LITHUANIAN SPORTS UNIVERSITY

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EFFECTS OF LOW CITRATE SYNTHASE ACTIVITY, MYOSTATIN DYSFUNCTION AND CALORIC RESTRICTION ON ENERGY METABOLISM AND BODY COMPOSITION IN MICE

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LIETUVOS SPORTO UNIVERSITETAS

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SUMAŽINTO CITRATO SINTAZĖS AKTYVUMO, MIOSTATINO DISFUNKCIJOS IR KALORIJŲ APRIBOJIMO ĮTAKA PELIŲ MEDŽIAGŲ APYKAITAI IR KŪNO KOMPOZICIJAI

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CONTENTS

ABBREVIATIONS

ACC	–	acetyl-CoA carboxylase
ACL	–	ATP citrate lyase
AKT	–	protein kinase B
ANOVA	–	analysis of variance
ANT	–	adenine nucleotide translocase
B6	–	C75BL/6J inbred strain of laboratory mice
B6.A	–	B6.A-(rs3676616-D10Utsw1)/Kjn (B6.A) mouse strain
B6.A10	–	consomic C57BL/6J-Chr 10A/J/NaJ strain of mice
BEH+/+	–	Berlin High mice strain with functional myostatin allele
BEH	–	Berlin High mice strain with myostatin dysfunction
CACT	–	carnitine/acylcarnitine translocase
CoA	–	coenzyme A
CPT	–	carnitine palmitoyltransferase
CR	–	caloric restriction
CrAT	–	carnitine acetyltransferase
CS	–	citrate synthase
DOX	–	deoxycholic acid
DTNB	–	5,5′-dithiobis (2-nitrobenzoic acid)
EDL	–	*extensor digitorum longus* muscle
EDTA	–	ethylenediaminetetraacetic acid
EE	–	energy expenditure
ERRα	–	estrogen-related receptor α
ETS	–	electron transport system
FA	–	fatty acid
FFA	–	free fatty acid
FAO	–	fatty acid oxidation
FD	–	food deprivation or food-deprived
GAS	–	*gastrocnemius* muscle
GDF-8	–	growth differentiation factor-8
GM	–	glutamate and malate
GR	–	glutathione reductase
H medium	–	homogenization medium
HFD	–	high fat diet

IT6 – incubation solution 6
LCFA – long-chain fatty acid
M – malate
MSTN – myostatin gene
Mstn – mice myostatin gene
NRF – nuclear respiratory factor
PC – palmitoyl-L-carnitine
PGC – peroxisome proliferator-activated receptor gamma coactivator
PM – pyruvate and malate
PPAR – peroxisome proliferator-activated receptors
RMR – resting metabolic rate
RO – rotenone
ROS – reactive oxygen species
RQ – respiratory quotient
S medium – suspension medium
SD – standard deviation
SOD – superoxide dismutase
SOL – *soleus* muscle
State 2 – mitochondrial metabolic state 2
State 3 – mitochondrial metabolic state 3
SU – succinate
TA – *tibialis anterior* muscle
TAG – triacylglycerol
TCA – tricarboxylic acid cycle
VCO_2 – carbon dioxide output
VO_2 – oxygen consumption

INTRODUCTION

Mitochondrial dysfunction may contribute to conditions and diseases such as weight gain, obesity, insulin resistance and type 2 diabetes (Christe et al., 2013; C. Yang et al., 2012). These metabolic abnormalities will remain the major challenge for public health in the foreseeable future (LeRoith, 2002). Both obesity and type 2 diabetes are associated with poor performance of mitochondria (Houmard, 2008). The imbalance between β-oxidation of free fatty acids (FFA) and substrate flux through the Krebs cycle seems to be of special importance for functioning of mitochondria and, therefore, the development of diabetes as well as excessive deposition of triglycerides and other lipids in various tissues (Bonora, Brangani, & Pichiri, 2008; I. Ioannidis, 2008; Koves et al., 2008). For instance, a high fat diet promotes insulin resistance and impairs glucose metabolism in mice (Terauchi et al., 2007). The balance between oxidation of FFA and oxidation of carbohydrates in the citric acid cycle is one of the key mechanisms to regulate performance of mitochondria (Fig. 1).

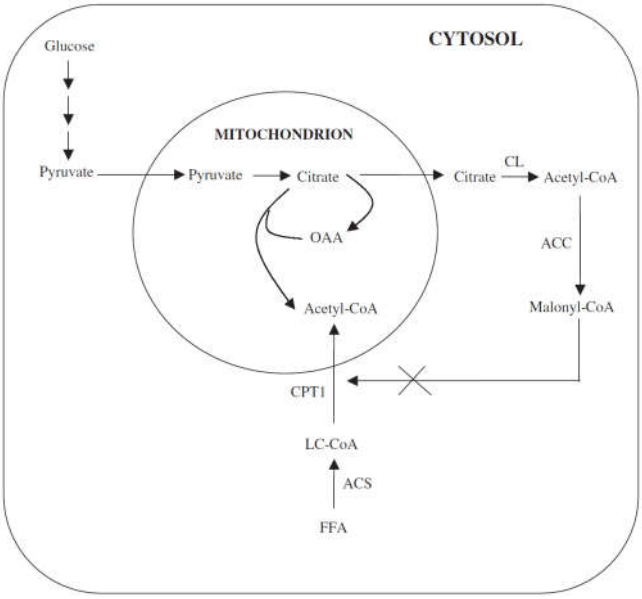

Fig. 1. Model of the balance between cytosolic free fatty acids (FFA) and carbohydrates in citric acid cycle. After pyruvate conversion to citrate in the mitochondria, a fraction of the citrate enters citric acid cycle, while the excess of citrate is converted by citrate lyase (CL) into acetyl-CoA, which in subsequent steps is used to form malonyl-CoA by acetyl-CoA carboxylase (ACC). Malonyl-CoA inhibits carnitine palmitoyltransferase-1 (CPT1) and blocks long-chain acyl-CoA (LC-CoA) transport into mitochondria. As a consequence, the levels of FFA elevate within cytosol (Figure was taken from: Zraika et al., 2002)

There is evidence that citrate or citric acid, an intermediate of the mitochondrial Krebs cycle, plays a role in controlling this balance (Ruderman, Saha, Vavvas, & Witters, 1999). Cytosolic citrate is converted by ATP citrate lyase (ACL) to acetyl-CoA, the substrate for acetyl CoA carboxylase (ACC) in the synthesis of malonyl-CoA. Malonyl-CoA can inhibit carnitine palmitoyltransferase 1 (CPT1) and thus interfere with FA oxidation (Fig. 1). Low rates of FFA oxidation contribute to the described metabolic disorders.

Elevated FFA levels affect mitochondrial function by dysregulation of biochemical pathways and through the alteration of key enzymes (Zraika, Dunlop, Proietto, & Andrikopoulos, 2002). One of the mechanisms propose, that elevated FFA levels cause a decrease in glucose oxidation via glucose-FFA cycle, where inhibition of pyruvate dehydrogenase, as an example, may play a major role in this process, because conversion of pyruvate to acetyl-CoA is limiting with prolonged exposure to FFAs. One of the other key enzymes is mitochondrial citrate synthase (CS), which catalyzes the conversion of acetyl-CoA and oxaloacetate into citrate. CS is a mitochondrial enzyme that has often been used as a mitochondrial marker in both animal and human studies (Hamilton & Booth, 2000). Mammalian CS is encoded by a single nuclear citrate synthase (*Cs*) gene. After translation to the cytosol, CS is transported into the mitochondrial matrix, where it functions as the first and rate-limiting enzyme of the tricarboxylic acid (TCA) cycle and thus plays a decisive role in regulating energy generation and reactive oxygen species (ROS) production through mitochondrial respiration. A missense mutation of *Cs* might therefore alter mitochondrial function (Johnson, Gagnon, Longo-Guess, & Kane, 2012). The liver, skeletal muscles, adipose tissue and pancreas are believed to play the major role in insulin resistance (Bouderba et al., 2012).

From another point of view, the problem of sex polymorphism in mice cannot be ignored, because there may be differences between males and females in mitochondria respiration. The understanding of a gender effect in mitochondria metabolism is a complicated issue because of many proteins involved in mitochondrial functioning. The differences between male and female mice may be also a crucial factor in analyzing effects of point mutations on energy metabolism (such as *Cs*). There are findings of no differences between male and female mice in oxygen consumption of mitochondria from heart, skeletal muscle and liver (Sanz et al., 2007). However, respiration mechanics of lungs differs between male and female mice. The larger lung volume of female mice could reflect adaptation to

higher metabolic and oxygen demands during pregnancies and lactation (Schulz et al., 2002).

In the first study we studied effects of reduced CS activity on oxygen consumption rates in isolated mitochondria from mouse liver and skeletal muscles. We investigated whether CS activity and respiration differ between mitochondria derived from C57BL/6J (B6) and congenic B6.A-(rs3676616-D10Utsw1)/Kjn (B6.A) mouse strains of both sexes. B6.A mice carry the A/J allele in the genomic region containing the *Cs* gene in chromosome 10 on otherwise B6 strain background. The A/J strain (Jackson Laboratory, Bar Harbor, United States) allele variant is predicted to cause an amino acid change of histidine to asparagine at position 55 (H55N) of the mouse CS protein (Johnson et al., 2012), which results in reduced CS activity in B6.A strain (Ratkevicius et al., 2010).

CS activity depends on many metabolic processes and can be increased, for instance, by endurance exercise training (Holloszy & Booth, 1976; Jaenisch, Bertagnolli, Borghi-Silva, Arena, & Lago, 2017). Our studies showed that mice with reduced CS activity had a lower endurance capacity compared to control mice with no sex effect on overall endurance performance (Kvedaras et al., 2017). It can be suggested that low levels of CS activity might be beneficial in promoting FA oxidation under conditions of excessive substrate supply and affect energy metabolism parameters, such as oxygen consumption, energy expenditure and respiratory quotient with possible gender effects. Furthermore, metabolic parameters of male and female mice (e.g., energy expenditure, respiratory quotient) may vary between strains.

In the second study we focused on the investigation of energy metabolism in control B6 and B6.A male and female mice with low CS activity (Ratkevicius et al., 2010). Chromosome substitution strains, called consomic, provide a model for exploring additional aspects of genetic influence on energy metabolism. They consist of a panel of strains where one chromosome of a host strain is replaced by a homologous chromosome of a donor strain by using backcross strategy (Ishii et al., 2011; Matin, Collin, Asada, Varnum, & Nadeau, 1999; Miller et al., 2020; Nadeau, Singer, Matin, & Lander, 2000). The research of mice with substitution of chromosome 10 (where our "focally pointed" CS gene exists) may open door to an additional information about other genes located in chromosome 10, which may influence energy metabolism or/and behave differently in absence of *Cs* gene. For instance, deletion of PTEN (phosphatase and tensin homolog deleted from

chromosome 10) activated protein kinase B (AKT), which affected regulatory network of phosphate metabolism (Kawai, Kinoshita, Ozono, & Michigami, 2020). Phosphate participates in a wide range of biochemical processes, including cell signaling and energy homeostasis (Quarles, 2012; Razzaque, 2012), and it's deficiency may result in long-term metabolic, skeletal complications and muscle weakness (Weber & Quarles, 2019). For these reasons we additionally examined metabolism parameters of C57BL/6J-Chr 10A/J/NaJ (B6.A10) consomic mice with the substitution of chromosome 10. Similarly, as B6.A, B6.A10 mice also carry the mutation in *Cs* gene, resulting in low CS activity levels.

Responses to different type of dietary interventions, such as food deprivation, caloric restriction and high fat diet, differ between mouse strains (West, Boozer, Moody, & Atkinson, 1992). There is a significant evidence suggesting that caloric restriction has a beneficial effect on health as judged by changes in body composition and lipoprotein profile (Anderson & Weindruch, 2012). Thus, food withdrawal could be associated with prevention of cardiovascular disease and diabetes.

In the third study we performed two experiments dealing with changes of energy metabolism and body composition in response to 48-h food deprivation. Our first investigation focused on mouse strains with wild type (B6 strain) and A/J allele with low CS activity (B6.A and B6.A10 strains). In the second experiment we compared effects of 48-h food deprivation in Berlin high mice with normal myostatin function (BEH+/+ strain) and myostatin dysfunction (BEH strain). In addition to the high muscularity, myostatin-deficiency has a positive effect on metabolic health by reducing adipose tissue and increasing insulin sensitivity (Hamrick, Pennington, Webb, & Isales, 2006; McPherron, Lawler, & Lee, 1997; Wilkes, Lloyd, & Gekakis, 2009), suggesting that myostatin dysfunction in comparison to low CS activity, could also prevent type II diabetes and obesity. That was the first reason of carrying out experiment on physiological effects of myostatin dysfunction during 48-h food deprivation. The second reason was that many chronic conditions, including cardiovascular heart diseases, diabetes, rheumatoid arthritis and various forms of muscular dystrophies affect energy metabolism and are associated with muscle wasting (Glass, 2005; Sakuma, Aoi, & Yamaguchi, 2014). The dilemma is that on one hand fasting improves metabolic health, but on the other hand leads to decrease in skeletal muscle mass (J. E. Donnelly, Jakicic, & Gunderson, 1991; Saris, 2001). This research aimed to

test if myostatin dysfunction improves energy metabolism and prevents loss of muscle mass and function during fasting.

In summary, the third study should provide an answer to the question whether or not low mitochondrial CS and myostatin dysfunction affect energy metabolism in mice. Another question will be addressed as to whether CS inhibition and myostatin dysfunction are viable therapeutic strategies in prevention of obesity and insulin resistance.

Not only food deprivation, genetic mutations or increase in physical activity could prevent weight gain and obesity, but adjustments in diet are often easier to implement on the population level (Westerterp, 2019).

A well-known fact is emphasized, that animals and humans gain weight when their energy intake exceeds energy expenditure (J. Galgani & Ravussin, 2008). **In the fourth study** we studied obese mice subjected to two different types of hypocaloric diets with equal protein content and partial caloric restriction. We compared changes in body composition and metabolic health of the C57BL/6J mouse strain in response to two energy-restricted diets with large differences in carbohydrate and fat content. The reason for performing this study is that it is still controversial whether proportions of carbohydrate and fat in the diet are important for metabolic health (Ge et al., 2020; Sacks et al., 2009). For instance, the so-called carbohydrate-insulin model proposes that dietary carbohydrates are inherently more obesogenic than fat due to strong effect on insulin secretion (Ludwig & Ebbeling, 2018), which is repeatedly criticized by other scientists (K. D. Hall, Guyenet, & Leibel, 2018). Recent randomized trial study with humans demonstrated that energy expenditure was significantly greater on a low-carbohydrate diet compared to high-carbohydrate diet for a similar energy intake (Ebbeling et al., 2018). Diets promoting energy expenditure while keeping energy input unchanged would be a promising strategy for weight regulation, and inbred mouse model is well suited to examine the controversial issue about the importance of dietary composition for weight loss and metabolic health, because it is much easier to thoroughly control food intake and the dietary interventions in mice, rather than in humans. The C57BL/6J mouse strain is prone to diet-induced obesity (Kleinert et al., 2018; Speakman, 2019b) and tolerates well diets with large differences in carbohydrate and fat content (Roberts et al., 2017, 2018). The strength of our fourth study was that we used distinct diets with precise macronutrient composition which is very difficult to achieve in human studies.

AIM, HYPOTHESES AND OBJECTIVES

The main aim of our studies was to examine effects of low citrate synthase activity, myostatin dysfunction and caloric restriction on energy metabolism and body composition of laboratory mice.

The hypotheses of our studies:

1. Low citrate synthase (CS) activity would alter substrate oxidation in mitochondria and promote lipid oxidation in C57BL/6J mouse strain (first study).

2. Low CS activity would increase fat oxidation at expense of carbohydrates by altering energy metabolism and respiratory quotient in freely moving mice (second study).

3. Low CS activity would enhance fat oxidation metabolism during food deprivation (third study).

4. Myostatin dysfunction due to higher glycolytic fiber content would be associated with greater muscle mass decrease and subsequent reliance on energy production from carbohydrate oxidation compared to wild-type controls during food deprivation (third study).

5. Changes in body composition and energy metabolism would not differ between carbohydrate and fat hypocaloric diets if they are matched for the total and protein-derived caloric content (fourth study).

The objectives of our studies:

1. Compare oxidation of carbohydrate and fatty substrates in mitochondria from male and female mice with low and normal CS activity.

2. Determine the effects of low CS activity on metabolic health and physical activity in B6, B6.A and B6.A10 mouse strains of both genders.

3. Analyze the impact of low CS activity on changes in energy metabolism and muscle mass after 48-h food deprivation.

4. Analyze the effect of myostatin dysfunction on changes in energy metabolism and muscle mass after 48-h food deprivation.

5. Examine if carbohydrate and fat hypocaloric diets with equal protein content induce different improvements in body composition, energy balance and glucose tolerance of obese mice during caloric restriction.

1. LITERATURE REVIEW

1.1. Role of citrate synthase in cellular metabolism

TCA cycle regulates energy production in mitochondrial respiration and plays a central role in substrate metabolism. The TCA cycle is efficient as it yields significantly greater amount of ATP compared to anaerobic glycolysis (Melendez-Hevia, Waddell, & Cascante, 1996). There are still some components of TCA cycle and chemical pathways which are not fully understood. One of such gray areas is the influence of mitochondrial enzymes, for example CS activity, on metabolism of FA and carbohydrates.

CS occurs exclusively in mitochondria (Reisch & Elpeleg, 2007). CS is a key enzyme in the Krebs cycle and a marker of mitochondrial content and function (Boushel et al., 2007; Larsen et al., 2012; Ruderman et al., 1999). Although it is the most common marker, but it is not necessarily the best one. There is evidence that coenzyme Q10 may be a better biomarker of mitochondrial content than C, because it is essential for electron transport from complex I and II to complex III of mitochondrial respiratory chain (Yubero et al., 2016). The activities of mitochondrial respiratory chain complexes are crucial in clinical diagnosis and are assayed spectrophotometrically.

CS activity can be increased by endurance exercise training (Holloszy & Booth, 1976; Jaenisch et al., 2017). Our recent studies showed that mice with ~35 % lower CS activity had 36 % lower endurance capacity compared to control mice with no sex effect on endurance performance (Kvedaras et al., 2017). These findings suggest that variation in CS activity may influence metabolic performance and fitness. On the other hand, there is evidence, that too high CS activity may have a negative effect on the energy metabolism. For example, after a meal, carbohydrate oxidation inhibits FA oxidation in mitochondria (Ranneries et al., 1998). It appears that high CS activity leads to high cytosolic levels of citrate which may indirectly inhibit the metabolism of fats, which leads to insulin resistance and obesity (Bays, Mandarino, & DeFronzo, 2004; Rogge, 2009). Therefore, the Cs gene (encoding CS protein) may be a pharmacological target to treat obesity, however there is still a lack of data as to how low CS activity affects energy expenditure and respiratory quotient in mice.

1.1.1. Citrate synthase structure and function

The mitochondrial CS is encoded by nuclear DNA and translated in the cytoplasm as a precursor (Alam, Finkelstein, & Srere, 1982; Harmey & Neupert, 1979). In humans the *Cs* gene is localized in chromosome 12 (12q13.3), while in mice it is localized in chromosome 10 (10 D3) (NCBI database: [citrate synthase]). After synthesis, CS is transported into the inner membrane of mitochondria (Addink, Boer, Wakabayashi, & Green, 1972). There is evidence that increased *Cs* gene expression leads to an increase in mitochondrial mass (E, Burns, & Swerdlow, 2014).

The CS structure consists of two monomeric subunits, each of which consists of 20 α-helical segments that make up 75 % of the 437 amino acid residues forming the α-helical secondary CS structure (J. Y. Wu & Yang, 1970). In the closed form of CS structure, binding sites for both citrate and oxaloacetate (OAA) and for coenzyme A (CoA) can be identified. Functional groups of the citrate molecule are involved in specific interactions with CS enzyme. Thus, the active site of CS is mainly polar, but nonpolar interactions probably help to determine high substrate specificity of CS and may serve as triggers for the conformational changes. A number of important substrates used in metabolic processes have structural features in common with the substrates of CS. Therefore, CS activity may be affected by such substrates as ATP, tricarboxylic acids, acyl-CoA (Beeckmans, 1984; Weitzman, 1981; Weitzman & Danson, 1976).

CS catalyzes condensation of acetyl-CoA with oxaloacetate to form citrate (Wiegand & Remington, 1986). The chemistry of this reaction was firstly elucidated by Eggerer et al. (Eggerer et al., 1970). CS catalyzes the first reaction of the TCA cycle and is generally assumed to be the rate-limiting enzyme of the cycle (Mukherjee, Srere, & Frenkel, 1976). CS performs two sequential reactions: a reversible condensation reaction converts acetyl-CoA and OAA into citryl-CoA (Cit-CoA), and an irreversible thioester hydrolysis then forms citrate and CoA. It is worth mentioning that CS is highly specific to its substrates. These pivotal metabolic reactions are performed by members of at least three enzyme superfamilies (Kobylarz, Grigg, Sheldon, Heinrichs, & Murphy, 2014). Since many conserved active-site residues participate in both the condensation and hydrolysis reactions, the central CS-Cit-CoA complex is expected to toggle among multiple configurations (Bayer, Bauer, & Eggerer, 1981).

CS also plays an important role in cell proliferation. The downregulation of CS stimulates metabolic dysfunction, which contributes to meiosis and cell division defects (Murray & Hynes, 2010; Rahman, Rosu, Joseph-Strauss, & Cohen-Fix, 2014; Song, Li, & Liu, 2013). Additionally, upregulation of CS (with hematopoietic cells in rat model) means that CS regulates metabolism in early phases of cell proliferation (Keast & Newsholme, 1991).

1.1.2. Citrate synthase and fatty acid metabolism

Balance between carbohydrate metabolism and FA oxidation is crucial and can lead to such metabolic disorders, as obesity. It has been long established that abnormalities in carbohydrate tolerance are associated with elevated plasma FA (Schalch & Kipnis, 1965). The concentration of FA is increased in obese humans with an exhibition of reduced insulin-stimulated glucose metabolism, as well as in high fat diet-induced insulin-resistant mice (J. M. Adams, 2nd et al., 2004; Bonnard et al., 2008; Dentin et al., 2006). Multiple studies have found that hepatic lipogenesis is associated with triglyceride synthesis and that this metabolic pathway is increased in individuals with obesity and insulin resistance (Diraison, Moulin, & Beylot, 2003; K. L. Donnelly et al., 2005; Schwarz, Linfoot, Dare, & Aghajanian, 2003). It is known, that accumulation of FA induces insulin resistance, inflammation, and cell apoptosis in skeletal muscles (Bonnard et al., 2008; Coll et al., 2008; Hulver et al., 2003). The toxic effects of FA are known to depend on their chain length and degree of saturation. Long-chain saturated fatty acids (LCFA), such as palmitate, are the most toxic. Consistently, palmitate induces not only insulin resistance, but also an apoptosis in many cell types (Paumen, Ishida, Muramatsu, Yamamoto, & Honjo, 1997; Sparagna, Hickson-Bick, Buja, & McMillin, 2001). However, the molecular mechanisms leading to excess FA accumulation and consequently insulin resistance have not been clearly resolved (Dentin et al., 2006).

One mechanism demonstrates the interaction between CS and energy metabolism of fats as a pathway of carnitine which also functions as an acyl group acceptor facilitating mitochondrial export of excess carbons in the form of acylcarnitine. Acylcarnitine is produced by the mitochondrial matrix enzyme, carnitine acetyltransferase (CrAT). Genetically engineered inhibition of FA oxidation lowered intramuscular acylcarnitine levels and preserved glucose tolerance in mice fed a high fat diet (An et al., 2004; Koves et al., 2008). CPT1,

which is the key regulatory enzyme of LCFA β-oxidation, executes the initial step in this process by catalyzing the reversible transesterification of long chain acyl-CoA with carnitine (McGarry & Brown, 1997). The long chain acylcarnitine product of CPT1 traverses the inner membrane via carnitine/acylcarnitine translocase (CACT) and is then delivered to CPT2, which regenerates acyl-CoA on the matrix side of the membrane where β-oxidation occurs.

Another mechanism is associated with excess of citrate under the influence of CS. In rats, a large amount of FA is synthesized in the liver (Brunengraber, Boutry, & Lowenstein, 1973). LCFA are major sources of energy and important components of the lipids that comprise the cellular membrane. They are either derived from food or are synthesized from acetyl-CoA in liver (Wakil, 1989).

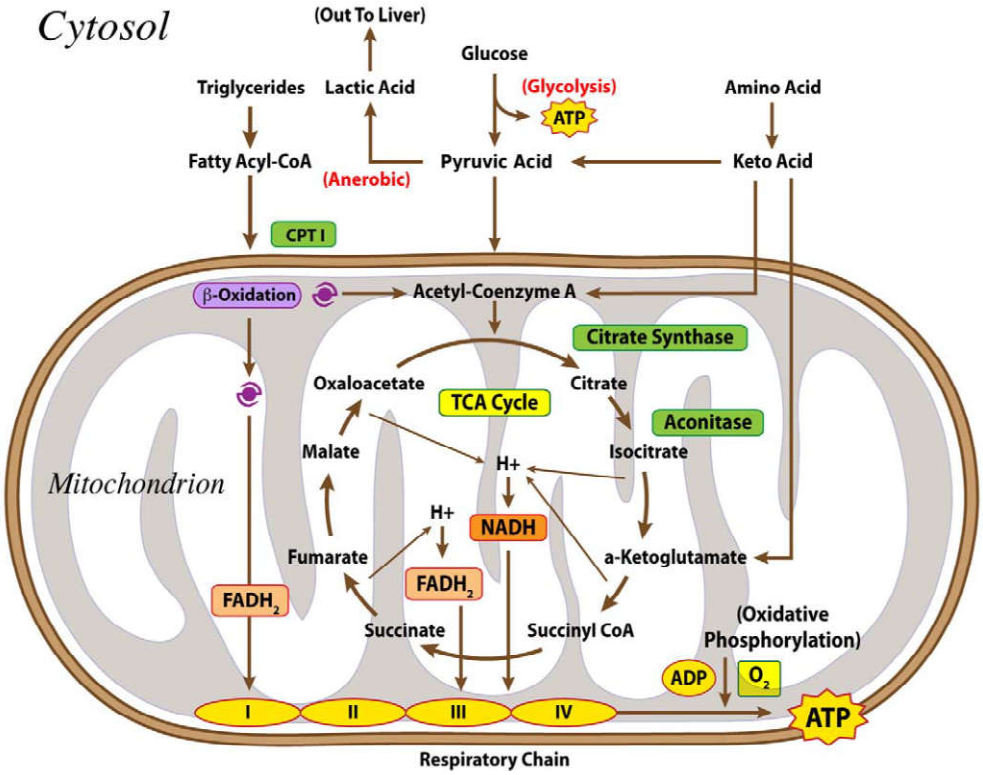

Fig. 2. Tricarboxylic acid (TCA) cycle and β-oxidation of fatty acids (FA). Aconitase and carnitine palmitoyltransferase 1 (CPT1) regulate the conversion of substrates to ATP. β-oxidation of FA involves a series of steps until all the carbons of the fatty acyl-CoA are converted to acetyl-CoA (Figure was taken from: Rogge, 2009)

FA synthesis requires malonyl-CoA, which is the CO_2 donor in the de novo synthesis of FA, and it plays an important role as an inhibitor of the carnitine/ palmitoyl shuttle system for FA oxidation. That means, malonyl-CoA may function as both precursor for FA synthesis and suppressor of FA oxidation (McGarry, Mannaerts, & Foster, 1977). In the first step of this cycle, CS catalyzes the condensation of oxaloacetate with acetyl CoA to form citrate (Fig. 2).

Citrate then acts as the substrate for aconitase and is converted into aconitic acid. The cycle ends with regeneration of oxaloacetate. Normally at rest more ATP is produced by β-oxidation of FA in the mitochondria than from carbohydrates. After FA are changed to fatty acyl-CoA, the fatty acyl-CoA is transported into the mitochondria by the enzyme carnitine palmitoyltransferase 1 (CPT1) (Fig. 2). The fatty acyl-CoA is processed through a series of enzymatic reactions similar to the TCA cycle (Smith et al., 2000). High CS activity results in excess of citrate inside mitochondria.

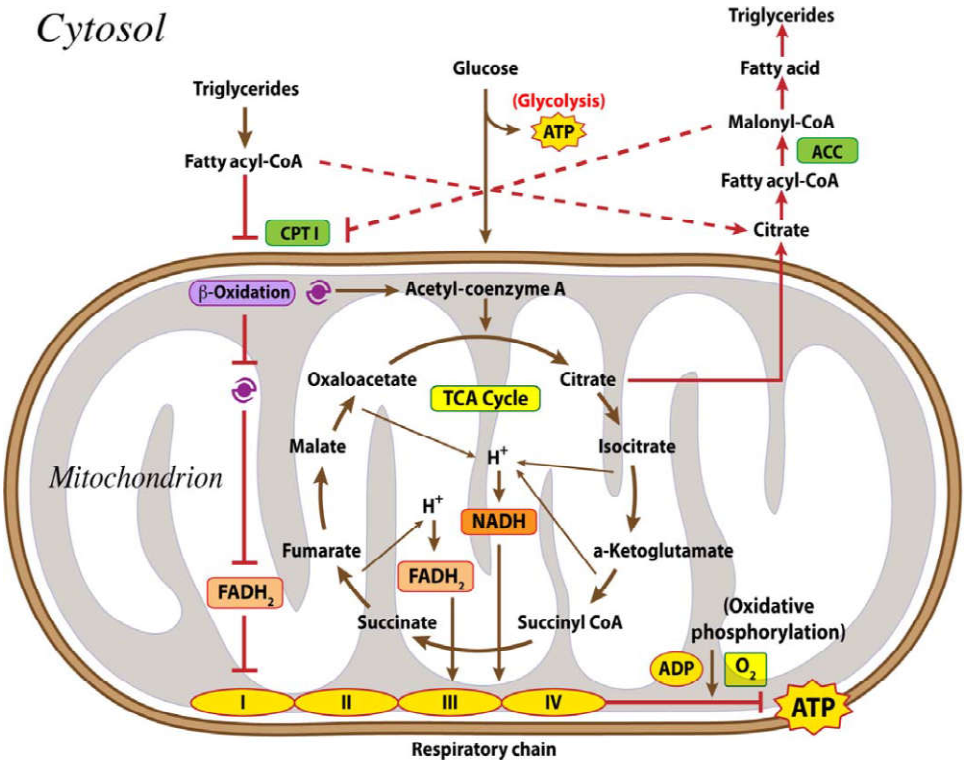

Fig. 3. Impaired mitochondrial metabolism. Tricarboxylic acid (TCA) cycle is highly dependent on malonyl-CoA. If citrate increases in the cytosol, more malonyl-CoA is formed. Malonyl-CoA diminishes carnitine palmitoyltransferase 1 (CPT1), and fatty acids are accumulated within the cytosol (Figure was taken from: Rogge, 2009)

Subsequently citrate is transported into the cytosol and transformed into fatty Acyl-CoA. Fatty acyl-CoA accumulates in the cytosol (Fig. 3), forming an excess of malonyl-CoA, which blocks CPT1 and suppresses beta-oxidation of fatty acyl-CoA.

Under the influence of the acetyl-CoA carboxylase (ACC), fatty acyl-CoA is converted to malonyl-CoA and committed to the re-synthesis of FA, which can accumulate within the cell or be transported to other tissues as triacylglycerol (TAG). Accumulation of TAG contributes to obesity, mitochondrial dysfunction, insulin resistance and as a consequence to type 2 diabetes. This "chain reaction" which accompanies the metabolic disorders is a result of several events. Mitochondrial dysfunction is probably not an initial event in the development of insulin resistance. In the case of skeletal muscle mitochondria of patients with type 2 diabetes, mitochondrial dysfunction is probably associated with increased oxidative stress due to lipid accumulation (Kelley, He, Menshikova, & Ritov, 2002; McGarry, 2002; Razak & Anand, 2004). Increased ROS levels also play an important role in altered insulin secretion thus compounding effects of insulin resistance (Evans, Goldfine, Maddux, & Grodsky, 2002). Control of ROS levels is highly complicated and difficult to interfere with directly. It appears that suppression of CS activity could be one of the indirect approaches. When CS activity is reduced, less citrate will accumulate, and less fatty acyl-CoA would be synthesized in cytosol. As a consequence, FA re-synthesis would be decreased producing an interference in the chain reaction.

CrAT is localized primarily within the mitochondrial matrix, prefers short-chain acyl-CoAs and catalyzes a reversible reaction (Farrell, Fiol, Reddy, & Bieber, 1984; Pieklik & Guynn, 1975). Thus, enzyme activity and substrate flux are thought to be predominantly regulated by substrate or product concentrations within the mitochondrial matrix. Regulatory factors controlling the relative distribution of carnitine are poorly understood. Because CPT1 resides on the outer mitochondrial membrane, this enzyme might gain first access to limited amounts of free carnitine, whereas the intramitochondrial carnitine pool could be more susceptible to depletion. Activities of CPT-1, CS and cytochrome *c* oxidase were significantly depressed in skeletal muscle samples obtained from obese compared with lean subjects (Simoneau, Veerkamp, Turcotte, & Kelley, 1999). These data are significant because it suggests defects at several key regulatory steps in FAO, such as lipid transfer into the mitochondria (by CPT-1), the Krebs cycle (by CS),

and the electron transport system (by cytochrome *c* oxidase).

Initial *in vivo* studies have reported a diminished CS/hexokinase (HK) ratio in diabetic skeletal muscles, where the oxidative/glycolytic ratio was shown to correlate with insulin sensitivity (Simoneau & Kelley, 1997). It was demonstrated that diabetic myotubes have an intrinsic defect with a lower CS activity and an attenuated response to insulin stimulation (Ortenblad et al., 2005) These data suggest that the CS activity is impaired irrespective of the mitochondrial content. An intriguing new observation was that myotubes generated from lean and obese control subjects, but not from type 2 diabetes subjects, express an insulin-mediated increased CS activity. In myotubes established from patients with type 2 diabetes, lipid oxidation and insulin-mediated glucose oxidation are reduced; in addition, palmitate impairs insulin-mediated glucose oxidation in myotubes of obese individuals with no diabetes pathology (Gaster & Beck-Nielsen, 2004; Gaster, Rustan, Aas, & Beck-Nielsen, 2004). Obesity-related insulin resistance shifts the metabolic capacity of skeletal muscle toward fat esterification rather than oxidation. In addition, dietary-induced weight loss does not correct this disposition (Simoneau et al., 1999).

1.1.3. Citrate synthase and caloric restriction

Calorie restriction (CR) and food deprivation (FD) have been known to greatly increase lifespan in multiple strains of mice (Weindruch, Naylor, Goldstein, & Walford, 1988). However, there is a lack of data about other positive metabolic changes, induced by CR and/or FD. The increase in lifespan during these interventions is associated with the reduction of adipose tissue (Barzilai & Gabriely, 2001; Barzilai & Gupta, 1999). The positive effect of CR on lifespan in mouse strains appears to be correlated with maintaining the level of adiposity under CR (Liao et al., 2011). The case favoring a role for reduced fat is based on the excess of adiposity in insulin resistance, type 2 diabetes and metabolic syndrome (Despres & Lemieux, 2006). Furthermore, removal of adiposity improves insulin sensitivity (Barzilai, Banerjee, Hawkins, Chen, & Rossetti, 1998; Das, Gabriely, & Barzilai, 2004).

Following a decrease in body mass in C57BL/6J mice due to CR, the activity of CS increased significantly (Hagopian, Soo Hoo, Lopez-Dominguez, & Ramsey, 2013). This means that relative mitochondrial content can also increase in response to CR or FD. On the other hand, different mouse strains respond differently to CR

(Liao, Rikke, Johnson, Diaz, & Nelson, 2010) or FD intervention: from life extension to life shortening outcomes. There is a lack of data on how mice with low CS activity respond to FD intervention, and how FD could affect carbohydrate and fatty substrates oxidation in mitochondria or other metabolism parameters in mice with low CS activity.

1.2. Mitochondrial respiration and genetic properties

Cellular respiration is a set of metabolic reactions and processes that take place in the cells of organisms to convert biochemical energy from substrates into ATP, and releasing CO_2 and H_2O. Cellular respiration takes place in mitochondria. Cellular organelles bound by a highly folded inner and fairly smooth outer membrane were recognized as hallmarks of eukaryotic cells providing the energy required for metabolism (Henze & Martin, 2003). Typically, a multicellular *Mammalia* has hundreds or thousands of mitochondria in each of their cells (Veltri, Espiritu, & Singh, 1990). These numbers not only vary among species but also among tissue types. The inner membrane is organized in cristae, harboring the respiratory chain and ATP synthase complexes which are responsible for the oxidative phosphorylation. The ultrastructure of the inner membrane provides a variety of morphological features that facilitate metabolism (Boldogh & Pon, 2006; Soubannier & McBride, 2009). This classical view of mitochondria as bean-shaped organelles prevailed until the last decade. Afterwards, new imaging techniques and genetic screens provided a more accurate description of a dynamic mitochondrial reticulum and mitochondrial proteins that fuse and divide continuously (Boldogh & Pon, 2007). Since then, significant findings have been made in the study of pathways responsible for fusion, fission and motility of mitochondrial proteins. However, the mechanisms and signals that regulate mitochondrial dynamics are only beginning to emerge. A growing body of evidence indicates that metabolic and cellular signals influence mitochondrial dynamics, leading to a new understanding of how changes in mitochondrial shape can have a profound impact on the functional output of the organelle. Mitochondria are between 0.75 and 3 μm in diameter but vary considerably in structural features (Wiemerslage & Lee, 2016). Unless specifically stained, mitochondria are not visible with an electron microscope. In addition to supplying cellular energy, mitochondria are involved in other tasks, such as signaling, cellular differentiation, cell death, as well as maintaining control of the cell cycle and cell growth (McBride, Neuspiel, &

Wasiak, 2006). The reactions involved in respiration are catabolic reactions break large molecules into smaller ones and release energy in the process, as weak so-called “high-energy” bonds are replaced by stronger bonds in the products.

1.2.1. Mitochondrial respiration substrates

Substrates that are commonly used by cells in respiration include sugar, amino acids, FA and oxygen (O_2) which is an oxidizing agent. ATP is then used to drive processes requiring energy, including biosynthesis, locomotion or transportation of molecules across cell membranes.

In our first study several substrates were used to measure mitochondrial respiration in mice. These include glutamate, pyruvate, malate and succinate (see 2.2.2). Palmitoyl-L-carnitine (PC) was taken as fatty substrate to study oxidation of fatty substrates in mitochondria isolated from liver and muscles.

Mitochondrial pyruvate metabolism requires transport across the impermeable inner mitochondrial membrane. It appears that mitochondrial pyruvate import is mediated by two proteins, the mitochondrial pyruvate carrier 1 and 2, which form a hetero-oligomeric complex in the inner mitochondrial membrane (Bricker et al., 2012; Herzig et al., 2012). Oxidation of pyruvate by the pyruvate dehydrogenase complex results in increased ATP production, which inhibits ATP-sensitive potassium (K_{ATP}) channels and promotes calcium influx to drive insulin release. In addition, several studies have shown that the production of anaplerotic products by pyruvate carboxylation in the mitochondrial matrix promotes insulin granule exocytosis by Ca^{2+}-independent mechanisms in beta-cells (Farfari, Schulz, Corkey, & Prentki, 2000; Hasan et al., 2008; Jensen et al., 2008). It must be emphasized that pyruvate is a substrate for gluconeogenesis in hepatocytes, and mitochondrial metabolism of pyruvate is required to convert pyruvate substrates in to a new glucose molecule, which may contribute to hyperglycemia when insulin is insufficient to suppress gluconeogenesis (McCommis et al., 2015).

Glutamate (G) and malate (M) are the substrates for respiration complex I, while succinate (SU) is a substrate for respiration complex II, and additional presence of rotenone in the medium with succinate prevent electron transfer from complex I (Baran et al., 2016). Rotenone (RO) is an inhibitor of respiratory chain complex I, therefore mitochondrial respiratory rate registered using SU + RO shows the activity of respiratory chain complex II (also known as succinate dehydrogenase or succinate-coenzyme Q reductase. Succinate-coenzyme Q

reductase is the only enzyme that functions in both electron transport chain and the Krebs cycle.

Palmitoyl carnitine (PC), which was used in current research as fatty substrate, is an ester derivative of carnitine (long-chain acylcarnitine). It is involved in the metabolism of FA. Within the cell, PC is transported into the mitochondria to deliver palmitate for FA oxidation and energy production (Goni, Requero, & Alonso, 1996). Carnitine can inhibit the activity of recombinant caspases, while PC can reverse this inhibition (Mutomba et al., 2000). Long-chain acylcarnitine is a well-known intermediate in mitochondrial FA oxidation which modifies myocardial levels of high-energy phosphates and FFA in the heart. It increases erythroid colony formation in culture and reduces surface negative charge of erythrocytes and myocytes. PC is able to disrupt the inner mitochondrial membrane barrier to solutes, leading to vesicle leakage, and, at higher concentrations, it produces complete membrane solubilization, while palmitoyl-coenzyme A (palmitoyl-CoA) produces no leakage. PC has the properties of many commonly used biochemical detergents (Requero, Goni, & Alonso, 1995).

1.2.2. Mitochondrial metabolic states

Mitochondrial metabolic states are defined in isolated mitochondria or permeabilized cells and tissues. In a medium without energy substrates, cells respire using endogenous substrate. Substrates can be added to the culture medium for detailed studies of respiration (Chance & Williams, 1955a).

Mitochondrial metabolic state 3 (state 3) respiration is defined as ADP-stimulated respiration (Sztark, Ouhabi, Dabadie, & Mazat, 1997). Under such condition's respiration increases only modestly but pyridine nucleotides become much more oxidized. Substrate deprivation is aggravated by high ATP demand. State 3 is a typical experiment showing the effect of oxidative phosphorylation, when endogenous substrate disappears in several minutes (Packer, 1961). State 3 can also be induced if creatine phosphokinase is added to the experimental medium containing ATP. This later approach was used in the current study. ATP is converted to ADP and ADP binds the enzyme complex ATP synthase. ADP binds the ATP synthase. In the presence of inorganic phosphate, which also binds ATP synthase, ADP binding opens a channel that permits diffusion of protons into the matrix from outside of the inner mitochondrial membrane. The energy that is released as protons are driven across is used to produce ATP. As energy in the

gradient is reduced the electron transport chain spontaneously speeds up. The synthesis of ATP by mitochondria is called oxidative phosphorylation. ATP synthase is not a part of the electron transport system (ETS), although it does float around in the same lipid bilayer as components of the ETS. The protons that pass-through ATP synthase simply recombine with hydroxyl ions in the matrix. They are ionization products of water in this system as they are in any aqueous system. Protons do not drive reactions, and they certainly do not reduce oxygen. Oxygen consumption in state 3 is caused by the same process as is oxygen consumption in state 4, when no ADP phosphorylation occurs (Sztark et al., 1997). It is catalyzed by cytochrome oxidase as the last step in electron transport. The passage of an electron pair through each of the proton-translocating complexes (I, III, and IV) is associated with a drop in free energy that somewhat exceeds that needed to phosphorylate one molecule of ADP.

In the early 1970s, three major hypotheses were proposed to explain the coupling of electron transport to ADP phosphorylation. The term coupling refers to how the energy released through electron transport is transferred to ADP and inorganic phosphate. One proposal called for a chemical intermediate to carry energy to an ATP synthetase enzyme, much like NADH carries energy to the ETS from Krebs reactions. A second proposal called for a conformational change in a membrane-bound complex that is directly associated with each complex. The change in conformation would store energy, and when ADP and phosphate bound the complex, the energy would be transferred. The last, the Mitchell hypothesis, was considered by many to be too far-fetched to be true. The proposal was that the energy was stored in the form of an electrochemical gradient, which was then utilized at a remote site to synthesize ATP.

Support for the first two hypotheses came in part from the observation that isolated mitochondria appear to produce three ATPs per electron pair via the “long” route, and two via the “short” (succinate) route. In current studies of this dissertation paper both “long” and short” routes of mitochondrial respiration were tested. It was observed that the extramitochondrial pH dropped when mitochondria were in the active state. Under ideal circumstances, isolated mitochondria could phosphorylate slightly more than three ADPs per oxygen atom. It can be verified NADH-supported respiration results in the phosphorylation of 1.5 times as much ADP as with succinate-supported respiration by examining the ratio of ADP molecules phosphorylated to atoms of oxygen consumed.

In vitro mitochondrial experiments were used to measure O_2 kinetics, due to the difficulty of measuring the mitochondrial matrix *in vivo* which has a high O_2 variability. The mitochondrial fraction of mouse liver and muscles provide a unique opportunity to study mitochondrial enzyme role in oxidative phosphorylation by calculating oxygen consumption rates using different mitochondrial substrates.

1.2.3. Mitochondrial genetic factors and diabetes

Mitochondria contain DNA and generate gene products that are necessary not only for their own function but for that of the entire cell. This, along with the transfer of genes from the organelle to nuclear genomes has changed over evolutionary time (K. L. Adams & Palmer, 2003; Blanchard & Schmidt, 1995). The biogenesis of mitochondria requires the expression of a large number of genes, most of which reside in the nuclear genome. The protein-coding capacity of mtDNA is limited to 13 respiratory subunits necessitating that nuclear regulatory factors play an important role in governing nucleo-mitochondrial interactions. Two classes of nuclear transcriptional regulators implicated in mitochondrial biogenesis have emerged in recent years. The first includes DNA-binding transcription factors, typified by nuclear respiratory factor (NRF) NRF-1, NRF-2 and others, that act on known nuclear genes that specify mitochondrial functions (Prakash & Kumar, 2016). A second, more recently defined class, includes nuclear coactivators typified by PGC-1 (for example PGC-1α) and related family members (PRC and PGC-1β) (Iacovelli et al., 2016). These molecules do not bind DNA but rather work through their interactions with DNA-bound transcription factors to regulate gene expression (Scarpulla, 2002). This system provides a mechanism for linking respiratory chain expression to environmental conditions and to integrate it with other functions related to the cellular energetics (Scarpulla, 2006). While several transcription/replication factors directly regulate mitochondrial genes, the coordination of these factors into a program responsive to the environment is not fully understood (Z. Wu et al., 1999).

Type 2 diabetes is characterized by two features: impaired insulin secretion by pancreatic β-cells and peripheral insulin deficiency (or insulin resistance by the influence of remote factors) (LeRoith, 2002; Polonsky, Sturis, & Bell, 1996; Taylor, Accili, & Imai, 1994). In turn, insulin resistance is a state of reduced insulin sensitivity, an inability of insulin to lower plasma glucose levels through suppression of hepatic glucose production and stimulation of glucose utilization in

skeletal muscle and adipose tissue, and its extent varies considerably among individuals (Fujimoto, 2000).

Direct impaired insulin secretion, associated with β-cell impairment, already has a strong correlation with type 2 diabetes. β-cell mitochondrial dysfunction is thought to play a key role in the pathogenesis of diabetes, as ATP production is necessary for optimal insulin secretion (Supale, Li, Brun, & Maechler, 2012). Type 2 diabetes develops when the beta-cells in pancreas fail to release appropriate amounts of insulin, causing metabolic dysregulation and hyperglycemia (Mulder & Ling, 2009). β-cell imbalance contributes to early phases of diabetes. For instance, mutant variants of the genes encoding the hepatic transcription factors HNF-1α and HNF-4α cause early-onset pathology of β-cells (Yamagata, Furuta et al., 1996; Yamagata, Oda et al., 1996). A large variety of mutations which are associated with type II diabetes have been found. Type II diabetes is probably a heterogeneous disease, with inheritable major and minor genes affecting obesity, insulin secretion, and insulin action (Malm, 1998). Diabetic β-cells exhibit decreased hyperpolarization of mitochondrial membranes and altered internal mitochondrial structure, but it is still not clear whether mitochondrial alterations are directly linked to the failure of β-cells. Furthermore, less is known about genetic factors or proteins, which are involved in type 2 diabetes development in other tissue, for instance, in skeletal muscle or the liver.

Peripheral control of insulin metabolism and its imbalance is much more complicated and requires significant analysis and further study. Some transcriptional coactivators regulate lipid metabolism and are essential for insulin secretion coupled to FA. For example, the peroxisome proliferator-activated receptor γ coactivators-1α and β (PGC-1α and PGC-1β) are important in the development of diabetes due to altered expression in highly oxidative tissues, such as skeletal muscle and the liver (Mootha et al., 2003; Patti et al., 2003). PGC-1α and PGC-1β are transcriptional co-activators that regulate activity of multiple transcription factors including nuclear respiratory factor 1 (NRF1), estrogen-related receptor α (ERRα) and peroxisome proliferator-activated receptors (PPAR) and are known to amplify expression of an extensive gene program controlling mitochondrial function and integrity (Liu & Lin, 2011; Villena, 2015). ERRα could be also one of the receptors, which are associated with gender differences in the mitochondrial level.

Mechanisms of mitochondrial dysfunction vary between tissues. In skeletal

muscles, there is evidence that reduced levels of oxidative enzymes associated to insulin resistance which is a hallmark for type 2 diabetes (Kelley et al., 2002).

1.2.4. Sex effects on mitochondria in mice

There is evidence for differences in mitochondrial physiology between males and females in most animal species across the lifespan. Lifespan of females is prolonged by reduced production of ROS and the associated decrease in damage to mitochondrial DNA (Vina, Sastre, Pallardo, & Borras, 2003). Comparative studies of higher vertebrates' animals with different aging rates have shown that the rate of mitochondrial ROS production is directly related to the steady-state level of oxidative damage to mitochondrial DNA and is inversely correlated with maximum longevity. Caloric, protein and methionine restriction decrease ROS production and oxidative damage (Sanz, Caro, & Barja, 2004; Seo et al., 2006). Interestingly, calorie restriction decreases the rate of aging and proportionately decreases mitochondrial ROS generation, especially at the level of complex I (Barja, 2004). That is one of the reasons why it would be sensible to additionally test mice after food deprivation (e.g., their mitochondrial respiration, and overall metabolism).

The problem of ROS cannot be ignored when talking about mitochondria. Mitochondria is one of the main sources of ROS along with peroxisomes, endoplasmic reticulum and enzyme systems (e.g. lipoxygenase, xanthine oxidase, NADPH oxidase) (Holmstrom & Finkel, 2014). There is an evidence, that ROS production is increased in mitochondria of fatty liver (S. Yang et al., 2000). The negative impact of ROS production is mitochondrial damage which leads to pathological states and aging, which differ between males and females. Mitochondrial dysfunction, which decreases cellular energy metabolism and increases ROS production, is thought to play a major role in the aging process of cochlear hair cells (Someya & Prolla, 2010; Yamasoba et al., 2007). Among C57BL6 mice, females have shorter lifespan than males (Ali et al., 2006), which can be also associated with mitochondrial physiology differences.

There are quite a few explanations for higher hydrogen peroxide synthesis in males. One of the explanations is the Meta theory of aging. It is suggested that free radicals are responsible for cellular impairment during aging (Harman, 1956). Estrogen is a common hormone to control hydrogen peroxide synthesis in females, but it is not the main reason for gender differences in mitochondria function. Interestingly, males and females of B6 mouse strain did not differ in oxygen

consumption rates of mitochondria from heart, skeletal muscle or liver when assessed in both state 3 and 4 and showed similar ATP levels in spite of markedly higher estrogen levels in females compared to females (Sanz et al., 2007). However, mitochondrial substrates and specific properties of some enzymes might be of importance here. This could be of importance in regulation of substrate-enzyme interactions that regulate oxidation-reduction reactions. For example, in B6 mice, mutation of nicotinamide nucleotide transhydrogenase leads to loss of the enzyme function and decreased oxidized glutathione ratio, a marker for oxidative stress (Ronchi et al., 2013). Glutathione reductase (GR), superoxide dismutase (SOD) and CS are other mitochondrial enzymes which are often studied to quantify differences between male and female animals. CS activity was higher in female than male mice (Ronchi et al., 2013). Also, in skeletal muscle mitochondria less hydrogen peroxide was produced in females compared to males in a response to isocaloric intake of high fat diet, but sex differences in hydrogen peroxide production from isolated liver mitochondria were not significant (Catala-Niell, Estrany, Proenza, Gianotti, & Llado, 2008). These data suggest that mitochondria of skeletal muscles show greater hydrogen peroxide differences between male and female animals liver mitochondria, and more attention should be paid to enzymes of mitochondrial Krebs cycle.

There is little data on respiration of mitochondria from skeletal muscles of humans. To our knowledge, the only study that compared respiration of mitochondria from skeletal muscle was done on samples of the gastrocnemius muscle from men and women. It did not reveal any differences in mitochondrial content or Complex I, II, III, and IV-dependent mitochondrial respiration between male and female subjects. CS activity did not differ between males and females in the same study (Thompson et al., 2013). Extraction and assessment of mitochondrial respiration in samples of human tissue have a variety of technical and ethical issues. It appears that mouse tissues are more suitable for detailed studies of mitochondria metabolism as repetition of experiments is often required (Jacobs, Diaz, Meinild, Gassmann, & Lundby, 2013). Previous study showed that oxygen consumption in muscle mitochondria was higher in female rats compared to males, but in liver mitochondria no gender-dependent positive results were observed (Catala-Niell et al., 2008).

Other research has found higher levels of the reduced form of glutathione (which has an increased antioxidant capacity) in liver mitochondria in female than

in male rats (Borras et al., 2003). These data suggest that in female rodents' antioxidant capacity may be higher than in males. Lower antioxidant capacity may contribute to stronger oxidative damage to mitochondria, leading to mitochondrial dysfunction.

Energy balance depends on the mechanisms that regulate basal metabolism, energy expenditure, respiration quotient and physical activity. These metabolism parameters may be gender dependent. For example, female rats have greater whole body O_2 consumption, which is indicative of higher energy expenditure than male rats (Rodriguez-Cuenca et al., 2002). As described above, females have different mitochondrial physiology. These could be responsible, at least in part, for the differences in energy expenditure found between genders (Quevedo, Roca, Pico, & Palou, 1998; Rodriguez-Cuenca et al., 2002).

1.3. Myostatin dysfunction

Myostatin, which belongs to transforming growth factor β-superfamily, is a protein which negatively regulates growth of skeletal muscle (Buehring & Binkley, 2013; Lee, 2004; McPherron et al., 1997). Myostatin, which is also known as growth differentiation factor-8 (GDF-8) (McPherron et al., 1997), is encoded by *MSTN* gene, which in humans is localized in chromosome 2 (2q32.2) and in mice it is localized in chromosome 1 (C1.1) (NCBI database: [MSTN myostatin]). Myostatin is primarily expressed in skeletal muscles, but low expression levels of myostatin were detected in mammary gland, heart muscle and adipose tissue (Ji et al., 1998; McPherron et al., 1997; Sharma et al., 1999).

Mice lacking myostatin have strikingly enlarged skeletal muscle mass due to the fiber hypertrophy and hyperplasia (McPherron et al., 1997; Whittemore et al., 2003). By the same token, myostatin overexpression in the mouse skeletal muscle decreases their mass significantly (Reisz-Porszasz et al., 2003; Zimmers et al., 2002). The myostatin signaling cascade suppresses protein synthesis and promotes protein breakdown, but the mechanism of myostatin activation remains uncertain (Lipina, Kendall, McPherron, Taylor, & Hundal, 2010; Schiaffino, Dyar, Ciciliot, Blaauw, & Sandri, 2013).

One known fact is that myostatin inhibition leads to partial improvements in muscle mass and function of dystrophic mice, a model for Duchenne muscular dystrophy (Bogdanovich et al., 2002). It is highly unlikely that myostatin dysfunction could lead to a cure of muscular dystrophy, since it does not address

the pathophysiological mechanisms related to the lack of dystrophin. Studies of other pharmaceutical targets are also required (Tsuchida, 2008). On the other hand, these findings speak for potential of myostatin inhibition therapies, which are still under intensive development. Some strategies including treatment with myostatin binding follistatin (Rajput et al., 2013; Tsuchida, 2008), myostatin antibodies (Bogdanovich et al., 2002; St Andre et al., 2017) and *Mstn* gene silencing with systemic siRNAs delivery (Khan et al., 2016) have shown promising results.

A great majority of studies of myostatin dysfunction were carried out using C57BL/6J strain. Studies of other mouse strains may be helpful for a better understanding of physiological effects of myostatin dysfunction. Berlin high mouse strain (BEH) presents such a model. BEH strain was derived from heterogeneous population of mice in 1970s by selection for protein content and body weight at the age of 60 days (Bünger et al., 2001). It was later discovered that BEH hypermuscularity is partially due to a mutation in myostatin gene (*Mstn*), in particular a 12bp deletion known as the compact allele (Varga et al., 1997). The BEH+/+ strain differs from BEH by 0.01 % of genome, ~0.4 Mb region on chromosome 1 engulfing the *Mstn* gene (Lionikas, Kilikevicius et al., 2013). This region in BEH+/+ contains wild type Mstn which was introgressed from the BEL strain by marker-assisted selection for many generations (Amthor et al., 2007). Interestingly, on the C57BL/6J background myostatin dysfunction induces both fiber hypertrophy and hyperplasia (Mendias, Marcin, Calerdon, & Faulkner, 2006), whereas on the BEH background it promotes fiber hypertrophy only (Lionikas, Kilikevicius et al., 2013). Thus, effects of myostatin dysfunction appear to be influenced by the genetic background.

1.3.1. Myostatin and insulin resistance

Metabolic studies of mice with myostatin deficiency suggest that the mutation may protect skeletal muscles against insulin resistance and obesity (Wilkes et al., 2009). Indeed, myostatin dysfunction inhibits body fat gain induced by high fat diet (Hamrick et al., 2006). That means, there is a strong association between myostatin and FA breakdown. Circulating adiponectin levels decrease with diet-induced obesity in mice with compact allele of *Mstn* gene, consistent with numerous studies showing a relationship between obesity and circulating adiponectin (Berg, Combs, & Scherer, 2002). Other study showed that transgenic overexpression of the inhibitory myostatin propeptide led to a decrease in

circulating adiponectin in mice fed a standard diet, meaning transgenic myostatin propeptide expression as a possible treatment method of diet-induced insulin resistance (Zhao, Wall, & Yang, 2005). Therefore, myostatin inhibition has a potential for the prevention of obesity and insulin resistance.

1.3.2. Myostatin and food deprivation

Quite a few studies of myostatin dysfunction focused on myostatin expression in high fat diet fed mice (Wilkes et al., 2009). However, there is little data on how myostatin inhibition is associated with insulin resistance and obesity with CR or FD interventions. It is well known that FD induces loss of body and muscle mass. Similarly, loss of muscle mass accompanies many chronic diseases, such as muscle dystrophies (Becker muscular dystrophy, Limb-girdle muscular dystrophy), cachexia-related diseases and sarcopenia, which ambiguously coexist with obesity (Emery, 2002; Hollander et al., 2016; Merlini, Vagheggini, & Cocchi, 2014; Ranjan, Ramachandran, Manikandan, & John, 2015; Rolland et al., 2008; Schiaffino et al., 2013; Wannamethee & Atkins, 2015).

On the other hand, humans subject themselves to muscle wasting states through deliberate FD or CR to target body fat. Despite the negative effect on muscle mass CR and FD, when applied appropriately, may improve overall health and longevity. For instance, mice subjected to CR have lost proportionally more white adipose tissue (WAT) mass than other types of tissue with positive longevity effect (Anderson & Weindruch, 2012). An excess of WAT was found to contribute to obesity and type 2 diabetes via WAT-derived cytokines (Lago, Dieguez, Gomez-Reino, & Gualillo, 2007). Although CR has strong potential in obesity prevention, it may negatively affect the mass of skeletal muscle (Hooper, 1984; Maxwell, Enwemeka, & Fernandes, 1992). Therefore, it is crucial to find means which could preserve muscle mass breakdown during these catabolic states.

It has been suggested that myostatin dysfunction prevents loss of mass of tibialis anterior muscle after 48 h, but not after 24 h of starvation (Allen, Cleary, Lindsay, Loh, & Reed, 2010). On the other hand, higher rates of muscle loss have been reported in C57BL/6J mice lacking myostatin compared to the wild type controls after 24 h of acute starvation and 5-week period of 40 % CR (Collins-Hooper et al., 2015a, 2015b; Matsakas et al., 2013). These findings are somewhat controversial and other models of myostatin dysfunction might be useful in resolving this controversy about effects of myostatin dysfunction on muscle

wasting during CR or acute starvation. The role of myostatin in muscle wasting during FD and/or CR is not fully understood (Allen et al., 2010; Collins-Hooper et al., 2015b). The effects of myostatin inhibition are likely to be strain dependent, because different mouse strains vary in body mass as well as muscle mass and composition (Lionikas, Kilikevicius et al., 2013). For example, Berlin High mice (BEH) even with functional myostatin have significantly greater muscle mass compared to B6 mice (Bunger et al., 2004). It is likely that BEH strain carries several gene variants to favor accretion of body and muscle mass (Varga et al., 2003). Different mouse strains with equal body masses can vary significantly in basal metabolic rate (Konarzewski & Diamond, 1995). Physiological mechanisms, triggered by these gene variants under FD conditions, might interact with myostatin deficient mice in distinct cell signaling pathways compared to B6 strain.

Myostatin dysfunction is associated with enhanced functioning of PI3-kinase/Akt/mTOR signaling pathway which stimulates protein synthesis in skeletal muscles (Lipina et al., 2010; Schiaffino et al., 2013). It appears that the same pathway is involved in control of proteasome and autophagic lysosome pathways responsible for protein breakdown (Glass, 2003). Overexpression of myostatin inhibits the activation of Akt (Trendelenburg et al., 2009).

It is important to identify physiological mechanisms responsible for the increase rate of muscle wasting in mice with myostatin dysfunction compared to wild-type mice. There is a need to maintain blood glucose levels for normal brain and other tissue function during starvation.

During FD, energy substrates are mobilized from adipose and other tissues, including skeletal muscles, to provide a continued source of nutrients for gluconeogenesis in liver (Allen et al., 2010). As myostatin-deficient mice have reduced adipose tissue mass, amino acids from skeletal muscles might be used for an energy to a greater extent than in wild-type individuals during FD (McPherron & Lee, 2002). Indeed, myostatin knock-out mice have reduced rates of lipid oxidation in skeletal muscles, high insulin sensitivity and high glucose uptake compared in wild-type mice in the fed state (Guo et al., 2009).

Skeletal muscle is known to be a tissue of high ATP energy demand when activated as in case of physical activity (Baker, McCormick, & Robergs, 2010). Reduction in levels of physical activity might be an important adaptation to acute starvation. This is probably reflected in a decrease of physical activity and body temperature during the restriction of caloric intake (Swoap, 2008).

As a consequence, muscle proteins are degraded and used as a source of substrate for gluconeogenesis under the energetic deficit. Also, abundance of adipose tissue (fat mass) could partially prevent from muscle wasting.

1.4. Importance of macronutrient distribution in a diet

According to the paradigm of energy balance, animals and humans gain weight when their energy intake exceeds energy expenditure (J. Galgani & Ravussin, 2009). Increase in physical activity could prevent weight gain, but adjustments in diet are often easier to implement on the population level (Westerterp, 2019). A key question is what diet is best suited for weight control. A popular belief is that macronutrient composition of food is important alongside reduction in food intake (Buchholz & Schoeller, 2004). Indeed, effect on satiety and dietary-induced thermogenesis are greater for dietary protein compared to carbohydrates or fat (Jequier, 2002; Leidy et al., 2015). Human overfeeding studies suggest that protein has a smaller detrimental effect on body composition compared to carbohydrates and fat which are usually the major candidates for restriction in various diets aimed for weight control (Leaf & Antonio, 2017). It is still controversial whether proportions of these two macronutrients are important for metabolic health (Ge et al., 2020; Sacks et al., 2009). One of the theories proposes that dietary carbohydrates are inherently more obesogenic than fat due to strong effect on insulin secretion (Ludwig & Ebbeling, 2018). The so-called carbohydrate-insulin model of obesity is often criticized as lacking strong evidence in support of it (K. D. Hall et al., 2018). Nevertheless, a recent randomized-controlled study in humans demonstrated that energy expenditure was by up to 478 kcal per day greater on a low-carbohydrate diet compared to high-carbohydrate diet for a similar energy intake (Ebbeling et al., 2018). Diets promoting energy expenditure while keeping energy input unchanged would be a promising strategy for weight management. However, concerns were raised about suitability of doubly labelled water technique to measure energy expenditure in diets of varying carbohydrate and fat content as in the above-mentioned study of Ebbeling et al. (2018) (Ebbeling et al., 2018; K. D. Hall & Guo, 2019; K. D. Hall et al., 2019). Nutrition epidemiology aimed at comparing different diets have also been plagued by methodological difficulties which mainly concern assessment of food intake (J. P. A. Ioannidis, 2018).

1.4.1. Mouse model suitability for dietary interventions

It appears that inbred mouse model is well suited to examine the controversial issue about the importance of dietary composition for weight loss and metabolic health. Key advantages of such studies are that food intake can be better controlled than in human studies and unpredictable effects of genetic factors are minimized. The C57BL/6J mouse strain is prone to diet-induced obesity (Speakman, 2019a) and tolerates well various diets with large differences in carbohydrate and fat content (National Research Council Subcommittee on Laboratory Animal, 1995; Roberts et al., 2017). A recent study of 29 diets has demonstrated that dietary fat content was associated with greater energy intake and preponderance to obesity of these mice fed *ad libitum* (Hu et al., 2018). According to this study, mice regulate their food consumption primarily to meet an energy rather than a protein target, but this system can be over-ridden by hedonic factors linked to fat, but not sucrose, consumption.

2. MATERIALS AND METHODS

2.1. Animals

All the experimental procedures concerning mice were approved by the Lithuanian State Food and Veterinary Service (no. 0223 in 2012 and no. 10 in 2014). Mice were kept in standard cages (cage dimensions: 267 × 207 × 140 mm) at 20–22 ºC temperature and 55 ± 10 % humidity with 12/12-h light/dark cycle. Mice fed for standard rodent diet (58.0 % kcal from carbohydrate, 28.5 % kcal from protein, 13.5 % kcal from fat; LabDiet 5001, LabDiet, St. Louis, USA) and received tap water ad libitum.

Five mouse strains were used in experiments: C57BL/6J (B6), B6.A-(rs3676616-D10Utsw1)/Kjn (B6.A), C57BL/6J-Chr $10^{A/J}$/NaJ (B6.A10) (Jackson laboratory, Bar Harbor, ME USA), Berlin High with myostatin dysfunction (BEH) and Berlin High with functional myostatin allele (BEH+/+). BEH and BEH+/+ breeding pairs were a kind gift of professor Lutz Bünger (Scotland's Rural College, Edinburgh, UK).

B6 mice represented control group with a native C57BL/6J strain genome. B6.A mice carried missense H55N mutation of *Cs* gene in chromosome 10, where A is substituted for C, rs29358506 on B6 mouse background. These B6.A mice with a point mutation in *Cs* gene are generated by the method of backcross and are called congenic (Johnson et al., 2012). A10 in B6.A10 strain refers to chromosome 10 of the A/J strain which had replaced the B6 strain's chromosome 10. Mice with substituted chromosome are called consomic, when two inbred strains are combined by chromosome replacement (one donor and one recipient strain) (Nadeau et al., 2000). Both B6.A and B6.A10 strains are characterized by a decrease in CS activity.

BEH mice were homozygous for MstnCmpt-dl1Abc (Compact; Cmpt) mutation which is associated with impaired function of myostatin due to mutation in *Mstn* gene (Amthor et al., 2007; Lionikas, Kilikevicius et al., 2013; Lionikas, Smith et al., 2013; Varga et al., 1997), while BEH+/+ strain had a wild-type functioning myostatin. BEH+/+ mice were generated by crossing BEH mice with the Berlin Low (BEL) strain and then repeatedly backcrossing the offspring to BEH by using marker assisted selection for the functional myostatin, as described in earlier studies (Amthor et al., 2007; Lionikas, Kilikevicius et al., 2013).

2.2. Experimental design

2.2.1. First study

The objective of this study was to compare oxidation of carbohydrate and fatty substrates in mitochondria expressing wild type and mutant CS activity. Steps of the study:

1. Mouse sacrifice and subsequent liver and muscle homogenization (see 2.3.1).
2. Isolation of mitochondria (see 2.3.1).
3. Protein concentration in mitochondrial suspensions (see 2.3.1).
4. Measurement of mitochondrial respiration (see 2.3.2).
5. Measurement of mitochondrial CS enzyme activity (see 2.3.3).
6. Analysis of the results.

2.2.2. Second study

The objective of this study was to determine the effects of low CS activity on metabolic health and physical activity in B6, B6.A and B6.A10 mouse strains of both genders. Steps of the study:

1. Mouse weighing before metabolism analysis (see 2.4).
2. Energy metabolism and physical activity measurements of freely moving mouse (see 2.4).
3. Analysis of the results.

2.2.3. Third study

The objective of this study was to analyze the impact of low CS activity (B6.A and B6.A10 strains) and myostatin dysfunction (BEH strain) on changes in energy metabolism and muscle mass after 48-h food deprivation. Steps of the study:

1. Mouse weighing before metabolism analysis (see 2.5.1).
2. Energy metabolism and physical activity measurements of freely moving mouse (see 2.5.1).
3. Mouse weighing before food deprivation intervention.
4. Mouse separation into single cage with subsequent 48-h food deprivation.
5. Mouse weighing at 24-h mark of food deprivation.
6. Mouse weighing after 48-h food deprivation

7. Energy metabolism and physical activity measurements of freely moving mouse (see 2.5.1).
8. Mouse sacrifice and dissection of hindlimb muscles (see 2.5.2).
9. Weighing of each of the dissected muscles.
10. Measurement of CS enzyme activity from muscle homogenate (see 2.5.3).
11. Muscle fiber type composition analysis (only for BEH+/+ and BEH mice, see 2.5.4).
12. Analysis of microscopic images of the cross sections (only for BEH+/+ and BEH mice, see 2.5.4).
13. Fat dissection and weighing (only for BEH+/+ and BEH mice, see 2.5.5).
14. Analysis of the results.

2.2.4. Fourth study

The objective of this study was to examine if carbohydrate and fat hypocaloric diets with equal protein content induce different improvements in body composition, energy balance and glucose tolerance of obese B6 mice during caloric restriction. Steps of the study:

1. 18-week mice exposure to the obesogenic diet and daily energy intake measurements (see 2.6.1).
2. 6 weeks of caloric restriction, which was gradually increased from 20 % (1 week) to 30 % (2–4 week) and 40 % (5–6 week) of the calculated energy intake on the *ad libitum* obesogenic diet (see 2.6.2).
3. Mouse weighing before glucose tolerance test.
4. Glucose tolerance test (see 2.6.3).
5. Mouse weighing before metabolism analysis
6. Energy metabolism and physical activity measurements of freely moving mouse
7. Mouse sacrifice.
8. dissection of hindlimb muscles and fats (see 2.6.4).
9. Analysis of the results (see 2.6.5).

2.3. Mitochondrial respiration. First Study

2.3.1. Isolation of mitochondria

Mitochondrial respiration was studied in 12-week-old B6 and B6.A mice (n = 16 of each strain, eight males and eight females). The following solutions were prepared for the isolation of mitochondria:

1) Homogenization medium (H medium: 250 mM sucrose, 10 mM TRIS, 3 mM EGTA, pH 7.7, 4 °C);

2) Suspension medium (S medium: 250 mM sucrose, 5 mM TRIS, pH 7.34, 4 °C);

3) Isolation medium A (2.5 ml of 150 mM sucrose, 75 mM KCl, 50 mM KH_2PO_4, 5 mM $MgCl_2$, 1 mM EGTA, pH 7.4, 4 °C) supplemented with 0.2 mg/ml bacterial proteinase (type XXIV, from *Bacillus licheniformis,* P8038, Sigma-Aldrich, Germany);

4) Isolation medium B (250 mM sucrose, 20 mM MOPS, 0.1 mM EGTA, pH 7.4, 4 °C) supplemented with 1 mg/ml bovine serum albumin (BSA) and 15 ml of isolation medium B without BSA.

The prepared solutions were kept on ice during procedure (4 °C). For mitochondria respiration measurements, incubation solution 6 (IT6: KCl 110 mM, creatine monohydrate 50 mM, TRIS 20 mM, KH_2PO_4 5 mM, Mg ($MgCl_2 \cdot 6H_2O$) 2.5 mM, pH 7.2, 37 °C) were prepared before mitochondria isolation procedure.

Following euthanasia by the cervical dislocation, liver and skeletal muscles of the hindlimb were quickly excised and placed into separate 40 ml ice-cold 0.9 % KCl solution.

For liver mitochondria isolation, 3 min of incubation in ice-cold KCI was implemented. Then liver was briefly minced with surgical scissors. Liver filled with H medium (liver mass with H medium ratio was 1:10 (m/V)) was homogenized by Potter-Elvehjem homogenizer (Thermo Fisher Scientific, United States of America) connected to electric T25 disperser (IKA, Germany) with 10 strokes at 750 rpm for 20–30 s. The homogenate was transferred into a centrifuge tube.

Mitochondria were isolated by differential centrifugation (Zukiene, Nauciene, Ciapaite, & Mildaziene, 2010). There were 3 steps of centrifugation for mitochondria isolation from liver: 1) 800 × g for 5 min; 2) 6800 × g for 10 min at

and 3) 6800 × g for 10 min at 4 °C (Allegra 64R centrifuge, Beckman Coulter, S. Drive, United States of America). After the first centrifugation the supernatant was filtered through double layered medical gauze fabric and centrifuged again. After the second step of centrifugation the pellet was resuspended in S medium. After the final third step of centrifugation mitochondrial precipitate was suspended again, the suspension was transferred into Eppendorf tube and put on ice before proceeding with the measurements of protein concentration.

For muscles mitochondria isolation, muscles were incubated for 5 min in ice-cold isolation A medium supplemented with 0.2 mg/ml proteinase (type XXIV, P8038, Sigma-Aldrich, Germany). After incubation, muscles were transferred to a shallow glass beaker on ice. After removal of connective and adipose tissue, muscles were cut into small pieces using surgical scissors until homogeneity. 20 ml of ice-cold isolation medium B supplemented with 1 mg/ml of BSA was added into minced muscles and the final mix was homogenized with 10 strokes at 750 rpm for 20–30 s. Homogenate was transferred into the centrifuge tube. The procedure for isolation of mitochondria from skeletal muscles was similar as in other studies (Garcia-Cazarin, Snider, & Andrade, 2011). Steps of differential centrifugation were as follows: 1) 800 × g for 10 min; 2) 10 000 × g for 10 min; 3) 10000 × g for 10 min at 4 °C (Allegra 64R centrifuge, Beckman Coulter, USA). After the first step of centrifugation the supernatant of muscles was filtered through medical gauze fabric, and after second step of centrifugation the supernatant was removed and 10 ml of ice-cold isolation medium B was added to resuspend the mitochondria pellet. After final third step of centrifugation the supernatant was discarded, 100–150 µl of ice-cold isolation medium B (without BSA) was added into tube with mitochondria. Afterwards mitochondrial pellet was resuspended again and prepared suspension (~100 mg/ml of protein) was transferred into Eppendorf tube. Both liver and isolated muscles mitochondrial suspensions were kept on ice throughout the experiment.

The protein concentration in mitochondrial suspensions was measured by a modified biuret method spectrophotometrically (Gornall, Bardawill, & David, 1949). BSA was used as a standard for biuret test: solutions of 0.25–10 mg of BSA were prepared using deionized H_2O (Milli-Q H_2O). Standard curve was drafted according to each standard value, measured spectrophotometrically at 536 nm wavelength. 1.6 ml of Biuret reagent, 380 µl of deoxycholic acid (DOX, 0.33 %)

and 20 µl of each standard were gently mixed and incubated for 15 min at 37 °C before spectrophotometric analysis. Mitochondrial protein concentration measurements went through the similar steps as BSA standards: 20 µl of protein, 380 µl of DOX (0.33 %) and 1.6 ml of biuret reagent are incubated for 15 min at 37 °C. Samples are prepared in duplicates with negative control (400 µl 0.33 % DOX and 1.6 ml of biuret reagent). Mitochondrial suspensions were used immediately for respiration measurement or stored at –80 ºC until CS enzyme activity analysis.

2.3.2. Measurement of mitochondrial respiration

Mitochondrial respiration was measured as oxygen consumption (O nmol $min^{-1} \cdot mg^{-1} \cdot$ protein) at 37 °C in 1.5 ml glass vessel equipped with Clark-type oxygen electrode by using polarography system (Rank Brothers LTD, UK). The technique of polarography is based on water-dissolved oxygen reduction (1.1, 1.2) and on fundamental biochemistry of oxidative phosphorylation (Chance & Williams, 1955b). Working oxygen electrode potential had been chosen with an estimation to not to exceed overall reaction speed of 0.7 V.

$$O_2 + 2\bar{e} + 2H^+ \rightarrow H_2O_2 \qquad (1.1)$$

$$O_2 + 4\bar{e} + 4H^+ \rightarrow 2H_2O \qquad (1.2)$$

The following respiratory substrates were used in experiment:

1. 5 mM glutamate (prepared from L-glutamic acid, G8415, Sigma-Aldrich, Germany) and 5 mM malate (prepared from L-(−)-malic acid disodium salt, M9138, Sigma-Aldrich, Germany) (GM).

2. 5 mM glutamate plus 5 mM malate plus 0.005 mM palmitoyl-L-carnitine (prepared from palmitoyl-L-carnitine chloride, P1645, Sigma-Aldrich, Germany) (GM + PC).

3. 5 mM pyruvate (prepared from sodium pyruvate, P2256, Sigma-Aldrich, Germany) and 5 mM malate (PM).

4. 5 mM pyruvate and 5 mM malate plus 0.005 mM palmitoyl-L-carnitine (PM + PC).

5. 0.005 mM palmitoyl-L-carnitine plus 0.25 mM malate (PC + M).

6. 5 mM succinate (prepared from sodium succinate dibasic hexahydrate, S2378, Sigma-Aldrich, Germany) plus 0.001 mM rotenone (prepared from rotenone, R8875, Sigma-Aldrich, Germany) (SU + RO).

Rotenone was used for respiratory chain complex I inhibition (Scholte, 1973) to measure oxygen consumption rates, which are associated with respiratory chain complex II activity (Bouderba et al., 2012) when succinate is used as a substrate.

1 ml of IT6 solution was added into glass vessel equipped with oxygen sensitive electrode together with one of the respiratory substrates and creatine phosphokinase (C3755, Sigma-Aldrich, Germany). Final concentration (prepared by the manufacturer protocol) of 0.05 mg/ml creatine phosphokinase was added. IT6 must be maintained at 37 °C throughout the experiment. The volume of mitochondrial suspension corresponding to 1 mg of liver mitochondria protein or to 0.15 mg of muscle mitochondria protein was added into IT6 medium (IT6 medium contains IT6 solution, 10 µl of respiratory substrate solution and 10 µl of prepared (0.05 mg/ml) creatine phosphokinase solution. Mitochondrial metabolic state 3 (state 3) respiration ("state 3 respiration" will also be used in results section) was initiated by adding 1 mM of ATP which is constantly converted to ADP by creatine phosphokinase that functions as ADP regenerating system (meaning high ADP and Pi concentrations) in medium together with creatine (Chance & Williams, 1955a; Kholodenko et al., 1987). Oxygen consumption of state 3 was calculated as nmol O · min^{-1} · mg $protein^{-1}$.

2.3.3. Mitochondrial CS enzyme activity

For assessment of CS activity in isolated mitochondria, 10 µl of mitochondria lysate was added to start the reaction in 990 µl of reaction reagent which then consisted of 100 mM triethanolamine-HCl, 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) (100 µM), 0.25 % Triton-X (vol/vol), 0.5 mM oxaloacetate, 0.31 mM acetyl CoA with pH adjusted to 8.0. Other steps of CS activity measurements were similar to the thoroughly described below muscles CS activity assay procedure (see 2.4.1).

2.4. Metabolism measurements, physical activity. Second study

Control B6 strain (n = 18, nine males and females, respectively) and two mouse strains with low CS activity, i.e., B6.A (n = 18, nine males and females, respectively) and B6.A10 (n = 18, nine males and females, respectively), were studied in the metabolic cage.

Assessment of absolute (ml · min^{-1}) and relative (ml · min^{-1} · $kg^{-0.75}$) oxygen consumption (VO_2) and carbon dioxide production (VCO_2) were carried as additional out to supplement respiration quotient (RQ) calculations. Absolute (kcal · day^{-1}) and relative (kcal · $kg^{-0.75}$ · day^{-1}) energy expenditure (EE) was also assessed.

The metabolic measurements were carried using in the metabolic cage (Physiocage 00, Panlab Harvard Apparatus, Barcelona, Spain) connected to the gas analyzer (LE405, Panlab Harvard Apparatus, Barcelona, Spain). Prior to measurements the gas analyzer (LE405, Panlab Harvard Apparatus, Barcelona, Spain) was calibrated with gas mixtures at the high point (50 % O_2, 1.5 % CO_2) and at the low point (20 % O_2, 0 % CO_2). During the metabolic measurements, the gas analyzer was coupled to the switching device (LE400, Panlab Harvard Apparatus, Barcelona, Spain) which controlled the air flow throw the metabolic cage and the analyzer. Air flow rate was set to 250 ml · min^{-1} as recommended by the manufacturer. Switching time was 3 min which refers to the duration of measurements O_2 and CO_2 concentrations in the metabolic cage and the external environment, respectively. Metabolism system produces whole body respiratory gas analysis in free moving animals. EE was calculated as the average values of the last 2 of 3 h measurements by Weir equation (2):

$$EE = [3.815 + (1.232 \cdot RQ)] \cdot VO_2 \cdot 1.44 \quad (2)$$

The average body mass measurements before and after EE assessment were used in EE adjustment to body mass. “Metabolism version 1.2” software (Panlab Harvard Apparatus, Barcelona, Spain) was applied for the acceptance of primary metabolism data.

Physical activity. The Physiocage system (Panlab Harvard Apparatus, Barcelona, Spain) was used to assess mouse physical activity and rearing parameters. The assessment of physical activity was done using strain gauges attached to the cage for measurements of ground reaction forces generated by mice in the cage. The rearing activity is assessed as lifts of the body above 10 cm height as detected by infrared barriers. Results were calculated by “Metabolism version 1.2” software (Panlab Harvard Apparatus, Barcelona, Spain).

2.5. Food deprivation. Third study

Food deprivation (FD) means that organism has no access to food. The control (CON) mice were provided with *ad libitum* access to water and food. The food-deprived (FD, identical abbr. for food-deprived and food deprivation) mice had *ad libitum* access to water, but did not receive any food for the subsequent 48 h.

2.5.1. Study design and metabolism analysis

Metabolic measurements were performed at light cycle (from 9 a.m. to 6 p.m.). For the control (CON metabolism) measurements, each of 16-week-old B6, B6.A, B6.A10 (n = 9 of each strain) male mouse was weighed (Kern, ABS 80-4, Germany) and tested in physiocage for 3 h (1 h of acclimation and 2 h of real-time measurements) before FD (as described thoroughly in the second study, see 2.3). After CON metabolism analysis, each mouse was weighed again and housed alone in a separate cage without food, but with tap water ad libitum for the next 48 h. During FD intervention, the mouse was weighed additionally at 24 h mark. After 48 h the mouse was weighed again followed by assessment of metabolism and physical activity (FD metabolism) using the identical conditions as for the control 3 h measurement. 18-week-old BEH+/+ (n = 45), BEH (n = 35) male mice were subdivided into the non-intervention control (for BEH+/+ n = 18, BEH n = 12) and experimental (for BEH+/+ n = 27, BEH n = 23) groups, respectively. Non-intervention control was used only for body composition analysis to have comparison material versus experimental group. For the metabolism measurements, each of experimental mouse was weighed and tested in physiocage before (CON) and after (FD) 48-h food withdrawal intervention. All the steps for BEH+/+ and BEH mice were matched to B6 lineage FD analysis. At the end of energy metabolism assessment, the mouse was weighed for the last time.

2.5.2. Single and aggregate muscle mass

Mice were sacrificed by the exposure to CO_2 at the end of all experiments. Immediately afterwards, as in our previous study (Kvedaras et al., 2017), the skeletal muscles of the left and right hindlimb muscles, including *gastrocnemius* (GAS), *plantaris* (PL), *soleus* (SOL), *tibialis anterior* (TA) and *extensor digitorum longus* (EDL), were dissected and weighed with a precision of 0.1 mg (Kern, ABS 80-4, Germany). Before weighing the muscles were freed from all visible

tendons and blotted dry rapidly on filter paper. The skeletal muscle mass was calculated as a sum of left and right leg masses (sum of muscle mass or aggregate muscle mass) of all five dissected muscles. Muscle to body mass ratio (3) was calculated as:

Sum of muscle mass · (body mass^{-1} *· 100) (3)*

Afterwards, muscles were having been immediately immersed in isobutanol (IUPAC nomenclature: 2-methylpropan-1-ol) and frozen in liquid nitrogen ("Litgenas", Kaunas, Lithuania).

2.5.3. CS enzyme activity from muscles

CS enzyme activity assay was determined from homogenized skeletal muscles as described previously (Kvedaras et al., 2017; Ratkevicius et al., 2010). From 40 to 70 mg samples (n = 8) of the GAS muscle were homogenized in ice-cold lysis buffer (50 mM Tris–HCl, 100 mM $KHPO_4$, 2 mM ethylenediaminetetraacetic acid (EDTA), 0.2 % w/v BSA, pH 7.0) and frozen liquid nitrogen. Then the homogenates were defrosted by shaking for 60 min and centrifuged at 13 000 g for 10 min at 4 °C. Bradford assay was used in assessment of protein concentration of supernatants. Measurements of CS activity were carried out using the reaction reagent (100 mM triethanolamine-HCl, dithiobis (2-nitrobenzoic acid), 0.5 mM Triton-X (0.25 % vol/vol), oxaloacetate, 0.31 mM acetyl CoA, pH 8.0) and spectrophotometer (GENESYS 10 Bio UV-Vis, Thermo Fisher Scientific Inc., Waltham, MA, USA) at room temperature of 21 °C. CS from a porcine heart was used as a standard (C3260-200UN, Sigma-Aldrich, Germany) for assay calibration. The molar extinction coefficient used was 13 600 M^{-1} cm^{-1} for CoA-5,5'-thiobis (2-nitrobenzoic acid) at wavelength of 412 nm. to assess the maximum CS activity during the first 2 min of the reaction.

2.5.4. Muscle fiber type composition

Fiber type composition and fiber size of SOL muscle were determined as described in our previous study (Kilikevicius et al., 2013). Transverse sections from the belly of the muscle were cut at a thickness of 10 μm with a cryotome (CM1850UV, Leica Biosystems, Germany) at −20 °C. The staining for myosin ATPase was performed after pre-incubation (at pH of 4.34) procedure. "ImageJ" software (NIH – version 1.43) was used in analysis of microscopic images of the

cross sections which were taken with four times magnification. The typical images are shown in Fig. 4. Total fiber number for type 1 and 2 fibers was counted. Average fiber cross sectional area was assessed using data from 25 % of randomly selected fibers.

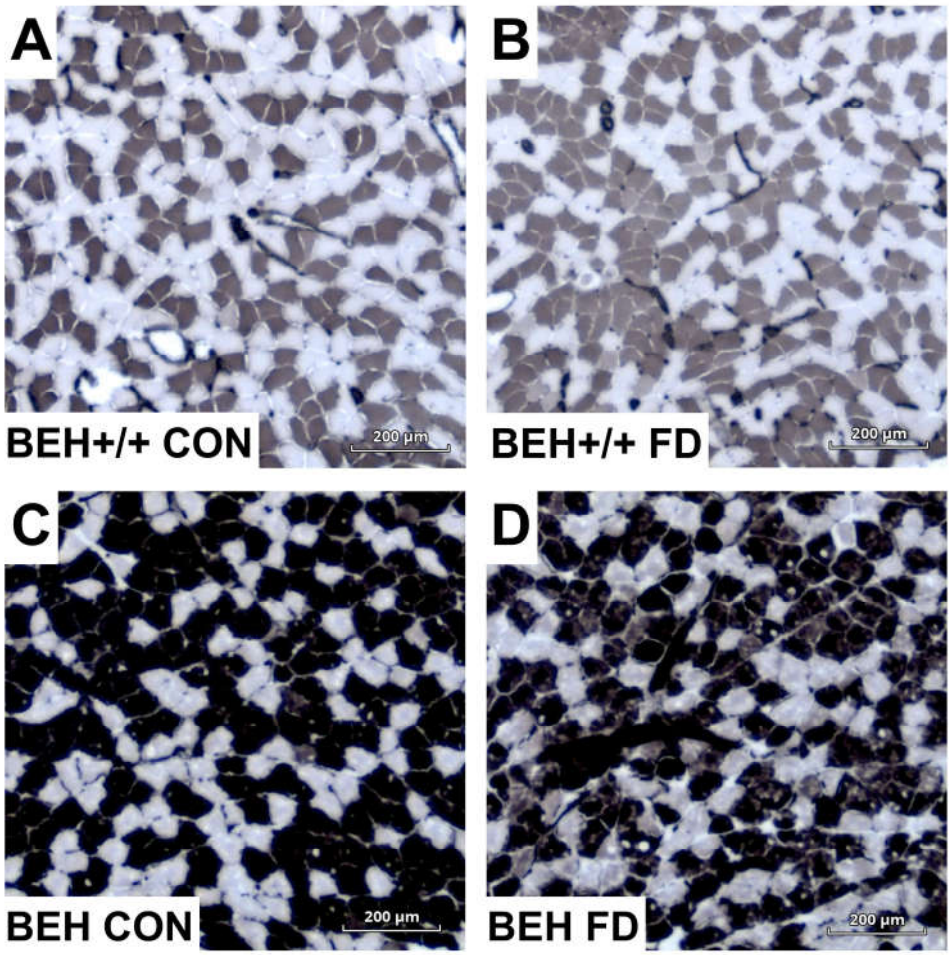

Fig. 4. Typical images of BEH+/+ mouse soleus muscle (SOL) cross section stained for ATPase. Images were obtained from the muscles of BEH+/+ and BEH control (CON) and food-deprived (FD) groups for the analysis of muscle fiber type composition. Type 1 fibers are darkly stained

2.5.5. Fat distribution

Fat distribution analysis was performed according to previously described methodology (Kvedaras, Minderis, Krusnauskas, Lionikas, & Ratkevicius, 2019). Fat from four different sites were also removed and weighed. The aggregate fat mass was assessed and included white adipose subcutaneous (sWAT) covering the hind limbs, gonadal (gWAT), visceral (vWAT, which includes mesenteric (mWAT) and perirenal (pWAT)) and intrascapular brown adipose tissue (iBAT) fats.

2.6. Weight loss dietary interventions. Fourth study

At 10 weeks of age B6 mice (n = 30) were switched to obesogenic high-fat and high-sugar diet (D12451, 45 % and 17.5 % kcal from fat and sugar, Research Diets, USA) for 18 weeks (Alhindi et al., 2019). This was followed by 6-week caloric restriction (CR for caloric restriction, *CR* for calorie-restricted group) on either low-fat diet (*Low-Fat,* n = 10) or low-carbohydrate diet (*Low-Carb,* n = 10). After weight-matching procedure ten mice were examined prior to CR as pre-diet obese controls (*Pre*).

2.6.1. Obesity phase

After 10 weeks of 18-week exposure to the obesogenic diet mice were moved into separate cages and food consumption was assessed every week for each mouse by subtracting food leftovers from initially provided food with corrections for humidity effect on the pellets weight. Daily energy intake (DEI) of mice was calculated by equation (2):

$$DEI\ (kcal \cdot g^{-1} \cdot day^{-1}) = (Weekly\ food\ consumption\ (g) \cdot Food\ energy\ density\ (kcal \cdot g^{-1})) \cdot (body\ mass\ (g) \cdot 7)^{-1} \quad (2)$$

Three-week average DEI of mice was 0.42 ± 0.04 kcal · g^{-1} · day^{-1} and only mice gaining at least 20 % of weight compared to the age-matched group on the regular chow diet (*Regular,* n = 10) were used for CR study.

2.6.2. Caloric restriction phase

Calorie restriction (CR) phase. 28-week-old obese mice were randomly assigned to one of the two *CR* groups and *Pre* group which was used for assessment of body composition and metabolism analysis. During 6 weeks mice were fed daily at 8 a.m. and CR was gradually increased from 20 % (1 week) to

30 % (2–4 week) and 40 % (5–6 week) of the calculated energy intake on the *ad libitum* obesogenic diet. Energy intake during CR phase was estimated for each mouse individually by reducing DEI by the extent of caloric deficit and multiply it by initial body mass of the animal prior CR. The amount of food was corrected for different caloric density of the diets (4.1 and 5.2 kcal · g^{-1} for *Low-Fat* and *Low-Carb*, respectively) to achieve equal total energy and protein content in the diets, i.e., 20, 60 and 20 % kcal from fat, carbohydrate and protein for *Low-Fat* (D17100401, Research Diets, New Brunswick, NJ, USA) and 20, 60 and 20 % kcal from carbohydrate, fat and protein for *Low-Carb* (D12492, Research Diets, New Brunswick, NJ, USA), respectively. Details of the macronutrient composition and sources of the diets are presented in Table 1.

Table 1. Detailed macronutrient composition of the diets provided by the manufacturer (Research Diets Inc., New Brunswick, NJ, USA)

Diet (mouse group on it)	**Obesogenic diet (*Pre*)**		**Low-fat diet (*Low-Fat*)**		**Low-carbohydrate diet (*Low-Carb*)**	
Group's energy state	*Ad libitum*		CR up to 40%		CR up to 40%	
Diet's manufacturer	Research Diets Inc., USA		Research Diets Inc., USA		Research Diets Inc., USA	
Diet's code	D12451		D17100401		D12492	
Protein (% kcal)	20		20		20	
Carbohydrate (% kcal)	35		60		20	
Fat (% kcal)	45		20		60	
Protein:	g	kcal	g	kcal	g	kcal
Casein	23.3	93.3	20.0	80.1	25.8	103.3
L-Cystine	0.35	1.4	0.30	1.2	0.35	1.4
Carbohydrate:						
Corn Starch	8.5	33.9	40.5	162.1	0	0
Maltodextrin	11.7	46.6	12.5	50.0	16.1	64.6
Sucrose	20.1	80.6	6.9	27.5	8.9	35.5
Fibre:						
Cellulose	5.83	0	5.0	0	6.5	0
Fat:						
Soybean Oil	2.9	26.2	2.5	22.5	3.2	29.1
Lard	20.7	186.3	6.5	58.5	31.6	284.8
Minerals:						
Mineral Mix S10026	1.17	0	1.00	0	1.29	0
DiCalcium Phosphate	1.52	0	1.30	0	1.68	0
Calcium Carbonate	0.64	0	0.55	0	0.71	0
Potassium Citrate	1.92	0	1.65	0	2.13	0
Vitamins:						
Vitamin Mix V10001	1.17	4.7	1.00	4.0	1.29	5.2
Choline Bitartrate	0.23	0	0.20	0	0.26	0
Total	100	473	100	406	100	524
kcal · g^{-1}	4.73		4.1		5.2	

2.6.3. Glucose tolerance

A 6-time point glucose tolerance test was carried out after an overnight fasting at 8–9 a.m. during the final 6th week of CR. Mice were subjected to an intraperitoneal injection of glucose solution (2 g glucose · kg body wt-1) and glucometer (Glucocard X-mini plus GT-1960, Arkray, Japan) was used to measure glucose in the blood samples from the tail vein at 0, 15, 30, 60, 90 and 120 min after injection. The area under curve (AUC) of glucose response was calculated using Prism 8.0 software (GraphPad Software Inc., CA, USA).

2.6.4. Energy metabolism and physical activity

During the final week of CR, mice were fasted overnight and subjected to assessment of total energy expenditure and physical activity as described in our previous study (Kvedaras et al., 2019) and in the method sections (see 2.3, 2.4.1) with several distinctive modifications. Briefly, all metabolism measurements were performed during a light period (from 9 a.m. to 3 p.m.). Each mouse was weighed (ABS 80-4, Kern, Germany) and transferred into metabolic cage for 3-h with no food and water provided. The respiratory quotient and total energy expenditure were calculated as the average values of the last 2-h spent in metabolic cage (Metabolism software version 1.2, Panlab Harvard Apparatus, Spain). Physical activity and the rearing of mice were assessed as described earlier (see 2.3).

2.6.5. Body composition

During CR mice were weighed weekly with a precision of 0.1 g (440-45N, Kern, Germany). After 6-week CR mice reached 34 weeks of age and were euthanized with an inhalation of CO_2. Immediately afterwards skeletal muscles and body fat were sampled and weighed with a precision of 0.1 mg (ABS 80-4, Kern, Germany). Combined hindlimb muscle mass was calculated as a sum of the GAS, PL, SOL, TA and EDL muscle masses. The muscles were trimmed from all visible tendons and blotted dry just before weighing. Combined body fat mass was assessed as the sum of the sWAT, gWAT, mWAT, pWAT and iBAT by the same difference procedure as mentioned earlier (see 2.4.3).

2.7. Statistical analysis

All the statistical analysis was performed with Prism 8.0 and SPSS 20 software. The data were tested for normality using the Shapiro–Wilk test. For all statistical tests, the level of significance was set a priori at $p < 0.05$. The data are presented as means ± standard deviation (*SD*).

First study. Two factor analysis of variance (2-way ANOVA) test and Bonferroni post hoc test were used to evaluate differences between the strains in ATP stimulated mitochondrial respiration. Unpaired T-test was performed to observe CS enzyme activity differences.

Second study. Energy metabolism results were analyzed by two-way ANOVA with Bonferroni's post hoc test were used to evaluate differences between strains (B6, B6.A, B6.A10). Unpaired t-test was performed to evaluate differences between the strains and between male and female mice.

Third study. For metabolism results two-way and/or three-way 2-way ANOVA with Bonferroni's post hoc test were used to evaluate differences between strains (B6, B6.A, B6.A10, BEH+/+, BEH). 2-way repeated measures ANOVA with Bonferroni's post hoc test were implemented to evaluate differences and between conditions (CON and after FD). One factor analysis of variance (1-way ANOVA) test was performed to evaluate strain differences with individual muscles. Covariance analysis with body mass as a covariate was used to compare energy expenditure (EE) between mouse strains. Student's t-test was used when assessing differences in baseline characteristics and FD induced changes in muscle mass between BEH and BEH+/+ mice. Relative values (in %) for body and muscle mass loss during FD were analyzed by 1-way of ANOVA with Bonferroni post hoc test.

Fourth study. Means were compared with one-way analysis of variance (ANOVA) using Bonferroni's post hoc test to assess differences between the studied groups of mice. Non-parametric Kruskal–Wallis test with Dunn's post hoc analysis was applied in the cases when means did not meet a criterion of normal distribution. Two-way repeated measures ANOVA was used for analysis of body mass change in the cases when it was assessed repeatedly on the same mice. Analysis of covariance (ANCOVA) was applied using linear models to determine effects of mouse groups on energy expenditure as previously recommended for this type of analysis (Tschöp et al., 2011). In this case body mass and physical activity were used as covariates. Linear regression analysis was also used on the plots of energy expenditure over physical activity. Pearson's correlation coefficient was calculated to evaluate strength of the association between variables.

3. RESULTS AND DISCUSSION

3.1. Effect of low CS activity on mitochondrial respiration. First study

3.1.1. Results

CS activity was measured in samples of liver mitochondria from B6 and B6.A male and female mice. CS activity was lower ($p < 0.01$) by ~32 % in B6.A compared to B6 mice (Fig. 5).

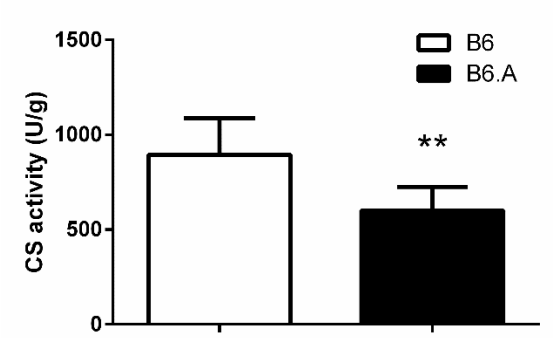

Fig. 5. CS activity in liver mitochondria from B6 (n = 8) and B6.A (n = 8) mice. Values are means ± *SD.* ** $p < 0.01$ – B6.A vs. B6 mice

There were no significant differences in state 3 respiration between B6 and B6.A mouse strains in mitochondria isolated from liver. However, there were state 3 differences, when analyzing PM with and without fatty substrate. For liver mitochondria of male mice, state 3 rates were higher ($p < 0.01$) with PM + PC compared to PM only (Fig. 6A). There were no such differences for female mice (Fig. 6B). There was also a tendency for higher state 3 rates with PM + PC in B6.A compared to B6 female mice.

In liver mitochondria the highest rates of state 3 respiration were measured using SU + RO substrate (134.1 ± 22.7 and 79.7 ± 15.5 nmol · min^{-1} · mg^{-1} for males and females of B6 strain; 118.1 ± 21.3 and 106.1 ± 17.5 nmol · min^{-1} · mg^{-1} for males and females of B6.A strain, respectively).

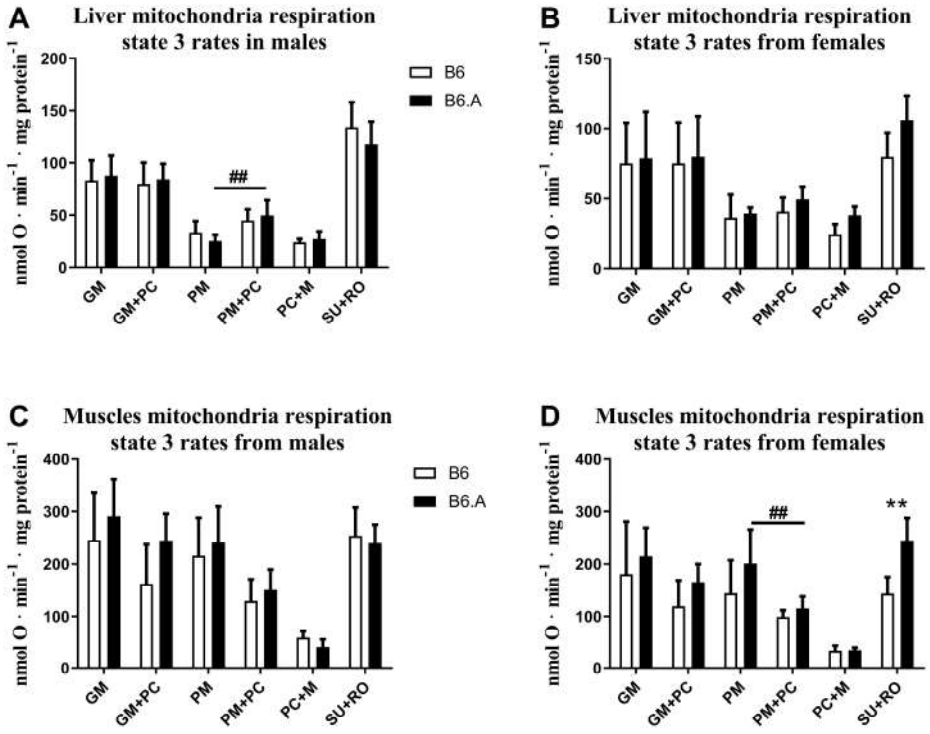

Fig. 6. Liver (A, B) and muscles (C, D) isolated mitochondria metabolic state 3 differences between B6 and B6.A strains of male (A, C) and female (B, D) mice. Oxygen consumption of state 3 was calculated as nmol O · min^{-1} · mg $protein^{-1}$. Following substrates were used: GM – glutamate-malate, GM + PC – glutamate-malate plus palmitoyl-L-carnitine, PM – pyruvate-malate, PM + PC – pyruvate-malate plus palmitoyl-L-carnitine, PC + M – palmitoyl-L-carnitine plus malate, SU + RO – succinate plus rotenone. Values are presented as means ± *SD*; ** $p < 0.01$ – B6 vs B6.A; ## $p < 0.01$ – PM vs. PM + PC

For mitochondria from muscles, state 3 rates were approximately two-fold higher than for liver mitochondria when GM, GM + PC, PM, PM + PC and SU + RO substrates were used, but PC + M substrate produced relatively low respiration rates in muscle mitochondria (Fig. 6C, D). In muscle-derived mitochondria, the only difference between B6 and B6.A strains was noted in females when using SU + RO (143.4 ± 31.7 vs 244.4 ± 43.2 nmol O · min^{-1} · mg $protein^{-1}$, respectively, $p < 0.01$, Fig. 6D). Also, in B6.A female's state 3 rates were higher with PM in comparison to PM + PC ($p < 0.01$). Strong tendency of increased state 3 rates with PM substrate compared to PM + PC remained in B6.A females ($p > 0.05$). Interestingly, this tendency was opposite for liver mitochondria: higher respiratory rate was with PM + PC compared to PM ($p > 0.05$).

Sex effect on mitochondrial respiration. For both liver ($p < 0.001$) and muscles ($p < 0.01$) mitochondria of B6 mice, state 3 rates with SU + RO substrate were significantly higher in males compared to females (Fig. 7A, C). State 3 rates did not differ with SU + RO in B6.A strain with a tendency of higher state 3 rates for liver of males compared to females ($p > 0.05$). No significant state 3 differences were identified with GM, GM + PC, PM, PM + PC, PC + M substrates between B6 male and female mice in both mitochondria isolated from liver and muscles.

For SU + RO, state 3 respiration of liver mitochondria was higher ($p < .05$) than for other substrates in males (Fig. 6A) compared to females, but there were no differences between SU + RO and GM or GM + PC for females (Fig. 7B) when B6 mice were studied.

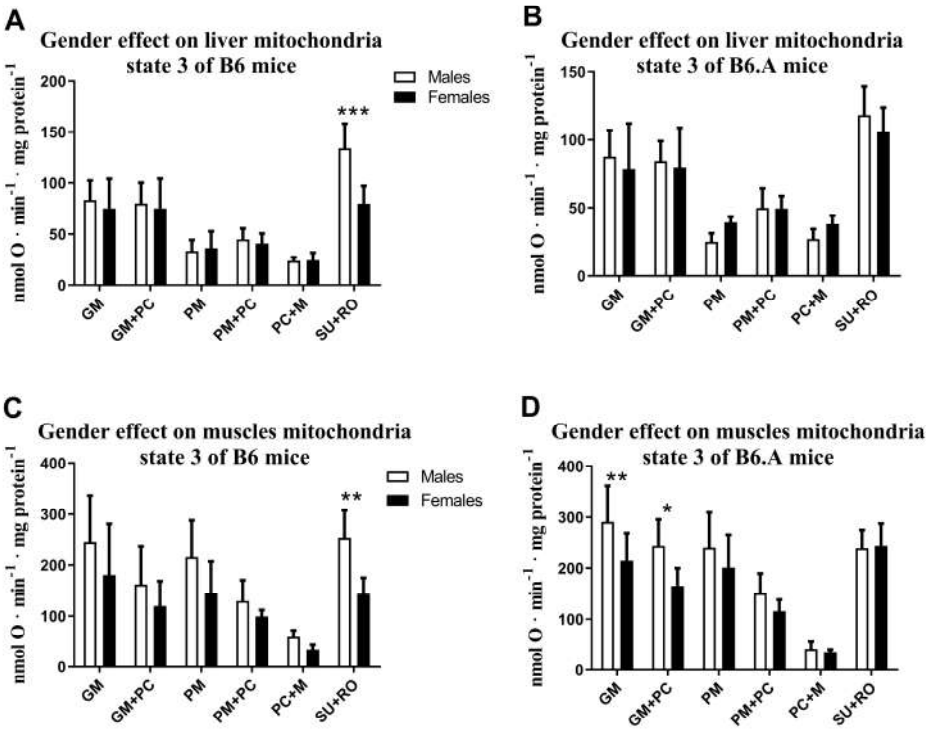

Fig. 7. Liver (A, B) and muscles (C, D) isolated mitochondria metabolic state 3 differences between males and females of B6 (A, C) and B6.A (B, D) mice. Oxygen consumption of state 3 was calculated as nmol O · min^{-1} · mg $protein^{-1}$. Following substrates were used: GM – glutamate-malate, GM + PC – glutamate-malate plus palmitoyl-L-carnitine, PM – pyruvate-malate, PM + PC – pyruvate-malate plus palmitoyl-L-carnitine, PC + M – palmitoyl-L-carnitine plus malate, SU + RO – succinate plus rotenone. Values are presented as means ± *SD*; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ – vs. female mice

In muscles mitochondria high rates of state 3 were observed not only for SU + RO, but also for GM, GM + PC and PM in both male and female mice of B6 strain. In case of B6 strain, female mice tended to show lower state 3 rates than males, but the difference was significant only for SU + RO (Fig. 7C). In case of B6.A males, state 3 rates were higher with GM ($p < 0.01$) and GM + PC ($p < 0.05$) compared to female mice (Fig. 7D).

3.1.2. Discussion

We hypothesized that low CS activity could improve mitochondrial respiration. We studied CS activity and respiration in mitochondria from B6 and B6.A mice. B6.A strain carries the A/J allele in the genomic region containing the *Cs* gene on otherwise B6 strain background. Previous studies showed that A/J mice show 50–65 % reduction in skeletal muscle tissue derived CS activity compared to other mouse strains despite similar levels of *Cs* mRNA and lack of differences in CS and cytochrome c protein content (Ratkevicius et al., 2010). In agreement with this study, we found ~32 % lower mitochondrial CS activity in B6.A compared to B6 mice. However, we did not observe any significant difference in mitochondrial respiration between these strains.

There was only one difference between the strains in mitochondrial respiration. It was for mitochondria from muscles of female mice, as state 3 rates were higher for B6.A mice compared to B6 mice when succinate and rotenone (SU + RO) was used. A strong trend for higher rate of state 3 respiration with SU + RO was also observed for mitochondria from liver of B6.A mice compared to B6 mice in female mice. Other studies showed, that in males' succinate-respiring mitochondria showed higher oxygen consumption rates in comparison to glutamate/malate-supplied mitochondria (Baran et al., 2016). In our research, in isolated muscle and liver mitochondria oxygen consumption rates at state 3 under SU + RO conditions were higher compared to state 3 rates with other substrates in B6 males and females. Surprisingly, oxygen consumption rates of high fat diet (HFD) male and female experimental groups were the same under equivalent conditions (Catala-Niell et al., 2008). HFD could not induce oxidative stress. It is suggested that UCP3 overexpression induced by lipids functions as antioxidative molecule (Catala-Niell et al., 2008).

No differences in state 3 were noticed with glutamate plus malate (GM),

pyruvate plus malate (PM) substrates in B6 mice liver and muscles mitochondria. Sanz et al. have used similar methods for liver, muscles and heart mitochondria isolation and respiration measurements from B6 mice with 2.5 mM pyruvate plus 2.5 mM malate (in heart and skeletal muscle), 2.5 mM glutamate plus 2.5 mM malate (in liver), 5 mM succinate (without or with 2 μM rotenone; in the three tissues). According to the oxygen consumption results, no respiration state 3 differences between B6 males and females were detected with the tested substrates (Sanz et al., 2007). The results with external interventions, e.g. hyperthermia, showed that with PM substrate (Zukiene et al., 2010).

In B6.A mice mitochondrial respiration differed significantly in presence and in absence of palmitoyl-L-carnitine (PC) which is a fatty substrate. Indeed, carbohydrate oxidation in liver mitochondria from males and in muscles mitochondria from females was faster with addition of PC (PM + PC) compared to PM alone. Those differences might exist because of strong link between β-oxidation of fatty substrates and oxidation of carbohydrates in citric acid cycle (Rogge, 2009). The results of our experiment showed, that there was a decline in respiration with PC + M substrate. In muscles mitochondrial respiration inhibition with PC can be explained by adenine nucleotide translocase (ANT) inhibition (Ciapaite et al., 2006).

CS activity may change after a variety of interventions. There is evidence showing that CS activity increases after cycling to exhaustion at 75 % of peak O_2 uptake, while activities of marker enzymes for FA oxidation (β-hydroxyacyl-CoA dehydrogenase) and glycolysis (phosphofructokinase) are unaffected (Tonkonogi, Harris, & Sahlin, 1997). It can be speculated, that dietary interventions, such as high fat diet feeding, might affect mitochondrial respiration differently in B6 compared to B6.A mice with low CS activity.

Gender differences in mitochondrial respiration. Limited number of studies addresses gender effect on metabolism, despite the fact that this effect exists, especially in mitochondrial metabolism (Thompson et al., 2013). Gender differences were identified in B6 mice with SU + RO (meaning inactive respiratory chain complex I and active complex II) respired mitochondria from both liver and muscles: in females' metabolic state 3 (state 3) was significantly lower than in males. The trend of lower state 3 with other substrates was also noticed in female mice compared to males. It can be explained by the fact, that females have greater tissue recruitment, which is reflected mainly in their mitochondrial respiration.

In summary, our study did not reveal any association between low CS activity and mitochondria respiration rates in mice with one exception in female mice with SU + RO under normal conditions without any external interventions. Experiments on isolated mitochondria from B6 and congenic B6.A mice suggest that low CS activity is associated with low rates of lipid oxidation under mixed substrate conditions, but not under all tested conditions. We also identified strong sex effect on mitochondria respiration under certain conditions (e.g., with SU + RO, PM vs PM + PC differences). It might be hypothesized that these differences in cellular metabolism would translate into different rates of fat gain during exposure to HFD.

3.2. Low CS activity and energy metabolism. Second study

3.2.1. Results

No strain effect on mice body weight was identified with an exception of B6.A males (29.2 ± 1.2 g), which were lighter than B6 (32.2 ± 1.1 g) and B6.A10 (33.0 ± 1.6 g) mice ($p < 0.001$) (Fig. 8). B6 females were 23 % lighter (24.9 ± 3.1 g of body weight) than males (32.2 ± 1.1 g of body mass). Furthermore, B6.A females were 23 % lighter (22.5 ± 0.9 g) than males (29.2 ± 1.2 g) and B6.A10 females were 20 % lighter (23.1 ± 1.4 g) than males (33.0 ± 1.6 g of body mass).

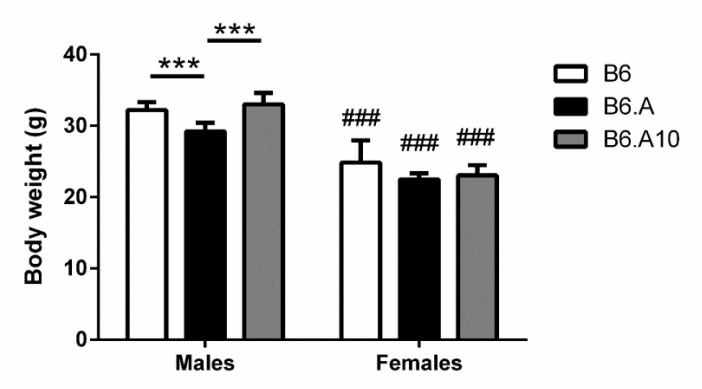

Fig. 8. Body mass in B6, B6.A and B6A.10 male and female mice.
Data is presented as means ± *SD*. *** $p < 0.001$ – vs. B6.A male mice;
$p < 0.001$ – females vs. males

There were no differences in VO_2 and VCO_2 between control B6 and B6.A, B6.A10 males (Fig. 8A, C). In male mice absolute VO_2 was higher in B6.A10 (1.9 ± 0.2 ml $\cdot$ min^{-1}) compared to B6.A (1.7 ± 0.2 ml $\cdot$ min^{-1}) ($p < 0.05$) (Fig. 9A). Interestingly, that absolute VCO_2 was also higher in B6.A10 (1.6 ± 0.1 ml $\cdot$ min^{-1}) compared to B6.A (1.4 ± 0.1 ml $\cdot$ min^{-1}) male mice ($p < 0.01$) (Fig. 9C). No significant strain effect association was identified in relative VO_2 and VCO_2 among males (Fig. 9B, D).

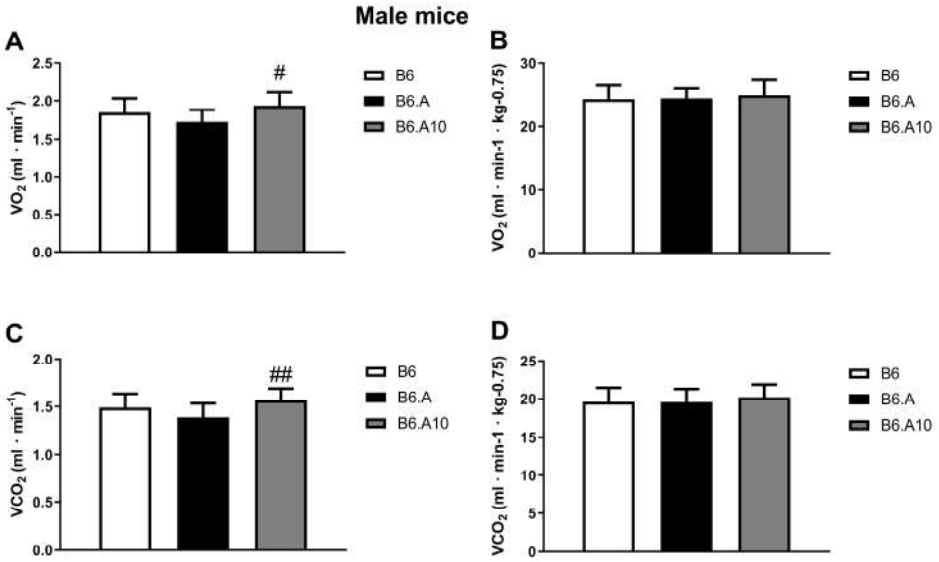

Fig. 9. Absolute (A, C) and relative (B, D) oxygen consumption (A, B) and carbon dioxide output (C, D) rates in B6, B6.A and B6A.10 male mice.
VO_2 – oxygen consumption, VCO_2 – carbon dioxide output. Values are means ± *SD*.
*** $p < 0.001$ – vs. B6.A male mice; # $p < 0.05$, ## $p < 0.01$ – vs. B6.A mice

Results were different for female mice. In case of absolute VO_2, B6 values (1.7 ± 0.2 ml $\cdot$ min^{-1}) were higher than for B6.A (1.5 ± 0.2 ml $\cdot$ min^{-1}, $p < 0.05$) and B6.A10 (1.4 ± 0.2 ml $\cdot$ min^{-1}, $p < 0.01$) (Fig. 10A). Similarly, absolute VCO_2 of B6 (1.4 ± 0.1 ml $\cdot$ min^{-1}) was higher (1.2 ± 0.2 ml $\cdot$ min^{-1}) than in B6.A10 (1.2 ± 0.1 ml $\cdot$ min^{-1}, $p < 0.01$) and B6 females, $p < 0.01$) (Fig. 10C). Interestingly, relative VO_2 was also higher ($p < .01$) in B6 (27.7 ± 2.1 ml $\cdot$ min^{-1} $\cdot$ kg$^{-0.75}$) compared to B6.A (25.3 ± 2.3 ml $\cdot$ min^{-1} $\cdot$ kg$^{-0.75}$) and B6.A10 (24.4 ± 2.4 ml $\cdot$ min^{-1} $\cdot$ kg$^{-0.75}$) mice (Fig. 10B). VCO_2 showed similar differences between the strains as VO_2 in female mice. VCO_2 was higher in B6 (22.2 ± 1.4 ml $\cdot$ min^{-1} $\cdot$ kg$^{-0.75}$) compared to

B6.A10 females (19.9 ± 2.1 ml · min^{-1} · $kg^{-0.75}$) ($p < 0.05$) (Fig. 10D). There was a tendency of higher relative VCO_2 rates in B6 mice compared to B6.A ($p = 0.052$). In overall, no significant difference was identified in either VO_2 or VCO_2 between B6.A and B6.A10 females (Fig. 10).

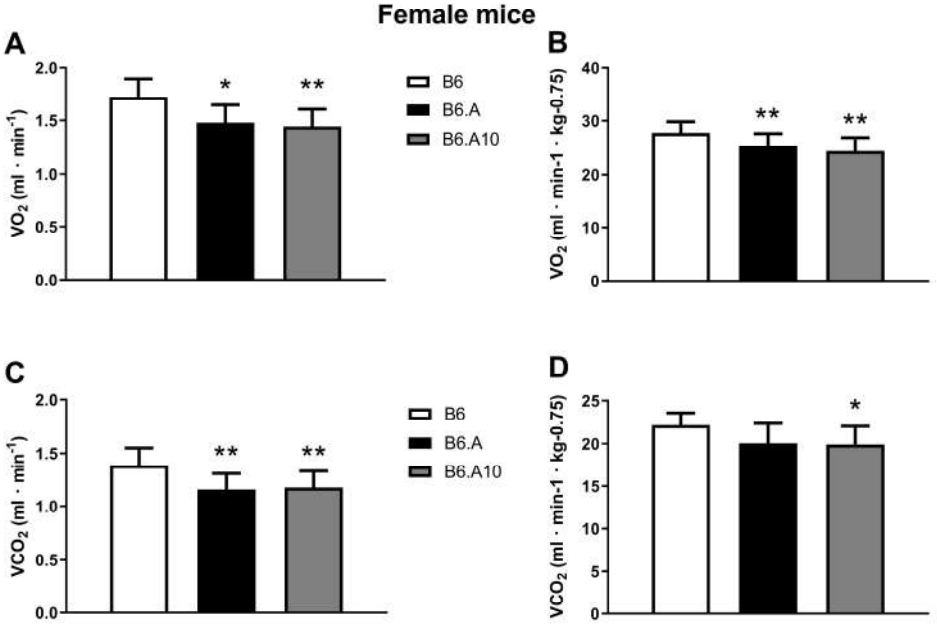

Fig. 10. Absolute (A, C) and relative (B, D) oxygen consumption (A, B) and carbon dioxide output (C, D) rates in B6, B6.A and B6A.10 female mice. VO_2 – oxygen consumption, VCO_2 – carbon dioxide output. Values are means ± *SD.*
* $p < 0.05$, ** $p < 0.01$ – vs. B6 mice

There were no significant differences between the strains in EE among male mice. However, absolute EE was higher in B6.A10 compared to B6.A (13.4 ± 1.1 vs 11.9 ± 1.1 kcal · day^{-1}, respectively, $p < 0.05$) (Fig. 11A). Absolute EE in female B6 (12.0 ± 1.1 kcal · day^{-1}) was higher in comparison to B6.A (10.2 ± 1.2 kcal · day^{-1}) and B6.A10 (10.0 ± 1.1 kcal · day^{-1}) mice ($p < 0.01$) (Fig. 11C). Relative EE in female mice was lower in B6.A10 (169.5 ± 17.0 kcal · $kg^{-0.75}$ · day^{-1}) ($p < 0.01$) compared to B6 (191.8 ± 13.7 kcal · $kg^{-0.75}$ · day^{-1}) mice (Fig. 11D).

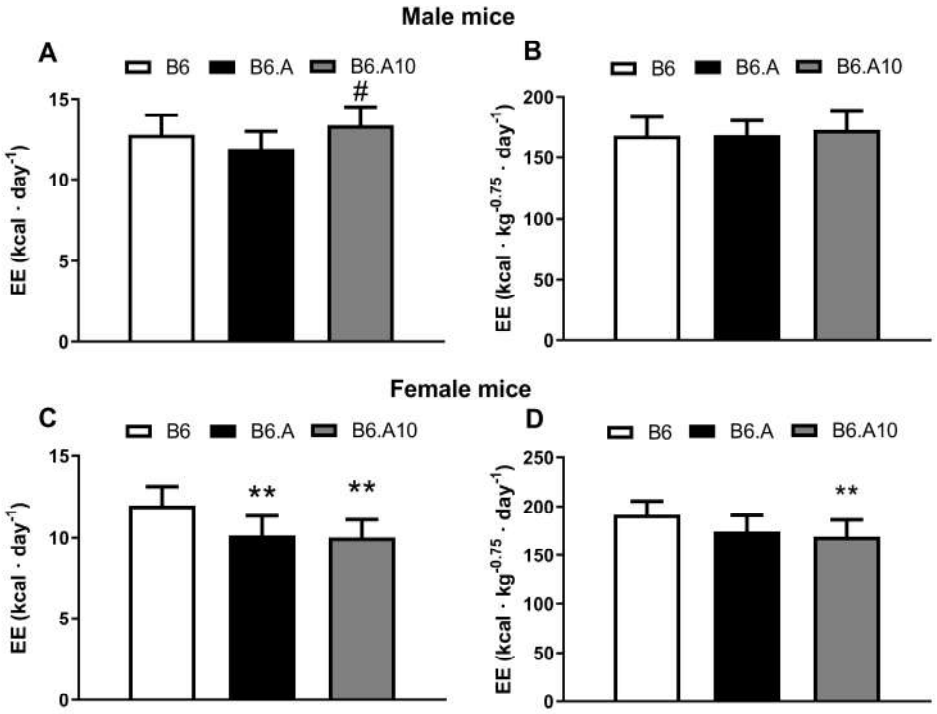

Fig. 11. Absolute (A, C) and relative (B, D) energy expenditure in B6, B6.A and B6A.10 male (A, B) and female (C, D) mice. EE – energy expenditure. Values are means ± *SD*. ** $p < 0.01$ – vs. B6 mice; # $p < 0.05$ is associated with differences between B6.A and B6.A10 mouse strains

In contrast to variation in EE, RQ values were similar among the strains of male (Fig. 12A) and female (Fig. 12B) mice and the average RQ was close to 0.8.

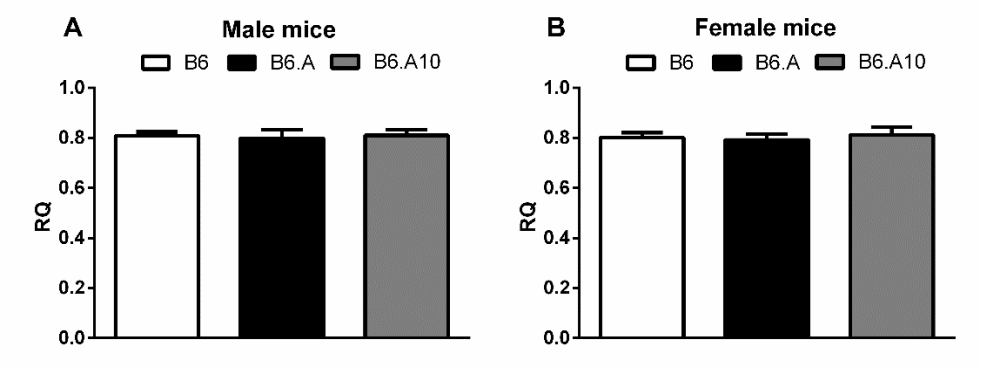

Fig. 12. Respiratory quotient in B6, B6.A and B6A.10 male (A) and female (B) mice. RQ – respiratory quotient. Values are means ± *SD*

Physical activity and rearing did not differ between the strains among male mice (Fig. 13A and 13B). In female mice, however, physical activity of B6.A was lower ($p < 0.05$) than in B6 mice and a similar trend of lower physical activity was observed for B6.A10 strain in comparison to B6 (Fig. 13C). No strain effect was noticed in rearing among female mice with a tendency of higher rearing in B6 compared to B6.A and B6.A10 mice (Fig. 13D).

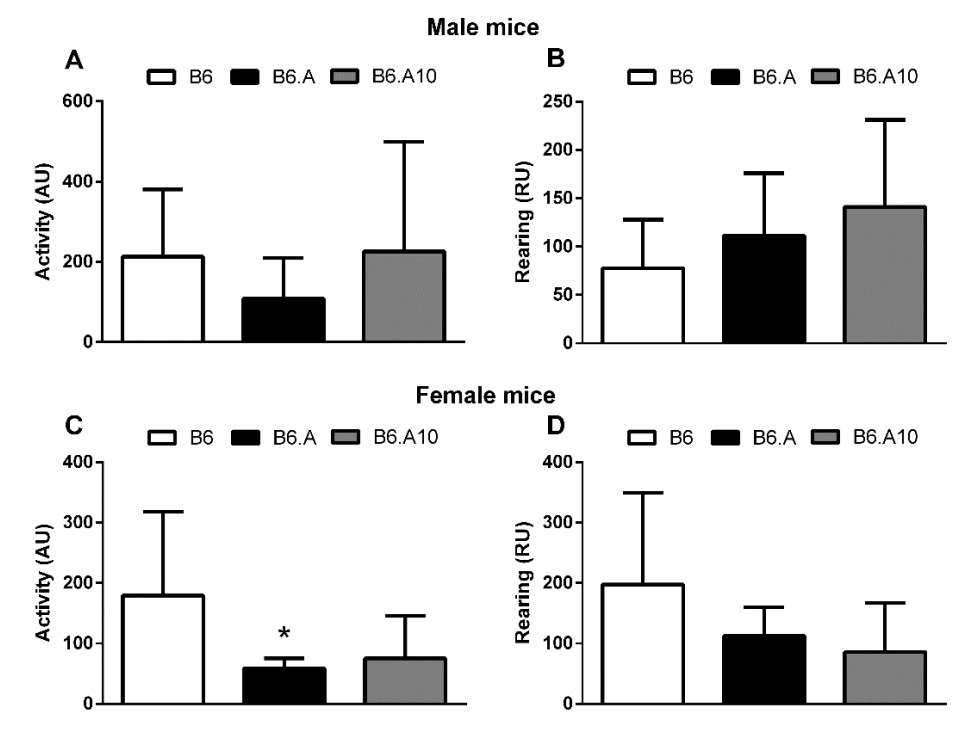

Fig. 13. Physical activity (A, C) and rearing (B, D) parameters in B6, B6.A and B6A.10 male (A, B) and female (C, D) mice. Activity – physical activity. Values are means ± *SD*. * $p < 0.05$ vs. B6 mice

Gender effect on metabolism, physical activity and rearing. Analysis of gender differences was also performed. In B6 mice relative EE was higher in females compared to males (191.8 ± 13.7 and 168.2 ± 15 kcal · $kg^{-0.75}$ · day^{-1}, respectively) ($p < 0.01$), no gender differences among B6.A and B6.A10 mice (Fig. 14A). RQ did not differ between male and female mice (Fig. 14B).

Gender effect on physical activity (Fig. 14C) and rearing (Fig. 14D) was also analyzed. No differences in physical activity were identified between male and female mice ($p > 0.05$) (Fig. 14C). However, there was a tendency for higher

physical activity in males compared to females. Interestingly, the number of rearing was significantly elevated in females ($p < 0.05$) of B6 mice compared to males (Fig. 14D).

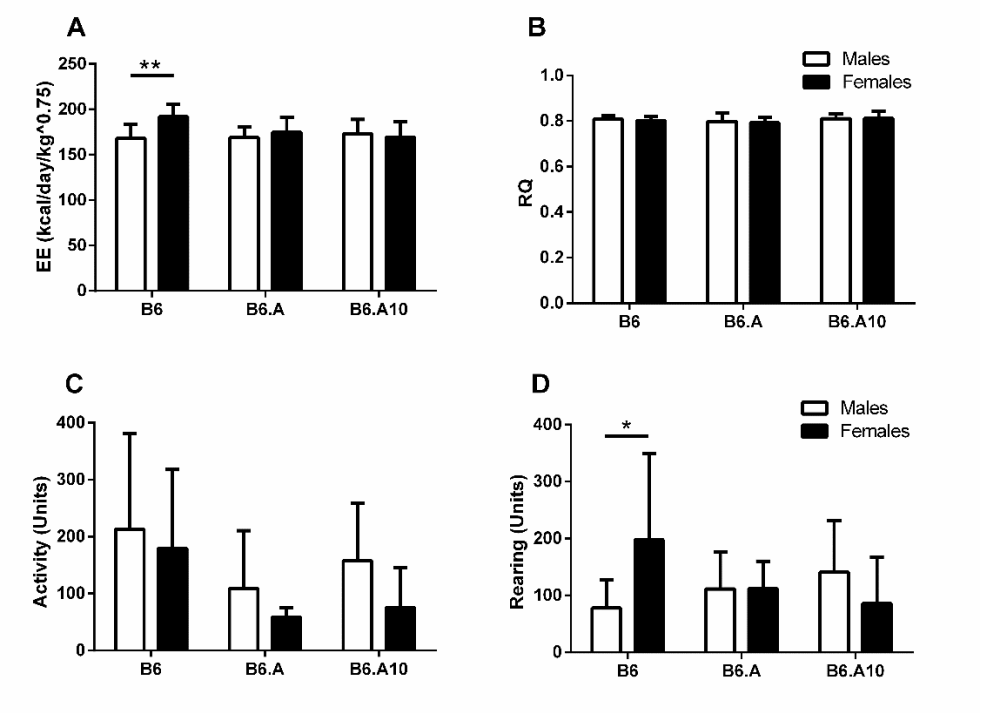

Fig. 14. Energy expenditure (A), respiratory quotient (B), physical activity (C) and rearing (D) differences between B6, B6.A and B6A.10 male and female mice. EE – energy expenditure; RQ – respiratory quotient; activity – physical activity. Values are means ± *SD*. * $p < 0.05$, ** $p < 0.01$ – vs. B6 mice

3.2.2. Discussion

The main aim of this study was to investigate if low CS activity could affect energy expenditure and substrate oxidation in mice. We studied B6 mice with normal CS activity as well as B6.A and B6.A10 strains with low CS activity. We did not observe any major differences between B6, B6.A and B6.A10 mice in metabolic parameters. One exception was detected in female B6.A10 mice, they had lower relative EE compared to B6 mice. Interestingly, EE did not differ between B6.A and B6 females. B6.A mice carry only a fragment of chromosome 10 from A/J strain with mutated *Cs* gene while B6.A10 mice have the whole chromosome 10 from A/J strain (Johnson et al., 2012; Kvedaras et al., 2017).

A genetic linkage map of chromosome 10 revealed many molecular markers (Justice et al., 1990) that could cause differences between B6.A and B6.A10 strains, i.e. genes of chromosome 10 from A/J strain could affect EE in B6.A10 mice without any association with CS activity. Therefore, we could speculate that other genes in chromosome 10 could affect EE in B6.A10 mice with no association with reduced CS enzyme activity. Also, in the current study no high fat diet intervention was performed to evaluate reduced CS performance and effect on oxidation of free fatty acids. For instance, we did not apply any high fat diet intervention which can have a greater effect on EE of B6 mice in comparison to other mouse strains (Cappelli et al., 2014). Stable RQ values estimated in all three tested mouse strains showed that reduced citrate synthase activity under normal conditions (standard rodent diet, no interventions) did not induce energy imbalance in mice metabolism.

Gender differences in metabolism parameters. B6 females weighed less than their B6 male littermates. Our results show that male mice were 20–23 % heavier than females, which is similar to findings of other study (Sanz et al., 2007). Interestingly, gender differences in body mass of rats can reach 70 % (Rollo, 2002). Such a large difference can probably explain differences in ROS production and respiration between sexes in rats (Valle et al., 2005).

We observed greater EE in B6 females compared to males. One study showed higher energy efficiency of male rodents compared to females (Catala-Niell et al., 2008). More studies are needed to confirm this finding. Interestingly, rearing was also significantly higher in B6 females compared to males. Differences in energy expenditure for rearing could be responsible, at least in part, for the differences in energy expenditure between genders (Quevedo et al., 1998; Rodriguez-Cuenca et al., 2002). The interesting part is that rearing was also significantly higher in B6 female mice ($p < 0.05$). Therefore, another possible mechanism of higher EE in B6 female mice compared to males may be higher energy demands because of increased number of rearing during the metabolism experiment.

In summary, this experiment has revealed 10–15 % EE reduction in female mice carrying mutant CS variant. There were no major differences in energy expenditure, substrate oxidation or physical activity between mice with normal and low CS activity.

3.3. Food deprivation. Third study

3.3.1. Results. Low CS activity and food deprivation

B6, B6.A and B6.A10 males were subjected to 48 h food deprivation (FD) (Fig. 15) with *ad libitum* access to water. B6.A mice were lighter than B6 and B6.A10 mice at all three time points, i.e., after 0, 24 and 48 h of FD ($p < 0.01$). FD had a strong negative effect on body mass of all three mouse strains ($p < 0.001$) (Fig. 15A). The loss of body mass during initial 24 h of FD was greater ($p < 0.001$) than during last 24 h ($p < 0.01$) (Fig. 15B). Mouse strains did not differ significantly in weight loss though B6 strain tended to show smaller loss of body mass than B6.A and B6.A10 ($p > 0.05$) (Fig. 15B).

Aggregate muscle mass in B6 was greater than in B6.A ($p < 0.001$), but smaller than in B6.A10 ($p < 0.01$) mice (Fig. 16A). Muscle to body mass ratio was greater in B6.A and B6.A10 mice compared to B6 ($p < 0.01$) (Fig. 16B).

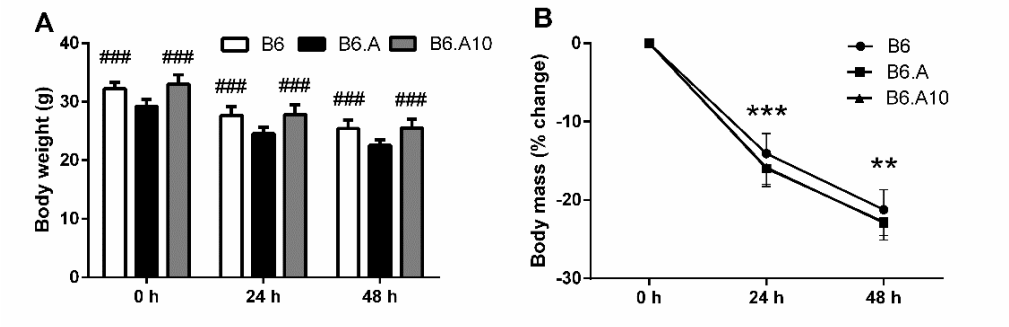

Fig. 15. Strain effect on body weight (A) and relative change of body mass in B6 (n = 9), B6.A (n = 9) and B6A.10 (n = 9) mouse strains during 48 h (0 h, 24 h and 48 h) of food deprivation. Values are means ± *SD*. ### $p < 0.001$ – vs. B6.A mice; ** $p < 0.01$, *** $p < 0.001$ is associated with the previous time point (initial 0 h or 24 h)

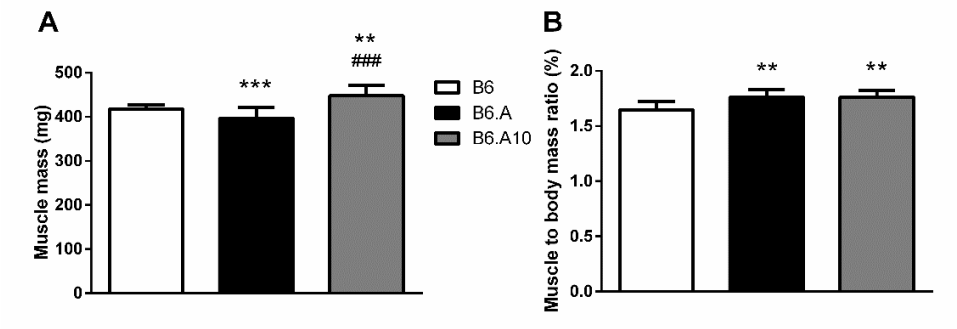

Fig. 16. Sum of right and left leg hindlimb skeletal muscle mass (A) and muscle to body mass ratio (B) in B6, B6.A and B6A.10 mouse strains after 48 h of food deprivation. Values are means ± *SD*. ### $p < 0.001$ – vs. B6.A; ** $p < 0.01$, *** $p < 0.001$ – vs. B6 mice

GAS was heavier in B6.A10 mice compared to B6 ($p < 0.05$) and B6.A ($p < 0.001$) mice (Fig. 17). PL and SOL were heavier in B6.A10 mice than in B6.A ($p < 0.001$) (Fig. 17B, 17C). PL and SOL muscles were lighter in B6.A mice compared to B6 mice ($p < 0.01$ and $p < 0.05$, respectively). TA and EDL showed different results than GAS, PL and SOL muscles. TA was heavier in B6.A mice than in B6 ($p < 0.05$) and in B6.A10 ($p < 0.01$) mice (Fig. 17D). EDL was significantly lighter in B6.A10 mice compared to B6 and B6.A ($p < 0.001$) mice (Fig. 17E). TA of B6.A mice was heavier than in B6 ($p < 0.05$). There was also a trend for higher EDL mass in B6.A than in B6 ($p > 0.05$).

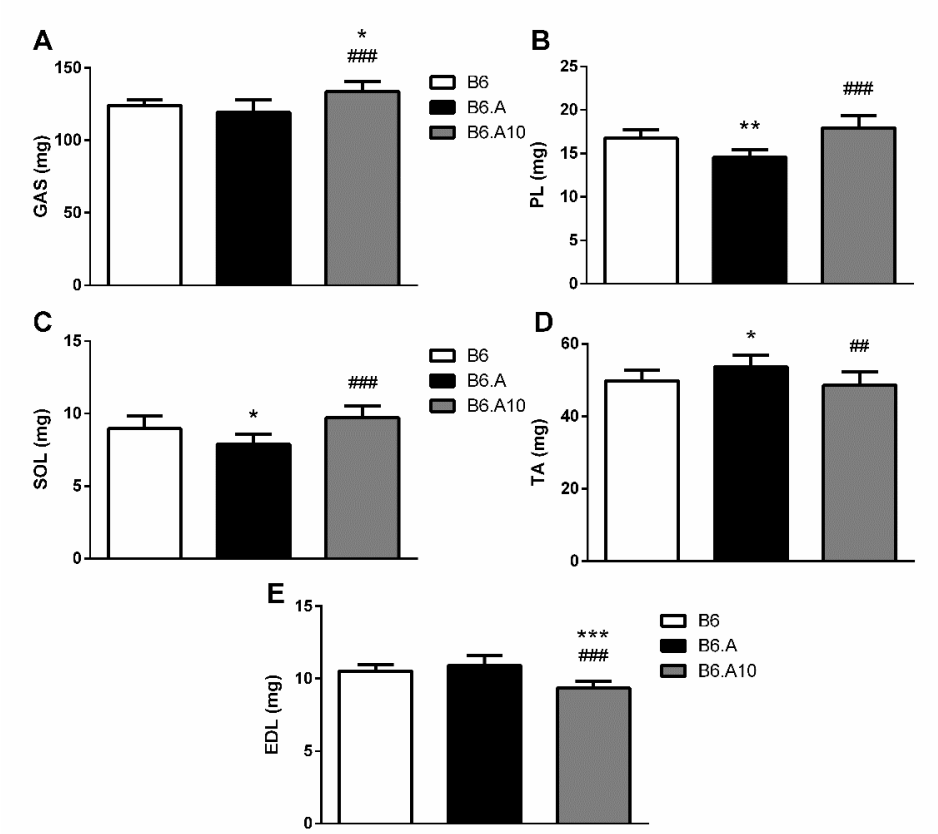

Fig. 17. Mass of hindlimb skeletal muscles in male mice: *gastrocnemius* (A), *plantaris* (B), *soleus* (C), *tibialis anterior* (D) and *extensor digitorum longus* (E) in B6, B6.A and B6A.10 mouse strains after 48 h of food deprivation. Hindlimb muscles: GAS – *gastrocnemius*, PL – *plantaris*, SOL – *soleus*, TA – *tibialis anterior*, EDL – *extensor digitorum longus*. Values are means ± *SD.* ## $p < 0.01$, ### $p < 0.001$ – vs. B6.A; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ – vs. B6 mice

Absolute VO_2 and VCO_2 decreased ($p < 0.001$) after FD in all mouse strains (Fig. 18A and 18C). It did not differ between B6.A and B6.A10 mice (1.7 ± 0.2 ml/min vs 1.9 ± 0.2 ml $\cdot$ min^{-1}, $p > 0.05$) before FD. After FD, VO_2 became lower in B6.A (0.8 ± 0.3 ml $\cdot$ min^{-1}) than in B6.A10 (1.2 ± 0.4 ml $\cdot$ min^{-1}, $p < 0.01$). Before FD VO_2 did not differ between strains, but after FD VO_2 decreased more in B6.A (52.9 %) compared to B6 mice (39 %, $p < 0.01$). Relative VO_2 also decreased after FD in all mouse strains ($p < 0.01$ in B6 and B6.A10 and $p < 0.001$ in B6.A mice). Before FD, relative VO_2 did not differ between strains, but after FD VO_2 decreased more ($p < 0.05$) in B6.A rather than in B6 mice (Fig. 18B). Relative VO_2 decreased by 19.7 % more in B6.A than in B6.A10 after FD. Relative VCO_2 was also significantly lower after FD in all mouse strains ($p < 0.001$) (Fig. 18D).

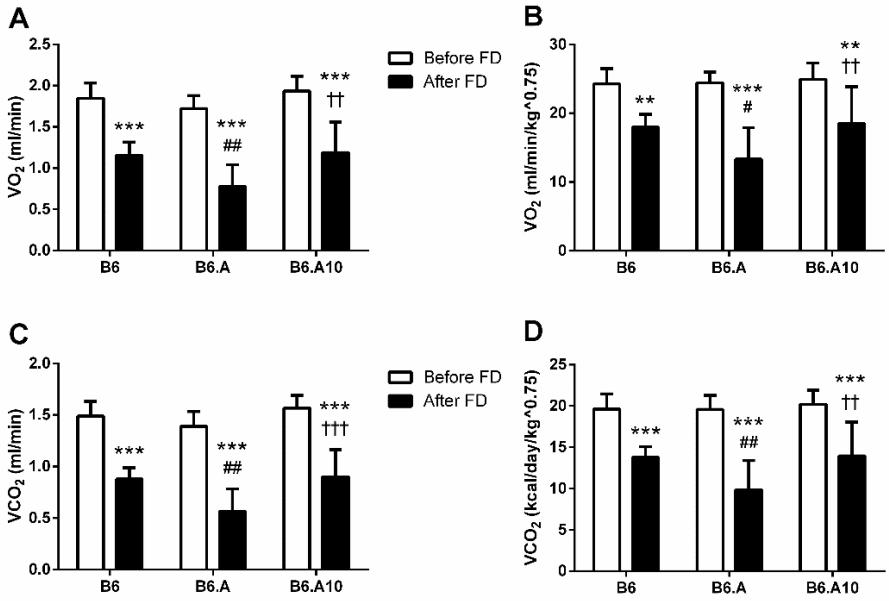

Fig. 18. Absolute (A, C) and relative (B, D) oxygen consumption (A, B) and carbon dioxide output (C, D) rates in B6, B6.A and B6A.10 male mouse strains before and after food deprivation. VO_2 – oxygen consumption, VCO_2 – carbon dioxide output, FD – food deprivation. Values are means ± *SD*. ** $p < 0.01$, *** $p < 0.001$ is associated vs. values before FD; # $p < 0.05$, ## $p < 0.01$ – vs. B6 mice; †† $p < 0.01$, ††† $p < 0.001$ – vs. B6.A

Before FD, absolute VCO_2 in B6.A mice (1.4 ± 0.1 ml $\cdot$ min^{-1}) did not differ compared to B6.A10 mice (1.6 ± 0.1 ml $\cdot$ min^{-1}) ($p > 0.05$). After FD, absolute VCO_2 became strongly decreased in B6.A (0.6 ± 0.2 ml $\cdot$ min^{-1}) in comparison to B6.A10 (0.9 ± 0.3 ml $\cdot$ min^{-1}) ($p < 0.001$). Despite no relative VCO_2 differences

between B6.A and B6.A10 mice observed before FD ($p > 0.05$), After FD VCO_2 decreased 19.7 % more in B6.A (9.9 ± 3.5 ml · min^{-1} · kg$^{-0.75}$) than in B6.A10 (14.0 ± 4.1 ml · min^{-1} · kg$^{-0.75}$) ($p < 0.01$) (Fig. 18D).

Absolute values of EE were greatly lower after FD in all mouse strains ($p < 0.001$) (Fig. 19). After FD values for relative EE were also significantly lower compared to the results before FD in all mice strains ($p < 0.01$). The lowest relative EE values were observed in B6.A (90.7 ± 31.4 kcal · kg$^{-0.75}$ · day^{-1}) mice. Relative EE was higher in B6 (123.1 ± 12.4 kcal · kg$^{-0.75}$ · day^{-1}) compared to B6.A (90.7 ± 31.4 kcal · kg$^{-0.75}$ · day^{-1}) strain after FD intervention ($p < 0.05$) (Fig. 19B).

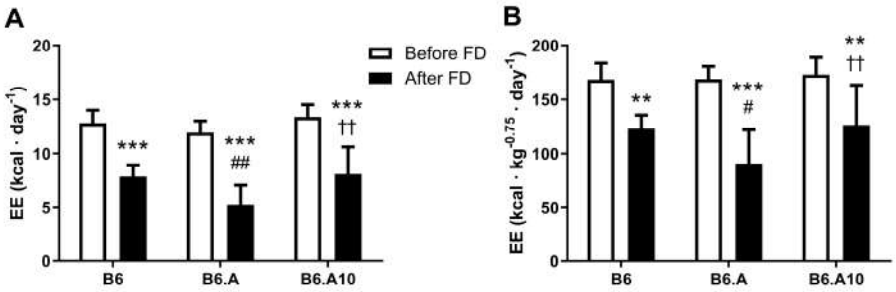

Fig. 19. Absolute (A) and relative (B) energy expenditure in B6, B6.A and B6A.10 male mouse strains before and after food deprivation. EE – energy expenditure, FD – food deprivation. Values are means ± *SD*. ** $p < 0.01$, *** $p < 0.001$ is associated vs. values before FD; # $p < 0.05$, ## $p < 0.01$ – vs. B6 mice; †† $p < 0.01$ – vs. B6.A mice

RQ was assessed before and after FD intervention (Fig. 20). FD was associated with reduction in RQ in all mice ($p < 0.05$ in B6, $p < 0.001$ in B6.A, $p < 0.01$ in B6.A10 mice, respectively).

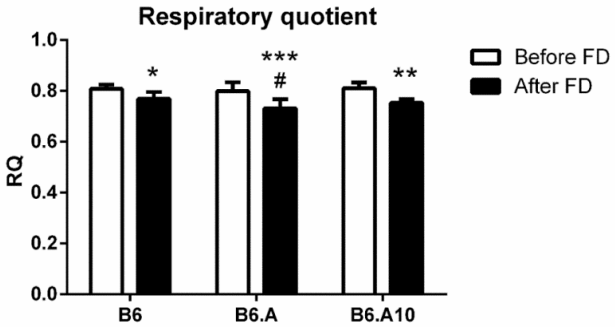

Fig. 20. Respiratory quotient in B6, B6.A and B6A.10 mouse strains before and after food deprivation. RQ – respiratory quotient, FD – food deprivation. Values are means ± *SD*; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ is associated vs. values before FD; # $p < 0.05$ – vs. B6 mice

RQ values were similar in all three mice strains ($p > 0.05$) and did not exceed 0.8 before FD. After FD RQ values have fallen down to approximately 0.7. The lowest RQ was noticed in B6.A strain (RQ = 0.73). The only slight but statistically significant difference was identified in B6.A (0.73 ± 0.04) strain compared to B6 (0.77 ± 0.03) ($p < 0.05$) (Fig. 20). After FD there was no difference in RQ between B6.A and B6.A10 strains (0.73 ± 0.04 vs 0.75 ± 0.01, $p > 0.05$).

There were no differences in physical activity between the strains ($p > 0.05$) (Fig. 21A). There was also a tendency for a decrease in physical activity after FD in B6 and B6.A mice, but not B6.A10. Similarly, mouse strains did not differ in rearing. However, B6.A strain had greater values for rearing before FD intervention compared to after FD ($p < 0.05$) (Fig. 21B). The trend of lower rearing values after FD were noticed in all three mice strains (B6, B6.A and B6.A10) compared to the rearing values before FD. The sum of rearing values of all three tested mice strains was significantly higher before FD compared to the rearing values after FD ($p < 0.05$).

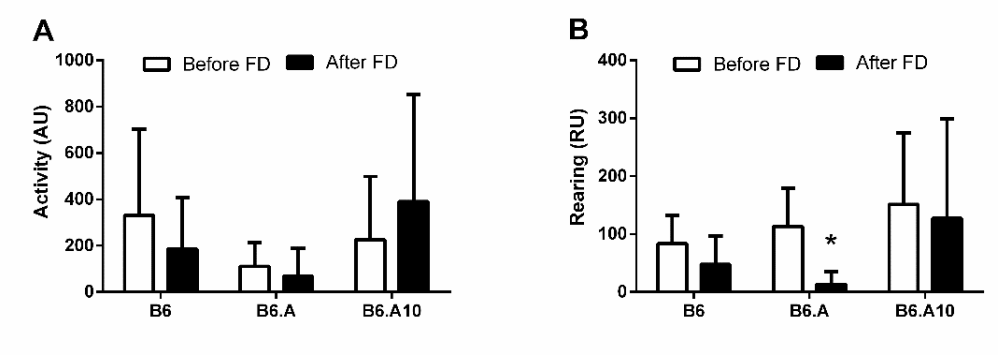

Fig. 21. Physical activity (A) and rearing (B) differences between B6, B6.A and B6.A10 mice strains before and after food deprivation. FD – food deprivation. Values are presented as means ± *SD*; * $p < 0.05$ is associated with significant FD effect in B6.A strain

CS activity in *gastrocnemius* muscle was assessed after FD from all mice of B6, B6.A and B6.A10 strains (Fig. 22). No differences were noticed between B6.A and B6.A10 strains, but both strain CS activity was lower than in control B6 group ($p < 0.001$).

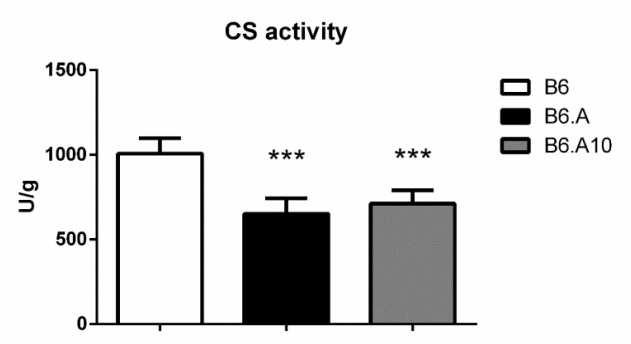

Fig. 22. Citrate synthase activity after food deprivation between B6, B6.A and B6.A10 mouse strains. CS – citrate synthase. Values are presented as means ± *SD*; *** $p < 0.001$ vs. B6 mice

3.3.2. Discussion

As expected, B6.A and B6.A10 strains had lower CS activity in the *gastrocnemius* muscle compared to B6 mice. A/J mice show 50–65 % reduction in CS activity compared to other mouse strains (Ratkevicius et al., 2010). Both B6.A and B6.A10 strains carry A/J variant *Cs* gene which resides in the telomeric region of mouse chromosome 10. Our results showed reduction in CS activity of 35 % for B6.A mice and 29 % for B6.A mice in comparison to B6 mice. CS has been used as a biomarker of mitochondrial functioning (Jacobs et al., 2013). Our results are not in conflict, that CS activity cannot be used as biomarker for mitochondria in B6.A and B6.A10 strains.

B6.A, B6.A10 and B6 mice did not differ in loss of body mass during 48 h of FD. B6.A and B6.A10 strains had greater muscle to body mass ratio compared to B6 mice. Also, aggregate muscle mass (sum of muscle mass) was greater in B6.A10 mice compared to B6 and B6.A. It appears that mouse chromosome 10 includes loci affecting neural development, bone development and cell growth pathways that may affect muscle weight (Justice et al., 1990).

The rate of RQ in mice usually ranges from 1.0 to 0.7. The value of 1.0 represents a value expected for oxidation of carbohydrates, whereas the value of 0.7 represents a value expected for oxidation of FA (Schutz, 1995). Fatty acids require more oxygen for their complete oxidation and are associated with lower respiratory quotients compared to carbohydrates (Kuo, Shiao, & Lee, 1993). FD induced a decrease in RQ to values below 0.8 in all three mouse strains suggesting that FA oxidation became the major source of energy during acute

starvation in all three mouse strains (Ellis, Hyatt, Hunter, & Gower, 2010).

One of the main aims of our study was to determine, if low CS activity could accelerate substrate oxidation in mitochondria and promote lipid oxidation in freely moving mice. If that is true, inhibition of CS activity would have a potential in treating insulin resistance. Individual differences in circulating insulin or insulin sensitivity may also confound reported associations between RQ and fatty substrate metabolism. Insulin is an anabolic hormone that acts to decrease fat oxidation and increase lipid storage (J. E. Galgani, Moro, & Ravussin, 2008). Accordingly, fasting serum insulin has been positively correlated RQ (Nagy et al., 1996). Human studies dictated, that enhanced tissue sensitivity to insulin has also been associated with FA accumulation (Hoffman, Stumbo, Janz, & Nielsen, 1995; Travers, Jeffers, & Eckel, 2002). These studies do not disagree with RQ results, obtained in our study before and after fasting.

After food deprivation EE (both absolute and relative) and RQ were lower in B6.A mice compared to B6. We can speculate, that lower CS activity disrupts process of energy production in B6.A mice, which enhances energy deficit during starvation. This is in line with the greater body mass decrement in B6.A mice. Lower RQ in mice with low CS activity indirectly shows, that fatty acid oxidation is more enhanced compared to control group as a main source of energy during long-term fasting.

No activity and rearing differences between strains were identified in our experiments. A significant variation of the data could have been the main factor here.

In summary, all the metabolic parameters decreased significantly after food deprivation in mice with both wild type and low CS activity. After food deprivation aggregate muscle mass and muscle to body mass ratio were lower in mice with wild type CS activity compared to mice with low CS activity. Hence, there were no data of muscle mass before food deprivation, which is a limitation of our study. After food deprivation relative EE and RQ were lower in B6.A mice compared to B6.

3.3.3. Results. Myostatin dysfunction and food deprivation

The Fig. 23 shows the morphometric data for BEH+/+ and BEH strains in the CON group and after 48-h FD. BEH mice were heavier, had greater hindlimb muscle mass, but less fat than BEH+/+ mice when fed *ad libitum* (Fig. 23A, C, D). FD led to a gradual decrease in body mass (Fig. 23A, B). This decrease was faster

in BEH+/++ than BEH strains during the first 24 h, but there was no difference between the strains in the relative weight loss after 48 h of FD. Both strains experienced a relatively small (~6 %) decrease in the combined hindlimb muscle mass (Fig. 23C) and a large (> 30 %) depletion of fat reserves (Fig. 23D). It is worth to mention, that there was a tendency of greater combined muscle mass decrease in BEH mice after food deprivation (Fig. 23C).

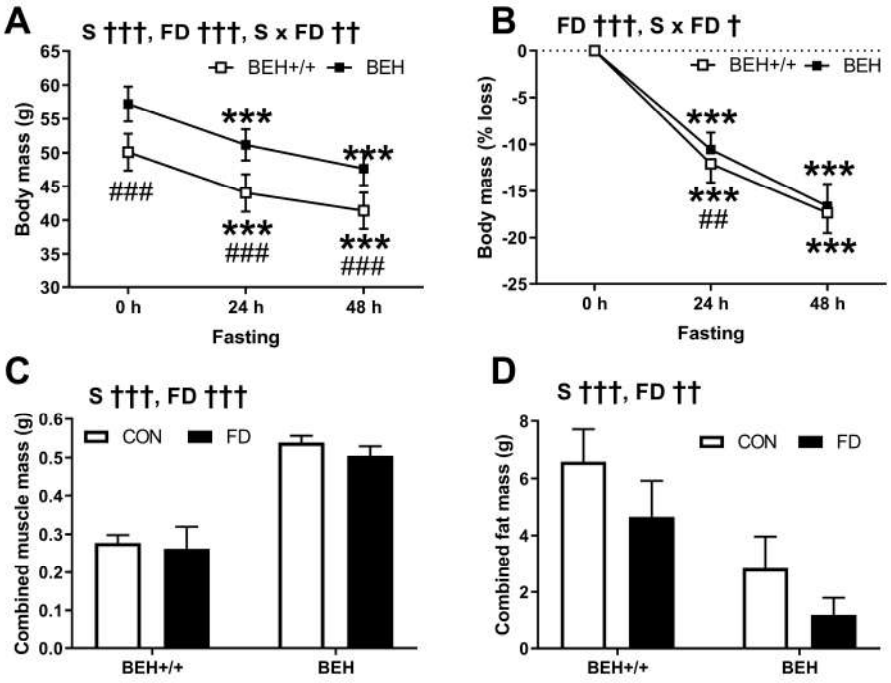

Fig. 23. The morphometric data for BEH+/+ and BEH strains including body mass (A, B) during 48-h food deprivation as well as the combined hindlimb muscle mass (C), the combined fat mass (D) in the control (CON) and food-deprived (FD) groups. Values are shown as mean ± *SD*; † $p < 0.05$, †† $p < 0.01$, ††† $p < 0.001$, two-way ANOVA for effects of strain (S), food deprivation (FD) and S × FD interaction, respectively; *** $p < 0.001$, post-hoc testing for differences from the initial value (0 h); ## $p < 0.01$, ## $p < 0.001$ differences between the strains, respectively

Data on fat from four different sampling sites is presented in Fig. 24. Three-way ANOVA showed significant effect of sampling site ($p < 0.001$), strain ($p < 0.001$) and FD ($p < 0.01$) with interactions of sampling site × strain ($p < 0.001$), sampling site × FD ($p < 0.001$), FD × strain ($p < 0.05$). Fat mass was larger in BEH+/+ mice compared to BEH mice and FD induced loss of fat at all

sampling sites. This FD induced fat depletion tended to be greater for vWAT and iBAT compared to sWAT and gWAT (Fig. 24).

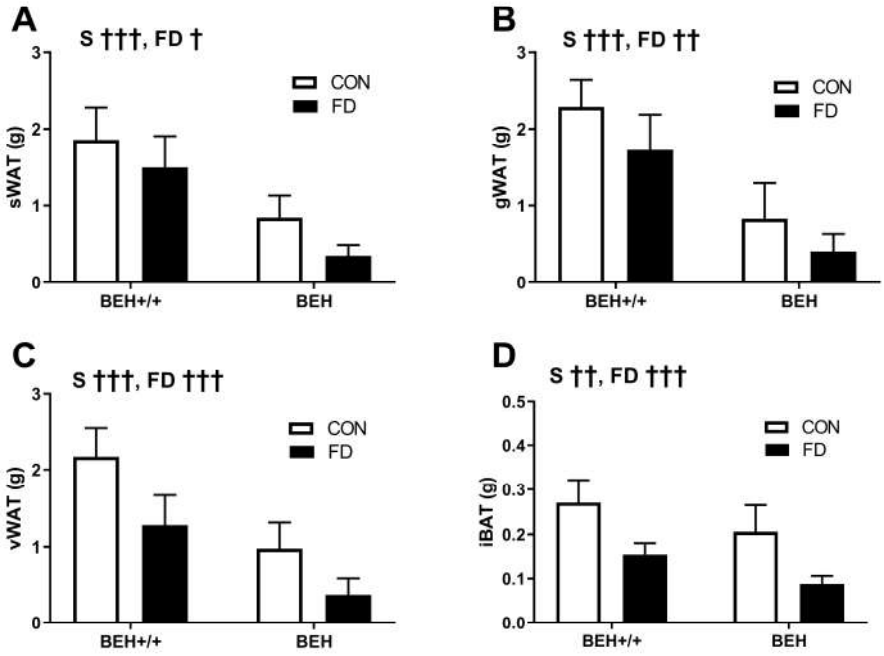

Fig. 24. Fat mass at four different sampling sites is shown for BEH+/+ and BEH strain in the control group (CON) and after 48-h food deprivation (FD). sWAT – adipose subcutaneous fat; gWAT – gonadal fat; vWAT – visceral fat; iBAT – intrascapular brown adipose tissue fat. Data are presented as mean ± *SD*; † $p < 0.05$, †† $p < 0.01$, ††† $p < 0.001$, two-way ANOVA for baseline effects of strain (S) and food deprivation (FD), respectively

Muscle fiber properties of SOL muscle are presented in Fig. 25. BEH+/+ and BEH mice did not differ in the total fiber number which was not affected by FD. BEH+/+ mice had greater percentage of type 1 fibers than BEH mice. FD did not affect muscle fiber type composition. Three-way ANOVA did not show any significant effect of fiber type, strain or FD on muscle fiber cross-sectional area. The FD effect was just below the significance level ($p = 0.065$) in this analysis. For CON group, however, two-way ANOVA showed a significant strain effect ($p = 0.036$) but no fiber type effect as cross-sectional area of type 1 and 2 fibers was larger by 15 % and 19 % in BEH mice compared to BEH+/+ mice, respectively.

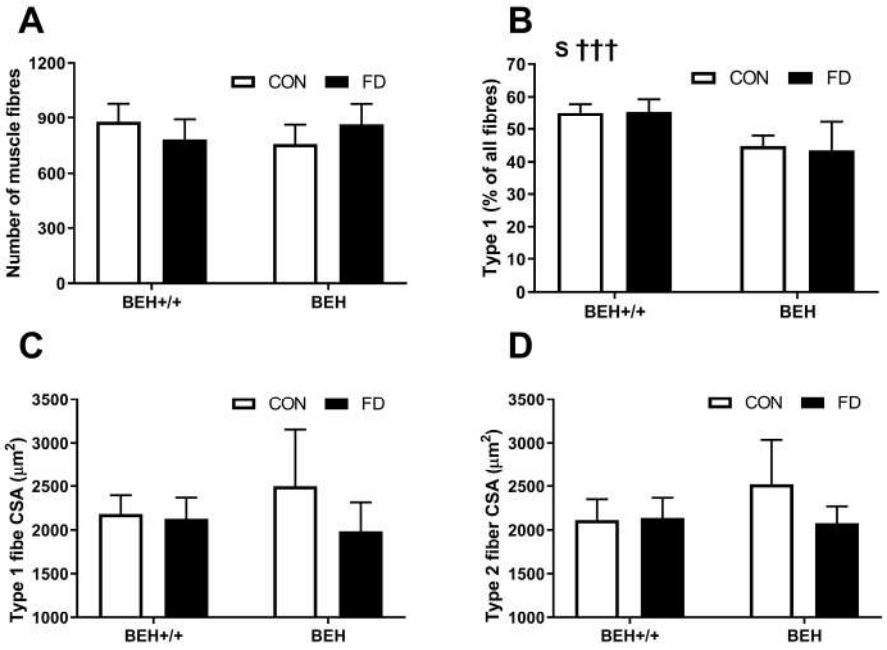

Fig. 25. Total number of soleus (SOL) muscle fibers (A), the muscle fiber type composition (B) and cross-sectional area for type 1 (C) and type 2 (D) fibers in the control (CON) group as well as in mice after 48-h of food deprivation (FD) for BEH+/+ and BEH strains, respectively. Data are shown as mean ± SD; ## $p < 0.01$, BEH+/+ versus BEH, respectively. Total number of muscle fibers (A), muscle fiber type composition (B) and cross-sectional area for type 1 (C) and type 2 (D) fibers in control (CON) mice as well as in mice after 48-h of food deprivation (FD) for BEH+/+ and BEH strains, respectively. ††† $p < 0.001$, two-way ANOVA for strain (S) effects.

Metabolic characteristics and physical activity of mice are presented in Fig. 26. Food intake did not differ between BEH+/+ and BEH strains. Energy expenditure (EE) was greater in BEH compared to BEH+/+ mice, but the covariance analysis using body mass as a covariate did not show any significant difference between the strains. FD induced a decrease in EE of both stains. RQ as well as physical activity did not differ between the strains and decreased after FD. CS enzyme activity of GAS muscle was lower in BEH mice compared to BEH+/+ mice and the negative effect of FD was below the significance level ($p = 0.089$).

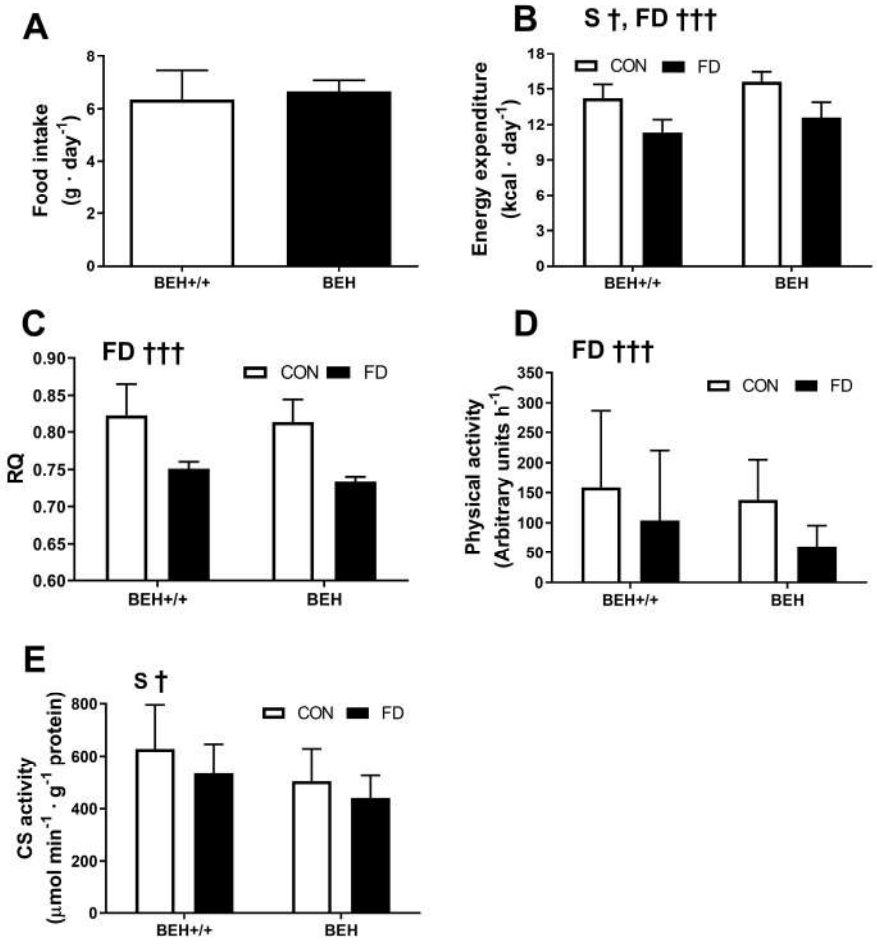

Fig. 26. Food intake (A), energy expenditure (B), respiratory quotient (RQ) (C), physical activity (D) and citrate synthase (CS) activity (E) in BEH+/+ and BEH mice under the control conditions (CON) of *ad libitum* feeding and after 48-h food deprivation (FD). Data are shown as mean ± *SD*; † $p < 0.05$, ††† $p < 0.001$, two-way ANOVA for effects of strain (S) and food deprivation (FD), respectively

3.3.4. Discussion

The main aim of the study was to evaluate effects of myostatin dysfunction on physiological response to FD which leads to acute starvation. We tested the hypothesis that myostatin dysfunction protects against muscle atrophy during fasting. Our results do not support this hypothesis. On the contrary, after 48-h food deprivation BEH mice with myostatin dysfunction tended to have a greater decrease in muscle mass compared to BEH+/+ mice which carry the wild type normally functioning myostatin.

Mice were fasted as in other studies of myostatin dysfunction (Collins-Hooper et al., 2015a, 2015b; Koves et al., 2008). After 48-h food deprivation there was ~17 % decrease in body weight of BEH+/+ and BEH mice. Allen et al. (2010) reported 25–28 % decrease in body mass of C57BL/6J mice which have two-fold smaller body mass than BEH mice. Body mass normalized metabolic rate decreases with increase in body mass of mammals (Hochachka, Darveau, Andrews, & Suarez, 2003). Obese women weighing approximately 80 kg lost only 2.5 % of body mass after 48-h fasting (Solianik & Sujeta, 2018). Our analysis of energy expenditure suggests that BEH mice expend at least 6 times more energy per unit of body mass than *homo sapiens* (Pontzer et al., 2016). Differences in weight loss between mice and humans suggest that metabolic rate is a key factor in food deprivation-induced weight loss.

RQ fell from 0.82 to 0.72 over the 48-h period fasting in both mouse strains suggesting that fat was the major source of energy during fasting. As in B6 mice, BEH mice with myostatin dysfunction had less body fat than BEH+/+ mice when fed ad libitum (Amthor et al., 2007; McPherron & Lee, 2002; Zhao et al., 2005). Differences in metabolism experimental groups might also affect degree of muscle atrophy. The previous studies showed that BEH and BEH+/+ strains did not differ in RQ while C57BL/6 mice null for myostatin show greater RQ compared to the wild type controls (Guo et al., 2009; McPherron & Lee, 2002). Thus, myostatin dysfunction is associated with a significant shift from fat to carbohydrate oxidation in C57BL/6 strain, but not in BEH mice. This shift is expected to promote gluconeogenesis from amino acids and increase loss of muscle mass in C57BL/6 mice null for myostatin compared to the wild type controls. This would provide another line of evidence contradicting findings of Allen et al. (2010). Unfortunately, no RQ data is available for C57BL/6 mice null for myostatin during food deprivation to support our contention. Nevertheless, our data provides another line of evidence that genetic background is of importance in mediating physiological effects of myostatin dysfunction on the whole-body metabolism in addition to skeletal muscle properties (Lionikas, Kilikevicius et al., 2013).

48-h food deprivation led to a decrease in energy expenditure in both BEH and BEH+/+ strains. Decreases in physical activity, diet induced thermogenesis as well as alteration in hormone profile could be of significance under these conditions (Hambly & Speakman, 2005; S. E. Mitchell et al., 2017; S. E. Mitchell et al., 2015). The observed decrease in physical activity is in contrast to feeding

anticipatory behavior which increases physical activity in mice subjected to 30 % caloric restriction (van Norren et al., 2015). We have also noted a significant reduction in interscapular fat which is dominated by a brown fat (Connolly, Morrisey, & Carnie, 1982). This reduction might also play a role in lower heat production and reduction in energy expenditure of the fasted mice (Connolly et al., 1982).

48-h food deprivation did not have a significant effect on CS activity which was lower in the gastrocnemius of BEH mice compared to BEH+/+ mice. CS is a popular marker of mitochondrial content in mice and humans (Jacobs et al., 2013; Vigelsø, Andersen, & Dela, 2014). Thus, it is unlikely that the decline in specific muscle force was due to impaired capacity for aerobic ATP resynthesis.

From the other point of view, fasting can be associated with upregulation of proteolytic and autophagy programs in skeletal muscles (Collins-Hooper et al., 2015a, 2015b). Lack of data on molecular mechanisms contributing to muscle atrophy might be considered as a limitation of the current study. On the other hand, extent of muscle wasting and differences between BEH and BEH+/+ strains were rather small and varied significantly between the muscles. It seems that muscle cell cultures with strict control of experimental conditions are better suited for studies of molecular mechanisms of myostatin effects (Manfredi, Paula-Gomes, Zanon, & Kettelhut, 2017).

In summary, the results of current study showed that myostatin dysfunction does not protect from skeletal muscle wasting during fasting. 48 h food deprivation is associated with a significant decrease in physical activity and energy expenditure of mice.

3.4. Weight loss dietary interventions. Fourth study

3.4.1. Results

In all the experiments of this study B6 mice were used. Data on energy intake is presented in Fig. 27. We aimed at maintaining similar energy intake in *Low-Fat* and *Low-Carb* groups during caloric restriction (CR). However, *Low-Carb* group did not consume all the food during the first week of CR, and the unconsumed food was left in the feeder with subsequent daily portion added on top of the leftovers. After two weeks of CR, *Low-Carb* group “caught-up” *Low-Fat* group for energy intake. For the entire 6-week CR, these groups did not differ in

the absolute (Fig. 27A) or body mass normalized energy intake when body mass before the start of CR was used for normalization (12.0 ± 0.4 and 12.1 ± 0.3 kcal · g initial body wt^{-1} for *Low-Fat* and *Low-Carb*, respectively; $p > 0.05$) (Fig. 27B). Mice in the *Regular* diet group had ~15 % greater energy intake compared to *Low-Fat* ($p = 0.007$) and *Low-Carb* ($p = 0.008$) groups during the same period (Fig. 27A).

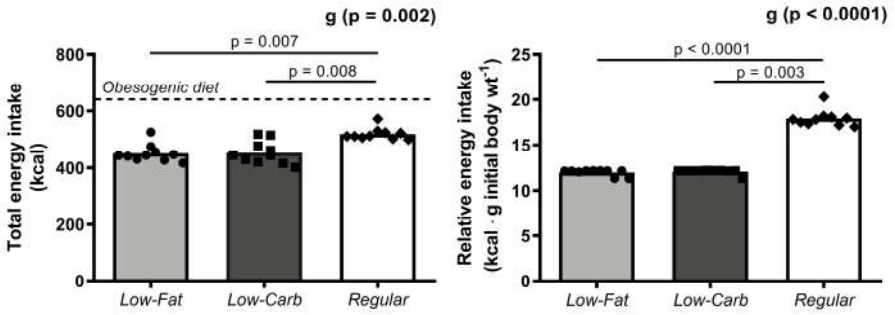

Fig. 27. Energy intake for *Low-Fat* and *Low-Carb* groups during 6-week caloric restriction (CR) and in *Regular* group fed standard chow diet *ad libitum*. Total energy intake is shown in absolute values (A) and normalized to body mass prior to CR (B). Data are presented as mean with each dot representing one mouse data sample. Non-parametric Kruskal–Wallis with Dunn's *post hoc* analysis was performed for group effect (g). Lines indicate significant differences between connected groups. Dotted reference line indicates average *ad libitum* energy intake of the obesogenic diet during a 6-week period before assignment to CR with *Low-Fat* or *Low-Carb* diet

The results of body mass are presented in Fig. 28. *Low-Fat* group tended to lose more weight than *Low-Carb* group during the first week of CR (Fig. 28A). This was probably due to the reduced food intake in *Low-Fat* group. Afterwards, however, *Low-Fat* group caught up with food intake and showed similar weight loss as *Low-Carb* group. Overall body mass loss did not differ between these two diet groups after 6-week CR (30.0 ± 5.6 % and 23.8 ± 7.5 % for *Low-Fat* and *Low-Carb*, $p > 0.05$, respectively, Fig. 28B). All mice showed clear reductions in body mass (Fig. 28C). Initially mice in the *Regular* diet group which was not subjected to obesogenic feeding had lower body mass ($p < 0.001$) than *Low-Fat* and *Low-Carb* groups, but the differences between the groups became absent of statistical significance during the final four weeks of CR which was applied to *Low-Fat* and *Low-Carb* groups only. *Regular* diet group showed a small reduction in body mass during a final week when measurements of energy metabolism and glucose tolerance were performed after the overnight fast.

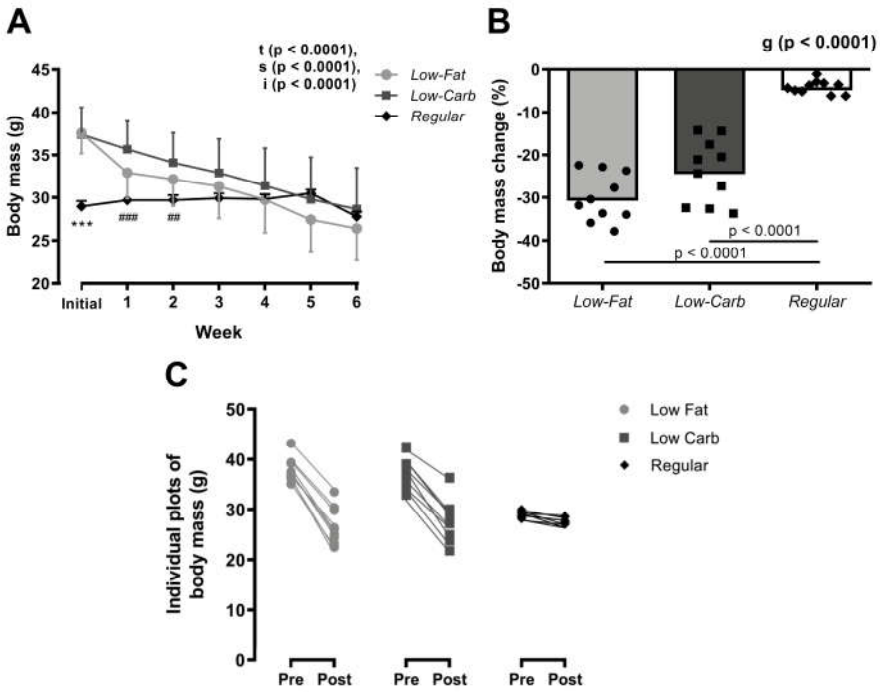

Fig. 28. Body mass during 6-week caloric restriction (CR) in *Low-Fat* and *Low-Carb* diet groups as well as in *Regular* group fed standard chow diet *ad libitum* for the same period. Data are shown as weekly measurements (A), percentage change from initial value (B) and individual plots for the 6-week CR period (C). Data are mean ± *SD* (A) or mean with plotted individuals dots (B, C). Each dot represents one mouse data sample. Two-way repeated measures ANOVA (A) with Bonferroni's *post hoc* analysis was performed for effects of group (g), time (t), subject (matching) (s) and interaction (i), respectively. One-way ANOVA (B) with Bonferroni's *post hoc* analysis was performed |for group effect (g). Lines indicate significant differences between connected groups. *** $p < 0.001$ vs. *Low-Fat* and *Low-Carb*, ## $p < 0.001$, ### $p = 0.005$ vs. *Low-Carb*

Data on muscle and fat mass is shown in Fig. 29. *Pre* group included mice that were subjected to obesogenic diet, but did not undergo CR. This group was used to assess effects of CR on muscle and fat mass in *Low-Fat* and *Low-Carb* groups. *Regular* diet group provided age-matched reference data. Combined muscle mass differed little between the groups though it was by ~5 % smaller ($p = 0.02$) in *Low-Fat* group compared to *Pre* group (Fig. 29A). Muscle mass per unit of body mass increased following 6-week CR in *Low-Fat* ($p < 0.001$) and *Low-Carb* ($p = 0.001$) groups (Fig. 29B). On the other hand, body fat for these groups decreased ($p < 0.0001$) to the level of *Regular* diet group (Fig. 29C) and became significantly lower than in *Pre* group (6.09 ± 2.73 % and 8.57 ± 4.55 % vs. 15.50 ± 3.28 % body mass, $p < 0.0001$, for *Low-Fat* and *Low-Carb* vs. Pre groups, respectively, Fig. 29D).

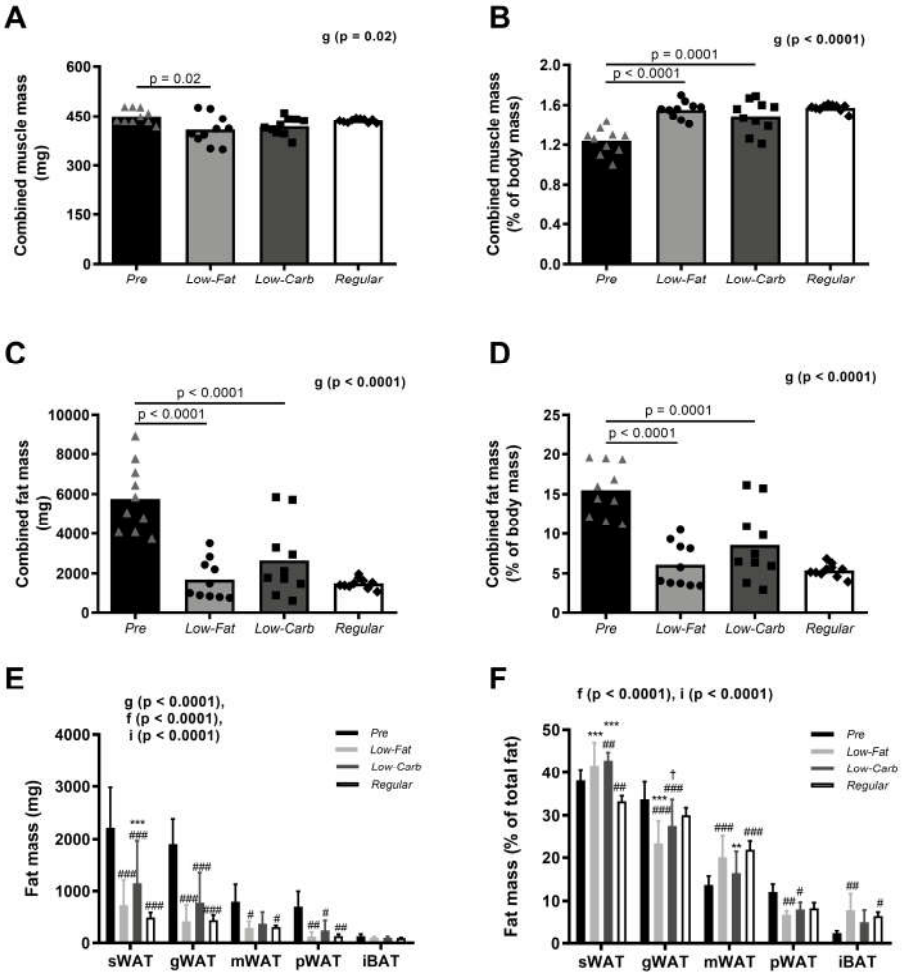

Fig. 29. Mass changes of skeletal muscle (A, B), body fat (C, D) and fat from different sampling sites (E, F) in *Low-Fat* and *Low-Carb* diet groups after 6-week caloric restriction (CR) compared to the obese group prior CR (*Pre*) as well as to the age-matched *Regular* group fed standard chow diet *ad libitum* for the same period. Abbreviations (E, F): sWAT, subcutaneous white adipose tissue; gWAT, gonadal white adipose tissue; mWAT, mesenteric white adipose tissue; pWAT, perirenal white adipose tissue; iBAT intrascapular brown adipose tissue. Data are mean ± *SD* (E, F) or mean with plotted individuals dots (A–D). Each dot represents one mouse data sample. One-way ANOVA (A–D) with Bonferroni's *post hoc* analysis was performed for group effect (g). Two-way repeated measures ANOVA (E, F) with Bonferroni's *post hoc* analysis was performed for effects of group (g), fat site (f) and interaction (i), respectively. Lines indicate significant differences between connected groups. # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ vs. Pre, ** $p < 0.01$, *** $p < 0.001$ vs. *Regular*; † $p = 0.04$ vs. *Low-Fat*

We have also examined body fat distribution by sampling fat from five different sites of the body. Both *Low-Fat* and *Low-Carb* diets reduced fat mass in four out of five sites to the level of *Regular* diet group (Fig. 29E). An exception was iBAT which was not significantly affected by the diets and did not differ between the studied groups. Thus, CR tended to increase relative iBAT mass compared to the values prior to CR (*Pre* group), but this increase was significant only for *Low-Fat* group ($p = 0.002$) (Fig. 29F). Relative mass of gWAT decreased more in *Low-Fat* than *Low-Carb* group ($p = 0.04$).

Data on glucose tolerance is presented in Fig. 30. Glucose AUC was similar in *Low-Fat* and *Low-Carb* groups ($p > 0.05$), but smaller compared to *Pre* group ($p < 0.01$) and *Regular* diet group ($p < 0.05$) (Fig. 30B). *Pre* and *Regular* diet groups did not differ in glucose AUC. However, *Regular* diet group demonstrated a large initial spike with subsequent normalization of blood glucose to baseline values whereas *Pre* group showed slow rise in blood glucose which did not show any decrease during the entire 2 h duration of the test (Fig. 30A).

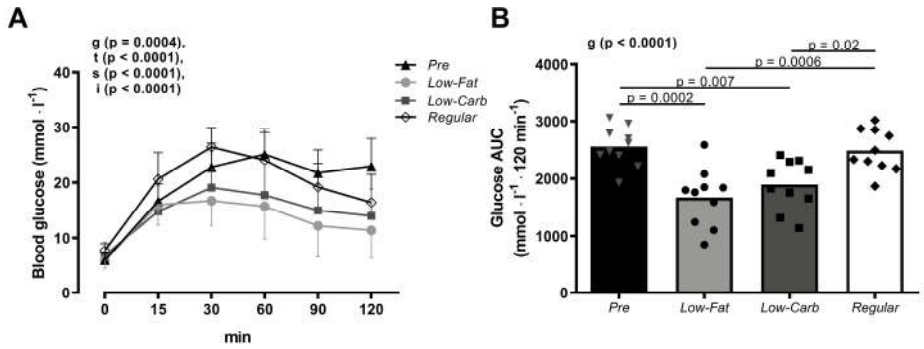

Fig. 30. Pattern of blood glucose clearance during a 120 min period (A) and glucose area under curve (AUC) (B) in *Low-Fat* and *Low-Carb* diet groups after 6-week caloric restriction (CR) compared to the obese group prior CR (*Pre*) as well as to the age-matched *Regular* group fed standard chow diet *ad libitum* for the same period. Data are mean ± *SD* (A) or mean with plotted individuals dots (B). Each dot represents one mouse data sample. Two-way repeated measures ANOVA (A) with Bonferroni's *post hoc* analysis was performed for effects of group (g), time (t) and interaction (i), respectively. One-way ANOVA (B) with Bonferroni's *post hoc* analysis was performed for group effect (g). Lines indicate significant differences between connected groups

Data on energy metabolism are presented in Fig. 31. Total energy expenditure did not differ between *Low-Fat* and *Low-Carb* groups ($p < 0.05$) (Fig. 31A). *Pre* group showed higher ($p = 0.02$) energy expenditure than *Regular* diet group, but ANCOVA analysis with body mass and physical activity as

covariates did not show any significant differences between the groups and showed that physical activity but not body mass had an effect on energy expenditure. There were no significant differences in physical activity between the groups which was probably due to rather large variations within the groups. *Low-Fat* and *Low-Carb* groups tended to be more active than *Pre* or *Regular* diet groups (Fig. 31B). Association between physical activity and energy expenditure was significant in all groups ($r = 0.70–0.80$, $p < 0.05–0.01$) (Fig. 31C). Linear regression analysis showed a tendency for a slightly greater predictive resting metabolic rate in *Low-Carb* compared to *Low-Fat* group (0.35 vs. 0.30 kcal · h^{-1}). On the other hand, respiratory quotient did not differ between the groups when measurements were performed in the fasted mice (Fig. 31D).

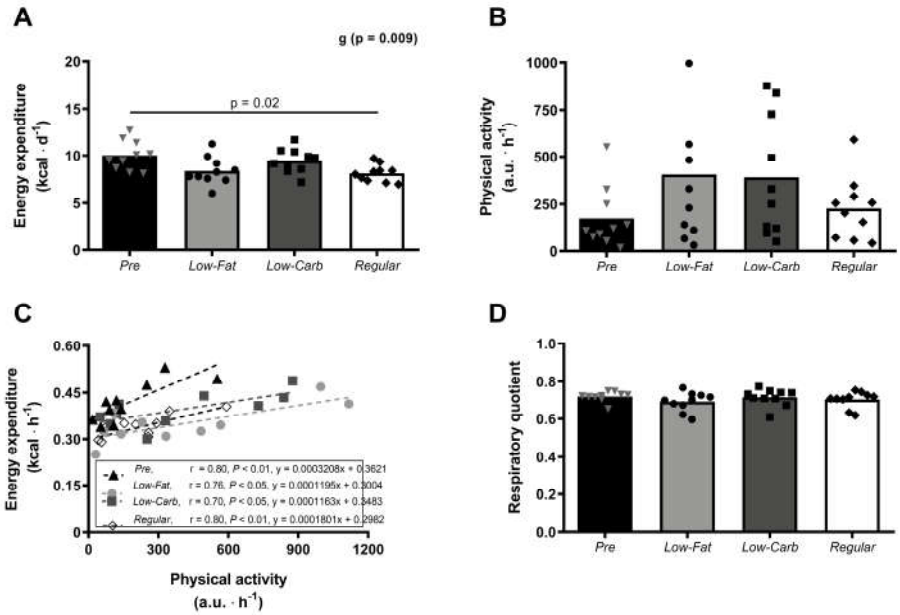

Fig. 31. Total energy expenditure (A), physical activity (B), plots of energy expenditure versus physical activity (C) and respiratory quotient (D) in *Low-Fat* and *Low-Carb* diet groups after 6-week caloric restriction (CR) compared to the obese group prior CR (*Pre*) as well as to the age-matched *Regular* group fed standard chow diet *ad libitum* for the same period. All the measurements were performed after overnight fasting. Data are presented as mean with each dot representing one mouse data sample. One-way ANOVA (A, B, D) with Bonferroni's *post hoc* analysis was performed for group effect (g). Lines indicate significant differences between connected groups. Pearson correlation coefficient (r) and linear regression equations for the plots of energy expenditure versus physical activity are also shown (C)

3.4.2. Discussion

The main aim of our study was to investigate if carbohydrate and fat content of diets affects physiological responses to caloric restriction in mice. Most of the previous studies have focused on effects of macronutrient content of diets on health and body composition in *ad libitum* fed mice (Hu et al., 2018; Solon-Biet et al., 2014) and there are only few studies under conditions of caloric restriction (Vangoitsenhoven et al., 2018). In agreement with recent findings on humans undergoing mild caloric restriction (Gardner et al., 2018), our results show that improvements in body composition and glucose tolerance do not differ between low-fat and low-carbohydrate diets with similar protein content under conditions of up to 40 % caloric restriction. This is important in view of the fact that differences between low-carbohydrate and low-fat diets have been widely discussed in relation to weight loss and metabolic health (Aragon et al., 2017).

Our focus on was not a test of the carbohydrate-insulin model of obesity (CIM), but rather a careful comparison of the effects of low-fat and low-carbohydrate diets on the parameters of body composition and metabolic changes after weight loss in mice. However, CIM has often been proposed in justification of health benefits of low-carbohydrate diets (Ludwig & Ebbeling, 2018). According to this line of reasoning high carbohydrate content of food leads to high blood insulin levels which acts to suppress the release of fatty acids from adipose tissue and directs circulating fat towards adipose tissue for storage rather than oxidation in metabolically active tissues. However, a large number of studies contradicts the carbohydrate-insulin model of obesity. Meta-analysis of 32 controlled feeding studies with substitution of carbohydrate for fat show that fat loss and energy expenditure were greater for low-fat diets compared to low-carbohydrate diets though differences between the diets in fat loss (16 $g \cdot d^{-1}$) and energy expenditure (26 $kcal \cdot d^{-1}$) were rather small (K. D. Hall & Guo, 2017). All the studies included in this meta-analysis have controlled dietary energy and protein intake between the diets. A recent randomized clinical trial which engaged over 600 participants showed no difference between low-fat and low-carbohydrate diets in weight loss during a 12-month period, and neither baseline insulin secretion nor genotype pattern relevant to carbohydrate and fat metabolism was associated with the dietary effects on weight change (Gardner et al., 2018). Self-reported energy intake was similar between the diet groups and protein intake did not differ much during this period (21 % and 24 % of kcal for low-fat and low-carbohydrate groups,

respectively). It appears that protein is a macronutrient which is particularly important for dietary-induced thermogenesis and satiety. The thermic effect of dietary protein is 25–30 % of its energy content compared to 5–10 % and 2–3 % for carbohydrate and fat, respectively (Leidy et al., 2015). Increase in protein intake from 15 to 30 % of total energy is associated with spontaneous reduction in total energy intake under conditions of *ad libitum* feeding (Weigle et al., 2005). High protein intake also led to increase in retention of lean body mass during caloric restriction (Pasiakos et al., 2013). Thus, comparison of high-fat and high-carbohydrate diets can be compromised by differences in protein content, as health benefits of protein-rich diets are often incorrectly assigned to carbohydrate and fat content of the diets (Soenen et al., 2012). In our study, we kept both the amount (20 % of total energy intake) and source (casein with addition of l-cystine) of dietary protein constant between the low-fat and low-carbohydrate diets. It appears that this amount of protein was adequate for skeletal muscle mass retention which did not change significantly during caloric restriction. Furthermore, mice were fed obesogenic diet for 18-week prior to caloric restriction. This diet induces minor changes in lean body and significant increase in body fat which might also help to preserve muscle mass during caloric restriction (Alhindi et al., 2019). Human weight loss studies show that approximately 25 % of weight loss is due to loss of lean body mass with major contribution of the skeletal muscles to this decline (Hoddy et al., 2014). People who are leaner tend to lose more of lean body mass under conditions of caloric restriction compared to those with greater body fat content (K. D. Hall, 2008). It appears that mice with diet-induced obesity show greater sparing of muscle mass during caloric restriction compared to humans. However, dissection of factor playing a role in preservation of muscle mass in mice and/or humans during caloric restriction was beyond the scope of our current study.

It appears that body fat was the main source of energy during caloric restriction and its loss did not differ between the two diets in our study. Increased fatty acid oxidation is a common feature of low-carbohydrate-high-fat diets which are sometimes perceived as more lipolytic and less obesogenic compared to low-fat-high-carbohydrate diets though human metabolic ward studies challenges this hypothesis (K. D. Hall et al., 2016). We did not observe any differences between the diets in respiratory quotient as the measurements were performed in the fasted state. Mice gorge on food and often consume all the food within less than 4 hours after feeding and spent significant periods of time in the fasted state when exposed

to caloric restriction (Acosta-Rodriguez, de Groot, Rijo-Ferreira, Green, & Takahashi, 2017; S. J. Mitchell et al., 2019). Food consumption early in the day might also be considered as study's limitation because it can affect circadian rhythms of nocturnal animals. Fasting is associated with high rate of fatty acid oxidation (Fokin et al., 2019). Measurements in the fasted state might be more representative of the overall metabolism compared to measurements in the post-absorptive state during caloric restriction. It appears that metabolic flexibility manifesting itself in switching between carbohydrate and fat oxidation allowed mice to maintain a similar net body fat balance independently of the macronutrient composition of the diets during caloric restriction (Goodpaster & Sparks, 2017).

Linear regression analysis of the plots for physical activity over energy expenditure allowed us to exclude effects of physical activity on energy expenditure and showed that predicted resting metabolic rate tended to be slightly greater under conditions of low-carbohydrate diet compared to the low-fat diet. However, this difference between the diets was not significant and can hardly be used as evidence in support of recent findings in human studies that low-carbohydrate diets lead to greater energy expenditure compared to low-fat diets (Ebbeling et al., 2018). Our results are in agreement with many human studies that reported no practically meaningful differences in energy expenditure between the isocaloric and isonitrogenous low-fat and low-carbohydrate diets (Gardner et al., 2018; K. D. Hall & Guo, 2017; K. D. Hall, Guo, & Speakman, 2019).

We have assessed glucose tolerance as a key indicator of metabolic health. After 6-week caloric restriction glucose tolerance improved similarly in both diets. It is likely that caloric restriction-induced loss of body fat was a key factor promoting better glucose control irrespective of the dietary carbohydrate and fat content. In contrast to our findings, a recent caloric restriction study of C57BL/6 mice showed smaller improvement in glucose tolerance for high-fat diet compared to chow diet which is high in carbohydrates in spite of similar weight loss for both diets (Goodpaster & Sparks, 2017). However, macronutrients and their sources were not strictly controlled in the latter study and the protein content differed substantially between both diets, i.e., 20 % kcal for high fat diet and 33 % kcal for chow diet low in fat. Dietary protein might influence postprandial glucose control due to its insulinotropic effects (Layman, Clifton, Gannon, Krauss, & Nuttall, 2008). There is evidence that consumption of the high-protein meal before the intake of carbohydrates attenuates the subsequent rise in the postprandial serum

glucose and results in lower glucose compared to isocaloric high-carbohydrate and high-fat meals (Meng, Matthan, Ausman, & Lichtenstein, 2017). Blood insulin levels were not measured and this might be considered as a shortcoming of our study. However, there were no differences in glucose tolerance between the caloric restricted diets, and it is unlikely that insulin levels differed much under such conditions. Indeed, differences in fasting insulin levels between diets are blunted during caloric restriction (Sacks et al., 2009).

In humans, weight loss is a priority target under the conditions of impaired glucose homeostasis as in case of type 2 diabetes (Magkos, Yannakoulia, Chan, & Mantzoros, 2009). Antidiabetic therapies that can control blood glucose levels but promote weight gain are less effective as greatest improvements in glucose control are observed in patients with greatest reductions in body mass (Blonde, Pencek, & MacConell, 2015).

Taken together, caloric restriction-induced body fat loss should be considered as a primary and most desirable target for positive management of blood glucose whereas macronutrient composition of isocaloric diets with equated protein probably plays a minor role at its best.

CONCLUSIONS

1. Mitochondrial respiration does not differ between mice with normal and low CS activity.

2. There are no major differences in energy expenditure, respiratory quotient or physical activity between mice with normal and low CS activity with the exception of female mice, where low CS activity is associated with reduced energy expenditure.

3. After 48-h food deprivation energy expenditure and respiratory quotient are lower in congenic mice with low CS activity compared to control mice with normal CS activity.

4. Myostatin dysfunction does not protect from skeletal muscle wasting during food deprivation. 48-h food deprivation is also associated with a significant decrease in physical activity, energy expenditure and fat reserves of mice with normal with no impact of myostatin dysfunction.

5. *Low-Fat* and *Low-Carb* diets has similar effect on body composition, energy metabolism, physical activity and glucose tolerance during caloric restriction. Fixed energy and protein intake rather than a distribution of dietary carbohydrate and fat is the main factor for improvement in body composition and metabolic health of obese mice. Similarly, improvements in glucose tolerance of obese mice are due to the reduction in body fat rather than dietary carbohydrate and fat content of the diets.

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SCIENTIFIC PUBLICATIONS

The thesis is based on the following articles:

1. **First study: Fokin, Andrej**; Žūkienė, Rasa; Ratkevičius, Aivaras. Reduced citrate synthase activity effect on oxygen consumption rates in isolated mitochondria from mice liver and muscles // Baltic Journal of Sport & Health Sciences. Kaunas: Lietuvos sporto universitetas. ISSN 2351-6496. eISSN 2538-8347. 2016, Vol. 2, No. 101, p. 26–30. doi:10.33607/bjshs.v2i101.52. [Central & Eastern European Academic Source (CEEAS); SPORTDiscus with Full Text; Index Copernicus] [M.k.: N 010, N 004] [Indėlis: 0,334]
2. **Second study: Fokin, Andrej**; Minderis, Petras; Žūkienė, Rasa; Ratkevičius, Aivaras. Metabolism analysis in mice with reduced citrate synthase activity // Baltic Journal of Sport & Health Sciences. Kaunas: Lietuvos sporto universitetas. ISSN 2351-6496. eISSN 2538-8347. 2017, Vol. 2, No. 105, p. 14–19. [Central & Eastern European Academic Source (CEEAS); SPORTDiscus with Full Text; Index Copernicus] [M.k.: N 004, N 010] [Indėlis: 0,250]
3. **Third study: Fokin, Andrej**; Minderis, Petras; Venckunas, Tomas; Lionikas, Arimantas; Kvedaras, Mindaugas; Ratkevicius, Aivaras. Myostatin dysfunction does not protect from fasting-induced loss of muscle mass in mice // Journal of Musculoskeletal and Neuronal Interactions. Nafplion: International Society of Musculoskeletal and Neuronal Interactions. ISSN 1108-7161. 2019, Vol. 19, No. 3, p. 342–353. [Embase; Scopus; BIOSIS Previews; MEDLINE; Science Citation Index Expanded (Web of Science)] [M.k.: N 010] [IF: 1,660, AIF: 3,589, IF/AIF: 0,462, kvartilis: Q4 (2019, InCites JCR SCIE)] [CiteScore: 2,30, SNIP: 0,729, SJR: 0,527 (2019, Scopus Sources)] [Indėlis: 0,170]. Link to the Pubmed database: https://pubmed.ncbi.nlm.nih.gov/31475942/
4. **Fourth study:** Minderis, Petras; **Fokin, Andrej**; Dirmontas, Mantas; Ratkevicius, Aivaras. Hypocaloric low-carbohydrate and low-fat diets with fixed protein lead to similar health outcomes in obese mice // Obesity. Hoboken: Wiley. ISSN 1930-7381. eISSN 1930-739X. 2020, Vol. 28, Iss. 8, p. 1494–1502. doi:10.1002/oby.22872. [SPORTDiscus; Embase; Scopus; MEDLINE; CAB Abstracts; Science Citation Index Expanded (Web of Science)] [M.k.: N 010] [IF: 3,742, AIF: 4,151, IF/AIF: 0,901, kvartilis: Q2 (2019, InCites JCR SCIE)] [CiteScore: 7,20, SNIP: 1,293, SJR: 1,687 (2019,

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5. Baltusnikas, Juozas; Venckunas, Tomas; Kilikevicius, Audrius; **Fokin, Andrej**; Ratkevicius, Aivaras. Efflux of creatine kinase from isolated soleus muscle depends on age, sex and type of exercise in mice // Journal of Sports Science and Medicine. Bursa: Department of Sports Medicine Medical Faculty of Uludag University. ISSN 1303-2968. 2015, Vol. 14, Iss. 2, p. 379–385. [DOAJ; Scopus; SPORTDiscus with Full Text; Index Copernicus; Science Citation Index Expanded (Web of Science)] [M.k.: N 010] [IF: 1,430, AIF: 2,277, IF/AIF: 0,628, kvartilis: Q3 (2015, InCites JCR SCIE)] [CiteScore: 1,56, SNIP: 1,036, SJR: 0,732 (2015, Scopus Sources)] [Indėlis: 0,200]
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7. Negaresh, Raoof; Ranjbar, Rouholah; Baker, Julien S.; Habibi, Abdoihamid; Mokhtarzade, Motahare; Gharibvand, Mohammad Momen; **Fokin, Andrej**. Skeletal muscle hypertrophy, insulin-like growth factor 1, myostatin and follistatin in healthy and sarcopenic elderly men: the effect of whole-body

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8. Negaresh, R.; Ranjbar, R.; Habibi, A.; Mokhtarzade, M.; Fokin, A.; Gharibvand, M. M. The effect of resistance training on quadriceps muscle volume and some growth factors in elderly and young men // Advances in Gerontology = Uspekhi Gerontologii. Sankt-Peterburg: Eskulap. ISSN 1561-9125. 2017, Vol. 30, No. 6, p. 880–887. [CINAHL Complete; PubMed; Scopus; MEDLINE] [M.k.: N 010] [CiteScore: 0,20, SNIP: 0,000, SJR: 0,123 (2017, Scopus Sources)] [Indėlis: 0,166]
9. Minderis, Petras; **Fokin, Andrej**; Ratkevičius, Aivaras. High growth dummerstorf mice have reduced specific force of slow and fast twitch skeletal muscle // Baltic Journal of Sport & Health Sciences. Kaunas: Lietuvos sporto universitetas. ISSN 2351-6496. eISSN 2538-8347. 2016, Vol. 2, No. 101, p. 44–52. doi:10.33607/bjshs.v2i101.55. [Central & Eastern European Academic Source (CEEAS); SPORTDiscus with Full Text; Index Copernicus] [M.k.: N 010] [Indėlis: 0,333]

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1. Minderis, P.; Dirmontas, M.; **Fokin, A.**; Libnickienė, I. Isocaloric low-carbohydrate and low-fat diets similarly improve body composition and glucose tolerance in obese mice // Sport Science for Sports Practice, Teacher Training and Health Promotion: 12th Conference of Baltic Society of Sport Sciences: April 25–26, 2019, Vilnius, Lithuania: abstracts. Kaunas: Vytautas Magnus University, 2019. ISBN 9786094673849. p. 109–110. doi:10.7220/9786094673849. [M.k.: N 010] [Indėlis: 0,250]
2. **Fokin, A.**; Dirmontas, M.; Libnickienė, I.; Minderis, P. Carbohydrate and fat ratio in hypocaloric diets with equal protein and calorie content does not affect energy metabolism in obese mice // Sport Science for Sports Practice, Teacher Training and Health Promotion: 12th Conference of Baltic Society

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3. **Fokin, Andrej**; Žūkienė, Rasa; Ratkevičius, Aivaras. Gender effect on metabolism and mitochondria respiration in mice with normal and reduced citrate synthesis activity // Multiplicity of Sports Science in Practice: Abstracts of the 10th Conference of the Baltic Sport Science Society, Riga, Latvia, 26–28 April 2017. Riga: Latvian Academy of Sport Education, 2017. ISBN 9789934520334. p. 51–52. [M.k.: N 010] [Indėlis: 0,334]
4. **Fokin, Andrej**; Bočkutė, Silvija; Venckūnas, Tomas; Mildažienė, Vida; Lionikas, Arimantas; Žukienė, R; Ratkevičius, Aivaras. Reduced citrate synthase activity effect on the mitochondrial respiration in absence and in presence of fatty substrates // 8th Conference of Baltic Society of Sport Sciences "Sport Science for Sports Practice and Teacher's Training": Abstracts, April 22–24, 2015, Vilnius, Lithuania. Vilnius: Lietuvos edukologijos universitetas, 2015. ISBN 9789955209881. p. 30–31. [M.k.: N 010] [Indėlis: 0,148]
5. **Fokin, Andrej**; Bočkutė, Silvija; Venckūnas, Tomas; Mildažienė, Vida; Lionikas, Arimantas; Žukienė, R.; Ratkevičius, Aivaras. Polymorphism in citrate synthase gene is associated with variable respiration in isolated mitochondria from liver and muscles // Physiology 2014 [Elektroninis išteklius]: Proceedings of the Physiological Society, London, UK, 30 June – 2 July 2014 / The Physiological Society. 2014, PCB173, p. 296P–297P. [M.k.: N 010] [Indėlis: 0,148]
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SANTRAUKA

ĮVADAS

Mitochondrijų disfunkcija gali prisidėti prie tokių patologijų kaip nutukimas, atsparumas insulinui ir II tipo diabetas (Christe et al., 2013; Yang et al., 2012). Nutukimas ir II tipo diabetas glaudžiai susiję su silpna mitochondrijų funkcija (Houmard, 2008). Disbalansas tarp riebalų rūgščių β-oksidacijos ir angliavandenių oksidacijos bei jų srauto Krebso cikle turi ypatingą reikšmę mitochondrijų funkcionavimui, pertekliniam trigliceridų ir kitų riebalų produktų kaupimuisi, nutukimo ir diabeto išsivystymui (Bonora, Brangani, & Pichiri, 2008; Ioannidis, 2008; Koves et al., 2008). Pavyzdžiui, riebaus pašaro dieta skatina atsparumą insulinui ir kenkia pelių gliukozės metabolizmui (Terauchi et al., 2007). Balansas tarp riebalų rūgščių ir angliavandenių oksidacijos Krebso cikle yra vienas iš mitochondrijų funkcijos reguliacijos raktinių mechanizmų. Yra faktų, kad citratas atlieka svarbų vaidmenį kontroliuojant šį balansą (Ruderman, Saha, Vavvas, & Witters, 1999). Citozolyje citratas per ATP citrato liazę konvertuojamas į acetil-kofermentą A (Acetil-CoA), reikalingą malonil-CoA sintezei. Malonil-CoA slopina karnitino palmitoiltransferazės 1 (CPT1) veiklą, taip trukdydamas riebalų rūgščių oksidacijai (Houmard, 2008). Nuslopinta riebalų rūgščių oksidacija veda link aptartų metabolinių sutrikimų (nutukimas, atsparumas insulinui, II tipo diabetas).

Padidėjusi laisvųjų riebalų rūgščių koncentracija neigiamai veikia mitochondrijų funkciją per biocheminių kelių disreguliaciją ir per sąveiką su raktiniais fermentais (Zraika, Dunlop, Proietto, & Andrikopoulos, 2002). Mes tyrėme mitochondrinį fermentą citrato sintazę (CS), kuris dažnai naudojamas kaip mitochondrijų žymuo tiriant žmones ir gyvūnus (Hamilton & Booth, 2000). Po transliacijos citozolyje CS pernešama į mitochondrijų matriksą, kur ji funkcionuoja Krebso cikle ir atlieka lemiamą vaidmenį energijos sintezės reguliavime bei reaktyviųjų deguonies radikalų (angl. *reactive oxygen species,* ROS) gamyboje per mitochondrinį kvėpavimą. Todėl taškinės *Cs* geno mutacijos gali keisti mitochondrijų funkciją (Johnson, Gagnon, Longo-Guess, & Kane, 2012). Manoma, kad kepenys, griaučių raumenys, riebalinis audinys ir kasa atlieka svarbų vaidmenį atsparumo insulinui procese (Bouderba et al., 2012). Todėl galima teigti, kad CS aktyvumo slopinimas gali būti naudingas skatinant riebalų rūgščių oksidaciją, veikiant įvairius medžiagų apykaitos parametrus, tokius kaip deguonies

suvartojimas, energijos eikvojimas ir kvėpavimo koeficientas.

Moteriškos ir vyriškos lyties atstovų medžiagų apykaitos parametrai, mitochondrijų kvėpavimas gali skirtis. Taip pat lytis yra kritinis veiksnys analizuojant taškinių mutacijų, tokių kaip H55N polimorfizmas, įtaką energijos metabolizmui. Buvo rasta lyties skirtumų mitochondrijų, išskirtų iš širdies, griaučių raumenų ir kepenų, kvėpavime (Sanz et al., 2007). Galima daryti prielaidą, kad pelių lytis gali turėti įtakos pelių linijų medžiagų apykaitos parametrams (energijos eikvojimui, kvėpavimo koeficientui).

Pirmame tyrime mes nagrinėjome sumažinto citrato CS aktyvumo įtaką deguonies suvartojimo greičiui izoliuotose mitochondrijose iš pelių kepenų ir griaučių raumenų. Mes tyrėme, ar mitochondrijų kvėpavimas skiriasi tarp kontrolinių C57BL/6J (B6) ir eksperimentinių B6.A-(rs3676616-D10Utsw1)/Kjn (B6.A) vyriškos ir moteriškos lyties pelių linijų. B6.A pelės turi A/J alelio variantą, kuris iššaukia aminorūgščių pasikeitimą CS baltymo histidino ir asparagino 55 (H55N) pozicijoje (Johnson et al., 2012). Tokio pasikeitimo rezultatas yra sumažintas B6.A pelių linijos CS aktyvumas. Tokios pelės vadinamos kongeninėmis.

CS aktyvumas priklauso nuo daugybės metabolinių procesų ir gali didėti, pvz., nuo ištvermės fizinių pratimų (Holloszy & Booth, 1976; Jaenisch, Bertagnolli, Borghi-Silva, Arena, & Lago, 2017). Ankstesni mūsų tyrimai parodė, kad pelės su sumažintu CS aktyvumu turėjo silpnesnius ištvermės rodiklius lyginant su kontrolė nepriklausomai nuo lyties (Kvedaras et al., 2017). Sumažėję CS aktyvumo lygiai gali turėti pranašumų skatindami riebalų rugščių oksidaciją esant perteklinio substratų tiekimo sąlygomis bei paveikdami energijos metabolizmo parametrus, tokius kaip deguonies suvartojimas, energijos eikvojimas ir kvėpavimo koeficientas.

Antrame tyrime mes susifokusavome ties galimų energijos metabolizmo skirtumų tarp B6 ir B6.A patinų ir patelių pelių su sumažintu CS aktyvumu (Ratkevicius et al., 2010). Visos chromosomos apsikeitimo pelių linija, vadinama konsomine, suteikia papildomas galimybės genų įtakos energijos metabolizmui. Konsominėse pelėse viena chromosoma iš recipiento linijos pakeista homologine iš donoro linijos taikant „backcrossing“ pelių kryžminimo strategiją (Ishii et al., 2011; Matin, Collin, Asada, Varnum, & Nadeau, 1999; Miller et al., 2020; Nadeau, Singer, Matin, & Lander, 2000). Pelių 10 chromosomos pasikeitimo tyrimai gali atskleisti kitų, esančių toje chromosomoje genų įtaką energijos metabolizmui. Pvz., PTEN (fosfokinazės ir tenzino homologo) pašalinimas iš 10 pelių chromosomos

aktyvavo proteino kinazę B raišką, ko pasekoje buvo paveiktas fosfato metabolizmas (Kawai, Kinoshita, Ozono, & Michigami, 2020). Fosfatas dalyvauja plačiame biocheminių procesų spektre, įskaitant ląstelių signalinių kelių reguliaciją ir energijos homeostazę (Quarles, 2012; Razzaque, 2012), o jo nepakankamumas gali sukelti ilgalaikes medžiagų apykaitos, skeleto raumenų silpnumo komplikacijas (Weber & Quarles, 2019). Dėl šitų priežasčių mes papildomai tyrinėjome energijos metabolizmo parametrus konsominių C57BL/6J-Chr 10A/J/NaJ (B6.A10) pelių su pakeista 10 chromosoma. Kaip ir B6, B6.A pelės turi mutaciją *Cs* gene, kas sukelia CS aktyvumo ilgalaikį sumažėjimą.

Skirtingo tipo maistinių intervencijų (tokių kaip pilnas maisto apribojimas, kalorijų apribojimas ir daug riebalų turinti dieta) atsakas gali skirtis tarp pelių linijų (West, Boozer, Moody, & Atkinson, 1992). Yra įrodymų, kad kalorijų apribojimas turi naudingą efektą sveikatos rodikliams, kadangi teigiamai įtakoja kūno kompozijciją bei lipoproteino profilį (Anderson & Weindruch, 2012). Todėl darome prielaidą, kad pilnas maisto apribojimas gali būti taikomas kaip prevencinis būdas kovoti su kraujagyslių ligomis ir II tipo diabetu.

Trečiame tyrime mes atlikome du eksperimentus, kurių bendras tikslas buvo nustatyti galimą metabolinį atsaką ir kūno kompozicijos pokyčius į 48 val. badavimą (pilną maisto apribojimą). Vienas eksperimentas apėme pelių linijas su normaliu (B6) ir sumažintu (B6.A) CS aktyvumu. Užpakalinių kojų griaučių raumenų masė buvo įvertinta po badavimo. Kitame eksperimente mes palyginome 48 val. badavimo įtaką tarp pelių su normaliai funkcionuojančiu miostatinu (BEH+/+) ir pelių suo miostatino disfunkcija (BEH). BEH pasižymi ne tik stipria skeleto raumenų hipertrofija, bet ir pati miostatino disfunkcija turi teigiamą įtaką metabolinei sveikatai, mažinandama riebalų kiekį bei padidindama jautrumą insulinui (Hamrick, Pennington, Webb, & Isales, 2006; McPherron, Lawler, & Lee, 1997; Wilkes, Lloyd, & Gekakis, 2009). Galima teigti, kad miostatino disfunkcija, panašiai kaip ir sumažintas CS aktyvumas, taip pat gali būti širdies II tipo diabeto bei nutukimo prevencijos priemonė. Tai buvo pirma priežastis, kodėl mes papildomai analizavome miostatino disfunkcijos įtaką esant 48 val. badavimui. Antra priežastis buvo ta, jog tokios chroninės patologijos, kaip kraujagyslių ir širdies ligų, skirtingų formų raumenų distrofijos paveikia energijos metabolizmą ir yra siejamos su raumenų masės netekimu (Glass, 2005; Sakuma, Aoi, & Yamaguchi, 2014). Dilemą kelia dvejopas badavimo poveikis: iš vienos pusės jis pagerina metabolinę sveikatą (geresnė medžiagų apykaita), iš kitos veda link

griaučių raumenų masės sumažėjimo (J. E. Donnelly, Jakicic, & Gunderson, 1991; Saris, 2001). Šitos tyrimų dalies tikslas buvo išsiaiškinti, ar miostatino disfunkcija pagerina energijos metabolizmą ir apsaugo griaučių raumenis nuo masės netekimo badavimo metu. Sumuojant, trečio tyrimo užduotis buvo atsakyti į klausimą, ar sumažintas CS aktyvumas ir miostatino disfunkcija paveikia energijos metabolizmą pėlese 48 val. badavimo intervencijos metu. Kitas klausimas adrestuotas į *Cs* geno funkcinį slopinimą, kuris gali būti tinkama strateginė terapeutinė priemonė kovojant su insulino rezistencija ir nutukimu.

Ne tik badavimas, genetinės mutacijos arba fizinio aktyvumo didėjimas gali apsaugoti nuo pertekliniaus kūno masės prieaugio ir nutukimo, bet ir pačių dietų sudėtis. Tokio tipo dietos lengviau implementuojamos populiacijos lygyje (Westerterp, 2019). Plačiai žinomas faktas, kad žmonės ir gyvūnai padidina savo kūno masę, kai jų energijos suvartojimo lygis viršija energijos eikvojimo (J. Galgani & Ravussin, 2008).

Ketvirtame tyrime mes studijavome dviejų skirtingo tipo hipokalorinių dietų su vienodu baltymo kiekiu kalorijų apribojimo įtaką nutukusioms pelėms. Mes palyginome C57BL/6J pelių linijos metabolinės sveikatos bei kūno kompozicijos pokyčius atsakui į dvi energijos restrikcijos dietas su drastiškais skirtumais angliavandenių ir riebalų santykyje. Šito tyrimo planavimo ir atlikimo priežastis ta, kad iki šiol yra stiprių kontroversijų, vyksta aktyvus moksliniai debatai dėl angliavandenių ir riebalų proporcijos dietos sudėtyje, ir kuri dieta labiau svarbi metabolinei sveikatai (Ge et al., 2020; Sacks et al., 2009). Pavyzdžiui, taip vadinamas angliavandenių-insulino modelis siūlo teoriją, kad dietos angliavandeniai yra labiau obesogeniniai negu riebalai dėl stipriai išreikšto neigiamo poveikio insulino sekrecijai (Ludwig & Ebbeling, 2018). Iš pirmo žvilgsnio kokybiškai pagrįsta teorija dažnai kritikuojama kitų mokslininkų (K. D. Hall, Guyenet, & Leibel, 2018). Nesenai atliktas atsitiktinių imčių tyrimas pademonstravo, kad energijos eikvojimas buvo reikšmingai didesnis mažai angliavandenių turinčioje dietoje, lyginant ją su daug angliavandenių turinčia dieta, atsižvelgiant į tą faktą, kad abiejų taikytų dietų energijos suvartojimas nesiskyrė (Ebbeling et al., 2018). Dietos, skatinančios energijos eikvojimą, išlaikant energijos suvartojimą nepakitusiu, yra perspektyvi strategija svorio reguliacijai, o pelės modelis yra puikus instrumentas tokios krypties studijoms realizavimui. C57BL/6J pelių linija yra linkusi į nutukimą sukeliančią maistinę intervenciją (Kleinert et al., 2018; Speakman, 2019b) ir gerai toleruoja dietas su stipriais skirtumais tarp

angliavandenių ir riebalų proporcijos dietų sudėtyje (Roberts et al., 2017, 2018). Mūsų ketvirto tyrimo stiprumas buvo tas, kad mes pelių modelyje taikėme hipokalorines dietas su tiksliai nustatyta makromedžiagų kompozicija, ką ypatingai sunku įgyvendinti žmogaus studijose.

TIKSLAS, HIPOTEZĖS IR UŽDAVINIAI

Pagrindinis tikslas buvo ištirti sumažinto citrato sintazės (CS) aktyvumo, miostatino disfunkcijos ir kalorijų apribojimo intervencijų įtaką pelių energijos metabolizmui ir kūno kompozicijai.

Tyrimų hipotezės:

1. Sumažintas CS aktyvumas gali pagerinti substratų oksidacijos greitį mitochondrijose ir paskatinti riebalų oksidaciją C57BL/6J pelėse (pirmas tyrimas).

2. Sumažintas CS aktyvumas gali paveikti energijos metabolizmą laisvai judančiose pelėse (antras tyrimas).

3. Sumažintas CS aktyvumas gali įtakoti energijos metabolizmą esant badavimui (trečias tyrimas).

4. Miostatino disfunkcija, kuri charakterizuojama hipertofuotais griaučių raumenimis dėl padidėjusių glikolitinių 2 tipo skaidulų kiekio ir masės bei sumažejusiu kūno riebalų kiekiu, energijos gamybai gali naudoti daugiau resursų iš raumenų palyginus su laukinio tipo kontrole esant badavimo sąlygomis (trečias tyrimas).

5. Medžiagų apykaitos rodiklių (tokių kaip energijos eikvojimas) ir kūno kompozicijos pokyčiai gali nesiskirti tarp angliavandenių ir riebalų hipokalorinių dietų, jiegu šitų dietų sudėtis yra kaloriškai suvienodinta pagal baltymo kiekį (ketvirtas tyrimas).

Tyrimų uždaviniai:

1. Palyginti mitochondrijų angliavandenių ir riebalų substratų oksidaciją abiejų lyčių pelėse su normaliu ir sumažintu CS aktyvumu.

2. Nustatyti sumažinto CS aktyvumo poveikį metabolinei sveikatai ir fiziniam aktyvumui B6, B6.A ir B6.A10 abiejų lyčių pelių linijose.

3. Išanalizuoti sumažinto CS aktyvumo įtaką energijos metabolizmo rodiklių pokyčiams ir raumenų masei po 48 val. badavimo.

4. Išanalizuoti miostatino disfunkcijos įtaką energijos metabolizmo pokyčiams ir raumenų masei prieš ir po 48 val. badavimo.

5. Ištirti nutukusių pelių daug angliavandenių ir daug riebalų turinčių hipokalorinių dietų su suvienodintu baltymo kiekiu įtaką energijos balansui, kūno kompozicijai ir gliukozės tolerancijai esant kalorijų apribojimui.

1.1. TYRIMO METODAI IR ORGANIZAVIMAS

1.1. Gyvūnai

Visi tyrimai buvo atlikti su laboratorinėmis inbredinėmis pelių linijomis. Visos procedūros buvo patvirtintos Valstybinės maisto ir veterinarijos tarnybos išduotais leidimais: nr. 0223 (2012 m.) ir nr. 10 (2014 m.). Pelės buvo veisiamos ir auginamos Lietuvos sporto universitetui priklausančiame vivariume. Jos buvo laikomos standartiniuose narvuose, esant 20–21 ºC temperatūrai, 55 ± 10 proc. drėgnumui ir kintančiam 12/12 val. (šviesos / tamsos) ciklui. Gyvūnai buvo maitinami *ad libitum* standartiniu, graužikams pritaikytu pašaru (58 proc. kcal iš angliavandenių, 28,5 proc. kcal iš baltymų, 13,5 proc. kcal iš riebalų; LabDiet 5001, LabDiet, Sent Luisas, JAV) ir girdomi vandeniu iš čiaupo.

Buvo tiriamos penkios pelių linijos: klasikinė C57BL/6J (B6), B6.A-(rs3676616-D10Utsw1)/KjnB6 (B6.A), C57BL/6J-Chr10$^{A/J}$/NaJ (B6.A10) (Bar Harboras, JAV), Berlin High (BEH) su miostatino disfunkcija ir Berlin High (BEH+/+) su normalia miostatino funkcija. BEH ir BEH+/+ pelių poros buvo dosni prof. Lutz Bünger dovana (Škotijos kaimo koledžas, Edinburgas, Jungtinė Karalystė). B6 pelės yra kontrolinė grupė su laukinio tipo C57BL/6J linijos genomu. B6.A pelės turi taškinę H55N mutaciją *Cs* gene (10 chromosomos 3 ekzone), kur A yra pakeistas į C, rs29358506. B6.A pelės su tokio tipo mutacija 10 chromosomoje vadinamos kongeninėmis ir yra išveistos „backrossing“ metodu (Johnson et al., 2012). B6.A10 pelių visa 10 chromosoma pakeista homologine C57BL/6J pelių chromosoma. Tokia linija su chromosomos pakeitimu vadinama konsomine, kai viena linija yra chromosomos donoras, kita – jos recipientas (Nadeau et al., 2000). 10 chromosomos pakeitimas veikia *Cs* geną kaip ir taškinė B6.A pelių mutacija, todėl lemia sumažintą abiejų pelių linijų CS aktyvumą. BEH+/+ linija su normalia miostatino funkcija buvo sukurta iš BEH linijos pelių, kurios turi MstnCmpt-dl1Abc (angl. *Compact,* Cmpt) mutaciją abiejuose miostatino (*Mstn*) aleliuose (Varga et al., 1997). Dėl šios mutacijos 12 bazių porų delecija yra įvykusi *Mstn* geno sekoje, koduojančioje propeptido sritį. BEH pelės neturi veikiančio miostatino ir pasižymi ypač raumeningu fenotipu (Amthor et al.,

2007; Lionikas et al., 2013). BEH+/+ su laukinio tipo miostatinu buvo išveistos kaip ir ankstesniuose tyrimuose (Amthor et al., 2007; Lionikas et al., 2013).

1.2. Mitochondrijų kvėpavimas. Pirmas tyrimas

1.2.1. Mitochondrijų išskyrimas

Buvo matuojamas 12 savaičių amžiaus B6 (n = 16, aštuoni patinai ir aštuonios patelės) ir B6.A (n = 16, aštuoni patinai ir aštuonios patelės) pelių mitochondrijų kvėpavimas. Mitochondrijų išskyrimui buvo paruoštos tokios terpės:

● homogenizavimo (H terpė: 250 mM cukrozės, 10 mM TRIS, 3 mM EGTA, pH 7,7; 4 ºC);

● suspendavimo (S terpė: 250 mM cukrozės, 5 mM TRIS, pH 7,34; 4 ºC);

● izoliacijos A (2,5 ml 150 mM cukrozės, 75 mM KCl, 50 mM KH_2PO_4, 5 mM $MgCl_2$, 1 mM EGTA, pH 7,4; 4 ºC) su pridėta 0,2 mg/ml bakterine proteinaze (XXIV tipas, iš *Bacillus licheniformis*, P8038, Sigma-Aldrich, Vokietija);

● izoliacijos B (250 mM cukrozės, 20 mM MOPS, 0,1 mM EGTA, pH 7,4; 4 ºC) su pridėtu 1 mg · ml^{-1} jaučio serumo albuminu (angl. *bovine serum albumin,* BSA) ir 15 ml izoliacijos terpės B be BSA.

Paruoštos terpės viso eksperimento metu buvo laikomos and ledo (4 ºC). Mitochondrijų kvėpavimui matuoti buvo paruošta inkubacijos terpė (IT6) (KCl 110 mM, kreatino monohidratas 50 mM, TRIS 20 mM, KH_2PO_4 5 mM, Mg ($MgCl_2 \cdot 6H_2O$) 2,5 mM, pH 7,2; 37 ºC). Tai buvo atlikta prieš pradedant mitochondrijų išskyrimą.

Atlikus pelės eutanaziją kervikaline dislokacija, kepenys ir galinių kojų griaučių raumenys buvo greitai išpreparuojami ir dedami į atskirus 40 ml lede atšaldytus 0,9 proc. KCl tirpalus. Kepenims reikalinga 3 min. inkubacija KCl tirpale. Po inkubacijos kepenys buvo kruopščiai susmulkintos žirklėmis ir užpiltos H terpe (kepenų ir H terpės santykis 1:10 (m/V)), visas mišinys homogenizuotas elektriniu Potter-Elvehjem homogenizatoriumi: 10 kartų po 20–30 sek. 750 rpm (angl. *rotations per minute,* rpm) greičiu.

Mitochondrijos buvo išskiriamos diferenciniu centrifugavimu (Zukiene, Ciapaite & Mildaziene, 2010) per 3 žingsnius: 800 × g 5 min., 6800 × g 10 min. ir 6800 × g 10 min. 4 ºC (Allegra 64R centrifuga, Beckman Coulter, Sent Draivas, JAV). Po pirmo centrifugavimo žingsnio supernatantas perfiltruotas. Po antro ir

trečio centrifugavimo žingsnio substancija resuspenduota S terpėje.

Raumenų mitochondrijų išskyrimui raumenys 5 min. buvo inkubuojami lede atšaldytoje izoliacijos terpėje A. Po inkubacijos raumenys buvo kruopščiai susmulkinti (išimtas jungiamasis audinys) ir užpilti izoliacijos terpe B. Gautas mišinys homogenizuotas elektriniu Potter-Elvehjem homogenizatoriumi: 10 kartų po 20–30 sek. 750 rpm greičiu. Raumenų mitochondrijų išskyrimo procedūra atlikta vadovaujantis kito tyrimo metodika (Garcia-Cazarin, Snider, & Andrade, 2011). Atlikti 3 diferencinio centrifugavimo žingsniai: 800 × g 10 min., 10000 × g 10 min., 10000 × g 10 min. 4 °C. Po pirmo centrifugavimo žingsnio supernatantas buvo perfiltruotas, o po antro žingsnio supernatantas buvo išpiltas ir įpilta 10 ml izoliacijos B terpės. Po trečio centrifugavimo žingsnio supernatantas buvo išpiltas ir įpilta 100–150 µl izoliacijos B terpės (~100 mg · ml^{-1} baltymo). Po centrifugavimo kepenų ir mitochondrijų suspensijos buvo laikomos lede, palaikant 4 °C temperatūrą.

Baltymo koncentracija mitochondrijų suspensijose buvo matuojama spektrofotometriškai, taikant modifikuotą Biureto metodą (Gornall, Bardawill, & David, 1949). Biureto testui BSA buvo naudojamas kaip standartas. Standartinė kreivė nubrėžta naudojant standarto rodiklius, pamatuotus spektrofotometriškai 536 nm bangos ilgyje. 1,6 ml Biureto reagento, 380 µl deoksicholinės rūgšties (DOX, 0,33 proc.) ir 20 µl kiekvieno standarto buvo sumaišyta ir inkubuojama 15 min. 37 °C. Mitochondrijų baltymo koncentracija matuota taip pat, kaip ir BSA standartai, tik vietoje 20 µl standarto buvo įpilta 20 µl mitochondrijų suspensijos. Kontrolei atlikti sumaišyta 400 µl DOX (0,33 proc.) ir 1,6 ml Biureto reagento.

1.2.2. Mitochondrijų kvėpavimo matavimai

Mitochondrijų kvėpavimas buvo matuojamas pagal deguonies suvartojimo greitį (O nmol min^{-1} · mg^{-1} baltymo) 37 °C 1,5 ml uždaroje stiklo kiuvetėje su įrengtu Klarko tipo deguoniui jautriu elektrodu, naudojant polarografijos sistemą (Rank Brothers LTD, Jungtinė Karalystė). Polarografijos technika pagrįsta ištirpusio vandenyje deguonies redukcija (1.1, 1.2) pagal fundamentalius oksidacinio fosforilinimo dėsnius (Chance & Williams, 1955).

$$O_2 + 2\bar{e} + 2H^+ \rightarrow H_2O_2 \qquad (1.1)$$

$$O_2 + 4\bar{e} + 4H^+ \rightarrow 2H_2O \qquad (1.2)$$

Mitochondrijų kvėpavimui matuoti buvo naudojami šie substratai ir jų mišiniai:

1. 5 mM glutamato (pagaminto iš L-glutamo rūgšties, G8415, Sigma-

Aldrich, Vokietija) ir 5 mM malato (pagaminto iš L-(−)-obuolių rūgšties dinatrio druskos, M9138, Sigma-Aldrich, Vokietija) (GM).

2. 5 mM glutamato ir 5 mM malato plius 0,005 mM palmitoil-L-karnitino (pagaminto iš palmitoil-L-karnitino chlorido, P1645, Sigma-Aldrich, Vokietija) (GM + PC).

3. 5 mM piruvato (pagaminto iš piruvato druskos, P2256, Sigma-Aldrich, Vokietija) ir 5 mM malato (PM).

4. 5 mM piruvato ir 5 mM malato plius 0,005 mM palmitoil-L-karnitino (PM + PC).

5. 0,005 mM palmitoil-L-karnitino plius 0,25 mM malato (PC + M).

6. 5 mM sukcinato (pagaminto iš sukcinato druskos dvibazinio heksahidrato, S2378, Sigma-Aldrich, Vokietija) plius 0,001 mM rotenono (pagaminto iš rotenono, R8875, Sigma-Aldrich, Vokietija) (SU + RO).

Rotenonas buvo naudojamas I kvėpavimo grandinės kompleksui slopinti (Scholte, 1973), matuojant deguonies suvartojimo greitį, susijusį su II kvėpavimo grandinės komplekso aktyvumu (Bouderba et al., 2012), kai sukcinatas naudotas kaip substratas.

1 ml IT6 buvo įpilama į kiuvetę su deguoniui jautriu elektrodu kartu su vienu iš nurodytų kvėpavimo substratų ir kreatino fosfokinaze (C3755, Sigma-Aldrich, Vokietija). Galutinė kreatino fosfokinazės koncentracija kiuvetėje (pagal gamintojo protokolą) buvo 0,05 mg · ml^{-1}. IT6 viso eksperimento metu buvo laikoma 37 ºC temperatūroje. Darbinis mitochondrijų suspensijos tūris, kuris buvo įšvirkščiamas į uždarytą kiuvetę, atitiko 1,0 mg kepenų mitochondrijų baltymo ir 0,15 mg raumenų mitochondrijų baltymo. Trečia metabolinė būsena (3 būsena) buvo inicijuojama pridedant 1 mM ATP, kuris kreatinfofsokinazės dėka konvertuotas į ADP. Gautame tirpale jis kartu su kreatinu funkcionavo kaip ADP regeneruojanti sistema (Kholodenko et al., 1987). 3 būsenos deguonies suvartojimas išreikštas kaip nmol O · min^{-1} · mg $baltymo^{-1}$.

1.3. Metabolizmo matavimai, fizinis aktyvumas. Antras tyrimas

Kontrolinės grupės B6 (n = 18) pelės, kurių CS aktyvumas normalus, B6.A (n = 18) ir B6.A10 (n = 18) pelės, kurių CS aktyvumas sumažintas, buvo įtrauktos į medžiagų apykaitos eksperimentą (po 9 patinus ir 9 pateles kiekvienoje linijoje). Medžiagų apykaitos matavimai atlikti naudojant metabolinį narvą (Physiocage 00, Panlab Harvard Apparatus, Barselona, Ispanija), sujungtą su dujų analizatoriumi

(LE405, Panlab Harvard Apparatus, Barselona, Spain), kuris buvo sukalibruotas naudojant dujų mišinius viršutiniame (50 proc. O_2, 1,5 proc. CO_2) ir apatiniame (20 proc. O_2, 0 proc. CO_2) kalibracijos taškuose. LE405 sujungtas su jungikliu LE400 (Panlab Harvard Apparatus, Barselona, Ispanija), kuriame nustatyta 250 ml · min^{-1} oro tėkmė. Metabolinė sistema atliko laisvai judančių gyvūnų viso kūno kvėpavimo dujų analizę. Buvo registruojami tris valandas trukusio eksperimento paskutinių dviejų valandų matavimų rodikliai (pirma valanda skirta pelių aklimacijai). Naudojant metabolinę sistemą, buvo matuojami šie medžiagų apykaitos parametrai: absoliutus (ml · min^{-1}) ir santykinis (ml · min^{-1} · $kg^{0.75}$ nuo kūno masės) deguonies suvartojimas (VO_2), anglies dioksido (VCO_2) išskyrimas, absoliutus (kcal · $diena^{-1}$) ir santykinis (kcal · $kg^{-0.75}$ · $diena^{-1}$) energijos eikvojimas (angl. *energy expenditure,* EE), kvėpavimo koeficientas (angl. *respiratory quotient,* RQ). Metabolizmo sistema (Panlab Harvard Apparatus, Barselona, Ispanija) atlieka viso kūno įkvėpiamų ir iškvėpiamų dujų analizę laisvai judančiose pelėse. EE skaičiuojamas pagal „Weir“ formulę (2):

$$EE = [3.815 + (1.232 \cdot RQ)] \cdot VO_2 \cdot 1.44 \quad (2)$$

Fizinis aktyvumas buvo nustatomas naudojant prie narvo apatinės dalies pritvirtintus judesiui jautrius matuoklius. Pasistiebimai buvo registruojami naudojant 10 cm aukštyje pritvirtintus infraraudonųjų spindulių barjerus.

Rezultatams analizuoti buvo naudojamas programinis paketas „Metabolism version 1.2“ (Panlab Harvard Apparatus, Barselona, Ispanija).

1.4. Badavimas. Trečias tyrimas

1.4.1. Eksperimento dizainas, medžiagų apykaitos analizė

Medžiagų apykaitos matavimai atlikti šviesos ciklo metu (nuo 9 iki 18 val.). 16 savaičių amžiaus B6, B6.A, B6.A10, BEH ir BEH +/+ linijų (n = 9 kiekvienoje linijoje) patinams buvo sukeltas 48 val. badavimas (angl. *food deprivation,* FD), naudojant ankstesniuose kitų mokslininkų tyrimuose taikytą metodologiją (Allen et al., 2010). FD metu organizmas gauna tik vandens. Prieš eksperimentą pelės buvo maitinamos *ad libitum* pašaru. Kontroliniam matavimui kiekviena pelė buvo pasverta (Kern, ABS 80-4, Vokietija) ir ištestuota fiziologiniame narve 3 val. (1 val. aklimacijos ir 2 val. matavimų) prieš 48 val. FD. Kiekviena pelė taip pat buvo pasverta praėjus 24 val. po FD. Praėjus 48 val. po FD pelė buvo pasverta dar kartą ir antrą kartą buvo atliekami medžiagų apykaitos matavimai. Po medžiagų

apykaitos matavimų pelė buvo sveriama paskutinį kartą. 18 savaičių amžiaus BEH+/+ (n = 45), BEH (n = 35) pelių patinai buvo suskirstyti į neintervencinę kontrolinę (BEH+/+ n = 18, BEH n = 12) ir eksperimentinę (BEH+/+ n = 27, BEH n = 23) grupes. Neintervencinė kontrolinė grupė buvo naudojama tik kūno kompozicijos analizei. Eksperimentinė grupė prieš badavimo intervenciją reprezentavo kontrolinę intervencinę grupę (angl. *control,* CON), o po badavimo ta pati grupė reprezentavo eksperimentinę intervencinę (angl. *food-deprived,* FD) grupę. Metabolizmo matavimo procedūros metu kiekviena iš eksperimentinių pelių buvo pasverta ir ištestuota fiziologiniame narve prieš (CON) ir po (FD) 48 val. badavimo. Visi medžiagų apykaitos analizės žingsniai sutapo su aukščiau aprašytais B6 pelių linijos. Energijos metabolizmo ir fizinio aktyvumo matavimų pabaigoje pelė buvo pasverta paskutinį kartą (Kern, ABS 80-4, Vokietija).

1.4.2. Atskiro raumens masė ir raumenų ir kūno masės santykis

Eksperimento pabaigoje pelės buvo nužudytos naudojant CO_2 dujas. Išpreparuoti visų tirtų pelių galinių kojų griaučių raumenys: dvilypis blauzdos raumuo (lot. *gastrocnemius,* GAS), padinis raumuo (lot. *plantaris*, PL), plekšninis blauzdos raumuo (lot. *soleus,* SOL), ilgasis tiesiamasis pirštų raumuo (lot. *extensor digitorum longus,* EDL), priekinis blauzdos raumuo (lot. *tibialis anterior,* TA). Išpreparuoti raumenys pasverti 0,1 mg tikslumu (Kern, ABS 80-4, Vokietija) ir užšaldyti skystame azote („Litgenas“, Kaunas, Lithuania). Kombinuota griaučių raumenų masė buvo paskaičiuota pagal kairės ir dešinės galinių kojų visų penkių (aukščiau išvardytų) išpreparuotų raumenų masė. Raumenų ir kūno masės santykis apskaičiuotas pagal formulę (3):

Raumenų masės suma (g) / kūno masė (g)·100 (3)

1.4.3. CS aktyvumo nustatymas iš raumenų audinio

CS aktyvumas buvo matuojamas iš homogenizuotų griaučių raumenų pagal ankstesniuose mūsų tyrimuose aprašytą metodiką (Kvedaras et al., 2017; Ratkevicius et al., 2010). 40–70 mg GAS raumens (n = 8) buvo homogenizuoti šaltame lizavimo buferyje (50 mM Tris–HCl, 100 mM KHPO4, 2 mM EDTA, 0,2 proc. w/v BSA, pH 7,0) ir užšaldyti skystame azote. Eksperimento dieną homogenatai buvo atšaldyti ir centrifuguoti 13 000 g 10 min. 4 °C temperatūroje. Bradfordo metodas (angl. *Bradford assay*) buvo taikomas baltymo koncentracijai nustatymui supernatante. CS aktyvumo matavimai buvo atlikti naudojant reakcijos

reagentą (100 mM trietanolaminas-HCl, ditiobis (2-nitrobenzoinė rūgštis), 0,5 mM Triton-X (0,25 proc. vol/vol), oksalacetatas, 0,31 mM acetyl CoA, pH 8,0) ir spektrofotometras (GENESYS 10 Bio UV-Vis, „Thermo Fisher Scientific“, Jungtinės Amerikos Valstijos) 21 °C temperatūroje. CS standartas (C3260-200UN, „Sigma-Aldrich“, Vokietija) naudotas kalibracijai. Molinis ekstinkcijos koeficientas buvo 13 600 M^{-1} cm^{-1} CoA-5,5'-tiobis (2-nitrobenzoinė rūgštis). Matavimai atlikti spektrofotomerte nustačius 412 nm bangų ilgį ir matuojant maksimalų CS aktyvumą pirmas 2 min.

1.4.4. Raumenų skaidulų kompozicija

SOL raumens skaidulų tipo kompozicija ir skaidulų dydis buvo nustatyti taikant ankstesniuose tyrimuose aprašytą ir prikaikytą metodiką (Kilikevicius et al., 2013). Buvo paskaičiuotos visos 1 ir 2 tipo raumenų skaidulos. Vidutinis skerspjūvio plotas buvo paskaičiuotas naudojant 25 proc. atsitiktiniu būdu parinktas skaidulas.

1.4.5. Riebalų pasiskirstymas

Riebalų pasiskirstymas buvo išanalizuotas pagal ansktesniuose mūsų tyrimuose aprašytą metodiką (Kvedaras, Minderis, Krusnauskas, Lionikas, & Ratkevicius, 2019). Riebalai iš keturių skirtingų pelės kūno vietų buvo išpreparuoti ir pasverti. Riebalų suminė masė buvo paskaičiuota iš baltųjų adipozinių poodinių (angl. *white adipose subcutaneous,* sWAT), gonadalinių (angl. *gonadal,* gWAT, visceralinių (angl. *visceral,* vWAT), kuriuos savo ruožtu sudaro mezenteriniai (angl. *mesenteric,* mWAT) ir perineriniai (angl. *perirenal,* pWAT). Taip pat buvo išpreparuoti rudieji riebalai (angl. *intrascapular brown adipose tissue,* iBAT), kurie taip pat buvo įskaičiuoti į suminę riebalų masę.

1.5. Svorio mažinimo maistinės intervencijos. Ketvirtas tyrimas

10 sav. amžiaus B6 pelės (n = 30), kurios visa savo gyvenimą valgė įprastą pašarą, buvo perjungtos į obesogeninę (daug riebalų ir daug angliavandenių turinčią) dietą (D12451, 45 proc. ir 17,5 proc. kcal iš riebalų ir angliavandenių, „Research Diets“, Jungtinės Amerikos Valstijos). Obesogeninės dietos trukmė buvo 18 sav. (Alhindi et al., 2019). Po nutukimo fazės sekė 6 sav. kalorijų apribojimas (angl. *caloric restriction,* CR), kuris buvo taikomas pelėse, kurios valgė mažai riebalų turintį pašarą (angl. *Low-Fat,* n = 10, D17100401, „Research

Diets“, Jungtinės Amerikos Valstijos) arba mažai angliavandenių turinčią dietą (*Low-Carb,* n = 10, D12492, „Research Diets“, Jungtinės Amerikos Valstijos). Kalorijų apribojimas palaipsniui didėjo nuo 20 proc. (1 sav.) iki 40 proc. (5–6 sav.). Po kontrolinių svorio matavimų buvo atrinktos aštuonios pelės, kurios reprezentavo predietinę nutukusią grupę (angl. *pre-diet obese controls, Pre*). *Regular* dietos grupė buvo naudojama kaip referentinė.

1.5.1. Gliukozės tolerancija, metabolizmas ir kūno kompozicija

Šešių kontrolinių taškų gliukozės tolerancijos testas buvo atliekamas 8–9 val. ryte po naktinio badavimo per paskutinę šeštą CR savaitę. Pelėms buvo atlikta gliukozės tirpalo (2 g glucose · kg body wt-1) injekcija, ir naudojant gliukomatį („Glucocard X-mini plus GT-1960“, Japonija) buvo matuojami gliukozės koncentracijos rodikliai tokiais laiko intervalais: 0, 15, 30, 60, 90 ir 120 min. po gliukozės injekcijos. Gliukozės atsako Plotas po kreive (angl. *the area under curve,* AUC) buvo paskaičiuotas naudojant Prism 8.0 kompiuterinę programą („GraphPad Software Inc.“, Jungtinės Amerikos Valstijos).

Energijos metabolizmo analizė atlikta pagal aukščiau aprašytą metodiką (žiūrėti 2.3). Kūno kompozicijos (raumenų masės bei riebalų kompozicijos) analizė atlikta pagal aukščiau aprašytus metodus (žiūrėti 1.4.2, 1.4.4).

1.6. Statistinė analizė

Duomenų analizė atlikta naudojant „Prism 8.0“ ir „SPSS 20.0“ programinius paketus. Duomenys buvo patikrinti, ar atitinka normalųjį skirstinį, taikant Shapiro–Wilk testą. Visiems statistiniams testams buvo taikomas statistinis $p < 0,05$ reikšmingumas. Duomenys reprezentuoti kaip reikšmės ± standartinis nuokrypis (*SN*).

Pirmas tyrimas. Dviejų veiksnių variacijos analizės (angl. *2-way ANOVA*) testas ir „Bonferroni post hoc“ testas buvo taikomi nustatant skirtumus tarp pelių linijų rezultatų ir skaičiuojant mitochondrijų kvėpavimo skirtumus tarp pelių linijų ir tarp skirtingų pelių lyčių. T-testas buvo taikytas vertinant CS aktyvumo skirtumus.

Antras tyrimas. Medžiagų apykaitos rezultatams įvertinti buvo naudojami „2-way ANOVA“ ir „Bonferroni post hoc“ testai, nustatant skirtumus tarp pelių linijų (B6, B6.A, B6.A10). Stjudento t-testas (t-testas) buvo taikomas analizuojant

skirtumus tap skirtingų pelių lyčių.

Trečias tyrimas. Energijos metabolizmo rezultatams įvertinti buvo naudojami „2-way ANOVA“ ir „3-way ANOVA“ testai kartu su Bonferroni post hoc testu, kurių pagalba buvo palyginti skirtumai tarp pelių linijų (B6, B6.A, B6.A10, BEH+/+, BEH). Dviejų veiksnių pasikartojančių reikšmių variacijos analizė (angl. *2-way repeated measures ANOVA*) su „Bonferroni post hoc“ testu buvo pritaikyti lyginant skirtumus tarp CON ir FD būsenų. Vienfaktorinė veiksnių variacijos analizė (angl. *one factor analysis of variance, 1-way ANOVA*) buvo atlikta skirtumams tarp atskirų raumenų apskaičiuoti. Kovariacijos (angl. *ANCOVA*) analizė atlikta tam, kad palyginti energijos eikvojimo (EE) skirtumus tarp pelių linijų. T-testas buvo taikomas analizuojant skirtumus tarp FD ir CON raumenų masių BEH ir BEH+/+ pelėse.

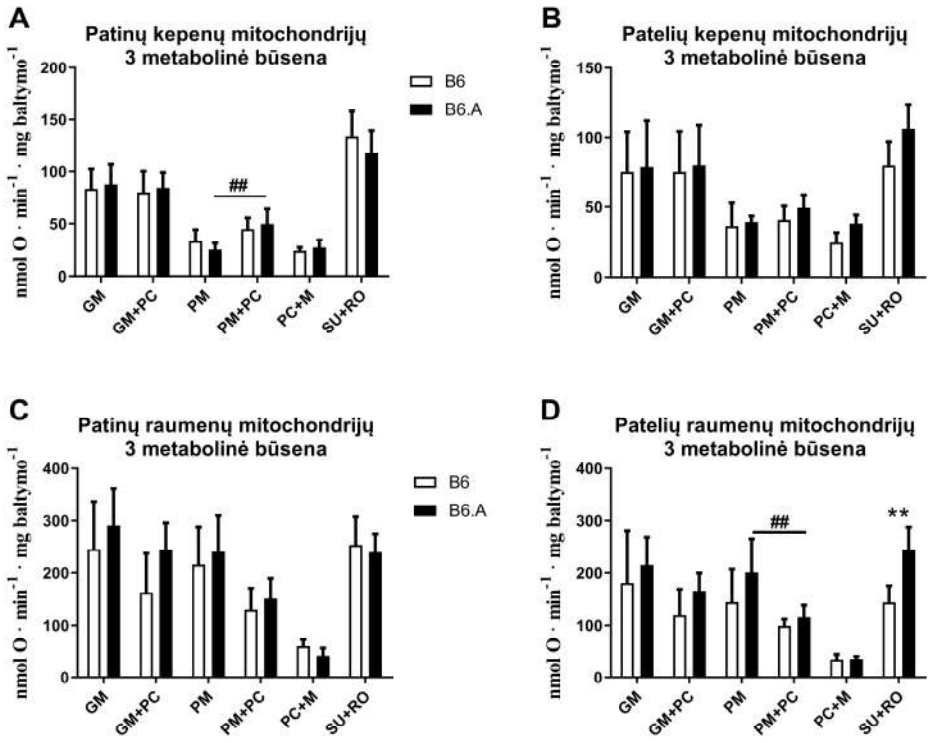
Ketvirtas tyrimas. Reikšmių įvertinimui buvo naudojamas „1-way ANOVA“ testas kartu su Bonferroni post hoc testu. Neparametrinis „Kruskal–Wallis“ testas su „Dunn post hoc“ analize pritaikytas atvejams, kurie neatitiko normalaus pasiskirstymo. „Two-way repeated measures ANOVA“ buvo taikoma kūno masės pokyčiams įvertinti, kai tos pačios buvo naudojamos kontrolinei ir eksperimentinei grupėms. „ANCOVA“ analizė atlikta nustatant EE skirtumus tarp pelių grupių pagal ankstesniame tyrime aprašytą metodinę rekomendaciją (Tschöp et al., 2011). Kūno masė ir fizinis aktyvumas „ANCOVA“ testui parinkti kaip kovariacinės reikšmės (angl. *covariates*).

2. REZULTATAI

2.1. Pirmas tyrimas

Buvo vertinamas B6 ir B6.A pelių kepenų mitochondrijų CS aktyvumas. B6.A pelių CS aktyvumas, lyginant su B6 pelėmis, buvo 32 proc. mažesnis ($p < 0,01$).

B6 ir B6.A pelių kepenų mitochondrijų 3 būsenos rodikliai nesiskyrė (1A ir 1B pav.). Didžiausi kepenų mitochondrijų 3 būsenos rodikliai nustatyti naudojant SU + RO substratą (B6 patinų ir patelių atitinkamai $134,1 \pm 22,7$ ir $79,7 \pm 15,5$ nmol · min^{-1} mg^{-1} baltymo; B6.A patinų ir patelių atitinkamai $118,1 \pm 21,3$ ir $106,1 \pm 17,5$ nmol · min^{-1} mg^{-1} baltymo).



Pastaba. Substratai: GM – glutamatas-malatas, GM + PC – glutamatas-malatas plius palmitoil-L-karnitinas, PM – piruvatas-malatas, PM + PC – piruvatas-malatas plius palmitoil-L-karnitinas, PC + M – palmitoil-L-karnitinas plius malatas, SU + RO – sukcinatas plius rotenonas. Dydžiai yra vidurkiai ± SN; ** $p < 0,01$ – B6, lyginant su B6.A linijos rodikliais; ## $p < 0,01$ – PM, lyginant su PM + PC substratu.

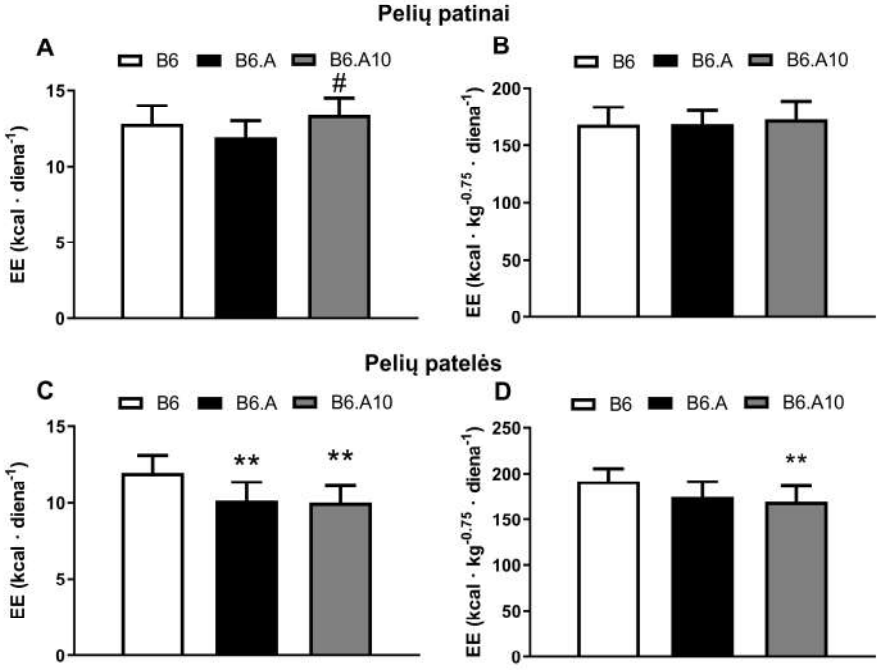
1 pav. Patinų (A, C) ir patelių (B, D) kepenų (A, B) ir raumenų (C, D) mitochondrijų 3 metabolinės būsenos skirtumai tarp B6 ir B6.A linijų

Raumenų mitochondrijų 3 būsenos rodikliai buvo mažiausi naudojant PC + M substratą (1C ir 1D pav.). B6.A pelių 3 būsenos rodikliai naudojant SU + RO buvo didesni nei B6. B6.A patelių 3 būsenos rodikliai naudojant PM + PC buvo didesni nei B6 ($p < 0,01$). Nustatyta, kad B6 patelių kepenų ir raumenų mitochondrijų 3 būsenos rodikliai naudojant SU + RO buvo didesni nei patinų ($p < 0,001$). O B6.A linijos 3 būsenos rodikliai naudojant SU + RO tarp patinų ir patelių nesiskyrė ($p > 0,05$). Patinų 3 būsenos rodikliai buvo didesni nei patelių naudojant GM ($p < 0,01$) ir GM + PC ($p < 0,05$) substratus.

2.2. Antras tyrimas

B6.A patinai (29,2 ± 1,2 g) buvo lengvesni nei B6 (32,2 ± 1,1 g) ir B6.A10 (33,0 ± 1,6 g) patinai. B6 (24,9 ± 3,1 g) ir B6.A (22,5 ± 0,9 g) patelės buvo 23 proc. lengvesnės nei B6 (32,2 ± 1,1 g) ir B6.A (29,2 ± 1,2 g) patinai. B6.A10 patelės (23,1 ± 1,4 g) buvo 20 proc. lengvesnės nei B6.A10 patinai (33,0 ± 1,6 g).

B6 ir B6.A bei B6.A10 patinų VO_2 ir VCO_2 reikšmės nesiskyrė. Tačiau absoliučios B6.A10 patinų VO_2 ($p < 0,05$) ir VCO_2 ($p < 0,01$) reikšmės buvo didesnės nei B6.A patinų. Priešingai nei patinų, B6 patelių VO_2 ir VCO_2 reikšmės tarp B6 ir B6.A bei B6.A10 linijų skyrėsi. Absoliučios B6 patelių VO_2 ir VCO_2 reikšmės buvo didesnės už B6.A (VO_2 – $p < 0,05$, VCO_2 – $p < 0,01$) ir B6.A10 ($p < 0,01$). Santykinės B6 patelių VO_2 reikšmės buvo taip pat didesnės nei B6.A ir B6.A10 ($p < 0,01$). Santykinės B6 patelių VCO_2 reikšmės buvo didesnės nei B6.A ($p < 0,05$), tačiau nesiskyrė nuo B6.A10 linijos VCO_2 reikšmių.



Pastaba. EE – energijos eikvojimas. Dydžiai yra vidurkiai ± SN;
** $p < 0,01$, lyginant su B6 linijos rodikliais; # $p < 0,05$ yra susijęs
su skirtumais tarp B6.A ir B6.A10 pelių linijų rodiklių.

2 pav. Absoliučių (A, C) ir santykinių (B, D) energijos eikvojimo reikšmių skirtumai tarp B6, B6.A ir B6A.10 patinų (A, B) ir patelių (C, D)

Visų linijų patinų santykinis EE buvo stabilus (B6, B6.A ir B6.A10; $p > 0,05$) (2A pav.), o absoliučios EE reikšmės B6.A10 pelių buvo didesnės nei B6.A (2B pav.). Absoliučios B6 patelių EE reikšmės buvo didesnės nei B6.A ($p < 0,01$) ir B6.A10 ($p < 0,01$) (2C pav.). Santykinės B6 patelių EE reikšmės buvo didesnės nei B6.A10 patelių ($p < 0,01$) (2D pav.).

Visų linijų (B6, B6.A ir B6.A10) ir lyčių RQ, fizinio aktyvumo ir pasistiebimų reikšmės nesiskyrė. B6.A pelės buvo mažiau aktyvios nei B6 pelės ($p < 0,05$). Nustatyta, kad B6 patelių santykinės EE reikšmės buvo didesnės nei B6 patinų ($p < 0,01$), tačiau B6.A ir B6.A10 linijose tokių lyčių EE skirtumų nenustatyta. Pabrėžtina, kad B6 patelių pasistiebimų reikšmės taip pat buvo didesnės nei B6 patinų ($p < 0,05$).

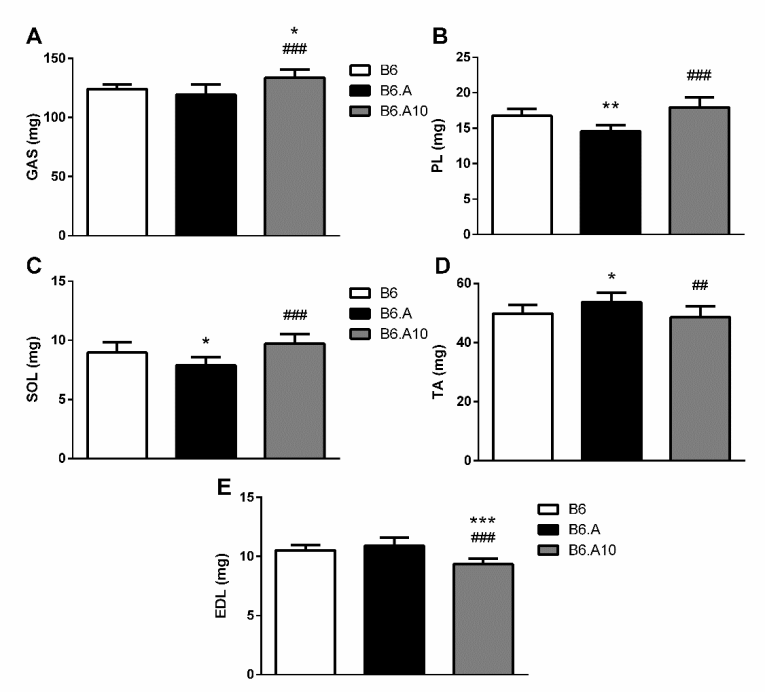
2.3. Trečias tyrimas

2.3.1. Sumažinto CS aktyvumo atsakas į badavimą

B6.A pelės buvo lengvesnės nei B6 ir B6.A10 visuose trijuose laiko taškuose: 0 val., 24 val. ir 48 val. FD ($p < 0,01$). FD turėjo įtakos visų tirtų pelių linijų kūno masei, lyginant su kūno mase prieš FD. Stipresnis FD poveikis procentiniam kūno masės pokyčiui nustatytas po pirmų 24 val. FD ($p < 0,001$) nei po paskutinių 24 val. FD ($p < 0,01$). Kūno masės pokyčių skirtumų tarp linijų visuose trijuose laiko taškuose nenustatyta ($p > 0,05$).

Raumenų masės suma buvo apskaičiuota po FD intervencijos. Nustatyta, kad B6 pelių raumenų masės suma buvo didesnė nei B6.A ($p < 0,001$), bet mažesnė nei B6.A10 pelių ($p < 0,01$). B6 pelių raumenų ir kūno masės santykis buvo mažesnis nei B6.A ir B6.A10 pelių ($p < 0,01$).

B6.A10 pelių GAS raumens masė buvo didesnė nei B6 ir B6.A pelių (atitinkamai $p < 0,05$ ir $p < 0,001$) (3A pav.). B6.A10 pelių PL ir SOL raumenų masė buvo didesnė nei B6.A pelių ($p < 0,001$) (3B ir 3C pav.). Įdomu, jog B6.A pelių PL ir SOL raumenys buvo lengvesni nei B6 pelių (atitinkamai $p < 0,01$ ir $p < 0,05$) (3B ir 3C pav.). TA ir EDL raumenų masės tendencijos ir skirtumai skyrėsi nuo GAS, PL ir SOL. B6.A pelių TA svėrė daugiau nei B6 ($p < 0,05$) ir B6.A10 pelių ($p < 0,01$) (3D pav.). B6.A10 pelių EDL raumens masė buvo mažesnė nei B6 ir B6.A pelių ($p < 0,001$) (3E pav.).



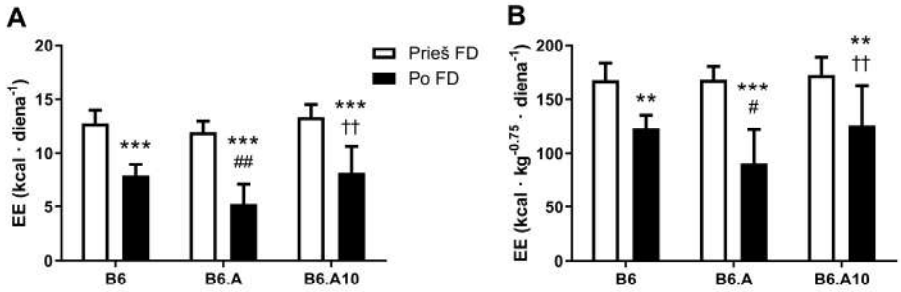
Pastaba. Dydžiai yra vidurkiai ± SN. GAS – dvilypis raumuo; PL – padinis raumuo; SOL – plekšninis raumuo; TA – priekinis blauzdos raumuo; EDL – ilgasis tiesiamasis pirštų raumuo. Dydžiai yra vidurkiai ± SD. ## $p < 0,01$; ### $p < 0,001$, lyginant su B6.A linijos rodikliais; * $p < 0,05$; ** $p < 0,01$; *** $p < 0,001$, lyginant su B6 linijos rodikliais.

3 pav. B6, B6.A ir B6.A10 linijų pelių griaučių raumenų masė po 48 val. badavimo

Prieš FD visų linijų absoliučios ir santykinės VO_2 bei VCO_2 reikšmės nesiskyrė ($p > 0,05$). Po FD visų linijų absoliučios ir santykinės VO_2 bei VCO_2 reikšmės sumažėjo ($p < 0,001$). Lyginant skirtumus tarp linijų nustatyta, kad po FD B6.A pelių absoliutus VO_2 tapo mažesnis nei B6.A10 pelių ($p < 0,01$). Po FD B6.A pelių VO_2 ir VCO_2 reikšmės tapo mažesnės nei B6 pelių ($p < 0,01$). B6 pelių santykinės VO_2 ir VCO_2 reikšmės po FD buvo mažesnės nei B6.A10 pelių ($p < 0,01$).

Prieš FD absoliučios ir santykinės pelių linijų EE reikšmės nesiskyrė ($p > 0,05$) (4 pav.). FD stipriai sumažino absoliučias ($p < 0,001$ – lyginant EE reikšmes prieš FD ir po jo) ir santykines ($p < 0,01$ – B6 ir B6.A10 linijų; $p < 0,001$ – B6.A, lyginant EE reikšmes prieš FD ir po jo) EE reikšmes. Absoliučios B6.A pelių EE reikšmės po FD tapo mažesnės nei B6 pelių ($p < 0,01$) (4A pav.). Po FD santykinės EE reikšmės B6.A pelių linijos sumažėjo labiau nei

B6 pelių linijos ($p < 0,05$) (4B pav.). Taip pat nustatyta, kad po FD absoliučios ir santykinės B6.A pelių EE reikšmės tapo mažesnės nei B6.A10 pelių ($p < 0,01$).

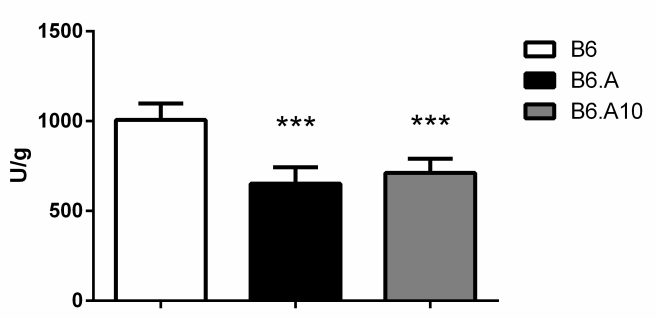


Pastaba. EE – energijos eikvojimas, prieš FD – reikšmės prieš badavimą, po FD – reikšmės po badavimo. Dydžiai yra vidurkiai ± SN; ** $p < 0,01$; *** $p < 0,001$ yra susijęs su reikšmėmis prieš FD; # $p < 0,05$; ## $p < 0,01$, lyginant su B6 linijos reikšmėmis; †† $p < 0,01$, lyginant su B6.A linijos reikšmėmis.

4 pav. B6, B6.A ir B6A.10 pelių linijų absoliučios (A) ir santykinės (B) energijos eikvojimo reikšmės prieš badavimą ir po jo

Prieš FD RQ skirtumų tarp linijų nenustatyta ($p > 0,05$). Po FD B6.A pelių RQ buvo mažesnis nei B6 pelių ($p < 0,05$). Svarbu pabrėžti, kad jokių fizinio aktyvumo ir pasistiebimų skirtumų prieš FD ir po jo tarp B6, B6.A ir B6.A10 linijų rezultatų nustatyta nebuvo ($p > 0,05$).

B6 pelių CS aktyvumas buvo didesnis nei B6.A ir B6.A10 pelių ($p < 0,001$) (5 pav.).

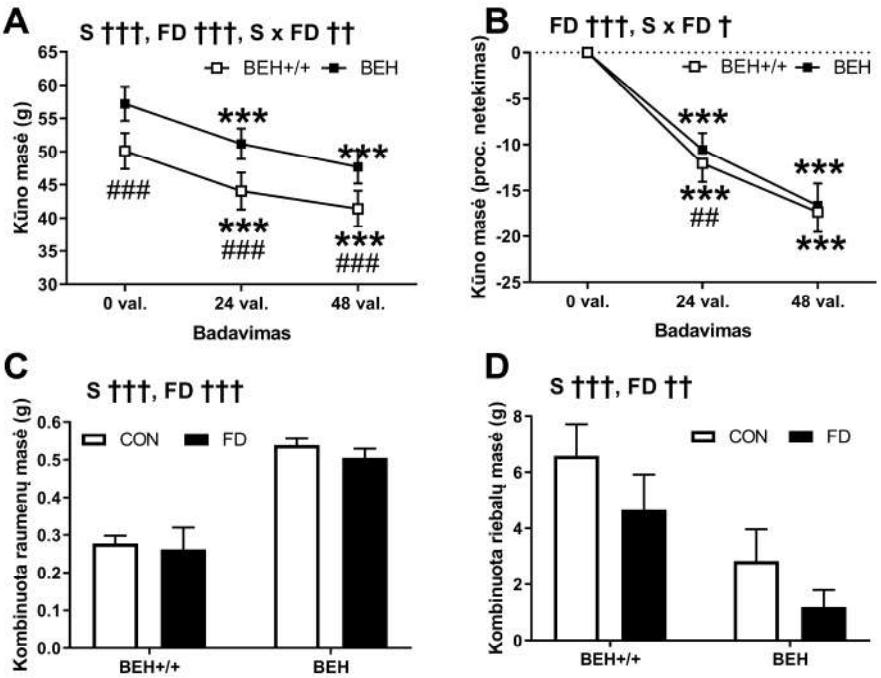


Pastaba. Dydžiai yra vidurkiai ± SN; *** $p < 0,001$, lyginant su B6 pelių rodikliais.

5 pav. Citrato sintazės aktyvumo skirtumai tarp B6, B6.A ir B6A.10 pelių po badavimo

2.3.2. Miostatino disfunkcijos atsakas į badavimą

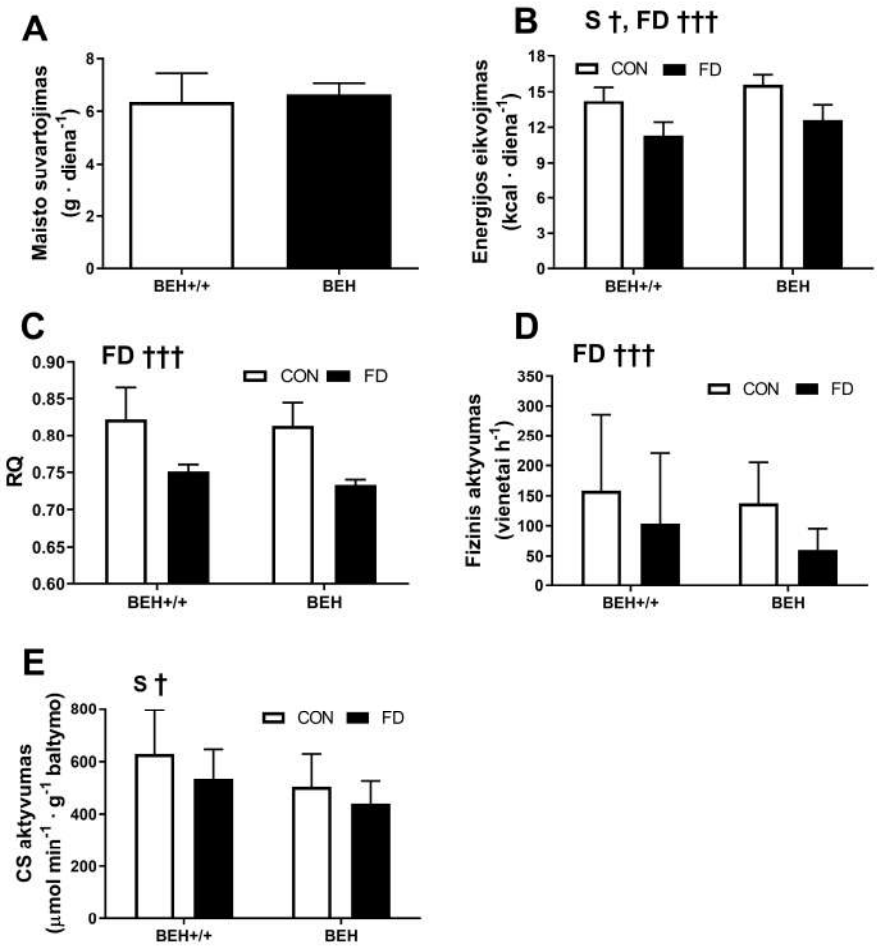
6 pav. parodyti morfometriniai BEH+/+ ir BEH pelių linijų duomenys kontrolinėje (angl. *control,* CON) prieš 48 val. badavimą ir po (angl. *food-deprived,* FD). BEH pelės buvo sunkesnės su daug didesne galinių kojų griaučių raumenų mase, bet turėjo mažesnę riebalų masę negu BEH+/+ *ad libitum* sąlygomis prieš badaujant (6A, C, D pav.). FD privedė prie polaipsninio kūno masės sumažėjimo (6A, B pav.), kuris buvo greitesnis BEH+/+ negu BEH linijose per pirmas 24 val., bet po 48 val. FD kūno masės mažėjimo skirtumai tarp šitų linijų išnyko. Abi pelių linijos patyrė santykinai mažą (~6 proc.) kombinuotos raumenų masės sumažėjimą (6C pav.) ir stiprų (> 30 proc.) riebalų sankaupų išeikvojimą (6D pav.).



Pastaba. Dydžiai yra vidurkiai ± SN; † $p < 0,05$, †† $p < 0,01$, ††† $p < 0,001$, pelių linijos (angl. *strain,* S), badavimo (FD) ir (arba) S × FD sąveikos poveikis; *** $p < 0,001$, skirtumai nuo atskaitos taško (0 val.); ## $p < 0,01$, ### $p < 0,001$, skirtumai tarp BEH+/+ ir BEH linijų

6 pav. Morfometriniai BEH+/+ ir BEH pelių duomenys: kūno masė (A, B) badavimo metu, kombinuota raumenų (C) ir riebalų (D) masės kontrolinėje (angl. *control,* CON) ir badavusioje (angl. *food-deprived,* FD) grupėse

Energijos metabolizmo charakteristikos ir fizinis aktyvumas parodyti 7 pav. Maisto suvartojimas nesiskyrė tarp BEH+/+ ir BEH linijų. EE buvo didesnis BEH negu BEH+/+, bet kovariacijos analizė (taikant kūno masę kaip kovariatą) neatskleidė skirtumų tarp šitų pelių linijų. FD sukelė EE sumažėjimą abiejose pelių linijose ($p < 0{,}001$) (7B pav.). RQ ir fizinis aktyvumas nesiskyrė tarp linijų ir sumažėjo po FD ($p < 0{,}001$) (7C, D pav.). GAS raumens CS fermento aktyvumas buvo mažesnis BEH pelių, lyginat jas su BEH+/+ ($p < 0{,}05$) (7E pav.).

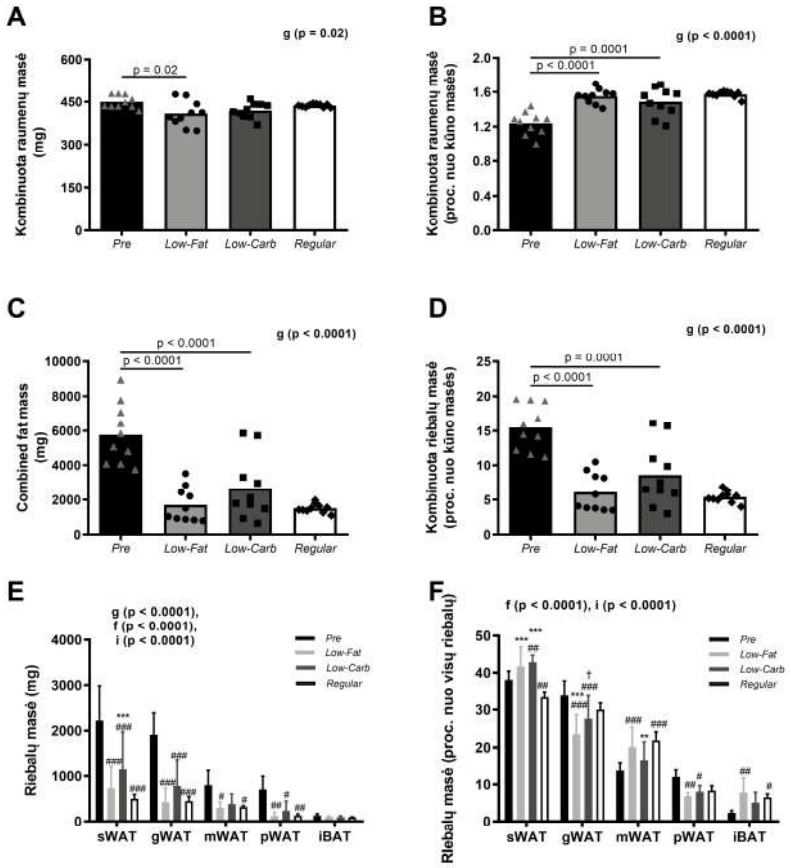


Pastaba. Dydžiai yra vidurkiai ± SN; † $p < 0{,}05$, ††† $p < 0{,}001$, pelių linijos (angl. *strain,* S) ir badavimo (FD) poveikis.

7 pav. Maisto suvartojimo (A), energijos eikvojimo (B), kvėpavimo koeficiento (RQ) (C), fizinio aktyvumo (D) ir citrato sintazės (CS) (E) palyginimas BEH+/+ ir BEH pelėse prieš (CON) ir po (FD) 48 val. badavimo

2.4. Ketvirtas tyrimas

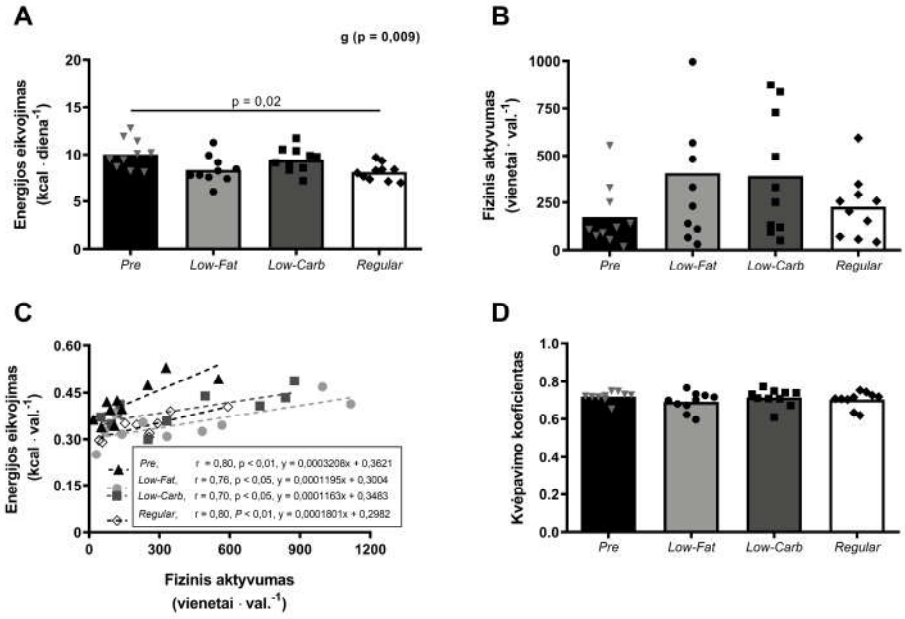
Mažai riebalų turinčios dietos (*Low-Fat*) grupė turėjo polinkį numesti daugiau svorio negu mažai angliavandenių (*Low-Carb*) turinčios dietos grupė. Kūno masės netekimas nesiskyrė tarp šitų dviejų grupių po 6 sav. kalorijų apribojimo (angl. *caloric restriction,* CR) ($p > 0,05$). Visų pelių kūno masė sumažėjo po CR.



Pastaba. Sutrumpinimai: (E, F): sWAT, poodiniai; gWAT, gonadaliniai; mWAT, mezenteriniai; pWAT, perineriniai; iBAT, rudieji. Dydžiai yra vidurkiai ± SN (E, F) arba dydžiai su individualiais taškais (A–D), kur kiekvienas taškas atstovauja vieną pelę. Linijos indikuoja reikšmingus skirtumus tarp sujungtų šitomis linijomis grupių. # $p < 0,05$, ## $p < 0,01$ and ### $p < 0,001$ lyginant su Pre, ** $p < 0,01$, *** $p < 0,001$ lyginant su Regular; † $p = 0,04$ lyginant su Low-Fat.

8 pav. Masės pokyčiai griaučių raumenyse (A, B), kūno riebaluose (C, D) ir riebaluose iš skirtingų pelės kūno paėmimo vietų (E, F) Low-Fat ir Low-Carb dietų grupėse po 6 sav. kalorijų apribojimo (angl. *caloric restriction,* CR). Low-Fat ir Low-Carb grupės palygintos su obesogenine Prie grupe prieš CR ir su referentine Regular grupe

Raumenų ir riebalų masių duomenys parodyti 8 pav. *Pre* grupei priklausė pelės, kurioms buvo taikyta obesogeninė dieta, bet nebuvo CR etapo. *Regular* dietos grupė buvo naudojama kaip referentinė. Kombinuota raumenų masė nedaug skyrėsi tarp grupių ir buvo ~5 proc. mažesnė *Low-Fat* ($p = 0,02$) grupėje negu *Pre* (8A pav.). Raumenų masė kūno masės vienetui padidėjo per 6 sav. CR *Low-Fat* ($p < 0,001$) ir *Low-Carb* ($p = 0,001$) grupėse (8B pav.). Iš kitos pusės, šitų grupių kūno riebalai sumažėjo ($p < 0,0001$) iki *Regular* grupės lygio (8C pav.) ir tapo reikšmingai mažesni negu *Pre* grupės ($6,09 \pm 2,73$ proc. ir $8,57 \pm 4,55$ proc. prieš $15,50 \pm 3,28$ proc. kūno masės, $p < 0,0001$, *Low-Fat* ir *Low-Carb* prieš *Pre* grupę, atitinkamai, 8D pav.). Taip buvo buvo išanalizuotas kūno riebalų pasiskirstymas išpreparuojant juos iš skirtingų pelės kūno vietų. Abi *Low-Fat* ir *Low-Carb* dietos sumažino riebalų masę iki *Regular* grupės lygio (8E pav.).



Pastaba. Visi matavimai atlikti po naktinio badavimo. Dydžiai yra reprezintuoti kaip individualūs taškai, kur kiekvienas taškas atstovauja vieną pelę. Linijos indikuoja reikšmingus skirtumus tarp sujungtų šitomis linijomis grupių.
Santrumpos: grupės poveikis (g); „Pearson" koreliacijos koeficientas (r) ir linijinių regresijų (y=…) lygtys (C)

9 pav. Absoliutus energijos eikvojimas (A), fizinis aktyvumas (B), energiojos eikvojimo ir fizinio aktyvumo sąsaja (C) ir kvėpavimo koeficientas (D) *Low-Fat* ir *Low-Carb* dietų grupėse po 6 sav. kalorijų apribojimo (angl. *caloric restriction,* CR). *Low-Fat* ir *Low-Carb* grupės palygintos su obesogenine *Pre* grupe prieš CR ir su referentine *Regular* grupe

Gliukozės tolerancija buvo panaši *Low-Fat* ir *Low-Carb* gupėse ($p > 0,05$), bet mažesnė negu *Pre* ($p < 0.01$) ir *Regular* dietų grupėse ($p < 0,05$). Absoliutus energijos eikvojimas nesiskyrė tarp *Low-Fat* ir *Low-Carb* grupių ($p < 0,05$) (9A pav.). *Pre* grupė parodė didesnį ($p = 0,02$) energijos eikvojimą negu *Regular* dietos grupė, bet ANCOVA analizė (kurioje kūno masė ir fizinis aktyvumas buvo parinkti kaip kovariatai) neparodė reikšmingų skirtumų tarp grupių bei paryškino stipresnę fizinio aktyvumo įtaką energijos eikvojimo pokyčiui. *Low-Fat* ir *Low-Carb* grupės turėjo polinkį į didesnį fizinį aktyvumą, lyginant jas su *Pre* ar *Regular* dietų grupėmis (9B pav.). Fizinio aktyvumo ir energijos eikvojimo sąsaja buvo reikšminga visose pelių grupėse ($r = 0,70–0,80$; $p < 0,05–0,01$) (9C pav.). Kvėpavimo koeficientas nesiskyrė tarp grupių (9D pav.).

3. REZULTATŲ APIBENDRINIMAS

Pagrindinis šio darbo tikslas buvo ištirti, ar CS aktyvumas gali pagreitinti substratų oksidaciją mitochondrijose, paskatinti laisvai judančių pelių lipidų oksidaciją ir išanalizuoti, ar sumažintas CS aktyvumas ir miostatino disfunkcija po visiško badavimo arba riebiosios dietos veikia pelių medžiagų apykaitą, raumenų bei kūno masę ir skatina laisvai judančių pelių gliukozės oksidaciją riebalų sąskaita. Darbas buvo suskirstytas į 4 tyrimus:

1. Sumažinto CS aktyvumo įtaka mitochondrijų kvėpavimui bei poveikis angliavandenių ir riebalų substratų oksidacijai mitochondrijose.

2. Sumažinto CS aktyvumo įtaka laisvai judančių pelių medžiagų apykaitai ir fiziniam aktyvumui.

3. Sumažinto CS aktyvumo ir miostatino disfunkcijos atsakas į badavimą, tiriant laisvai judančių pelių medžiagų apykaitą, fizinį aktyvumą, kūno ir raumenų masę bei jų pokytį FD metu.

4. Nutukusių pelių maistinių intervencijų įtaka kalorijų apribojimo sąlygomis.

1. Sumažinto CS aktyvumo įtaka mitochondrijų kvėpavimui. Buvo ištirtas B6 ir B6.A pelių linijų mitochondrijų CS aktyvumas ir mitochondrijų kvėpavimas. B6.A linija turėjo mutaciją *Cs* gene, o B6 buvo kontrolinė pelių linija. Šio tyrimo metu nustatyta, kad B6.A pelių mitochondrijų CS aktyvumas buvo ~32 proc. mažesnis nei B6 pelių.

Mitochondrijų kvėpavimo rezultatai parodė vienintelį reikšmingą skirtumą

tarp B6 ir B6.A patelių: B6.A raumenų mitochondrijų 3 būsenos rodikliai naudojant SU + RO buvo didesni nei B6 ir turėjo tendenciją didėti kepenų mitochondrijose. Rotenonas nėra substratas. Jis atlieka I kvėpavimo komplekso slopinimo funkciją. Kitas tyrimas parodė, kad mitochondrijų deguonies suvartojimo greitis naudojant SU substratą buvo didesnis nei GM (Baran et al., 2016). Šio tyrimo B6 patinų ir patelių kepenų mitochondrijų 3 būsenos rodikliai naudojant SU + RO buvo didesni nei naudojant kitus substratus. Įdomu, kad B6 patinų ir patelių deguonies suvartojimo greičio po riebiosios dietos (HFD) rezultatai buvo panašūs (Catala-Niell et al., 2008). B6.A pelių mitochondrijų kvėpavimas skyrėsi, lyginant 3 būsenos rodiklius naudojant angliavandenių substratus su riebiuoju PC substratu ir 3 būsenos rodiklius be PC. B6.A patinų kepenų ir raumenų mitochondrijose angliavandenių substratų oksidacija buvo efektyvesnė, į terpę pridedant PC (šiuo atveju PM + PC), nei naudojant vien PC (PC + M) substratą. Tokie skirtumai gali būti todėl, kad yra stiprus ryšys tarp riebalų substratų β-oksidacijos ir angliavandenių oksidacijos Krebso cikle (Rogge, 2009). Šiame darbe pristatyti rezultatai parodė kvėpavimo nuosmukį naudojant PC substratą. Raumenų mitochondrijų kvėpavimo slopinimą naudojant PC substratą galima paaiškinti adenino nukleotido translokazės slopinimas (Ciapaite et al., 2006).

Lyties skirtumai nustatyti tiriant kepenų ir raumenų mitochondrijų kvėpavimą naudojant SU + RO: B6 patelių 3 būsenos rodikliai buvo mažesni nei patinų.

Sumažinto CS aktyvumo įtaka medžiagų apykaitai. Pagrindinis šio tyrimo tikslas buvo išanalizuoti laisvai judančių kontrolinių B6 (kurių CS aktyvumas normalus) bei B6.A ir B6.A10 (kurių CS aktyvumas sumažintas) pelių medžiagų apykaitą ir fizinį aktyvumą bei nustatyti galimus skirtumus tarp šių pelių linijų.

Nustatyta, kad medžiagų apykaitos parametrų skirtumų tarp linijų nėra. Viena išimtis buvo nustatyta patelių EE: B6.A10 pelių santykinis EE buvo mažesnis nei B6. Įdomus faktas, kad tarp B6.A ir B6 pelių tokių EE skirtumų nustatyta nebuvo. Vienas galimas paaiškinimas yra skirtingas mutacijos tipas B6.A10 pelių linijoje (Johnson, Gagnon, Longo-Guess, & Kane, 2012), apimantis ne tik *Cs* geno, bet ir kitų genų mutaciją. Genetinis 10 chromosomos žemėlapis parodė įvarius molekulinius markerius (Justice et al., 1990), kurie gali lemti tirtų pelių linijų medžiagų apykaitos skirtumus. Analizuojant sumažinto CS aktyvumo

įtaką medžiagų apykaitai, šiame tyrime nebuvo taikoma riebioji dieta, kuri galėtų paveikti riebalų rūgščių oksidaciją. Vienas tyrimas parodė, kad riebioji dieta reikšmingai veikė EE (jis buvo matuojamas naudojant tą pačią Panlab metabolinę sistemą, kaip ir šiame tyrime), C57BL/6 lyginant su kontrolinėmis pelėmis (Cappelli et al., 2014). Stabilios visų trijų pelių linijų RQ reikšmės parodė, kad, esant normalioms sąlygoms (standartinis graužikų pašaras, nėra intervencijų), sumažintas CS aktyvumas nesukelia energijos disbalanso pelių medžiagų apykaitoje. Analizuojant lyčių skirtumus nustatyta, kad B6 patelės yra lengvesnės nei B6 patinai. Tai patvirtina ir ankstesni tyrimai (Sanz et al., 2007; Schulz et al., 2002). B6 patelių EE buvo didesnis už patinų. Tai rodo, kad šios linijos pelių patelės eikvoja daugiau energijos nei patinai. Ankstesnis tyrimas parodė didesnį patinų energijos pasisavinimą (Catala-Niell et al., 2008).

Sumažinto CS aktyvumo ir miostatino disfunkcijos atsakas į badavimą. Kaip ir buvo tikėtasi, kongeninių B6.A ir konsominių B6.A10 pelių raumenų CS aktyvumas buvo sumažintas, lyginant su kontrolinėmis B6 pelėmis. B6.A10 linijos pelių CS koduojantis genas, esantis telomeriniame 10 chromosomos regione, yra susijęs su sumažintu CS aktyvumu. Ankstesni tyrimų rezultatai parodė, kad A/J pelių raumens audiniuose CS aktyvumas yra sumažėjęs 50–65 proc., lyginant su kitomis pelių linijomis, nors linijų CS mRNR koncentracija buvo panaši (Ratkevicius et al., 2010). Šio tyrimo rezultatai parodė 35 proc. CS aktyvumo sumažėjimą B6.A ir 29 proc. sumažėjimą B6.A10 pelių linijoje, rezultatus lyginant su B6 pelių linijos rezultatais. Šio tyrimo rezultatai neprieštarauja ankstesnio tyrimo rezultatams (Ratkevicius et al., 2010).

Nors kūno masės skirtumų praėjus 24 ir 48 val. po FD tarp linijų nenustatyta, B6 pelių raumenų ir kūno masės santykis buvo mažesnis nei B6.A ir B6.A10 pelių. Galima iškelti hipotezę, kad B6.A ir B6.A10 pelės turi mažiau riebalų ir daugiau raumeninės masės nei kontrolinė grupė, kurios CS aktyvumas normalus. B6.A10 linijos pelių kojų raumenų masės suma buvo didesnė nei B6 ir B6.A pelių. Ankstesniame tyrime pelių 10 chromosoma buvo išanalizuota, joje identifikuoti įvairūs lokusai, susiję su neuronų vystymusi, kaulo išsivystymu ir augimu (Justice et al., 1990), kurie gali lemti raumenų masę.

FD smarkiai sumažino B6, B6.A ir B6.A10 pelių medžiagų apykaitos parametrų (VO_2, VCO_2, EE ir RQ) reikšmes. Svarbiausia šio tyrimo rezultatų dalis parodė, kad B6.A pelės, kurių EE ir RQ po FD žemesni, labiau atsparios visiškam badavimui nei kontrolinės B6 pelės. Tai gali reikšti, kad taškinė *Cs* geno mutacija

apsaugo energijos atsargas nuo stipraus katabolinio stimulo.

RQ rodiklis paprastai varijuoja nuo 1,0 iki 0,7, kaip aprašyta Panlab metabolinės sistemos protokole. RQ = 1,0 rodo gryną angliavandenių oksidaciją, RQ = 0,7 rodo gryną riebalų oksidaciją (Schutz, 1995). Labiau oksiduojamos molekulės (pvz., riebalų rūgštys) pilnam metabolizmui reikalauja daugiau deguonies ir pasižymi mažesniais RQ rodikliais (Kuo, Shiao, & Lee, 1993). Šiame tyrime po FD B6.A pelių RQ reikšmė nukrito iki ~0,75, o kontrolinių B6 pelių RQ sumažėjo tik iki ~0,77. Tai yra netiesioginis įrodymas fakto, kad pelės, kurių CS aktyvumas sumažintas, po FD intervencijos naudojo lipidus kaip pagrindinį energijos šaltinį, ir tai gali nulemti energijos balansą organizme (Ellis, Hyatt, Hunter, & Gower, 2010). Fizinio aktyvumo skirtumų tarp B6, B6.A ir B6.A10 prieš badavimo ir po jo nenustatyta. Tokie fizinio aktyvumo rezultatai atlieka „kontrolinio punkto“ vaidmenį, kai pašalinama „klaidinga“ *Cs* mutacijos įtaka tokiems medžiagų apykaitos parametrams, kaip EE ir RQ. Šiame tyrime fizinis aktyvumas nepaveikė tarp linijų nustatytų medžiagų apykaitos parametrų skirtumų po FD.

Miostatino disfunkcijos atsakas į badavimą. Pagrindinis šio tyrimo objektas buvo BEH ir BEH+/+ pelės, kurios yra atitinkamai su funkcionaliu miostatinu ir be jo. Pagrindinis šio tyrimo tikslas buvo įvertinti šių pelių linijų adaptacijos atsaką į stiprų raumenų atrofinį stimulą (48 val. FD). FD poveikis, kuris dažnai taikomas sukeliant raumenų nykimą, buvo analizuojamas tiriant pelių galinių kojų griaučių raumenų savybes (Allen et al., 2010). Šio tyrimo rezultatai parodė, kad miostatino disfunkcija siejama su didesniu griaučių raumenų masės netekimu. Miostatino slopinimas yra susijęs su padidintu PI3-kinazės/Akt/mTOR signaliniu keliu, kuris griaučių raumenyse stimuliuoja baltymo sintezę (Lipina et al., 2010; Schiaffino et al., 2013). Atrodo, kad tas pats signalinis kelias yra įtrauktas į autofaginių lizosomų kelią ir yra atsakingas už baltymo išsekimą (Glass, 2003). Ir atvirkščiai, padidinta miostatino geno raiška nuslopina Akt aktyvaciją (Trendelenburg et al., 2009).

Šiuo tyrimu įrodyta, kad pelės, turinčios miostatino disfunkciją (BEH), nėra apsaugotos nuo raumens masės nykimo po FD, net atvirkščiai – miostatino disfunkcija turi tendenciją sustiprinti raumens masės nykimą. Šie duomenys sutapo su ankstesnio tyrimo rezultatais, kurie parodė, kad pelės su nuslopintu miostatinu buvo labiau jautrios raumenų nykimui, lyginant su kontrolinėmis pelėmis (Allen et al., 2010).

Šio tyrimo rezultatai neparodė RQ skirtumų tarp BEH+/+ ir BEH linijų prieš

FD ir po jo, tačiau BEH+/+ pelių RQ sumažėjo nuo ~0,83 iki ~0,75, o BEH pelių – nuo ~0,82 iki ~0,73. Tokie rezultatai parodė, kad BEH pelės naudoja riebalinius substratus kaip pagrindinį energijos šaltinį po FD. Šie medžiagų apykaitos rezultatai neprieštarauja hipotezei, kad miostatino disfunkcija gali apsaugoti organizmą nuo atsparumo insulinui. Rezultatai taip pat parodė, kad B6 pelių medžiagų apykaita greitesnė nei BEH linijos. Žinoma, kad griaučių raumenys reikalauja daug ATP energijos (Baker, McCormick, & Robergs, 2010). Tai leidžia manyti, kad FD gali sukelti stipresnį efektą pelėms, kurių raumenų masė didelė. Jų EE taip pat bus didesnis. McPherron ir Lee (McPherron & Lee, 2002) bei Guo ir kt. (Guo et al., 2009) taip pat nenustatė medžiagų apykaitos skirtumų tarp pelių su nuslopintu miostatinu.

Po FD CS aktyvumas reikšmingai nesumažėjo, todėl funkcionalus mitochondrijų pajėgumas nenukentėjo. Tai rodo, kad griaučių raumenys išsaugojo aerobinės ATP resintezės pajėgumą, o šiuo tyrimu nustatyti skirtumai tarp pelių linijų galimai nėra susiję su energijos pokyčiais po badavimo.

4. Nutukusių pelių maistinių intervencijų įtaka kalorijų apribojimo sąlygomis. Pagrindinis mūsų tyrimo tikslas buvo išnagrinėti angliavandenių ir riebalų skirtingą sudėtį dietose, kurios paveikia fiziologinius procesus pelėse kalorijų apribojimo metu. Dauguma ankstesnių tyrimų buvo susikoncentruoti ties markomedžiagų sudėties dietose ir sveikatos bei kūno kompozicijos rodikliais valgant *ad libitum* (Hu et al., 2018; Solon-Biet et al., 2014), ir tik keli tyrimai buvo susiję su kalorijų apribojimų taikant tokio tipo dietas (Vangoitsenhoven et al., 2018). Neprieštaraujant ankstesnių atradimų, susijusių su žmogaus lengvu kalorijų apribojimu (Gardner et al., 2018), mūsų pelių tyrimų rezultatai parodė, kad kūno kompozicijos ir gliukozės tolerancijos pagerėjimai nesiskyrė tarp mažai riebalų turinčios (*Low-Fat*) ir mažai angliavandenių turinčios (*Low-Carb*) dietinių grupių, kurių baltymo kiekis buvo suvienodintas, ir kurios praėjo 40 proc. kalorijų apribojimo etapą. Panašu, kad ne angliavandenių ir riebalų santykis dietoje, bet pats sumažėjęs energijos suvartojimas su fiksuotu baltymų kiekiu yra pagrindinis veiksnys, lemiantis šiuos pagerėjimus. Padidėjusi riebalų oksidacija yra pagrindinis veiksnys *Low-Carb* dietos, kuris yra dažnai suvokiamas kaip daugiau lipolitinis ir mažiau obesogeninis lyginant su *Low-Fat* dieta, nors žmogaus metabolizmo studijos meta iššukį šiai hipotezei (K. D. Hall et al., 2016). Mes taip pat nenustatėme kvėpavimo koeficiento skirtumų tarp šitų dviejų dietų esant kalorijų apribojimo sąlygomis.

IŠVADOS

1. Mitochondrijų kvėpavimo rodikliai pelių, kurių CS aktyvumas normalus, reikšmingai nesiskyrė nuo tų, kurių CS aktyvumas buvo sumažintas.

2. CS aktyvumo lygis neįtakoja energijos eikvojimo, kvėpavimo koeficiento ir fizinio aktyvumo, kurie nesiskiria tarp pelių su normaliu ir sumažintu CS aktyvumu, išskyrus pateles, kurių absoliutus energijos eikvojimas yra mažesnis esant sumažintam CS aktyvumui. Pabrėžtina, kad abiejų pelių grupių su sumažintu CS aktyvumu, konsominių ir kongeninių, absoliutus energijos eikvojimas reikšmingai skiriasi nuo kontrolinių su normaliu CS aktyvumu. Konsominių pelių su pakeista 10 chromosoma šis rodiklis yra didesnis negu kongeninių, turinčių taškinę *Cs* geno mutaciją.

3. Pagrindiniai netiesioginės kalometrijos medžiagų apykaitos rodikliai sumažėja po 48 val. badavimo esant normaliam ir sumažintam pelių CS aktyvumui. Vis dėlto, energijos eikvojimas ir kvėpavimo koeficientas sumažėja labiau kongeninėse pelėse su sumažintu CS aktyvumu negu kontrolinėse pelėse su normaliu CS aktyvumu.

4. Miostatino disfunkcija lemia didesnę pelių raumeninę ir mažesnę riebalinę masę. Tačiau 48 val. badavimo metu miostatino disfunkcija neapsaugo pelių griaučių raumenų masės nuo sumažėjimo. Badaujant taip pat reikšmingai sumažėja riebalų atsargos, energijos eikvojimas, fizinis aktyvumas nepriklausomai nuo miostatino aktyvumo.

5. Mažai riebalų ir mažai angliavandenių turinčios dietos panašiai veikia nutukusių pelių kūno kompoziciją, energijos metabolizmą, fizinį aktyvumą ir gliukozės toleranciją esant kalorijų apribojimui. Panašu, kad ne angliavandenių ir riebalų santykis dietoje, bet pats sumažėjęs energijos suvartojimas su fiksuotu baltymų kiekiu yra pagrindinis veiksnys, lemiantis šiuos metabolinės sveikatos pagerėjimus.

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Original Article
OBESITY BIOLOGY AND INTEGRATED PHYSIOLOGY

Obesity

Hypocaloric Low-Carbohydrate and Low-Fat Diets with Fixed Protein Lead to Similar Health Outcomes in Obese Mice

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Objective: It is controversial whether low-carbohydrate diets are better suited for weight control and metabolic health than high-carbohydrate diets. This study examined whether these diets induce different improvements in body composition and glucose tolerance in obese mice during caloric restriction (CR).
Methods: Male C57BL/6J mice were fed an obesogenic diet ad libitum for 18 weeks and then subjected to 6-week progressive CR of up to 40%, using either a low-fat or low-carbohydrate diet with equal protein content. Mice fed a regular chow diet ad libitum served as controls. Body mass, hindlimb muscle mass, fat mass, energy expenditure, and glucose tolerance were compared between the groups.
Results: Initially low-fat and low-carbohydrate groups had similar body mass, which was 30% greater compared with controls. CR induced similar weight loss in low-fat and low-carbohydrate groups. This weight loss was mainly due to fat loss in both groups. Energy expenditure of freely moving mice did not differ between the groups. Glucose tolerance improved compared with the values before CR and in controls but did not differ between the diets.
Conclusions: Dietary carbohydrate or fat content does not affect improvements in body composition and metabolic health in obese mice exposed to CR with fixed energy and protein intake.

Study Importance

What is already known?

- Caloric restriction is an effective intervention against obesity.
- Caloric restriction can be applied by using various diets. Whether low-carbohydrate or low-fat diets are more effective is still not clearly established.

What does this study add?

- Carbohydrate to fat ratio in hypocaloric diets with equal energy and protein content does not affect improvements in body composition and glucose tolerance in obese mice.
- Thus, under conditions of caloric restriction, both diets are effective.
- Overall energy intake should be targeted, whereas dietary carbohydrate and fat content can be left to personal preferences for adherence purposes in diets for weight loss.

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Introduction

Obesity is a risk factor for many noninfectious chronic diseases, including cardiovascular heart disease, stroke, diabetes, and cancer, which are the major causes of premature death (1). The prevalence of obesity is steadily increasing, and the disease has become a threat to economic prosperity as well as the national security of many countries; thus, identification of solutions to the obesity epidemic is high on the agenda worldwide (2,3).

According to the paradigm of energy balance, animals and humans gain weight when their energy intake exceeds energy expenditure (4). Increases in physical activity could prevent weight gain, but adjustments in diet are often easier to implement on the population level (5). A key question is what diet is best suited for weight control. A popular belief is that macronutrient composition of food is important alongside reduction in food intake (6). Indeed, effect on satiety and dietary-induced thermogenesis are greater for dietary protein compared with carbohydrates or fat (7,8). Human overfeeding studies have suggested that protein has a smaller detrimental effect on body composition compared with carbohydrates and fat, which are usually the major candidates for restriction in various diets aimed for weight control (9). It is still controversial whether proportions of these two macronutrients are important for metabolic health (10,11). One of the theories proposes that dietary carbohydrates are inherently more obesogenic than fat because of the strong effect on insulin secretion (12). The so-called carbohydrate-insulin model of obesity is often criticized as lacking strong evidence in support of it (13). Nevertheless, a recent randomized controlled study with humans demonstrated that energy expenditure was up to 478 kcal/d greater on a low-carbohydrate diet compared with a high-carbohydrate diet for a similar energy intake

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(14). Diets promoting energy expenditure while keeping energy input unchanged would be a promising strategy for weight management. However, concerns were raised about the suitability of the doubly labeled water technique to measure energy expenditure in diets of varying carbohydrate and fat content as in the study of Ebbeling et al. (15). Nutrition epidemiology aimed at comparing different diets has also been plagued by methodological difficulties, which mainly concern assessment of food intake (16).

It appears that the inbred mouse model is well suited to examine the controversial issue about the importance of dietary composition for weight loss and metabolic health. Key advantages of such studies are that food intake can be better controlled than in human studies and unpredictable effects of genetic factors are minimized. The C57BL/6J mouse strain is prone to diet-induced obesity (17,18), and it tolerates various diets with large differences in carbohydrate and fat content well (19,20). A recent study of 29 diets demonstrated that dietary fat content was associated with greater energy intake and preponderance to obesity of these mice fed ad libitum (21). Our aim was to compare changes in body composition and metabolic health of the C57BL/6J mouse strain in response to two energy-restricted diets with large differences in carbohydrate and fat content. The strength of our study was that we used well-defined diets, which is difficult to achieve in human studies. We hypothesize that changes in body composition would not differ between these two diets if they are matched for the total and protein-derived caloric content.

Methods

Animals

The study was carried out at the Lithuanian Sports University with approval of all the procedures by the Lithuanian State Food and Veterinary Service in 2018 (reference number G2-90). The breeding pairs of C57BL/6J mouse strain were obtained from the Jackson Laboratory, and only males were used in the experiment. Mice were housed at ambient temperature of 20°C to 21°C and 40% to 60% humidity with an alternating 12-hour light/dark cycle. After weaning, mice were housed two to five animals per cage and fed ad libitum with a regular grain-based rodent chow diet (Kombi, Joniskio grudai, Lithuania), and the mice had unrestricted access to tap water. At 10 weeks of age, mice ($n = 30$) were switched to the obesogenic high-fat and high-sugar diet (D12451, 45% and 17.5% of kilocalories from fat and sugar; Research Diets Inc.) for 18 weeks (22). This was followed by 6-week caloric restriction (CR) on either a low-fat diet (low-fat, $n = 10$) or a low-carbohydrate diet (low-carb, $n = 10$). Ten weight-matched mice were examined prior to CR as prediet obese controls (Pre).

Dietary intervention

Obesity phase. After 10 weeks of the 18-week exposure to the obesogenic diet, mice were moved into separate cages, and food consumption was assessed every week for each mouse by subtracting food leftovers from initially provided food with corrections for humidity effect on the weight of pellets. Daily energy intake (DEI) of mice was calculated as follows:

$$DEI\left(\text{kcal}\cdot\text{g}^{-1}\cdot\text{d}^{-1}\right) = \frac{\text{Weekly Food Consumption (g)} \times \text{Food Energy Density}\left(\text{kcal}\cdot\text{g}^{-1}\right)}{\text{Body Mass (g)}\cdot 7\,\text{(days)}}$$

The 3-week average DEI of mice was 0.42 (0.04) kcal · g^{-1} · d^{-1}, and only mice gaining at least 20% of weight compared with the age-matched group on the regular chow diet (regular, $n = 10$) were used for the CR study.

CR phase. The 28-week-old obese mice were randomly assigned to one of the two CR groups and the Pre group, which was used for assessment of body composition and metabolism at the baseline. For 6 weeks, mice were fed daily at 8 AM, and CR was gradually increased from 20% (1 week) to 30% (2-4 weeks) and 40% (5-6 weeks) of the calculated energy intake on the ad libitum obesogenic diet. Energy intake during the CR phase was estimated for each mouse separately by reducing DEI by the extent of the caloric deficit and multiplying it by the initial body mass of the animal prior to CR. The amount of food was corrected for the different caloric density of the diets (4.1 and 5.2 kcal · g^{-1} for low-fat and low-carb, respectively) to achieve equal total energy and protein content in the diets (i.e., 20%, 60%, and 20% of kilocalories from fat, carbohydrates, and protein for low-fat [D17100401; Research Diets] and 20%, 60%, and 20% of kilocalories from carbohydrates, fat, and protein for low-carb [D12492; Research Diets]). Details of the macronutrient composition and sources of the diets are presented in Table 1.

Glucose tolerance

A glucose tolerance test was carried out after an overnight fasting at 8 to 9 AM during the 6th and final week of CR. Mice were subjected to an intraperitoneal injection of glucose solution (2 g glucose · kg body weight^{-1}), and a glucometer (Glucocard X-mini plus GT-1960) was used to measure glucose in the whole blood samples from the tail vein at 0, 15, 30, 60, 90, and 120 minutes after injection. The area under curve (AUC) for blood glucose was calculated by using Prism 6.0 software (GraphPad Software Inc.).

Body composition

During CR, mice were weighed weekly with a precision of 0.1 g (440-45N, Kern). After the 6-week CR, the 34-week-old mice were euthanized with an inhalation of CO_2. Immediately afterward, skeletal muscles and body fat were sampled and weighed with a precision of 0.1 mg (ABS 80-4, Kern). Combined hindlimb muscle mass was calculated as a sum of the gastrocnemius, plantaris, soleus, tibialis anterior, and extensor digitorum longus muscle mass. The muscles were trimmed from all visible tendons and blotted dry just before weighing. Combined body fat mass was assessed as the sum of the hindlimb subcutaneous, gonadal, mesenteric, and perirenal white adipose tissue and intrascapular brown adipose tissue (iBAT) as in previous studies (23,24).

Energy expenditure and physical activity

During the final week of CR, mice were fasted overnight and subjected to assessment of total energy expenditure and physical activity (24). Briefly, the metabolic cage of standard size was connected to the gas analyzer (LE405; Panlab Harvard Apparatus) and the switching device (LE400; Panlab Harvard Apparatus) for the control of the air flow. The gas analyzer was calibrated at the high point (50% O_2,

TABLE 1 Detailed macronutrient composition provided by diet manufacturer

Diet (mouse group)	Obesogenic diet (Pre)		Low-fat diet (low-fat)		Low-carbohydrate diet (low-carb)	
Group's energy state	Ad libitum		CR up to 40%		CR up to 40%	
Diet's manufacturer	Research Diets Inc.		Research Diets Inc.		Research Diets Inc.	
Diet's code	D12451		D17100401		D12492	
Protein (% kcal)	20		20		20	
Carbohydrate (% kcal)	35		60		20	
Fat (% kcal)	45		20		60	
Protein	g	kcal	g	kcal	g	kcal
Casein	23.3	93.3	20.0	80.1	25.8	103.3
L-Cystine	0.35	1.4	0.30	1.2	0.35	1.4
Carbohydrate						
Corn starch	8.5	33.9	40.5	162.1	0	0
Maltodextrin	11.7	46.6	12.5	50.0	16.1	64.6
Sucrose	20.1	80.6	6.9	27.5	8.9	35.5
Fiber						
Cellulose	5.83	0	5.0	0	6.5	0
Fat						
Soybean oil	2.9	26.2	2.5	22.5	3.2	29.1
Lard	20.7	186.3	6.5	58.5	31.6	284.8
Minerals						
Mineral Mix S10026	1.17	0	1.00	0	1.29	0
DiCalcium phosphate	1.52	0	1.30	0	1.68	0
Calcium carbonate	0.64	0	0.55	0	0.71	0
Potassium citrate	1.92	0	1.65	0	2.13	0
Vitamins						
Vitamin Mix V10001	1.17	4.7	1.00	4.0	1.29	5.2
Choline bitartrate	0.23	0	0.20	0	0.26	0
Total	100	473	100	406	100	524
kcal · g^{-1}	4.73		4.1		5.2	

1.5% CO_2) and at the low point (20% O_2, 0% CO_2). Air flow was set to 250 mL · min^{-1} with a 3-minute switching time between measurements of O_2 and CO_2 concentrations in the metabolic cage and the external environment. All metabolism measurements were performed during a light period (from 9 AM to 3 PM). Each mouse was weighed (440-45N, Kern) and transferred into metabolic cage for 3 hours with no food provided and ad libitum access to water. The respiratory quotient and total energy expenditure were calculated as the average values of the last 2 hours spent in the metabolic cage (Metabolism software version 1.2; Panlab Harvard Apparatus). The physical activity of mice was assessed by using strain gauges mounted on the supporting constructions of the metabolic cage. The integral of ground reaction forces was used as an indirect measure of physical activity.

Statistical analysis

All data are presented as mean (SD) or mean with plotted individual data points. The statistical analysis was performed using Prism 6.0 and IBM SPSS Statistics version 20 software. Normality of data distribution was verified with Shapiro-Wilk test. Means were compared with one-way ANOVA, using Bonferroni post hoc test to assess differences between the studied groups of mice. Nonparametric Kruskal-Wallis test with Dunn post hoc analysis was applied in the cases in which means did not meet a criterion of normal distribution. Two-way repeated-measures ANOVA was used for analysis of body mass change when it was assessed repeatedly on the same animals. ANCOVA was applied using linear models to assess effects of mouse groups on energy expenditure as previously recommended for this type of analysis (25). In this case, body mass and physical activity were used as covariates. Linear regression analysis was also used on the plots of energy expenditure over physical activity. Pearson correlation coefficient was calculated to assess the strength of the association between the variables. The level of significance was set at $P<0.05$.

Results

Energy intake was similar in low-fat and low-carb groups

Data on energy intake are presented in Figure 1. We aimed at maintaining similar energy intake in low-fat and low-carb groups during CR. However, the low-fat group did not consume all the food during the first week of CR, and the unconsumed food was left in the feeder with subsequent daily portions added on top of the leftovers. After 2 weeks of CR, the low-fat group matched the low-carb group for energy intake. For the entire 6-week CR, these groups did not differ in the absolute (Figure 1A) or body mass normalized energy intake when body mass before the start of CR was used for normalization (12.0 [0.4] and 12.1 [0.3] kcal · g initial body weight^{-1} for low-fat and low-carb, respectively; $P>0.05$) (Figure 1B). Mice in the regular diet group had ~15% greater energy intake compared with low-fat ($P=0.007$) and low-carb ($P=0.008$) groups during the same period (Figure 1A).

Body mass decreased similarly during CR in both diet groups

Data on body mass are presented in Figure 2. The low-fat group tended to lose more weight than the low-carb group during the first week of CR (Figure 2A). This was probably due to the reduced food intake in the low-fat group. Afterward, however, the low-fat group caught up with food intake and showed similar weight loss as the low-carb group. Overall body mass loss did not differ between these two diet groups after the 6-week CR (30.0% [5.6%] and 23.8% [7.5%] for low-fat and low-carb, $P>0.05$, respectively, Figure 2B). All mice showed clear reductions in body mass (Figure 2C). Initially, mice in the regular diet group, which was not subjected to obesogenic feeding, had lower body mass ($P<0.001$) than the low-fat and low-carb groups, but the differences between the groups became insignificant during the final 4 weeks of CR, which was applied to low-fat and low-carb groups only. The regular diet group showed a small reduction in body mass during a final week when measurements of energy metabolism and glucose tolerance were performed after the overnight fast.

Body fat but not skeletal muscle as main energy donor during CR

Data on muscle and fat mass are shown in Figure 3. The Pre group included mice that were subjected to the obesogenic diet but did not undergo CR. This group was used to assess effects of CR on muscle and fat mass in the low-fat and low-carb groups. The regular

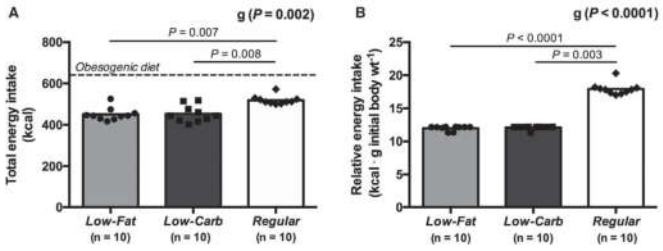

Figure 1 Energy intake for low-fat and low-carb groups during 6-week caloric restriction (CR) and in a regular group fed standard chow diet ad libitum. Total energy intake is shown (**A**) in absolute values and (**B**) normalized to body mass prior to CR. Data are presented as mean with each dot representing one mouse data sample. Nonparametric Kruskal-Wallis with Dunn post hoc analysis was performed for group effect (g). Lines indicate significant differences between connected groups. Dotted reference line indicates average ad libitum energy intake of the obesogenic diet during a 6-week period before assignment to CR with low-fat or low-carb diet.

diet group provided age-matched reference data. Combined muscle mass differed little between the groups, though it was ~5% smaller ($P=0.02$) in the low-fat group compared with the Pre group (Figure 3A). Muscle mass per unit of body mass increased following the 6-week CR in low-fat ($P<0.001$) and low-carb ($P=0.001$) groups (Figure 3B), while body fat decreased ($P<0.0001$) to the level of the regular diet group (Figure 3C) and became significantly lower than in the Pre group (6.09% [2.73%] and 8.57% [4.55%] vs. 15.50% [3.28%] body mass, $P<0.0001$, for low-fat and low-carb vs. Pre groups, respectively; Figure 3D). We also examined body fat distribution by sampling fat from five different sites of the body. Both low-fat and low-carb diets reduced fat mass in four out of five sites to the level of the regular diet group (Figure 3E). An exception was iBAT, which was not significantly affected by the diets and did not differ between the studied groups. Thus, CR tended to increase relative iBAT mass compared with the values prior to CR (Pre group), but this increase was significant only for the low-fat group ($P=0.002$) (Figure 3F). Relative mass of gonadal white adipose tissue decreased more in the low-fat than the low-carb group ($P=0.04$).

Glucose tolerance improved similarly independently of diets after CR

Data on glucose tolerance are presented in Figure 4. Glucose AUC was similar in the low-fat and low-carb groups ($P>0.05$) but smaller compared with the Pre group ($P<0.01$) and regular diet group ($P<0.05$) (Figure 4B). Pre and regular diet groups did not differ in glucose AUC. However, the regular diet group demonstrated a large initial spike with subsequent normalization of blood glucose to baseline values, whereas the Pre group showed a slow rise in blood glucose, which did not show any decrease during the entire 2-hour duration of the test (Figure 4A).

Low-fat and low-carb diets had same effect on energy metabolism and physical activity

Data on energy metabolism are presented in Figure 5. Total energy expenditure did not differ between the low-fat and low-carb groups ($P<0.05$) (Figure 5A). The Pre group showed higher ($P=0.02$) energy expenditure than the regular diet group, but ANCOVA analysis with body mass and physical activity as covariates did not show any significant differences between the groups and showed that physical activity but not body mass had an effect on energy expenditure. There were no significant differences in physical activity between the groups, which was probably because of rather large variations within the groups. Low-fat and low-carb groups tended to be more active than Pre or regular diet groups (Figure 5B). The association between physical activity and energy expenditure was significant in all groups ($r=0.70$-0.80, $P<0.05$-0.01) (Figure 5C). Linear regression analysis showed a tendency for a slightly greater predictive resting metabolic rate in the low-carb compared with the low-fat group (0.35 vs. 0.30 kcal · h^{-1}). On the other hand, respiratory quotient did not differ between the groups when measurements were performed in the fasted mice (Figure 5D).

Discussion

The main aim of our study was to investigate whether the carbohydrate and fat content of diets affects physiological responses to CR in mice. Most of the previous studies have focused on effects of macronutrient content of diets on health and body composition in ad libitum–fed mice (19,26), and there have been only a few studies under conditions of CR (27). In agreement with recent findings on humans undergoing mild CR (28), our results show that improvements in body composition and glucose tolerance do not differ between low-fat and low-carbohydrate diets with similar protein content under conditions of up to 40% CR. This is important in view of the fact that differences between low-carbohydrate and low-fat diets have been widely discussed in relation to weight loss and metabolic health (29).

Our aim was not to test the carbohydrate-insulin model of obesity but rather to carefully compare effects of low-fat and low-carbohydrate diets on body composition and metabolic health after weight loss. However, the carbohydrate-insulin model has been proposed in justification of the health benefits of low-carbohydrate diets (10). According to this line of reasoning, high carbohydrate content of

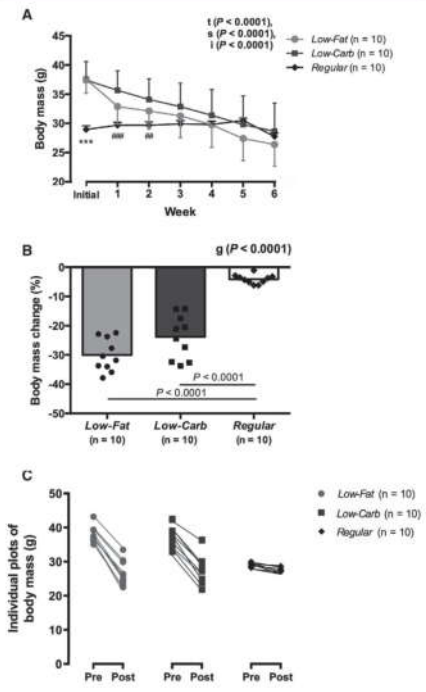

Figure 2 Body mass during 6-week caloric restriction (CR) in low-fat and low-carb diet groups as well as in a regular group fed standard chow diet ad libitum for the same period. Data are shown as (**A**) weekly measurements (mean ± SD), (**B**) percentage change from initial value, and (**C**) individual plots for the 6-week CR period (mean with plotted individuals dots). Each dot represents one mouse data sample. In panel A, two-way repeated-measures ANOVA with Bonferroni post hoc analysis was performed for effects of group (g), time (t), subject (matching) (s), and interaction (i). In panel B, one-way ANOVA with Bonferroni post hoc analysis was performed for group effect (g). Lines indicate significant differences between connected groups. ***$P<0.001$ vs. low-fat and low-carb; ##$P<0.001$, ###$P=0.005$ vs. low-carb.

food leads to high blood insulin levels, which act to suppress the release of fatty acids from adipose tissue and direct circulating fat toward adipose tissue for storage rather than oxidation in metabolically active tissues. Nevertheless, a large number of studies have contradicted the carbohydrate-insulin model of obesity. A meta-analysis of 32 controlled feeding studies with substitution of carbohydrate for fat showed that fat loss and energy expenditure were greater for low-fat diets compared with low-carbohydrate diets, though differences between the diets in fat loss (16 $g \cdot d^{-1}$) and energy expenditure (26 $kcal \cdot d^{-1}$) were rather small (30). All the studies included in this meta-analysis had controlled dietary energy and protein intake between the diets. A recent randomized clinical trial that engaged more than 600 participants showed no difference between low-fat and low-carbohydrate diets in weight loss during a 12-month period, and neither baseline insulin secretion nor genotype pattern relevant to carbohydrate and fat metabolism was associated with the dietary effects on weight change (28). Self-reported energy intake was similar between the diet groups, and protein intake did not differ much during this period (21% and 24% of kilocalories for low-fat and low-carbohydrate groups, respectively). It appears that protein is a macronutrient that is particularly important for dietary-induced thermogenesis and satiety. The thermic effect of dietary protein is 25% to 30% of its energy content compared with 5% to 10% and 2% to 3% for carbohydrates and fat, respectively (8). An increase in protein intake from 15% to 30% of total energy is associated with spontaneous reduction in total energy intake under conditions of ad libitum feeding (31). High protein intake also led to an increase in retention of lean body mass during CR (32). Thus, comparison of high-fat and high-carbohydrate diets can be compromised by differences in protein content, as health benefits of protein-rich diets are often incorrectly assigned to carbohydrate and fat content of the diets (33). In our study, we kept both the amount (20% of total energy intake) and source (casein with addition of L-cystine) of dietary protein constant between the low-fat and low-carbohydrate diets. It appears that this amount of protein was adequate for skeletal muscle mass retention, which did not change significantly during CR. Furthermore, mice were fed an obesogenic diet for 18 weeks prior to CR. This diet induces minor changes in lean body and significant increases in body fat, which might also help to preserve muscle mass during CR (20). Human weightloss studies have shown that approximately 25% of weight loss is due to loss of lean body mass with major contribution of the skeletal muscles to this decline (34). People who are leaner tend to lose more lean body mass under conditions of CR compared with those with greater body fat content (35). It appears that mice with diet-induced obesity show greater sparing of muscle mass during CR compared with humans. However, dissection of factors playing a role in preservation of muscle mass in mice and/or humans during CR was beyond the scope of our current study.

It appears that body fat was the main source of energy during CR, and its loss did not differ between the two diets in our study. Increased fatty acid oxidation is a common feature of low-carbohydrate high-fat diets, which are often perceived as more lipolytic and less obesogenic compared with low-fat high-carbohydrate diets, though human metabolic ward studies have challenged this hypothesis (36). We did not observe any differences between the diets in respiratory quotient, as the measurements were performed in the fasted state. Mice gorge on food and often consume all the food within less than 4 hours after feeding and they spend significant periods of time in the fasted state when exposed to CR (37,38). Food consumption early in the day might also be considered as a limitation of our study as it can affect circadian rhythms of nocturnal animals. Fasting is associated with a high rate of fatty acid oxidation (23). Measurements in the fasted state might be more representative of the overall metabolism compared with measurements in the postabsorptive state during CR. It appears that metabolic flexibility manifesting itself in switching between carbohydrate and fat oxidation allowed mice to maintain a similar net body fat balance independently of the macronutrient composition of the diets during CR (39).

Linear regression analysis of the plots for physical activity over energy expenditure allowed us to exclude effects of physical activity on energy

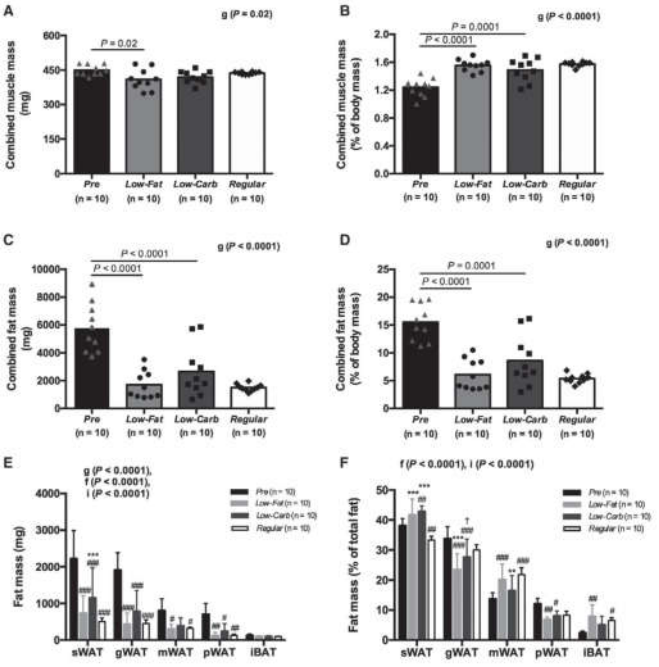

Figure 3 Mass changes of (**A,B**) skeletal muscle, (**C,D**) body fat (mean with plotted individual dots), and (**E,F**) fat from different sampling sites (mean ± SD) in low-fat and low-carb diet groups after 6-week caloric restriction (CR) compared with the obese group prior to CR (Pre) as well as the age-matched regular group fed standard chow diet ad libitum for the same period. Each dot represents one mouse data sample. In panels A-D, one-way ANOVA with Bonferroni post hoc analysis was performed for group effect (g). In panels E-F, two-way repeated-measures ANOVA with Bonferroni post hoc analysis was performed for effects of group (g), fat site (f), and interaction (i). Lines indicate significant differences between connected groups. $^{\#}P<0.05$, $^{\#\#}P<0.01$, and $^{\#\#\#}P<0.001$ vs. Pre; $^{**}P<0.01$, $^{***}P<0.001$ vs. regular; $^{\dagger}P=0.04$ vs. low-fat. sWAT, subcutaneous white adipose tissue; gWAT, gonadal white adipose tissue; mWAT, mesenteric white adipose tissue; pWAT, perirenal white adipose tissue; iBAT intrascapular brown adipose tissue.

expenditure and showed that predicted resting metabolic rate tended to be slightly greater under conditions of the low-carbohydrate diet compared with the low-fat diet. However, this difference between the diets was not significant and it can hardly be used as evidence in support of recent findings in human studies that low-carbohydrate diets lead to greater energy expenditure compared with low-fat diets (12). Our results are in agreement with many human studies that have reported no practically meaningful differences in energy expenditure between the isocaloric and isonitrogenous low-fat and low-carbohydrate diets (28,30,40).

We assessed glucose tolerance as a key indicator of metabolic health. After the 6-week CR, glucose tolerance improved similarly in both diets. It is likely that CR-induced loss of body fat was a key factor promoting better glucose control irrespective of the dietary carbohydrate and fat content. In contrast to our findings, a recent CR study of C57BL/6 mice showed a smaller improvement in glucose tolerance for a high-fat diet compared with chow diet, which is high in carbohydrates, in spite of similar weight loss for both diets (27). However, macronutrients and their sources were not strictly controlled in the latter study, and the protein content differed substantially between both diets (i.e., 20% of kilocalories for high-fat diet and 33% of kilocalories for chow diet low in fat). Dietary protein might influence postprandial glucose control because of its insulinotropic effects (41,42). There is evidence that consumption of the high-protein meal before the intake of carbohydrates

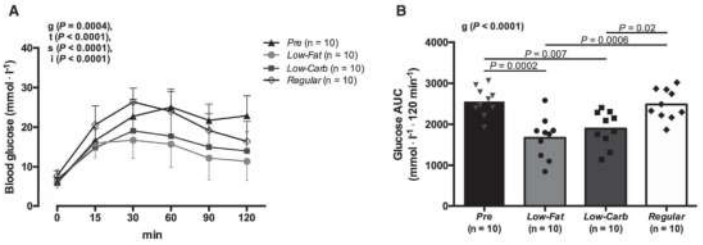

Figure 4 (**A**) Pattern of blood glucose clearance during a 120-minute period (mean ± SD) and (**B**) glucose area under curve (AUC) (mean with plotted individual dots) in low-fat and low-carb diet groups after 6-week caloric restriction (CR) compared with the obese group prior to CR (Pre) as well as with the age-matched regular group fed standard chow diet ad libitum for the same period. Each dot represents one mouse data sample. In panel A, two-way repeated measures ANOVA with Bonferroni post hoc analysis was performed for effects of group (g), time (t), subject (matching) (s), and interaction (i). In panel B, one-way ANOVA with Bonferroni post hoc analysis was performed for group effect (g). Lines indicate significant differences between connected groups.

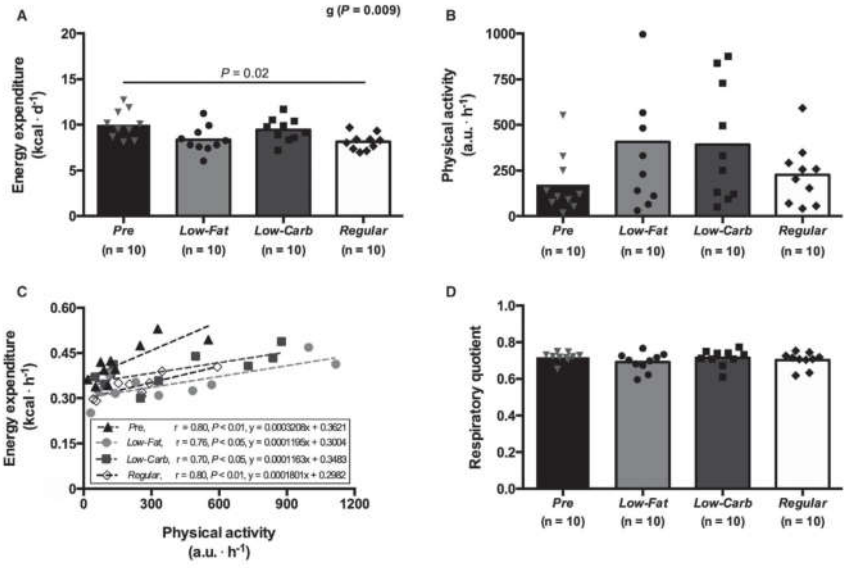

Figure 5 (**A**) Total energy expenditure, (**B**) physical activity, (**C**) plots of energy expenditure versus physical activity, and (**D**) respiratory quotient in low-fat and low-carb diet groups after 6-week caloric restriction (CR) compared with the obese group prior to CR (Pre) as well as the age-matched regular group fed standard chow diet ad libitum for the same period. All the measurements were performed after overnight fasting. Data are presented as mean with each dot representing one mouse data sample. One-way ANOVA with Bonferroni post hoc analysis was performed for group effect (g). Lines indicate significant differences between connected groups. In panel C, Pearson correlation coefficient (r) and linear regression equations for the plots of energy expenditure versus physical activity are also shown.

attenuates the subsequent rise in the postprandial serum glucose and results in lower glucose compared with isocaloric high-carbohydrate and high-fat meals (43). Blood insulin levels were not measured, and this might be considered as a shortcoming of our study. However, there were no differences in glucose tolerance between the CR diets, and it is unlikely that insulin levels differed significantly under such conditions. Indeed, differences in fasting insulin levels between diets are blunted during CR (10).

In humans, weight loss is a priority target under the conditions of impaired glucose homeostasis, as in the case of type 2 diabetes (44). Antidiabetic therapies that can control blood glucose levels but promote weight gain are less effective, as the greatest improvements in glucose control are observed in patients with the greatest reductions in body mass (45). Taken together, CR-induced body fat loss should be considered as the primary and most desirable target for positive management of blood glucose, whereas macronutrient composition of isocaloric diets with equated protein probably plays a minor role at best.

Conclusion

CR with fixed energy and protein intake rather than a distribution of dietary carbohydrate and fat was the main factor for improvement in body composition of obese mice. Similarly, improvements in glucose tolerance of obese mice were due to the reduction in body fat rather than dietary carbohydrate and fat content of the diets. It appears that the overall energy intake should be targeted when the aim is to improve body composition and glucose control, while dietary carbohydrate and fat content should be left to personal preference for adherence purposes.O

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Author contributions: PM conceived and designed research. PM, AF, and MD performed experiments. PM, AF, and AR analyzed data. PM, AF, and AR interpreted results of experiments. PM and AF prepared figures. PM and AF drafted manuscript. PM, AF, and AR edited and revised manuscript. PM, AF, MD, and AR approved final version of manuscript.

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