated the difference between diets across genotypes ($P < 0.05$).

The glycolytic enzymes HK, PFK1, PK, and LDH were also assayed in skeletal muscle of WT and ShcKO animals (Figure 3). No interaction between the diets and genotypes was observed. No effects of either diet or genotype were observed on the activities of HK and LDH. In both genotypes, PFK-1 activity tended to be higher on the HFD with this increase being significant ($P < 0.05$) in the WT mice. PFK-1 activity was also increased ($P < 0.05$) in the ShcKO versus WT mice on the chow diet. PK activity was also decreased ($P < 0.05$) in the ShcKO compared to

WT mice on both diet, and PK activity was significantly increased ($P < 0.05$) with HFD feeding in the ShcKO animals.

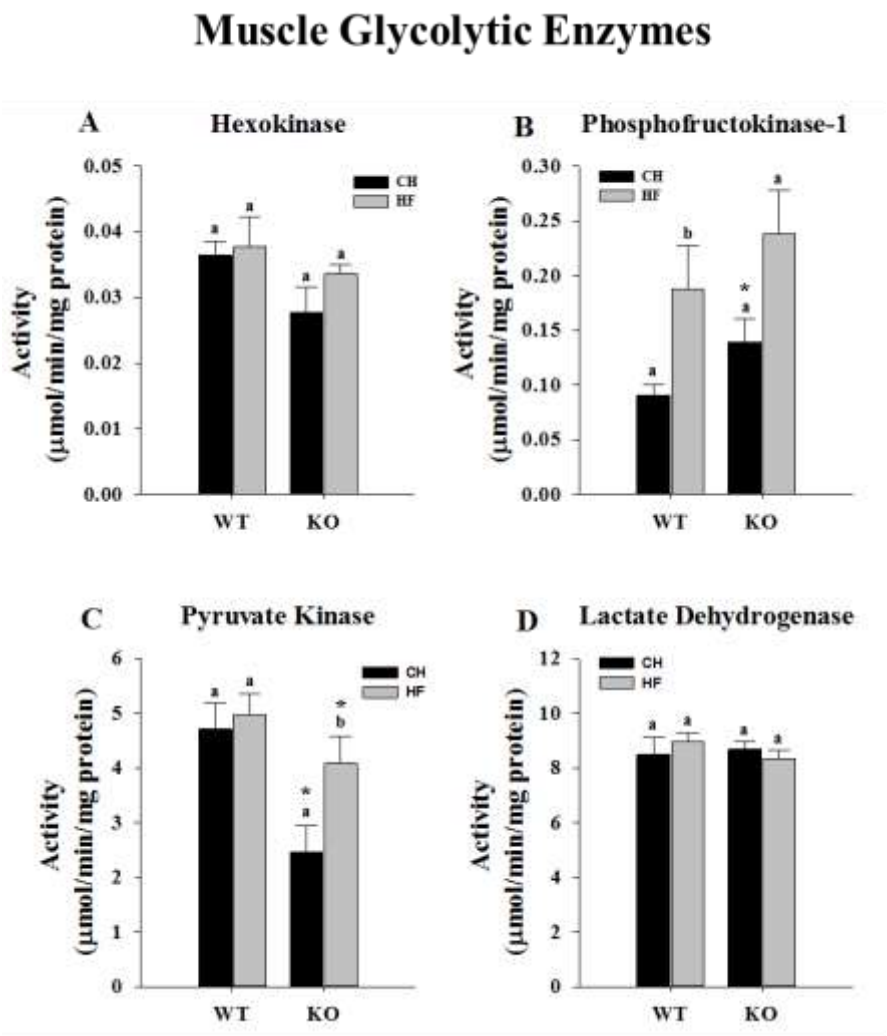

Figure 3. Activities of Hexokinase (**A**), phosphofructokinase-1 (**B**), pyruvate kinase (**C**) and lactate dehydrogenase (**D**) in skeletal muscle of wild-type (WT) and Shc knockout (KO) male mice fed on chow (CH) or high fat diet (HFD). All values are mean ± SEM (n = 8 -- 11). Different letters are used to indicate a diet effect ($P < 0.05$) within a genotype. The symbol (*) indicated the difference between diets across genotypes ($P < 0.05$).

3.3.4. Activity of citric acid cycle and ETC enzymes

The activities of the citric acid cycle (CS and AC) and electron transport chain (Complex I and IV) enzymes in liver and muscle are summarized in Figures 4 and 5, respectively. An Interaction between diet and genotype was observed for liver CS activity ($P < 0.05$) with the ShcKO mice showing a trend toward an increase ($P < 0.10$) in CS activity with the HFD while no change was observed with diet in the WT

animals. For liver AC, diet did not alter enzyme activity in either genotype while AC activity was decreased (P< 0.05) in the ShcKO compared to WT mice on both diets.

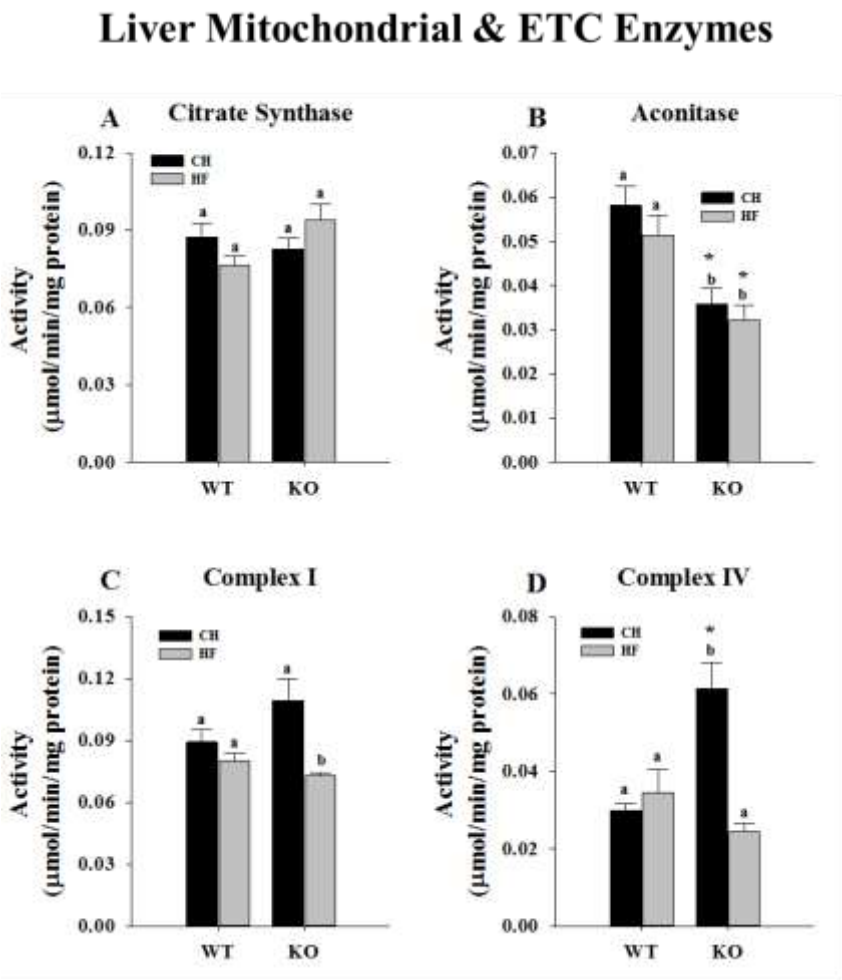

Figure 4. Activities of citrate synthase (**A**), aconitase (**B**) and electron transport chain enzymes (ETC) complex I (**C**) and complex IV (**D**) in hepatic tissue of wild-type (WT) and Shc knockout (KO) male mice fed on chow (CH) or high fat diet (HFD). All values are mean ± SEM (n = 8 -- 11). Different letters are used to indicate a diet effect (P < 0.05) within a genotype. The symbol (*) indicated the difference between diets across genotypes (P < 0.05).

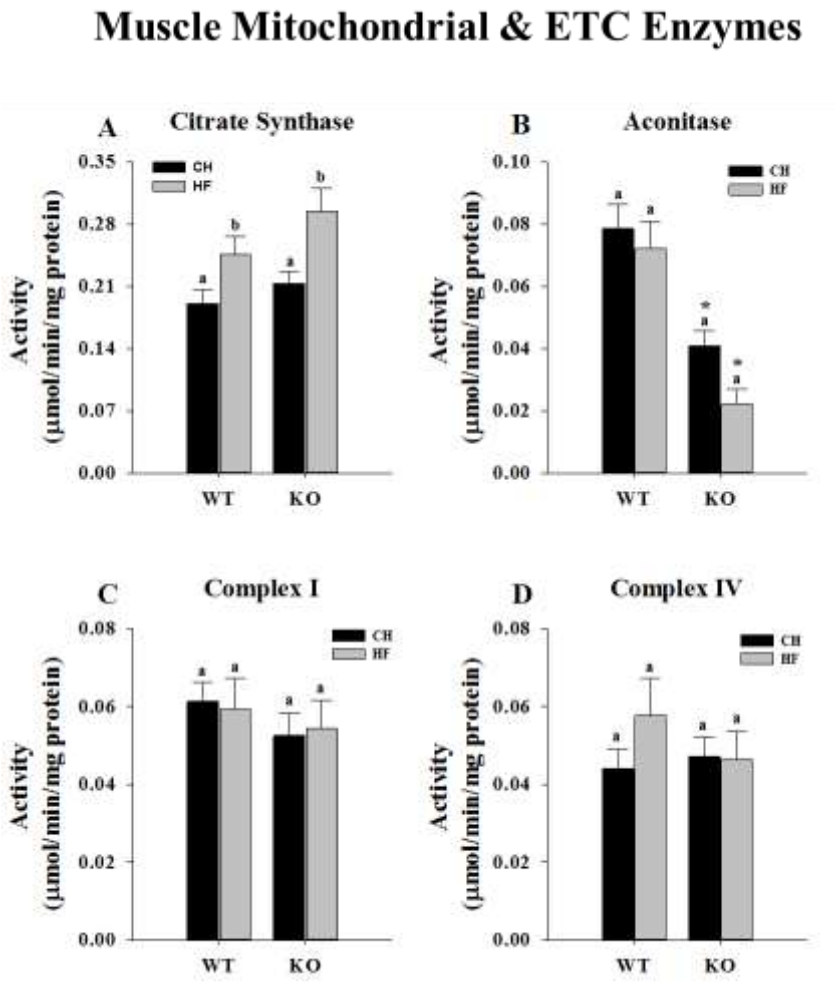

Figure 5. Activities of citrate synthase (**A**), aconitase (**B**) and electron transport chain enzymes (ETC) complex I (**C**) and complex IV (**D**) in skeletal muscle of wild-type (WT) and Shc knockout (KO) male mice fed on chow (CH) or high fat diet (HFD). All values are mean ± SEM (n = 8 -- 11). Different letters are used to indicate a diet effect ($P < 0.05$) within a genotype. The symbol (*) indicated the difference between diets across genotypes ($P < 0.05$).

The interaction between diet and genotype was observed ($P < 0.05$) for both ETC enzymes (Complexes I and IV) in liver. ShcKO showed lower activities ($P < 0.05$) of complexes I and IV on a HFD compared to the chow diet, while no differences with diet were observed for the WT animals. The ShcKO mice also had higher ($P < 0.05$) Complex IV activities than the WT mice when consuming chow, but no differences were observed between genotypes with the HFD.

No diet and genotype interactions were observed for any of the mitochondrial enzymes in skeletal muscle. Both WT and ShcKO mice showed an increase ($P < 0.05$) in response to feeding a HFD. Diet had no impact on the activities of aconitase, Complex I or Complex IV in either genotype. However, similar to hepatic tissue,

aconitase activity was decreased ($P < 0.05$) in ShcKO compared to WT mice on both chow and HFD.

3.3.5. Activities of β-oxidation and ketone body metabolism enzymes

The activities of Acyl-CoA dehydrogenase (ACDH) in liver and skeletal muscle and HMG-CoA synthase in liver of WT and ShcKO animals are summarized in Figures 6 and 7. An Interaction between diet and genotype was observed for ACDH activity ($P < 0.05$) in both liver and skeletal muscle with only the ShcKO mice showing an increase ($P < 0.05$) in ACDH activity on the HFD versus chow diet. Also, ShcKO mice on the HFD showed higher ACDH activity ($P < 0.05$) than WT mice in both liver and skeletal muscle. In liver, no effects for diet, genotype or interaction were obtained for HMG-CoA synthase (Figure 7).

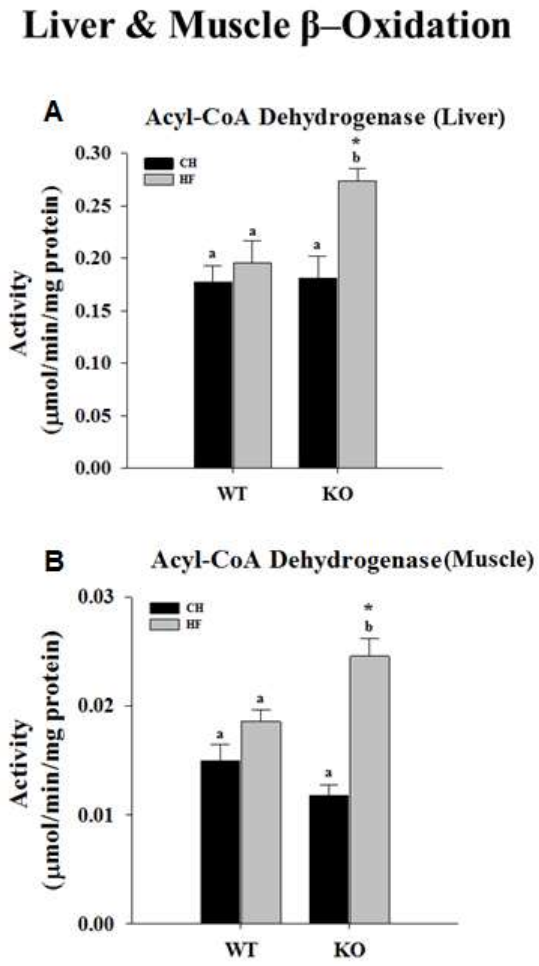

Figure 6. Activities of acyl-CoA dehydrogenase in liver (**A**) and muscle (**B**) of wild-type (WT) and Shc knockout (KO) male mice fed on chow (CH) or high fat diet (HFD). All values are mean ± SEM (n = 8 -- 11). Different letters are used to indicate a diet effect ($P < 0.05$) within a genotype. The symbol (*) indicated the difference between diets across genotypes ($P < 0.05$).

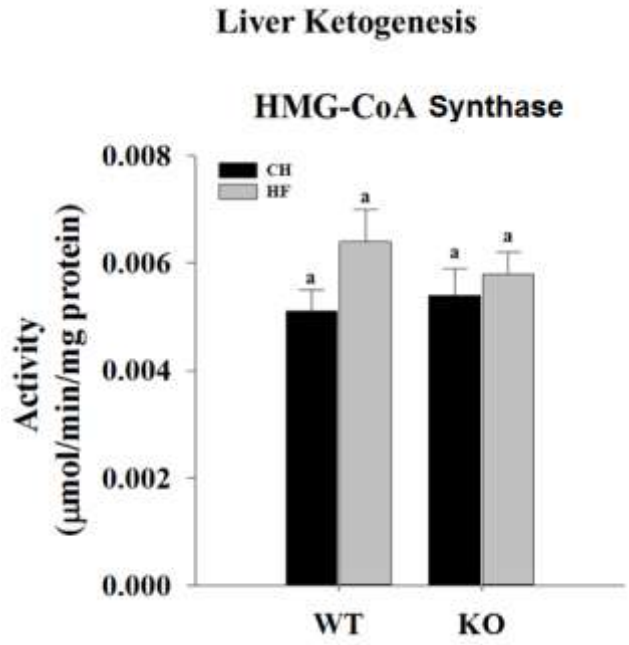

Figure 7. The activity of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA synthase) in the liver of wild-type (WT) and Shc knockout (KO) male mice fed on chow (CH) or high fat diet (HFD). All values are mean ± SEM (n = 8 -- 11). Different letters are used to indicate a diet effect ($P < 0.05$) within a genotype. The symbol (*) indicated the difference between diets across genotypes ($P < 0.05$).

3.4. Discussion

This study used ShcKO mice to determine the influence of Shc proteins on the metabolic response to acute feeding of a HFD. The study showed that ShcKO increases ($P < 0.05$) energy expenditure adjusted for either total body weight or lean mass (Table 2). This change in energy expenditure could contribute to the previously reported decrease in weight gain in ShcKO versus WT mice fed a HFD (5). Moreover, the decrease in weight and fat mass gain previously reported in ShcKO mice following feeding of a HFD could be due to better ability to alter substrate oxidation in response to a shift in diet composition and/or increased energy expenditure. However, the study did not find shifts in metabolic pathways similar to those observed in ShcKO mice with fasting. Although decreased levels of Shc proteins influenced the activity of some enzymes in response to high fat feeding, such as increasing the activity of acyl-CoA dehydrogenase, it did not produce concerted changes in enzymes of glycolysis, citric acid cycle or the ETC. Furthermore, low Shc levels did not alter whole animal substrate oxidation, as shown by RER, with acute (5-7 days) high fat feeding.

3.4.1. Energy expenditure

In order to study the substrate oxidation in ShcKO mice, we investigated this in two ways of RER and found no evidence that Shc proteins alter whole animal substrate oxidation in response to short-term feeding of a HFD (Figure 1 B). A study showed that ShcKO mice are resistant to weight gain on a HFD despite consuming the same kcal as controls (5). Also, ShcKO mice on a leptin-deficient ob/ob mouse background are also resistant to weight gain compared to ob/ob WT mice, despite the fact that they consumed the same amount of energy per day (7). An increase in energy expenditure is one mechanism that could explain this resistance to weight gain. The Berniakovich et al. paper (5) suggests that energy expenditure may be increased in the ShcKO mice, but they do not provide calorimetry data from mice consuming a HFD. Previous work has indicated that energy expenditure adjusted for either body weight or lean mass is not increased in ShcKO mice maintained on a standard rodent chow diet or subjected to calorie restriction on the same chow diet (17-19). However, there is evidence that ShcKO mice can show increased activation of brown adipose tissue (20), suggesting that energy expenditure could be increased in these mice under certain circumstances.

The results of the present study indicate that energy expenditure is increased in ShcKO versus WT mice with high fat feeding. It remains to be determined if brown adipose tissue, or other tissues, are responsible for this increase in energy expenditure. Our results indicate that Shc proteins should be considered as potential targets for developing interventions to mitigate weight gain on HFD by stimulating energy expenditure. Additionally, an increase in mass-adjusted energy expenditure may be a mechanism that contributes to the decreased weight gain previously reported in ShcKO mice fed a HFD. This would fit with previous studies indicating that brown fat activity is increased in ShcKO mice (20). Future studies should investigate brown fat in ShcKO mice to determine if this is the main mechanism that contributes to the metabolic response to a HFD in these mice.

3.4.2. Glycolysis

Decreased levels of Shc proteins have been shown to produce concerted shifts in the activities of enzymes of intermediary metabolism toward an increase in

capacity for fatty acid oxidation and a decrease in capacity for glycolysis (9-10). These changes are most pronounced with fasting. Similar to fasting, adaptation to a HFD requires a shift in metabolism toward fatty acid oxidation and away from glucose. However, the present study does not provide support for Shc proteins induce the same decrease in capacity for glycolysis observed with fasting in the HFD fed mice. In liver, ShcKO differed from WT mice in that the activities of GK and PK were increased with a HFD and the activity of PFK-1 was not decreased in these animals. Thus, the change in glycolysis is nearly opposite of what was observed in the liver of fasted ShcKO mice and ShcKO mice shortly after feeding (3 hrs) (9).

In contrast, the activities of glycolytic enzymes, with the exception of PK, were not changed with a HFD in skeletal muscle of ShcKO mice. Thus, Shc proteins appear to play distinct roles in regulating glycolytic enzyme activities depending on either physiological state (fed versus fasted) or diet (chow versus HFD). Therefore, unlike fasting, ShcKO did not change enzymes activities of a particular pathway. Response to HFD clearly shows tissue differences in the ShcKO animals and there is no evidence in either skeletal muscle or liver that ShcKO decreases capacity for glycolysis with high fat feeding. Thus, more studies are needed to determine how Shc proteins specifically change the activities of glycolytic enzymes in response to a HFD.

3.4.3. Fatty acid oxidation and ketogenesis

With sustained high fat feeding, there is evidence for increased activity of the β-oxidation enzyme acyl-CoA dehydrogenase in both skeletal muscle (21, 22) and liver (23, 24). Such a change could be beneficial for increasing capacity for fatty acid β-oxidation. The present study indicates that ShcKO dramatically increases the activity of skeletal muscle and liver acyl-CoA dehydrogenase in response to high fat feeding. This results suggest that Shc proteins influence β-oxidation pathway and may contribute to the lower body fat in ShcKO mice. However, in the present study ShcKO mice do not increased the activity of HMG-CoA synthase, a key enzyme in ketogenesis. Thus, ShcKO does not have uniform impact on all enzymes involved in lipid metabolism and it remains to be determined if ShcKO stimulates the activities of other enzymes involved in fatty acid oxidation. Previous work indicated that the low Shc levels result in increased liver and muscle β-oxidation enzyme activities in response to fasting and induce chronic increases in the activity of liver ketogenic

enzymes. The activities of acyl-CoA dehydrogenase, 3-hydroxyacyl-CoA dehydrogenase and ketoacyl-CoA thiolase were increased in fasted ShcKO compared to WT mice (8). However, additional studies are needed to determine how Shc proteins changes the activities of enzymes involved in lipid metabolism and ketogenesis in mice fed a HFD.

3.4.4. Mitochondrial enzymes (citric acid cycle and ETC)

Maintenance of mitochondrial content is important with high fat feeding, since mitochondria are a major site of lipid β-oxidation. However, the impact of high fat feeding on mitochondrial enzyme activities is complex. Citrate synthase (CS), an enzyme frequently used as a marker of mitochondrial content (25), has been previously reported in skeletal muscle to show either no change (26-29) or an increase (21, 22, 27, 29-31) in activity with consumption of a HFD. In liver, CS activity has often been found to not change with sustained feeding of a HFD (24, 28, 32, 33). In the present study, we found no impact of ShcKO on changes in CS activity in response to a HFD with both genotypes showing an increase in CS activity in skeletal muscle and no change in the activity of this enzyme in liver.

Caution should be taken, however, in relying solely on CS activity as an indicator of mitochondrial content or changes in the major energy metabolism pathways in mitochondria. In our study, the activities of Complexes I and IV (NADH: Ubiquinone oxidoreductase and Cytochrome C oxidase, respectively) did not mirror the activities of CS in response to a HFD in the ShcKO mice for either liver or muscle. These results suggest that Shc proteins may play a role in modulating ETC enzyme activity in response to a HFD. Aconitase activity was also decreased in the ShcKO compared to WT mice for both the chow and HFD. ShcKO is considered to decrease oxidative stress (34, 35), and thus, it seems unlikely that the decreased aconitase activities in young ShcKO mice consuming chow is due to oxidative damage. Here, the effect of genotype was clear on the aconitase activity and, therefore, ShcKO mice may decrease the flow of electrons to oxygen, leading to decreased oxidative phosphorylation and this result indicates the importance of Shc proteins to prevent oxidative stress [34, 35]. However, future studies are also needed to determine if Shc proteins are an important regulator of aconitase.

3.4.5. Limitations

With the ShcKO mouse, it is not possible to determine which specific Shc isoform is responsible for observed changes in enzyme activities or energy expenditure. Additional models that allow controlled expression of specific Shc isoforms are needed to further dissect the contribution of the individual isoforms to changes in metabolism following consumption of a HFD. In addition, the extremely low Shc expression may alter the whole system to a dysfunctional state, which is possible in knockout models. The present study only investigated changes in metabolism in response to consumption of a HFD for 7 days. This period of time is sufficient for changes in the expression of most proteins and enzymes, however, our study is not capable of determining the influence of Shc proteins on the metabolic response to long term consumption of a HFD.

3.5. Conclusion

There is an overall effect of high fat diet increasing energy expenditure in ShcKO versus WT mice. In particular, ShcKO increases energy expenditure adjusted for either body weight or lean mass in response to feeding a HFD.

It is possible that this increase in energy expenditure contributes to the decrease in weight gain previously reported for these mice when consuming a HFD (5). ShcKO also influenced, in a tissue specific manner, the activities of some enzymes of intermediary metabolism in response to consumption of a HFD.

Thus, short-term feeding of HFD was enough to partially alter enzyme activities in liver and muscle of ShcKO, leading to changes in energy metabolism. However, the physiological significance of these changes in enzyme activities remains to be determined.

Acknowledgments

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4. ESTIMATED HEAT PRODUCTION, BLOOD PARAMETERS AND MITOCHONDRIAL DNA COPY NUMBER OF NELLORE BULLS WITH HIGH AND LOW RESIDUAL FEED INTAKE

Abstract

Our hypothesis was that heat production, blood parameters, liver protein profile, and mitochondrial DNA (mtDNA) copy number differed among Nellore bulls with high and low residual feed intake (RFI). The RFI values were obtained by regression of dry mater intake (DMI) in relation to average daily gain (ADG) and mid-test metabolic body weight. Calculations of RFI were not corrected with any estimate of body composition. Eighteen animals (9 low and 9 high RFI) were individually fed in a feedlot for 98 days. Heart rate (HR) was monitored for 4 consecutive days and used to calculate the estimated heat production (EHP) in 12 bulls (6 high *versus* 6 low RFI). Electrodes were fitted to bulls with stretch belts and oxygen consumption was determined using a facemask connected to the gas analyzer as HR was simultaneously measured for 15 minutes. Daily EHP was calculated multiplying individual oxygen pulse (O_2P) by the average HR of each animal, assuming 4.89 kcal/L of O_2. Blood parameters such as hematocrit, hemoglobin, and glucose were assayed between 45 and 90 days. Immediately after slaughter, liver, muscle and adipose tissues (subcutaneous and visceral fat) were collected and, subsequently, the amounts of mtDNA were quantified by quantitative real-time PCR. The protein profile of hepatic tissue and levels of mitochondrial uncoupling proteins (UCPs) in liver, muscle, and fat were also investigated. The study showed that EHP (expressed in Mcal/day or in relation to gain), O_2 consumption, and mtDNA copy number per cell were similar ($P > 0.10$) between RFI groups, while HR, hemoglobin and hematocrit concentration were lower ($P < 0.05$) in the efficient phenotype (low RFI). This results suggest that blood parameters and heart rate may be useful tools to determine feed efficiency. In addition, we found 71 protein spots in liver differentially expressed between RFI groups, suggesting that several proteins are related to the phenotypic expression of RFI. Finally, UCPs protein abundances were similar, indicating no differences for uncoupling and mitochondrial function between RFI groups.

Keywords: Bioenergetics; *Bos indicus;* Energy metabolism; Mitochondria; Uncoupling

4.1. Introduction

Because feed represents the largest production cost there has always been a search for animals that are more efficient. In recent years however, feed efficiency

(FE) became even more important because of environmental concerns. Improving FE reduce the use of limited natural resources as well as reduce the release of pollutants (including green house gases) per unit of meat delivered to a hungry world. In this context, world population should increase by 2.5 billion and per capita meat consumption should increase by around 45% up to 2050 (FAO, 2009). Among the existing methods to measuring FE, gross feed efficiency (G:F) and residual feed intake (RFI) are the most common ones (Bottje and Carstens, 2012). However, the major difficulty in incorporating FE traits into beef cattle breeding programs is the measurement of individual animal dry matter intake (DMI). Additionally, variation in FE traits and DMI are due to unknown differences in several processes including physiological mechanisms as digestion of feed, heat increment, protein turnover, feeding behavior, activity, body composition and rate of gain (Nkrumah et al., 2006).

Thus, an alternative methods that have been proposed, would be selecting animals that produce less heat. The calorimetric chamber is often used to measure the estimated heat production (EHP), however, this methodology is very laborious and expensive (Brosh, 2007; Chaves et al., 2014). Therefore, heat production can be indirectly estimated from heart rate (HR) after calibration to oxygen consumed per heart beat, based on the fact that oxygen used by homoeothermic animals is transported to the tissues by the heart (Brosh, 2007). In association with heat production, some physiological indicators of FE such as blood parameters have been used, including hematocrit content (Gomes et al., 2011). Since high RFI animals commonly consume more feed than the low RFI ones, this should increase oxygen consumption and thus require a higher capacity of oxygen transportation in high RFI animals (Nkrumah et al., 2006; Chaves et al., 2014; Chaves et al., 2015). However, increasing oxygen consumption should not necessarily change red blood cell parameters, in order to enhance oxygen carrying capacity (Gomes et al., 2011). Thus, the physiological significance of these changes in blood parameters remains to be determined.

Additionally, there is the challenge of identifying molecular mechanism that modulates FE traits in beef cattle. Several studies have focused on the association between mitochondrial function and FE (Bottje and Carstens, 2009). These organelles are responsible for producing approximately 90% of the energy for the cell, and how efficiently this process is conducted has implications for animal growth and development (Bottje and Carstens, 2009; Bottje and Carstens, 2012). Moreover,

greater protein oxidation observed in less efficient animals is likely due to greater basal mitochondrial reactive oxygen species (ROS) production (Divakaruni and Brand, 2011). Increased ROS production could oxidize nuclear and mitochondrial DNA (mtDNA) (Baughman and Mootha, 2006). The damage in mtDNA is undesirable in terms of FE because the key function of mitochondria is the oxidative phosphorylation (OXPHOS), which has two main outputs: generation of cellular ATP and heat (Bottje and Carstens, 2009; Bottje and Carstens, 2012). Thus, mtDNA degradation and repair would represent an additional energetic cost to the cell and, consequently, increased heat production for the same rate of growth.

The purpose of this study was to investigate the relationships among EHP, the concentration of blood parameters (hemoglobin, hematocrit, and glucose) and amounts of mtDNA in liver, muscle and adipose tissue (subcutaneous and visceral depots) in Nellore bulls classified as low or high RFI. Additionally, because hepatic tissue play a key role in energy metabolism we also study the protein profile of liver samples and the content of mitochondrial uncoupling proteins (UCPs) in order to understand the molecular mechanisms associated with RFI phenotypes.

4.2. Material and Methods

The experimental procedures were conducted in accordance with guidelines on animal welfare and humane slaughter and were approved by the Ethics Committee on Animal Use of Instituto de Zootecnia (CEUA/IZ) (Protocol 213-15).

4.2.1. Animals and RFI calculation

The experiment was conducted at Centro Avançado de Pesquisa Tecnológica dos Agronegócios de Bovinos de Corte, an organ of the Instituto de Zootecnia, São Paulo State, Brazil. The Nellore herd of the Center consists of 500 dams divided into three selection lines: Control Nellore (NeC), Selection Nellore (NeS), and Traditional Nellore (NeT). These lines originated from a breeding program designed to increase the postweaning weight of the animals, in which selection is performed based on individual performance. The NeC line is being selected based on selection differentials of zero or close to zero for weight at 378 days of age in

males and 550 days in females, while the NeS and NeT lines are being selected based on the highest possible selection differentials for the same traits (Bonilha et al., 2008).

One-hundred and twenty-eight Nellore calves were submitted to a feed efficiency test after weaning to identify their RFI class. RFI was calculated as the error term of the equation: $DMI = \beta_0 + \beta_P * BW^{0.75} + \beta_G * ADG + \varepsilon$ (RFI), where DMI is the dry matter intake observed during the test, β_0 is the intercept of the equation, $BW^{0.75}$ is the mid-test metabolic live weight, ADG is the average daily weight gain during the test, and β_P and β_G are the regression coefficients of $BW^{0.75}$ and ADG, respectively. ADG was estimated by the linear regression coefficient of live weight as a function of days in test. Mid-test metabolic live weight was calculated by the equation $BW^{0.75} = [\alpha + \beta^* (DIT/2)]^{0.75}$, where α is the intercept of the regression equation corresponding to the initial live weight, β is the linear regression coefficient corresponding to the average daily gain, and DIT are the days in test. No composition of growth parameter was used in the regression.

After the efficiency test, animals were classified into low (<0.5 standard deviation below the mean; more efficient), medium (±0.5 standard deviation from the mean), and high (>0.5 standard deviation above the mean; less efficient) RFI groups. From this total, 18 animals from low and high RFI groups were randomly chosen for the finishing experiment for slaughter, of which 9 were classified as low RFI and 9 as high RFI, having initial mean live weight and age of 430 ± 44 kg and 709 ± 24 days, respectively.

4.2.2. Feed intake, performance and efficiency measures in finishing period

For the finishing period, animals were housed in individual 4 x 2 m pens, with a feed bunk in front of the pen and a waterer between two pens. The finishing diet was formulated with corn silage, Brachiaria hay, ground corn, soybean meal, and a mineral mixture, with a roughage-to-concentrate ratio of 35:65, having 14% crude protein and 80% total digestible nutrients. Animals had *ad libitum* access to feed. Dry matter intake was calculated as the difference between the amount of feed offered and leftovers expressed as kilograms and percentage of body weight. Feed was dried and consumption expressed as dry matter weight.

The experimental period was of 98 days (28 days of adaptation + 70 days of data collection). The criteria adopted for slaughter was a mean body weight of 550 kg, with all animals being slaughtered on the same day. Animals were weighed at the beginning and at the end of the experiments after 16 h fasting. No fasting was necessary for intermediate weight recordings that were performed weekly.

Average daily gain was estimated by linear regression coefficient of live weight as a function of days in test. Feed efficiency (G:F) was calculated as the ratio between ADG and DMI. At the end of finishing period, RFI was re-calculated in order to verify the level of re-ranking between experimental groups.

4.2.3. Heat production by oxygen pulse methodology

Estimated heat production (EHP) was determined using oxygen pulse (O2P) methodology as previously described (Brosh et al., 1998; Chaves et al., 2015), with minor adaptations. Briefly, six low RFI and six high RFI bulls were randomly chosen from the experimental groups and their heart rate (HR) per minute was recorded for at least 4 consecutive days, between 62 and 72 d after the beginning of the finishing period. Electrodes were fitted to the animals with stretch belts (POLAR RS400; Kempele, Finland). This data of HR during 4 days was essential to the calibration period.

To calculate O2P, an open respiratory system previously described (Taylor et al., 1982) was used. In briefly, a facemask connected to a gas analyzer (EXHALYZER, ECOMedics, Zurich, Switzerland) was used on different days and periods. Simultaneously, the HR of each animal was measured for 15 minutes. The gas analyzer was previously calibrated with gasses with a known oxygen concentration and a syringe of known volume (3 L).

To avoid biased results, calibration of oxygen consumption per beat was taken only when HR was close to normal conditions (maximum of 20% greater than those previously obtained under 4 days) (Brosh, 2007). Daily oxygen consumption was calculated by multiplying the volume of O_2 per beat by total daily beats. The result was multiplied by a constant 4.89 kcal/L (Nicol and Young, 1990) of O_2 to measure daily EHP that was expressed in kilocalories/kilogram $BW^{0.75}$ as well as in kilocalories/kg gain.

4.2.4. Blood parameters

Blood samples were collected using vacuum tubes containing EDTA between days 45 and 90th after the beginning of the finishing period in order to measure the concentration of hemoglobin, hematocrit, and glucose. Hemoglobin was assayed by colorimetric methodology using commercial kits (Labtest Diagnóstica, Lagoa Santa, Brazil) and a spectrophotometer (UV-1601PC, Shimadzu, Japan) at 525 nm. In addition, each blood sample was partially transferred into a capillary tube and centrifuged using a microhematocrit centrifuge at 1,077g for 5 minutes at 25˚ C. The packed cell volume as a percentage of each specimen was determined using a graduated scale in millimeters. Additionally, plasma glucose was determined using the enzymatic colorimetric kit *Glicose* GOD-PAP (Laborlab Ltda., Guarulhos, SP, Brazil) at 505 nm and 37˚ C according to manufacturer instructions.

4.2.5. Slaughter, tissue collection and sample preparation

Animals were slaughtered on average with 557.65 ± 10.39 kg of BW, as planned. Harvesting was carried out in an experimental slaughterhouse (University of São Paulo, Pirassununga, São Paulo, Brazil) and was preceded by fasting for 16 h in accordance to Brazilian government inspection procedures. Animals were stunned by cerebral concussion, suspended, and exsanguinated through the jugular vein.

Immediately after slaughter, a section of liver, a portion of *longissimus thoracis* (LT) muscle, plus a portion of subcutaneous fat (surrounding LT muscle, between the 9–11th ribs) and portions of visceral adipose tissue (from kidney, pelvic and inguinal fat) were collected, placed in a sterilized aluminum foil and frozen in liquid nitrogen for transport. Subsequently, all samples were stored at -80° C freezer until processing.

4.2.6. Mitochondrial DNA quantification

Samples of liver, muscle and adipose tissues (subcutaneous and visceral depots) were used to quantify mtDNA copy number per cell. Samples of visceral adipose tissue of each animal were homogenized and then aliquoted prior to the DNA extraction procedures. Total DNA was isolated using the Qiagen DNeasy

Tissue kit (Valencia, CA, USA) according to manufacturer instructions. Subsequently, the relative quantification of mtDNA was performed by a quantitative real-time PCR (qPCR) method as previously described (Chiaratti et al., 2010; Machado et al., 2015).

The 7500 Fast Real-Time PCR System (Life Technologies, Foster City, CA) was used for qPCR reactions with minor modifications. Briefly, a fragment of mtDNA was amplified in a 15 µl PCR reaction composed of 4.5 µM bMT3010-f (5'-GCCCTAGAACAGGGCTTAGT-3') and bMT3096-r (5'-GGAGAGGATTTGAATCTCTGG-3') primers plus 1.5 µM TaqMan probe bMT3030-Fam (5'-FAM-AAGGTGGCAGAGCCCGGTAATTGC-BHQ1-3`; Promega) plus 1X TaqMan Gene Expression Master Mix (Applied Biosystems) plus 5 µl of template.

The number of mtDNA copies per cell was determined in all tissues by normalization of mtDNA amount against a single copy nuclear gene (B2M). The conditions used to amplify the B2M gene were the same described above, but using the bovB2M-f (5'-TGGGCCTTCAAAGGTGAACT-3') and bovB2M-r (5'-ACTCTTCTCTTCAGGCTACCTAGCA-3') primers and the bovB2M-FAM probe (5'-FAM-TCCCTCCCTTTGCGTGGCAGC-BHQ1-3'). Amplification of both mtDNA and B2M were performed in parallel. The amplification conditions and amplification efficiencies were analyzed following previously published methods (Livak and Schmittgen, 2001). Thus, the following cycling conditions were applied for amplification: initial denaturation at 95˚ C for 15 min followed by 40 cycles consisting of 95˚ C for 20 seconds and 63˚ C for 1 minute. The probe fluorescence was read at the end of each extension step (63˚ C) and the number of mtDNA copies per cell was then determined as reported (Nicklas et al., 2004).

4.2.7. Liver protein profile by two-dimensional electrophoresis

The proteins present in hepatic tissue was studied by two-dimensional electrophoresis (2D-PAGE). For each RFI group (high and low), a pooled sample was created from equal amount from all samples of the liver. This standard was also used for protein identification gels. Approximately 375 µg of protein extracts were loaded into 13-cm isoelectric focusing strips which contained the precast gel with an ampholyte-immobilised pH 3–10 gradient as previously described (Baldassini et al., 2015). The first dimension runs were performed and the strips were subsequently

placed on a polyacrylamide gel (12.5% w/v) in the second dimension of the electrophoretic process. The gels of the experimental groups (all in triplicate) were then scanned and the images analyzed using the ImageMaster Platinum (v. 7.0) software to measure parameters such as the number of spots, the percentage of matching between gels, isoelectric point (pI) and molecular mass (MW) of the protein spots.

4.2.8. UCPs levels

Samples of liver, muscle, and adipose tissue were used to study UCP1, UCP2 and UCP3 protein levels by Western blotting methods as previously described (Velloso et al., 2009) with minor modifications. In briefly, samples were homogenized in buffer (1% Triton X-100, 100mM Tris-HCl, pH 7.4, 100mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium orthovanadate, 2.0mM phenylmethylsulfonylfluoride, and 0.1 mg aprotinin/mL) at 4° C with a Polytron homogenizer (model PT MR 2100; Brinkmann Instruments). Insoluble material was removed by centrifugation for 15 minutes (9000 g at 4° C) and protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA), with BSA as the standard.

Thirty micrograms of protein extract were diluted 1:1 (vol:vol) in Laemmli buffer (Laemmli, 1970), boiled (100 ˚C) for 1 min and separated in 10% nongradient SDS-PAGE using mini-protean apparatus (Bio-Rad, Hercules, California). Running started with 70 mV and subsequently was increased to 100 mV, after samples completely aligned into the gel. Once separation was concluded, proteins were transferred to nitrocellulose membranes and blotted with antibodies, using 10 mL antibody in 10 mL blotting buffer.

The primary antibodies were UCP1 (sc-6529, goat polyclonal, Santa Cruz Biotechnology), UCP2 (sc-6525, goat polyclonal, Santa Cruz Biotechnology) and UCP3 (sc-31387, goat polyclonal, Santa Cruz Biotechnology). The concentrations and incubation time used were 1:750 dilution and overnight at 4°C, respectively. Goat anti-rabbit IgG-HRP (sc-2030, Santa Cruz Biotechnology) was used as secondary antibody in combination with ECL Western Blotting Detection Reagents (GE Amersham) for detection of specific bands by chemiluminescence. Visualization was performed by exposure of the membranes to RX-films and quantification of the bands

was performed using the Scion software (ScionCorp, Frederick, Maryland) (Velloso et al., 2009).

4.2.9. Statistical analysis

A completely randomized design was adopted. Variables measured were DMI, ADG, BW, RFI, G:F (ADG/DMI), HR, O2P, EHP, blood parameters (hemoglobin, hematocrit, and glucose), protein levels (Western-blotting), protein spots (2D-PAGE), and mtDNA copy number per cell in liver, muscle, and adipose tissues. For all variables, *P*-values ≤ 0.05 were declared significant, and values ≤ 0.10 were considered tendencies.

Data were analyzed with the GLM procedure of SAS software (SAS Inst. Inc version 9.3) considering the fixed effects of RFI class, and the covariate initial body weight. The PDIFF option was applied to comparisons between RFI classes. mtDNA copy number per cell, protein spots of liver and levels of UCPs in the liver, muscle, and adipose tissues were also compared by t-tests (high versus low RFI). Spearman correlations were computed to compare ADG, DMI, metabolic BW ($BW^{0.75}$), and RFI across the two test periods (growing and finishing).

The normality and homogeneity of variances were verified (Shapiro-Wilk and studentized Breusch-Pagan test, respectively). The traits EHP (kcal/kg gain) and glucose concentration (mg/dL) did not show normality and log transformation was applied. The inverse transformation was applied on variable mtDNA copy number in visceral fat.

4.3. Results

4.3.1. Performance and efficiency measures

The difference between RFI means from high and low RFI groups found in the present study was roughly 2.0 kg DM/d. Low-RFI animals (more efficient) consumed -1.16 kg/d and high-RFI animals (less efficient) consumed +1.22 kg/d (Table 1). No significant differences between RFI groups (*P* > 0.10) were observed during the finishing period for age, BW traits, and ADG (Table 1). Significant

differences ($P < 0.01$) between high and low RFI animals were observed for DMI and gross FE (G:F). Nellore bulls with low RFI (efficient) showed lower DMI and higher G:F than high RFI ones ($P < 0.01$).

Table 1. Performance and feed efficiency traits during the finishing period of Nellore bulls classified as high and low residual feed intake (RFI).

Trait[1]	High RFI (inefficient)		Low RFI (efficient)		
	Mean	SEM	Mean	SEM	*P*-value
Finishing period[2]					
Initial age, d	685.8	8.2	685.5	7.6	0.970
Age at slaughter, d	783.8	8.2	783.5	7.6	0.978
Initial BW, kg	434.6	8.1	435.1	13.4	0.975
Final BW, kg	557.4	12.6	563.9	17.1	0.564
Empty BW, kg	318.9	7.2	321.2	9.7	0.738
ADG, kg/d	1.75	0.6	1.84	0.10	0.562
DMI, kg/d	15.2	0.6	13.2	0.7	0.014
DMI, % BW	2.7	0.1	2.3	0.1	<0.001
RFI, kg/d	1.22	0.22	-1.16	0.30	<0.001
G:F	0.120	0.004	0.139	0.004	<0.001

[1] BW = body weight; $BW^{0.75}$ = metabolic body weight; ADG = average daily gain; DMI = dry matter intake; RFI = residual feed intake; G:F = gross feed efficiency (ADG/DMI)
[2] Previous to data analysis, RFI values of Nellore bulls were re-calculated at the end of finishing period in order to verify the evidence of re-ranking of RFI

In the current study there were some re-ranking of RFI from the growing to the finishing periods as data illustrated in Figure 1. Therefore, previous to data analysis, performance and FE traits were re-calculated within experimental groups.

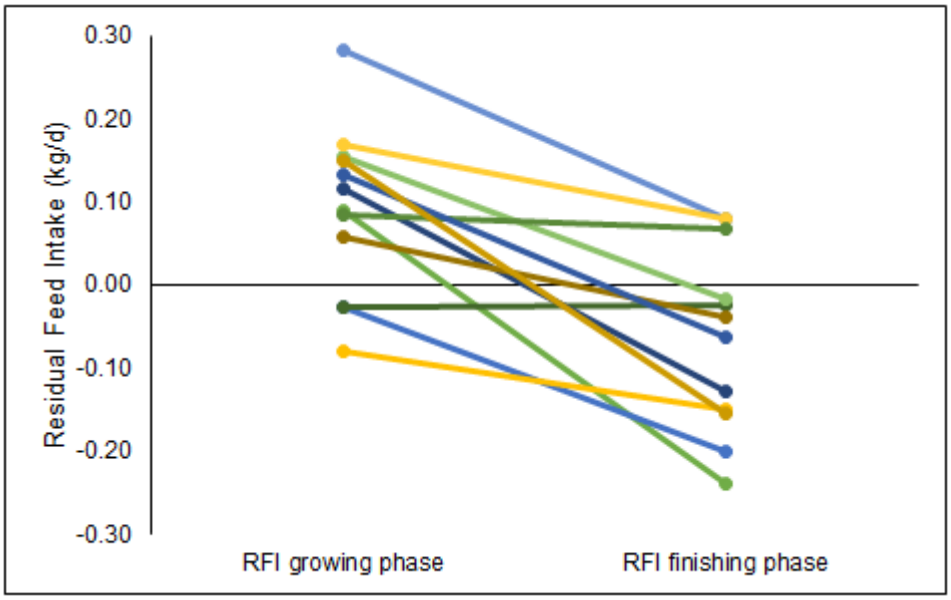

Figure 1. Re-ranking of Nellore bulls for residual feed intake (RFI) from the growing to the finishing period evaluation.

Spearman correlation data were summarized in Table 2. There is a strong and positive association (r = 0.722; *P* < 0.001) between RFI at the growing (RFI_g) and finishing (RFI_f) periods. In addition, there is a substantial and positive correlation (r = 0.634; *P* <0.01) between gross FE in the growing and finishing periods, while correlations close to zero (*P* >0.05) were observed for ADG and metabolic BW between tests periods.

Table 2. Spearman correlation coefficients between performance traits and residual feed intake (RFI) of Nellore bulls obtained in two evaluation periods of RFI (growing and finishing).

	Performance trait $_{finishing}$
$BW^{0.75}_{growing}$	0.012 (*P* = 0.961)
$ADG_{growing}$	-0.044 (*P* = 0.861)
$DMI_{growing}$	0.323 (*P* = 0.191)
$RFI_{growing}$	0.722 (*P* < 0.001)
$G:F_{growing}$	0.634 (*P* < 0.01)

$BW^{0.75}$ = metabolic body weight; ADG = average daily gain; DMI - dry matter intake; G:F = gross feed efficiency (ADG/DMI).

4.3.2. Estimated heat production measured by the oxygen pulse methodology

HR, oxygen consumption and EHP data are summarized in Table 3. During the calibration period (15 minutes), the HR was lower ($P < 0.05$) in efficient animals (low RFI) compared to inefficient (high RFI). However, oxygen consumption (VO_2) and oxygen pulse (O_2P) were similar ($P > 0.05$) between RFI groups. At the trial period (4 days), analogous variation ($P < 0.10$) for HR was observed between high and low RFI animals, while no difference ($P > 0.05$) was detected between high and low RFI animals for VO_2. Additionally, similar results ($P > 0.05$) were also observed between RFI groups for EHP. Although low RFI bulls showed EHP (Mcal/d) 15 to 20% lower than high RFI, this difference did not reach statistical significance ($P = 0.230$). Thus, differences in EHP were not detected regardless as how it was expressed (Mcal/d; kcal/kg $BW^{0.75}$; kJ/d $BW^{0.75}$; or kcal/kg gain).

Table 3. Heart rate, oxygen consumption and estimated heat production traits of Nellore bulls classified as high and low residual feed intake (RFI) in the finishing period[1].

Trait[2]	High RFI (inefficient)		Low RFI (efficient)		
	Mean	SEM	Mean	SEM	*P*-value
O_2 calibration period[3]					
Heart rate, bpm	153.0	10.5	100.2	7.6	0.002
O_2 consumption, mL/beat	12.5	1.3	12.3	1.0	0.956
VO_2, mL·min^{-1}·kg $BW^{-0.75}$	15.3	1.4	13.5	0.7	0.612
O_2P, µL O_2·beat^{-1}·kg $BW^{-0.75}$	159.5	19.9	189.1	22.0	0.392
Trial period[4]					
Heart rate, bpm	136.2	14.6	93.8	5.8	0.066
O_2 consumption, mL·d^{-1}	2202.2	220.1	1789.3	88.41	0.230
EHP, Mcal·d^{-1}	10.8	1.1	8.7	0.4	0.230
EHP, kcal·d^{-1}·kg $BW^{-0.75}$	98.8	9.1	83.8	3.7	0.339
EHP, kcal/kg gain	97.4	12.1	70.2	5.1	0.146

[1] Evidence re-ranking of RFI from the growing to the finishing periods was observed. Thus, heart rate, O_2 consumption, and EHP were re-calculated within experimental groups.
[2] bpm = beats per minute; VO_2 = O_2 volume; BW= Body weight; $BW^{-0.75}$ = metabolic BW; O_2P = oxygen pulse; EHP* = estimated heat production. * EHP was determined by oxygen pulse methodology (Chaves et al., 2015) using an open respiratory system. All values are mean ± SEM (n = 6 per group).
[3] Minimum 15 minutes
[4] Minimum 4 days

The profile of HR of animals in the current study (Figure 2) was considered within a normal pattern and are consistent with previous research with Nellore cattle and *Bos taurus* breeds as described (Brosh et al., 1998; Chaves et al., 2015).

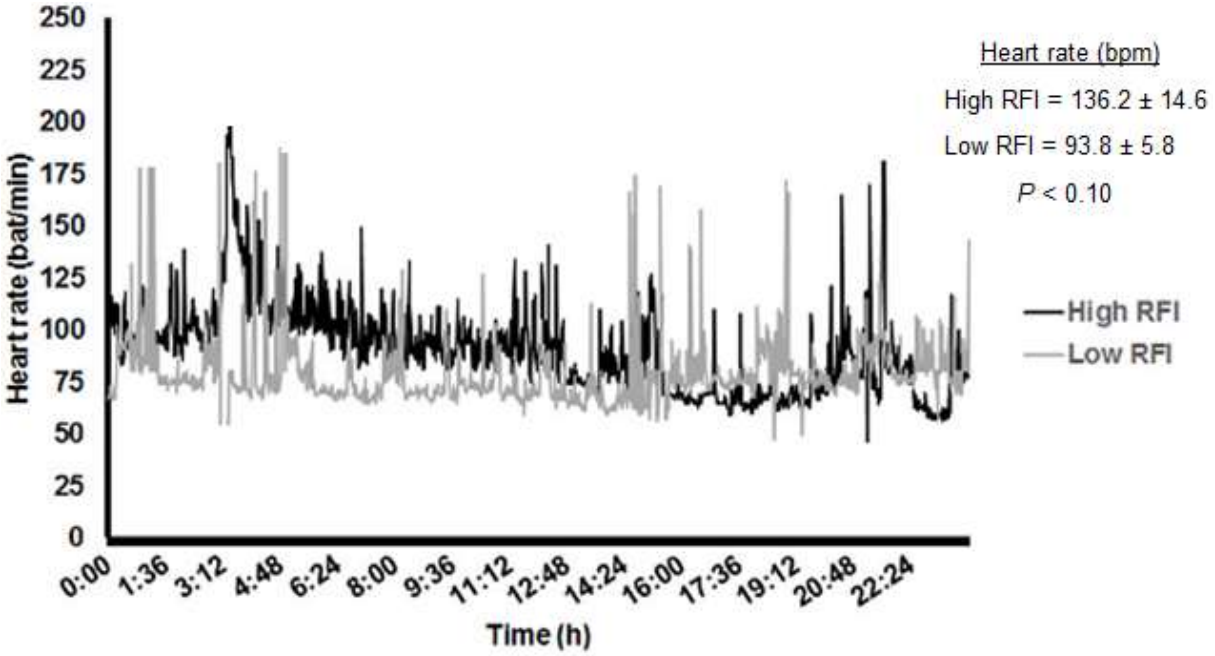

Figure 2. Profile of diurnal heart rate (HR) of Nellore bulls classified as high and low residual feed intake (RFI). HR data of two animals randomly chosen within each RFI group are shown.

4.3.3. Blood parameters

The low RFI group showed lower hemoglobin concentration and hematocrit percentage ($P < 0.05$). For glucose concentration, a trend ($P < 0.10$) was observed between RFI groups (Table 4).

Table 4. Blood parameters of Nellore bulls classified as high and low residual feed intake (RFI).

Blood variable	High RFI (inefficient)		Low RFI (efficient)		
	Mean	SEM	Mean	SEM	*P*-value
Hemoglobin, g/dL	14.8	0.4	12.7	0.6	0.014
Hematocrit, %	42.5	1.6	38.4	1.1	0.048
Glucose, mg/dL	60.1	3.4	74.2	7.0	0.079

4.3.4. mtDNA copy number

No differences were observed for copy number of mtDNA per cell in liver, muscle and adipose tissue (subcutaneous and visceral depots) of Nellore bulls with high and low RFI (Figure 3).

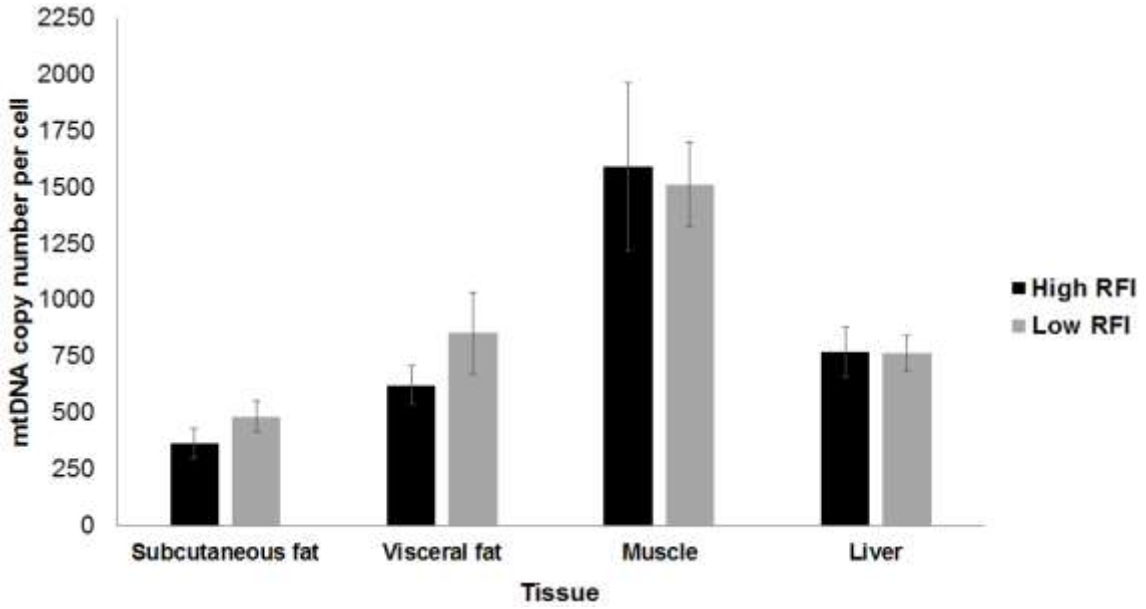

Figure 3. Mitochondrial DNA (mtDNA) copy number in tissues of Nellore bulls (n = 18) classified as high and low residual feed intake (RFI). Bars depict mtDNA copy number per cell from animals slaughtered with 558 ± 10.4 kg of body weight and aged 25 months. Values are reported as mean ± SEM. No significant difference between high and low RFI bulls was observed ($P > 0.05$).

4.3.5. Liver protein profile by 2D-PAGE

The gels of electrophoretic runs showed both good resolution and protein separation (matching > 97%). There was a great diversity of protein spots, and most protein were found in the 20–66 kDa MW range, with the most frequent isoeletric point (pIs) in the approximate range of 4–8 (Figure 4). However, the presence of protein spots with higher molecular weights (MW > 66 kDa) was also noteworthy. A total of 279 and 215 liver protein spots were found in high and low RFI bulls, respectively. From this total, 71 spots were found to be differentially expressed ($P < 0.05$) (Figure 4 C).

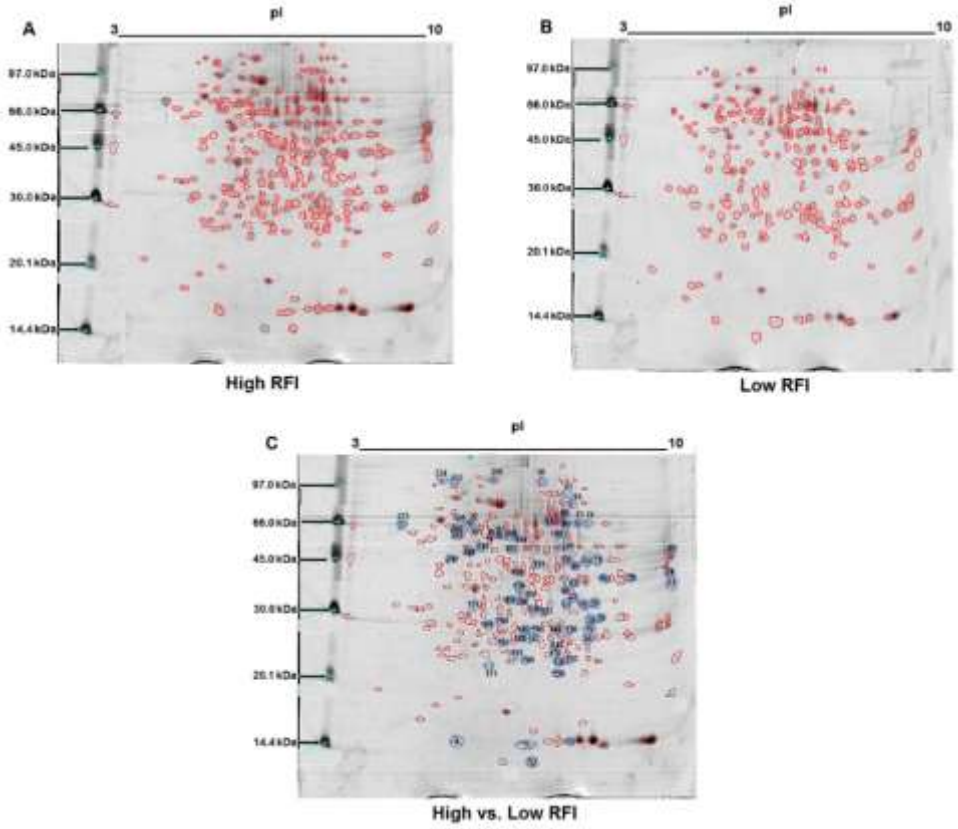

Figure 4. Representative 2D-PAGE gels from the hepatic tissue of Nellore bulls classified as high (**A**) and low (**B**) residual feed intake (RFI). The protein spots that showed differential expression (**C**) were highlighted with blue circles (high vs. low RFI) and were selected using the ImageMaster Platinum (v. 7.0) software.

4.3.6. UCPs levels

UCP2 and UCP3 protein levels in liver and muscle were not different ($P > 0.05$) between Nellore bulls classified as high and low RFI. Thus, protein levels of UCP2 and UCP3 were not affected by RFI phenotypes.

In the present study, the protein levels of UCP1 were not measurable in liver and skeletal muscle, while similar amounts were obtained in subcutaneous and visceral fat. Representative results obtained in Western blot experiments were illustrated in Figure 5.

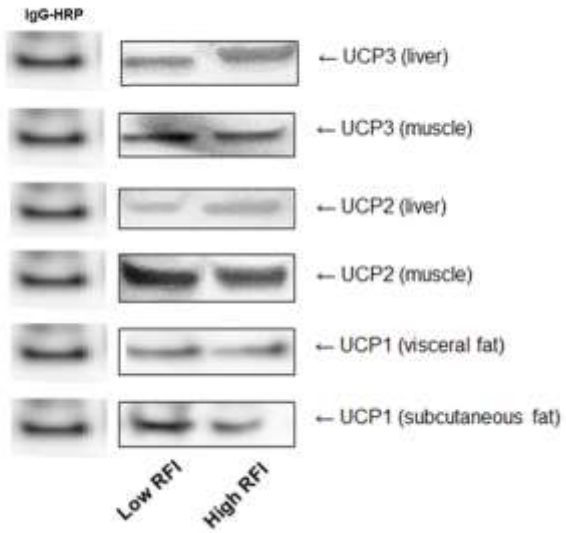

Figure 5. Western-blotting membranes from liver, skeletal muscle, and adipose tissues of Nellore bulls classified as high and low residual feed intake (RFI). Samples containing 30 mg protein were separated by SDS-PAGE, transferred to nitrocellulose membranes and blotted with anti-UCP specific antibody. Figures are representative of typical experiments.

4.4. Discussion

Our hypothesis was that heat production, blood parameters, liver protein profile, UCPs levels, and mtDNA copy number in liver, muscle, and adipose tissue would differ between RFI phenotypes. To this end, we used Nellore bulls divergently ranked according to RFI during the growing and finishing periods. The study showed that EHP (expressed in Mcal/day or in relation to gain) and mtDNA copy number per cell are similar between RFI groups, while hemoglobin and hematocrit concentration may be useful traits to identify the efficient phenotype (low RFI). In addition, the protein profile in liver showed differences between RFI groups. Thus, these results may allow a better understanding of the organ effects and the consequences it can have on the RFI. Also, we also investigated mitochondrial function by measuring the levels of UCPs in the liver, muscle, and adipose tissue and found that UCP2 and UCP3 abundances were similar between RFI groups.

4.4.1. Performance and efficiency measures

The results obtained for RFI (kg/d) were similar to those previously observed in Nellore cattle ranked for RFI (Gomes et al., 2012; Bonilha et al., 2013),

emphasizing the phenotypic differences and the ability of feed use between experimental groups. The average amplitude between low and high RFI was consistently close to values reported in the literature (Fitzsimons et al., 2013; Mcgee et al., 2014). As expected, when RFI is used as a measure of FE, efficient animals (low RFI) consume less than their inefficient counterparts for the same production performance (Fitzsimons et al., 2013). This is in agreement with the results for DMI and G:F observed in the current study. In contrast, a study with Nellore bulls reported similar DMI between high and low RFI in finishing period (Bonilha et al., 2017).

Since RFI is a measure of metabolic efficiency, therefore, independent of ADG (Koch et al., 1963; Nkrumah et al., 2006), similar results were observed for ADG between the Nellore bulls classified as high and low RFI. Additionally, as RFI is also independent of growth rate, mature body size, and weight characteristics (Koch et al., 1963), no significant differences were detected between experimental groups for initial, final or empty BW. Our results are in agreement with others previously reported for *Bos indicus* (Gomes et al., 2012; Bonilha et al., 2013) and *Bos taurus* (Nkrumah et al., 2006) genotypes.

In the current study, the Spearman correlations between traits obtained in growing and finishing periods (RFI and G:F) is an evidence of re-ranking of the animals for RFI (Table 2). Similar evidence were observed with Nellore cattle in two performance tests: growth phase and measurement of the methane emission phase (Mercadante et al., 2015). Studies have shown that comparing RFI values on individual animals over different biological time periods as well as on different planes of nutrition may cause re-ranking in cattle (Kelly et al., 2010; Durunna et al., 2011). According to these authors, re-ranking occurs by several reasons such as errors in BW and DMI measurements, compensatory gain, and animal differences in efficiency with animal maturity and diet digestibility. Moreover, re-ranking for RFI, DMI and ADG may occurs due the changes in the diet from a grower to finisher diet (Basarab et al., 2013). Factors such as changes in body composition, compensatory gain, and diet may explain the re-ranking of Nellore bulls for RFI observed in the current study (Figure 1).

4.4.2. Heart rate and estimated heat production

As expected for the oxygen pulse methodology (Brosh et al., 1998), the HR of animals during the calibration period was similar to the average daily mean during the 4 days of the measurement period. These results suggest that the animals were not under stress condition during oxygen measurement, indicating that daily pattern of HR was similar over time throughout the days (Brosh, 2007).

The results for HR at the calibration and trial (4 days) period are in agreement to studies whereby greater HR was observed in steers classified as high RFI compared to low RFI (Hafla et al., 2013; Chaves et al., 2015). In these studies, the differences between high and low RFI animals were related to increased requirements for maintenance or greater response to stress in high RFI animals (Brosh, 2007; Chaves et al., 2014). Our results for HR are consistent with those reported in the literature, and are possibly related to higher DMI, and greater response to stress in the inefficient phenotype (high RFI).

The results observed for oxygen consumption and O_2P between RFI animals are in contrast to another study with heifers (Paddock, 2010), where high RFI animals presented greater oxygen consumption and O_2P compared to their counterparts (low RFI). The large variation in oxygen consumption may have precluded the rather large numerical difference to be proven significant. Studies with Nellore steers (Chaves et al., 2015) and beef cows (Brosh et al., 2002) did find differences in oxygen consumption between high and low RFI animals. Differences among studies may also be due to age, sex and compensatory growth of the animals.

Variation in heat production and thus energy available for maintenance and growth occurs as a result of differences in energy expenditure (Herd and Arthur, 2009). Also, variation in heat production accounts for 95% of the differences in energy retained in the body, while only 5% of the difference is due to feed intake (Herd and Arthur, 2009). When we calculated the EHP, we expected that low RFI animals (efficient) would produce less heat compared to high RFI. This could be an alternative method to identify efficient animals, considering that the calorimetric chamber is a laborious and expensive method (Chaves et al., 2014).

Although low RFI bulls showed EHP numerically lower than high RFI (regardless as it was expressed), this difference did not reach statistical significance. These results may are due to the small number of animals used in EHP methodology

(n = 12) in the current study. Taken together, these results suggest that a greater number of animals should be evaluated by O_2P methodology in future studies, especially because variables such as HR and oxygen consumption are high influenced by individual behavior, environment, and animal husbandry.

To study energy expenditure, high and low RFI beef calves were used to determine differences responses such as heat production measured using two different methods (head box and Greenfeed™) (Sainz et al., 2016). In this study, heat production (Mcal/day) was also estimated as O_2 uptake (L/day) using the constant 4.825 kcal/L of O_2. Results from this study showed that based only on oxygen uptake and only from the Greenfeed™ system, no difference between RFI groups were observed. Although Sainz et al. (2016) used a different methodology, these results are in similar to the present study, whereby efficient animals (low RFI) showed similar EHP compared to inefficient bulls (high RFI).

4.4.3. Blood parameters

In the current study, low RFI Nellore bulls showed lower hemoglobin and hematocrit and this is consistent with our observations in previous study (Chaves et al., 2015). Based on these results, we suggest that hemoglobin and hematocrit concentration are useful blood traits to identify efficient animals (low RFI). We are the first research group to find consistent results for hemoglobin and hematocrit concentration in Nellore cattle. This results may be related to the oxygen transport and blood flow, which may explain differences in efficiency. When passing through pulmonary blood capillaries, the hemoglobin inside these red blood cells binds oxygen, forming oxyhemoglobin (Hsia, 1998). Thus, the differences observed here may be associated with oxygen-carrying capacity because the number of red blood cells influences the oxygenation capacity of tissues. Another study with young Nellore males and females reported significant differences ($P < 0.05$) between sexes for platelets, red blood cells and hemoglobin (Bonilha et al., 2015). The authors reported that since males have a greater body volume than females, they may carry a larger number of red blood cells, platelets, and hemoglobin concentration.

In contrast, studying Nellore steers and bulls, no effects of RFI class were observed for the blood parameters hemoglobin and hematocrit (Gomes et al., 2011).

Additionally, variables such as red blood cells, mean corpuscular volume, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration were also similar ($P > 0.05$). However, there was no difference between most and least efficient Nellore cattle for plasma glucose. We found opposite results for glucose concentration between high and low RFI bulls ($P < 0.10$). The divergent results found in the literature regarding differences in blood variables such as glucose, hemoglobin, and hematocrit may be caused by differences in age, sex, management, and hormonal concentrations of the animals.

4.4.4. mtDNA copy number

Once mitochondrial function depends on an orchestrated communication between nuclear DNA and mtDNA, which encodes 13 peptides involved in oxidative phosphorylation (OXPHOS) (Bottje and Carstens, 2009; Divakaruni and Brand, 2011; Bottje and Carstens, 2012), we investigated the amounts of mtDNA in liver, muscle and adipose tissue of Nellore bulls. Quantification of mtDNA copy number was estimated in relation to a single copy nuclear gene, providing a rapid and sensitive analysis (Chiaratti et al., 2010; Machado et al., 2015). The results found in the current study is in accordance with experiments measuring mtDNA copy number in the hepatic and adipose tissue of dairy cows (Laubenthal et al., 2016). In this study, the greatest number of mtDNA copies was observed in liver, in which the calculated mean mtDNA content ranged between 400 to 500 copies per cell, corresponding well to values obtained in the present study.

However, no differences were observed between the mtDNA copy number in liver, muscle or adipose tissue of animals (Figure 3). This result suggests that differences in RFI were not due to alterations in mitochondrial content (and possibly OXPHOS) between high and low RFI groups. However, it is still possible that mitochondrial function differed between groups. Our results suggest that electron transport chain (ETC) would be similar between low and high RFI groups, though additional studies measuring protein amount or enzyme activity would be needed to truly determine if ETC enzymes (and citrate synthase) differs between groups.

A study measured the cell number and volume and showed that mitochondrial content varied as a function of cell size at constant mitochondrial density (Renner et al., 2003). In the current study, we do not measure the cell size

and density of mitochondria in the bovine tissues, and this measurement should be take into account for future approaches. The body composition and organ mass will be also considered, since the number of mitochondria in a particular cell type can also vary depending on many factors, including the stage in the cell cycle, the environment, the stage of differentiation, and a number of cell signaling mechanisms (Malik and Czajka, 2013). However, in contrast to the results observed by Renner et al. (2003), the adipocyte mitochondrial oxidative capacity was reduced in obese compared with nonobese humans and this difference was not due to cell size variation (Yin et al., 2014). In their study, the adipocyte mitochondrial content was not significantly different between obese and nonobese individuals. Additionally, mitochondrial function and content (represented by mtDNA copy number) were also not different (Yin et al., 2014). Thus, the physiological significance of cell size and mitochondrial content and function are not completely clear.

Furthermore, proliferation and differentiation of mitochondria and mtDNA are likewise regulated and maintained by genes involved in the mitochondrial biogenesis, such as peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC1- α) (Al-Kafaji and Golbahar, 2013; Tsai and John, 2016). Moreover, the mitochondrial biogenesis and division are controlled by cellular factors such as the adaptors Shc proteins. These proteins have been implicated in the regulation of receptor signaling, mitochondrial metabolism, and energy expenditure. In particular, p66Shc isoform appears to be part of the signaling pathway that regulates mtDNA replication (Trinei et al., 2006).

As an indicator of mitochondrial abundance, mtDNA copy number can vary according to the energy demands of the cell (Al-Kafaji and Golbahar, 2013). In addition, the changes in mtDNA content could be used as a biomarker to detect mitochondrial dysfunction as altered mtDNA levels may contribute to enhanced oxidative stress and inflammation and could play a pathogenic role in mitochondrial dysfunction as previously reviewed (Malik and Czajka, 2013). Here, we performed the investigation of mtDNA copy number not only because mtDNA is associated with mitochondrial content and OXPHOS, but also due to mtDNA modulates the thermal regulation in endothermic animals as previously reported (Tsai and John, 2016).

It is expected that more efficient animals (low RFI) produce less heat and also shows difference in mtDNA copy number compared to inefficient (high RFI) in

main metabolic tissues. These differences were not detectable and more studies are needed to investigate the physiological significance of mtDNA copy number in the liver, muscle, and adipose tissues, which may help to understand the complex nature of the interaction of nuclear and mtDNA-encoded proteins that comprise the fully functional organelle. However, the small magnitude of the differences in efficiency, around 3 to 5% among RFI groups observed in previous work (Chaves et al., 2015; Nascimento et al., 2016), makes difficult to find changes of similar magnitudes in gene expression, mitochondria content, and/or heat production.

4.4.5. Liver protein profile

Since the liver is a central organ of metabolism which is responsible for an important function such as protein synthesis and its proteomic approaches is poorly documented (Molette et al., 2012) we studied the liver protein profile of high and low RFI bulls. We found seventy one protein spots differentially expressed between RFI groups, suggesting that a greater number of proteins are related to the phenotypic expression of RFI. However, the present study does not provide support for associate a specific protein or peptides to the differences observed in FE or blood variables. Additional studies using mass spectrometry would need to truly identification of liver proteins associated with phenotypic expression of RFI. This approach will be considered for future studies.

Others studies have indicated that a large number of proteins are differentially expressed in liver and muscle of FE phenotype (Grubbs et al., 2013; Ramos and Kerley, 2013). Additionally, the increase of ROS may also alter gene expression and, therefore, transcription factors and other genes encoding mitochondrial proteins may be critical determinants of cellular function associated with the phenotypic expression of FE (Bottje and Carstens, 2009).

4.4.6. UCPs levels

The UCPs play a key role in physiological processes, therefore, the protein levels in liver, muscle, and adipose tissue may account for variation in energy expenditure (Bouillaud et al., 2016). However, we measured UCPs by Western

blotting technique and found no differences in protein levels in main metabolic tissues (high versus low RFI groups). This could be attributed to posttranslational modifications or an increased protein turnover rate, making it difficult to detect a change. Differences of similar magnitude to variation in heat production would be difficult to be detected by this technique. Moreover, this could be due to limitation of the Western blotting technique such as antibody specificity and protein extraction methods used in the current study.

In mitochondria, since UCP activity (uncoupling) might become a proton “short-circuit” and a dangerous competitor to ATP production that jeopardizes cell viability (Bouillaud et al., 2016), we expected that low RFI animals (efficient) would presented less UCPs levels compared to high RFI. We also investigated UCPs levels because mitochondria are responsible for producing approximately 90% of the energy for the cell, how efficiently this process is conducted has implications in animal growth and development (Bottje and Carstens, 2009; Bottje and Carstens, 2012; Bouillaud et al., 2016). Thus, several studies focus on the association between mitochondrial function (and UCPs) and FE of beef cattle (Kolath et al., 2006a; Kolath et al., 2006b), poultry and livestock species (Bottje and Carstens, 2009; Grubbs et al., 2013).

In this context, a study evaluated the differential expression of genes involved in mitochondrial function such as PGC1α, TFAM, and UCPs, and showed differences in the mRNA expression levels of the UCP2 gene in hepatic tissue of Nellore cattle divergently ranked by RFI (Fonseca et al., 2015). Thus, the investigation of genes involved in mitochondrial function and energy metabolism such as UCP1, UCP2 and UCP3 should be considered in future studies. However, typical results relating to UCP mRNA levels must be taken with extremely caution, since it was demonstrated that UCP mRNA does not produce heat (or it is related to energy expenditure), and UCP protein levels would be the more relevant parameter to measure. The increase in total UCP1 amount, for example, correlates temporally with the increase in nonshivering thermogenesis, whereas changes in UCP1 mRNA or specific UCP1 protein levels do not correlate (Nedergaard and Cannon, 2013).

4.5. Conclusion

The performance and feed efficiency traits observed in the current study emphasizing the phenotypic differences and the ability of feed use between efficient and inefficient animals (low and high RFI, respectively). We also showed that correlations between traits in growing and finishing periods are an evidence of re-ranking of Nellore bulls for RFI, which may occur due to effects of compensatory gain, body composition, and animal differences in efficiency.

Efficient animals (low RFI) showed heat production 20% lower than the inefficient (high RFI), but this difference did not reach statistical significance due to the small number of animals evaluated. These results may also due to oxygen consumption data collection, which is more influenced by individual behavior and environment. The higher daily heart rate observed in inefficient animals (high RFI) is possibly related to greater DMI. Lower hemoglobin and hematocrit concentration were observed in the efficient phenotype (low RFI). This is consistent with previous study by our group with Nellore cattle and may be useful to identify efficient animals for beef cattle breeding programs.

Differences in RFI were not associated with alterations in mitochondrial content (mtDNA copy number per cell) between high and low RFI bulls. However, it is still possible that mitochondrial function differed between efficient and inefficient animals. Although mitochondrial content (mtDNA) was similar between low and high RFI, additional studies measuring protein amount, enzyme activity per weight of tissue, cell size and density are needed to truly determine if electron transport chain enzymes and mitochondrial function differs between efficient and inefficient animals. Finally, the investigation of the liver proteome suggests that several proteins are associated to RFI, while the abundance of all three UCPs proteins unchanged in these animals.

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5. CONCLUSION

The study performed in Chapter 3 shows that Shc proteins should be considered as potential targets for developing interventions (e.g. genetic selection or molecular markers) to control both weight gain and body composition in animals consuming high-density energy diets by stimulating energy expenditure. Although energy metabolism and heat production was changed, no clear and concerted changes in enzymes and mitochondrial functions were observed. Activity of Acyl CoA dehydrogenase, a beta-oxidation enzyme, was increased in both liver and muscle of mice fed HFD only in Shc knockout mice.

In chapter 4 we demonstrated no changes in mtDNA copy number per cell. However, changes in mitochondrial content per gram of tissue would still prove to explain changes in the efficiency of growth (high versus low RFI). We were also unable to demonstrate differences in UCP abundance on a per cell basis in groups of animals inferior or superior for feed efficiency.

We evaluated parameters of energy metabolism and mitochondrial function as related to feed efficiency and growth. The results suggest no apparent differences in mitochondria abundance, or in electron transport chain and oxidative phosphorylation in liver, muscle, and adipose tissue from these groups of animals differing in efficiency. However, additional studies measuring specific genes, protein amount or enzyme activity are needed.

Changes of very small magnitude in either mitochondrial function or enzyme activities could greatly alter energy metabolism and cause the changes in feed efficiency observed *in vivo*. Most of our biochemical studies are unable to detect the magnitude of the changes in residual feed intake observed in beef cattle. These small energy expenditure changes are of enormous economic significance.