UNIVERSITY OF CALIFORNIA SAN DIEGO

Time Restricted Feeding on Age-related Physiological Decline and Skeletal Muscle Function

A Thesis submitted in partial satisfaction of the requirements

for the degree Master of Science

in

Biology

by

Raghav Bhardwaj

Committee in charge:

Professor Satchidananda Panda, Chair

Professor Randolph Hampton, Co-Chair

Professor Chris Jon Armour

Professor Amir Zarrinpar

2020

The Thesis of Raghav Bhardwaj is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

_____

_____

Co-chair

Chair

University of California San Diego

2020

DEDICATION

For my parents

EPIGRAPH

You are what you do repeatedly, excellence therefore, is not an act but a habit

Aristotle

TABLE OF CONTENT

LIST OF FIGURES

LIST OF TABLES

LIST OF ILLUSTRATIONS

ABBREVIATIONS

TRF – Time Restricted Feeding

ALF – Ad libitum Feeding

HFD – High Fat Diet

CCG – Clock Controlled Genes

SCN – Suprachiasmatic Nucleus

TTFL – Transcription Translation Feedback Loop

CTS – Circadian Timing System

CVD – Cardiovascular Disease

BAT – Brown Adipose Tissue

WAT – White Adipose Tissue

IMTG – Intramyocellular Triglycerides

NAD – Nicotinamide Adenine Dinucleotide

ACKNOWLEDGEMENTS

I would like to acknowledge the guidance and support I received from Dr. Amandine Chaix, without whom this thesis would not be possible. Her wisdom and advice at every step of this project helped me in an immeasurable fashion. Her constant faith in me proved to be the catalyst in my evolution as a scientist. For a mentor like her, I will forever be grateful.

I would like to acknowledge Professor Satchidananda Panda for his support as the chair of my committee. I'd like to thank him for giving me the opportunity to work in his lab and for providing me with all the resources to conduct my research. His guidance has proved to be invaluable for this project and for my personal growth.

I want to thank Professor Randolph Hampton for his support as the co-chair of my committee and for teaching me everything I know about muscle physiology

I would like to thank Dr. Shaunak Deota for dedicating his valuable time to teaching me technical skills which allowed me produce meaningful data.

I'd like to acknowledge the work of all members of Panda Lab in making this project a success. I thank them for providing a supportive environment throughout my time in lab.

I'm extremely grateful for my parents who have always lead by example and are phenomenal role models. I want to thank them for always supporting me in my endeavors and for inspiring me to become the best I can be.

ABSTRACT OF THE THESIS

Time Restricted Feeding on Age-related Physiological Decline and Skeletal Muscle Function

by

Raghav Bhardwaj

Master of Science in Biology

University of California San Diego, 2020

Professor Satchidananda Panda, Chair

Professor Randolph Hampton, Co-Chair

Aging is a natural part of an organism's life cycle. It is, however, associated with increased risks for many chronic diseases including obesity and sarcopenia. Aging is also associated with dampened functions of the circadian clock, an internal timing system that orchestrates physiological function and behavior and displays recurrent daily 24h rhythms. The circadian clock has an intricate relationship with metabolic regulators and plays a key role in the daily partitioning of energy producing/consuming processes for the efficient functioning of metabolism. Time-

restricted feeding (TRF) – a feeding regime that restricts caloric intake to an 8-9 hour feeding window in the active phase of an organism – is conceptualized as a method of synchronizing feeding-fasting cycles with endogenous circadian clock. TRF is a particularly innovative dieting strategy because it does not require alterations in caloric quantity/quality, thus rendering TRF an easy to adopt and yet highly efficacious intervention. The ability of TRF to holistically improve numerous health parameters in young mice makes it an attractive candidate for the prevention (or delay) of sarcopenia and age-related metabolic decline. Here we showed that TRF prevents body weight gain, reduces fat mass, preserves lean mass, bolsters glucose regulation, improves endurance and strength, and enhances muscle clock gene expression, all without caloric restriction and irrespective of age in male C57BL/6J mice fed a western diet. Thus, TRF can delay age-related physiological decline in middle-aged mice and our results set stage for further exploration of the benefits of TRF across lifespan.

INTRODUCTION

Aging

Aging is a natural part of an organism's life cycle. Aging, however, is associated with poor quality of life and increased risk for many diseases including sarcopenia, obesity, non-alcoholic fatty liver disease, diabetes, cardiovascular disease, atherosclerosis, and cancer. With advances in biomedical research in the last few decades, the lifespan of individuals has increased. Life expectancy at birth in the United states, for example, has increased from 47.3 to 78.8 between 1990 and 2015 (CDC/NCHS, 2016). Thus, the number of individuals surviving to experience older ages is increasing. According to a study conducted by the United Nations department of economic and social affairs, the global population aged 60 and over was 962 million in 2017 and is expected to double up to 2.1 billion by 2050. Increments in survival rates and lifespans are desirable outcomes of scientific research. Unfortunately, however, research targeted at adding years to life has created a gap between lifespan and healthspan such that healthspan, or the part of lifespan during which a person is healthy, lags behind lifespan in terms of total increment (Brown et al., 2014). Increasing lifespans but more or less stagnant healthspans leads to increased morbidity, arguably because individuals simply live long enough to be affected by age-related diseases (Hansen and Kennedy, 2016). Therefore, as the percent of elderly population increases, so do incidents of age-related illnesses. This is a concerning public health problem because increased rates of age-related disorders are associated with increased healthcare costs and have direct socioeconomic implications burdening both, the individual and the system (Janssen et al., 2004; Yang and Hall, 2008; Atella et al., 2015). Furthermore, despite the significant progress made in the field of biomedical research, the phenomenon of aging remains elusive. Numerous theories attempting to explain aging have emerged, however, mechanisms of aging are complex and a unified theory of

aging is currently non-existent (Sergiev et al., 2015). Hence, it is imperative that we strive to better understand the underlying causes of age-related physiological decline and increase research targeted at healthspan extension. Finally, with predictions of massive increments in the older population and associated disease burden in the coming decades, the need for research and development of novel approaches for holistically treating age-related disorders is urgent.

Sarcopenia

Sarcopenia or age-related muscle is an age-related disease which is a significant contributor of shorter healthspans, poor quality of life, and socioeconomic burden. In the United States, the healthcare cost of sarcopenia was estimated at $18.5 billion in the year 2000 (Janssen et al., 2004). Sarcopenia is the incremental decline in skeletal muscle strength and mass with age (Santilli et al., 2014). Symptoms of sarcopenia can arise as early as the third decade but are more severe after the seventh decade of life. Skeletal muscle mass decreases at a rate of about 3-8% per decade after the age of 30 and the rate of decrease increases up to 10% per decade after the age of 70 (Volpi et al., 2004; Siparsky et al., 2014; Melton et al., 2000). Interestingly, the rate of muscle strength decline seems to exceed the concomitant rate of muscle mass decline. Skeletal muscle strength decreases at a rate of about 10-15% every decade until the age of 70 and the rate of decrease can accelerate up to 40% per decade afterwards (Goodpaster et al., 2006; Hughes et al., 2001). Altogether, sarcopenia is a muscle wasting disease which can manifest in middle-ages and its severity increases with age.

Sarcopenia is a product of muscle atrophy and muscle dysfunction. Studies show that skeletal muscle undergoes both quantitative and qualitative deteriorations with age (Thompson, 2009; Larson et al., 2019). Sarcopenic loss of muscle mass is caused by a decrease in total number

of muscle fibers along with a decrease in mass of each individual muscle fiber (Thompson, 1994). Sarcopenic loss of muscle strength, although, can occur from loss of muscle mass itself, is better explained through alterations in muscle quality – defined as the force produced per volumetric unit of muscle tissue (Shaffer et al., 2017; Barbat-Artigas et al., 2012). Age-related deteriorations in muscle quality can cause reductions in muscle strength as a function of reduced force production from individual muscle fibers. Interestingly, although age-related reduction in muscle mass can affect the magnitude of changes in muscle strength, deterioration of muscle strength is prevalent despite maintained muscle mass (Keller and Englehardt et al., 2014; Hughes et al., 2001).

Sarcopenia affects both muscle strength and mass, but it is important to understand the severity and consequences of each. In the context of age-related deteriorations, muscle strength appears to be the more clinically relevant parameter. Adverse physical outcomes that are generally associated with sarcopenia are predominantly influenced by muscle strength which is predicted by muscle quality. Loss of muscle strength is associated with an increased likelihood of falls and risk of injuries thus resulting in disabilities and/or functional dependence (Wolfson et al., 1995; Bean et al., 2002; Cruz-Jentoft et al., 2019). Muscle mass or muscle quantity, on the other hand, appears to have relevance for metabolic regulation. Skeletal muscle is one of the largest organs responsible for insulin-stimulated glucose uptake. Thus, muscle mass can exert a significant influence over glucose metabolism, such that reductions in muscle mass may produce states of insulin resistance. Therefore, maintenance of both muscle strength or quality and muscle mass or quantity are highly important consideration for the aging population.

The mechanisms behind sarcopenia are multifactorial and the pathophysiology is not fully understood. Maintenance of skeletal muscle is a complex physiological process that is dependent on a delicate balance between anabolic and catabolic pathways. An important anabolic pathway

that can upregulate muscle protein synthesis is the phosphatidylinositol 3-kinase (PI3K)/serine threonine kinase (Akt) pathway which stimulate the mammalian target of rapamycin (mTOR) and can contribute to retention of muscle mass/function. The ubiquitin-proteasome system – regulated by the activity forkhead box transcription factor O (FoxO) – is a catabolic pathway that has downstream effects on muscle protein degradation and contributes to deteriorations in muscle mass/function. Evidence suggests that aging is associated with a decrease in the anabolism driven pathways and an increase in catabolism driven pathways, thus causing a potential double whammy for muscle maintenance (Ebner et al., 2015). Known causative agent that can contribute towards age-related downregulation of the PI3K/Akt/mTOR pathway and upregulation of FoxO activity include reduced physical inactivity, hormonal changes, nutritional deficiencies etc. (Gomes et al., 2017). Moreover, age-related changes like shifts in muscle composition (loss of type II fibers), adipose infiltration (increased intramyocellular triglyceride stores), neuromuscular decline etc. (Mcgregor et al., 2014, Picca et al., 2018) can have detrimental consequences for muscle quality and function. Overall, several physiological changes that are involved in the pathology of sarcopenia can produce age-related decline in muscle physiology.

Obesity

Obesity also worsens with age and significantly contributes to poor quality of life and socioeconomic burden. The healthcare cost of obesity, in the year 2008, was estimated at $147 billion in the United States (Finkelstien et al., 2009). Obesity is characterized by increments in abdominal fat stores. Aging is associated with a loss of lean mass and a gain of fat mass, which can contribute towards age-related obesity and insulin resistance (Batsis and Villareal, 2018; Cleasby et al., 2016; Zamboni et al., 2008). Accretion of fat mass and adiposity begin to increase

around middle-age and can accelerate until the seventh decade of life (Flegal et al., 2009; Heo et al, 2009). Moreover, aging is associated with a redistribution of fat from subcutaneous to abdominal depots and ectopic sites like the skeletal muscle (Tchkonia et al., 2010; Kuk et al., 2009) which can contribute towards insulin resistance and metabolic syndrome. Thus, the association of obesity with aging is important to recognize since it has consequences for metabolic health and disease.

Additionally, increased BMI in advanced aging worsens adipose tissue dysfunction having detrimental consequences for energy metabolism. For example, increased secretion of pro-inflammatory cytokines like TNFa, IL-6, NO by the obese adipose tissue can promote systemic inflammation and impaired regulation of adipokines secretion which can affect appetite regulation and alter metabolic regulation . Under physiological conditions, adipokines leptin and adiponectin regulate satiety and fat/glucose metabolism respectively. However, under obesogenic conditions, leptin increases to abnormal levels eventually leading to leptin resistance, whereas abnormal decrease in adiponectin levels can be detrimental for energy metabolism and result in type 2 diabetes (Nakamura et al., 2014). Like Sarcopenia, obesity is a multifactorial disease and its causative factors are intertwined with aging. Obesity is often correlated with body weight gain which occurs as function of energy imbalance : when energy intake exceeds energy expenditure. Aging-associated physical inactivity – potentially resulting from post-retirement shifts in lifestyle from active to sedentary – can produce states of chronic positive energy balance which can lead to accumulation of excess fat. Altogether, obesity is a complex disorder which can worsen with aging and certain lifestyles.

Sarcopenia and Obesity

Importantly, sarcopenia and obesity can potentiate each other and function synergistically to produce physiological declension (Santilli et al., 2014). Skeletal muscle is a highly metabolically active organ and it can take up to 40-50% of total body mass (Kinney et al., 2011). Skeletal muscle also regulates glucose homeostasis and is the predominant site of insulin-mediated glucose uptake and glycogen storage (DeFronzo and Tripathy 2009; Jansen et al., 2011). Since muscle is the largest glucose metabolizing/insulin-responsive tissue, sarcopenic reductions in skeletal muscle mass can induce insulin resistance and obesity. On the other hand, older individuals that are resistant to the anabolic functions of insulin have impairments in muscle protein synthesis which can cause muscular atrophy and thus sarcopenia (Rasmussen et al., 2010). Further, age-related obesity and the associated increase in abdominal fat mass induces inflammation which can negatively affect muscle mass and function. Elevated visceral fat is associated with increased secretion of leptin and pro-inflammatory cytokines like IL-6 and TNF-alpha (Tilg and Moschen et al., 2006) along with decreased action of adiponectin induced anti-inflammatory signals. Pro-inflammatory cytokine augmentation can have catabolic consequences for the skeletal muscle and may even promote insulin resistance (Santilli et al., 2014). Studies indicate that a direct link may exist between inflammation and sarcopenia (Cesari et al., 2005; Schaap et al., 2009). Moreover, obesity can also increase deposition of fatty acids into the skeletal muscle in the form of intramyocellular triglycerides (IMTGs). Although IMTGs are harmless if regularly depleted through physical activity and exercise (Goodpaster et al., 2001), accumulation of IMTGs (observed in sarcopenic and overweight individuals) can contribute to muscular dysfunction and insulin resistance (Li et al., 2015). The term sarcopenic obesity – defined as obesity with a loss of muscle mass and function (Baumgartner, 2000) – appropriately characterizes

the intertwined relationship of sarcopenia and obesity. In a nutshell, aging associated sarcopenia and obesity synergistically potentiate each other to cause physical disabilities, cardiometabolic disorders, and mortality (Figure A, Zamboni et al., 2008)

Limitations in Treatments for Sarcopenia and Obesity

Finally, both sarcopenia and obesity are complex disorders and arise from age-related dysfunction of multiple organ systems. Mechanisms of sarcopenia and obesity are multifactorial. Environmental factors such as decreased physical activity and inappropriate nutritional intake, along with, inflammation, insulin resistance, neuromuscular decline, hormonal dysregulation, mitochondrial abnormalities are all potentiators of sarcopenia and obesity (Choi, 2016; Waltson et al., 2012). Thus, pharmacological interventions targeted at specific mechanisms might only be effective at treating a part of these multifaceted problems. In fact, treating singular aspects of such complex metabolic diseases can worsen other symptoms, for example, adiposity is elevated with the thiazolidinedione class of insulin sensitizers (Bray and Ryan, 2014). Also, pharmacological agents can produce unwanted side-effects, for example, hyperglycemia and edema seen with growth hormone therapy (Liu et al., 2007). Hence, to tackle the intrinsic complications that accompany age-related disorders, innovative strategies with multi-dimensional outcomes are needed.

For treatment of obesity and sarcopenia, physical inactivity and nutritional inadequacies have been targets of lifestyle interventions such as exercise and reducing/altering caloric intake. However, physical inactivity in older adults may be a function of fatigue and pain associated with increased chronic disease burden (Egerton et al., 2015; Zhou et al., 2018) and inappropriate nutrition can have physical/physiological and psychosocial basis (Leslie and Hankey, 2015). While

behavioral interventions aimed at increasing physical activity and improving nutrition have been first line therapies for prevention/delay of sarcopenia and obesity, such interventions require constant effort and have remained elusive to a large percentage of individuals (Anderson et al., 2001). Additionally, the effects of timing of food intake and fasting interventions against sarcopenia have remained unclear. Hence, flexible interventions capable of producing a pleiotropic effect on multiple organ systems while improving various age-related physiological parameters without altering physical activity and caloric intake are required.

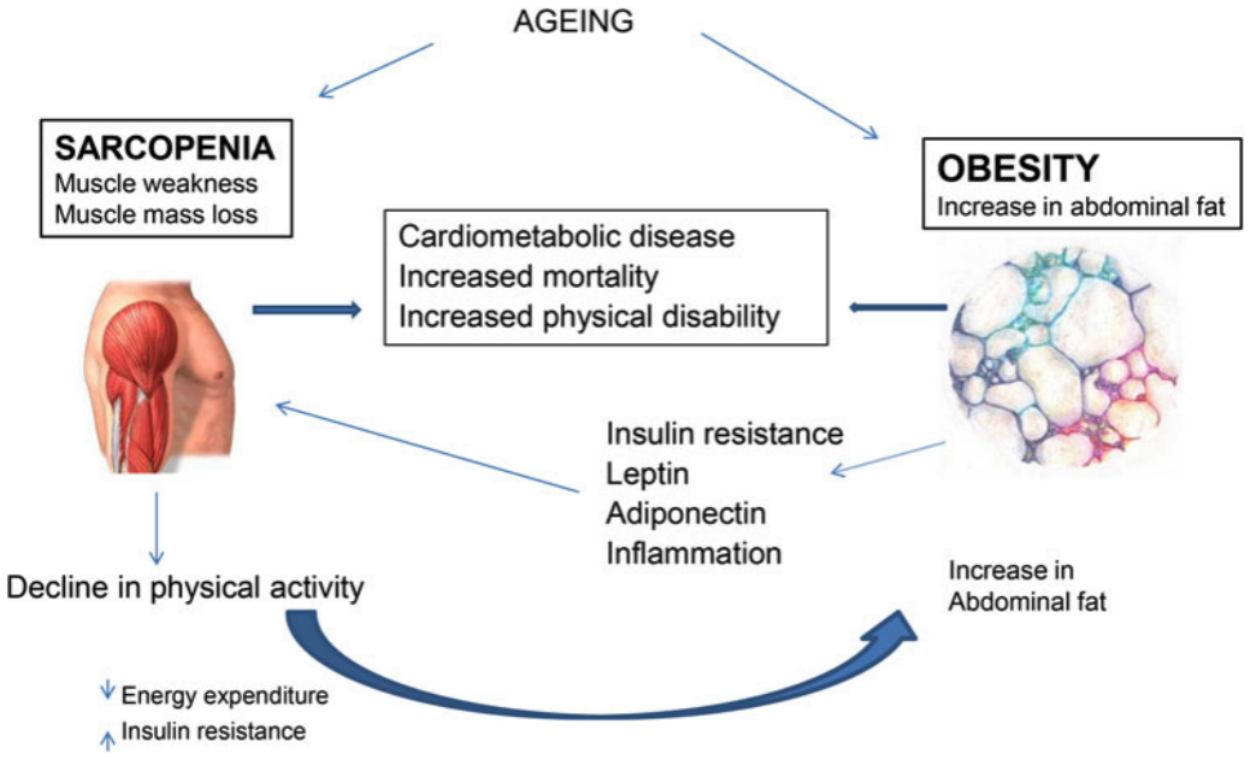

Illustration 1 : **Aging, Sarcopenia, and Obesity**.

Aging-induced sarcopenia/obesity and associated mechanims (Reprint from Zamboni et al., 2008)

Circadian Rhythms

An important aspect of healthspan and longevity are circadian rhythms. Circadian rhythms are biological rhythms with a 24 h period that guide and control physiology and behavior based on environmental light (generated by the rotation of Earth) and other timing cues such as nutrient availability. The circadian system is composed of a master clock in the hypothalamus and peripheral clocks in peripheral tissues and other areas of the brain. The hypothalamic suprachiasmatic nucleus (SCN) serves as the central circadian pacemaker or the master clock of the body. The SCN sits above the optic chiasm and receives light information (relayed through the retinohypothalamic tract) directly from the intrinsically photosensitive retinal ganglion cells in the eyes. Besides its intrinsic ability to produce robust circadian outputs (Welsh et al., 2010), the SCN uses light as a zeitgeber to entrain peripheral clocks in the body, thus coordinating circadian rhythms in a hierarchical fashion.

Peripheral clocks are biological clocks present in nearly all peripheral tissues including skeletal muscle, liver, adipose tissue, etc. These peripheral clocks receive SCN input via autonomic nervous system, hormonal signals, alterations in body temperature, and behavioral modulations etc. (Illustration 2; reprint from Stenvers et al., 2018). Given that the peripheral clocks do not have direct access to light information, they can get entrained by other zeitgebers such as nutrient availability (Shi and Zeng, 2013); this is especially relevant for tissues involved in metabolism such as the liver which can get entrained to metabolic signals produced as function of food intake (Stenvers et al., 2018). Interestingly, it has been shown that metabolic signals can bypass signals from the SCN to entrain peripheral clocks (Shi & Zeng, 2013; Dallmann et al., 2012). This hints at the importance of maintaining set patterns feeding-fasting cycles for sustenance of robust circadian rhythms.

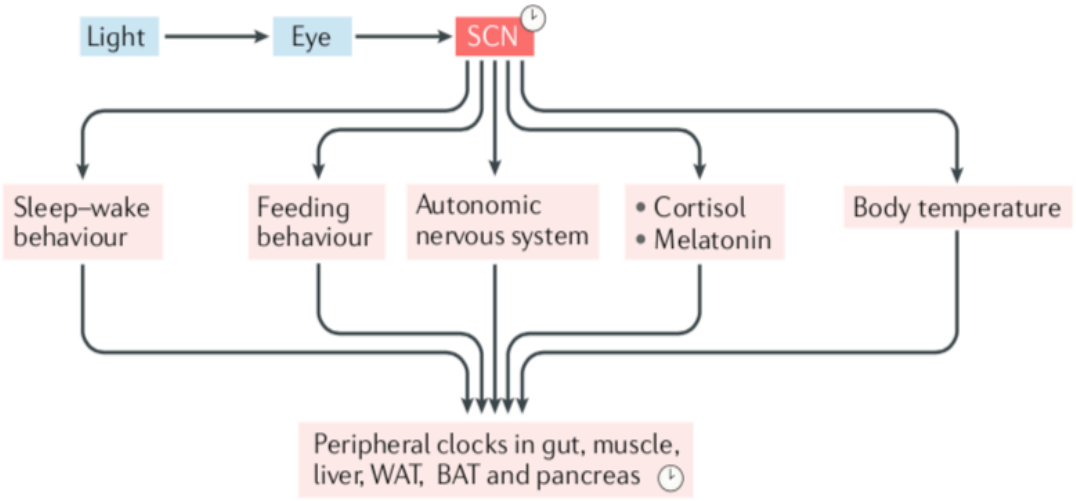

Illustration 2 : **SCN and peripheral clocks**.

The SCN receives light information through eyes and entrains peripheral clocks in muscle, liver, BAT etc. through various signals (Reprint from Stenvers et al., 2018)

Skeletal Muscle Clocks

Skeletal muscles are integral for energy homeostasis and are involved in critical aspects of health such as locomotion, oxygen consumption, substrate turnover and storage. In skeletal muscle, the circadian clock system drives circadian expression of genes important for numerous aspects of muscle biology such as muscle metabolism, muscle performance, and general muscle maintenance. Since the skeletal muscle can constitute 40-50% of body mass, it can be considered one of the largest collections of peripheral clocks in the body (Janssen et al., 2000). Efficient functioning of molecular clocks play a key role in healthy aging since proper functioning of the muscle is important for whole-body metabolism. It is known that the skeletal muscle can regulate energy storage/use depending on feeding-fasting and activity/rest patterns, however, it has recently been shown that muscle metabolism is further regulated by endogenous skeletal muscle clocks (Hodge et al., 2015). In fact, molecular clocks in the muscle can be modulated by the presence of

molecules that play a role in the metabolic sensing machinery. These metabolic sensors can be influenced by nutrient presence, contractile activity, energy balance, etc. (Lira et al., 2010; Muoio and Koves et al., 2007; Witczak et al., 2008) and display a diurnal pattern in activity levels indicating that muscle cell metabolism and circadian rhythms might be mutually dependent (Asher et al., 2008; Lamia et al., 2009; Liu et al., 2007). Interestingly, it has been proposed that interactions between circadian and metabolic oscillators can be modulated via the actions of adenosine monophosphate-activated protein kinase (AMPK), peroxisome proliferator-activator receptor gamma coactivator-1 alpha/beta (Pgc1a/b), and Sirtuins (Sirt1) (Lefta et al., 2015).

Disruption of clocks in the skeletal muscle can have detrimental consequences for numerous parameters of metabolism and physical performance. Muscle-specific knockdown of Bmal1, a core clock component, can reduce expression levels of GLUT4 and can negatively impact insulin-stimulated glucose uptake (Dyar et al., 2013). Moreover, muscle-specific Bmal1 knockout (KO) mice result in downregulation of glucose catabolism enzymes like pyruvate dehydrogenase and hexokinase 2 (Hodge et al., 2015). The metabolic dysfunction that results from muscle clock disruption can cause impairments in physical performance (Gabriel and Zierath, 2019). Additionally, global Bmal1 KO mice display pathological changes in the skeletal muscle such that they have reduced force generation capacity, mitochondrial abnormalities, as well as myofilament and sarcomere disorganization (Andrews et al., 2010). Moreover, global Bmal1 KO mice displayed hallmarks of premature aging including skeletal muscle atrophy, arthropathy, age-related body and organ reductions (Kondratov et al., 2006; Rudic et al., 2004). These studies highlight the importance of skeletal muscle clocks for maintenance of skeletal muscle metabolism and functionality along with their relevance for aging. They also point at the importance of

understanding molecular mechanisms governing muscle clocks and that of recognizing differences in clock function in health and disease.

Molecular Clock Mechanisms

Efficient functioning of the circadian timing system is crucial for maintaining health and vitality since it regulates important aspects of physiological and behavioral wellbeing including sleep, cognition, metabolism, digestion, etc. The proper functioning of the aforementioned parameters becomes an even more important consideration with advancing age. Understanding how circadian clocks change with age and uncovering the molecular mechanisms underlying circadian rhythms is of great clinical relevance given that disruptions in these rhythms are associated with a plethora of chronic diseases such as obesity, diabetes, sleep disorders, depression, anxiety, etc. Central to the molecular mechanisms of circadian biology is the cyclic but tissue-specific expression of clock genes regulated through a network of transcriptional-translational feedback loops (TTFL). TTFL networks play a key role in allowing the cell-autonomous circadian oscillator machinery to produce 24 hour rhythmic patterns of expression of clock controlled genes (CCGs) which can be described as genes whose protein products are needed to generate and regulate circadian rhythms at the cellular level and thus at the organismal level (Lowery and Takahashi, 2004).

The TTFL (Figure C; Chaix et al. 2019) is a network of positive and negative feedback loops that operate around the clock, utilizing internal timing cues from the SCN/peripheral clocks and numerous external zeitgebers. The primary feedback loop involves transcription and translation of BMAL1 (ARNTL) and CLOCK, which produce proteins that serve as transcription activators. BMAL1 and CLOCK heterodimers bind to E-box motifs of PER1, PER2, PER3, and

CRY1, CRY2 thus initiating PER/CRY transcription. PER and CRY heterodimerize in the cytoplasm and translocate back into the nucleus to act as repressors of BMAL1/CLOCK to inhibit transcriptional activation, thereby inhibiting their own expression and that of other CCGs. As the PER/CRY repressor proteins build up over a period of time, they start getting degraded via ubiquitin-dependent pathways. This relieves the repression on BMAL1/CLOCK allowing them to activate PER/CRY once again, completing the cycle with 24 hour rhythmicity (Takahashi, 2015). Other axillary regulatory loops involve the competitive binding of nuclear receptors such as ROR (α, β) and REV-ERB (α, β, γ) to the promoter region of BMAL1. This transcriptional activation of Bmal1 by ROR and repression by REV-ERB allows additional fine-tuning of the period and amplitude of the integration with other cellular signaling pathways. Ultimately, BMAL1 and CLOCK regulate the expression of other clock controlled genes (CCGs) which produce clock output and manifest rhythmic biological processes (Partch et al., 2014).

Overall, while the TTFL networks are highly intricate, they provide important insights regarding the mechanisms of circadian rhythms-related complications. Studying these mechanisms and uncovering the underlying biology, like the TTFLs, can prove to be very beneficial in understanding the effects of circadian interventions.

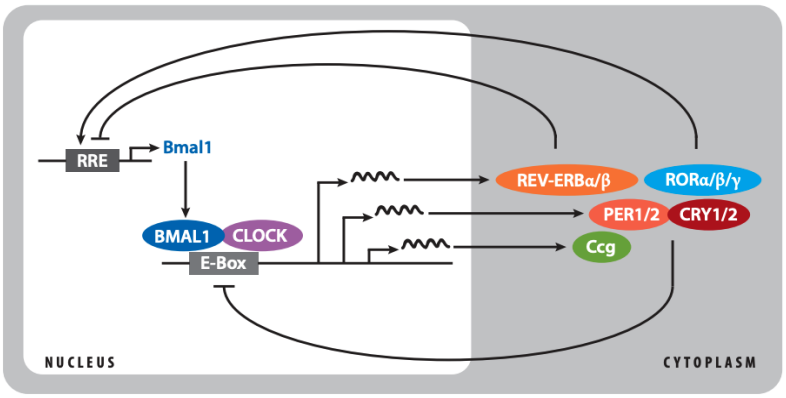

Illustration 3 : **Representation of TTFL.**

Visual representation of the transcriptional-translational feedback loops (Chaix et al., 2019)

Ageing and Circadian Rhythms

Besides numerous age-related disorders, aging is associated with alterations in the normal functioning of the circadian clock. Physiological rhythmic outputs of the circadian timing system (CTS) such as temperature, sleep and hormonal rhythms dampen with age in both animals and humans (Nakamura et al., 2011; Hood and Amir, 2017). Critically, this dampening may be not only a result of aging but also a contributor to mortality. Landmark experiments showed that circadian disruption in hamsters decreased lifespan while fetal SCN transplants in old hamsters increased lifespan (Hurd and Ralph, 1998). Another key evidence supporting the role of the circadian clock in aging came from the finding that Bmal1 KO mouse model show a dramatic reductions in lifespan and display pathologic alterations similar to age-related dysfunctions including sarcopenia, cataracts, less subcutaneous fat and organ shrinkage (Kondratoy et al., 2006).

The molecular underpinnings of circadian clock aging are still unresolved. In particular, it is important to consider that different mechanisms might be at play in the central versus peripheral clock. For example, in the SCN, aging does not seem to be associated with dampened oscillations of clock components (Nakamura et al., 2011). The SCN consists of a network of individual cell-autonomous circadian oscillators. Inter-neuronal coupling is required for phase-coherence and the generation of coherent and robust rhythms that controls downstream circadian rhythmic behavior. During aging, the efficiency of SCN neurons coupling deteriorates leading to desynchrony in the network and dampening in output signals (Farajnia et al., 2012). In tissue stem cells and in liver, aging is associated with a different set of diurnal transcripts despite similar profile of core clock genes oscillations (Sato et al., 2017; Solanas et al., 2017). It is unknown how other tissues' clocks respond to aging and how this response is affected by different feeding regimen. Overall, while

we do not completely understand the mechanisms, it seems that aging is associated with dampening of the activity of the central clock.

Circadian Rhythms and Metabolism

Apart from regulating activity : sleep rhythms, the circadian clock is intricately connected to metabolic rhythms (Asher and Schibler, 2011; Eckel-Mahan and Sassone-Corsi, 2013; Bass, 2012). Thus, age-related disruption of circadian rhythms can have immense metabolic consequences. Studies using mouse models with clock gene mutations and the consequential metabolic dysfunction have provided clear evidence regarding the relationship between metabolism and circadian rhythms (Sahar and Sassone-Corsi, 2012). One way the clock influences metabolism is through transcriptional control of metabolic genes. For example, genes that regulate lipid and glucose metabolism as well as oxidative phosphorylation demonstrate 24 oscillations in expression (Panda et al., 2002). Some metabolic genes that are under clock control also play a key role in ageing and longevity. For instance, CLOCK/BMAL1 cyclically regulate the expression of the gene nicotinamide phosphorybosil transferase (NAMPT) – a rate limiting enzyme in conversion of nicotinamide and 5′-phosphoribosyl-pyrophosphate to nicotinamide mononucleotide (NMN) in the NAD^+-salvage pathway (Ramsey et al., 2009). Apart from its metabolic rate, NAD^+ is a cofactor of NAD-dependent protein deacetylase such as sirtuins. (Asher and Sassone-Corsi, 2015). Sirtuin 1 (SIRT1) has been implicated in ageing, longevity, inflammation, cellular metabolism and SIRT1 mutations in many species results in shorter lifespans (Kim et al., 2019; Guarente, 2011; Wang et al., 2016). Sirtuin3 (SIRT3), which is a mitochondrial NAD^+-dependent deacetylase, plays a role in entrainment of peripheral clocks to feeding patterns (Asher et al., 2010) by exerting circadian influence over mitochondrial function

(Peek et al., 2013). SIRT6 play a role in obesity and inflammation since it controls lipid metabolism (Kuang et al., 2018). Additionally, important regulators of nutrient homeostasis like cAMP response element-binding protein (CREB) and AMP-activated protein kinase (AMPK) that are coupled with clock proteins are driven by feeding-fasting patterns (Zarrinpar et al., 2016). Therefore, the timing of food can have important consequences for the metabolism at molecular and physiological levels.

The diurnal regulation of metabolism is a highly important consideration in the context of circadian rhythms and ageing. Metabolic dysregulation is linked with numerous age-related diseases including type 2 diabetes, dyslipidemia, obesity etc. Metabolism is synchronized by the interaction between feeding-fasting routines and endogenous circadian clocks. This results in a metabolism that is diurnally regulated by the aforementioned metabolite concentrations, the microbiome, and hormones of the endocrine system (Manoogian and Panda, 2017). Insulin is arguably the most important metabolic hormone under circadian regulation. The temporal regulation of insulin and glucagon is modulated by patterns of feeding and fasting (external zeitgeber) along with circadian rhythms (internal zeitgeber). Intrinsically, insulin levels peak around 1700 h and trough around 0400h (Goel et al., 2009). However, feeding patterns are strong signals that can override endogenous circadian cues to accommodate for the altered blood nutrient levels (Manoogian and Panda, 2017) thus making the system more adaptable. Although this adaptability allows an organism to better adjust to environmental conditions, if external cues constantly contradict intrinsic clocks, the ensuing circadian disruption can be physiologically detrimental. Conversely, if such powerful external zeitgebers like feeding-fasting patterns are synchronized with internal circadian clocks, the consequential robust circadian rhythms have the potential to restore metabolic vitality. Prompting a synchronous relationship between feeding-

fasting patterns and internal biological clocks can therefore serve as therapeutic agents to combat the progression and potentially even reverse the pathology of metabolic diseases.

For long, the importance of cyclical expression of metabolic regulators for an efficiently functioning metabolism has been theorized. Many studies in murine models have shown the implications of feeding-fasting patterns for circadian rhythms and metabolism (Kobayashi et al., 2004; Fonken et al., 2010; Asher et al., 2010). Mouse models with genetic modifications have also proved useful in highlighting the correlation between altered feeding schedules and metabolic syndrome. Turek et al., showed that clock mutant mice have alterations in their diurnal feeding pattern, become obese and develop the pathology numerous metabolic disorders (Turek et al., 2005). Adipocyte-specific BMAL1 knockout mouse models also demonstrate alterations in the diurnal rhythms of food intake along with increased body weight. Compared to wildtype (WT), mice with disrupted adipocyte clocks also had lower circulating concentrations polyunsaturated fatty acids (PUFA) in hypothalamic neurons, which consequently led to alterations in feeding-fasting rhythms (Paschos et al., 2012). Moreover, it has been shown through gene expression studies, metabolomics profiling, and assays targeting various metabolic regulators, that a maintained daily pattern of feeding-fasting can be a determining factor in the diurnal regulation of metabolism (Barclay et al., 2012; Adamovich et al., 2014; Vollmers et al., 2009; Eckel-Mahan et al., 2012;) These studies highlight the importance of adjusting feeding-fasting cycles to be in tune with circadian rhythms for an efficient metabolism. Hence, through vast research on the intricately intertwined relationship between circadian rhythms, metabolism, and feeding-fasting patterns, the idea of time-restricted feeding (TRF) was born.

Time-Restricted Feeding

TRF is conceptualized as a method of synchronizing feeding-fasting cycles with endogenous circadian clock outputs with the rationale of restricting feeding events to a window wherein the body is best prepared (based on circadian clock function) to process food. Given what is known about circadian rhythms, metabolism, and feeding-fasting patterns, TRF has the potential to optimize metabolism and to generate robust circadian rhythms thus improving and even enhancing health. TRF is a particularly interesting intervention since it does not require alterations in caloric quantity and/or quality. It has been shown that TRF is capable of preventing metabolic disease in mice fed a high-fat diet without reducing caloric intake (Hatori et al., 2012). Unlike their ad libitum fed counterparts, mice on TRF were protected from the detrimental effects of a high-fat diet. TRF mice had improved rhythms in nutrient homeostasis and energy expenditure. TRF reduced adiposity, serum cholesterol, and liver steatosis while improving glucose tolerance and bile acid production. Further, efficacy of TRF against preexisting obesity, diverse nutritional challenges, and different durations of the feeding window have also been assessed (Chaix et al., 2014). TRF proved to be an outstanding intervention as it was able to stabilize and even reverse metabolic disease progression in mice with pre-existing obesity and type II diabetes. TRF was effective in preventing excessive body weight gain and in improving body composition against a variety of obesogenic diets. TRF works remarkably well irrespective of time schedule, however, benefits of TRF seem to be proportional to the duration of fasting window. TRF was also effective at reducing whole-body fat accumulation as well as fat-associated inflammation. Metabolic benefits of TRF can also have significant implications for skeletal muscle function. A recently conducted study demonstrated that subjecting drosophila to obesogenic challenges including circadian rhythm disruption produces metabolic disease phenotypes in skeletal muscle (Villanueva

et al., 2019). 12 h TRF regimen improved muscle performance through a suppression of intramuscular fat infiltration, markers of insulin resistance, mitochondrial abnormalities, regulation of phospho-AKT levels. Moreover, TRF has been shown to protect and preserve lean mass and is associated with enhanced motor coordination and increased endurance (Chaix et al., 2014; Hatori et al., 2012). Altogether, TRF has wide-spread implications for metabolic regulation, muscle function, circadian rhythms and physiology. It has been denoted as an efficacious non-pharmacological intervention against obesity and associated diseases.

Based on what is currently known about the time-restricted feeding regimens ability to holistically impact multiple organ systems thus improving numerous health parameters, it is an attractive candidate for treatment of sarcopenia and age-related obesity. Additionally, the effects of TRF on middle age mice are unknown, thus warranting investigation regarding TRF's ability to sustain benefits across lifespan. Therefore, the broad question this thesis addresses is whether TRF can be used as an intervention to prevent/delay age-related physiological decline. More specifically, we assessed (i) effects of TRF on body weight (ii) TRF on body composition, (iii) TRF on glucose tolerance, (iv) TRF on strength, endurance, and coordination, (v) TRF on muscle tissue physiology, (vi) TRF on muscle metabolism gene expression, and (vi) TRF on muscle clock gene expression. In lieu of the multifactorial complications that arise with aging, it is important to characterize the effects of TRF for the middle aged population. Symptoms of sarcopenia start arising around middle-ages and preventative measures might be more effective before symptoms worsen. Therefore, it is essential to understand the effects of TRF on body composition and muscle function in middle-aged mice. Additionally, it is important to validate the efficacy/limitations of TRF in the middle-aged mice models before proceeding to older mice models. Addressing these

questions will offer novel insights regarding the role of eating patterns in age-related physiological decline.

This thesis study evaluated the efficacy of time-restricted feeding on numerous parameters age-related physiological decline and skeletal muscle function. We investigated weather TRF could serve as a therapeutic intervention against sarcopenia, obesity, and circadian dysfunction (illustration 4). We evaluated differences in whole body physiology, physical performance, skeletal muscle physiology and gene expression. The results of this study suggested that TRF can serve as a powerful intervention to attenuate age-related physiological decline. The pleiotropic effects of TRF showed improvements in age-affected body weight, body composition, and glucose regulation indicating that TRF is capable of preventing age- and HFD-induced obesity simultaneously. TRF also improved endurance performance and certain parameters of muscle strength in old mice, thus pointing at the therapeutic potency of TRF against sarcopenia. Although the "old" animals used for experiments were really middle-age and not already sarcopenic, the TRF-mediated improvements in muscle retention and function imply that TRF holds potential to delay if not prevent sarcopenia. Finally, improvements in the rhythmicity of muscle clock gene expression suggests that TRF could prevent age-related deteriorations in circadian rhythms. Altogether, TRF holds promise to prevent obesity and delay sarcopenia while curbing age-related circadian deteriorations, thereby serving as a powerful intervention for reinstating health and vitality.

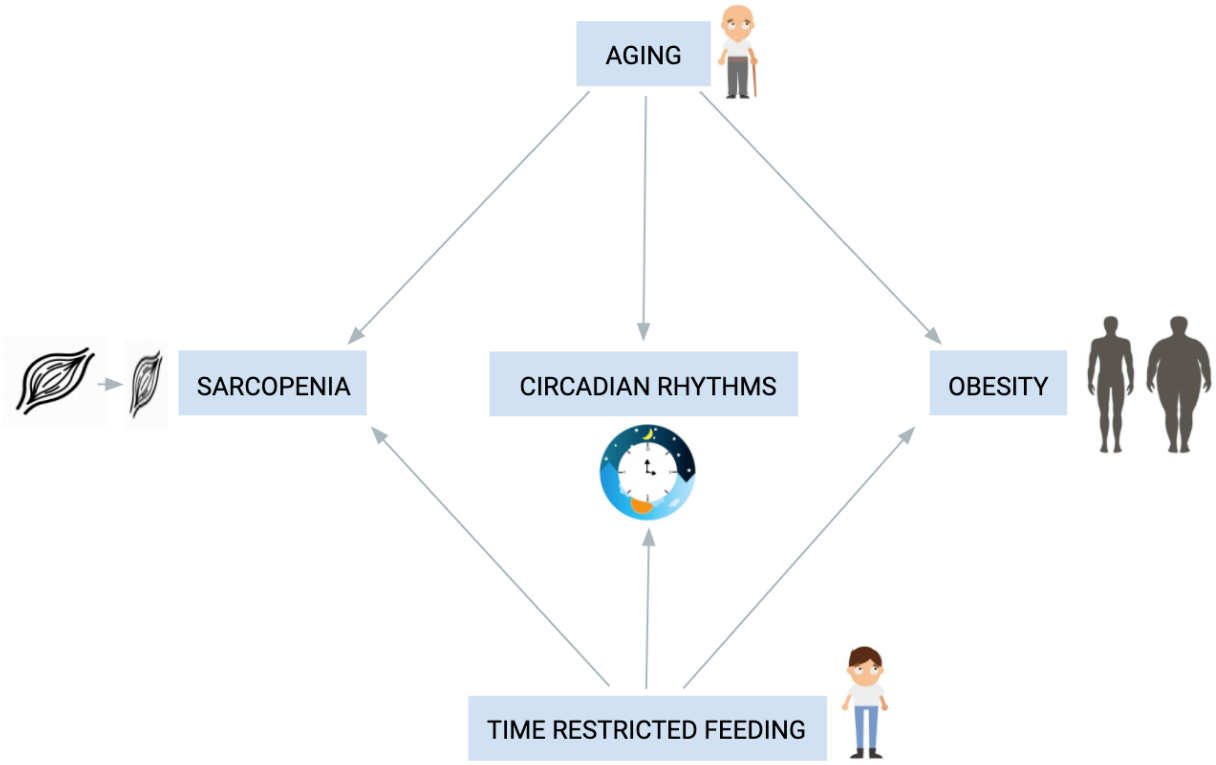

Illustration 4 : **Representation of thesis question**

. This thesis study evaluated the efficacy of time-restricted feeding on numerous parameters age-related physiological decline and skeletal muscle function. Particularly, we assessed whether TRF could serve as a therapeutic intervention against sarcopenia, obesity, and circadian dysfunction.

RESULTS

TRF Prevents BW Gain on Western Diet Without Caloric Reduction Irrespective of Age

Previous studies in young mice have shown that TRF is a preventive and therapeutic intervention to combat excessive body weight gain and associated-metabolic diseases upon various nutritional challenges including a high fat diet (60% fat) and high fructose diet (32% fat, 25% fructose) (Hatori et al., 2012; Chaix et al., 2014). We set out to test the therapeutic effects of TRF against age-related physiological decline. Importantly, we analyzed, in parallel, the benefits of short–term TRF in both young and old mice on a high-fat-high-sucrose diet or "western diet" (4.7 kcal/g – 45% fat, 20% protein, 35% carbohydrates of which 17% was sucrose). We adopted this diet for our experiments because its nutritional content is a close representation of a typical American diet (hence the name "western diet") and because the effects of this diet have not been previously characterized in young mice. We subjected 12-week-old (young) and 1-year-old (old) male wild-type C57BL/6J mice to either Ad libitum Feeding (ALF) or Time-Restricted Feeding (TRF). ALF mice were given 24 h access to food and TRF mice had food access restricted to a 9h window (ZT13 – ZT22, wherein ZT0 denotes light on). Specifically, we had 4 groups including 1. young male mice on ALF (**young mALF**), 2. young male mice on TRF (**young mTRF**), 3. old male mice on ALF (**old mALF**), and 4. old male mice on TRF (**old mTRF**). Animals were maintained on the feeding regimens for 10-14 weeks and experiments were conducted between weeks 9 through 12. Specifics of study design and timeline including the length of experiment and the specific weeks during which different assays were performed can be found in Figure 1. For numerical values associated with body weight and food consumption refer to Tables 1 and 2.

Young mice that were fed the western diet on a 9h time-restricted feeding window (young mTRF) had a lower total body weight at the end of 10 weeks as compared to young mice fed the western diet ad libitum (young mALF)(33.12 ± 4.27g for young mTRF Vs. 38.21 ± 3.87g for young mALF; mean ± SD; Figure 2A). Also, young mTRF only gained half as much body weight (relative to baseline) over the 10 week period compared to their ALF counterparts (14% increase in young mTRF Vs. 32% in young mALF). Moreover, the young mTRF mice consumed an almost identical amount of calories over each week of the study as the young mALF mice (Figure 2C; for numerical values refer to Table 2). *Altogether, young mice on TRF had a lower total body weight and lesser percent increase in body weight than young mice on ALF despite isocaloric food intake between the two groups. These results are in agreement with previous studies and indicate that body weight-associated benefits of TRF are sustained against a western diet.*

Old mice that were fed the western diet on a 9h time-restricted feeding window (old mTRF) had a lower total body weight at the end of 14 weeks as compared to old mice fed the western diet ad libitum (old mALF)(41.40 ± 0.96g for old mTRF Vs. 49.71 ± 0.98g for old mALF; mean ± SEM; Figure 2B). Old mice on TRF gained a significantly lower amount of body weight (relative to baseline) over the 14 week period compared to their ALF counterparts (6% increase in old mTRF Vs. 29% in old mALF). Furthermore, the old mTRF mice consumed an almost identical amount of calories over each week of the study as the old mALF mice (Figure 2D; for numerical values refer to Table 2). *Overall, old mice on TRF had a lower total body weight and a lower percent increase in body weight than old mice on ALF despite isocaloric food intake between the two groups. These novel results indicate that TRF prevents excessive body weight gain without caloric reduction in old mice on western diet suggesting that benefits of TRF are potentially sustained across lifespan.*

Interestingly, when comparing the effect of TRF in young and old animals, TRF-mediated weight gain protection is greater in old mice. Indeed, the percent increase in body weight (relative to baseline) was lower in old mALF compared to young mice (6% increase in old mTRF Vs. 14% in young mTRF) despite isocaloric consumption., potentially because of the age-related increases in body weight. Thus TRF seems more efficacious at preventing excessive body weight gain in old mice. Ad libitum feeding of the western diet was equally detrimental for both young and old mice. Both young and old mice on ALF had a high and comparable percent increments in body weight (relative to baseline) (29% increase in old mALF Vs. 32% in young mALF). Mice in all 4 groups consumed roughly the same amount of calories during the duration of the study (Table 2). Note that, at the beginning of the study, old mice weighed more than young mice. Old mice on both ALF and TRF started at a much higher body weight than young mice on ALF and TRF (38.58 ± 0.83g for old mALF Vs. 28.99 ± 1.72g for young mALF; 38.92 ± 0.70g for old mTRF Vs. 29.17 ± 2.56g for young mTRF; mean ± SEM; refer to Table 1 for numerical values). This is a normal observation and was expected as result of age-related increments in body weight.

In summary, TRF successfully prevents excessive body weight gain upon consumption of western diet for both young and old mice. Interestingly, TRF seems even more efficacious at attenuating age-associated body weight increments in old mice.

TRF Reduces Fat Mass and Preserves Lean Mass Regardless of Age

Aging is also associated with several body composition changes such as increase in body weight (Stenholm et al., 2015) and total body fat (Guo et al., 1999; Coin et al., 2008;) along with reduction in lean mass (Jackson et al., 2012) and sarcopenia (Goodpaster et al., 2006). Since TRF has proven beneficial in reducing whole body fat accumulation and preserving lean mass in young

mice (Hatori et al., 2012; Chaix et al., 2014), we set out to determine if TRF could have similar body composition benefits in old mice. Whole-body composition of young and old mice on ALF and TRF was determined in live mice with a small animal MRI machine (body composition analyzer) using NMR relaxometry (see methods) at week 8. Numerical values associated with body composition can be found in Table 3.

Young mTRF had improved body composition parameters compared to young mALF. Young mTRF had a mean lean mass of 25.41 ± 2.39g (mean ± SD) and a mean fat mass of 5.46 ± 2.83g (mean ± SD; Figure 3A; Table 3). Young mALF had a mean lean mass of 24.17 ± 1.36g (mean ± SD), and a mean fat mass of 10.87 ± 4.29g (mean ± SD; Figure 3A; Table 3). In comparison, young mTRF had slightly higher lean mass and significantly lower fat mass than young mALF. Moreover, differences between TRF and ALF became more apparent when parameters of body composition were plotted as a percentage of total body weight. Lean mass as a percentage of total body weight was significantly higher in young mTRF compared to young mALF (74% in young mTRF Vs. 62% in young mALF; *$p < 0.05$; Figure 3D). Fat mass as a percentage of total body weight, on the other hand, was significantly lower in young mTRF compared to young mALF (16% for young mTRF Vs. 28% for young mALF; ***$p < 0.001$; Figure 3C). *These results are congruent with previous studies and indicate that TRF is effective at reducing fat mass and preserving lean mass in young mice.*

Old mice on TRF also had highly significant improvements in body composition parameters compared to old mice on ALF. Old mTRF had a mean lean mass of 28.47 ± 2.40g (mean ± SD) and a mean fat mass of 9.48 ± 5.07g (mean ± SD; Figure 3B; Table 3). Old mALF had a mean lean mass of 26.61 ± 1.44g (mean ± SD) and a mean fat mass of 20.36 ± 1.55g (mean

± SD; Figure 3A; Table 3). In comparison, old mTRF had a higher lean mass and a significantly lower fat mass compared to old mALF. Furthermore, as with young mice, differences in body composition between old mice on TRF and ALF became more apparent when parameters were plotted as a percentage of total body weight. Lean mass as a percentage of total body weight was significantly higher in old mTRF compared to old mALF (70% for old mTRF Vs. 52% for old mALF; ***$p < 0.001$; Figure 3F). Fat mass as a percentage of total body weight, on the other hand, was significantly lower in old mTRF compared to old mALF (22% in old mTRF Vs. 40% in old mALF; ***$p < 0.001$; Figure 3E). *These novel results indicate that TRF is highly effective at reducing fat mass and preserving lean mass in old mice sugesting that TRF-mediated body composition improvements might be maintained over lifespan.*

Body composition differences between ALF and TRF are prominent in both young and old mice. In juxtaposition, both young and old mice retain benefits of TRF in a comparable fashion. Lean mass and fat mass as a percentage of body weight were comparable between old mTRF and young mTRF (70% for old mTRF Vs. 74% for young mTRF; 22% for old mTRF Vs. 16% for young mTRF). Additionally both young and old animals in the ad libitum feeding groups experienced the western diet-associated detriments in body composition. In comparison, old animals on ALF were worse off than young animals on ALF. Lean mass as a percent of body weight was lower in old mALF compared to young mALF (52% for old mALF Vs. 62% for young mALF). Fat mass as a percent of body weight was significantly higher in the old mALF as compared to the young mALF (40% for old mALFVs. 28% for young mALF).

Altogether, this demonstrates that TRF is effective at preventing excessive body weight gain, reducing fat mass, and preserving lean mass in young and old mice hinting at the therapeutic capabilities of TRF against age-related diseases like sarcopenia and obesity.

TRF Bolsters Glucose Regulation Irrespective of Age

Obesity is associated with the development of glucose intolerance and insulin resistance (Kahn and Flier, 2000). Since TRF can prevent excessive body weight gain in young and old mice on western diet, we set out to test the protective effects of TRF against sarcopenia- and obesity-associated insulin resistance. To achieve this, we tested whether TRF could improve serum glucose regulation in old mice (and young mice), when faced with the nutritional challenge of a whole-meal. Previously it was shown that TRF improves glucose regulation in young mice (Chaix et al., 2014); however, this was determined through an intraperitoneal glucose tolerance test (ipGTT). While this is a good method of measuring the body's ability to regulate glucose, it fails to simulate a real-life feeding scenario, since most consumable food items will likely contain more than just glucose. Therefore, in order to better understand glucose metabolism with TRF, we conducted a meal tolerance test (MTT), wherein the regulation of glucose after consumption of a complete meal with proteins, carbohydrates, and fats was tested (Figure 4)

Young and old mice on ALF and TRF (all 4 groups) were gavaged with a constant bolus of 700 cal (approximatly 5% of daily intake) of a complete meal (5.5% protein, 21% carbohydrate, 5% fat) after 16h of fasting. Body weight (in grams) (Figure 4A) and fasting glucose (mg/dL)(Figure 4B) of young and old mice on ALF and TRF were measured prior to the test. After administering bolus dose of the complete meal, blood glucose (mg/dL) measurements were taken every 10 minutes up to 120 minutes (Figure 4, C). Finally, to quantify the differences in peak blood glucose levels and the time required to restore normoglycemia, area under the curve (AUC) above baseline levels of glucose was calculated (Figure 4D).

Young mice on TRF had better glucose regulation compared to young mice on ALF. Differences in fasting glucose between young mALF and young mTRF were insignificant (134.30

± 11.34 mg/dl for young mTRF Vs. 144.40 ± 8.18 mg/dl for young mALF; mean ± SEM; n = 8/group; Figure 4B). The similarities in fasting glucose can also be observed at time 0 in Figure 4C. 10 minutes after initial bolus dose of a complete meal, young mTRF mice show a slightly lower peak blood glucose (247.00 ± 37.94 mg/dl, mean ± SD) compared to young mALF (295.38 ± 57.19 mg/dl, mean ± SD); Further, young mTRF demonstrate a quicker drop in blood glucose levels (202.00 ± 2.45 mg/dl at 45 minutes, mean ± SD); however normoglycemia was still not restored at the 60 minute mark (198.87 ± 30.48 mg/dl at 60 minutes, mean ± SD). Young mALF mice, on the other hand, had a higher initial peak and showed a much slower drop in blood glucose levels (275.13 ± 47.84 mg/dl at 45 minutes, mean ± SD). Normoglycemia was not restored at the 60 minute mark (275.13 ± 47.84 mg/dl at 60 minutes, mean ± SD) and young mALF were still had high blood glucose levels at th 120 minute mark (203.63 ± 37.89 mg/dl at 120 minutes, mean ± SD). To quantify these differences, area under the curve (AUC) was calculated. Young mTRF mice had a significantly lower AUC above baseline glucose compared to young mALF mice (**$p < 0.01$; n = 8/group; Figure 4D). *These results indicate that TRF improves glucose regulation in young mice.*

Old mice on TRF had significantly improved glucose regulation compared to old mice on ALF. Differences in fasting glucose between old mALF and old mTRF were insignificant (198.00 ± 14.47 mg/dl for old mTRF Vs. 208.30 ± 11.36 mg/dl for old mALF; mean ± SEM; n = 8/group; Figure 4B). The similarities in fasting glucose can also be observed at time 0 in Figure 4C. 10 minutes after initial bolus dose of a complete meal, old mTRF mice show a slightly lower peak blood glucose (321.25 ± 36.80 mg/dl, mean ± SD) compared to old mALF (356.13 ± 58.74 mg/dl, mean ± SD); Further, young mTRF demonstrate a very quick and steep drop in blood glucose levels such that normoglycemia was restored within 20 minutes (195.50 ± 45.72 mg/dl at 20

minutes, mean ± SD) and was maintained over 60 minutes (205.37 ± 30.80 mg/dl at 60 minutes, mean ± SD). old mALF mice, on the other hand, had a high initial peak and showed a much slower drop in blood glucose levels and had much higher glucose levels at 20 minutes (294.00 ± 45.71 mg/dl at 20 minutes, mean ± SD); further, it took old mALF mice 120 minutes to restore normoglycemia (208.38 ± 35.11 mg/dl at 120 minutes, mean ± SD). These differences were reflected in the AUC. Young mTRF mice had a significantly lower AUC above baseline glucose compared to young mALF mice (*$p < 0.05$; n = 8/group; Figure 4D). *These results indicate that TRF improves glucose regulation in old mice.*

Both young and old animals in the TRF groups have batter glucose responses compared to their ALF counterparts. In comparison, young mTRF showed a much lower initial peak in blood glucose compared to old mTRF, but young mTRF also started at lower fasting glucose levels than old mTRF. The decline in blood glucose levels was slower in young mTRF than in old mTRF, however, AUC for young mTRF was non-significantly different from old mTRF (Figure 4D). Ad libitum feeding, on the other hand, seemed to affect young animals more adversely than old animals. Young mALF had a lower initial peak in blood glucose compared to old mALF, but young mALF also started at lower fasting glucose levels than old mALF. Moreover, while both young and old mice demonstrated a very slow decline in blood glucose level, AUC for young mALF was significantly higher than that for old mALF (*$p < 0.05$; n = 8/group; Figure 4D) indicating that ALF has more drastic effects on glucose regulation for young mice. *Altogether, these results suggest that TRF exerts a strong influence over glucose regulation and equally benefits both young and old mice*.

Effects of TRF on Endurance, Motor Coordination, and Grip Strength in Old Mice

In order to understand the effects of TRF on physical performance, we set out to test endurance capacity, motor coordination, and grip strength in old mice (Figure 5). These are highly relevant tests for the purposes of this study since aging and sarcopenia are associated with decreased grip strength (sheth et al., 2018), impaired motor coordination (Kwon and Yoon, 2017), and reduced endurance capacity due to VO2max reductions (Prior et al., 2016). Therefore, we wanted to determine if TRF could attenuate the aforementioned age-related impairments in physical performance. Previous studies have shown that TRF does enhance endurance capacity and motor coordination in young mice (Chaix et al., 2014; Hatori et al., 2012), thus we hypothesized endurance and motor coordination benefits in old mice. Although, no difference in grip strength between ALF and TRF was observed in young mice (Chaix et al., 2014), we decided to test grip strength in old mice based on the hypothesis that strength would be more susceptible to dietary changes with age.

To determine endurance capacity, a one-time medium intensity run to exhaustion assay (without prior training) was chosen (Figure 5A). ALF and TRF mice were run side by side on a 6 lane treadmill (see methods). In order to determine endurance capacity on the treadmill, mice were run until exhaustion, which was defined as the inability to continue running despite repeated stimulation (Narkar et al., 2008). The time to exhaustion was significantly higher for old mTRF (200.20 ± 26.79 minutes; mean ± SEM) as compared to old mALF (97.89 ± 21.25 minutes; mean ± SEM) ($n = 9$, $^{**}p < 0.01$; Figure 5A; for numerical values refer to table 5). *Thus old mTRF mice had better treadmill performance compared to old mALF mice thus indicating enhanced endurance capacity with TRF.*

In order to assess motor coordination, we used a rotarod test was used (Figure 5B). A four compartment rotarod machine was used to test ALF and TRF side by side (Deacon, 2013; see methods). Mice were placed on a constantly accelerating rotating rod and the time to fall (latency in sec) was recorded. The time to fall was not significantly different between old mTRF (86.50 ± 7.09 minutes; mean ± SEM) and old mALF (87.98 ± 7.31 minutes; mean ± SEM) (n = 8; Figure 5B; for numerical values refer to table 5). The non-significant difference in rotarod performance between ALF and TRF indicates that old mice do not experience the motor coordination benefits associated with TRF.

To test for muscle strength, a grip strength meter and Kondziela's inverted screen test were used. The grip strength meter was used to quantify the grip strength of forelimb muscles by measuring the amount of force applied on the meter by the mice (see methods). Forelimb grip strength was not significantly different between old mTRF (50.02 ± 1.90 g; mean ± SEM) and old mALF (50.08 ± 1.20 g; mean ± SEM) (n = 16; Figure 5C; for numerical values refer to table 5). Further, to get a better picture of the strength differences in the old mice on TRF we decided to do the Kondziela's inverted screen test. This assay uses a wire grid system to non-invasively measure the ability of mice to sustain constant limb tension against their own gravitational force thus allowing for strength determination of all four limbs (Deacon, 2013). The time to fall off the screen was significantly higher for old mTRF (24.22 ± 7.56 minutes; mean ± SEM) as compared to old mALF (4.89 ± 0.34 minutes; mean ± SEM) (n = 15, *$p < 0.05$; Figure 5D; for numerical values refer to table 5). Better performance on the Kondziela's inverted screen test indicates that the combined total strength in all four limbs compared is higher in old mice on TRF compared to old mice on ALF; however, the latency to fall values from the inverted screen test were highly

correlated to body weight. *These results indicate that TRF improves certain parameters of strength in old mice.*

Effects of TRF on Skeletal Muscle Tissue Physiology

Based on the difference in performance assays, we examined the tissue level variation in the skeletal muscle. The quality of muscle is an essential consideration for sarcopenia and obesity. Age-associated fat infiltration into the muscle and alterations in muscle glycogen levels are strong determinants of muscle quality and functional capacity (Addison et al., 2014; Knuiman et al., 2015). In order to assess the differences in muscle quality of old mice ALF and TRF, we analyzed levels of triglyceride and glycogen in the gastrocnemius and soleus muscle of old animals. Skeletal muscle triglyceride content of old mALF was 3.82 ± 0.48 (mean ± SEM, n = 12) and that of old mTRF was 4.17 ± 0.37 (mean ± SEM, n = 12)(Figure 6A); the differences between ALF and TRF were non-significant. Similarly, skeletal muscle glycogen levels of old mALF were 2.21 ± 0.10 (mean ± SEM) and that of old mTRF were 4.17 ± 0.15 (mean ± SEM), thus failing to produce differences of statistical significance (Figure 6B; for numerical values refer to table 6). Therefore, differences in triglyceride content and glycogen levels in the skeletal muscle of old mice on ALF and TRF were not significantly different. These results indicate that differences in performance assays between ALF and TRF were not due to muscle triglyceride or glycogen variations.

Next, we wanted to assess histological differences in the skeletal muscle to analyze alteration in tissue architecture between ALF and TRF. To achieve this, we planned to perform histological assays such as H&E, Cox staining, and iHC to learn more about muscle tissue anatomy, mitochondrial activity, and fiber-type differences respectively, between mice on ALF and TRF. These results could provide valuable insights into age-related alteration in muscle quality

and function along with the potential impacts of TRF on attenuating sarcopenia. Unfortunately however, histological assessments could not be executed due to the overlap between this thesis study and the Covid-19 pandemic.

TRF on Skeletal Muscle Metabolism- and Performance-Associated Gene Expression

To understand the molecular mechanisms responsible for producing physiological differences in metabolism and physical performance we analyzed mRNA expression of genes associated with metabolism and performance in skeletal muscle samples. Because previous studies suggested differences in long-term lipid and glucose homeostasis as a function of transcriptional regulation in adipose and liver tissues (Chaix et al., 2014), we sought to analyze metabolic activity of skeletal muscle by determining mRNA levels of genes involved in fat and glucose metabolism such as Ppard, Pgc1a, Cpt1b, Fasn, Scd1, Pkm, Pdk4.

To analyze lipid metabolism we analyzed genes involved in both fatty acid synthesis as well as oxidation. We tested mRNA expression of fatty acid synthase (Fasn) and steroyl-CoA denaturase-1(Scd1) which are 2 genes whose protein products are key enzymes of the de novo lipogenesis (DNL) pathway thus making them good candidates for understanding fatty acid synthesis. qRT-PCR analysis resulted in non-significant differences in levels of mRNA expression of Fasn and Scd1 between ALF and TRF (Figure 7). Next, we looked at peroxisome proliferator-activated receptor delta (Ppard), peroxisome proliferator-activated receptor gamma co-activator 1 alpha (Pgc1a), and carnitine palmitoyl transferase-1b (Cpt1b) expression which are genes involved in fatty acid oxidation. Ppard is master regulator of metabolic activity in the skeletal muscle with multiple downstream effects including fatty acid transport and basal lipid oxidation (Stancic et al., 2013; Ravnskjaer et al., 2010). Pgc1a is a transcriptional co-activator of multiple genes with

downstream effects ranging from fat degradation, mitochondrial biogenesis, muscle fiber-type determination, angiogenesis, etc. (Arany et al., 2008). qRT-PCR analysis revealed drastic differences in mRNA levels of Pgc1a between ALF and TRF (****$p < 0.0001$; Figure 7). Unexpectedly, no differences were observed in levels of mRNA expression of Ppard between ALF and TRF (Figure 7). Cpt1b, a skeletal muscle isoform of Cpt1, is yet another key component of fatty acid oxidation since it facilitates fatty acid transport into the mitochondria. qRT-PCR analysis resulted in non-significant differences in mRNA levels of Cpt1b between ALF and TRF (Figure 7).

Finally, to understand differences in glucose metabolism between ALF and TRF, we looked at pyruvate kinase muscle isozyme (Pkm) and pyruvate dehydrogenase kinase 4 (Pdk4) expression. Pkm encodes the pyruvate kinase enzyme which is a key enzyme in final step of the glycolytic pathway. Pdk4, on the other hand, encodes pyruvate dehydrogenase kinase which regulates pyruvate dehydrogenase complex (PDC) thereby regulating glucose as well as fat metabolism. qRT-PCR analysis revealed a statistically significant difference in mRNA levels of Pkm (*$p<0.05$; Figure 7). Pdk4 mRNA expression was plotted as a time series because it demonstrated rhythmicity. Although Pdk4 had similar levels of relative mRNA expression between ALF and TRF, it was completely phase shifted in the ALF mice indicating circadian impairments of glucose metabolism (shown as time-series; Figure 7). All data generated from qRT-PCR analysis were initially plotted as a time series because previous studies observed rhythmicity in expression of metabolic genes like Fasn and Scd1 in liver and adipocytes (Hatori et al., 2012). However, after conducting a 2-way Anova test with time of day (TOD) and feeding groups (ALF/TRF) as parameters, the results were non-significant for all tested genes except Pdk4.

Therefore, samples collected at different time points were treated as replicates and plotted as shown (Figure 7).

Overall, these results indicate that TRF induces changes in gene expression with implications for physical performance and age-related alterations in glucose and lipid metabolism in the skeletal muscle.

TRF on Skeletal Muscle Clock Gene Oscillations

TRF is a dieting strategy that synchronizes feeding-fasting rhythms with the endogenous circadian clock. Previous studies have confirmed that TRF can prevent the dampening of transcriptional rhythmicity of core clock components in livers of young mice otherwise observed under HFD feeding (Hatori et al., 2012). Here, we set out to determine whether young and old mice also experienced improvements in transcriptional rhythmicity of clock genes as a function of TRF in another key metabolic organ that is the skeletal muscle.

Using qRT-PCR, we analyzed the diurnal profile of mRNA expression of Bmal1, Clock, Cry1 and Per2 in skeletal muscle of young mice. All clock genes displayed a diurnal pattern of expression, confirmed by MetaCycle statistical analysis for Bmal1, Clock and Per2 (Figure 8). As expected, we observed that Bmal1 and Clock expression are in phase, with coincident peak and trough of expression. Per2, on the other hand, cycle in anti-phase. We did not observe a significant difference in amplitude or phase between young mTRF and young mALF for Clock and Bmal1. Per2 elicited slightly higher amplitude and an early peak in young mTRF. Cry1 showed an ultradian rhythm with 2 peaks of higher amplitude in young mTRF.

In old mice, Bmal1, Clock, and Cry1 mRNA expression were also rhythmic (Figure 9). Statistical analysis using MetaCycle confirmed that Bmal1 and Cry1 were indeed cycling, however

Clock was not. Both Bmal1 and Cry1 cycled at a higher amplitude in mice on TRF. Similar to what was observed in young mice, Cry1 displayed an ultradian profile with 2 daily peaks for which the relative amplitude was different between ALF and TRF. Clock profile was the most different between young and old mice with absence of rhythmicity in old mALF and somehow preserved rhythms in TRF.

Interestingly, all clock genes seem to oscillate at higher amplitude in the young muscle than the old, suggesting that the muscle clock is subject to age-related dampening of the clock. Moreover, the circadian-enhancement effect of TRF was higher in old than in young. *Since Bmal1, Clock, Cry1, and Per2 along with other clock components, play an important role in generating robust circadian rhythms, our results suggest that TRF has the potential to prevent age-related circadian deteriorations.*

TRF Improves Rhythmicity of Clock Genes at Transcriptional and Translational levels

To understand whether TRF could improve circadian rhythmicity of gene expression at the mRNA as well as the protein level, we performed simultaneous analysis of mRNA and protein expression in liver of young mice and skeletal muscle of young and old mice. We chose to look at liver samples because of the known significant alterations in hepatic metabolism in young mice (Hatori, 2012, Chaix, 2014). As previously described, Bmal1 display a clear diurnal rhythm of expression in the liver, with slightly higher amplitude in mice on TRF (Figure 10A). Since TRF can act on nutrient-sensing pathways which mostly act at the post-translational (PTM) level, and since PTM can affect clock protein stability, we analyzed BMAL1 protein expression by western blot in liver samples of young mice. We observed a clear rhythmicity of BMAL1 protein expression in mice on TRF (Figure 10B) as visualized by the quantification of the signal in panel

10C.. mRNA expression was in-phase with the protein expression of Bmal1 in mice on TRF. ALF mice, on the other hand, displayed lower amplitude rhythmicity at the mRNA level (Figure 10A) and severely dampened rhythmicity at the protein level with no discernable phase relationship between transcriptional and translational levels.

Next, we performed a similar mRNA/protein level analysis in the skeletal muscle of old mice. In ALF mice, if Bmal1 mRNA level was rhythmic, at the protein level, this rhythmicity was severely dampened (Figure 11C). ALF mice also had a slight phase shift between mRNA and protein expression. In TRF mice, on the contrary, Bmal1 was highly rhythmic both at the mRNA and protein level in the skeletal muscle (Figure 11). TRF mice also had higher peaks and low troughs in protein expression of Bmal1. Interestingly, mRNA expression seemed almost anti-phase to the protein expression of Bmal1 in mice on TRF.

These results indicate that TRF is capable of improving clock gene rhythmicity at the transcriptional and translational levels through mechanisms that are yet to be determined.

DISCUSSION

The process of aging is associated with a decline in health and vitality. Age-related diseases along with deteriorating circadian rhythms can have devastating consequences for the quality of life of aging individuals. Currently, the aging population is growing at a high rate thus increasing risk of consequential age-related disorders like sarcopenia and obesity. Time-restricted feeding, a behavioral intervention that restricts caloric intake to an 8-9 hour window, is a unique intervention because it does not require alterations in caloric quantity/quality thus making it easy to adopt for individuals of all ages. TRF has been shown to improve numerous health parameters in young

mice (Hatori et al., 2012), however effects TRF on middle-aged and older mice are not clear. In this thesis study, we demonstrated that TRF is a holistic intervention with potent effects on various measures of metabolic fitness, physical performance, and muscle function in middle-aged mice, all of which hints at the therapeutic potential of TRF for prevention (or delay) of sarcopenia and age-related metabolic decline. Here we discuss (1) Age-independent benefits of TRF on metabolic homeostasis, (2) Benefit of TRF on muscle function and implications for Sarcopenia, (3) Benefits of TRF on maintenance of skeletal muscle clock function.

Age-Independent Benefits of TRF on Metabolic Homeostasis

Young and old mice fed a western diet (45% fat, 17% sucrose) experienced obesity and general metabolic decline with ad-libitum access to food. Young and old mice on a 9h TRF intervention, however, were protected from the adverse effects of the western diet despite consuming the same amount of food as the ALF mice. In particular, our results show that TRF successfully prevented excessive body weight gain associated with consumption of a high fat and high sucrose diet in young and old mice. In both cases, the difference in body weight between ALF and TRF was attributable to lower body fat and higher lean mass. These desirable changes in body weight and composition that occur as a function of feeding in a time-restricted fashion can partially be explained through the improvements in metabolic regulation which are intrinsic to aligning feeding events with endogenous circadian function. The circadian clock guides cyclical activation and repression of metabolic and nutrient-sensing pathways, which exercise temporal control over anabolic and catabolic signals for nutrient utilization (Villanueva et al., 2019). Therefore, timing feeding intervals during the active phase of the mice can synchronize feeding-fasting rhythms with output of circadian clocks such that circadian physiology of metabolism is optimized to process

food (Panda, 2016). TRF can also likely improve rhythms in central regulation of metabolic hormones and nutrient homeostasis, thereby improving transmission of feeding-fasting signals to peripheral clocks. This, in turn, allows for better anticipation of feeding-fasting periods by the peripheral clocks thus priming metabolic responses suitably.

Moreover, benefits of TRF on body composition can also be explained through fasting-mediated adaptations on whole-body physiology and fat mobilization. Extended fasting (15h in our study) can support fatty acid utilization (Chaix et al., 2014) by increasing mobilization of free-fatty acids and upregulating beta-oxidation (Hatori et al., 2012) subsequently shifting the source of energy substrate from glucose to fat (Owen et al., 1979). This likely explains the demonstrated reductions in adiposity with TRF in both young and old mice. Baseline lean mass was not measured, but we observed higher lean mass in mice on TRF compared to their ALF counterparts. Although changes in gene expression explained the preservation of lean mass observed with TRF (see section 2), fasting-mediated hormonal changes such as upregulation of growth hormone (Vendelbo et al., 2010; Nørrelund et al. 2001) along with autophagy (Masiero et al., 2009) might have a role to play in lean mass differences between ALF and TRF.

Interestingly, we observed a greater effect of TRF on body compositional changes in the old mice. A possible hypothesis accounting for the differences in efficacy of TRF between the young and the old might be found in age-related alterations in the circadian timing system. Aging is associated with an increased susceptibility to circadian dysfunction (Brown et al., 2011). Circadian dysfunction, in turn, is linked with detrimental effects on body weight and composition (Shi et al., 2013; Baron et al., 2017). TRF is a circadian intervention that has been shown to improve circadian clock function down to the molecular level (Hatori et al., 2012). Therefore, a circadian modulator like TRF is likely to be more effective at preventing the combined detriments

of age-related circadian dysfunction and a high fat/high sucrose diet on body weight and composition in old mice. Young mice, on the other hand, are also protected from the harmful effects of the western diet; however, since they do not undergo age-related alterations in circadian function, the effects of TRF are less pronounced than in the old.

Aging, obesity, and the loss of lean mass are also associated with the development of glucose intolerance and insulin resistance (Shou et al., 2020; Luca and Olefsky, 2007). Since TRF could prevent age-related alterations in body composition on the western diet, we assessed the protective effects of TRF against sarcopenia- and obesity-associated insulin resistance. We used a meal tolerance test (MTT) to determine whether TRF impacted whole-body glucose regulation when mice were confronted with the nutritional challenge of a whole-meal that not only contains carbohydrates but also lipids and proteins. We chose this method over traditional intraperitoneal or oral glucose tolerance test (ipGTT/oGTT) to simulate a real-life feeding scenario, since most consumable food items will likely contain more than just glucose. ALF resulted in impairments in glucose regulation and both young and old ALF mice. Feeding a high fat diet ad libitum can induce circadian disruption (Kohsaka et al., 2007) which is linked to insulin resistance and obesity (Shi et al., 2013), thus resulting in impaired glucose tolerance observed in mice on ALF. TRF, on the contrary, resulted in better glucose tolerance in both young and old TRF mice. TRF-mediated improvements in glucose regulation can be partially explained by underlying circadian regulation of glycemic control. It has been shown that glucose tolerance and insulin sensitivity are under circadian control (Fleur et al., 2001). In mice, glucose uptake shows a peak at the beginning of the dark/active phase and a trough at the beginning of the light/inactive phase. 24 h rhythms in insulin sensitivity contribute to the 24 h rhythms in glucose uptake such that, peaks in insulin sensitivity are correlated with peaks in glucose uptake. Therefore, the impact for endogenous circadian

rhythms on glucose regulation might be mediated through improved beta-cell response during the active phase (Morris et al., 2015). Mice on TRF have food access restricted to a 9h window in dark/active phase, which means that they consistently feed at times when glucose uptake is optimized via heightened insulin sensitivity and strong beta-cell response. Thus explaining improved glycemic control in mice on TRF during the meal tolerance tests.

Another hypothesis for TRF-mediated improvements in glycemic control is that differences in body composition between ALF and TRF resulted in improved glucose regulation. Fat mass was significantly higher in ALF mice compared to TRF mice. Increased fat mass, obesity, and obesity-induced inflammation contribute to the pathogenesis of insulin resistance and metabolic diseases (Luca and Olefsky, 2007; Kahn and Flier, 2000). This was reflected through the poor glucose response in the fatty ALF mice. Conversely, mice on TRF had lower fat mass which is correlated with the demonstrated improvements in glucose tolerance. TRF mice also had higher lean mass or the muscle tissue mass than the ALF mice. Since the skeletal muscle is the largest organ responsible for post-prandial glucose uptake and decreased muscle mass is associated with impaired glucose tolerance (Kalyani et al., 2012), having a higher lean mass can result in improved glucose response. These observations pointed at the benefits of TRF on muscle function and its therapeutic implications for sarcopenia.

Benefits of TRF on Muscle Function and Implications for Sarcopenia

Sarcopenia is a complex disorder that is characterized by age-related loss of muscle mass and muscle function. Current strategies to combat this loss of muscle mass and function are primarily focused on quality and quantity of caloric intake as well as physical exercise. Since the primary anabolic stimuli for preservation of skeletal muscle mass and upregulation of muscle

protein synthesis are mechanical tension and dietary protein intake (Traylor et al., 2018), the aforementioned interventions make intuitive sense for treatment of sarcopenia. However, age-related functional restrictions limit the feasibility of exercise and social factors like bereavement/social isolation paired with chronic illness and physiological changes as a function of age affect nutritional quality (Leslie and Hankey, 2015). Benefits of maintaining regular and consistent patterns of feeding-fasting have been explored for combating numerous age-related diseases and for maintaining good health (Mattson et al., 2017; Longo and Panda, 2016). However, the effects of controlling the timing of food intake and fasting as a preventative intervention for sarcopenia have remained unclear. Previous TRF studies hinted at the therapeutic efficacy of TRF in improving muscle physiology thus indicating its potential relevance for sarcopenia (Villanueva et al., 2019). Moreover, sarcopenia is potentiated by obesity and TRF was effective at preventing and even reversing obesity and metabolic disease, all while maintaining lean mass (Hatori et al., 2012; Chaix et al., 2014). Since aging is associated with both sarcopenia and obesity, we explored the effects of TRF on age-related alterations in physiology and skeletal muscle function.

Symptoms of sarcopenia start arising around middle age and worsen progressively. Thus, we characterized the effects of TRF in middle-aged mice (which we refer to old mice for the sake of simplicity) because we wanted to determine the efficacy of TRF as a measure to prevent the exacerbation of sarcopenia. Moreover, we speculated that if TRF could help with delaying sarcopenia in middle-aged mice then it might have implications for older mice as well. In accordance with the revised European consensus (EWGSOP) regarding key characteristics of sarcopenia (Cruz-Jentoft et al., 2019), we assessed effects of TRF on i. muscle quantity (MRI), ii. muscle strength (grip strength and inverted screen test), iii. endurance performance (treadmill test), and iv. muscle quality (tissue physiology – couldn't achieve due to covid-19).

TRF-mediated preservation of lean mass (as determined through the MRI) along with improved glucose tolerance (discussed above) were strong indicators of maintained muscle quantity in the old TRF mice. Further, we looked at effects of TRF on muscle strength. Given that muscle strength is a highly clinically relevant parameter (Wolfson et al., 1995), we utilized two different approaches to fully understand variations in strength: grip strength test and Kondziela's inverted screen test. The grip strength test only measures the strength of the forelimbs, whereas the inverted screen test measures the combined strength of all four limbs against their own gravitational force. Interestingly, there were no differences in grip strength between ALF and TRF mice, but TRF mice performed better than ALF mice on the inverted screen test. One interpretation of the insignificant difference in forelimb grip strength and the significant difference in all four limb grip strength could be that hindlimbs account for the true differences in strength between ALF and TRF. Moreover, differences in the results of the inverted screen test might have resulted due to underlying differences in muscle fatigue – defined as the exercise-induced reduction in force production (Wan et al., 2017). Increased hang time as seen with TRF mice might be due to an increased time to muscle fatigue. Another explanation might be that TRF mice perform better on the inverted screen assay simply because they have lower body weights and a lower consequential force of gravity against which their limbs are tested thus allowing them to hang for longer durations than the heavier ALF mice. Regardless, we demonstrate that TRF exerts a positive influence over certain parameters of muscle strength. Further investigation is required to determine whether these effects are direct or indirect.

Next, we characterized the effects of TRF on endurance performance and motor coordination, both of which are implicated in increased healthspans. While no differences in motor coordination were observed, old mice on TRF had significantly better performance on the treadmill

run-to-exhaustion assay than the old mALF mice. We hypothesized that differences in endurance performance could result as a function of (1) differences in skeletal muscle architecture, (2) differences in skeletal muscle fuel sources, (3) differences in muscle metabolic capacity. First, to compare skeletal muscle architecture between ALF and TRF, we planned to look at tissue anatomy along with fiber type differences and mitochondrial content and activity. Unfortunately however, histological assessments could not be executed due to the overlap between this thesis study and the Covid-19 pandemic. Second, in order to get an insight into possible differences in fuel sources, we measured glycogen and triglycerides stores. However, we found no differences in glycogen and TG levels between ALF and TRF. Third, in order to get a molecular understanding of the observed differences in muscle function, we examined gene expression signature of metabolic and performance genes in skeletal muscle.

To characterize the effects of TRF on *lipid metabolism* in the skeletal muscle, we looked at the de-novo lipogenesis (DNL) pathway. DNL is a complex and highly regulated metabolic process wherein excess carbohydrates are converted into fatty acids and then esterified into trigacylglycerols which can be broken down via beta-oxidation. The skeletal muscle is an active site of DNL and is known to utilize stored IMTGs during exercise (Hollands and Cawthorne, 1981; Goodpaster et al., 2001). Consuming a diet that is rich in both fat and sugars combined with reduced physical activity leads to increased deposition of fat in muscle tissues as observed in pronounced sarcopenic obesity. Ectopic deposition of fat in non-canonical fat storing tissues is under the control of DNL, which itself is stimulated by presence of excess dietary fat and sugars. Thus, we looked at the gene expression of fatty acid synthase (Fasn) and Stearoyl-CoA desaturase-1 (Scd1) which are key enzymes of DNL.

Fasn is a key rate-limiting enzyme of DNL that converts malonyl-CoA to palmitate (Song et al., 2018). Palmitate then undergoes elongation and desaturation to produce more complex fatty acids like palmitoleic acid, stearic acid, and oleic acid. Stearoyl-CoA desaturase-1 (Scd1) is a desaturase that converts palmitate to palmitoleic acid. Build-up of these fatty acids can have detrimental consequences such as increased triglyceride stores, inflammation, steatosis, fibrosis etc. There was no difference in Fasn and Scd1 gene expression between ALF and TRF. This was in accordance with the insignificant differences in muscle triglyceride content between ALF and TRF. Although unexpected, these results suggest that there is was difference in skeletal muscle DNL enzyme expression between old ALF and TRF.

Next, we assessed possible differences in lipolysis through fatty acid beta-oxidation (FAO). FAO is metabolic process wherein long chain acyl-CoA molecules – the primary components of fatty acids – are broken down to acetyl-CoA molecules and are used up for energy (Houten and Wanders 2010). Studying the FAO pathway was particularly relevant for this study because fasting is associated with lipolysis and utilization of fats as an energy source (Owen et al., 1979). Moreover, in tissues with high energy requirements, such as the skeletal muscle, fats can serve as efficient fuel sources. Thus, we looked at mRNA expression of carnitine palmitoyl transferase-1b (Cpt1b) and peroxisome proliferator-activated receptor delta (ppard).

Cpt1b is a key rate-limiting component in FAO pathway in the skeletal muscle. It encodes a mitochondrial transporter protein that modifies long-chain fatty-acyl-CoA to acylcarnitine molecules and transports them across the mitochondrial membrane thus controlling mitochondrial entry of activated fatty acids. Ppard is a master transcriptional regulator of metabolic activity in the skeletal muscle that controls fat degradation by regulating expression of genes involved in fatty acid transport, beta-oxidation and mitochondrial respiration (Takahashi et

al., 2007). Surprisingly, as for FAO, there was no difference in mRNA expression of Cpt1b or Ppard between ALF and TRF. Further analysis would be necessary to evaluate differences in enzyme expression and/or activity. The balance between DNL and beta-oxidation is regulated by intermediary metabolites such as malonyl-CoA. Thus, measuring the activity of key regulatory metabolites would provide a clearer picture regarding the dynamics of these pathways and whether they are temporally affected by the feeding paradigm.

To analyze the contribution of *glucose metabolism* to the observed differences in muscle physiology, we looked at key enzymes of glycolysis namely pyruvate dehydrogenase kinase 4 (Pdk4) and pyruvate kinase muscle isozyme (Pkm). Pdk4 encodes pyruvate dehydrogenase kinase which is a mitochondrial protein that inhibits the pyruvate dehydrogenase complex (PDC – key for glucose homeostasis during fed/fasted states) by phosphorylating one of its subunits, thereby regulating glucose metabolism and controlling metabolic flux through the citric acid cycle (Peterson et al., 2019; Bethesda, 2004). Pdk4 can also be coactivated by Pgc1a to exert transcriptional control over skeletal muscle glucose metabolism (Wende et al., 2005). Pkm, on the other hand, expresses a key enzyme that catalyzes the conversion of phospoenolpyruvate to pyruvate while generating ATP in the final and irreversible step of the glycolytic pathway. We observed an increase in steady state levels of Pkm with TRF. Fasting-induced decrease in the flux through the glycolytic pathway can elevate glucose levels in the skeletal muscle thus explaining Pkm upregulation (Yamada and Noguchi, 1999). Interestingly, the relative level of expression of Pdk4 was not different between ALF and TRF. However, it has been shown previously that Pdk4 expression is affected by both meal timing and diet composition (Goede et al., 2017). We observed similar changes in diurnal expression profile of Pdk4 : expression was higher during the light/fasting phase under TRF whereas it was higher in the dark/feeding phase

in ALF. Although, fat metabolism wasn't different and carbohydrate metabolism was slightly different in the skeletal muscle of ALF and TRF; we hypothesized that temporal aspect maybe critical to muscle metabolism and function as suggested by phase shifted expression of Pdk4 (section 3).

Finally, to further gauge the differences in muscle performance as a function of differential gene expression, we looked at mRNA levels of peroxisome proliferator-activated receptor gamma co-activator 1 alpha (Pgc1a). Pgc1a is nuclear transcriptional coactivator that regulates essential metabolic processes including mitochondrial biogenesis, adaptive thermogenesis, fatty acid oxidation, angiogenesis, and insulin sensitivity by controlling expression of numerous transcription factors. (Handschin and Spiegelman, 2006; Wu et al., 1999; Arany et al., 2008; Bonen, 2009). Moreover, Pcg1a expression has been shown to decrease with age and has great implications age-related changes in muscle function (Gill et al., 2018; Anderson and Prolla, 2009). Interestingly, we observed significantly elevated mRNA expression of Pgc1a old TRF mice as compared to old ALF mice. Upregulation of Pgc1a in the skeletal muscle can potentially explain observed benefits of TRF on (i) preservation of lean mass, (ii) endurance performance, and (iii) glucose tolerance.

Studies have suggested that certain fasting interventions can cause loss of lean mass and skeletal muscle atrophy (Bak et al., 2018) while others suggest lean mass is preserved while fasting and only fat mass is lost (Norren et al., 2015; Gotthardt et al., 2016). By subjecting mice to a 15 h fasting window (predominantly in the inactive phase), we observed higher lean mass compared to the ALF mice. The observed preservation of lean mass could be directly linked with Pgc1a upregulation through TRF. High levels of Pgc1a during catabolic conditions can spare muscle mass during fasting by suppressing FoxO-dependent loss of muscle mass (Sandri et al.,

2006; Geng et al., 2011). It has been shown that Pgc1a influences muscle protein turnover by reducing protein degradation without affecting protein synthesis (Brault et al., 2010) thus preventing muscle atrophy during fasted states. Additionally, Pgc1a can induce skeletal muscle remodeling resulting in an increased percentage of type I muscle fibers along with mitochondrial biogenesis thus boosting oxidative capacity of the skeletal muscle (Zhang et al., 2017; Liang and Ward, 2006). Skeletal muscle expression of Pcg1a is also associated with increased exercise capacity and peak oxygen uptake (Tadaishi et al., 2011). Therefore, endurance enhancements with TRF might have resulted from downstream effects of Pgc1a upregulation. Moreover, since endurance exercise itself is a known stimulator of Pgc1a and downstream influence on muscle function, our results suggest that TRF can mimic exercise-induced physiology (to some degree) through Pgc1a upregulation. It should be noted that the contribution of other organs such as the heart can also be involved in the observed enhancements in endurance performance with TRF and it would be interesting to test whether similar upregulation of Pgc1 happens in cardiac muscle. Finally, Pgc1a-mediated switch in fiber-type to the more oxidative type I fibers can itself influence glucose regulation by increasing skeletal muscle glucose uptake (Stuart et al., 2013; Zhang et al., 2017). Although, overexpression (beyond physiologic limits) of Pgc1a has paradoxically been linked to insulin resistance (Choi et al., 2008), Bonen and colleagues showed that overexpression of Pgc1a, within physiologic ranges, can improve insulin sensitivity through GLUT4 upregulation (Bonen, 2009). Moreover, Pgc1a dysfunction is associated with impaired glucose tolerance and type 2 diabetes (Łukaszuk et al., 2015; Mootha et al., 2003). Thus, we speculate that improvements in glucose regulation seen with TRF might be linked to Pgc1a-dependent increments in type I muscle fibers and/or GLUT4 expression which are associated with enhanced insulin stimulated glucose transport (Michael et al., 2001).

Overall, TRF had numerous implications for skeletal muscle function and sarcopenia. TRF had a positive influence on aspects of muscle function that are relevant for sarcopenia such as muscle quantity, muscle strength, and endurance performance. TRF-mediated improvements in muscle function were likely linked to underlying differences in skeletal muscle gene expression. Specifically, Pgc1a, a key regulator of energy metabolism, could play an important role in retention of muscle mass, enhanced endurance, and improved glucose regulation. Previous studies have shown that Pgc1a regulates gluconeogenesis and other aspects of the fasting response in the liver (Yoon et al., 2001; Herzig et al., 2001). Here, we show that benefits of fasting (9h TRF) on physiology and performance could be mediated via upregulation of Pgc1a in the skeletal muscle. Increased Pgc1a expression can also prove beneficial against age-related muscle loss and dysfunction as well as whole-body metabolism. Pgc1a upregulation and its numerous downstream effects can prevent mitochondrial dysfunction, neuromuscular decline, and other degenerative processes thus maintaining muscle integrity with age. Additionally, benefits of TRF on muscle integrity protection resulted in improved glucose metabolism and prevented age-related obesity. However, mechanisms through which TRF affects muscle function require further investigation. Therefore, while TRF is effective at preventing obesity and holds promise for delaying sarcopenia, a complete understanding of the mechanistic interaction between TRF and muscle physiology is required before recommending TRF as a therapeutic intervention against sarcopenia.

Benefits of TRF on Maintenance of Skeletal Muscle Clock Function

Age-related dampening of circadian rhythms combined with poor nutritional intake (western diet) can cause numerous diseases and contribute toward shorter healthspans. Aging and obesity likely potentiate each other to produce dampened whole body circadian rhythms, as well as

rhythms of the skeletal muscle. Given the importance of proper muscle function for whole-body metabolism and given the recently described role of the clock in controlling many aspect of muscle physiology, efficient functioning of molecular clocks in the skeletal muscle are essential for healthy aging. This also has strong implications for cause and progression of muscle dysfunction and sarcopenia (Miyazaki et al., 2011).

Age-related reduction in physical activity, the prevention of which is vital for sarcopenia, can modulate the molecular clock in muscle by affecting both the phase and amplitude of circadian rhythms (Wolff and Esser, 2012). Disruption of the core clock can also alter skeletal muscle metabolism causing impairments in physical performance (Gabriel and Zierath, 2019). On the other hand, exercise can optimize circadian entrainment of the muscle clock to improve muscle performance (Chaix and Panda, 2019). This highlights the importance of maintaining muscle clock function with age. TRF is linked with improved circadian rhythms and has been shown to synchronize peripheral clocks in numerous tissues including liver and adipose thus increasing metabolic efficiency (Hatori et al., 2012; Chaix et al., 2014). TRF was also effective at restoring muscle function in drosophila models of obesity and circadian disruption (Villanueva et al., 2019) thus making TRF a strong candidate to prevent age-related alterations in molecular clock function in skeletal muscle of old mice.

We assessed effects of TRF on the rhythmicity of mRNA expression of core clock components in skeletal muscle of young and old mice. In agreement with previous results from our lab, we observed slight, but consistent improvements in rhythmicity of core clock components at the mRNA level with TRF. Given that aging is associated with circadian deterioration, TRF-mediated improvements in muscle clock rhythmicity were highly relevant for the old mice. In particular, we observed a different temporal profile in Cry1 expression between old ALF and TRF.

Cry 1 has been shown to suppress Ppard and its target genes including Pgc1a thus limiting exercise performance (Jordan et al., 2017). A temporal shift in Cry1 expression, therefore, might contribute to impaired physical performance in ALF mice. Additionally, mRNA level of core clock component Bmal1 was dampened in old ALF mice. Bmal1, a key component of the skeletal muscle core clock, exerts transcriptional control over lipid and amino acid metabolism and depletion of muscle-specific Bmal1 is associated with insulin resistance, decreased GLUT4, and obesity (Schiaffino et al., 2016). Bmal1 deficiency is also associated with premature aging, age-related pathologies and muscle wasting (Kondratov et al., 2006). Given the relevance of Bmal1 for muscle function, we assessed differences in Bmal1 expression at the protein level.

Interestingly, the dampening of Bmal1 rhythmicity was even more obvious at the protein level. In ALF mice there was a complete absence of rhythmic protein expression of BMAL1 in the skeletal muscle. On the contrary, TRF mice demonstrated maintained rhythmicity of Bmal1 expression at mRNA and protein levels. These results prompted us to analyze whether some of the mechanisms behind the benefits of TRF might be governed at the post-transcriptional level and whether aging and/or obesity affected these mechanisms to produce circadian declension and muscle dysfunction. Thus, we assessed Bmal1 expression at the protein level in the liver (central to metabolic activity) of young mice. Interestingly, similar to observations made in skeletal muscle of old mice, protein expression of Bmal1 displayed dampened rhythmicity despite rhythmic mRNA expression in young mice on ALF. These results suggest that HFD-induced obesity, and not necessarily aging, act at both the translational and post transcriptional level to blunt diurnal rhythms in clock protein expression. TRF, on the other hand, can act both at the transcriptional and post transcriptional level to improve rhythmicity at the protein level in both young and old mice (in both liver and muscle samples).

Altogether, we suggest that TRF-mediated improvements in skeletal muscle clock function could prevent deteriorations in circadian rhythms with age. We demonstrated that TRF has a very strong impact on BMAL1 protein oscillations both in the liver and the skeletal muscle. Our results suggest that TRF mechanistically improves circadian rhythms and combats aging by enhancing muscle clock rhythmicity both at the transcriptional and post-transcriptional level. We further speculated that there might exist a link between improved muscle clock rhythmicity (as demonstrated) and improved lean mass, glucose uptake, and physical performance. This study indicates that benefits of TRF on physiology, muscle function, and performance are potent enough to state TRF as a therapeutic intervention against aging, obesity, and circadian decline with implications for sarcopenia.

CONCLUSIONS

In conclusion, our results demonstrate the therapeutic benefit of time-restricted feeding on age-related physiological decline and skeletal muscle dysfunction. We show that TRF exerts multifaceted influence over muscle biology including muscle metabolism, muscle circadian clock, and muscle PGC1a expression, all of which likely contribute to lean mass preservation and improved muscle function. TRF-mediated protection against sarcopenia likely plays a key role in the increased glucose tolerance of TRF mice. Further, we highlight age-independent benefits of TRF on metabolic homeostasis and suggest that it holds promise to prevent obesity and attenuate sarcopenia while preventing age-related circadian deteriorations. Further mechanistic analysis will elucidate the mechanisms through which TRF influences muscle physiology.

LIMITATIONS

The study presented in this thesis was a short-term study. To fully understand the mechanisms and physiological impacts of a holistic intervention like TRF, a longer-term study would be more revealing. It would also be informative to determine whether TRF can restore muscle function and reverse sarcopenia, in older (18-24 months old) and/or sarcopenic mouse models and/or models of muscle injury. Ideally, we would like to use or develop better techniques to assess differences in muscle strength. Current methods (including the ones used) are limited in their ability to fully characterize all aspects of muscle strength. This is important for assessing effects of TRF on sarcopenia. Therefore, further work is required before qualifying TRF as a therapeutic intervention against sarcopenia. Nonetheless, TRF has immense therapeutic potential for improving physiology and is a unique intervention that can be easily inculcated in the lifestyle of individuals of all ages. Thus rendering TRF a powerful intervention for reinstating health and vitality for both the young and the old.

METHODS

Animals and Diets

All animal experiments were carried out in accordance with the guidelines of IACUC of the Salk Institute. 32 one year old and 40 twelve weeks old male C57BI/6J mice from JAX labs were acclimatized to a reverse night-dark cycle for 2 weeks on a normal chow diet. All mice were fed the 'western diet' (Research Diet-D12451 – 4.7 kcal/g – 45% fat, 20% protein, 35% carbohydrates of which 17% was sucrose). At the starting point of the study, mice were randomly assigned to

Time-Restricted Feeding (TRF) regimen (9 hours feeding window) or Ad-Libitum Feeding (ALF) regimen (24 hour food access). TRF mice had food access restricted to a 9 hour window during ZT13 – ZT22 for 7 days of the week. ALF mice had ad-libitum access to food for 24 hours of the day, 7 days a week. Food intake and body weight were monitored weekly throughout the experiments.

Animal Cohorts

2 independent animal cohorts for both young and old mice

Young Animals : 2018 and 2020 TRF	**Old Animals : 2019 and 2020 TRF**
2018 TRF → Circadian take down, every 2 hours for 48h	2019 TRF → Circadian take down, every 2 hours for 24h
2020 TRF → metabolic phenotyping	2020 TRF → metabolic phenotyping

Organ Collection

Within each feeding group, 2-4 mice were sacrificed every 2 hours for 48h or 24h (depending on the cohort) after completion of study. Organs from individual mice were extracted and ground to fine powder in liquid nitrogen and aliquots were used for RNA and protein analyses.

Body Composition

Body composition was analyzed via NMR relaxometry in live mice using a body composition analyzer (EchoMRITM- 100H) during week 12 of the study.

Performance Assay

The **rotarod** performance test was performed using the Rotamex rotarod (Columbus Instruments) at ZT 15. On day 1 (training), first, mice were given 3 attempts to maintain position on non-rotating rod up to 60 seconds. Second, mice were given 2 attempts to balance on rotating (3rpm) but non-accelerating rod up to 60 seconds. Third, mice were given one attempt to balance on rotating (3rpm) but non-accelerating rod up to 60 seconds and the time to fall during this attempt was recorded as training values. On day 2 (testing), mice were placed on an accelerating rotating rod (0-300rpm) and the time to fall was recorded. Mice were trained for 1 day and performance was recorded on day 2. Data represent the average time spent on the rod for 5 trials on day 2. Rotarod test was done during weeks 6 and 11 of the study.

The **treadmill** exhaustion test was performed using the Exer 6M first generation treadmill (Columbus Instruments) at ZT 15. On day 1, mice were trained at low speed for 15 minutes with speed ramping up every 5 minutes. On day 2, mice were trained for 15 minutes at intermediate speed with 5 minutes of ramping. On day 3, maximal speed was reached by ramping up every 5 minutes. 5 degree angle of incline was applied to the treadmill when running time exceeded 1 hour and 10 degree angle of incline was applied when running time exceeded 1 hour 30 minutes. Mice were run until exhaustion, which was defined as the inability to continue running despite repeated stimulation by brushes and compressed air. Treadmill test was done during week 10.

Grip strength was determined using a digital grip strength meter (Columbus Instruments). Each animal was tested three times on 2 separate days at ZT16. Data represents the average of the 3 measurements on day 2. Grip strength test was done during week 12 of the study.

Kondziela's Inverted screen test was performed by placing mice on a flat wire-mesh screen and inverting the screen such that mice have to use all 4 limbs to hang on to the screen against the force of gravity. Time to fall off the screen was recorded and data was normalized to body weight. Kondziela's Inverted screen test was performed as described (Deacon, 2013) during week 12.

Triglyceride Quantification

Skeletal muscle tissue powder was homogenized in RIPA buffer, and triglyceride concentration was measured using an enzymatic assay (Triglycerides LiquiColor, Stanbio). Data were normalized to respective tissue weight.

Glycogen Quantification

Glycogen was quantified in skeletal muscle tissue powder using an enzymatic assay (Sigma) according to the manufacturer's instruction. Data were normalized to respective tissue weights.

MTT

Mice were gavaged with a constant bolus of 700 cal (approximately 5% of daily intake) of a complete meal (5.5% protein, 21% carbohydrate, 5% fat) after 16h of fasting (ZT21-ZT13) nine weeks into the feeding regimen. Blood glucose level was measured using OneTouch Ultra glucose meter prior to gavaging and several times post meal bolus as indicated.

RT-qPCR

RNA and cDNA were prepared, and RT-qPCR (reverse-transcription-quantitative polymerase chain reaction) was performed as described (Vollmers et al., 2009). Absolute transcript expression was calculated using the standard curve method (using two technical replicates), normalized to

HPRT1 expression (which does not show circadian- or diet-dependent changes in mRNA levels), and finally median normalized groupwise. Primer sequences can be found in Table 7.

Western Blotting

Total lysates were prepared in RIPA buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 1 mM Na3VO4) supplemented with protease and phosphatase inhibitor cocktails (cOmplete and PhosSTOP tablets, ThermoFischer). Western blots were conducted as described (Vollmers et al., 2009). Membranes were cut and probed simultaneously with antibodies directed against BMAL1 (A3012-616A - Bethyl Lab), ERK (p44/42 MAPK [Erk1/2] Antibody #9102 – CST). Signals were quantified using ImageJ.

Statistical Analyses

The Student's test was used for pairwise comparisons, and ANOVA with post hoc tests was used to compare to the control group. For time series, repeated- measured ANOVA with post hoc tests was used to compare to the control group. For examining two variables, two-way ANOVA was used. Statistics were calculated as appropriate using Prism. Throughout all figures, data are presented as mean ± SEM with statistical result of the statistical test, with *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. Statistical significance was concluded at $p < 0.05$.

REFERENCES

1. National Center for Health Statistics. Health, United States, 2016: With Chartbook on Long-term Trends in Health. Hyattsville, MD. 2017.
2. United Nations, Department of Economic and Social Affairs, Population Division (2017). World Population Ageing 2017 - Highlights (ST/ESA/SER.A/397).
3. Brown G. C. (2015). Living too long: the current focus of medical research on increasing the quantity, rather than the quality, of life is damaging our health and harming the economy. *EMBO reports*, *16*(2), 137–141. https://doi.org/10.15252/embr.201439518
4. Hansen, M., & Kennedy, B. K. (2016). Does Longer Lifespan Mean Longer Healthspan?. *Trends in cell biology*, *26*(8), 565–568. https://doi.org/10.1016/j.tcb.2016.05.002
5. Janssen, I., Shepard, D. S., Katzmarzyk, P. T., & Roubenoff, R. (2004). The healthcare costs of sarcopenia in the United States. *Journal of the American Geriatrics Society*, *52*(1), 80–85. https://doi.org/10.1111/j.1532-5415.2004.52014.x
6. Yang, Z., & Hall, A. G. (2008). The financial burden of overweight and obesity among elderly Americans: the dynamics of weight, longevity, and health care cost. *Health services research*, *43*(3), 849–868. https://doi.org/10.1111/j.1475-6773.2007.00801.x
7. Atella, V., Kopinska, J., Medea, G., Belotti, F., Tosti, V., Mortari, A. P., Cricelli, C., & Fontana, L. (2015). Excess body weight increases the burden of age-associated chronic diseases and their associated health care expenditures. *Aging*, *7*(10), 882–892. https://doi.org/10.18632/aging.100833
8. Sergiev, P. V., Dontsova, O. A., & Berezkin, G. V. (2015). Theories of aging: an ever-evolving field. *Acta naturae*, *7*(1), 9–18.
9. Finkelstein, E. A., Trogdon, J. G., Cohen, J. W., & Dietz, W. (2009). Annual medical spending attributable to obesity: payer-and service-specific estimates. *Health affairs (Project Hope)*, *28*(5), w822–w831. https://doi.org/10.1377/hlthaff.28.5.w822
10. Santilli, V., Bernetti, A., Mangone, M., & Paoloni, M. (2014). Clinical definition of sarcopenia. *Clinical cases in mineral and bone metabolism : the official journal of the Italian Society of Osteoporosis, Mineral Metabolism, and Skeletal Diseases*, *11*(3), 177–180.
11. Volpi, E., Nazemi, R., & Fujita, S. (2004). Muscle tissue changes with aging. *Current opinion in clinical nutrition and metabolic care*, *7*(4), 405–410. https://doi.org/10.1097/01.mco.0000134362.76653.b2
12. Siparsky, P. N., Kirkendall, D. T., & Garrett, W. E., Jr (2014). Muscle changes in aging: understanding sarcopenia. *Sports health*, *6*(1), 36–40. https://doi.org/10.1177/1941738113502296
13. Melton, L. J., 3rd, Khosla, S., Crowson, C. S., O'Connor, M. K., O'Fallon, W. M., & Riggs, B. L. (2000). Epidemiology of sarcopenia. *Journal of the American Geriatrics Society*, *48*(6), 625–630.
14. Goodpaster, B. H., Park, S. W., Harris, T. B., Kritchevsky, S. B., Nevitt, M., Schwartz, A. V., Simonsick, E. M., Tylavsky, F. A., Visser, M., & Newman, A. B. (2006). The loss of skeletal muscle strength, mass, and quality in older adults: the health, aging and body composition study. *The journals of gerontology. Series A, Biological sciences and medical sciences*, *61*(10), 1059–1064. https://doi.org/10.1093/gerona/61.10.1059

15. Hughes, V. A., Frontera, W. R., Wood, M., Evans, W. J., Dallal, G. E., Roubenoff, R., & Fiatarone Singh, M. A. (2001). Longitudinal muscle strength changes in older adults: influence of muscle mass, physical activity, and health. *The journals of gerontology. Series A, Biological sciences and medical sciences*, *56*(5), B209–B217. https://doi.org/10.1093/gerona/56.5.b209
16. Keller, K., & Engelhardt, M. (2014). Strength and muscle mass loss with aging process. Age and strength loss. *Muscles, ligaments and tendons journal*, *3*(4), 346–350.
17. Thompson L. V. (2009). Age-related muscle dysfunction. *Experimental gerontology*, *44*(1-2), 106–111. https://doi.org/10.1016/j.exger.2008.05.003
18. Larsson, L., Degens, H., Li, M., Salviati, L., Lee, Y. I., Thompson, W., Kirkland, J. L., & Sandri, M. (2019). Sarcopenia: Aging-Related Loss of Muscle Mass and Function. *Physiological reviews*, *99*(1), 427–511. https://doi.org/10.1152/physrev.00061.2017
19. Thompson L. V. (1994). Effects of age and training on skeletal muscle physiology and performance. *Physical therapy*, *74*(1), 71–81. https://doi.org/10.1093/ptj/74.1.71
20. Chiles Shaffer, N., Fabbri, E., Ferrucci, L., Shardell, M., Simonsick, E. M., & Studenski, S. (2017). Muscle Quality, Strength, and Lower Extremity Physical Performance in the Baltimore Longitudinal Study of Aging. *The Journal of frailty & aging*, *6*(4), 183–187. https://doi.org/10.14283/jfa.2017.24
21. Barbat-Artigas, S., Rolland, Y., Zamboni, M., & Aubertin-Leheudre, M. (2012). How to assess functional status: a new muscle quality index. *The journal of nutrition, health & aging*, *16*(1), 67–77. https://doi.org/10.1007/s12603-012-0004-5
22. McGregor, R. A., Cameron-Smith, D., & Poppitt, S. D. (2014). It is not just muscle mass: a review of muscle quality, composition and metabolism during ageing as determinants of muscle function and mobility in later life. *Longevity & healthspan*, *3*(1), 9. https://doi.org/10.1186/2046-2395-3-9
23. Picca, A., Mankowski, R. T., Burman, J. L., Donisi, L., Kim, J. S., Marzetti, E., & Leeuwenburgh, C. (2018). Mitochondrial quality control mechanisms as molecular targets in cardiac ageing. *Nature reviews. Cardiology*, *15*(9), 543–554. https://doi.org/10.1038/s41569-018-0059-z
24. Wolfson, L., Judge, J., Whipple, R., & King, M. (1995). Strength is a major factor in balance, gait, and the occurrence of falls. *The journals of gerontology. Series A, Biological sciences and medical sciences*, *50 Spec No*, 64–67. https://doi.org/10.1093/gerona/50a.special_issue.64
25. Bean, J. F., Kiely, D. K., Herman, S., Leveille, S. G., Mizer, K., Frontera, W. R., & Fielding, R. A. (2002). The relationship between leg power and physical performance in mobility-limited older people. *Journal of the American Geriatrics Society*, *50*(3), 461–467. https://doi.org/10.1046/j.1532-5415.2002.50111.x
26. Cruz-Jentoft, A. J., Bahat, G., Bauer, J., Boirie, Y., Bruyère, O., Cederholm, T., Cooper, C., Landi, F., Rolland, Y., Sayer, A. A., Schneider, S. M., Sieber, C. C., Topinkova, E., Vandewoude, M., Visser, M., Zamboni, M., & Writing Group for the European Working Group on Sarcopenia in Older People 2 (EWGSOP2), and the Extended Group for EWGSOP2 (2019). Sarcopenia: revised European consensus on definition and diagnosis. *Age and ageing*, *48*(1), 16–31. https://doi.org/10.1093/ageing/afy169

27. Batsis, J. A., & Villareal, D. T. (2018). Sarcopenic obesity in older adults: aetiology, epidemiology and treatment strategies. *Nature reviews. Endocrinology*, *14*(9), 513–537. https://doi.org/10.1038/s41574-018-0062-9
28. Cleasby, M. E., Jamieson, P. M., & Atherton, P. J. (2016). Insulin resistance and sarcopenia: mechanistic links between common co-morbidities. *The Journal of endocrinology*, *229*(2), R67–R81. https://doi.org/10.1530/JOE-15-0533
29. Zamboni, M., Mazzali, G., Fantin, F., Rossi, A., & Di Francesco, V. (2008). Sarcopenic obesity: a new category of obesity in the elderly. *Nutrition, metabolism, and cardiovascular diseases : NMCD*, *18*(5), 388–395. https://doi.org/10.1016/j.numecd.2007.10.002
30. Nair K. S. (2005). Aging muscle. *The American journal of clinical nutrition*, *81*(5), 953–963. https://doi.org/10.1093/ajcn/81.5.953
31. Slawik, M., & Vidal-Puig, A. J. (2006). Lipotoxicity, overnutrition and energy metabolism in aging. *Ageing research reviews*, *5*(2), 144–164. https://doi.org/10.1016/j.arr.2006.03.004
32. Flegal, K. M., Carroll, M. D., Kit, B. K., & Ogden, C. L. (2012). Prevalence of obesity and trends in the distribution of body mass index among US adults, 1999-2010. *JAMA*, *307*(5), 491–497. https://doi.org/10.1001/jama.2012.39
33. Heo, M., Faith, M. S., Pietrobelli, A., & Heymsfield, S. B. (2012). Percentage of body fat cutoffs by sex, age, and race-ethnicity in the US adult population from NHANES 1999-2004. *The American journal of clinical nutrition*, *95*(3), 594–602. https://doi.org/10.3945/ajcn.111.025171
34. Kuk, J. L., Saunders, T. J., Davidson, L. E., & Ross, R. (2009). Age-related changes in total and regional fat distribution. *Ageing research reviews*, *8*(4), 339–348. https://doi.org/10.1016/j.arr.2009.06.001
35. Tchkonia, T., Morbeck, D. E., Von Zglinicki, T., Van Deursen, J., Lustgarten, J., Scrable, H., Khosla, S., Jensen, M. D., & Kirkland, J. L. (2010). Fat tissue, aging, and cellular senescence. *Aging cell*, *9*(5), 667–684. https://doi.org/10.1111/j.1474-9726.2010.00608.x
36. Kinney, J. M. (1992). *Energy metabolism: tissue determinants and cellular corollaries*. raven Press.
37. DeFronzo, R. A., & Tripathy, D. (2009). Skeletal muscle insulin resistance is the primary defect in type 2 diabetes. *Diabetes care*, *32 Suppl 2*(Suppl 2), S157–S163. https://doi.org/10.2337/dc09-S302
38. Jensen, J., Rustad, P. I., Kolnes, A. J., & Lai, Y. C. (2011). The role of skeletal muscle glycogen breakdown for regulation of insulin sensitivity by exercise. *Frontiers in physiology*, *2*, 112. https://doi.org/10.3389/fphys.2011.00112
39. Glynn, E. L., Fry, C. S., Drummond, M. J., Dreyer, H. C., Dhanani, S., Volpi, E., & Rasmussen, B. B. (2010). Muscle protein breakdown has a minor role in the protein anabolic response to essential amino acid and carbohydrate intake following resistance exercise. *American journal of physiology. Regulatory, integrative and comparative physiology*, *299*(2), R533–R540. https://doi.org/10.1152/ajpregu.00077.2010
40. Tilg, H., & Moschen, A. R. (2006). Adipocytokines: mediators linking adipose tissue, inflammation and immunity. *Nature reviews. Immunology*, *6*(10), 772–783. https://doi.org/10.1038/nri1937
41. Cesari, M., Kritchevsky, S. B., Baumgartner, R. N., Atkinson, H. H., Penninx, B. W., Lenchik, L., Palla, S. L., Ambrosius, W. T., Tracy, R. P., & Pahor, M. (2005). Sarcopenia,

obesity, and inflammation--results from the Trial of Angiotensin Converting Enzyme Inhibition and Novel Cardiovascular Risk Factors study. *The American journal of clinical nutrition*, *82*(2), 428–434. https://doi.org/10.1093/ajcn.82.2.428

42. Schaap, L. A., Pluijm, S. M., Deeg, D. J., Harris, T. B., Kritchevsky, S. B., Newman, A. B., Colbert, L. H., Pahor, M., Rubin, S. M., Tylavsky, F. A., Visser, M., & Health ABC Study (2009). Higher inflammatory marker levels in older persons: associations with 5-year change in muscle mass and muscle strength. *The journals of gerontology. Series A, Biological sciences and medical sciences*, *64*(11), 1183–1189. https://doi.org/10.1093/gerona/glp097
43. Goodpaster, B. H., He, J., Watkins, S., & Kelley, D. E. (2001). Skeletal muscle lipid content and insulin resistance: evidence for a paradox in endurance-trained athletes. *The Journal of clinical endocrinology and metabolism*, *86*(12), 5755–5761. https://doi.org/10.1210/jcem.86.12.8075
44. Li, Y., Xu, S., Zhang, X., Yi, Z., & Cichello, S. (2015). Skeletal intramyocellular lipid metabolism and insulin resistance. *Biophysics reports*, *1*, 90–98. https://doi.org/10.1007/s41048-015-0013-0
45. Baumgartner R. N. (2000). Body composition in healthy aging. *Annals of the New York Academy of Sciences*, *904*, 437–448. https://doi.org/10.1111/j.1749-6632.2000.tb06498.x
46. Choi K. M. (2016). Sarcopenia and sarcopenic obesity. *The Korean journal of internal medicine*, *31*(6), 1054–1060. https://doi.org/10.3904/kjim.2016.193
47. Walston J. D. (2012). Sarcopenia in older adults. *Current opinion in rheumatology*, *24*(6), 623–627. https://doi.org/10.1097/BOR.0b013e328358d59b
48. Bray, G. A., & Ryan, D. H. (2014). Update on obesity pharmacotherapy. *Annals of the New York Academy of Sciences*, *1311*, 1–13. https://doi.org/10.1111/nyas.12328
49. Liu, H., Bravata, D. M., Olkin, I., Nayak, S., Roberts, B., Garber, A. M., & Hoffman, A. R. (2007). Systematic review: the safety and efficacy of growth hormone in the healthy elderly. *Annals of internal medicine*, *146*(2), 104–115. https://doi.org/10.7326/0003-4819-146-2-200701160-00005
50. Egerton, T., Chastin, S. F., Stensvold, D., & Helbostad, J. L. (2016). Fatigue May Contribute to Reduced Physical Activity Among Older People: An Observational Study. *The journals of gerontology. Series A, Biological sciences and medical sciences*, *71*(5), 670–676. https://doi.org/10.1093/gerona/glv150
51. Zhou, P., Hughes, A. K., Grady, S. C., & Fang, L. (2018). Physical activity and chronic diseases among older people in a mid-size city in China: a longitudinal investigation of bipolar effects. *BMC public health*, *18*(1), 486. https://doi.org/10.1186/s12889-018-5408-7
52. Leslie, W., & Hankey, C. (2015). Aging, Nutritional Status and Health. *Healthcare (Basel, Switzerland)*, *3*(3), 648–658. https://doi.org/10.3390/healthcare3030648
53. Anderson, J. W., Konz, E. C., Frederich, R. C., & Wood, C. L. (2001). Long-term weight-loss maintenance: a meta-analysis of US studies. *The American journal of clinical nutrition*, *74*(5), 579–584. https://doi.org/10.1093/ajcn/74.5.579
54. Welsh, D. K., Takahashi, J. S., & Kay, S. A. (2010). Suprachiasmatic nucleus: cell autonomy and network properties. *Annual review of physiology*, *72*, 551–577. https://doi.org/10.1146/annurev-physiol-021909-135919

55. Shi, M., & Zheng, X. (2013). Interactions between the circadian clock and metabolism: there are good times and bad times. *Acta biochimica et biophysica Sinica*, *45*(1), 61–69. https://doi.org/10.1093/abbs/gms110
56. Stenvers, D. J., Scheer, F., Schrauwen, P., la Fleur, S. E., & Kalsbeek, A. (2019). Circadian clocks and insulin resistance. *Nature reviews. Endocrinology*, *15*(2), 75–89. https://doi.org/10.1038/s41574-018-0122-1
57. Lowrey, P. L., & Takahashi, J. S. (2004). Mammalian circadian biology: elucidating genome-wide levels of temporal organization. *Annual review of genomics and human genetics*, *5*, 407–441. https://doi.org/10.1146/annurev.genom.5.061903.175925
58. Chaix, A., Manoogian, E., Melkani, G. C., & Panda, S. (2019). Time-Restricted Eating to Prevent and Manage Chronic Metabolic Diseases. *Annual review of nutrition*, *39*, 291–315. https://doi.org/10.1146/annurev-nutr-082018-124320
59. Takahashi J. S. (2015). Molecular components of the circadian clock in mammals. *Diabetes, obesity & metabolism*, *17 Suppl 1*(0 1), 6–11. https://doi.org/10.1111/dom.12514
60. Partch, C. L., Green, C. B., & Takahashi, J. S. (2014). Molecular architecture of the mammalian circadian clock. *Trends in cell biology*, *24*(2), 90–99. https://doi.org/10.1016/j.tcb.2013.07.002
61. Nakamura, T. J., Nakamura, W., Yamazaki, S., Kudo, T., Cutler, T., Colwell, C. S., & Block, G. D. (2011). Age-related decline in circadian output. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, *31*(28), 10201–10205. https://doi.org/10.1523/JNEUROSCI.0451-11.2011
62. Hood, S., & Amir, S. (2017). The aging clock: circadian rhythms and later life. *The Journal of clinical investigation*, *127*(2), 437–446. https://doi.org/10.1172/JCI90328
63. Hurd, M W, and M R Ralph. "The significance of circadian organization for longevity in the golden hamster." *Journal of biological rhythms* vol. 13,5 (1998): 430-6. doi:10.1177/074873098129000255
64. Farajnia, S., Michel, S., Deboer, T., vanderLeest, H. T., Houben, T., Rohling, J. H., Ramkisoensing, A., Yasenkov, R., & Meijer, J. H. (2012). Evidence for neuronal desynchrony in the aged suprachiasmatic nucleus clock. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, *32*(17), 5891–5899. https://doi.org/10.1523/JNEUROSCI.0469-12.2012
65. Sato, S., Solanas, G., Peixoto, F. O., Bee, L., Symeonidi, A., Schmidt, M. S., Brenner, C., Masri, S., Benitah, S. A., & Sassone-Corsi, P. (2017). Circadian Reprogramming in the Liver Identifies Metabolic Pathways of Aging. *Cell*, *170*(4), 664–677.e11. https://doi.org/10.1016/j.cell.2017.07.042
66. Solanas, G., Peixoto, F. O., Perdiguero, E., Jardí, M., Ruiz-Bonilla, V., Datta, D., Symeonidi, A., Castellanos, A., Welz, P. S., Caballero, J. M., Sassone-Corsi, P., Muñoz-Cánoves, P., & Benitah, S. A. (2017). Aged Stem Cells Reprogram Their Daily Rhythmic Functions to Adapt to Stress. *Cell*, *170*(4), 678–692.e20. https://doi.org/10.1016/j.cell.2017.07.035
67. Asher, G., & Schibler, U. (2011). Crosstalk between components of circadian and metabolic cycles in mammals. *Cell metabolism*, *13*(2), 125–137. https://doi.org/10.1016/j.cmet.2011.01.006
68. Eckel-Mahan, K. L., Patel, V. R., de Mateo, S., Orozco-Solis, R., Ceglia, N. J., Sahar, S., Dilag-Penilla, S. A., Dyar, K. A., Baldi, P., & Sassone-Corsi, P. (2013). Reprogramming

of the circadian clock by nutritional challenge. *Cell*, *155*(7), 1464–1478. https://doi.org/10.1016/j.cell.2013.11.034
69. Bass J. (2012). Circadian topology of metabolism. *Nature*, *491*(7424), 348–356. https://doi.org/10.1038/nature11704
70. Sahar, S., & Sassone-Corsi, P. (2012). Regulation of metabolism: the circadian clock dictates the time. *Trends in endocrinology and metabolism: TEM*, *23*(1), 1–8. https://doi.org/10.1016/j.tem.2011.10.005
71. Panda, S., Antoch, M. P., Miller, B. H., Su, A. I., Schook, A. B., Straume, M., Schultz, P. G., Kay, S. A., Takahashi, J. S., & Hogenesch, J. B. (2002). Coordinated transcription of key pathways in the mouse by the circadian clock. *Cell*, *109*(3), 307–320. https://doi.org/10.1016/s0092-8674(02)00722-5
72. Ramsey, K. M., Yoshino, J., Brace, C. S., Abrassart, D., Kobayashi, Y., Marcheva, B., Hong, H. K., Chong, J. L., Buhr, E. D., Lee, C., Takahashi, J. S., Imai, S., & Bass, J. (2009). Circadian clock feedback cycle through NAMPT-mediated NAD+ biosynthesis. *Science (New York, N.Y.)*, *324*(5927), 651–654. https://doi.org/10.1126/science.1171641
73. Kim, D. H., Jung, I. H., Kim, D. H., & Park, S. W. (2019). Knockout of longevity gene Sirt1 in zebrafish leads to oxidative injury, chronic inflammation, and reduced life span. *PloS one*, *14*(8), e0220581. https://doi.org/10.1371/journal.pone.0220581
74. Guarente L. (2011). Sirtuins, aging, and metabolism. *Cold Spring Harbor symposia on quantitative biology*, *76*, 81–90. https://doi.org/10.1101/sqb.2011.76.010629
75. Wang, R. H., Zhao, T., Cui, K., Hu, G., Chen, Q., Chen, W., Wang, X. W., Soto-Gutierrez, A., Zhao, K., & Deng, C. X. (2016). Negative reciprocal regulation between Sirt1 and Per2 modulates the circadian clock and aging. *Scientific reports*, *6*, 28633. https://doi.org/10.1038/srep28633
76. Asher, G., Reinke, H., Altmeyer, M., Gutierrez-Arcelus, M., Hottiger, M. O., & Schibler, U. (2010). Poly(ADP-ribose) polymerase 1 participates in the phase entrainment of circadian clocks to feeding. *Cell*, *142*(6), 943–953. https://doi.org/10.1016/j.cell.2010.08.016
77. Peek, C. B., Affinati, A. H., Ramsey, K. M., Kuo, H. Y., Yu, W., Sena, L. A., Ilkayeva, O., Marcheva, B., Kobayashi, Y., Omura, C., Levine, D. C., Bacsik, D. J., Gius, D., Newgard, C. B., Goetzman, E., Chandel, N. S., Denu, J. M., Mrksich, M., & Bass, J. (2013). Circadian clock NAD+ cycle drives mitochondrial oxidative metabolism in mice. *Science (New York, N.Y.)*, *342*(6158), 1243417. https://doi.org/10.1126/science.1243417
78. Kuang, J., Chen, L., Tang, Q., Zhang, J., Li, Y., & He, J. (2018). The Role of Sirt6 in Obesity and Diabetes. *Frontiers in physiology*, *9*, 135. https://doi.org/10.3389/fphys.2018.00135
79. Zarrinpar, A., Chaix, A., & Panda, S. (2016). Daily Eating Patterns and Their Impact on Health and Disease. *Trends in endocrinology and metabolism: TEM*, *27*(2), 69–83. https://doi.org/10.1016/j.tem.2015.11.007
80. Manoogian, E., & Panda, S. (2017). Circadian rhythms, time-restricted feeding, and healthy aging. *Ageing research reviews*, *39*, 59–67. https://doi.org/10.1016/j.arr.2016.12.006
81. Goel, N., Stunkard, A. J., Rogers, N. L., Van Dongen, H. P., Allison, K. C., O'Reardon, J. P., Ahima, R. S., Cummings, D. E., Heo, M., & Dinges, D. F. (2009). Circadian rhythm profiles in women with night eating syndrome. *Journal of biological rhythms*, *24*(1), 85–94. https://doi.org/10.1177/0748730408328914

82. Kohsaka, A., Laposky, A. D., Ramsey, K. M., Estrada, C., Joshu, C., Kobayashi, Y., Turek, F. W., & Bass, J. (2007). High-fat diet disrupts behavioral and molecular circadian rhythms in mice. *Cell metabolism*, *6*(5), 414–421. https://doi.org/10.1016/j.cmet.2007.09.006
83. Fonken, L. K., Workman, J. L., Walton, J. C., Weil, Z. M., Morris, J. S., Haim, A., & Nelson, R. J. (2010). Light at night increases body mass by shifting the time of food intake. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(43), 18664–18669. https://doi.org/10.1073/pnas.1008734107
84. Turek, F. W., Joshu, C., Kohsaka, A., Lin, E., Ivanova, G., McDearmon, E., Laposky, A., Losee-Olson, S., Easton, A., Jensen, D. R., Eckel, R. H., Takahashi, J. S., & Bass, J. (2005). Obesity and metabolic syndrome in circadian Clock mutant mice. *Science (New York, N.Y.)*, *308*(5724), 1043–1045. https://doi.org/10.1126/science.1108750
85. Paschos, G. K., Ibrahim, S., Song, W. L., Kunieda, T., Grant, G., Reyes, T. M., Bradfield, C. A., Vaughan, C. H., Eiden, M., Masoodi, M., Griffin, J. L., Wang, F., Lawson, J. A., & Fitzgerald, G. A. (2012). Obesity in mice with adipocyte-specific deletion of clock component Arntl. *Nature medicine*, *18*(12), 1768–1777. https://doi.org/10.1038/nm.2979
86. Adamovich, Y., Rousso-Noori, L., Zwighaft, Z., Neufeld-Cohen, A., Golik, M., Kraut-Cohen, J., Wang, M., Han, X., & Asher, G. (2014). Circadian clocks and feeding time regulate the oscillations and levels of hepatic triglycerides. *Cell metabolism*, *19*(2), 319–330. https://doi.org/10.1016/j.cmet.2013.12.016
87. Barclay, J. L., Husse, J., Bode, B., Naujokat, N., Meyer-Kovac, J., Schmid, S. M., Lehnert, H., & Oster, H. (2012). Circadian desynchrony promotes metabolic disruption in a mouse model of shiftwork. *PloS one*, *7*(5), e37150. https://doi.org/10.1371/journal.pone.0037150
88. Bray, M. S., Tsai, J. Y., Villegas-Montoya, C., Boland, B. B., Blasier, Z., Egbejimi, O., Kueht, M., & Young, M. E. (2010). Time-of-day-dependent dietary fat consumption influences multiple cardiometabolic syndrome parameters in mice. *International journal of obesity (2005)*, *34*(11), 1589–1598. https://doi.org/10.1038/ijo.2010.63
89. Eckel-Mahan, K. L., Patel, V. R., Mohney, R. P., Vignola, K. S., Baldi, P., & Sassone-Corsi, P. (2012). Coordination of the transcriptome and metabolome by the circadian clock. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(14), 5541–5546. https://doi.org/10.1073/pnas.1118726109
90. Vollmers, C., Gill, S., DiTacchio, L., Pulivarthy, S. R., Le, H. D., & Panda, S. (2009). Time of feeding and the intrinsic circadian clock drive rhythms in hepatic gene expression. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(50), 21453–21458. https://doi.org/10.1073/pnas.0909591106
91. Hatori, M., Vollmers, C., Zarrinpar, A., DiTacchio, L., Bushong, E. A., Gill, S., Leblanc, M., Chaix, A., Joens, M., Fitzpatrick, J. A., Ellisman, M. H., & Panda, S. (2012). Time-restricted feeding without reducing caloric intake prevents metabolic diseases in mice fed a high-fat diet. *Cell metabolism*, *15*(6), 848–860. https://doi.org/10.1016/j.cmet.2012.04.019
92. Chaix, A., Zarrinpar, A., Miu, P., & Panda, S. (2014). Time-restricted feeding is a preventative and therapeutic intervention against diverse nutritional challenges. *Cell metabolism*, *20*(6), 991–1005. https://doi.org/10.1016/j.cmet.2014.11.001
93. Villanueva, J. E., Livelo, C., Trujillo, A. S., Chandran, S., Woodworth, B., Andrade, L., Le, H. D., Manor, U., Panda, S., & Melkani, G. C. (2019). Time-restricted feeding restores muscle function in Drosophila models of obesity and circadian-rhythm disruption. *Nature communications*, *10*(1), 2700. https://doi.org/10.1038/s41467-019-10563-9

94. Stenholm, S., Vahtera, J., Kawachi, I., Pentti, J., Halonen, J. I., Westerlund, H., Razak, F., Subramanian, S. V., & Kivimäki, M. (2015). Patterns of weight gain in middle-aged and older US adults, 1992-2010. *Epidemiology (Cambridge, Mass.)*, *26*(2), 165–168. https://doi.org/10.1097/EDE.0000000000000228
95. Guo, S. S., Zeller, C., Chumlea, W. C., & Siervogel, R. M. (1999). Aging, body composition, and lifestyle: the Fels Longitudinal Study. *The American journal of clinical nutrition*, *70*(3), 405–411. https://doi.org/10.1093/ajcn/70.3.405
96. Coin, A., Sergi, G., Minicuci, N., Giannini, S., Barbiero, E., Manzato, E., Pedrazzoni, M., Minisola, S., Rossini, M., Del Puente, A., Zamboni, M., Inelmen, E. M., & Enzi, G. (2008). Fat-free mass and fat mass reference values by dual-energy X-ray absorptiometry (DEXA) in a 20-80 year-old Italian population. *Clinical nutrition (Edinburgh, Scotland)*, *27*(1), 87–94. https://doi.org/10.1016/j.clnu.2007.10.008
97. Goodpaster, B. H., Park, S. W., Harris, T. B., Kritchevsky, S. B., Nevitt, M., Schwartz, A. V., Simonsick, E. M., Tylavsky, F. A., Visser, M., & Newman, A. B. (2006). The loss of skeletal muscle strength, mass, and quality in older adults: the health, aging and body composition study. *The journals of gerontology. Series A, Biological sciences and medical sciences*, *61*(10), 1059–1064. https://doi.org/10.1093/gerona/61.10.1059
98. Jackson, A. S., Janssen, I., Sui, X., Church, T. S., & Blair, S. N. (2012). Longitudinal changes in body composition associated with healthy ageing: men, aged 20-96 years. *The British journal of nutrition*, *107*(7), 1085–1091. https://doi.org/10.1017/S0007114511003886
99. Kahn, B. B., & Flier, J. S. (2000). Obesity and insulin resistance. *The Journal of clinical investigation*, *106*(4), 473–481. https://doi.org/10.1172/JCI10842
100. Sheth, K. A., Iyer, C. C., Wier, C. G., Crum, A. E., Bratasz, A., Kolb, S. J., Clark, B. C., Burghes, A., & Arnold, W. D. (2018). Muscle strength and size are associated with motor unit connectivity in aged mice. *Neurobiology of aging*, *67*, 128–136. https://doi.org/10.1016/j.neurobiolaging.2018.03.016
101. Kwon, Y. N., & Yoon, S. S. (2017). Sarcopenia: Neurological Point of View. *Journal of bone metabolism*, *24*(2), 83–89. https://doi.org/10.11005/jbm.2017.24.2.83
102. Prior, S. J., Ryan, A. S., Blumenthal, J. B., Watson, J. M., Katzel, L. I., & Goldberg, A. P. (2016). Sarcopenia Is Associated With Lower Skeletal Muscle Capillarization and Exercise Capacity in Older Adults. *The journals of gerontology. Series A, Biological sciences and medical sciences*, *71*(8), 1096–1101. https://doi.org/10.1093/gerona/glw017
103. Narkar, V. A., Downes, M., Yu, R. T., Embler, E., Wang, Y. X., Banayo, E., Mihaylova, M. M., Nelson, M. C., Zou, Y., Juguilon, H., Kang, H., Shaw, R. J., & Evans, R. M. (2008). AMPK and PPARdelta agonists are exercise mimetics. *Cell*, *134*(3), 405–415. https://doi.org/10.1016/j.cell.2008.06.051
104. Addison, O., Marcus, R. L., Lastayo, P. C., & Ryan, A. S. (2014). Intermuscular fat: a review of the consequences and causes. *International journal of endocrinology*, *2014*, 309570. https://doi.org/10.1155/2014/309570
105. Knuiman, P., Hopman, M. T., & Mensink, M. (2015). Glycogen availability and skeletal muscle adaptations with endurance and resistance exercise. *Nutrition & metabolism*, *12*, 59. https://doi.org/10.1186/s12986-015-0055-9
106. Deacon R. M. (2013). Measuring motor coordination in mice. *Journal of visualized experiments : JoVE*, (75), e2609. https://doi.org/10.3791/2609

107. Deacon R. M. (2013). Measuring the strength of mice. *Journal of visualized experiments : JoVE*, (76), 2610. https://doi.org/10.3791/2610
108. Arany Z. (2008). PGC-1 coactivators and skeletal muscle adaptations in health and disease. *Current opinion in genetics & development*, *18*(5), 426–434. https://doi.org/10.1016/j.gde.2008.07.018
109. Stancic, A., Buzadzic, B., Korac, A., Otasevic, V., Jankovic, A., Vucetic, M., Markelic, M., Velickovic, K., Golic, I., & Korac, B. (2013). Regulatory role of PGC-1α/PPAR signaling in skeletal muscle metabolic recruitment during cold acclimation. *The Journal of experimental biology*, *216*(Pt 22), 4233–4241. https://doi.org/10.1242/jeb.089334
110. Ravnskjaer, K., Frigerio, F., Boergesen, M., Nielsen, T., Maechler, P., & Mandrup, S. (2010). PPARdelta is a fatty acid sensor that enhances mitochondrial oxidation in insulin-secreting cells and protects against fatty acid-induced dysfunction. *Journal of lipid research*, *51*(6), 1370–1379. https://doi.org/10.1194/jlr.M001123
111. Panda S. (2016). Circadian physiology of metabolism. *Science (New York, N.Y.)*, *354*(6315), 1008–1015. https://doi.org/10.1126/science.aah4967
112. Owen, O. E., Reichard, G. A., Jr, Patel, M. S., & Boden, G. (1979). Energy metabolism in feasting and fasting. *Advances in experimental medicine and biology*, *111*, 169–188. https://doi.org/10.1007/978-1-4757-0734-2_8
113. Vendelbo, M. H., Jørgensen, J. O., Pedersen, S. B., Gormsen, L. C., Lund, S., Schmitz, O., Jessen, N., & Møller, N. (2010). Exercise and fasting activate growth hormone-dependent myocellular signal transducer and activator of transcription-5b phosphorylation and insulin-like growth factor-I messenger ribonucleic acid expression in humans. *The Journal of clinical endocrinology and metabolism*, *95*(9), E64–E68. https://doi.org/10.1210/jc.2010-0689
114. Nørrelund, H., Nair, K. S., Jørgensen, J. O., Christiansen, J. S., & Møller, N. (2001). The protein-retaining effects of growth hormone during fasting involve inhibition of muscle-protein breakdown. *Diabetes*, *50*(1), 96–104. https://doi.org/10.2337/diabetes.50.1.96
115. Masiero, E., Agatea, L., Mammucari, C., Blaauw, B., Loro, E., Komatsu, M., Metzger, D., Reggiani, C., Schiaffino, S., & Sandri, M. (2009). Autophagy is required to maintain muscle mass. *Cell metabolism*, *10*(6), 507–515. https://doi.org/10.1016/j.cmet.2009.10.008
116. Brown, S. A., Schmitt, K., & Eckert, A. (2011). Aging and circadian disruption: causes and effects. *Aging*, *3*(8), 813–817. https://doi.org/10.18632/aging.100366
117. Baron, K. G., Reid, K. J., Kim, T., Van Horn, L., Attarian, H., Wolfe, L., Siddique, J., Santostasi, G., & Zee, P. C. (2017). Circadian timing and alignment in healthy adults: associations with BMI, body fat, caloric intake and physical activity. *International journal of obesity (2005)*, *41*(2), 203–209. https://doi.org/10.1038/ijo.2016.194
118. Shou, J., Chen, P. J., & Xiao, W. H. (2020). Mechanism of increased risk of insulin resistance in aging skeletal muscle. *Diabetology & metabolic syndrome*, *12*, 14. https://doi.org/10.1186/s13098-020-0523-x
119. de Luca, C., & Olefsky, J. M. (2008). Inflammation and insulin resistance. *FEBS letters*, *582*(1), 97–105. https://doi.org/10.1016/j.febslet.2007.11.057

120. la Fleur, S. E., Kalsbeek, A., Wortel, J., Fekkes, M. L., & Buijs, R. M. (2001). A daily rhythm in glucose tolerance: a role for the suprachiasmatic nucleus. *Diabetes*, *50*(6), 1237–1243. https://doi.org/10.2337/diabetes.50.6.1237
121. Morris, C. J., Yang, J. N., Garcia, J. I., Myers, S., Bozzi, I., Wang, W., Buxton, O. M., Shea, S. A., & Scheer, F. A. (2015). Endogenous circadian system and circadian misalignment impact glucose tolerance via separate mechanisms in humans. *Proceedings of the National Academy of Sciences of the United States of America*, *112*(17), E2225–E2234. https://doi.org/10.1073/pnas.1418955112
122. Kohsaka, A., Laposky, A. D., Ramsey, K. M., Estrada, C., Joshu, C., Kobayashi, Y., Turek, F. W., & Bass, J. (2007). High-fat diet disrupts behavioral and molecular circadian rhythms in mice. *Cell metabolism*, *6*(5), 414–421. https://doi.org/10.1016/j.cmet.2007.09.006
123. Shi, S. Q., Ansari, T. S., McGuinness, O. P., Wasserman, D. H., & Johnson, C. H. (2013). Circadian disruption leads to insulin resistance and obesity. *Current biology : CB*, *23*(5), 372–381. https://doi.org/10.1016/j.cub.2013.01.048
124. Kalyani, R. R., Varadhan, R., Weiss, C. O., Fried, L. P., & Cappola, A. R. (2012). Frailty status and altered glucose-insulin dynamics. *The journals of gerontology. Series A, Biological sciences and medical sciences*, *67*(12), 1300–1306. https://doi.org/10.1093/gerona/glr141
125. Basse, A. L., Dalbram, E., Larsson, L., Gerhart-Hines, Z., Zierath, J. R., & Treebak, J. T. (2018). Skeletal Muscle Insulin Sensitivity Show Circadian Rhythmicity Which Is Independent of Exercise Training Status. *Frontiers in physiology*, *9*, 1198. https://doi.org/10.3389/fphys.2018.01198
126. Aras, E., Ramadori, G., Kinouchi, K., Liu, Y., Ioris, R. M., Brenachot, X., Ljubicic, S., Veyrat-Durebex, C., Mannucci, S., Galié, M., Baldi, P., Sassone-Corsi, P., & Coppari, R. (2019). Light Entrains Diurnal Changes in Insulin Sensitivity of Skeletal Muscle via Ventromedial Hypothalamic Neurons. *Cell reports*, *27*(8), 2385–2398.e3. https://doi.org/10.1016/j.celrep.2019.04.093
127. Traylor, D. A., Gorissen, S., & Phillips, S. M. (2018). Perspective: Protein Requirements and Optimal Intakes in Aging: Are We Ready to Recommend More Than the Recommended Daily Allowance?. *Advances in nutrition (Bethesda, Md.)*, *9*(3), 171–182. https://doi.org/10.1093/advances/nmy003
128. Leslie, W., & Hankey, C. (2015). Aging, Nutritional Status and Health. *Healthcare (Basel, Switzerland)*, *3*(3), 648–658. https://doi.org/10.3390/healthcare3030648
129. Mattson, M. P., Longo, V. D., & Harvie, M. (2017). Impact of intermittent fasting on health and disease processes. *Ageing research reviews*, *39*, 46–58. https://doi.org/10.1016/j.arr.2016.10.005
130. Longo, V. D., & Panda, S. (2016). Fasting, Circadian Rhythms, and Time-Restricted Feeding in Healthy Lifespan. *Cell metabolism*, *23*(6), 1048–1059. https://doi.org/10.1016/j.cmet.2016.06.001
131. Wolfson, L., Judge, J., Whipple, R., & King, M. (1995). Strength is a major factor in balance, gait, and the occurrence of falls. *The journals of gerontology. Series A, Biological sciences and medical sciences*, *50 Spec No*, 64–67. https://doi.org/10.1093/gerona/50a.special_issue.64

132. Wan, J. J., Qin, Z., Wang, P. Y., Sun, Y., & Liu, X. (2017). Muscle fatigue: general understanding and treatment. *Experimental & molecular medicine*, *49*(10), e384. https://doi.org/10.1038/emm.2017.194
133. Hollands, M. A., & Cawthorne, M. A. (1981). Important sites of lipogenesis in the mouse other than liver and white adipose tissue. *The Biochemical journal*, *196*(2), 645–647. https://doi.org/10.1042/bj1960645
134. Houten, S. M., & Wanders, R. J. (2010). A general introduction to the biochemistry of mitochondrial fatty acid β-oxidation. *Journal of inherited metabolic disease*, *33*(5), 469–477. https://doi.org/10.1007/s10545-010-9061-2
135. Takahashi, S., Tanaka, T., & Sakai, J. (2007). New therapeutic target for metabolic syndrome: PPARdelta. *Endocrine journal*, *54*(3), 347–357. https://doi.org/10.1507/endocrj.kr-99
136. Tanaka, T., Yamamoto, J., Iwasaki, S., Asaba, H., Hamura, H., Ikeda, Y., Watanabe, M., Magoori, K., Ioka, R. X., Tachibana, K., Watanabe, Y., Uchiyama, Y., Sumi, K., Iguchi, H., Ito, S., Doi, T., Hamakubo, T., Naito, M., Auwerx, J., Yanagisawa, M., … Sakai, J. (2003). Activation of peroxisome proliferator-activated receptor delta induces fatty acid beta-oxidation in skeletal muscle and attenuates metabolic syndrome. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(26), 15924–15929. https://doi.org/10.1073/pnas.0306981100
137. Pettersen, I., Tusubira, D., Ashrafi, H., Dyrstad, S. E., Hansen, L., Liu, X. Z., Nilsson, L., Løvsletten, N. G., Berge, K., Wergedahl, H., Bjørndal, B., Fluge, Ø., Bruland, O., Rustan, A. C., Halberg, N., Røsland, G. V., Berge, R. K., & Tronstad, K. J. (2019). Upregulated PDK4 expression is a sensitive marker of increased fatty acid oxidation. *Mitochondrion*, *49*, 97–110. https://doi.org/10.1016/j.mito.2019.07.009
138. Gene [Pdk4]. Bethesda (MD): National Library of Medicine (US), National Center for Biotechnology Information; 2004 – [cited 2020 06 07]. Available from: https://www.ncbi.nlm.nih.gov/gene/5166#reference-sequences
139. Wende, A. R., Huss, J. M., Schaeffer, P. J., Giguère, V., & Kelly, D. P. (2005). PGC-1alpha coactivates PDK4 gene expression via the orphan nuclear receptor ERRalpha: a mechanism for transcriptional control of muscle glucose metabolism. *Molecular and cellular biology*, *25*(24), 10684–10694. https://doi.org/10.1128/MCB.25.24.10684-10694.2005
140. Yamada, K., & Noguchi, T. (1999). Nutrient and hormonal regulation of pyruvate kinase gene expression. *The Biochemical journal*, *337 (Pt 1)*(Pt 1), 1–11.
141. de Goede, P., Sen, S., Oosterman, J. E., Foppen, E., Jansen, R., la Fleur, S. E., Challet, E., & Kalsbeek, A. (2017). Differential effects of diet composition and timing of feeding behavior on rat brown adipose tissue and skeletal muscle peripheral clocks. *Neurobiology of sleep and circadian rhythms*, *4*, 24–33. https://doi.org/10.1016/j.nbscr.2017.09.002
142. Handschin, C., & Spiegelman, B. M. (2006). Peroxisome proliferator-activated receptor gamma coactivator 1 coactivators, energy homeostasis, and metabolism. *Endocrine reviews*, *27*(7), 728–735. https://doi.org/10.1210/er.2006-0037
143. Wu, Z., Puigserver, P., Andersson, U., Zhang, C., Adelmant, G., Mootha, V., Troy, A., Cinti, S., Lowell, B., Scarpulla, R. C., & Spiegelman, B. M. (1999). Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. *Cell*, *98*(1), 115–124. https://doi.org/10.1016/S0092-8674(00)80611-X

144. Bonen A. (2009). PGC-1alpha-induced improvements in skeletal muscle metabolism and insulin sensitivity. *Applied physiology, nutrition, and metabolism = Physiologie appliquee, nutrition et metabolisme*, *34*(3), 307–314. https://doi.org/10.1139/H09-008
145. Gill, J. F., Santos, G., Schnyder, S., & Handschin, C. (2018). PGC-1α affects aging-related changes in muscle and motor function by modulating specific exercise-mediated changes in old mice. *Aging cell*, *17*(1), e12697. https://doi.org/10.1111/acel.12697
146. Anderson, R., & Prolla, T. (2009). PGC-1alpha in aging and anti-aging interventions. *Biochimica et biophysica acta*, *1790*(10), 1059–1066. https://doi.org/10.1016/j.bbagen.2009.04.005
147. Bak, A. M., Vendelbo, M. H., Christensen, B., Viggers, R., Bibby, B. M., Rungby, J., Jørgensen, J., Møller, N., & Jessen, N. (2018). Prolonged fasting-induced metabolic signatures in human skeletal muscle of lean and obese men. *PloS one*, *13*(9), e0200817. https://doi.org/10.1371/journal.pone.0200817
148. van Norren, K., Rusli, F., van Dijk, M., Lute, C., Nagel, J., Dijk, F. J., Dwarkasing, J., Boekschoten, M. V., Luiking, Y., Witkamp, R. F., Müller, M., & Steegenga, W. T. (2015). Behavioural changes are a major contributing factor in the reduction of sarcopenia in caloric-restricted ageing mice. *Journal of cachexia, sarcopenia and muscle*, *6*(3), 253–268. https://doi.org/10.1002/jcsm.12024
149. Gotthardt, J. D., Verpeut, J. L., Yeomans, B. L., Yang, J. A., Yasrebi, A., Roepke, T. A., & Bello, N. T. (2016). Intermittent Fasting Promotes Fat Loss With Lean Mass Retention, Increased Hypothalamic Norepinephrine Content, and Increased Neuropeptide Y Gene Expression in Diet-Induced Obese Male Mice. *Endocrinology*, *157*(2), 679–691. https://doi.org/10.1210/en.2015-1622
150. Sandri, M., Lin, J., Handschin, C., Yang, W., Arany, Z. P., Lecker, S. H., Goldberg, A. L., & Spiegelman, B. M. (2006). PGC-1alpha protects skeletal muscle from atrophy by suppressing FoxO3 action and atrophy-specific gene transcription. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(44), 16260–16265. https://doi.org/10.1073/pnas.0607795103
151. Geng, T., Li, P., Yin, X., & Yan, Z. (2011). PGC-1α promotes nitric oxide antioxidant defenses and inhibits FOXO signaling against cardiac cachexia in mice. *The American journal of pathology*, *178*(4), 1738–1748. https://doi.org/10.1016/j.ajpath.2011.01.005
152. Brault, J. J., Jespersen, J. G., & Goldberg, A. L. (2010). Peroxisome proliferator-activated receptor gamma coactivator 1alpha or 1beta overexpression inhibits muscle protein degradation, induction of ubiquitin ligases, and disuse atrophy. *The Journal of biological chemistry*, *285*(25), 19460–19471. https://doi.org/10.1074/jbc.M110.113092
153. Liang, H., & Ward, W. F. (2006). PGC-1alpha: a key regulator of energy metabolism. *Advances in physiology education*, *30*(4), 145–151. https://doi.org/10.1152/advan.00052.2006
154. Zhang, L., Zhou, Y., Wu, W., Hou, L., Chen, H., Zuo, B., Xiong, Y., & Yang, J. (2017). Skeletal Muscle-Specific Overexpression of PGC-1α Induces Fiber-Type Conversion through Enhanced Mitochondrial Respiration and Fatty Acid Oxidation in Mice and Pigs. *International journal of biological sciences*, *13*(9), 1152–1162. https://doi.org/10.7150/ijbs.20132

155. Tadaishi, M., Miura, S., Kai, Y., Kano, Y., Oishi, Y., & Ezaki, O. (2011). Skeletal muscle-specific expression of PGC-1α-b, an exercise-responsive isoform, increases exercise capacity and peak oxygen uptake. *PloS one*, *6*(12), e28290. https://doi.org/10.1371/journal.pone.0028290
156. Stuart, C. A., McCurry, M. P., Marino, A., South, M. A., Howell, M. E., Layne, A. S., Ramsey, M. W., & Stone, M. H. (2013). Slow-twitch fiber proportion in skeletal muscle correlates with insulin responsiveness. *The Journal of clinical endocrinology and metabolism*, *98*(5), 2027–2036. https://doi.org/10.1210/jc.2012-3876
157. Choi, C. S., Befroy, D. E., Codella, R., Kim, S., Reznick, R. M., Hwang, Y. J., Liu, Z. X., Lee, H. Y., Distefano, A., Samuel, V. T., Zhang, D., Cline, G. W., Handschin, C., Lin, J., Petersen, K. F., Spiegelman, B. M., & Shulman, G. I. (2008). Paradoxical effects of increased expression of PGC-1alpha on muscle mitochondrial function and insulin-stimulated muscle glucose metabolism. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(50), 19926–19931. https://doi.org/10.1073/pnas.0810339105
158. Mootha, V. K., Lindgren, C. M., Eriksson, K. F., Subramanian, A., Sihag, S., Lehar, J., Puigserver, P., Carlsson, E., Ridderstråle, M., Laurila, E., Houstis, N., Daly, M. J., Patterson, N., Mesirov, J. P., Golub, T. R., Tamayo, P., Spiegelman, B., Lander, E. S., Hirschhorn, J. N., Altshuler, D., … Groop, L. C. (2003). PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. *Nature genetics*, *34*(3), 267–273. https://doi.org/10.1038/ng1180

159. Michael, L. F., Wu, Z., Cheatham, R. B., Puigserver, P., Adelmant, G., Lehman, J. J., Kelly, D. P., & Spiegelman, B. M. (2001). Restoration of insulin-sensitive glucose transporter (GLUT4) gene expression in muscle cells by the transcriptional coactivator PGC-1. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(7), 3820–3825. https://doi.org/10.1073/pnas.061035098
160. Yoon, J. C., Puigserver, P., Chen, G., Donovan, J., Wu, Z., Rhee, J., Adelmant, G., Stafford, J., Kahn, C. R., Granner, D. K., Newgard, C. B., & Spiegelman, B. M. (2001). Control of hepatic gluconeogenesis through the transcriptional coactivator PGC-1. *Nature*, *413*(6852), 131–138. https://doi.org/10.1038/35093050
161. Herzig, S., Long, F., Jhala, U. S., Hedrick, S., Quinn, R., Bauer, A., Rudolph, D., Schutz, G., Yoon, C., Puigserver, P., Spiegelman, B., & Montminy, M. (2001). CREB regulates hepatic gluconeogenesis through the coactivator PGC-1. *Nature*, *413*(6852), 179–183. https://doi.org/10.1038/35093131
162. Janssen, I., Heymsfield, S. B., Wang, Z. M., & Ross, R. (2000). Skeletal muscle mass and distribution in 468 men and women aged 18-88 yr. *Journal of applied physiology (Bethesda, Md. : 1985)*, *89*(1), 81–88. https://doi.org/10.1152/jappl.2000.89.1.81
163. Miyazaki, M., Schroder, E., Edelmann, S. E., Hughes, M. E., Kornacker, K., Balke, C. W., & Esser, K. A. (2011). Age-associated disruption of molecular clock expression in skeletal muscle of the spontaneously hypertensive rat. *PloS one*, *6*(11), e27168. https://doi.org/10.1371/journal.pone.0027168
164. Wolff, G., & Esser, K. A. (2012). Scheduled exercise phase shifts the circadian clock in skeletal muscle. *Medicine and science in sports and exercise*, *44*(9), 1663–1670. https://doi.org/10.1249/MSS.0b013e318255cf4c

165. Gabriel, B. M., & Zierath, J. R. (2019). Circadian rhythms and exercise - re-setting the clock in metabolic disease. *Nature reviews. Endocrinology*, *15*(4), 197–206. https://doi.org/10.1038/s41574-018-0150-x
166. Jordan, S. D., Kriebs, A., Vaughan, M., Duglan, D., Fan, W., Henriksson, E., Huber, A. L., Papp, S. J., Nguyen, M., Afetian, M., Downes, M., Yu, R. T., Kralli, A., Evans, R. M., & Lamia, K. A. (2017). CRY1/2 Selectively Repress PPARδ and Limit Exercise Capacity. *Cell metabolism*, *26*(1), 243–255.e6. https://doi.org/10.1016/j.cmet.2017.06.002
167. Schiaffino, S., Blaauw, B., & Dyar, K. A. (2016). The functional significance of the skeletal muscle clock: lessons from *Bmal1* knockout models. *Skeletal muscle*, *6*, 33. https://doi.org/10.1186/s13395-016-0107-5
168. Kondratov, R. V., Kondratova, A. A., Gorbacheva, V. Y., Vykhovanets, O. V., & Antoch, M. P. (2006). Early aging and age-related pathologies in mice deficient in BMAL1, the core componentof the circadian clock. *Genes & development*, *20*(14), 1868–1873. https://doi.org/10.1101/gad.1432206
169. Dallmann, R., Viola, A. U., Tarokh, L., Cajochen, C., & Brown, S. A. (2012). The human circadian metabolome. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(7), 2625–2629. https://doi.org/10.1073/pnas.1114410109
170. Nakamura, K., Fuster, J. J., & Walsh, K. (2014). Adipokines: a link between obesity and cardiovascular disease. *Journal of cardiology*, *63*(4), 250–259. https://doi.org/10.1016/j.jjcc.2013.11.006
171. Gomes, M. J., Martinez, P. F., Pagan, L. U., Damatto, R. L., Cezar, M., Lima, A., Okoshi, K., & Okoshi, M. P. (2017). Skeletal muscle aging: influence of oxidative stress and physical exercise. *Oncotarget*, *8*(12), 20428–20440. https://doi.org/10.18632/oncotarget.14670
172. Ebner, N., Sliziuk, V., Scherbakov, N., & Sandek, A. (2015). Muscle wasting in ageing and chronic illness. *ESC heart failure*, *2*(2), 58–68. https://doi.org/10.1002/ehf2.12033
173. Hodge, B. A., Wen, Y., Riley, L. A., Zhang, X., England, J. H., Harfmann, B. D., Schroder, E. A., & Esser, K. A. (2015). The endogenous molecular clock orchestrates the temporal separation of substrate metabolism in skeletal muscle. *Skeletal muscle*, *5*, 17. https://doi.org/10.1186/s13395-015-0039-5
174. Lira, V. A., Benton, C. R., Yan, Z., & Bonen, A. (2010). PGC-1alpha regulation by exercise training and its influences on muscle function and insulin sensitivity. *American journal of physiology. Endocrinology and metabolism*, *299*(2), E145–E161. https://doi.org/10.1152/ajpendo.00755.2009
175. Muoio, D. M., & Koves, T. R. (2007). Skeletal muscle adaptation to fatty acid depends on coordinated actions of the PPARs and PGC1 alpha: implications for metabolic disease. *Applied physiology, nutrition, and metabolism = Physiologie appliquee, nutrition et metabolisme*, *32*(5), 874–883. https://doi.org/10.1139/H07-083
176. Witczak, C. A., Sharoff, C. G., & Goodyear, L. J. (2008). AMP-activated protein kinase in skeletal muscle: from structure and localization to its role as a master regulator of cellular metabolism. *Cellular and molecular life sciences : CMLS*, *65*(23), 3737–3755. https://doi.org/10.1007/s00018-008-8244-6
177. Asher, G., Gatfield, D., Stratmann, M., Reinke, H., Dibner, C., Kreppel, F., Mostoslavsky, R., Alt, F. W., & Schibler, U. (2008). SIRT1 regulates circadian clock gene

expression through PER2 deacetylation. *Cell*, *134*(2), 317–328. https://doi.org/10.1016/j.cell.2008.06.050

178. Lamia, K. A., Sachdeva, U. M., DiTacchio, L., Williams, E. C., Alvarez, J. G., Egan, D. F., Vasquez, D. S., Juguilon, H., Panda, S., Shaw, R. J., Thompson, C. B., & Evans, R. M. (2009). AMPK regulates the circadian clock by cryptochrome phosphorylation and degradation. *Science (New York, N.Y.)*, *326*(5951), 437–440. https://doi.org/10.1126/science.1172156

179. Liu, C., Li, S., Liu, T., Borjigin, J., & Lin, J. D. (2007). Transcriptional coactivator PGC-1alpha integrates the mammalian clock and energy metabolism. *Nature*, *447*(7143), 477–481. https://doi.org/10.1038/nature05767

180. Lefta, M., Wolff, G., & Esser, K. A. (2011). Circadian rhythms, the molecular clock, and skeletal muscle. *Current topics in developmental biology*, *96*, 231–271. https://doi.org/10.1016/B978-0-12-385940-2.00009-7

181. Dyar, K. A., Ciciliot, S., Wright, L. E., Biensø, R. S., Tagliazucchi, G. M., Patel, V. R., Forcato, M., Paz, M. I., Gudiksen, A., Solagna, F., Albiero, M., Moretti, I., Eckel-Mahan, K. L., Baldi, P., Sassone-Corsi, P., Rizzuto, R., Bicciato, S., Pilegaard, H., Blaauw, B., & Schiaffino, S. (2013). Muscle insulin sensitivity and glucose metabolism are controlled by the intrinsic muscle clock. *Molecular metabolism*, *3*(1), 29–41. https://doi.org/10.1016/j.molmet.2013.10.005

182. Andrews, J. L., Zhang, X., McCarthy, J. J., McDearmon, E. L., Hornberger, T. A., Russell, B., Campbell, K. S., Arbogast, S., Reid, M. B., Walker, J. R., Hogenesch, J. B., Takahashi, J. S., & Esser, K. A. (2010). CLOCK and BMAL1 regulate MyoD and are necessary for maintenance of skeletal muscle phenotype and function. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(44), 19090–19095. https://doi.org/10.1073/pnas.1014523107

183. Rudic, R. D., McNamara, P., Curtis, A. M., Boston, R. C., Panda, S., Hogenesch, J. B., & Fitzgerald, G. A. (2004). BMAL1 and CLOCK, two essential components of the circadian clock, are involved in glucose homeostasis. *PLoS biology*, *2*(11), e377. https://doi.org/10.1371/journal.pbio.0020377

184. Chaix, A., & Panda, S. (2019). Timing tweaks exercise. *Nature reviews. Endocrinology*, *15*(8), 440–441. https://doi.org/10.1038/s41574-019-0229-z

FIGURES :

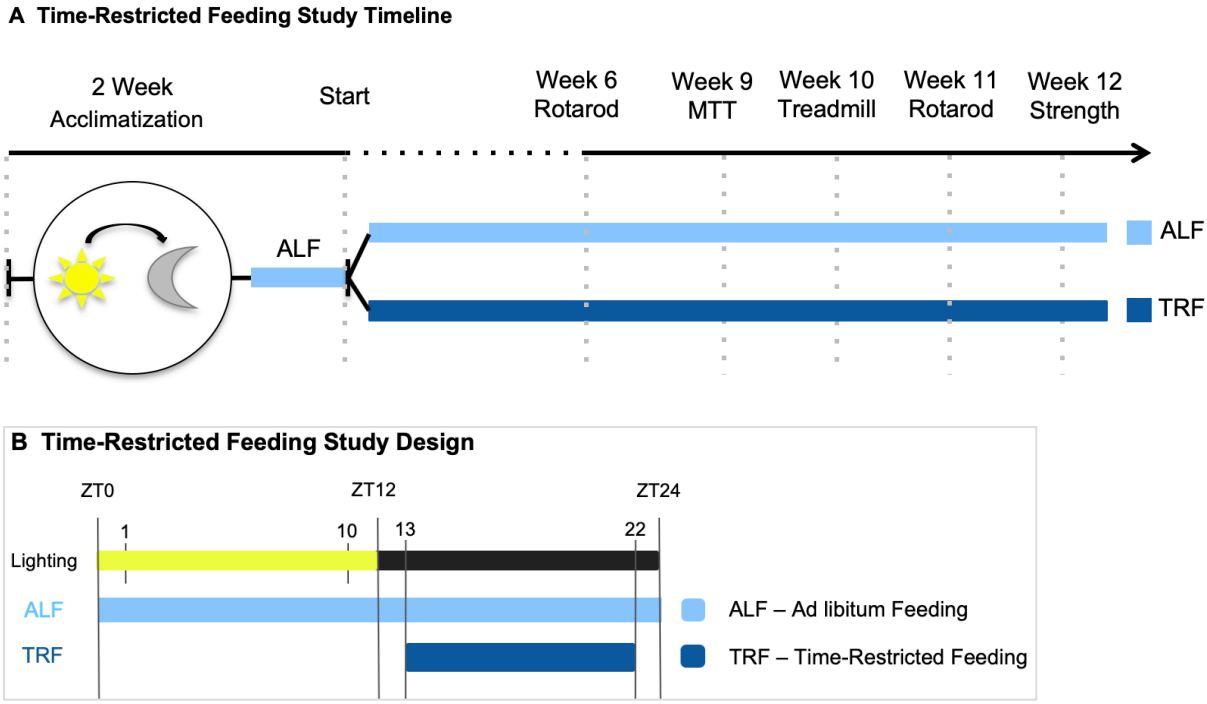

Figure 1 : Time-Restricted Feeding Study design and Timeline.

Schematic representation of complete **(A)** study timeline and **(B)** study design. **(A)** Mice were acclimatized to a reverse light-dark cycle for 2 weeks on a normal chow diet. All mice were given ad-libitum access to the 'western diet' 5 days prior to being randomly assigned to Time-Restricted Feeding (TRF) or Ad-Libitum Feeding (ALF) groups. First rotarod assay was performed during week 6 and the second was performed during week 11. Meal Tolerance test (MTT), treadmill test, and grip strength test were performed during week 4, week 10, and week 12 respectively. **(B)** TRF mice had food access restricted to a 9 hour window during the dark phase from ZT13 – ZT22 (where ZT0 denotes lights on), 7 days a week. ALF mice had ad-libitum access to food for 24 hours of the day (ZT0 – ZT24), 7 days a week.

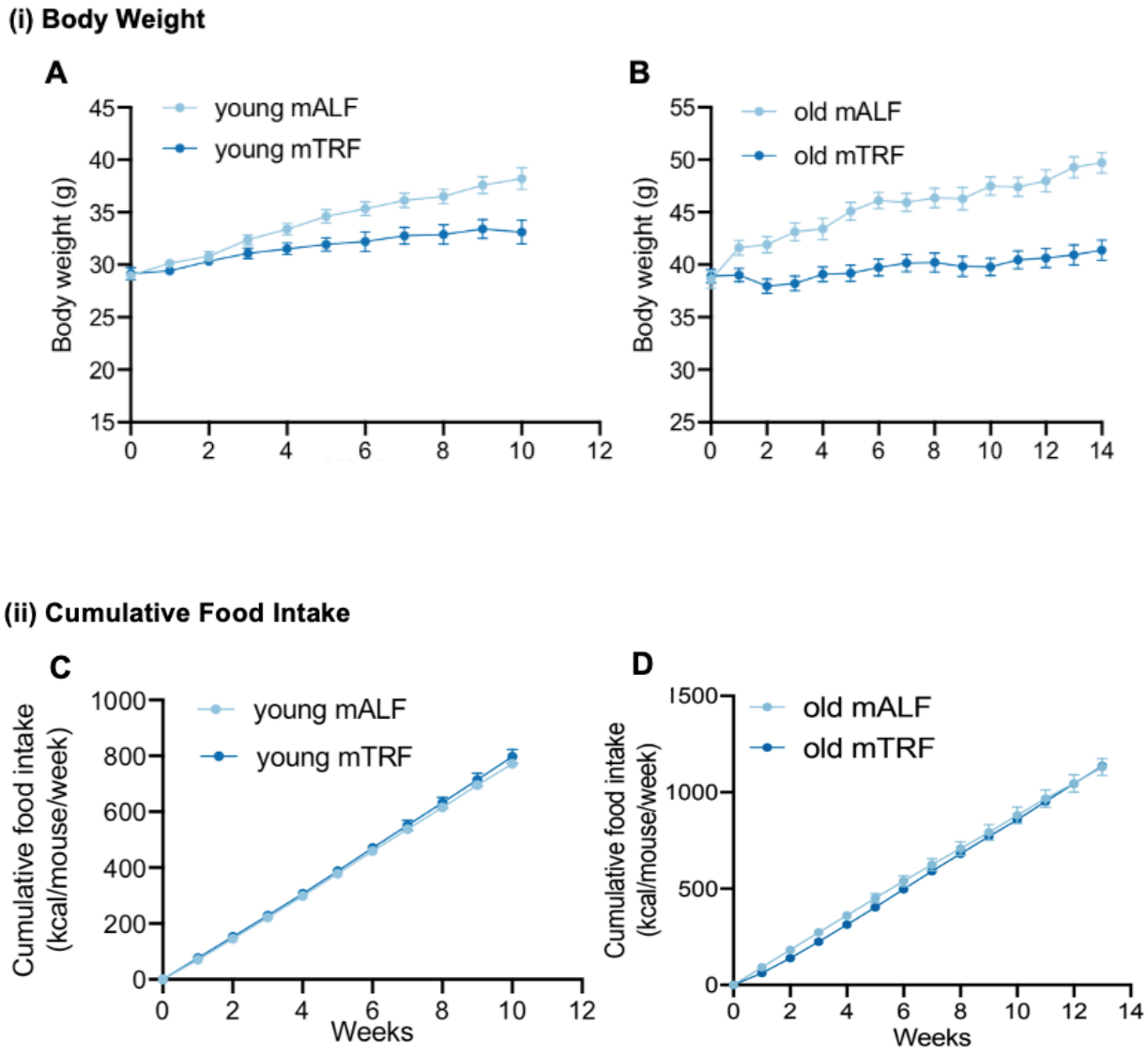

Figure 2 : Body weight and cumulative food consumption of young/old mice on TRF/ALF. **(i)** Body weight in grams of **(A)** young male ALF mice (young mALF) vs. young male TRF mice (young mTRF) (mean ± SD) and **(B)** old male ALF mice (old mALF) vs. old male TRF mice (old mTRF) (mean ± SEM). **(ii)** Cumulative food intake (mean ± SD) represented as kcal/mouse/week for **(C)** young mALF vs. young mTRF and **(D)** old mALF vs. old mTRF. Number of mice (n) for each group was (A and C) n = 20, (B and D) n = 16.

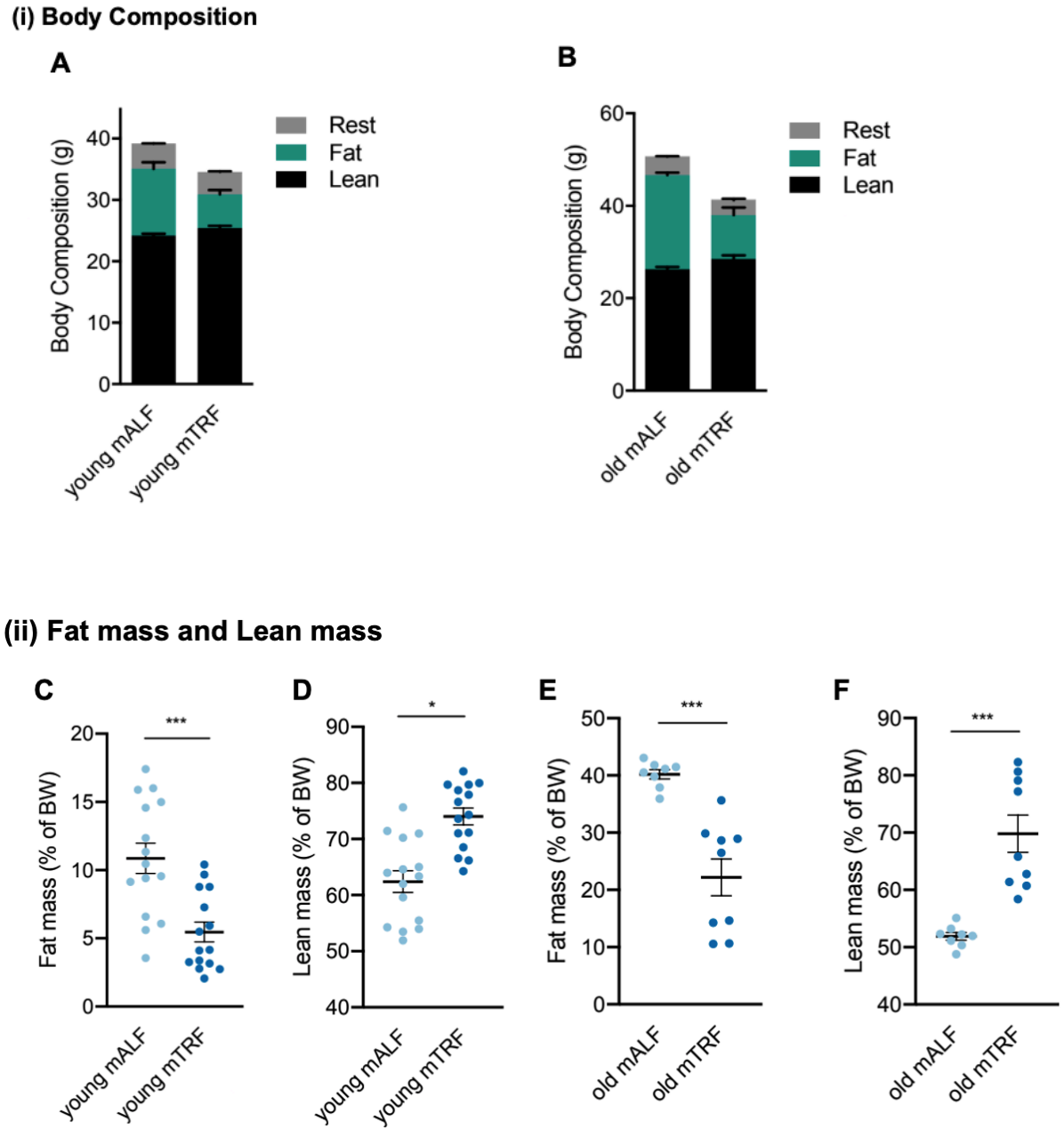

Figure 3 : Body composition, fat mass, and lean mass of young/old mice on TRF/ALF.

(i) Body composition (mean ± SD) for **(A)** young mALF vs. young mTRF and **(B)** old mALF vs. old mTRF. **(i)** Body fat mass **(C,E)** and lean mass **(D,F)** as a percentage of total body weight for young and old mice on ALF and TRF (mean ± SEM). n = 20 for groups A,C,D and n = 16 for groups B,E,F; Data presented as mean ± SEM and *p < 0.05, **p < 0.01, ***p < 0.001.

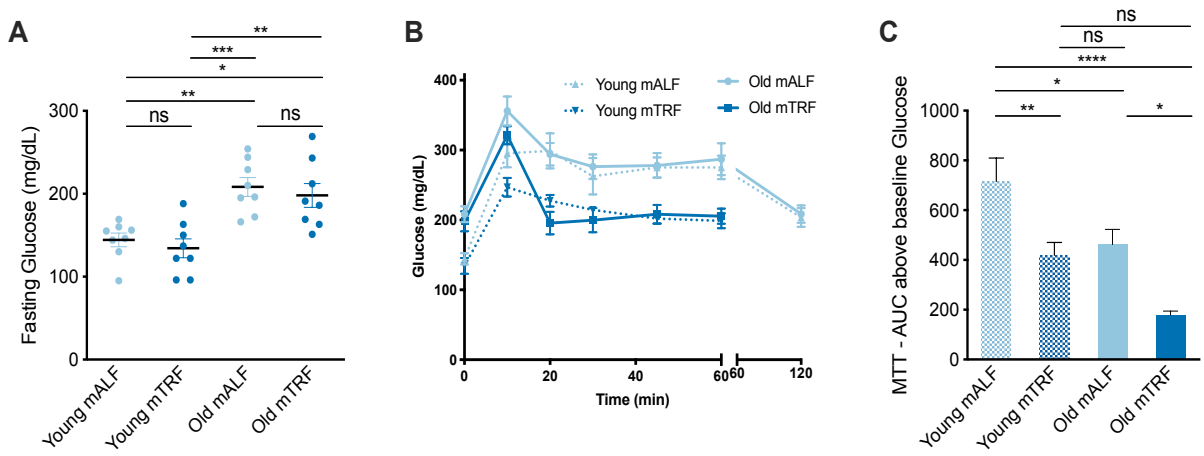

Figure 4: **Meal tolerance test (MTT) in young/old mice on ALF/TRF**.
(A) fasting glucose (mg/dl) after 16h of fasting for young and old mice on ALF and TRF. **(B)** Blood glucose measurements (mg/dL) taken every 10 minutes for 120 minutes post-bolus and **(C)** quantification of area under curve (AUC) above baseline levels of glucose for young and old mice on ALF and TRF. Mice were gavaged with a constant bolus of 700 cal (~ 5% of daily intake) of complete meal (5.5% protein, 21% carbohydrate, 5% fat) nine weeks into feeding regimen. n = 8 for A,B,C,D (1-way ANOVA with Tukey's multiple comparisons tests). Data presented as mean ± SEM and *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

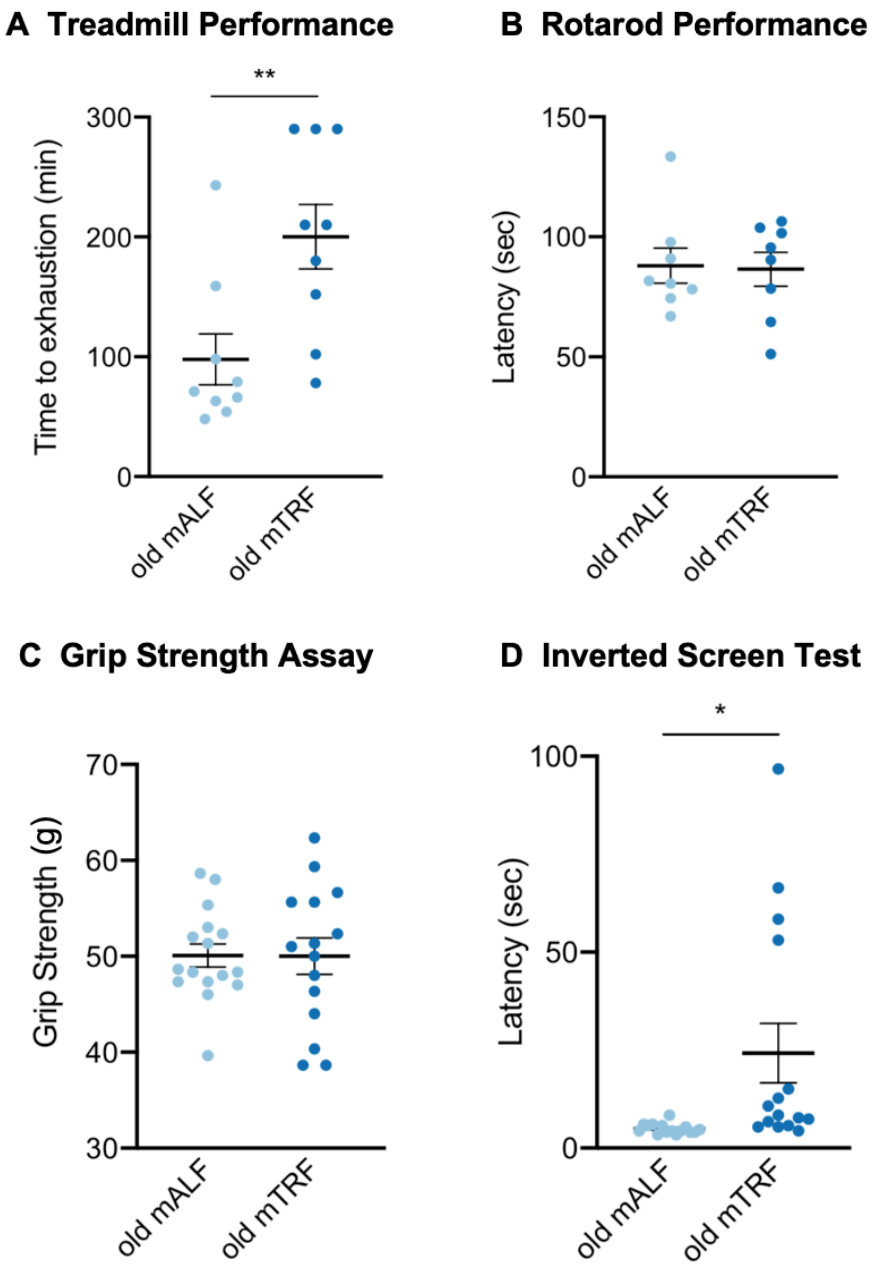

Figure 5 : **Treadmill, rotarod, grip strength, wire hang assay in old mice on ALF/TRF.** **(A)** Running time (min) on treadmill in a run-to-exhaustion assay (n = 9), **(B)** time to fall (latency in sec) on accelerating rotarod (n = 8), **(C)** forelimb muscle grip force (g) applied on grip strength meter (n = 16), **(D)** time to fall (latency in sec) on Kondziela's inverted screen test (n = 15) for old mice on ALF and TRF. Data presented as mean ± SEM and *p < 0.05, **p < 0.01, ***p < 0.001.

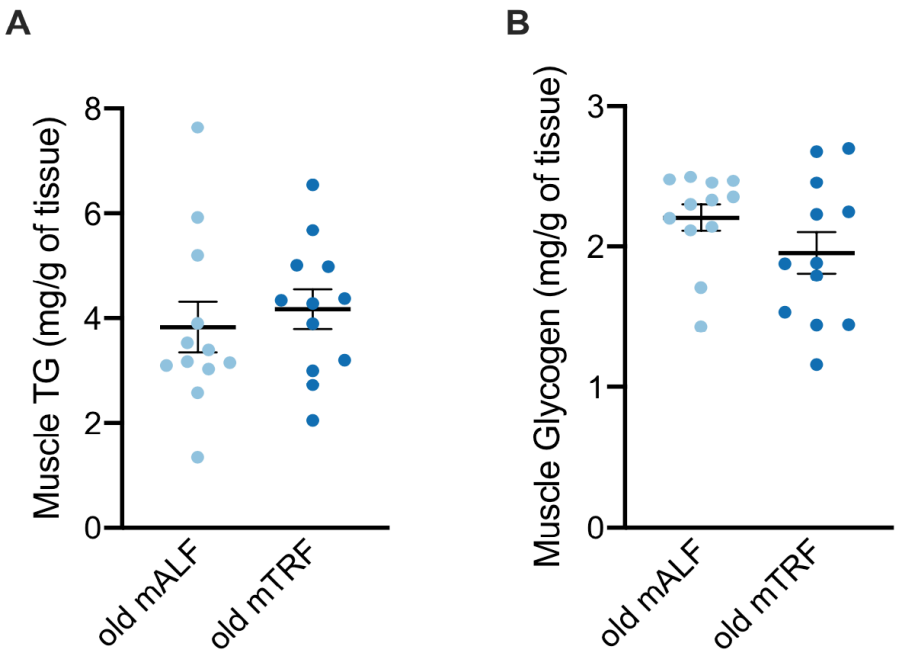

Figure 6 : **Triglyceride and glycogen stores in skeletal muscle of old mice on ALF/TRF.** **(A)** Mg of triglyceride content per gram of skeletal muscle tissue in old mice on ALF and TRF (n = 12). Mg of triglyceride content per gram of skeletal muscle tissue in old mice on ALF and TRF (n = 12). Data presented as mean ± SEM and *p < 0.05, **p < 0.01, ***p < 0.001 for ALF vs. TRF

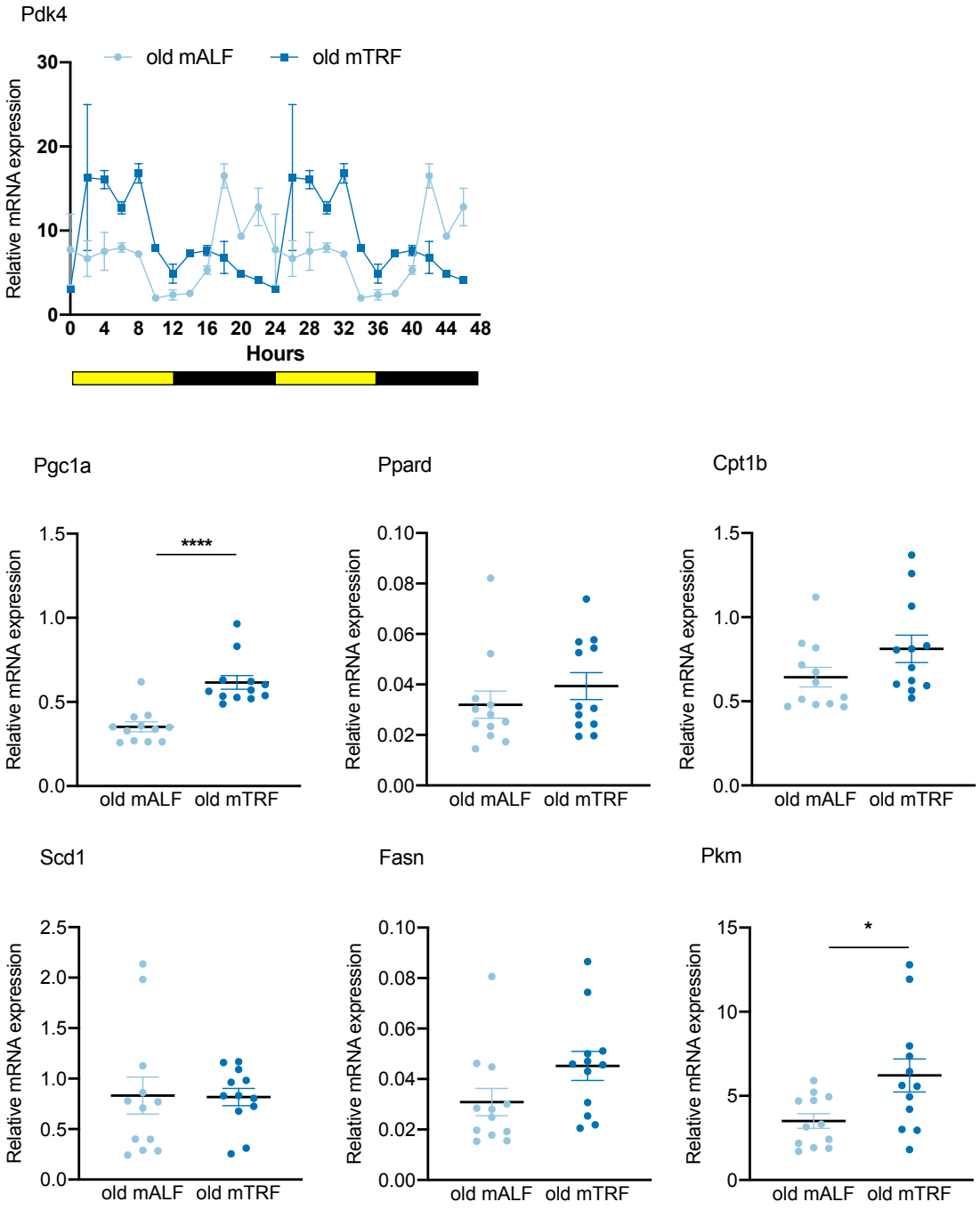

Figure 7 : Metabolism- and performance-associated gene expression in skeletal muscle of old mice of ALF/TRF.

mRNA levels of metabolism and performance genes *Pgc1a, Ppard, Cpt1b, Scd1, Fasn, Pkm,* and *Pdk4* in skeletal muscle determined via qPCR analysis. *Pdk4* shown as a time series plot because it demonstrates rhythmicity in mRNA expression. Absolute transcript expression was calculated using the standard curve method, data was normalized to HPRT1 expression and median was normalized group wise. n = 12 for ALF and TRF. Data presented as mean ± SEM and *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

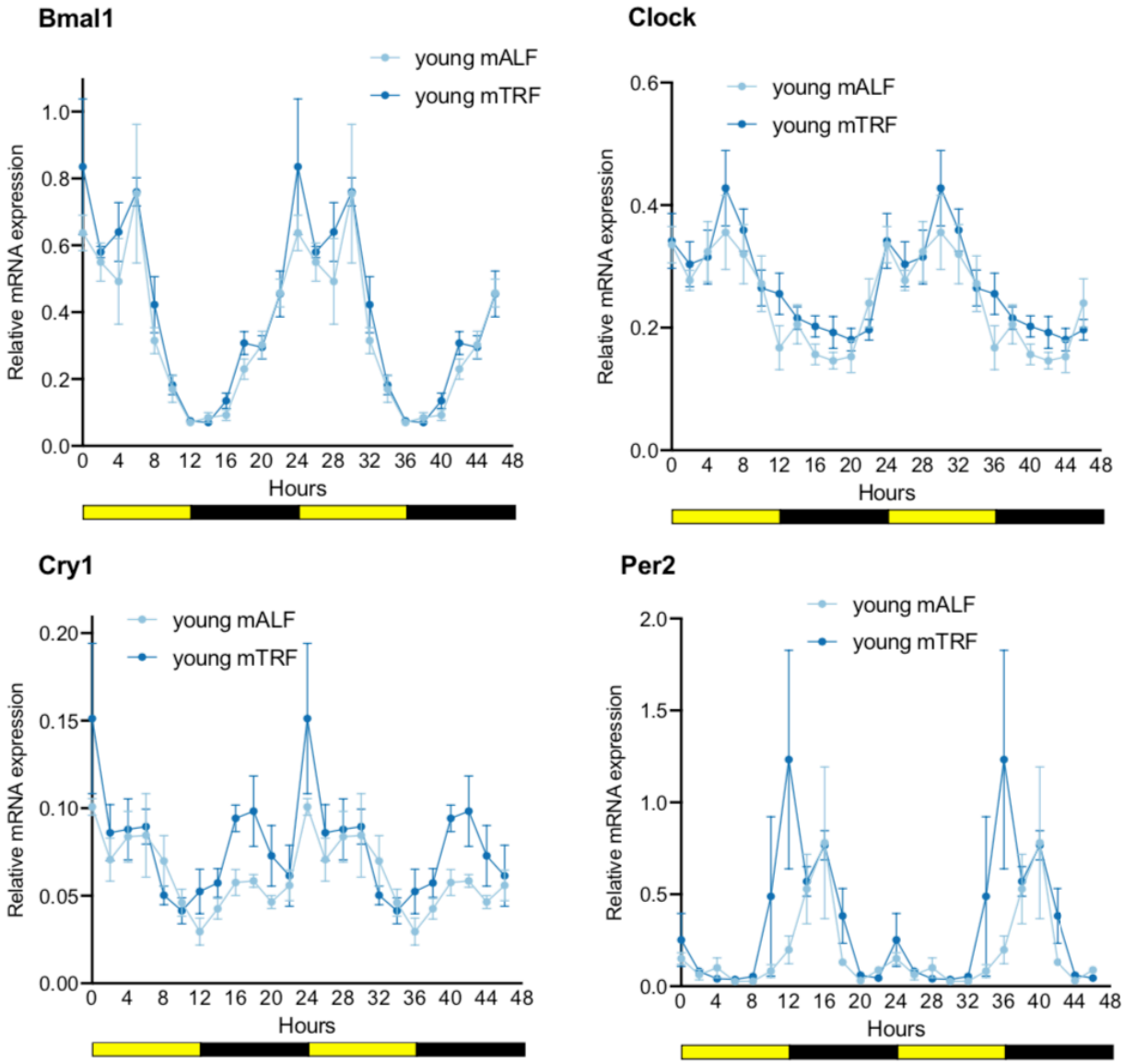

Figure 8 : Circadian clock gene expression in skeletal muscle of young mice on ALF/TRF. Time series of mRNA expression of circadian clock genes *Bmal1, Clock, Cry1, Per2* in young mTRF/mALF skeletal muscle determined via qPCR analysis double plotted for 48 hours. Absolute transcript expression was calculated using the standard curve method, data was normalized to HPRT1 and median was normalized group wise. Each time point shown as mean ± SEM; n = 2/time point/feeding group.

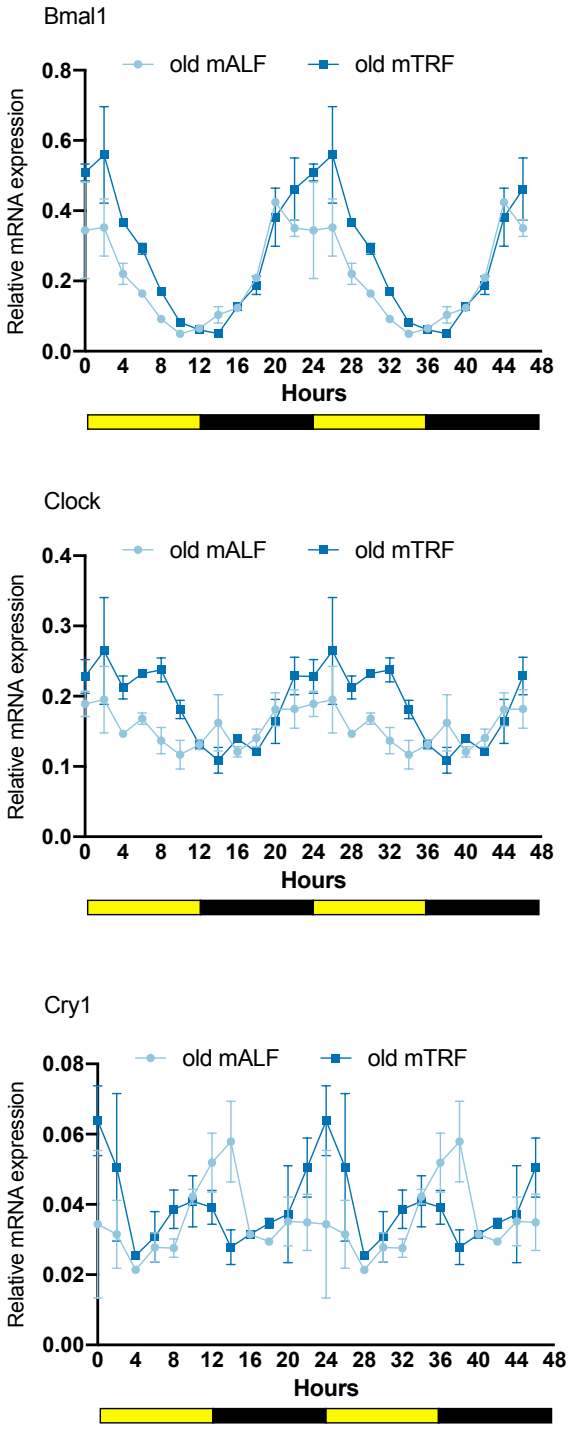

Figure 9 : Circadian clock gene expression in skeletal muscle of old mice on ALF/TRF.
Time series of mRNA expression of circadian clock genes *Bmal1, Clock, Cry1* in old mTRF/mALF skeletal muscle determined via qPCR analysis double plotted for 48 hours. Absolute transcript expression was calculated using the standard curve method, data was normalized to HPRT1 and median was normalized group wise. Each time point shown as mean ± SEM; n = 2/time point/feeding group.

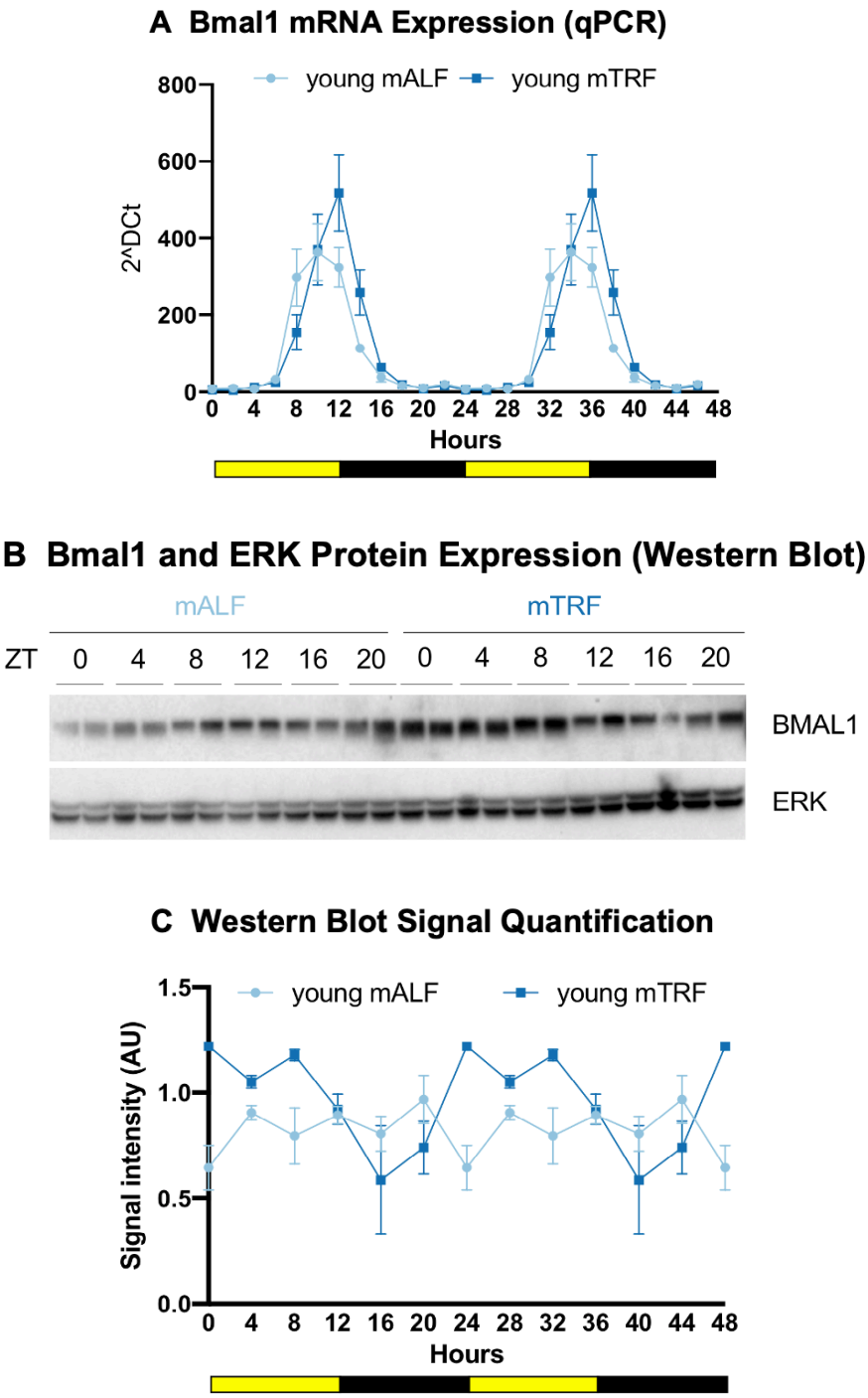

Figure 10 : **mRNA/Protein expression of Bmal1 in liver of young mice on ALF/TRF.**
(A) mRNA levels of BMAL1 over 48 hours in liver obtained via qRT-PCR; Absolute transcript expression was calculated using the standard curve method, data was normalized to HPRT1 expression and median was normalized group wise. **(B)** Protein levels of BMAL1 in liver samples obtained via western blotting. Data normalized to ERK expression **(C)** BMAL1 protein expression was quantified by measuring the pixel intensity (AU) on imageJ. Each time point shown as mean ± SEM; n = 2/time point/feeding group.

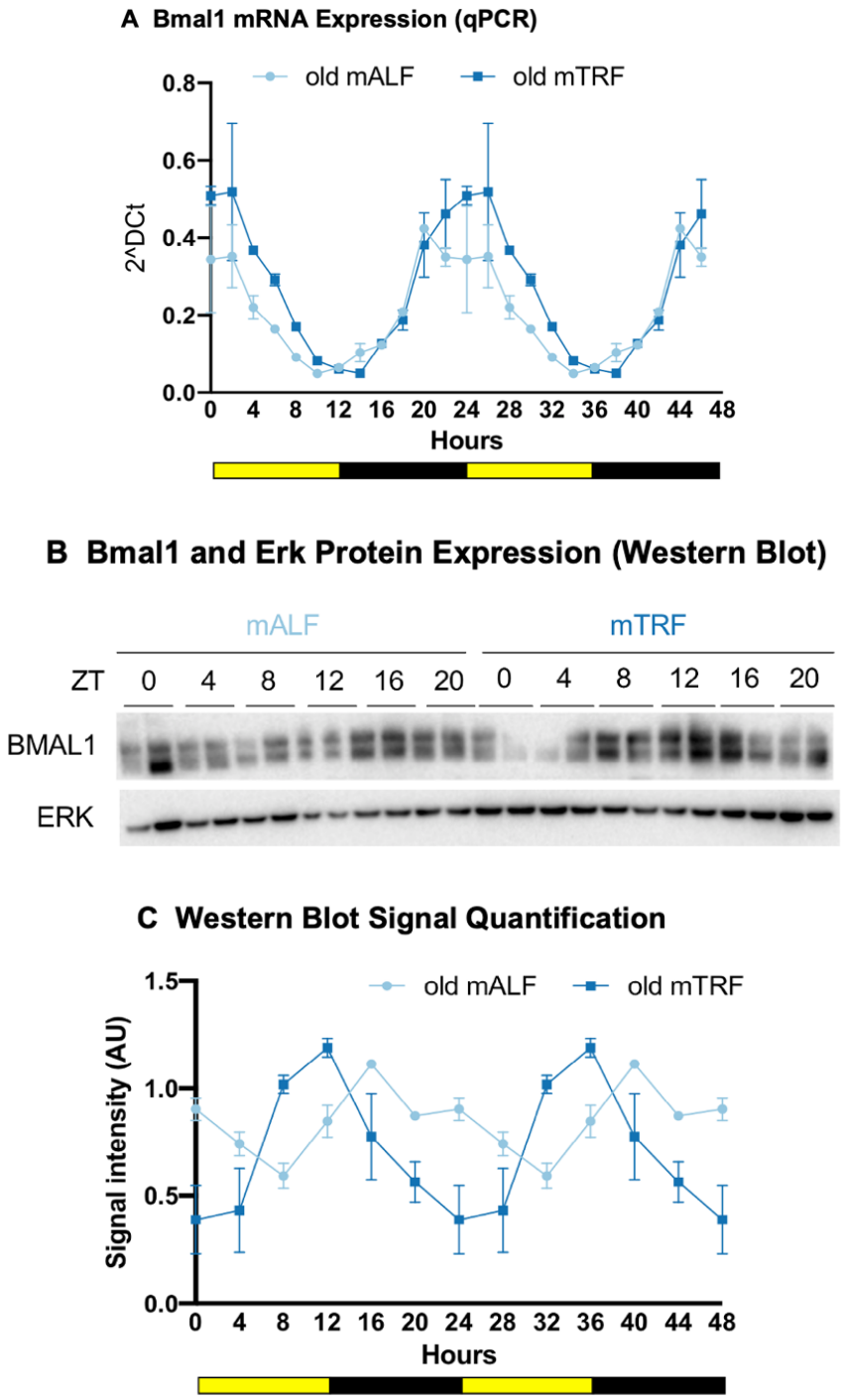

Figure 11 : **mRNA/Protein expression of Bmal1 in skeletal muscle of old mice on ALF/TRF.** **(A)** mRNA levels of BMAL1 over 48 hours in skeletal muscle obtained via qPCR; Absolute transcript expression was calculated using the standard curve method, data was normalized to HPRT1 expression and median was normalized group wise. **(B)** Protein levels of BMAL1 in muscle samples obtained via western blotting. Data normalized to ERK expression **(C)** BMAL1 protein expression was quantified by measuring the pixel intensity (AU) on imageJ. Each time point shown as mean ± SEM; n = 2/time point/feeding group.

TABLES :

BODY WEIGHT

Table 1 : Mean body weight in grams (Mean), standard error of mean (SEM)/ standard deviation (SD), and sample size (N) of young and old mice on ALF and TRF over 10-14 weeks.

Weeks	young mALF			young mTRF		
X	Mean	SD	N	Mean	SD	N
0.000	28.995	1.718	20	29.165	2.560	20
1.000	30.125	1.940	20	29.410	1.727	20
2.000	30.825	2.096	20	30.330	1.902	20
3.000	32.390	2.087	20	31.080	2.219	20
4.000	33.385	2.319	20	31.520	2.446	20
5.000	34.610	2.765	20	31.935	2.860	20
6.000	35.340	2.935	20	32.200	4.181	20
7.000	36.135	3.034	20	32.765	3.619	20
8.000	36.500	3.071	20	32.895	4.128	20
9.000	37.580	3.624	20	33.400	4.033	20
10.000	38.214	3.867	14	33.121	4.270	14

Weeks	old mALF			old mTRF		
X	Mean	SEM	N	Mean	SEM	N
0.000	38.584	0.827	50	38.920	0.695	50
1.000	41.618	0.695	50	39.002	0.634	49
2.000	41.902	0.788	50	37.967	0.677	48
3.000	43.144	0.859	50	38.208	0.701	48
4.000	43.410	1.039	50	39.079	0.719	48
5.000	45.092	0.864	50	39.181	0.782	48
6.000	46.106	0.779	50	39.727	0.816	48
7.000	45.935	0.841	49	40.157	0.826	47
8.000	46.369	0.932	49	40.221	0.900	47
9.000	46.284	1.077	49	39.832	0.964	47
10.000	47.494	0.879	49	39.779	0.833	47
11.000	47.402	0.921	49	40.474	0.862	47
12.000	47.979	1.068	48	40.631	0.901	45
13.000	49.281	1.001	48	40.938	0.947	45
14.000	49.706	0.984	48	41.396	0.963	45

FOOD

Table 2 : Average food consumed in grams (Mean), standard deviation (SD), and sample size (N) for young and old mice on ALF and TRF over 10-14 weeks.

Weeks	young mALF			young mTRF		
X	Mean	SD	N	Mean	SD	N
0.000	0.000	0.000	4	0.000	0.000	4
1.000	70.218	3.781	4	76.775	3.462	4
2.000	145.136	6.204	4	152.609	7.050	4
3.000	221.911	6.267	4	228.726	11.563	4
4.000	298.826	5.369	4	306.746	15.752	4
5.000	378.139	8.624	4	387.821	22.221	4
6.000	459.096	5.444	4	470.682	26.929	4
7.000	537.704	9.001	4	551.263	37.031	4
8.000	615.348	8.153	4	632.385	38.653	4
9.000	695.036	9.193	4	714.048	46.070	4
10.000	771.599	10.827	4	797.073	50.951	4

Weeks	old mALF			old mTRF		
X	Mean	SD	N	Mean	SD	N
0.000	0.000	0.000	12	0.000	0.000	11
1.000	91.807	14.565	12	61.903	7.922	11
2.000	181.870	35.010	12	139.767	12.473	11
3.000	273.640	51.067	12	224.741	14.770	11
4.000	360.134	69.130	12	312.740	16.394	11
5.000	450.676	83.813	12	403.487	18.993	11
6.000	539.099	95.656	12	497.318	20.586	11
7.000	623.958	111.626	12	590.169	22.109	11
8.000	708.499	124.559	12	680.686	22.857	11
9.000	792.048	139.470	12	770.001	26.636	11
10.000	881.174	147.591	12	856.572	32.895	11
11.000	966.734	157.182	12	951.837	30.028	11
12.000	1045.402	156.365	12	1044.931	27.733	11
13.000	1130.222	151.872	12	1135.961	29.858	11

BODY COMPOSITION (g)

Table 3 : Body composition (in grams), percent fat mass, and percent lean mass in young and old mice on ALF and TRF over 10-14 weeks.

	Lean			Fat			Rest		
	Mean	SD	N	Mean	SD	N	Mean	SD	N
young mALF	24.171	1.361	15	10.865	4.285	15	4.090	0.253	15
young mTRF	25.413	1.440	15	5.464	2.829	15	3.697	0.411	15
	Lean			Fat			Rest		
	Mean	SD	N	Mean	SD	N	Mean	SD	N
old mALF	26.261	1.437	8	20.357	1.552	8	3.995	0.371	8
old mTRF	28.472	2.391	9	9.485	5.066	9	3.287	0.795	9

% FAT MASS

	young mALF	young mTRF
Number of values	15	15
Minimum	3.550	2.061
Maximum	17.42	10.41
Range	13.87	8.347
Mean	10.87	5.464
Std. Deviation	4.285	2.829
Std. Error of Mean	1.106	0.7303

	old mALF	old mTRF
Number of values	8	9
Minimum	35.93	10.55
Maximum	43.05	35.66
Range	7.118	25.10
Mean	40.21	22.18
Std. Deviation	2.299	9.583
Std. Error of Mean	0.8128	3.194

% LEAN MASS

	young mALF	young mTRF
Number of values	15	15
Minimum	51.93	64.26
Maximum	75.63	82.04
Range	23.70	17.78
Mean	62.40	74.01
Std. Deviation	7.508	5.777
Std. Error of Mean	1.938	1.492

	old mALF	old mTRF
Number of values	8	9
Minimum	48.79	58.41
Maximum	55.10	82.30
Range	6.309	23.89
Mean	51.89	69.82
Std. Deviation	1.886	9.767
Std. Error of Mean	0.6668	3.256

MEAL TOLERANCE TEST (MTT)

Table 4 : Values associated with the meal tolerance test (MTT) conducted on young and old mice on ALF and TRF.

Fasting glc	Young mALF	Young mTRF	Old mALF	Old mTRF
Number of values	8	8	8	8
Minimum	95.00	96.00	166.0	151.0
Maximum	169.0	188.0	254.0	269.0
Range	74.00	92.00	88.00	118.0
Mean	144.4	134.3	208.3	198.0
Std. Deviation	23.13	32.07	32.14	40.94
Std. Error of Mean	8.179	11.34	11.36	14.47

	Old mALF			Old mTRF			Young mALF			Young mTRF		
X	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N
0.000	208.250	32.137	8	198.000	40.935	8	144.375	23.133	8	134.250	32.066	8
10.000	356.125	58.740	8	321.250	36.796	8	295.375	57.186	8	247.000	37.940	8
20.000	294.000	45.707	8	195.500	45.717	8	299.000	71.150	8	227.625	23.573	8
30.000	276.375	49.379	8	199.750	48.673	8	262.625	73.756	8	214.375	15.445	8
45.000	277.875	50.400	8	208.250	38.048	8	275.250	40.496	8	202.000	20.445	8
60.000	287.000	64.790	8	205.375	30.799	8	275.125	47.843	8	198.875	30.489	8
120.000	208.375	35.108	8				203.625	37.894	8			

PERFORMANCE ASSAY

Table 5 : Values associated with treadmill test, wirehang test (inverted screen assay), grip strength test, and rotarod test conducted on old mice on ALF and TRF.

Treadmill	old mALF	old mTRF
Number of values	9	9
Minimum	48.00	78.00
Maximum	243.0	290.0
Range	195.0	212.0
Mean	97.89	200.2
Std. Deviation	63.76	80.38
Std. Error of Mean	21.25	26.79

Wirehang	old mALF	old mTRF
Number of values	15	15
Minimum	3.333	4.333
Maximum	8.333	96.67
Range	5.000	92.33
Mean	4.889	24.22
Std. Deviation	1.307	29.26
Std. Error of Mean	0.3375	7.556

Grip	old mALF	old mTRF
Number of values	16	15
Minimum	39.67	38.67
Maximum	58.67	62.33
Range	19.00	23.67
Mean	50.08	50.02
Std. Deviation	4.796	7.367
Std. Error of Mean	1.199	1.902

Rotarod	old mALF	old mTRF
Number of values	8	8
Minimum	66.88	51.20
Maximum	133.5	106.4
Range	66.58	55.23
Mean	87.98	86.50
Std. Deviation	20.68	20.04
Std. Error of Mean	7.313	7.087

Table 6 : Values associated with triglyceride and glycogen quantification in skeletal muscle of old mice on ALF and TRF.

TRIGLYCERIDE

	old mALF	old mTRF
Number of values	12	12
Minimum	1.341	2.050
Maximum	7.634	6.538
Range	6.293	4.487
Mean	3.828	4.171
Std. Deviation	1.675	1.291
Std. Error of Mean	0.4836	0.3726

GLYCOGEN

	old mALF	old mTRF
Number of values	12	12
Minimum	1.432	1.162
Maximum	2.497	2.699
Range	1.065	1.537
Mean	2.207	1.955
Std. Deviation	0.3299	0.5090
Std. Error of Mean	0.09522	0.1469

PRIMER SEQUENCES

Table 7 : Primer sequences used in qRT-PCR analysis.

Gene Symbol	Official full name	mRNA	Forward Primer	Reverse Primer
Pgc1a	Peroxisome Proliferative Activated Receptor, Gamma, Coactivator 1 alpha	NM_008904.2	GAAAGGGCCAAACAGAGAGA	GTAAATCACACGGCGCTCTT
Ppard	Peroxisome Proliferator Activated Receptor delta	NM_011145.3	CAAACCCACGGTAAAGGCAG	TGGCTGTTCCATGACTGACC
Cpt1b	carnitine palmitoyl transferase-1b	NM_009948.2	CCTGGTGCTCAAGTCATGGT	CCATGACCGGCTTGATCTCT
Scd1	Stearoyl-CoA desaturase-1	NM_009127.4	GTGCCGTGGGCGAGG	AGCCCAAAGCTCAGCTACTC
Fasn	Fatty acid synthase	NM_007988.3	CGGATTCGGTGTATCCTGCT	CCTCGGGTGAGGACGTTTAC
Pdk4	Pyruvate dehydrogenase kinase 4	NM_001253283.1	CCTGGTGCTCAAGTCATGGT	CCATGACCGGCTTGATCTCT
Pkm	pyruvate kinase muscle isozyme	NM_001253883.1	CCACACAGATGCTGGAGAGC	TTCAAACAGCAGACGGTGGA
Bmal1	Brain And Muscle ARNT-Like 1	NM_007489.4	GCCCCACCGACCTACTCT	TGTCTGTGTCCATACTTTCTTGG
Clock	Circadian Locomoter Output Cycles Protein Kaput	NM_007715.5	CCAGTCAGTTGGTCCATCATT	TGGCTCCTAACTGAGCTGAAA
Cry1	ryptochrome Circadian Regulator 1	NM_007771.3	ATCGTGCGCATTTCACATAC	TCCGCCATTGAGTTCTATGAT
Per2	Period Circadian Regulator 2	NM_011066.3	TCCGAGTATATCGTGAAGAACG	CAGGATCTTCCCAGAAACCA