PROTEIN FRACTIONAL SYNTHESIS RATES WITHIN TISSUES OF HIGH- AND LOW-ACTIVE MICE

A Thesis

by

KRISTINA MARIE CROSS

Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Chair of Committee,	Nicolaas E. P. Deutz
Committee Members,	Mariëlle P.K.J. Engelen
	J. Timothy Lightfoot
Head of Department,	Melinda Sheffield-Moore

May 2020

Major Subject: Kinesiology

Copyright 2020 Kristina Cross

ABSTRACT

With the rise in physical inactivity and its related diseases, it is necessary to understand the mechanisms involved in physical activity regulation. Scientists have explored physical activity regulation by investigating various physiological mechanisms involving hormones, neurotransmitters, and genetics; however, little is known about the role of metabolism on physical activity level. We hypothesize that protein turnover in specific organs is higher in mice previously exhibiting high physical activity levels, as a mechanism to adapt to the increased demand. Therefore, we studied protein fractional synthesis rate (FSR) in tissues of inherently high and low active mice. In order to study protein FSR of various organs, we assessed 12-week-old male inherently low-active (LA) mice (n=14, lean body mass: 21.0±1.1g, C3H/HeJ strain) and high active (HA) mice (n=14, lean body mass: 22.5±1.3, C57L/J strain). One day before tissue collection, a D_2O bolus was administered via intraperitoneal injection, and mice were provided D_2O enriched drinking water to enrich the total body water to about 5% D_2O. Blood samples and eleven tissues (kidney, heart, lung, muscle, fat, jejunum, ileum, liver, brain, skin, and bone) were collected and analyzed for enrichment of alanine in the plasma and protein-bound pool (LC-MS/MS). FSR was calculated as -ln(1-enrichment) as fraction per day. Data are mean±SE (unpaired t-test: GraphPad Prism 8.3.1). The kidney protein FSR in the low-active mice were 7.82% higher than the high active mice (low-active 0.1863±0.0018, high-active 0.1754±0.0028, p= 0.0030). No significant differences were found between protein FSR of high-active and low-active mice in any of the other

measured organ. Although the kidney tissue resulted in significant differences, we do not deem the findings as physiologically different because a protein synthesis difference of only 7.82% is not physiologically relevant. The observed lack of significant differences in high and low-active mice suggests that differences in specific organ tissue protein turnover may not be a mechanism regulating inherent physical activity level. Since protein turnover is representative of the ability to adapt through upregulation and downregulation of metabolic processes, these results show that high-active mice are inherently no more equipped for metabolic regulation than the low active mice.

DEDICATION

First, I dedicate this thesis to God who gives me hope, strength and endless love. He guides my path each and every day and is the reason for my success. I leaned on Him in every high and low throughout this thesis process. In addition, I am dedicating this thesis to my family. My mom is my role model, best friend, and the woman who has shown me how to be positive in all situations. My dad and brother offered encouragement and believed in me every step. Thank you, family, for your constant support. I love you all.

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Deutz, and my committee members, Dr. Engelen and Dr. Lightfoot for their guidance and support throughout the course of this research. I appreciate the time they have given to help me achieve my goals and agreeing to mentor me with 5 months before the thesis deadlines. In addition, thank you to Dr. Fluckey for exposing me to research and encouraging my growth as a young scientist. I would not have started the masters program or even stepped foot in the research world without your guidance.

Thank you also to the CTRAL staff and students who welcomed me in like a family member. Specifically, Sarah, JK, Clayton, and Raven Agata for answering my questions daily and taking the time to explain the ways of the lab, statistics, and providing examples of writing. All the students became not only colleagues, but also my friends.

Thanks also go to my friends, colleagues, and the department faculty and staff for making my time at Texas A&M University a great experience.

Finally, thanks to my mother and father for their encouragement and Jorge, not only for his participation in the project but the support he offered along the way.

CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supervised by a thesis committee consisting of Dr. Nicolaas Deutz, Dr. Mariëlle Engelen, and Dr. Timothy Lightfoot of the Department of Health and Kinesiology.

The study was designed in collaboration by Dr. Nicolaas Deutz and Dr. Timothy Lightfoot and conducted by Dr. Jorge Granados. Tissue collection was conducted by Dr. Gabrie Ten Have and Dr. Jorge Granados. All samples were prepared in part by Dr. Jorge Granados, Dr. Ayland Letsinger, and the students of the Biology of Physical Activity Laboratory. All intracellular alanine samples used for the results were prepared and analyzed by Dr. Jorge Granados. All sample analysis was done with the help of Dr. John Thaden.

All other work conducted for the thesis was completed by the student independently.

Funding Sources

This work was also made possible in part by the Sydney and J.L. Huffines Institute for Sports Medicine, Human Performance Student Research Grant, Center for Translational Research in Aging and Longevity Grant, and College of Education and Human Development Student Research Grant. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the Sydney and J.L. Huffines Institute for Sports Medicine.

NOMENCLATURE

CI	Confidence Interval
CV	Coefficient of Variation
FMOC	Flourenylmethoxycarbonyl chloride
FSR	Fractional Synthesis Rates
HA	High Active
icALA	Intracellular Alanine
LA	Low Active
LC-MS/MS	Lipid Chromatography-Tandem Mass Spectrometer
pbALA	Protein-bound Alanine
plaALA	Plasma Alanine
SE	Standard Error
TTR	Tracer-to-Tracee Ratio

TABLE OF CONTENTS

Page

ABSTRACT ii

DEDICATION iv

ACKNOWLEDGEMENTS v

CONTRIBUTORS AND FUNDING SOURCES vi

NOMENCLATURE vii

TABLE OF CONTENTS viii

LIST OF FIGURES x

LIST OF TABLES xi

1. INTRODUCTION 1

1.1. Metabolism Driving Physical Activity Regulation 1
1.2. Tissue Protein Synthesis 3
1.3. Protein Synthesis in Specific Organs 6
1.3.1. Skeletal Muscle 6
1.3.2. Liver 6
1.3.3. Brain 7
1.3.4. Skin 7
1.3.5. Kidney 8
1.3.6. Heart 8
1.3.7. Ileum and Jejunum 9
1.3.8. Bone 9
1.3.9. Lungs 9
1.3.10. Fat 10
1.4. Using D_2O for FSR Measurement 11
1.5. Use of high- and low-active mice for physical activity level model 12

2. METHODS 14

2.1. Animal Care 14
2.2. D_2O administration 15

2.3. Study Day....15
2.4. Sample Collection....16
2.5. Plasma Analysis....17
2.6. Tissue Analysis....17
2.7. Calculations....19
2.8. Statistical Analysis....20

3. RESULTS....21

3.1. Subject Characteristics....21
3.2. Enrichment Curve....21
3.3. D_2O enrichment....23
3.4. Using Plasma for Fractional Synthesis Rates....30
3.5. Tissue Protein Fractional Synthesis Rates....34
3.6. Literature Comparison....37

4. DISCUSSION....40

4.1. Enrichment of D_2O....40
4.2. Using Plasma Alanine TTR as the precursor pool....43
4.3. Fractional Synthesis Rate of High and Low Active Mice....43
4.4. Literature Comparison....46
4.5. Considerations....47
4.6. Future Studies....48

5. CONCLUSIONS....49

REFERENCES....50

LIST OF FIGURES

Page

Figure 1. D_2O labeling of alanine. 12

Figure 2. Bland-Altman plot of the run vs the rerun. 19

Figure 3. Estimated ratio (f) between alanine TTR in protein (pbALA) and in the precursor pool and the average f of the tissues. 23

Figure 4. Comparison of plasma alanine TTR in 1% D_2O enrichment (4A) and 5% D_2O enrichment (4B) obtained at various blood draw time points (3, 5, 30, and 40 minutes). 29

Figure 5. Plasma alanine TTR and mean intracellular alanine TTR according to percentage of D_2O enrichment. 32

Figure 6. Protein FSR of low-active and high-active groups in the kidney. 36

Figure 7. Ranked P-values from unpaired t-test of protein FSR of each tissue. 37

Figure 8. Protein FSR of each tissue. 38

Figure 9. Comparison of the observed skeletal muscle protein FSR in the present study compared to reported skeletal muscle protein FSR values found in literature. 39

LIST OF TABLES

Page

Table 1. Characteristics of mice. 21

Table 2. Comparison of precursor TTR and protein-bound alanine (pbALA) TTR in mice given 1% and 5% D_2O enrichment. 25

Table 3. Protein FSR of mice given 1% and 5% D_2O enrichment using intracellular alanine TTR as the precursor pool. 27

Table 4. Protein FSR of mice given 1% and 5% D_2O enrichment using plasma alanine TTR as the precursor pool. 28

Table 5. Plasma TTR reported according to blood sample time and percentage of D_2O enrichment. 30

Table 6. Comparison of the precursor pools used to calculate FSR. 33

Table 7. Protein FSR of tissues of low-active (C3H/HeJ) and high-active mice (C57L/J). 35

1. INTRODUCTION

With physical inactivity rising at epidemic rates, researchers have studied the biological factors regulating physical activity to establish a possible target for improving physical activity level [1]. This work is important, given the detrimental implications associated with the declining physical activity level in the United States [2] and consequently, the increase in hypokinetic related diseases in most Western cultures [3]. Physical inactivity is linked to more than 35 chronic diseases [4, 5], accounts for 70% of U.S. deaths [3], and produces an estimated $53.8 billion in yearly health care costs [6] but less than 5% of American adults achieve the recommended physical activity guidelines [2]. In order to better understand the cause of physical inactivity, scientists have explored numerous avenues to find the factors that regulate physical activity, including environmental factors [7, 8], hormonal factors [9-11], and genetic influences [1, 12, 13]. The common thread between all the previous investigations is changes in metabolism; however, there is a lack of knowledge about metabolic variation between physically active and physically inactive individuals.

1.1. Metabolism Driving Physical Activity Regulation

Differences in the genetic makeup account for only 11%-34% of the physical activity distance, duration, and speed variation between a high-active and low-active mouse strain [14], suggesting that other biological factors contribute to physical activity regulation. Previously, scientists have provided evidence that metabolism within specific

tissues may be related to physical activity level [15, 16]. It was demonstrated that inherently high-active mice (C57L/J) when compared to an inherently low-active mouse strain (C3H/HeJ) have an overexpression of proteins associated with metabolism (creatine kinase B and succinyl CoA ligase) in the nucleus accumbens, an area of the brain thought to be involved in physical activity regulation via the dopaminergic reward system [15]. Furthermore, to evidence metabolism's possible role in physical activity regulation, we examined the skeletal muscle proteome of high- and low-active mice and concluded that high-active mice (C57L/J) had overexpression of proteins associated with the Krebs Cycle (TCA cycle) and calcium regulation, while low-active mice (C3H/HeJ) had overexpression of proteins associated with the electron transport chain and cytoskeletal structure [16]. Ferguson et al. suggests that the high-active mice were more capable to be active at least partially due to an increased efficiency in calcium release and uptake, which is a potential mechanism to decrease fatigability of skeletal muscle. Finding higher protein concentrations of some metabolically related proteins in both brain and muscle of high-active mice indicates that alterations in protein synthesis, degradation, or some combination of the two in specific organs may be contributing to inherent differences in physical activity.

Elevated amounts of metabolic related proteins in the skeletal muscle may infer an increased anabolism of these proteins in the specific mice. The relation between physical activity levels and the amount of certain proteins, is important in metabolism and supports the notion that the capacity to increase metabolism in certain organs may be related to the physical activity level of an organism.

In addition, studies have shown that differences in metabolism exists between physical activity levels when examining amino acids associated with protein synthesis [17]. Differences have been reported between both nonessential [18] and essential amino acids [19] in high- and low-active mice. Skeletal muscle concentration of glutamate, an important substrate for alanine [20] and glutamine [21], which has a positive relationship to protein synthesis [17], in active healthy older adults when compared to sedentary healthy older adults [20]. These findings support our hypothesis that there will be differences in metabolism between animals displaying differing physical activity levels.

1.2. Tissue Protein Synthesis

Fractional synthesis rate (FSR) is defined as the rate at which a precursor compound is incorporated into a product, such as fatty acids incorporated into triglycerides, nucleic acids incorporated into DNA, or amino acids incorporated into proteins. Quantification of tissue protein synthesis by measuring the protein FSR has provided vital insight in the understanding of protein turnover [22, 23] and is a powerful approach for characterizing the dynamics of protein synthesis [24]. Protein FSR provides explanatory power about the processes and pathways that underlie the phenotype and adaptations of tissues by providing a quantitative measure of the functional processes and disturbances in protein metabolic systems. Metabolic regulation is dependent on the rate of protein turnover because an increased turnover represents the ability to adapt by upregulation or downregulation of processes controlled by protein concentrations. For instance, the intestines have a high protein FSR which allows for the intestinal system to

quickly react to stimuli or environmental change. By accounting for time, the protein FSR allows for characterization of kinetic processes, active synthesis and dynamically altered proteins rather than solely a snapshot of one time point such as a concentration measurement. In addition, the protein FSR provides knowledge about the growth of an organ as growth results from the balance of protein breakdown and protein synthesis [22].

Protein synthesis rate measurements are suitable for identification of organs' protein turnover in varying physical activity levels. Collaborators on this proposal (Deutz, et al.) and others have measured protein synthesis utilizing stable isotope tracer methodology with amino acid tracers in the lung, liver [25], jejunum [25, 26], ileum [25, 26], pancreas [27], stomach [27], spleen [27], thymus [27], and heart [27]. D2O labelled specific amino acids were used to measure tissue-specific protein FSR in the heart, gastrocnemius, and liver in various feeding states [28]. In regard to physical activity, multiple groups report significantly different protein FSR measurements in skeletal muscle after exercise bouts [22, 29], but have failed to measure the protein dynamics of other organs exposed to physical activity.

Utilizing animal models has been deemed acceptable for human physiology research. The inbred mouse model we employ in this study, allows for more control of genetic factors and environmental factors when compared to human studies. In addition, the mouse model allows for the examination of more tissues than accessible in humans. Therefore, the focus of this thesis is to determine the differences in protein FSR between tissues of inherently high- and low-active inbred mouse strains (C57L/J and C3H/HeJ,

respectively) [13, 14, 16, 30]. We hypothesize that protein FSR in skeletal muscle, brain, liver, skin, kidney, heart, ileum, and jejunum is higher in the high-active mice when compared to the low-active mice because these organ sites have been shown to have higher metabolically associated protein expression [15, 16, 31]. and amino acid concentration findings [15, 16, 31]. When utilizing protein FSR to analyze metabolic differences between physical activity levels, it can be hypothesized that in a more physically active organism, protein FSR will be higher in the major energy-producing and energy utilizing organs during physical activity such as skeletal muscle, liver, and brain, because the demand for growth and renewal will be higher. The growth leads to increased protein synthesis due to the demand for enzymes in the energy utilization processes and the growth of structural proteins. Increased proteins, due to an increased anabolic rate, provide for cells to be more responsive to the stress of physical activity, allowing the individual to be more active. Since protein FSR is a measure of protein turnover, organs most responsive to physical activity are expected to have higher protein FSRs to provide more capability of physical activity.

In addition, we hypothesize that there are no significant differences between protein FSR of the high-active mice and low-active mice in the lung and the skull bone because these organs do not actively respond to physical activity nor undergo large physiological changes in response to physical activity. Furthermore, we predict that fat will have a decreased protein FSR in high-active mice because fat is broken down for energy for physical activity thus expected to be in a catabolic state.

1.3. Protein Synthesis in Specific Organs

In brief, I will discuss the changes in protein synthesis in more detail that are known to occur in the different organs.

1.3.1. Skeletal Muscle

Previous evidence has shown that skeletal muscle protein FSR increases after introduced exercise [22]; however, there is a lack of evidence measuring protein synthesis differences of various physical activity levels. Skeletal muscle is predicted to have a higher metabolic rate in more physically active organisms due to an increased need for energy for repair and growth from exercise bouts. Growth occurs when anabolism is at a higher rate, thus will result in an increased FSR. In addition, previous studies have shown that skeletal muscle of high-active mice have higher concentrations of proteins associated with metabolism when compared to low active mice, suggesting a higher protein turnover [16, 31]. Measuring protein FSR of the skeletal muscle will allow for the understanding of how protein turnover affects adaptation of high and low active mice.

1.3.2. Liver

The liver is hypothesized to have a higher FSR because of its role in maintaining energy substrates, like glucose and lactate in the blood, possibly more in a physically active organism to allow greater availability of energy substrates. With the liver being a site for glycogenolysis and gluconeogenesis, it is the organ responsible for controlling

the energy substrates available for more physical activity. Regular aerobic exercise upregulates enzymes involved in gluconeogenesis and carbohydrate metabolism [32] suggesting that we may observe a more active liver in the more physically active mice so the liver can fulfill the energy demand.

1.3.3. Brain

The brain is hypothesized to have a higher protein FSR due to previous evidence showing increased concentrations of metabolic proteins in the more physically active mouse brain [15]. In addition, previous findings suggest that amino acid levels and metabolism plays a role in mental fatigue which may drive the physical activity level regulation [33]. The "central fatigue" hypothesis supports the hypothesis that the brain is the primary source of fatigue signaling due to the buildup of free tryptophan in the brain leading to an increased synthesis of serotonin, a neurotransmitter associated with sleep, mood, and aggression [33]. The increased serotonin may force athletes to stop exercising or reduce intensity. This metabolic reason for decreased physical activity may be examined by analyzing the brain's ability to respond to stressors, such as physical activity and a concomitant increase in neurotransmitters, via increasing protein turnover.

1.3.4. Skin

Skin thickness has been shown to have a relationship with the number of hours spent exercising per week which is speculated to be due to the mechanical tension, increased cutaneous oxygen and nutrient delivery and enhanced growth hormone

secretion [34]. Therefore, thicker skin which is an actively anabolic state of the more physically active would be expected to have a higher protein turnover.

1.3.5. Kidney

Previously, it has been shown that with an increase in time spent performing physical activity, kidney function parameters are increased, and high levels of physical activity are associated with less kidney damage [35]. With the evidence that the kidney is responsive to physical activity levels, we hypothesize that the kidneys of the high active mice will have a higher protein turnover because the kidney must utilize increased protein dynamics to adapt to pressure, hormonal, and osmotic changes induced by physical activity.

1.3.6. Heart

The heart is predicted to have a higher FSR, because a heart with an inherently higher protein turnover would be more resistant to fatigue of physical activity, have an increased adaptability and better at repair after an exercise bout, allowing the organism to be more physically active. As known, the heart physically and mechanically changes in response to physical activity resulting in an increased FSR [36] and it would be logical if a higher active mouse had an inherent ability in the heart tissue to adapt to the exercise.

1.3.7. Ileum and Jejunum

The jejunum is the location for digestion and absorption of proteins and fully-digested carbohydrates and the ileum absorbs vitamin B12, bile salts, and digested products not absorbed by the jejunum. The ileum is responsible for the final stages of protein and carbohydrate digestion. Since the intestines are the location for nutrient uptake [37], they are hypothesized to be actively growing and creating more proteins to keep up with the nutrient uptake demand of a more physically active individual, thus resulting in a higher protein FSR in more active individuals.

1.3.8. Bone

We do not expect the bone (skull) to have differing FSRs between physical activity levels because after development, the skull does not grow larger or respond to physical activity. Adult skull bone cells, calvarial cells, have reduced proliferation, mineralization, and growth factor responsiveness than juvenile osteoblasts [38] and are less susceptible to the modest estrogen decreases caused by regular physical activity [39, 40].

1.3.9. Lungs

Although it is known that oxygen uptake increases with physical activity due to better uptake at the organs and better distribution by the vessels, the lungs do not grow or increase in capacity with exercise. We hypothesize that the lung protein turnover of high active and low active mice is not significantly different due to the fact that the lungs

do not change with activity. One group found weak positive associations of increased lung function when comparing more active adults with lower active adults, each with healthy lungs [41]; however, the results found using spirometry measurements, such as FEV1, can be confounded by diaphragm and other expiratory muscle strength. In addition, no evidence was found for the hypothesis that sustained exercise enhances lung growth when measuring lung volume, alveolar surface area, and alveolar number in mice [42].

1.3.10. Fat

It can be hypothesized that fat will have a decreased FSR in the more active mice because fat is in a catabolic state to be broken down for energy substrate instead of building more proteins. Physical activity is a key component of energy expenditure; therefore, it is reasonable to hypothesize a decrease in fat mass with more physical activity [43]. It has been reported that individuals with higher physical activity tend to report lower weight gain over time, allowing for the conclusion that fat protein turnover will be lower in a more physically active organism due to less growth of the organ system [44]. In addition, trained women have been found to have smaller adipocytes than age-matched sedentary controls [45] and others have shown evidence that there is little change in the adipocyte number, but rather a decrease in cell size with fat mass loss [46, 47]. A reduction in size of the cell would result in a decreased FSR in the high active mice because there would be a reduction in growth and less protein turnover.

1.4. Using D_2O for FSR Measurement

Methods to measure FSR of protein are based on the use of radioactive or stable isotopes [48]. Primed constant infusion of tracers of essential amino acids like phenylalanine or leucine will progressively label protein and the percent of labeling is used to calculate the protein FSR. Previously, the administration of heavy water (D_2O), as a way to create labelled amino acids, was introduced to measure protein FSR [49]. D_2O uniformly distributes throughout the body [49, 50] and is not incorporated into C-H bonds of post-translational amino acids because C-H bonds do not exchange hydrogen spontaneously with body water. Instead, D_2O labels amino acids and many other small molecules during their synthesis. Amino acids then incorporate into their subsequent polymers, making it a promising label to measure protein synthesis and turnover [51]. D_2O provides a deuterium for natural alanine transaminase exchange to label the alpha- and beta-hydrogens of alanine with a deuterium molecule (Figure 1) [52]. A maximum of 4 four hydrogen atoms in alanine can be exchanged to deuterium in vivo; however, the average hydrogen labeling of alanine is 3.7 times that of water [23, 48, 50, 51]. Alanine is synthesized from pyruvate by alanine transaminase during the conversion of glutamate to alpha-ketoglutarate, a metabolite in the TCA cycle. The labeling of labelled alanine is the highest after intake of D_2O and therefore is used to calculate for protein FSR. The exchange, quantified as a measured ratio of the labelled and unlabeled alanine within the tissue, is representative of the synthesis rate of protein. Protein FSR measurement using D_2O provides a method for quantifying protein synthesis through incorporation where the system naturally labels the proteins without introduction of

additional substrate. Stable isotopic water also has been used to quantify rates of synthesis of nucleic acids [53], fatty acids [49], and cholesterol [51] in mice and humans; however, this method of FSR measurement has been mostly used to measure in skeletal muscle FSR analysis [22, 23].

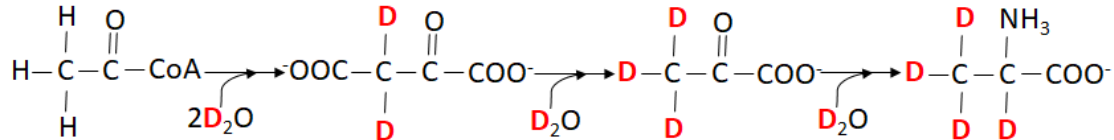

Figure 1. D_2O labeling of alanine. Deuterium can take the place of 4 hydrogens on an alanine molecule. Deuterium molecules are incorporated into oxaloacetate through the TCA cycle via aconitase and fumarase. Another deuterium is added during the conversion of oxaloacetate to pyruvate. The synthesis of alanine via alanine transaminase adds a deuterium molecule to pyruvate.

We propose this study would be the first to utilize D_2O to measure protein FSR in several tissues as a multi-organ approach using a mouse. We will focus on the FSR differences between organs of high- and low- active mice. The identification of organs with higher FSR will allow for targeted future studies to determine physical activity regulation.

1.5. Use of high- and low-active mice for physical activity level model

Scientists have extensively utilized animal models for physiological research and have deemed it to be reasonable to apply biological data collected from rodents to human physiology [9]. With the mouse genome having approximately 99% of genes with a homologue in the human genome [54], the inbred mouse allows for modelling of human physiology and control of the genetic differences that could factor into physical

activity regulation [9]. Previously, mice strains have been determined to be inherently more or less physically active based on wheel running duration, distance and speed mice from each strain per day [55]. By stratifying the wheel running duration of each mouse, we can use a mouse that is evidenced to be lower or higher active to analyze differing physical activity level. In order to specifically examine the factors that contribute to a higher physical activity level, we utilized tissues from control (C57Bl6/J), low-active (C3H/HeJ), and high-active mice (C57L/J) which have been determined to have varying inherent physical activity levels based on duration of wheel running per day (232.8±70.6 min/day, 75.6±99.0 min/day, 351.1±61.6 min/day, respectively) [55]. We have repeatedly shown that C3H/HeJ inbred mice are low active and the C57L/J inbred mice are high active [14, 55, 56] and activity levels within the strains are highly repeatable [57]. In addition, the use of inherently high- and low-active inbred mice reduces the extrinsic effects of cultural, environmental and emotional stimuli which can cause complexity in untangling the factors regulating human physical activity, while also allowing for the collection of tissues difficult to acquire in humans (e.g. brain tissue). Previously, the high- and low-active mouse model has been used in to examine factors regulating the biological variation of physical activity, such as gene expression [13, 58], hormonal control, neurotransmitter differences [30, 57, 59], and aging [60]. In summary, the high- and low-active mouse model can be used to examine differences in various metabolic pathways that may be associated with the differential activity levels while controlling genetic and environmental variation.

2. METHODS

We analyzed samples from 24 male C3H/HeJ mice (inherently low-active inbred strain) and 24 male C57L/J mice (inherently high-active inbred strain), purchased from The Jackson Laboratory (Bar Harbor, ME, USA). In addition, we studied C57Bl6/J (n=13) as a control for background enrichment. These tissues were collected under a previously approved Texas A&M IACUC protocol (AUP # 2015-0159).

2.1. Animal Care

The mice were obtained at 10-weeks of age and group-housed in standard mouse-cages in a light and temperature-controlled housing facility (12-hour light-dark cycle, room temperature 22-24°C) with ad libitum water and a standard chow diet (Harlan Labs, Houston TX; 25.2% protein, 4.0 % fat, 39.5% carbohydrate, 3.3% crude fiber, 10% neutral fiber, and 9.9% ash) diet. Food consumption was measured daily by measuring unconsumed food weights. During the acclimation period, mice were not exposed to wheel-running activity because we have shown that multiple day exposure to running wheels can result in exercise adaptation induced physiological changes [13]. In addition, average physical activity levels of these two mouse strains have previously been described [55] and are on average a wheel running distance of LA = 0.6 ± 1.1 km/day and HA = 9.5 ± 2.0 km/day (mean±SD). The physical activity level of the mice strains used in this study are highly reproducible [57].

2.2. D_2O administration

After a two-week acclimation period, we performed the metabolic phenotyping procedures via a terminal surgery. The day before tissue collection, we gave the mice an intraperitoneal injection of a pre-calculated amount of D_2O according to body weight to raise the body water enrichment to a level that will sufficiently enrich alanine to be able to calculate the protein FSR at a sufficient precision in all the organs of interest. We first gave the mice D_2O: 73uL/10g to obtain a 1% enrichment but decided to increase the D_2O intake to 365uL/10g to obtain a 5% enrichment to increase the precision of the protein FSR measurements. The mice were also given the same respective percentage of D_2O in their drinking water to maintain steady state D_2O enrichment.

2.3. Study Day

Four hours before commencement of surgical procedures, food was removed from mouse cages to study animals in a post-absorptive condition. Bodyweight was assessed immediately after food withdrawal using a digital beam scale. Lean body mass, fat mass, and total body water were measured using echo MRI (EchoMRI LLC, Houston, TX 77079).

We anesthetized the mice with a mixture of ketamine (1.25 mg/10g BW) and medetomidine (2 µg/10g BW) via intraperitoneal (IP) injection (0.1 ml/10g body weight) [61] and maintained anesthesia using a continuous subcutaneous pump infusion of ketamine (0.35 mg/ 10 g bw/h) and medetomidine (0.35 µg/10g bw/h) at a rate of 0.1 ml/10 g bw/h [61]. We maintained the fluid balance and blood pressure by an initial 1.5

ml IP saline injection (0.9% sterile, NaCl), and by continuous subcutaneous pump infusion (Harvard PHD2000) of normal saline at a rate of 2.5 ml/hour [61]. Breathing was continuously monitored, and body temperature was maintained at 37°C using a heating pad and lamp.

2.4. Sample Collection

Under anesthesia, a peripheral catheter was placed in the right jugular vein for blood sampling and infusion of a stable isotope tracer pulse as described [25]. Blood samples (0.05- 0.1 ml per sample) were collected on two sampling time schedules (schedule 1: t = 1 and 30 minutes; schedule 2: t = 3 and 40 minutes) after pulse administration to provide a wider range of temporal points. Blood samples were replaced with an equal volume of sterile normal saline. We collected blood in lithium-heparinized tubes (microtube; Sarstedt, Newton, NC) and immediately placed them on ice (4° C) then centrifuged the samples (4 °C, 3120 x g for 5 min) to obtain plasma within the hour. The plasma was deproteinized with 0.1 volume of 33% (w/w) trichloroacetic acid and stored at −80 °C for later analysis.

After blood sampling, eleven tissues were collected from each mouse in the following order: skeletal muscle, fat (peri-testicular), jejunum, ileum, liver, kidney, lung, heart, brain (forebrain), skin (ear), and bone (skull). Each tissue was freeze clamped, snap-frozen in liquid nitrogen, and stored in -80 °C until processing.

2.5. Plasma Analysis

The plasma samples were previously analyzed using an LC-MS/MS by methods previously described [25]. The samples were each aliquoted (20uL) then had 20uL of amino acid internal standard (high mass stable isotopes) added. The samples were derivatized using 9-flourenylmethoxycarbonyl (Fmoc-chloride) in acetonitrile (Sigma Aldrich, St. Louis, Missouri). After being neutralized, the samples were analyzed using a liquid chromatography tandem-mass spectrometry (LC-MS/MS) then dried down using a speedvac. In order to study the samples for the present study, we reconstituted the samples using 150uL of 0.25M acetic acid in acetonitrile and analyzed the samples for labelled and unlabeled alanine using the LC-MS/MS. We calculated the mass signal areas to enable tracer/tracee ratio (TTR) calculations.

2.6. Tissue Analysis

Before analysis, the tissue homogenizing and processing was completed as previously described [25]. The tissue hydrolysates from the biobank were aliquoted (20uL) into 0.5mL plastic polypropylene tubes (Chemglass, Inc Vineland, NJ). Each sample also had 20uL of internal standard (Valine-8) to be used to recognize machine drift from the LC-MS/MS. We also prepared standards of known concentration in the range of expected TTR (0.432, 0.173, 0.0865, 0.0432, 0.0173, 0.00865, 0.00432 and 0.00086) in order to create a calibration curve for TTR of the samples. The aliquoted hydrolysate samples and internal standard solution are treated with 35uL of a borate buffer (50 mL of 1 M sodium borate, pH 10.25, 4.286 ml of 6M NaOH). Next, 75uL of a

5 mg/mL solution of Fmoc-chloride in acetonitrile (Sigma Aldrich, St. Louis, Missouri) is added to the samples for 15 minutes and the reaction is stopped with 50uL of stop solution (12mL of acetonitrile and 5.72mL of glacial acetic acid adjusted to 100 mL with water). Samples are vortexed between each step and centrifuged after the reaction to be analyzed using LC-MS/MS. We performed simultaneous mass analysis for unlabeled alanine and labelled alanine to calculate the mass signal areas for labelled to unlabeled alanine ratio calculations.

When measuring the protein-bound alanine levels, 47 of the samples (various tissues and physical activity levels) were rerun through the LC-MS/MS if the original run resulted in peaks higher than the window of measurement. If the peak was higher than the window of measurement cutting off the top of the peak, then the calculation of the peak area would be a lower amount than the true value. When this was realized, the samples were rerun using a smaller injection amount to gain a full measurement of the peak area. The original run and rerun are compared (**Figure 2**). The plot is as expected because a difference between the rerun and run will be a higher value as the measurement obtained by the rerun would account for the total peak area.

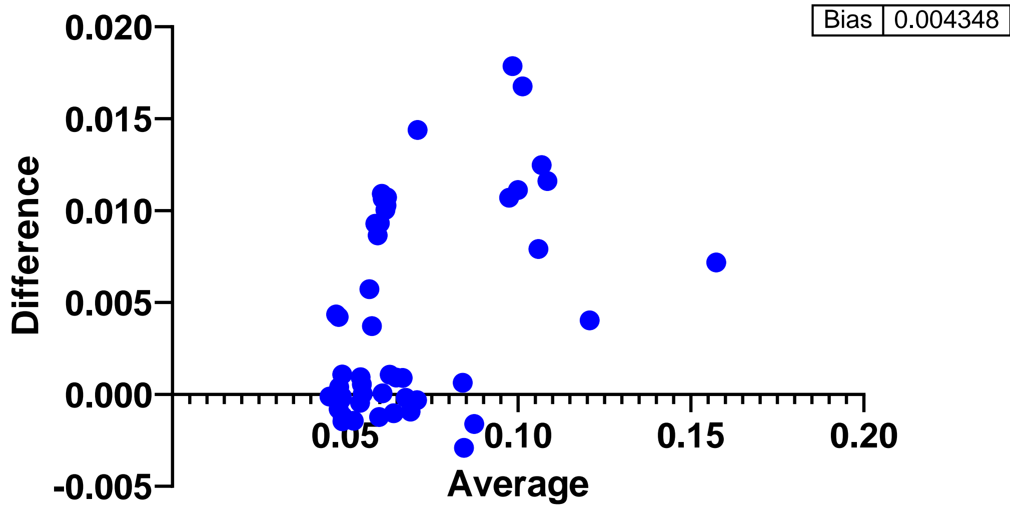

Figure 2. Bland-Altman plot of the run vs the rerun. Difference is rerun-run**.**

2.7. Calculations

We calculated the labelled to unlabeled ratio (tracer-tracee ratio =TTR= enrichment) of each organ corrected for background measurements provided by the C57Bl6/J mice that were not given D_2O. Using the TTR, we calculated protein FSR using equation (1) where f is calculated as protein-bound alanine TTR divided by the precursor pool alanine TTR. f will be between 0 and 1. The precursor pool can be either intracellular or plasma measurements. The amount of time (t) is the duration between the first bolus administration of D_2O and the collection of tissue.

Equation 1

$$FSR = \frac{-ln(1-f)}{t}$$

2.8. Statistical Analysis

Results are expressed as mean±SE. If data failed normality or equal variance tests, they were log-transformed. Unpaired t-tests were used to determine significant differences of the characteristics of the high and low active mice, between the percent enrichment of D_2O administered, and the protein FSR of each organ and the difference in tissue protein FSR between the high-active and low-active mice groups with the level of significance set a priori at $p < 0.05$. The p-values from the t-test were analyzed using false discovery rate (FDR) approach controlled with Q=0.05 and set to use the two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli [62]. FDR adjusts the p-values to recognize if the significance is a true discovery. GraphPad Prism (Version 8.3.1) was used for data analysis. Reported coefficient of variation is an output of Prism calculated as the percent of standard deviation divided by the mean. All mean differences are calculated as the percentage of 1 minus the ratio of the two means.

3. RESULTS

3.1. Subject Characteristics

We analyzed a total of 48 male mice (24 low-active, and 24 high-active) at twelve-weeks of age (**Table 1**). The high-active mice were characterized by 6.2% higher total body weight ($p<0.0001$) with a 6.8% higher lean mass ($p=0.0003$); however, we found no differences in total fat mass ($p=0.5255$) or average daily food consumption ($p=0.3211$). In addition, the high-active mice had 6.8% higher total-body water observed.

Table 1. Characteristics of mice. Body weight and lean mass were significantly different. Statistics: unpaired t-test, *significance ($p<0.05$). Data are expressed as mean±SE in grams.

Characteristic	High-active mice (n=24)	Low-active Mice (n=24)	p-value
Body weight	27.49±0.26	25.88±0.26	**<0.0001***
Lean Mass	22.50±0.20	21.07±0.22	**0.0003***
Fat Mass	2.48±0.15	2.61±0.14	0.5255
Avg. Daily Food Consumption	3.10±0.21	3.37±0.15	0.3211
Total-body water	1.92±0.03	1.79±0.02	**0.0006***

3.2. Enrichment Curve

Deuterium from D_2O incorporates into alanine through biochemical pathways and reactions where H_2O is usually utilized. The incorporation of enriched alanine into protein (protein-bound = pbALA) allows us to measure the amount of protein created over time. With time, the enrichment of alanine incorporated into protein will reach the enrichment of alanine in the precursor pool (plasma=plaALA or intracellular=icALA),

eventually resulting in equal TTR in the precursor pool and protein-bound alanine (f ratio of 1 or 100%; see equation (1)). The calculated f of the different organs measured are graphed on the theoretical enrichment curve **(Figure 3)**. The theoretical curve shows that the higher the f the more the tissue is incorporating the labelled alanine into proteins and approaching equivalence to the labelled alanine in the precursor pool. These results indicate that tissues such as jejunum, ileum, and liver incorporated the deuterium at a faster rate than others and approached f=1. Analyzing the enrichment of tissues can also be used to speculate which organ tissues will have higher FSR since FSR is a function of f and time. It is assumed that when f is less than 0.7, equation (1) can be used to calculate FSR correctly with sufficient precision.

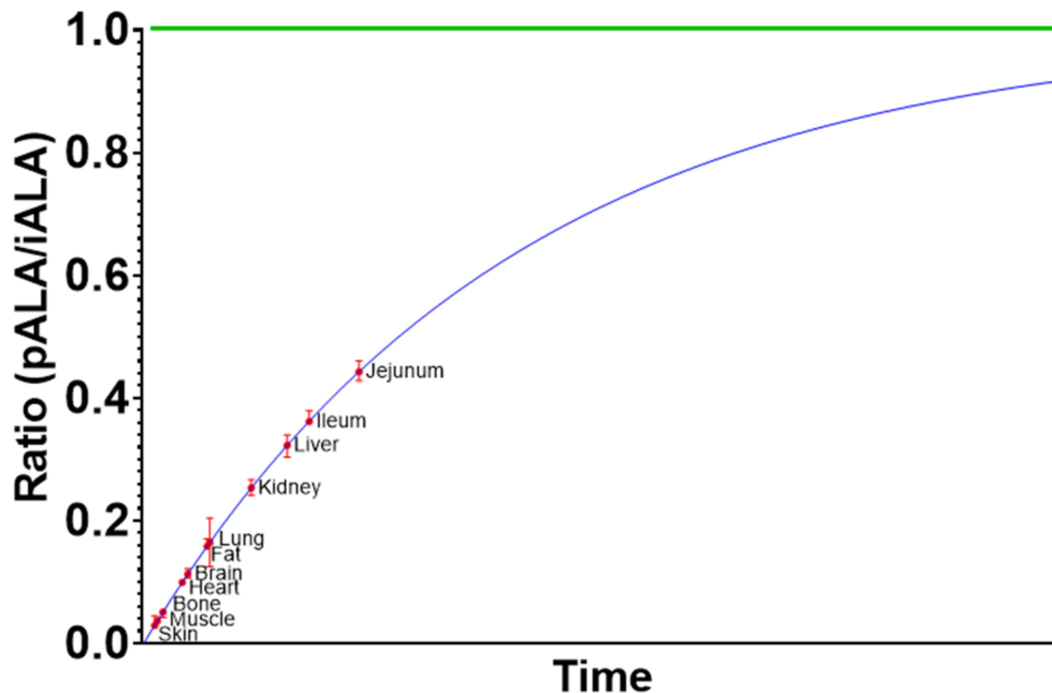

Figure 3. **Estimated ratio (f) between alanine TTR in protein (pbALA) and in the precursor pool and the average f of the tissues.** The blue line is depicting the estimated rate of enrichment calculated as a ratio (f) of the protein-bound alanine TTR to the intracellular alanine TTR. Each tissue point is the calculated average enrichment values placed along the estimated curve. Note that the enrichment values are not graphed according to real-time. The green line represents when protein-bound alanine TTR is equal to intracellular alanine TTR (f ratio of 1). Data are mean [95% CI].

3.3. D_2O enrichment

We gave 21 mice (mix of control, high-active, and low active mice) a bolus to reach 1% D_2O enrichment of total body water and 34 mice (mix of control, high-active, and low active mice) 5% D_2O enrichment of total body water. The reason for this change was due to the necessary balance between the amount of D_2O given and time in order to achieve detectable amounts of protein-bound labelled alanine. For example, one could

give a large amount of D_2O such as 50% and measure the turnover in 2 hours or could give low amounts such as 1% for several days in order to achieve detectable amounts of protein-bound labelled alanine. Using previous literature, we decided that 1% would not be enough over 24 hours to measure the labelled alanine properly in the organ expected to have the lowest protein turnover.

We then analyzed the TTR to estimate the effects of the anticipated enrichment level on the variation. As expected, the TTR values of mice given 1% vs 5% D_2O enrichment were significantly different in the precursor pool of all tissues ($p<0.0001$ in all tissues) (**Table 2**) as well as the TTR values of protein-bound alanine of mice given 1% or 5% D_2O enrichment ($p<0.0001$ in all tissues) (**Table 2**). These results show, also as expected, that 5% alanine TTRs are approximately five times the amount of 1% alanine TTRs.

The intracellular and plasma TTR of alanine should theoretically be the same if given the same D_2O enrichment in the mice over 24h, regardless of the tissue, thus allowing for either icALA or pbALA to act as the precursor pool. However, it should be noted that the precursor alanine TTR were not the same. The mice given 5% enrichment resemble the 5% enriched plaALA TTR more than the 1% icALA TTR resemble the plaALA TTR. The 1% mean of the icALA TTR differed 80.62% while the 5% enriched had a lower average mean difference of 12.58%.

Table 2. Comparison of precursor TTR and protein-bound alanine (pbALA) TTR in mice given 1% and 5% D_2O enrichment. Each of the reported precursor TTRs from organ tissues are measured from the intracellular fraction (icALA). The mean difference was calculated as the percentage of 1- (icALA TTR/plaALA TTR). Data are expressed as mean±SE. n=21 for both 1% precursor TTR and 1% pbALA TTR. n=54 for both 5%

Organ Tissue	1% precursor TTR	5% precursor TTR	1% pbALA TTR	5% pbALA TTR	Mean difference of 1%	Mean difference of 5%
Plasma	0.0407 ±0.0007	0.2001 ±0.0021	-	-		
Kidney	0.0364 ±0.0007	0.1827 ±0.0062	0.0094 ±0.0003	0.04471 ±0.0010	80.55%	18.84%
Heart	0.0342 ±0.0007	0.1881 ±0.0042	0.0035 ±0.0001	0.0189 ±0.0006	81.72%	14.29%
Fat	0.0374 ±0.0008	0.1997 ±0.0032	0.0064 ±0.0004	0.0318 ±0.0008	80.03%	4.55%
Lung	0.0372 ±0.0009	0.1931 ±0.0050	0.0126 ±0.0020	0.0187 ±0.0005	80.11%	13.09%
Jejunum	0.0376 ±0.0007	0.1900 ±0.0053	0.0179 ±0.0003	0.0813 ±0.0020	79.88%	14.04%
Ileum	0.0373 ±0.0014	0.1976 ±0.0066	0.0130 ±0.0006	0.0698 ±0.0017	80.07%	6.60%
Muscle	0.0428 ±0.0037	0.2112 ±0.0036	0.0019 ±0.0002	0.0073 ±0.0002	77.13%	0.10%
Brain	0.0336 ±0.0008	0.1885 ±0.0032	0.0051 ±0.0003	0.0193 ±0.0004	82.07%	12.24%
Liver	0.0406 ±0.0007	0.2012 ±0.0064	0.0154 ±0.0009	0.0600 ±0.0010	78.28%	10.79%
Bone	0.0329 ±0.0016	0.1905 ±0.0044	0.0024 ±0.0002	0.0071 ±0.0003	82.43%	14.09%
Skin	0.0288 ±0.0009	0.1451 ±0.0033	0.0002 ±0.0002	0.0070 ±0.0005	84.63%	29.74%

Although the individual values of precursor pool alanine TTR and pbALA TTR were expected to be different if given different D_2O levels. The ratio of pbALA TTR and precursor alanine TTR of the 1% and of the 5% should have been similar resulting in similar FSR values, regardless of D_2O level administered. We calculated and compared the protein FSR of 1% D_2O enriched mice and 5% D_2O enriched mice using icALA TTR as the precursor pool (**Table 3**). Unexpectedly, lung, muscle, brain, liver, bone, and skin tissue protein FSR resulted in significant differences. In order to further compare the information obtained from 1% and 5% enriched mice, we analyzed the variation of the FSR using plasma as the precursor pool (**Table 4**). Kidney, lung, jejunum, muscle, brain, liver, bone, and skin tissue protein FSR were significantly different between the 1% D_2O enriched mice and 5% D_2O enriched mice.

In most of the significantly different organ tissues, the 1% had an increased variability. For example, the lung and brain protein FSR values of the 1% D_2O enriched mice resulted in a variation 3 times greater than the protein FSR variation of 5% D_2O enriched mice and the muscle variation was 2 times greater in the 1% than the 5% (**Table 3**). The same observation was found in plasma with the variation of the kidney and muscle at 1% being about 2 times higher than the 5% and the brain with 1% having a variation approximately 5 times higher than 5% enriched (**Table 4**). The average of the coefficient of variation of the protein FSR is higher in the 1% enriched mice in calculations with both precursor pools (**Table 3 and 4**).

Table 3. Protein FSR of mice given 1% and 5% D_2O enrichment using intracellular alanine TTR as the precursor pool. Lung, muscle, brain, liver, bone, and skin tissue protein FSR were significantly different in the 1% D_2O enriched mice and 5% D_2O enriched mice. The average is calculated as the average of the reported CV for each tissue. Statistics: unpaired t-test, *significance ($p<0.05$). Data are expressed as mean±SE in fraction/day.

Organ Tissue	1% FSR (n=21)	1% FSR Coefficient of Variation (%)	5% FSR (n=30)	5% FSR Coefficient of Variation (%)	p-value
Kidney	0.2395±0.0079	13.23%	0.2515±0.0141	29.03%	0.5374
Heart	0.0870±0.0021	10.44%	0.0799±0.0030	19.18%	0.0822
Fat	0.1539±0.0143	35.89%	0.1384±0.0039	14.91%	0.1878
Lung	0.3730±0.0651	76.05%	0.0861±0.0038	25.25%	**<0.0001***
Jejunum	0.5099±0.0090	7.66%	0.4735±0.0202	24.88%	0.1993
Ileum	0.3483±0.0261	31.76%	0.3601±0.0173	25.91%	0.6965
Muscle	0.0366±0.0029	33.20%	0.0299±0.0010	17.06%	**0.0188***
Brain	0.1336±0.0073	21.92%	0.0858±0.0014	8.23%	**<0.0001***
Liver	0.3841±0.0248	25.79%	0.3269±0.0162	24.75%	**0.0500***
Bone	0.0643±0.0054	34.82%	0.0333±0.0025	36.97%	**<0.0001***
Skin	0.0082±0.0072	349.30%	0.0353±0.0044	59.86%	**0.0017***
Average		58.19%		26.00%	

Table 4. Protein FSR of mice given 1% and 5% D_2O enrichment using plasma alanine TTR as the precursor pool. Kidney, lung, jejunum, muscle, brain, liver, bone, and skin tissue protein FSR were significantly different in the 1% D_2O enriched mice and 5% D_2O enriched mice. The average is calculated as the average of the reported CV for each tissue. Statistics: unpaired t-test, *significance ($p<0.05$). Data are expressed as mean±SE in fraction/day.

Organ Tissue	1% FSR (n=21)	1% FSR Coefficient of Variation (%)	5% FSR (n=35)	5% FSR Coefficient of Variation (%)	p-value
Kidney	0.2127±0.0063	11.51%	0.1818±0.0018	5.716%	**<0.0001***
Heart	0.0712±0.0025	14.65%	0.069±0.0030	22.95%	0.5584
Fat	0.1372±0.0116	34.79%	0.1307±0.0033	13.80%	0.5109
Lung	0.3364±0.0567	71.45%	0.0734±0.0032	24.95%	**<0.0001***
Jejunum	0.4588±0.0095	9.06%	0.3706±0.0079	12.20%	**<0.0001***
Ileum	0.3025±0.0192	26.87%	0.3105±0.0080	14.74%	0.6511
Muscle	0.0355±0.0030	34.20%	0.0298±0.0009	16.58%	**0.0311***
Brain	0.1038±0.0059	22.64%	0.0742±0.0006	4.564%	**<0.0001***
Liver	0.3868±0.0265	26.50%	0.2729±0.0041	8.188%	**<0.0001***
Bone	0.0490±0.0041	33.21%	0.0287±0.0021	35.79%	**<0.0001***
Skin	0.0025±0.0046	717.2%	0.0247±0.0028	54.15%	**<0.0001***
Average		91.10%		19.42%	

In addition to the intracellular alanine TTR, plasma can be used as a precursor pool. We measured the alanine TTR within plasma at various blood sampling time points (3 and 30 minutes or 5 and 40 minutes) after the mice were given a stable isotope pulse. The alanine TTRs at each time point can be compared according to the amount of

D_2O enrichment (**Figure 4a and 4b**). The parameters of the data expressed in **Figure 4a and 4b** are found in **Table 5**.

We observed a higher mean plaALA TTR at the time points more immediately after the stable isotope pulse was given (3 and 5 minutes) when compared to later times after the pulse was given (30 and 40 minutes) (**Table 5**), thus suggesting the stable isotope pulse affected the measurements of labelled alanine. The change in the means between time points evidences the stable isotope pulse influence. The effect is observed more in the 1% enriched mice, most likely, due to the deuterium of the stable isotopes making a larger change in total deuterium available when the enrichment was a lower amount.

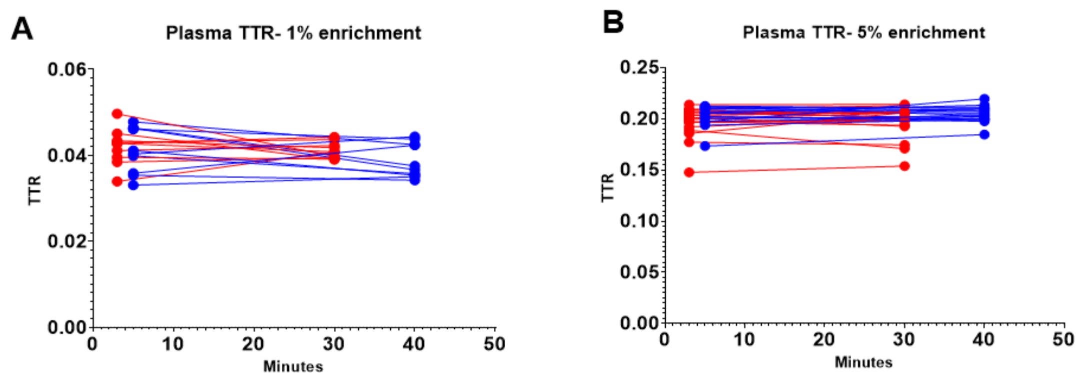

Figure 4. Comparison of plasma alanine TTR in 1% D_2O enrichment (4A) and 5% D_2O enrichment (4B) obtained at various blood draw time points (3, 5, 30, and 40 minutes). 1% enrichment 3 min and 30 min n=10; 1% enrichment 5 min and 40 min n=11; 5% enrichment 3 min and 30 min n=16; 5% enrichment 5 min and 40 min n=17. Data are expressed as plaALA TTR for each mouse sampled at the given time point.

Table 5. Plasma TTR reported according to blood sample time and percentage of D_2O enrichment. The change in variation is the difference between the means of the two time points (3 min-30 min and 5 min-40 min) divided by the mean of the second time point expressed as a percentage. Statistics: Data are expressed as mean [95% CI].

Enrichment	3 minute	30 minutes	Mean Difference	5 minute	40 minute	Mean Difference
1%	0.0420 [0.0390, 0.0449]	0.0411 [0.0398, 0.0424]	2.20%	0.0532 [0.0262, 0.0802]	0.0520 [0.0223, 0.0817]	2.34%
5%	0.1962 [0.1876, 0.2049]	0.1964 [0.1876, 0.2051]	0.10%	0.2044 [0.2014, 0.2075]	0.2054 [0.2023, 0.2085]	0.49%

Taking into account the fact that: 1) the precursor pools of 1% enriched mice were less similar than the 5% precursor pool (**Table 2**); 2) the 1% enriched mice resulted in increased variation in protein FSR measurements using either precursor pool (**Table 3 and 4**); and 3) that the 1% enriched mice had more variation in plaALA TTR after the stable isotope pulse (**Figure 3 and Table 5**), we used only the 5% enriched mice for FSR measurements.

3.4. Using Plasma for Fractional Synthesis Rates

Since we suspected that the stable isotope pulse interfered with labelled alanine values obtained, we also suspected that the stable isotopes affected the icALA TTR more, because the metabolism of the amino acids given take place in the cell creating an inflated level of labelled icALA.

Due to the variation found in both precursor pools (**Table 3 and 4**) and the possible effect of the stable isotope pulse (**Figure 4**), we analyzed both plaALA and

icALA TTR together to observe if the variation was the same per mouse in each precursor pool. We observed that within each mouse the precursor pool is reasonably consistent (**Figure 5).** As expected, if the plaALA TTR in the plasma was elevated in a mouse, the icALA TTR was also.

However, when utilizing the intracellular or plasma as the precursor pool to calculate FSR, the results showed that the protein FSR of the two calculations are significantly different in all tissues except fat, muscle, and bone (**Table 6**). The mean protein FSR using icALA TTR was higher than protein FSR calculated with plaALA TTR in each tissue. This is evidence that the icALA TTR was inflated more by the stable isotope pulse.

Since 1) the protein FSR between the two precursor pools were mostly significantly different and 2) there was evidence of inflation in the icALA TTR from the stable isotope pulse, the plaALA TTR was used as the precursor pool to calculate protein FSR.

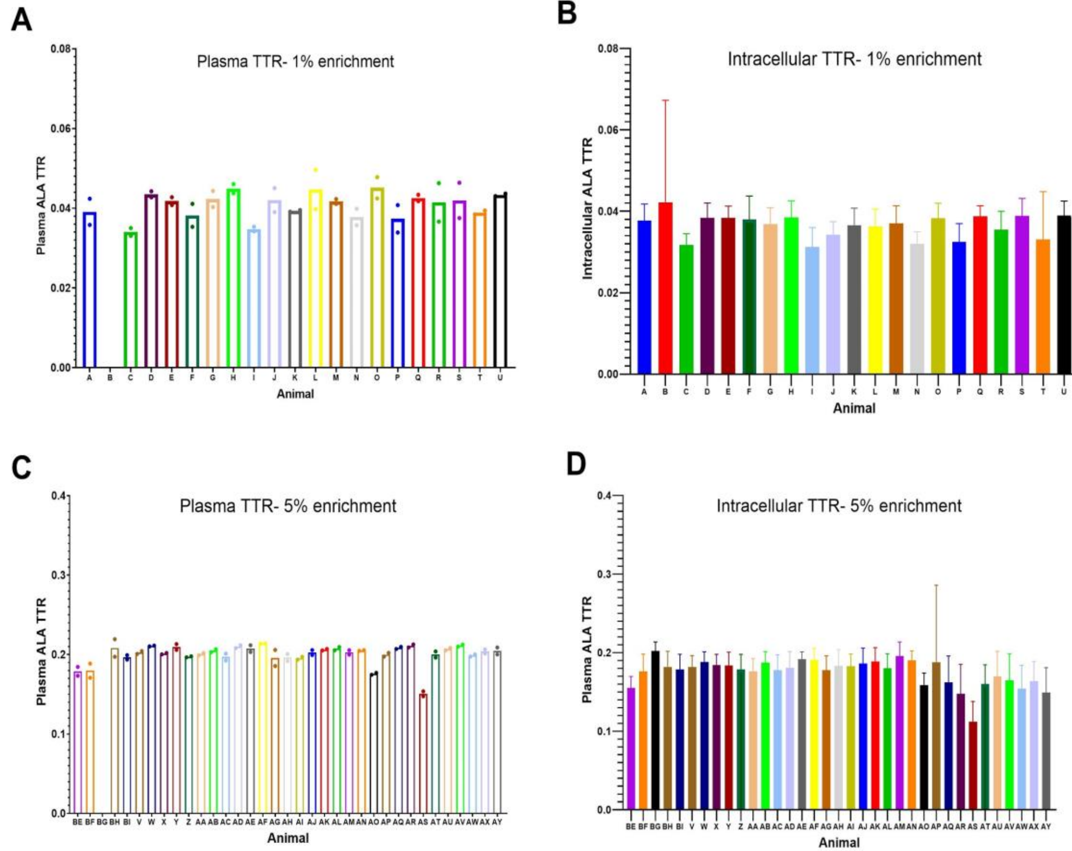

Figure 5. Plasma alanine TTR and mean intracellular alanine TTR according to percentage of D_2O enrichment. plaALA TTR (A and C) and icALA TTR (B and D) are expressed as mean with 95% CI.

Table 6. Comparison of the precursor pools used to calculate FSR. Data include only animals given 5% D_2O enrichment. FSR (plasma) indicated that plasma alanine TTR was used as the precursor pool during FSR calculation and FSR (intracellular) indicated that intracellular alanine TTR was used. P-value is reported for t-test between FSR (plasma) and FSR (intracellular). Statistics: unpaired t-test, *significance ($p<0.05$). Data are expressed as mean [95% CI]. FSR is fraction/day. n=35 for all.

Tissue	plaALA TTR	icALA TTR	pbALA TTR	FSR (plasma)	FSR (intracellular)	P-value
Kidney	0.2001 [0.1957, 0.2045]	0.1624 [0.1504, 0.1744]	0.04073 [0.0396, 0.0418]	0.1818 [0.1780, 0.1855]	0.2445 [0.2195, 0.2694]	**<0.0001***
Heart		0.1715 [0.1664, 0.1767]	0.0164 [0.0150, 0.0178]	0.0687 [0.0624, 0.0749]	0.0799 [0.0737, 0.0860]	**0.0116***
Fat		0.1910 [0.1865, 0.1955]	0.0303 [0.0287, 0.0318]	0.1307 [0.1238, 0.1375]	0.1384 [0.1304, 0.1464]	0.1372
Lung		0.1739 [0.1654, 0.1824]	0.0178 [0.0164, 0.0193]	0.0734 [0.0669, 0.0799]	0.0861 [0.0784, 0.0938]	**0.0125***
Jejunum		0.1720 [0.1622, 0.1817]	0.0744 [0.0716, 0.0772]	0.3706 [0.3546, 0.3867]	0.4735 [0.4324, 0.5147]	**<0.0001***
Ileum		0.1869 [0.1684, 0.2054]	0.0646 [0.0616 0.0677]	0.3105 [0.2943, 0.3268]	0.3599 [0.3294, 0.3903]	**0.0053***
Muscle		0.2003 [0.1956, 0.2050]	0.0074 [0.0069, 0.0078]	0.0298 [0.0279, 0.0317]	0.0299 [0.0281, 0.0317]	0.9378
Brain		0.1756 [0.1719, 0.1793]	0.0179 [0.0174, 0.0183]	0.0742 [0.0729 0.0755]	0.0860 [0.0834, 0.0886]	**<0.0001***

Table 6 continued.

Tissue	plaALA TTR	icALA TTR	pbALA TTR	FSR (plasma)	FSR (intracellular)	P-value
Liver		0.1785 [0.1663, 0.1906]	0.0578 [0.0560, 0.0597]	0.2729 [0.2644 0.2814]	0.3176 [0.2890 0.3462]	**0.0038***
Bone		0.1719 [0.1655, 0.1784]	0.0070 [0.0060, 0.0086]	0.0287 [0.0244 0.0331]	0.0333 [0.0281 0.0385]	0.1684
Skin		0.1406 [0.1347, 0.1465]	0.0060 [0.0046, 0.0074]	0.0247 [0.0189 0.0305]	0.0353 [0.0262 0.0444]	**0.0475***

3.5. Tissue Protein Fractional Synthesis Rates

Utilizing the plasma alanine TTR, the protein-bound alanine TTR and time to calculate FSR of the mice given 5% D_2O enrichment, we found significant differences between the kidney, fat, lung, and skin of control, high active, and low active mice, but not in any of the other measured organ tissues (**Table 7** and **Figure 6**). The kidney protein FSR in the low-active mice were 7.82% higher than the high active mice. No significant differences were found between protein FSR of high-active and low-active mice in any of the other measured organ. The p-values were analyzed to correct for multiple comparison using a false discovery rate approach controlled for Q=5%, using the two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli [62] **(Figure 7).**

Table 7. Protein FSR of tissues of low-active (C3H/HeJ) and high-active mice (C57L/J). These FSR are calculated using mice given 5% D_2O enrichment. Plasma alanine TTR was used as the precursor pool. Statistics: unpaired t-test, *significance of FDR (q<0.05). Data are expressed as mean [95% CI] in fraction/day.

Tissue	Low-active (n=15)	High-active (n=15)	P-value	Q-value
Kidney	0.1863 [0.1824, 0.1902]	0.1754 [0.1694, 0.1815]	**0.0030***	**0.0315***
Heart	0.06491 [0.0621, 0.0678]	0.0712 [0.0548, 0.0875]	0.4375	0.6241
Fat	0.1314 [0.1265, 0.1364]	0.1230 [0.1104, 0.1356]	0.1327	0.3483
Lung	0.0711 [0.0677, 0.0744]	0.0643 [0.0600, 0.0686]	0.0128	0.0672
Jejunum	0.3688 [0.3525, 0.3851]	0.3602 [0.3308, 0.3896]	0.5944	0.6241
Ileum	0.3083 [0.2828, 0.3338]	0.3057 [0.2810, 0.3305]	0.8774	0.8375
Muscle	0.0305 [0.0274, 0.0337]	0.0292 [0.0258, 0.0325]	0.4911	0.6241
Brain	0.0734 [0.0717, 0.0750]	0.0750 [0.0721, 0.0778]	0.2999	0.6241
Liver	0.2754 [0.2595, 0.2912]	0.2690 [0.2555, 0.2825]	0.5321	0.6241
Bone	0.0320 [0.0248, 0.0392]	0.0293 [0.0229, 0.0357]	0.5495	0.6241
Skin	0.0236 [0.0192, 0.0280]	0.0170 [0.0120, 0.0220]	0.0225	0.0788

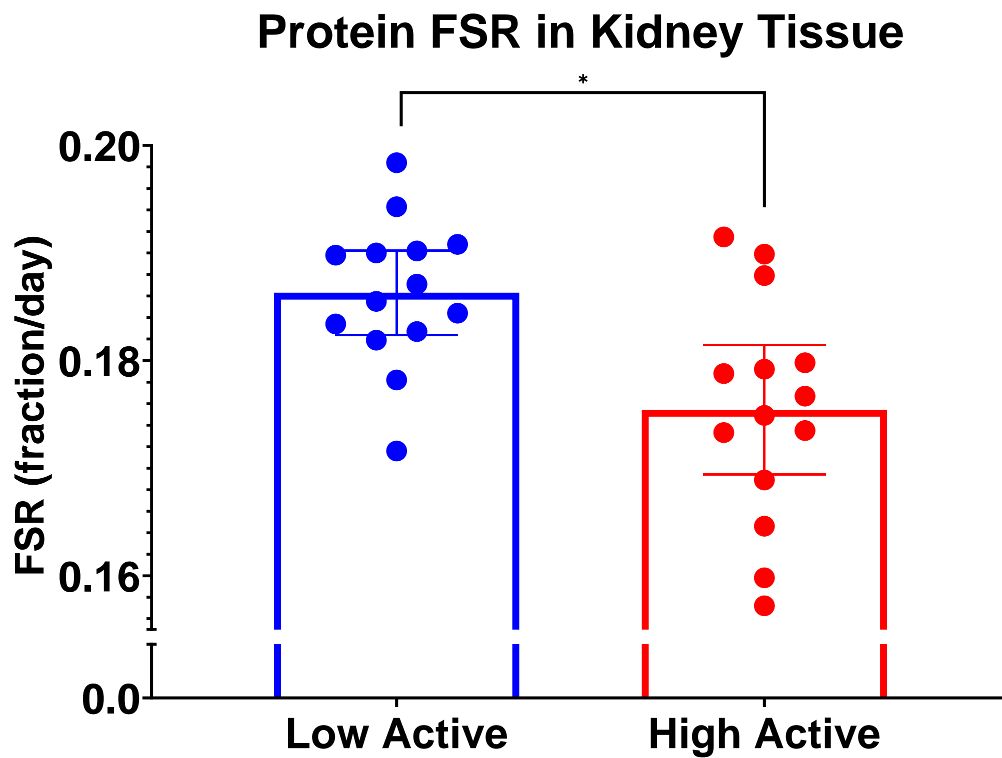

Figure 6. Protein FSR of low-active and high-active groups in the kidney. Statistics: t-test corrected with false discovery rate (significance: $q<0.05$). Data are expressed as mean [95% CI] in fraction/day.

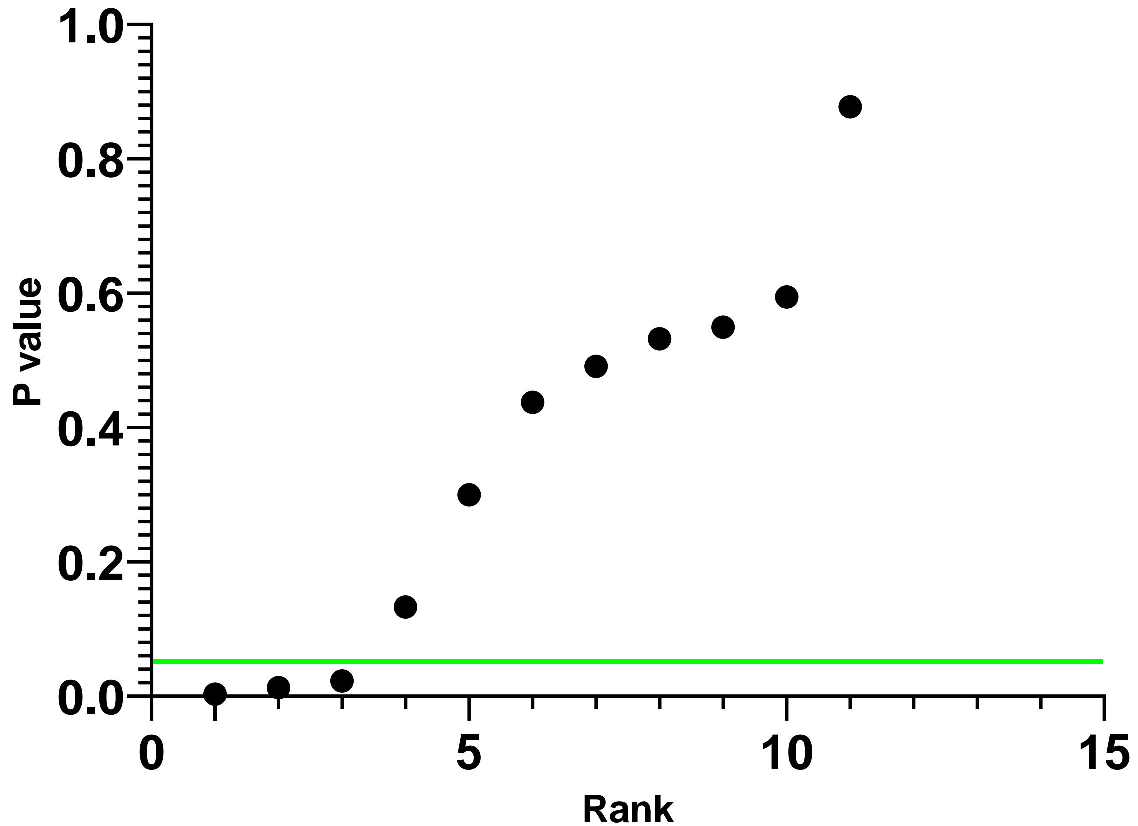

Figure 7. Ranked P-values from unpaired t-test of protein FSR of each tissue. False discovery rate approach was controlled with Q=0.05 (green line) and used the two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli [62].

3.6. Literature Comparison

First, we analyzed the protein FSR between tissues to the protein FSR of tissues found in literature. In the present study, we report that the jejunum resulted in the highest protein FSR followed by the ileum and liver (**Figure 8**). This finding is consistent with previous findings [25, 28] reporting that jejunum has the highest protein FSR followed by liver, ileum, heart and skeletal muscle.

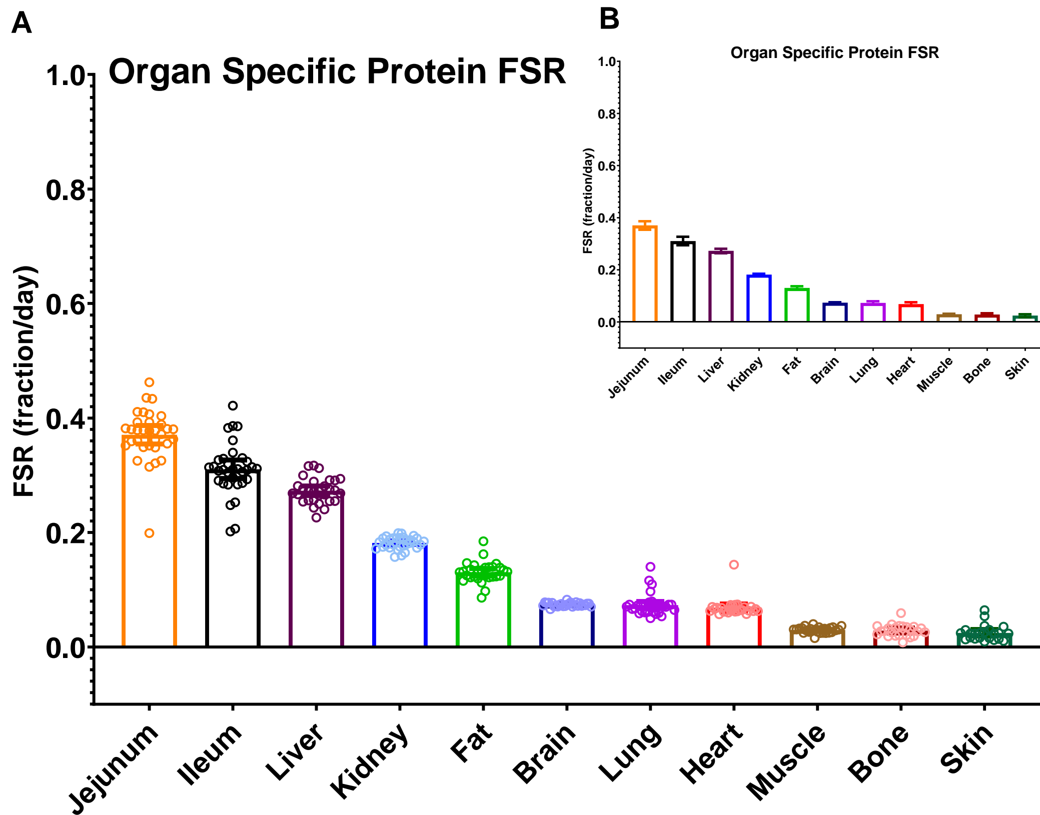

Figure 8. Protein FSR of each tissue. 6A shows protein FSR of the tissue from each mouse administered 5% D_2O enrichment. 6B shows the mean and 95% CI without the specific data points. Data is mean with 95% confidence interval.

The values obtained for the protein FSR of specific organs are consistent with literature that reported values of the same specific organ. For example, when comparing the observed mean skeletal muscle protein FSR values in the present study to the skeletal muscle FSR values found in literature (**Figure 9**), our value was significantly different than all other studies we tested against. The literature we compared our skeletal muscle results to were Marini 2105 (mouse)[27], Yuan 2008 (rats)[28], Wilkinson 2014 (human)[23], Robinson 2011 (human)[63] and Trommelen (human)[64].

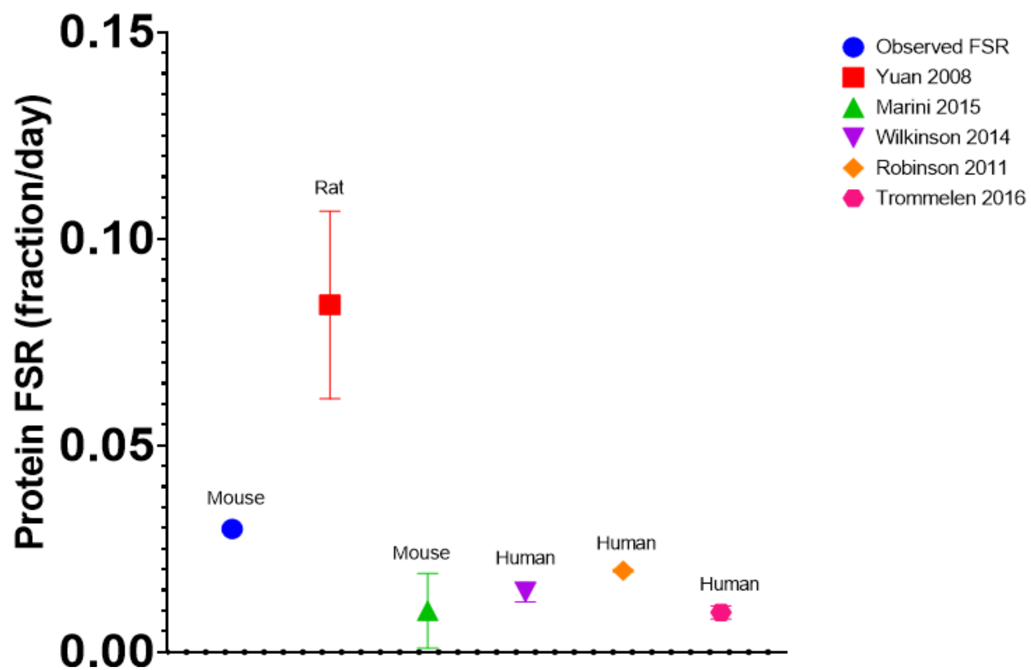

Figure 9. Comparison of the observed skeletal muscle protein FSR in the present study compared to reported skeletal muscle protein FSR values found in literature. Data from literature were mathematically converted to fraction/day. Statistics: One-way ANOVA, mean [95% CI], significance $p<0.05$

4. DISCUSSION

The purpose of the present study was to determine the differences in fractional synthesis rates (FSR) of various organ tissues between high and low active mice. We used D_2O to analyze the FSR of various tissues in high- and low-active mice in order to examine if tissues have a difference in protein synthesis according to physical activity level. We found significant differences between protein fractional synthesis rate of high and low active mice in the kidney tissue.

4.1. Enrichment of D_2O

Fractional synthesis rate is measured in a variety of methods, including administration of stable isotope and radioisotope labelled amino acids. We used D_2O, which distributes equally through all body water [50], and results in a natural labeling of proteins with a deuterium. The deuterium is utilized by biochemical pathways equivalent to a hydrogen in H_2O and is incorporated on amino acids such as alanine [51]. Subsequently, we can measure the amount of the labelled amino acids incorporated into proteins compared to the amount of unlabeled amino acids, resulting in a tracer-to-tracee ratio (TTR). The comparison of the TTR from protein-bound alanine to the TTR of alanine from the precursor pool in the intracellular compartment or plasma, represents the enrichment of D_2O. With time the TTR of protein-bound alanine increases to approach the TTR of the precursor pool, eventually resulting in a ratio equal to 1. By plotting the calculated enrichment values of the tissues on the curve, we can analyze

which organs are incorporating the deuterium at a faster rate (**Figure 3**). Jejunum, ileum, and liver resulted in the highest enrichment rates and bone, muscle and skin had the lowest enrichment rates as expected from literature [25].

By measuring enrichment of D_2O, we can determine if 1% D_2O could be used in future studies. Most studies use 4% and 5% [65, 66]} enrichment; however, studies with human subjects must use a lower enrichment because higher levels induce nausea or vertigo [66, 67]. It has been speculated that a lower enrichment could be considered with advanced technology [68]. The actual values of intracellular TTR and protein-bound TTR were expected to be different if given different D_2O enrichment levels (**Table 2**) but when calculated in a ratio the comparison between the incorporated in the tissue and the precursor pool would be the same. Therefore, it was expected that 1% and 5% enrichment would not be different but many of the tissues resulted in significant differences of the protein FSR between the 1% and 5% enriched mice when using either precursor source (**Table 3 and 4**). In most of the significantly different organ tissues, the 1% had a higher ratio possibly due to the increased variability. For example, the lung and brain protein FSR values of the 1% D_2O enriched mice resulted in a variation 3 times greater than the 5% D_2O enriched mice and the muscle variation was 2 times greater in the 1% than the 5% (**Table 3**). The same issue is found in plasma with the variation of the kidney and muscle at 1% being about 2 times higher than the 5% and the brain at 1% having a variation about 5 times higher than 5% enriched (**Table 4**).

In addition, according to the equivalent diffusion of D_2O just as water across all areas of the body [50], we would expect the precursor pool alanine TTR to be similar if

measured in the plasma or the intracellular compartments. Analysis of the icALA TTR of each tissue vs the plaALA TTR resulted in differences in both the mice given 1% enrichment and 5% enrichment. However, it should be noted that the average mean difference of the 1% enriched mice were almost 3 times higher than the 5% enriched mice. Based on the increased variability in the 1% D_2O enriched mice, we caution the use of a low enrichment amount due to the variability.

Furthermore, we suspect that the values resulting from the 1% enriched mice may show more variability due to the stable isotope pulse given. First, as seen in **Table 5** and **Figure 4**, the variability in the plasma TTR at the time closest to the pulse (minute 3 and 5) is more variable than the later time points (30 and 40 minutes). This change in variation allows for the conclusion that the pulse of stable isotopes may interfere with the amount of labelled alanine detected. Second, **Figure 4** and **Table 5** displays that the suspected effect of the pulse is more profound in the 1% than the 5% percent. This effect could be because the portion of deuterium being created by the metabolism of stable isotope molecules represents a larger amount in the overall deuterium amount than in the 5% enrichment, thus affecting the ratios to a larger degree.

Based on 1) the increased difference between precursor pools in the 1% enriched mice, 2) the increased variability of the protein FSR and 3) the greater effect of the stable isotope pulse given, we decided to exclude the measurements taken from the 1% enriched animals.

4.2. Using Plasma Alanine TTR as the precursor pool

As discussed, FSR is a measure of the ratio between the protein-bound alanine TTR and precursor pool alanine TTR over a given time. Typically, the intracellular alanine TTR is used as the precursor pool [65, 69], but we examined if plasma could act as the precursor pool. We concluded that the stable isotope pulse may affect the data obtained, because a higher variability is observed in plaALA TTR immediately after the pulse is administered (**Figure 4**). It can be expected that if there is a disturbance in the plasma caused by the pulse then the change will be amplified in the intracellular as the location for metabolism is within the cell. Thus, an amplified change in the intracellular would result in an inflated FSR as seen in **Table 6**. Due to this inflation in the intracellular alanine TTR, we utilized plasma alanine TTR in further FSR calculations.

4.3. Fractional Synthesis Rate of High and Low Active Mice

Fractional synthesis rate (FSR) is calculated as a function of enrichment and time, representing protein turnover in a given time. As the deuterium molecules are incorporated into proteins from the time D_2O is administered to the time of tissue collection, the measured amount of labelled amino acid isolated from protein in a cell is the amount of newly made protein in the given amount of time. FSR is important to measure in order to gain an understanding of metabolic regulation. The faster the organ can make protein, the more control the tissue has through upregulation and downregulation of metabolic pathways. FSR also represents the organ’s ability to adapt. For example, the ileum, jejunum, and liver have the highest measured mean protein FSR

(Figure 8). A heightened ability for the intestine to adapt is most likely due to the changing of the intestinal environment caused by variation in food intake. The liver may have a higher regulation as one of the main metabolic filters, because of the necessity to adapt to pharmaceutical and substance introduction in order to make harmful substances more hydrophilic for excretion. Both of these organs may be able to adapt quickly due to a faster FSR.

In addition, measuring a dynamic parameter of metabolism, such as protein FSR, is beneficial when compared to a single static measurement, the same way a video would provide more context than a still picture. In this case, measuring tissue-specific protein FSR allows for an understanding of the spatial and temporal differences of metabolic rates. Calculating protein FSR allows for the advantage to not only measure the amount of alanine present in the tissue as a static measurement but also the amount of newly created over time giving insight into the activity of the tissue.

We measured protein FSR in high-active and low-active mice in order to understand the differences in metabolism in various physical activity levels **(Table 7)**. We hypothesized that protein FSR in skeletal muscle, brain, liver, skin, kidney, heart, ileum, and jejunum are higher in the high-active mice when compared to the low-active mice because these organs are expected to be able to adapt with change in physical activity. In addition, we hypothesized that there are no significant differences in the lung and the skull bone because these organs do not undergo physiological changes in response to physical activity nor actively grow after adolescence [38, 42]. We also

predicted that fat would have a decreased protein FSR in high-active mice because fat is broken down for energy for physical activity thus expected to be in a catabolic state.

In contradiction to most of our hypothesis, we found that only the kidney of the low active mice resulted in significantly higher protein FSR than the high active mice (**Table 7)** and no significant differences in all other measured tissue. When analyzing the p-values for false discovery rate (**Figure 7**) we found that the adjusted p-values remain significant discoveries. The kidney had mean difference of 7.82%/day. Although these organ tissues show a statistical difference, we believe the results are not physiologically different because the low difference between the groups would not make a large impact on the actual amount of protein present. Other authors have reported that a physiological difference exists in their observed protein FSR with mean differences of approximately 12% [68], ~20% [25], and ~36% [70]. The mean difference we observe are lower than those reported in other literature. It is also important to note that the high active mice have lower FSR in each organ tissue except for the heart. Since the difference between high and low active mice are not enough to make a large impact on metabolic regulation, it can be concluded that the high-active mice organs are no more equipped for adaptation through protein turnover rates than the low-active mice. These results show that the source of physical activity regulation is not an increase in protein turnover causing the ability of certain organs to adapt to physical activity.

In addition to our findings, previous evidence supports that there is a metabolic connection to physical activity level and more studies are necessary to understand the relationship. Previous evidence shows that protein expression of proteins associated with

metabolic pathways are significantly different in high and low active mice skeletal muscle and brain [15, 16]. Furthermore, high and low active mice had significantly different levels of some essential amino acids and non-essential amino acids in blood [18, 19]. If the high-active mice are believed to have more metabolic regulation, the present study results show that the source of the metabolic regulation is not from an overall increase in ability to synthesize protein at a higher rate.

4.4. Literature Comparison

Before comparing specific values to literature, we compared the whole organ mean order to the means found in literature to establish if our findings resemble the order of organ protein FSR found in literature. The order we observed with jejunum having the highest protein FSR and skin having the lowest protein FSR is constant with the reported protein FSR found in multi-organ analyses in literature [25, 28].

When comparing the skeletal muscle protein FSR values obtained from the current study to other studies' control group skeletal muscle protein FSR values, our observed mean value was significantly different than all compared means (**Figure 8**). The studies chosen to compare to utilize the D_2O method [23, 28, 63] and [$^2H_5$]phenylalanine flood dose method [27, 64] to obtain FSR values. The studies include mice[27], rats[28], and human[23] subjects to show that the muscle protein synthesis is conserved across animals.

4.5. Considerations

One consideration posed by Daurio et al [68] is an issue regarding the consequences of using a single time point to infer the protein turnover rate, because the error between the apparent rate and expected rate at 24 hours of D_2O administration was ~20%. Daurio et al goes on to explain that converting the data from a linear to a logarithmic scale reduces the size of the error and is a more reliable estimation of the rate regardless of the amount of time after D_2O administration [68]. We attempted to minimize this limitation by utilizing an equation that incorporates a natural log transformation of the enrichment data. In addition, inaccuracy can be tolerated in this case since the data is used as a comparison between groups rather than used as a definite value. Although the explicit values may be needed in some situations, the values in the present study are sufficient to demonstrate a significant difference or not between the high and low active mice.

The mice in this experiment were also given a pulse of stable isotope tracers [18, 19] about an hour before tissue collection. There is a possibility that the stable isotope tracers increased the amount of deuterium available as the labelled hydrogens of the given amino acids were cleaved by normal metabolic reactions. The interference caused by the pulse affected the mice given 1% D_2O enrichment when compared to the 5%. The observed inflation and increases in variation lead to the conclusion that only 5% enriched mice would be used when calculating FSR. Although we used 5% D_2O, we believe that 1% can be used in future studies with the conditions that blood sampling must happen before the pulse of stable isotopes is given and plasma is used as the

precursor pool. If intracellular is to be used as the precursor pool, then the subject should not be exposed to a stable isotope pulse.

Furthermore, the C57BL6/J mice were used as a "control" group; however, an argument can be made that they serve more as a moderate physical activity group, because their reported physical activity level, based on daily running duration [55], is almost exactly halfway between the duration ran by the low active mice and high active mice. We deemed this group as a control group because we used the measurements for background (naturally occurring) D_2O.

4.6. Future Studies

The present study utilized intracellular alanine TTR to represent the precursor pool as most scientists use [65, 69] but we tested if plasma would make an adequate precursor pool with this methodology. Plasma analysis requires less time and is more convenient than measuring in the intracellular fraction. In conclusion from our studies, we recommend when designing an experiment to avoid the variability caused by a stable isotope pulse, obtain plasma before the isotopes are given, and use plasma as the precursor pool.

More studies are needed to find what, if any, metabolic parameters differentiate physical activity level. The methods used in this study can be used to explore utilization of energy substrates such as measuring fatty acid oxidation and glycogen storage in the liver and muscle [68]. Others have measured fatty acid and lipid synthesis using D_2O [71, 72] which can be applied to study physical activity level.

5. CONCLUSIONS

To understand the variation in metabolism between high and low physical activity levels, we measured protein fractional synthesis rates of tissues from inherently high and low active mice. The kidney protein FSR in low active mice was 7.82% higher than in the high-active mice. However, we suggest that the differences are not physiologically relevant. In conclusion, the high active mice do not have an advantage from protein turnover to adapt to physical activity.

In addition, we examined if 1% D_2O enrichment allowed for reasonable protein FSR measurements when compared to the normally used 5% D_2O enrichment; however, the 1% enrichment values contained more variability than the 5% enrichments and may have been affected by the stable isotope pulse administered before measurement. We also discovered that plasma is a more ideal precursor pool to be used for calculation of protein FSR in this study, because of the possible effect of the stable isotope pulse on the intracellular precursor pool.

REFERENCES

1. Lightfoot, J.T., et al., *Biological/Genetic Regulation of Physical Activity Level: Consensus from GenBioPAC.* Med Sci Sports Exerc, 2018. **50**(4): p. 863-873.
2. Troiano, R.P., et al., *Physical Activity in the United States Measured by Accelerometer.* Medicine & Science in Sports & Exercise, 2008. **40**(1): p. 181-188.
3. Mokdad, A.H., et al., *Actual Causes of Death in the United States, 2000.* JAMA, 2004. **291**(10): p. 1238-1245.
4. Booth, F.W., C.K. Roberts, and M.J. Laye, *Lack of exercise is a major cause of chronic diseases.* Comprehensive Physiology, 2011. **2**(2): p. 1143-1211.
5. Booth, F.W., et al., *Role of inactivity in chronic diseases: evolutionary insight and pathophysiological mechanisms.* Physiological reviews, 2017. **97**(4): p. 1351-1402.
6. Ding, D., et al., *The economic burden of physical inactivity: a global analysis of major non-communicable diseases.* The Lancet, 2016. **388**(10051): p. 1311-1324.
7. Vellers, H.L., et al., *High Fat High Sugar Diet Reduces Voluntary Wheel Running in Mice Independent of Sex Hormone Involvement.* Front Physiol, 2017. **8**: p. 628.
8. Schmitt, E.E., et al., *Environmental Endocrine Disruptor Affects Voluntary Physical Activity in Mice.* Med Sci Sports Exerc, 2016. **48**(7): p. 1251-8.

9. Bowen, R.S., M.J. Turner, and J.T. Lightfoot, *Sex hormone effects on physical activity levels: why doesn't Jane run as much as Dick?* Sports Med, 2011. **41**(1): p. 73-86.

10. Bowen, R.S., et al., *Effects of Supraphysiological Doses of Sex Steroids on Wheel Running Activity in Mice.* J Steroids Horm Sci, 2012. **3**(2): p. 110.

11. Lightfoot, J.T., *Sex hormones' regulation of rodent physical activity: a review.* Int J Biol Sci, 2008. **4**(3): p. 126-32.

12. Lightfoot, J.T., *Why control activity? Evolutionary selection pressures affecting the development of physical activity genetic and biological regulation.* Biomed Res Int, 2013. **2013**: p. 821678.

13. Dawes, M., et al., *Differential gene expression in high- and low-active inbred mice.* Biomed Res Int, 2014. **2014**: p. 361048.

14. Lightfoot, J.T., et al., *Quantitative trait loci for physical activity traits in mice.* Physiol Genomics, 2008. **32**(3): p. 401-8.

15. Ferguson, D.P., et al., *Differential protein expression in the nucleus accumbens of high and low active mice.* Behav Brain Res, 2015. **291**: p. 283-288.

16. Ferguson, D.P., et al., *Differential skeletal muscle proteome of high- and low-active mice.* J Appl Physiol (1985), 2014. **116**(8): p. 1057-67.

17. MacLennan, P.A., R.A. Brown, and M.J. Rennie, *A positive relationship between protein synthetic rate and intracellular glutamine concentration in perfused rat skeletal muscle.* 1987. **215**(1): p. 187-191.

18. Granados, J.Z., et al., *Metabolic Differences in Nonessential Amino Acid Plasma Concentrations in High- and Low-Active Mice.* The FASEB Journal, 2017. **31**(1_supplement): p. lb210-lb210.

19. Letsinger, A.C., et al., *Metabolic Differences in Essential Amino Acid Plasma Concentrations in High- and Low-Active Mice.* The FASEB Journal, 2017. **31**(1_supplement): p. lb211-lb211.

20. Engelen, M.P., et al., *Exercise-induced lactate increase in relation to muscle substrates in patients with chronic obstructive pulmonary disease.* American journal of respiratory and critical care medicine, 2000. **162**(5): p. 1697-1704.

21. Engelen, M.P., et al., *Altered glutamate metabolism is associated with reduced muscle glutathione levels in patients with emphysema.* Am J Respir Crit Care Med, 2000. **161**(1): p. 98-103.

22. Phillips, S.M., et al., *Mixed muscle protein synthesis and breakdown after resistance exercise in humans.* 1997. **273**(1): p. E99-E107.

23. Wilkinson, D.J., et al., *A validation of the application of D2O stable isotope tracer techniques for monitoring day-to-day changes in muscle protein subfraction synthesis in humans.* American Journal of Physiology-Endocrinology and Metabolism, 2014. **306**(5): p. E571-E579.

24. Holmes, W.E., et al., *Chapter Seven - Dynamic Proteomics: In Vivo Proteome-Wide Measurement of Protein Kinetics Using Metabolic Labeling*, in *Methods in Enzymology*, M.M. Christian, Editor. 2015, Academic Press. p. 219-276.

25. Ten Have, G.A.M., et al., *Inhibition of jejunal protein synthesis and breakdown in Pseudomonas aeruginosa-induced sepsis pig model.* Am J Physiol Gastrointest Liver Physiol, 2019. **316**(6): p. G755-G762.

26. Ten Have, G.A.M., *Pig interorgan balance studies in health and disease.* Thesis, Maastricht University, 2017: p. 1-301.

27. Marini, J.C. and I.C. Didelija, *Arginine Depletion by Arginine Deiminase Does Not Affect Whole Protein Metabolism or Muscle Fractional Protein Synthesis Rate in Mice.* PLOS ONE, 2015. **10**(3): p. e0119801.

28. Yuan, C.L., et al., *Preserved protein synthesis in the heart in response to acute fasting and chronic food restriction despite reductions in liver and skeletal muscle.* 2008. **295**(1): p. E216-E222.

29. Chesley, A., et al., *Changes in human muscle protein synthesis after resistance exercise.* Journal of Applied Physiology, 1992. **73**(4): p. 1383-1388.

30. Jung, A.P., et al., *Physical activity and food consumption in high- and low-active inbred mouse strains.* Med Sci Sports Exerc, 2010. **42**(10): p. 1826-33.

31. Schild, M., et al., *Basal and exercise induced label-free quantitative protein profiling of m. vastus lateralis in trained and untrained individuals.* Journal of Proteomics, 2015. **122**: p. 119-132.

32. Shephard, R.J. and N.J.E.J.o.A.P. Johnson, *Effects of physical activity upon the liver.* 2015. **115**(1): p. 1-46.

33. Gleeson, M., *Interrelationship between Physical Activity and Branched-Chain Amino Acids.* The Journal of Nutrition, 2005. **135**(6): p. 1591S-1595S.

34. Whitmore, S.E. and M.A. Levine, *Risk factors for reduced skin thickness and bone density: Possible clues regarding pathophysiology, prevention, and treatment.* Journal of the American Academy of Dermatology, 1998. **38**(2): p. 248-255.

35. Martens, R.J.H., et al., *Amount and pattern of physical activity and sedentary behavior are associated with kidney function and kidney damage: The Maastricht Study.* PLOS ONE, 2018. **13**(4): p. e0195306.

36. Sanchis-Gomar, F., et al., *Endurance Exercise and the Heart: Friend or Foe?* Sports Medicine, 2016. **46**(4): p. 459-466.

37. Miller, D. and R.K. Crane, *The digestive function of the epithelium of the small intestine: I. An intracellular locus of disaccharide and sugar phosphate ester hydrolysis.* Biochimica et Biophysica Acta, 1961. **52**(2): p. 281-293.

38. Cowan, C.M., et al., *Age-related changes in the biomolecular mechanisms of clvarial osteoblast biology affect fibroblast growth factor-2 signaling and osteogenesis.* Journal of Biological Chemistry, 2003. **278**(34): p. 32005-32013.

39. Gharibi, B., et al., *Site-specific differences in osteoblast phenotype, mechanical loading response and estrogen receptor-related gene expression.* Molecular and Cellular Endocrinology, 2018. **477**: p. 140-147.

40. Ennour-Idrissi, K., E. Maunsell, and C. Diorio, *Effect of physical activity on sex hormones in women: a systematic review and meta-analysis of randomized controlled trials.* Breast Cancer Research, 2015. **17**(1): p. 139.

41. Luzak, A., et al., *Association of physical activity with lung function in lung-healthy German adults: results from the KORA FF4 study.* BMC Pulmonary Medicine, 2017. **17**(1): p. 215.

42. D. Bartlett, J. and J.G. Areson, *Quantitative lung morphology in Japanese waltzing mice.* Journal of Applied Physiology, 1978. **44**(3): p. 446-449.

43. Thompson, D., et al., *Physical Activity and Exercise in the Regulation of Human Adipose Tissue Physiology.* Physiological Reviews, 2012. **92**(1): p. 157-191.

44. Littman, A., A. Kristal, and E. White, *Effects of physical activity intensity, frequency, and activity type on 10-y weight change in middle-aged men and women.* International journal of obesity, 2005. **29**(5): p. 524.

45. Mauriege, P., et al., *Regional differences in adipose tissue metabolism between sedentary and endurance-trained women.* American Journal of Physiology-Endocrinology And Metabolism, 1997. **273**(3): p. E497.

46. Spalding, K.L., et al., *Dynamics of fat cell turnover in humans.* Nature, 2008. **453**(7196): p. 783.

47. Björntorp, P., et al., *Effect of an energy-reduced dietary regimen in relation to adipose tissue cellularity in obese women.* The American journal of clinical nutrition, 1975. **28**(5): p. 445-452.

48. MacDonald, A.J., et al., *A novel oral tracer procedure for measurement of habitual myofibrillar protein synthesis.* Rapid Communications in Mass Spectrometry, 2013. **27**(15): p. 1769-1777.

49. Brook, M.S., et al., *Recent developments in deuterium oxide tracer approaches to measure rates of substrate turnover: implications for protein, lipid, and nucleic acid research.* Current Opinion in Clinical Nutrition & Metabolic Care, 2017. **20**(5): p. 375-381.

50. Dufner, D.A., et al., *Using 2H2O to study the influence of feeding on protein synthesis: effect of isotope equilibration in vivo vs. in cell culture.* Am J Physiol Endocrinol Metab, 2005. **288**(6): p. E1277-83.

51. Busch, R., et al., *Measurement of protein turnover rates by heavy water labeling of nonessential amino acids.* Biochim Biophys Acta, 2006. **1760**(5): p. 730-44.

52. Previs, S.F., et al., *Quantifying rates of protein synthesis in humans by use of 2H2O: application to patients with end-stage renal disease.* American Journal of Physiology-Endocrinology and Metabolism, 2004. **286**(4): p. E665-E672.

53. Brook, M.S., et al., *A novel D2O tracer method to quantify RNA turnover as a biomarker of de novo ribosomal biogenesis, in vitro, in animal models, and in human skeletal muscle.* American Journal of Physiology-Endocrinology and Metabolism, 2017. **313**(6): p. E681-E689.

54. Waterston, R.H., et al., *Initial sequencing and comparative analysis of the mouse genome.* Nature, 2002. **420**(6915): p. 520-562.

55. Lightfoot, J.T., et al., *Strain screen and haplotype association mapping of wheel running in inbred mouse strains.* J Appl Physiol (1985), 2010. **109**(3): p. 623-34.

56. Lightfoot, J.T., et al., *Genetic influence on daily wheel running activity level.* Physiol Genomics, 2004. **19**(3): p. 270-6.

57. Knab, A.M., et al., *Repeatability of exercise behaviors in mice.* Physiol Behav, 2009. **98**(4): p. 433-40.

58. Dawes, M., et al., *Differential miRNA expression in inherently high- and low-active inbred mice.* Physiological Reports, 2015. **3**(7): p. e12469.

59. Knab, A.M., et al., *Pharmacological manipulation of the dopaminergic system affects wheel-running activity in differentially active mice.* J Biol Regul Homeost Agents, 2012. **26**(1): p. 119-29.

60. Turner, M.J., S.R. Kleeberger, and J.T. Lightfoot, *Influence of genetic background on daily running-wheel activity differs with aging.* Physiol Genomics, 2005. **22**(1): p. 76-85.

61. Hallemeesch, M.M., G.A. Ten Have, and N.E. Deutz, *Metabolic flux measurements across portal drained viscera, liver, kidney and hindquarter in mice.* Lab Anim, 2001. **35**(1): p. 101-10.

62. Benjamini, Y., A.M. Krieger, and D. Yekutieli, *Adaptive linear step-up procedures that control the false discovery rate.* Biometrika, 2006. **93**(3): p. 491-507.

63. Robinson, M.M., et al., *Long-term synthesis rates of skeletal muscle DNA and protein are higher during aerobic training in older humans than in sedentary young subjects but are not altered by protein supplementation.* The FASEB Journal, 2011. **25**(9): p. 3240-3249.

64. Trommelen, J., et al., *Resistance Exercise Augments Postprandial Overnight Muscle Protein Synthesis Rates.* Medicine & Science in Sports & Exercise, 2016. **48**(12): p. 2517-2525.

65. Busch, R., et al., *Measurement of protein turnover rates by heavy water labeling of nonessential amino acids.* Biochimica et Biophysica Acta (BBA) - General Subjects, 2006. **1760**(5): p. 730-744.

66. Neese, R.A., et al., *Measurement in vivo of proliferation rates of slow turnover cells by ²H₂O labeling of the deoxyribose moiety of DNA.* Proceedings of the National Academy of Sciences, 2002. **99**(24): p. 15345-15350.

67. Jones, P.J.H. and S.T. Leatherdale, *Stable isotopes in clinical research: safety reaffirmed.* Clinical Science, 1991. **80**(4): p. 277-280.

68. Daurio, N.A., et al., *Spatial and temporal studies of metabolic activity: contrasting biochemical kinetics in tissues and pathways during fasted and fed states.* American Journal of Physiology-Endocrinology and Metabolism, 2019. **316**(6): p. E1105-E1117.

69. Dufner, D.A., et al., *Using 2H2O to study the influence of feeding on protein synthesis: effect of isotope equilibration in vivo vs. in cell culture.* American Journal of Physiology-Endocrinology and Metabolism, 2005. **288**(6): p. E1277-E1283.

70. Wilkinson, D.J., et al., *A validation of the application of D(2)O stable isotope tracer techniques for monitoring day-to-day changes in muscle protein*

subfraction synthesis in humans. Am J Physiol Endocrinol Metab, 2014. **306**(5): p. E571-79.

71. Strawford, A., et al., *Adipose tissue triglyceride turnover, de novo lipogenesis, and cell proliferation in humans measured with 2H2O.* Am J Physiol Endocrinol Metab, 2004. **286**(4): p. E577-88.

72. Previs, S.F., et al., *Using [2H] water to quantify the contribution of de novo palmitate synthesis in plasma: enabling back-to-back studies.* American Journal of Physiology-Endocrinology and Metabolism, 2018. **315**(1): p. E63-E71.