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Effect of Varying Feeding Windows on an Acute Response to Exercise Following Chronic Exercise and Time-Restricted Feeding

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EFFECT OF VARYING FEEDING WINDOWS ON AN ACUTE RESPONSE TO EXERCISE FOLLOWING CHRONIC EXERCISE AND TIME-RESTRICTED FEEDING

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Abstract

Time-restricted feeding (TRF) involves splitting the day into two windows, a feeding window and a fasting window. TRF positively affects mitochondria and metabolic health. Aerobic exercise is shown to have the same effect. However, the combination of the two in healthy subjects is not as extensively studied. The purpose of this study was to examine how changing the window of feeding in relation to exercise changes performance and mitochondrial biogenesis. Healthy, young mice were divided into 3 groups: an ad libitum control group (CON), a 16-hour fast with an 8-hour feed immediately post exercise (IM), and a 16-hour fast with an 8-hour feed starting 5 hours after exercise (DG). No differences in mitochondrial biogenesis and performance were found between groups. Our results suggest that the timing of ones fast in relation to exercise doesn't seem significantly affect muscle adaptation from aerobic exercise.

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List of Abbreviations

Abbreviation	Definition
AMP	Adenosine monophosphate
AMPK	5' adenosine monophosphate actived protein kinase
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
cDNA	Complementary DNA
Con	Control
DG	Delayed group
DNA	Deoxyribonucleic acid
ETC	Electron transport chain
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
IM	Immediate group
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
mtDNA	Mitochondrial DNA
mTOR	Mechanistic target of rapamycin
NRF1	Nuclear respiratory factor 1
OXPHOS	Oxidative Phosphorylation
P-ampk	Phospho 5' adenosine monophosphate actived protein kinase
PGC1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
RNA	Ribonucleic acid

rtPCR	reverse transcription polymerase chain reaction
SDS-page	Sodium dodecyl sulfate and polyacrylamide gel
TBST	Tris buffered saline tween
TFAM	Mitochondrial transcription factor A
TNFα	Tumor necrosis factor alpha
TRF	Time-restricted feeding

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Introduction

Over the past 5 years, interest in intermittent fasting has increased rapidly. At the time of writing, searches are as much as 25% percent more popular for terms related to intermittent fasting compared to even just one year ago (Trends, 2020). Unlike caloric restriction, where total calories consumed are reduced, intermittent fasting restricts the timing of when calories are consumed. The duration of the fast can be as long as a day, termed alternate day fasting, or as short as part of the day, termed time-restricted feeding. Alternate day fasting restriction varies from as low as 0 calories (Smith et al., 2019) to a fourth of general consumption on fasting days (Patterson & Sears, 2017). TRF also varies in length of restriction, with some research having restrictions as short as 8 hours (Chaix, Zarrinpar, Miu, & Panda, 2014; Megumi. Hatori et al., 2012) or as long as 18 hours (Stockman, Thomas, Burke, & Apovian, 2018).

Time-restricted feeding (TRF) is shown to reduce body weight as well as improve metabolic health (Patterson & Sears, 2017). Metabolic health is closely regulated by skeletal muscle mitochondria (Argiles, Lopez-Soriano, & Busquets, 2015; Carson, Hardee, & VanderVeen, 2016; H. Lee & Song, 2018). TRF can both improve mitochondrial function and promote mitochondrial biogenesis in healthy mice and preserve mitochondrial function in diseased mice (S. D. Anton et al., 2018; Mattson, Longo, & Harvie, 2017). TRF induces phosphorylation of 5' adenosine monophosphate-activated protein kinase (AMPK), the master energy sensor in the cell (Paoli, Tinsley, Bianco, & Moro, 2019). Activation of AMPK has been shown to signal mitochondrial biogenesis, which may be one mechanism for the metabolic benefits of TRF (Marin et al., 2017; Reznick & Shulman, 2006).

Another popular method of metabolic health improvement is exercise. Although there are many variations, one of the most accessible ways to exercise is aerobic training. The energy for

aerobic exercise primarily comes from energy produced from the mitochondria. One adaptation from aerobic exercise is increases in mitochondrial content and function. This is regulated by mitochondrial transcription factor (TFAM) which is controlled by peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α). Muscles contracting create a signal that alters mitochondrial function (Hood, Tryon, Carter, Kim, & Chen, 2016; Huertas, Casuso, Agustín, & Cogliati, 2019). The process of mitochondrial biogenesis is also regulated by mammalian target of rapamycin (mTOR) (Morita et al., 2013) which is acutely activated after exercise. AMPK and mTOR play major roles in promoting metabolic flexibility through their interactions with the mitochondrial energy creation pathways thus maintaining metabolic health and regulating the replenishment of energy stores post exercise (Andreux, Houtkooper, & Auwerx, 2013).

Replenishment of used energy stores after exercise is theorized to be improved by the consumption of a meal within a certain time frame of completion. Although the idea of a metabolic/anabolic window is mostly used in strength training, it is commonly used with aerobic exercise as well. Research is limited when it comes to aerobic exercise and is further complicated by the composition and timing of the diet. One study posits that the existence of a window depends on the fasting state of the exerciser, with fasted exercise creating a net negative protein synthesis balance (Kumar, Atherton, Smith, & Rennie, 2009). There is some research that suggests exercising in a fed state leads to improved aerobic performance if the exercise is long enough. However, if the exercise is short (~60 minutes or less) there is limited difference in performance (Aird, Davies, & Carson, 2018). Problematically, much of the research on intermittent fasting, exercise, and the combination of the two focuses on diseased populations. There is a lack of research on the effects of fasting and exercise on healthy populations.

Chapter 1

Literature Review

Living creatures eat, it is an unavoidable part of being alive. Consumption, and ultimately digestion and metabolism, are required for healthy living. According to the World Health Organization, health is defined as "a state of complete physical, mental and social well-being and *not merely the absence of disease or infirmity* [emphasis added]." (World Health Organization, 2020). However, overconsumption can lead to health problems such as obesity. Obesity leads to increased mortality from diabetes, coronary heart disease, and diabetes (Kissebah, Freedman, & Peiris, 1989). Therefore, to be healthy, one should avoid obesity. However, simply not being obese is not enough to be considered healthy. Therefore, to truly be considered healthy, any efforts must do more than simply reduced fat in the body. With this in mind, there are numerous methods related to regulating consumption to promote health.

Caloric Restriction

The NIH defines caloric restriction as "reducing average daily caloric intake below what is typical or habitual, without malnutrition or deprivation of essential nutrients" (N.I.A, 2020). Although the exact mechanisms are unknown, caloric restriction appears to induce mitochondrial biogenesis (Aris et al., 2013; Dutta, Calvani, Bernabei, Leeuwenburgh, & Marzetti, 2012; Kayo, Allison, Weindruch, & Prolla, 2001; C. K. Lee, Klopp, Weindruch, & Prolla, 1999; Rangaraju et al., 2009; Wohlgemuth et al., 2007) and also results in mitochondria that produce less reactive oxygen species and utilize less oxygen (Jornayvaz & Shulman, 2010).

Increased mitochondrial biogenesis is important because mitochondria are used to produce energy from fat. Obese individuals can have dysfunction in their mitochondria (Bournat & Brown, 2010; Breininger et al., 2019). Mitochondrial biogenesis is important to

protecting the mitochondria by fixing the damage caused by obesity. In cells, that damage downregulates expression of numerous genes involved in mitochondrial biogenesis, such as NRF1, PGC1α, and TFAM (Gao et al., 2010). In mouse muscle high fat feeding led to reduced expression of PGC1α mRNA and protein concentration (Sparks et al., 2005) as well as mitochondrial dysfunction (Breininger et al., 2019; Devarshi, McNabney, & Henagan, 2017; Stewart et al., 2009).

With caloric restriction alone and caloric restriction and exercise, researchers found an increase in mitochondrial DNA (mtDNA) content in skeletal muscle, a marker for mitochondrial mass (Civitarese et al., 2006; Wang, Hiatt, Barstow, & Brass, 1999), compared to control groups (Civitarese et al., 2007). Caloric restriction has also been shown to reduce free radical generation in the mitochondria and induce mitochondrial proliferation (Jornayvaz & Shulman, 2010). Beyond just the creation of new mitochondria, the function of the ubiquitin-proteosome system is also improved (S. Anton & Leeuwenburgh, 2013). The ubiquitin-proteosome system functions to remove malfunctioning mitochondria. However, individuals can have difficulty sticking with caloric restriction for an extended amount of time (Scheen, 2008). Therefore, other methods that can yield the same results have been sought after (S. Anton & Leeuwenburgh, 2013).

Intermittent Fasting

Intermittent fasting has become a popular method to reduce caloric intake. Intermittent fasting refers to having a period of fasting or reduced caloric consumption paired with a period of unregulated regular caloric consumption. This can be done with the intention of restricting caloric intake overall, for religious reasons, or even without health benefits in mind. One example of religious intermittent fasting is the Islamic religious fasting during Ramadan. This

fast lasts from sunrise to sunset. The current research suggests that caloric restriction and intermittent fasting can trigger similar pathways in the body (S. Anton & Leeuwenburgh, 2013; C. Lee & Longo, 2011). One study compared numerous variations of intermittent fasting and found that when consuming a high fat diet any variation of restricted caloric consumption resulted less fat gain than a control group (Smith et al., 2019). Regardless of the method, it seems that the fast should be at least 12 hours to prevent weight gain because a fast of 8, 9, or 12 hours results in similar amounts of weight gain (Chaix et al., 2014; Megumi. Hatori et al., 2012). If that is true, then individuals may be able to substitute their preferred method in to reap any potential benefits. There are numerous variations of intermittent fasting, but they can be simplified into two different categories: alternate day fasting and time-restricted feeding.

Alternate Day Fasting

Alternate day fasting is a variation of intermittent fasting where the individual alternates days of consuming no calories one day and ad libitum calorie consumption on the next. One variation of this diet involves consuming approximately a fourth of one's regular calorie needs on fasting days, in place of consuming nothing (Patterson & Sears, 2017). A more stringent alternate day fasting involves consumption of 0 calories on the fasting day, and *ad libitum* access on regular days (Smith et al., 2019).

One study found that C57BL/6 mice consumed the same amount of food overall and still have the benefits from alternate day fasting. The fasting mice ate almost twice as much on their regular consumption days (Anson et al., 2003). Therefore, it seems that the health benefits do not just appear from having restricted caloric intake overall and result from some kind of time-gate.

To determine if alternate day fasting is something that healthy individuals would adhere to, one study performed 21 days of alternate day fasting. They had subjects fast for 22 hours every other day. They found hunger feelings did not change, but daily fat oxidation increased. Overall, they found that alternate day fasting is feasible in non-obese populations. However, due to the feelings of hunger on fasting days, it is potentially unlikely to be adhered to long term (Heilbronn et al., 2019). In fact, this is a problem with fasting diets. People are going to get hungry when they increase their fasting duration. Feeling hungry is generally an unpleasant feeling, and people tend to not willingly make themselves uncomfortable unless they need to. Because of this, fasts lasting an entire day may be unreasonable for the average person.

Time-Restricted Feeding

Time-restricted feeding is a version of intermittent fasting. During time-restricted feeding, the individual will consume an unregulated number of calories for a limited amount of time every day. The rest of the day will be spent abstaining from caloric consumption. There are numerous durations used in time restricted feeding, with some studies as short at 8 hours (Chaix et al., 2014; M. Hatori et al., 2012) and some as long as 18 hours (Stockman et al., 2018). Overall, time restricted feeding has positive benefits on health with or without weight loss (Patterson & Sears, 2017). In one time-restricted feeding study, mice consumed equal calories whether doing an 8 hour fast or not of a high fat diet; however, the restricted feeding group had improved protection against numerous metabolic diseases (Megumi. Hatori et al., 2012). Another group that underwent time restricted feeding had food access for 6 hours a day fasted for 18 hours (Smith et al., 2019;Delahaye et al., 2018). At least in mice, 8 hours of fasting has a positive effect (Megumi. Hatori et al., 2012). In humans an 8 hour fast results in reduced blood pressure, mild caloric restriction, and weight loss (Gabel et al., 2018). Therefore, time restricted

feeding and alternate day fasting have similar positive effects on health. The fasting time to experience health benefits does not need to be an entire day. A possible reason for fasting groups to have improved health is through mitochondrial changes.

Mitochondrial Biogenesis

Mitochondria are famously known as the powerhouse of the cell. This is due to the role they play in ATP production, specifically in aerobic environments. Oxidative phosphorylation, OXPHOS, is the name of the process mentioned above. The body can create more mitochondria, called mitochondrial biogenesis, in response to increased energy demands, such as after exercise (Hood, Uguccioni, Vainshtein, and D'souza, 2011). AMPK is involved in the regulation of ATP production and use, and through this process is involved in the overall regulation of mitochondrial biogenesis (Hardie, 2007; Jornayvaz & Shulman, 2010). If supplies of energy are low while demands are high, AMPK phosphorylation results in mitochondrial biogenesis through stimulation of PGC1α (Vaughan, Mermier, Bisoffi, Trujillo, & Conn, 2014). PGC1α is considered the major regulator of mitochondrial biogenesis (Puigserver et al., 1998). PGC1α activates nuclear respiratory factors 1 and 2 which increase TFAM expression (Jornayvaz & Shulman, 2010; Sergi et al., 2019; Vaughan et al., 2014). TFAM regulates mitochondrial gene expression by directly interacting with the mitochondrial genome (Gleyzer, Vercauteren, & Scarpulla, 2005; Wu et al., 1999). PGC1α expression is enhanced by fasting through AMPK activation (Carles Cantó et al., 2010).

There does not appear to be a consensus in the research regarding caloric restriction and mitochondrial biogenesis. A reduction in reactive oxygen species is a consistent consequence of caloric restriction, however there is controversy regarding an increase in mitochondrial biogenesis. *In vivo*, there was no increase in mitochondrial protein synthesis and there are

variable results regarding an increase in PGC1α between studies (Gouspillou & Hepple, 2013). Six months of caloric restriction causes an increase in TFAM and PGC1α expression levels (Civitarese et al., 2007) with (12.5% reduced energy intake and +12.5% increased energy demand) or without (25% reduced energy intake) exercise. Combining weight loss with exercise in overweight individuals increased mitochondrial function (Menshikova et al., 2005) and PGC1α gene expression (Russell, Hesselink, Lo, & Schrauwen, 2005). In humans, 4 weeks of single leg training lead to a larger acute increase in PGC1α muscle mRNA compared to an untrained leg (Pilegaard, Saltin, & Neufer, 2019). Therefore, with training, PGC1α mRNA increases in the muscle meaning trained individuals should expect a greater muscle specific reaction compared to a non-trained individual. Thus, we expect exercise training to alter mitochondrial biogenesis.

Fasting and Exercise

There are many variations of exercise. Aerobic exercise is one variation with a majority of the energy being produced by the mitochondria. Aerobic exercise tends to be low to moderate intensity over a longer duration compared to anaerobic exercise. Exercise causes an increase in total mitochondrial protein content, including enzymes involved in energy production (Holloszy, 1967; Lundby & Jacobs, 2016; Scalzo et al., 2014). Exercise also causes an induction of PGC1α (Uguccioni, 2010). Exercise and metabolism are interconnected, where a change in diet and metabolism can alter exercise performance.

With fasting, one of the issues is whether there is differences in performance when exercising in a fed state or a fasted state. When coupled with caloric restriction, 6 months of aerobic training led to an increase in TFAM and PGC1α expression levels (Civitarese et al., 2007). Therefore, at the very least there is reason to believe modified consumption can lead to

altered mitochondria. Aird et. Al found numerous studies that showed a fed state improves performance, but also found a number of studies that showed no difference between fasted and fed groups (Aird et al., 2018).

Despite these adaptations, it seems there is no benefit to athletic performance while fasting (Levy & Chu, 2019). However, there might be changes in metabolism that do not lead to changes in performance. One such change in metabolism is a shift in fat oxidation. When mitochondria produce energy, it is primarily through fat oxidation. A fed state following carbohydrate ingestion results in decreased rates of fat oxidation compared to a fasted state (Achten & Jeukendrup, 2004). Fasted aerobic exercise leads to higher rates of fat oxidation than fed aerobic exercise (Vieira, Costa, Macedo, Coconcelli, & Kruel, 2016). Fat is stored in your body to provide energy. If carbohydrates are not readily available, like when fasting, it stands to reason the body would shift towards metabolizing fats for energy. Therefore, it seems like there is some window of feeding that affects metabolism of substrates.

Feeding Window

If there is a time period surrounding exercise that alters metabolism, we would expect to see a difference in exercise response between fasted and fed subjects. When exercising in a fasted state, muscle protein synthesis increases immediately after, however so does muscle protein breakdown (Biolo, Maggi, Williams, Tipton, & Wolfe, 1995). When not fasted, groups that consumed a supplement with protein pre and post exercise had increased adaptations to exercise compared to a group with the same supplement with a 5 hour fasted window before and after the workout (Cribb & Hayes, 2006). Therefore, in terms of protein synthesis numerous studies seem to show consumption of excess protein will contribute to more muscle protein synthesis (Aragon & Schoenfeld, 2013; Kumar et al., 2009). A mitigating factor in these studies

is the content of the consumed supplement. When supplemented with creatine and protein, subjects experienced greater grains in muscle mass compared to just creatine (Candow, 2011). Further, another study found protein supplementation compared to carbohydrate supplementation resulted in greater muscle cross sectional area (Anderson et al., 2017). Many of the studies above had a resistance training protocol and looked at muscle protein synthesis. While logical, seeing as how resistance exercise targets muscle, the crossover to aerobic exercise is not clear cut.

Research is limited on how the response to non-resistance exercise changes with fed and fasted individuals. Further, much of the research that is done that focuses on fasting exercise is done in untrained individuals. So, while there is some research done on exercising in a fasted state, a bulk of it is either looking at specifically protein synthesis following resistance exercise or is done on untrained individuals. The amount of studies done looking at aerobic exercise specific changes on trained individuals is minimal.

Purpose and Hypothesis

The purpose of this study was to determine if the timing of fasting in relationship to exercise time affects mitochondrial biogenesis and performance. The hypothesis is that timing of the fast will not negatively affect mitochondrial biogenesis and will not negatively impact aerobic exercise performance. We hypothesize that there will be no differences between groups.

Chapter 2

Materials and Methods

Animals and Experimental Design

All animal experiments were approved by the University of Memphis IACUC (protocol #0833). Six-week-old C57BL/6 male mice (n=36) were purchased from Envigo (Indianapolis, Indiana). Animals were housed in a USDA-approved animal facility on the university of Memphis campus. Upon arrival at the animal facility, the mice were acclimated for two weeks during which time they were entrained to a reverse light cycle with lights off (active phase) from 6am-6pm. During this time all mice had ad libitum access to food and water. Mice were housed two per cage. After these 2 weeks, the mice were randomly assigned their fasting and exercise routines. Mice were divided into 3 groups which differed in timing of food availability. Two groups followed a time-restricted feeding protocol where they had 6 hours of food access and 18 hours of fasting with ad libitum access to water. The third group had ad libitum access to food 24h a day. Mice were kept on their respective diet protocol for 8 weeks. All mice were weighed twice a week, starting the week before the diet and exercise began. Once a week the mice underwent an MRI.

Diet and feeding protocol

Mice were provided a growing rodent chow, which was approximately 20% protein, 63% carbohydrate, and 7% fat (diet composition listed in table 1) (AIN-93G, Research Diets, New Brunswick, New Jersey) for the duration of the study. The TRF-delayed group, which also was the first group to run, had food access from 12:00pm until 6:00pm; food access was provided approximately 5 hours following the cessation of exercise. The TRF-immediate group, which ran second, had food access from 8:00am until 2:00 pm. The final group (Control), which ran third, had ad libitum access to food. Food was weighed daily, and the amount consumed per cage

recorded.

Exercise

All mice exercised 5 days a week for the 8 weeks of the study. The first week of exercise all mice underwent a familiarization protocol. This familiarization protocol included the standard warm-up consisting of 5 minutes at 5 meters/minute, 5 minutes at 10 meters/minute and 5 minutes at 15 meters/minute at a 10% incline followed by 30 minutes at 20 meters/minute. Starting at week 2 through the end of the study all mice ran a protocol that consisted of the same 15-minute warm-up. After this warm-up period, the mice ran for 45 minutes at 20 meters/minute for a total of 1 hour running. All mice completed the exercise training within the first three hours of the active phase. TRF-delayed group began running at 6:00am, TRF-immediate started at 7:00am, and the control group began running at 8:00am.

Run to Fatigue

After the week of familiarization with the treadmill (Columbus Instruments, Columbus, Ohio), the mice underwent a run to fatigue. The run to fatigue involved the same warm-up protocol as training. After 20 minutes at 20 meters/minute the speed was increased to 25 meters/minute. The mice then ran until fatigue set in. Mice were deemed fatigued if they, upon nudging, did not run and fell to the back of the treadmill consecutively. The incline was set at 5%. To encourage the mice to run a gloved hand was used to push the mice away from the end of the belt towards the front of the treadmill. Mice performed two total runs to fatigue, one at the end of week 0 and the other at the end of week 8. Mice were given two days off between their last bout of exercise and their run to fatigue.

MRI Protocol

Once a week, starting the one week before initiation of exercise and diet protocols, mice

underwent an MRI. The mice were fasted 6h prior to their MRI. The two (TRF-delayed and TRF-immediate) time restricted feeding groups underwent their usual fasting. The *ad libitum* group had their food removed at 10:00pm the night prior to testing. The mice were tested in the following order: TRF-delayed, TRF-immediate, and then control. Mice were placed in a holding tube. The tubes were then placed into the MRI machine (EchoMRI, Houston, Texas). The results from the machine were recorded. Mice were then removed from the tubes and placed back in their cages. MRI was conducted prior to running.

Sacrifice

On the day of the sacrifice the mice completed their standard exercise protocol. Upon finishing their run the mice were injected with puromycin (0.04µMol/ g BW). 30 minutes later the mice were sacrificed. Hindlimb muscles were collected from each hind leg weighed and placed in liquid nitrogen until they could be stored in a -80° Celsius freezer for later use.

Protein Expression/Isolation

A portion of the right gastrocnemius was homogenized on ice with Mueller Buffer (composition provided in table 3) that contains protease and phosphatase inhibitors. The samples were placed in a centrifuge for ten minutes at 10,000 g's at 4 ºC. Next the supernatant was removed and placed in a new tube combined with Diluent Buffer (composition provided in table 4). A Bradford assay was used to quantify the amount of protein in each sample. Samples were then diluted to 1.5ug/ul with diluent buffer and a 5X Lane Marking Reducing Sample Buffer and then heated the samples at 95° Celsius for five minutes. The samples were then loaded into a Western Blot apparatus to be ran through sodium dodecyl sulfate polyacrylamide gel electrophoresis [SDS-PAGE] on a 4-15% gradient gel and subsequently transferred on to a PVDF membrane. The transfer was checked with a ponceau stain. The ponceau was removed

and the blot was washed three time for five minutes in Tris- buffered-saline-0.1% Tween 20 (TBST). The membrane was blocked in 5% Bovine Serum Albumin in TBST for an hour and then placed in their primary antibody overnight, PAMPK and AMPK (Cell Signaling Technology, Danvers, Massachusetts), PGC1α and ETC components- total OXPHOS (ABCAM, Cambridge, United Kingdom), and GAPDH (Santa Cruz Biotechnology, Dallas, Texas). The next day, the membranes were placed in the appropriate animal based secondary for two hours, either mouse based or rabbit based (Cell Signaling Technology, Danvers, Massachusetts). The washing step was repeated afterwards and then the blots were photographed using chemiluminescent agent and imaged with an iBright FL1500 imaging system (Thermo Fisher Scientific, Waltham, Massachusetts). ImageJ software was used to quantify the bands.

Gene Expression/RNA Isolation

RNA was extracted from the gastrocnemius using Trizol (Ambion, Austin, Texas) following the manufacturers specifications. cDNA was prepared using a high capacity cDNA kit (Applies Biosystems, Waltham, Massachusetts, USA). RT PCR was run on the following genes: TFAM and PGC1α, sequences can be found in table 2. Primers were ordered from IDT (Coralville, Iowa, USA). The PCR was run using PowerUp SYBER green (Applies Biosystems, Waltham, Massachusetts, USA.). All PCR was run on a QuantStudio 6 instrument (Applies Biosystems, Waltham, Massachusetts, USA). Data were analyzed using the ddct method.

Statistical Analysis

All data are presented as mean ± SEM. A repeated measures ANOVA was used to access differences across time and condition. A one-way ANOVA was used to compare across conditions. Tukey post hoc analysis was used to examine interactions. GraphPad Prism 8 (San Diego, CA, USA) was used to analyze and graph all data.

Chapter 3

Results

Body Weight Changes

Body weight was measured twice a week starting the week before the exercise and fasting protocol started (figure 1A). There was an effect of time ($p<0.0001$) and the interaction of time and diet ($p<0.0001$) on body weight. On day 42, the start of week 7, the TRF-immediate group on average weighed 2.1 grams less than the control group ($p=0.023$) and 1.9 grams less than the TRF- delayed group ($p=0.037$). On day 51, the end of the study, the TRF-immediate group weighed on average 2.1 grams less than the control group ($p=0.039$).

To discern what might be causing the differences, MRIs were performed weekly on the mice. Lean mass (figure 1B) varied significantly based on week ($p<0.0001$), diet ($p=0.046$) and the interaction of week and diet ($p=0.003$). In weeks 1, 2, 4, and 7 the TRF-immediate group was significantly lower than the control group. In week 7 the TRF-delayed group was also significantly lower than the control group. Body fat percentage was also measured (figure 1C). Week ($p<0.0001$) and the interaction of week and diet ($p<0.0001$) were found to be potential sources of variation in body fat percentage. In weeks 2 and 4 the TRF-delayed group was significantly greater than the control group. In week 5 the TRF-delayed group was significantly greater than the TRF-immediate group.

To identify if the differences in lean body mass were attributed to alterations in muscle, hindlimb muscle mass was measured as the sum of the gastrocnemius, tibialis anterior, extensor digitorum longus, soleus, and plantaris (figure 1D). Hindlimb mass in the TRF-immediate group was 9%, ($p=0.029$) lower than the control group on average. Similarly, the hindlimb mass of the

TRF-delayed group was 10 % (p=0.50) lower than the control group.

Transcriptional regulation of mitochondrial biogenesis

We examined if the acute response of mitochondrial biogenesis through transcriptional regulation was altered by varying feeding windows and exercise. PGC1α expression was significantly lower in the TRF-delayed compared to the control group (p=0.008) (figure 2A). We next looked at markers of mitochondrial biogenesis to see if the different fasting times altered mitochondrial biogenesis.

There were no statistical differences in PGC1α protein expression, (figure 4A), however there was a trend towards an increase in the TRF-immediate group compared to the control group. (p=0.072). There was also a trend towards a decrease of PGC1α protein expression in the TRF- delayed group compared to the TRF-immediate group (p=.057). TFAM activates mitochondrial transcription and is a known target of PGC1α. There were no significant differences in TFAM mRNA expression between groups, (figure 2B); however, there was a trend towards a decrease in the TRF-delayed group compared to the control (p=.087).

Marker of energy status and mitochondrial biogenesis

P-AMPK is activated in periods of high energy demand when ADP levels are elevated. We looked at P-AMPK to see if TRF altered the energy signaling after a bout of exercise in trained mice (figure 3A). We found no statistical differences in P-AMPK levels between groups. Next components of the electron transport chain were analyzed as an indicator of mitochondrial content, (figure 4B-4F). There were no statistical differences in any of the ETC components between groups.

Run to exhaustion

Finally, we looked at run to exhaustion time to see if overall performance was altered by

TRF, (figure 5). Mice performed a run to exhaustion before and after 8 weeks of training. There was a main effect of time on performance ($p=0.04$). The control group improved by 14%±43%. The TRF-immediate group increased by 13% ±33%, and the TRF-delayed group improved by 55% ±86%; however, there was no significant group effect, suggesting that the timing of feeding had no effect on performance in trained mice.

Chapter 4

Discussion, Conclusion, Limitations

Discussion

Over the last few years, lifestyle modifications that restrict the timing of food have become popular methods to prevent metabolic disorders and control weight. In addition, some athletes utilize time restricted feeding to enhance performance. There is limited evidence demonstrating the effect of timing of fasting on performance and aerobic adaptations in skeletal muscle.

TRF has been shown to affect body composition. We found that mice eating *ad libitum* had the largest increase in lean mass. Others have also demonstrated a greater increase in lean mass and lower body weights in rodents following a 10 week exposure to *ad libitum* feeding of a high fat and sugar diet (Wilson, Deasy, Stathis, Hayes, & Cooke, 2018). In comparing various methods of restricted consumption including time restricted feeding and alternate day fasting, all restricted groups had less fat gain than a control group (Smith et al., 2019). Although our control mice were leaner, this could be due to the fact that they were on a healthy diet instead of a high fat diet. Another study done in humans with an 8-hour feeding window found that resistance training of 4 workouts a week combined with intermittent fasting can result in an enhanced body composition via decreased fat mass and increased lean mass (Hayward et al., 2014).

While, many studies done on intermittent fasting focus on a high fat diet, disease, or both (Gotthardt & Bello, 2017; Park, Yoo, Hyun, & Kang, 2017; Wilson et al., 2018). Our data supports the idea that intermittent fasting can alter body composition even in exercising mice consuming a healthy diet. This data is further supported in heathy humans during Ramadan fasting who lost weight whether or not they exercised in a fasted or a fed state (Trabelsi et al.,

2012). Both of these groups underwent fasting, with the main difference being if they ate prior to exercise. Body fat percentage was not statistically different in our groups at the end of the study. This result could be because our mice, even the control group, underwent a healthy diet with exercise. A study looking at healthy, lean adults during Ramadan fasting (28 days of 14 hours) found no changes in body composition comparing pre and post fast (Harder-Lauridsen et al., 2017). This aligns with our results at the end of the study; however early in the study we demonstrated greater body fat in the fasting groups compared to the *ad libitum* group and lower lean mass. It could be that the first few weeks of the intervention the mice were adapting to the increased stress caused by both the exercise and the fasting. Both of our TRF groups exercised in a fasted state. Ramadan fasting combined with aerobic exercise in humans shows that exercising in a fasted state compared to a fed state results in a lowered body fat percentage (Trabelsi et al., 2012). This is different from our findings; however, the timing or refeeding after exercise and the length and time of day of the fasting and exercise may impact body composition. Sato et al. demonstrate differential substrate utilization base on the time of day exercise is conducted. While they did not look at fasting directly exercise early in the active phase, after a period with reduced feeding activity, showed a greater metabolic response including glycolysis and lipid oxidation (Sato et al., 2019). More research is needed to identify the interactions of time of feeding pre and post exercise on body composition in healthy populations.

Metabolic flexibility is regulated by activation the energy sensor AMPK. We found no difference in AMPK phosphorylation levels between our groups after exercise. AMPK is

activated following an increase in the AMP/ATP ratio (Hardie, 2007) AMPK activity is increased when muscle glycogen is low, both at rest and during exercise however AMPK is also believed to increase fatty acid uptake and oxidation (Richter & Ruderman, 2009). Both fasting (Bujak et al., 2015; Steinberg & Jorgensen, 2007) and exercise activate AMPK acutely in skeletal muscle (Chen et al., 2003; Fujii et al., 2000; Wojtaszewski, Nielsen, Hansen, Richter, & Kiens, 2000). Studies have shown that exercising as low as approximately 60% of max aerobic capacity is sufficient to activate AMPK (Chen et al., 2003; Musi et al., 2001; Stephens et al., 2002; Wojtaszewski et al., 2003; Wojtaszewski et al., 2000). In humans trained individuals have a higher acute expression of AMPK than untrained individuals (Nielsen et al., 2003).

Although our study was conducted with chronic training, due to the timing of the sacrifice we see that there was no change in the acute response following training and fasting. While training should impact AMPK levels, all the mice did the same training. The difference in groups was the timing of the fast that they did. If fasted for long enough, the metabolic switch can be flipped (C. Cantó & Auwerx, 2009). The metabolic switch is the point at which the glycogen in the liver is depleted and the body mobilizes fatty acids. It is suggested that this switch is triggered beyond 12 hours of fasting (S. D. Anton et al., 2018). Despite our two fasting groups both fasting for at least 12 hours before exercise, and 16 hours total, we did not find an acute change in AMPK levels after exercise.

AMPK activation is also shown to regulate mitochondrial biogenesis (C. Cantó & Auwerx, 2009; Jørgensen et al., 2007; Suwa, Nakano, & Kumagai, 2003; Terada et al., 2002; Winder et al., 2000). Although we did not see acute changes in AMPK, one adaptation to aerobic training in increases in skeletal muscle mitochondrial biogenesis. We found no significant difference in protein expression of PGC1α. However, we did find a trend towards an increase in

TRF-immediate compared to control and a decrease in TRF-delayed compared to TRF-immediate in protein expression levels. Gene expression of PGC1α was significantly lower in the TRF-delayed group compared to the control group after exercise. PGC1α expression has previously been shown to increased 24-36 hours after exercise (Kuhl et al., 2006). Additionally, PGC1α gene expression has been shown to acutely increase within 6 hours of food removal and return to baseline after 48 hours (de Lange et al., 2006). All of our groups were at least 6 hours removed from food prior to their harvest. However, the delayed group had its food removed earlier than the control group prior to tissue harvest. This could explain the difference in the delayed group compared to control, as expression could peak and begin to decrease over time. Long-term fasted state aerobic training leads to increased mitochondrial capacity and function in healthy subjects (Hansen, De Strijcker, & Calders, 2017). PGC1α plays an important role in maintaining high fat oxidation when the body is in a fasted state, but it is not as important for substrate switching in a fed state (Gudiksen & Pilegaard, 2017). PGC1α mRNA levels are increased following exercise both acutely (Pilegaard et al., 2019) and chronically (Short et al., 2003). Excess stress placed on the muscle after exercise and inability to replenish energy stores by delaying feeding after exercise may contribute to the suppressed PGC1α levels in the TRF-delayed group. In much the same way that protein ingestion after resistance exercise increases protein synthesis, it could be that immediate consumption in the TRF-immediate group promotes mitochondrial biogenesis, whereas the TRF-delayed group does not have that stimulus.

Although we found differences in PGC1α, we found no statistical differences in its target gene, TFAM, expression between groups. However, similar to the PGC1α expression there was a trend towards a decrease in TFAM expression in the TRF-delayed group versus the control group ($p=0.087$). Independently both fasting and exercise increase TFAM and PGC1α expression levels (Civitarese et al., 2007). TFAM is increased following acute bouts of exercise (Saleem & Hood,

2013) as well as chronic training (de las Heras et al., 2018). One study found that alternate day fasting by itself did not increase TFAM expression, but combining exercise with alternate day fasting resulted in an increase in TFAM expression compared to control (Marosi et al., 2018). These mice had access to food immediately post exercise on feeding days meaning the mice either exercised on a fed state, like our control group, or in a fasted state with immediate access post exercise, like our TRF-immediate group. Unlike our study, their mice alternated between these states daily. This alteration could explain the differences in results between our study and theirs. However, unlike our TRF-immediate group, they found an increase in TFAM mRNA levels. One reason for our differing results could be the length of fast. Our mice underwent an 18 hour fast, compared to a 24 hour fast and the TRF-immediate group was entrained to receive food after exercise. In regard to our TRF-delayed group, the decrease in TFAM gene expression is consistent with the suppression of PGC1α levels we observed.

In addition to regulating mitochondrial biogenesis muscle PGC1α is shown to affect exercise performance (Calvo et al., 2008). In our run to exhaustion testing, we found no significant difference between our dietary groups, but we did find time to have a main effect. This shows that our mice successfully adapted to the training stimulus. However, the lack of difference between groups suggests that the timing of fast does not alter performance. Marosi et. al found that mice performed better on a run to exhaustion after a month of alternate day fasting compared to *ad libitum* feeding (Marosi et al., 2018). The difference here could be in the duration of fast. Our mice fasted for a total of 16 hours, whereas their mice fasted for 24 hours. Researchers also found a lowered RER in their ADF group compared to their *ad libitum* group, showing a shift towards using fats as fuel (Marosi et al., 2018). Interestingly, researchers

looking at exercise at various times of day found that early active phase exercise, leads to diminished transcripts related to mitochondrial respiratory function following acute exercise (Sato et al., 2019). Marosi et. al exercised their mice during the mice's rest phase (Marosi et al., 2018). Sato et. al found an increase in lipid metabolites during activity in the rest phase (Sato et al., 2019) which matches the RER data from the Marosi et. al study. By comparison, our mice exercised and tested during the active phase, in which Sato et. al found an increase in glycolytic metabolites, indicating increased glycolysis (Sato et al., 2019). This matches our results and may help explain why we found limited differences in mitochondrial biogenesis. Further work in needed to determine if the window of feeding during time restricted feeding alters substrate utilization and metabolic flexibility when combined with exercise.

Limitations

The present study was designed to examine the effects of timing of feeding during TRF in a healthy exercising population. Our results present the acute alterations in skeletal muscle mitochondrial biogenesis in trained mice after eight weeks of time restricted feeding. Future research is needed to identify the acute changes that occur when starting a TRF protocol with exercise. Our study presented several limitations. In our study, the lack of a group that performed no exercise limits the comparisons we can draw. Although the design allows for a comparison between trained responses, any decreases compared to control can't truly show to what level they are decreased compared to an untrained population. Additionally, we were limited to one time point after exercise to make our comparisons. Future studies should examine a time course to identify if responses are blunted or delayed.

Research supports the idea that intermittent fasting improves markers of health [13]. Weight loss has been a benefit extensively shown, but the existence of other believed benefits

suggests that even those who are not seeking weight loss can benefit from intermittent fasting. It seems that, when fasting, immediate consumption of your meal leads to an increased metabolic response compared to delayed consumption. However, when it comes to aerobic performance, it doesn't seem to matter when the fasting window occurs.

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Appendix

List of Tables

Table 1. Diet per 1000g

Class description	Ingredient	Grams	
Protein	Casein, Lactic, 30 Mesh	200	g
Protein	Cystine, L	3	g
Carbohydrate	Starch, Corn	397.49	g
Carbohydrate	Lodex 10	132	g
Carbohydrate	Sucrose, Fine Granulated	100	g
Fiber	Solka Floc, FCC200	50	g
Fat	Soybean Oil, USP	70	g
Anti-oxidant	tert-Butylhydroquinone (tBHQ)	0.01	g
Mineral	S10022G	35	g
	Sucrose, Fine Granulated	7.73597264	g
	Calcium Carbonate, Light, USP	12.49487505	g
	Potassium Phosphate, Monobasic	6.859931401	g
	Sodium Chloride	2.5899741	g
	Potassium Citrate, Monohydrate	2.477275227	g
	Potassium Sulfate	1.63098369	g
	Magnesium Oxide, Heavy, DC USP	839.9916001	mg
	Ferric Citrate	212.097879	mg
	Zinc Carbonate	57.74942251	mg
	Sodium Metasilicate	50.74949251	mg
	Manganese Carbonate Hydrate	22.0497795	mg
	Copper Carbonate	10.499895	mg
	Chromium Potassium Sulfate	9.799902001	mg
	Boric Acid	2.799972	mg
	Sodium Fluoride	2.099979	mg
	Nickel (II) Carbonate	1.0499895	mg
	Lithium Chloride, anhydrous	0.699993	mg
	Sodium Selenate	0.3499965	mg
	Potassium Iodate	0.3499965	mg
	Ammonium Molybdate Tetrahydrate	0.3499965	mg
	Ammonium (meta)vanadate	0.3499965	mg
Vitamin	V10037	10	g
	Sucrose, Fine Granulated	9.719203	g
	Vitamin E Acetate, 50%	149.9985	mg
	Niacin (a.k.a. B3)	29.9997	mg
	Vitamin B12, 0.1% Mannitol	24.99975	mg
	Biotin, 1%	19.9998	mg
	Pantothenic Acid, d, Calcium (a.k.a. B5)	15.99984	mg
	Vitamin D3, 100,000 IU/gm	9.9999	mg
	Vitamin A Acetate, 500,000 IU/gm	7.99992	mg
	Pyridoxine HCl (a.k.a. B6)	6.99993	mg
	Riboflavin (a.k.a. B2)	5.99994	mg
	Thiamine HCl (a.k.a. B1)	5.99994	mg
	Folic Acid	1.99998	mg
	Phylloquinone (a.k.a. Vitamin K1)	0.799992	mg
	Choline Bitartrate	2.5	g

Table 2. Gene primers for qPCR analysis.

Gene Symbol	Forward (5'-3')	Reverse (5'-3')	Amplicon size (bp)
PGC1-α	AAGACGGATTGCCCTCATTT	AGTGCTAAGACCGCTGCATT	191
TFAM	TCCCCTCGTCTATCAGTCTTG	GGGCTGCAATTTTCCTAACC	171
GAPDH	GTTGTCTCCTGCGACTTCA	TGCTGTAGCCGTATTCA	124

Table 3. Composition of stock Muller Buffer

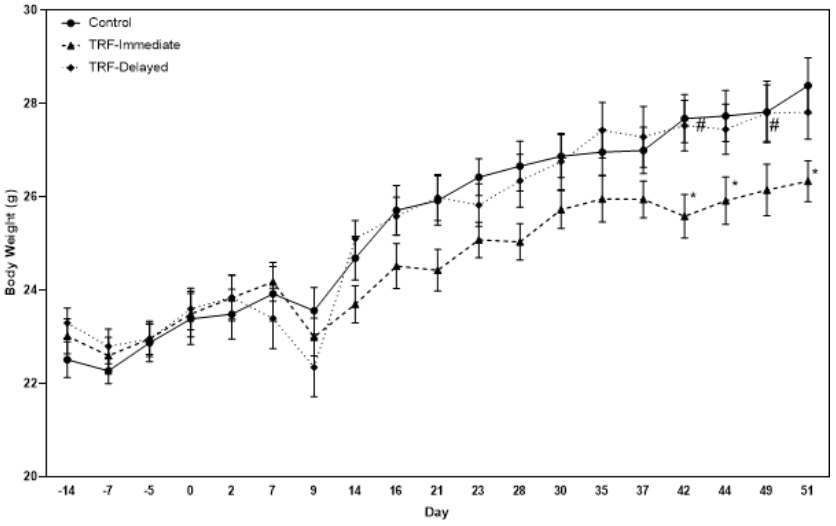
Muller Buffer	Stock Concentration	Desired Concentration	Volume needed (ul)
Hepes	500mM	50mM	600
Triton-X	100%	0.10%	6
EGTA (pH 8.0)	500mM	4mM	48
EDTA (pH 8.0)	500mM	10mM	120
$Na_4P_2O_7$	100mM	15mM	900
β-glycerophosphate	2M	100mM	300
NaF	500mM	25mM	300
NaVO4	1M	5mM	30
dH2O	-	-	3585
Protease Inhibitor	-	-	60

Table 4. Composition of Diluent Buffer

Diluent Buffer	Stock Concentration	Desired Concentration	Volume needed (ul)
glycerol	100%	50%	1500
$Na_4P_2O_7$	100mM	50mM	1500
EGTA (pH 8.0)	500mM	2.5mM	15
β-mercaptoethanol	500mM	1mM	6
Protease Inhibitor	-	-	30

List of Figures

A



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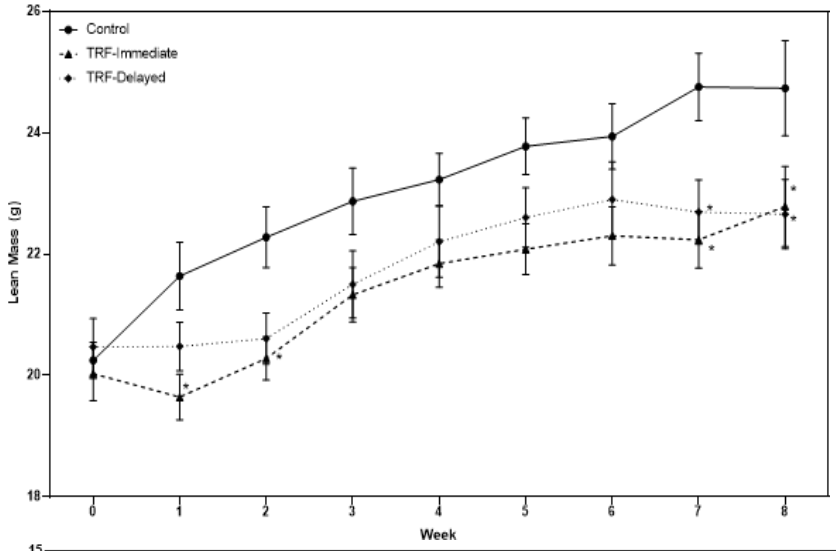

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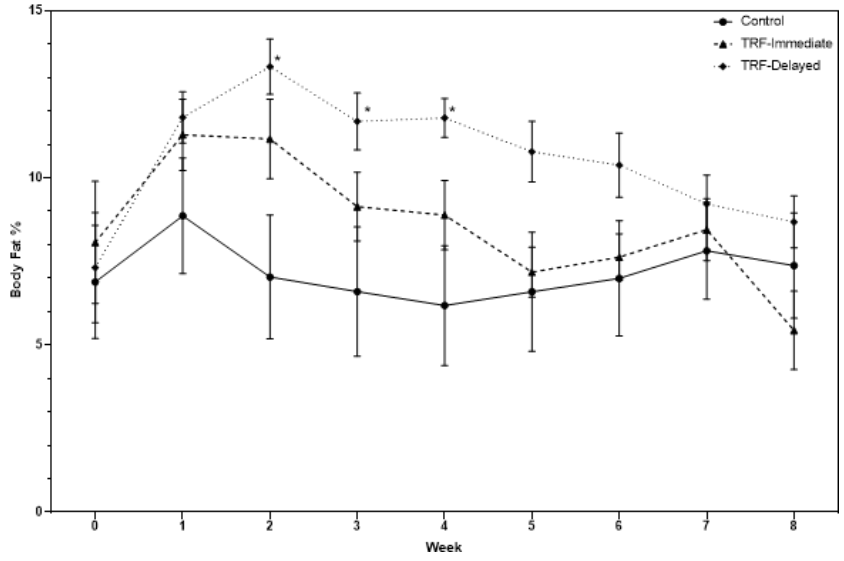

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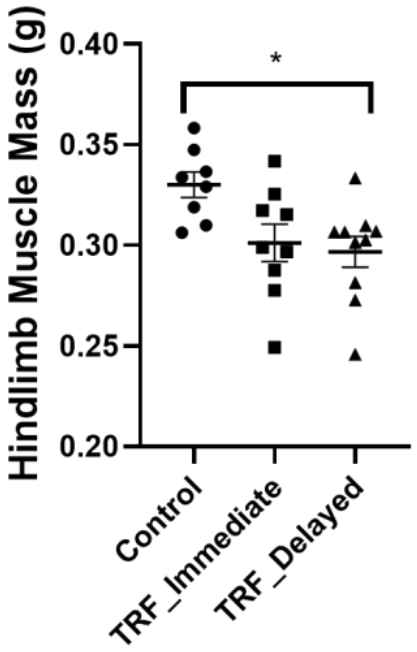

Figure 1: Body weight changes during exercise with time restricted feeding. A) Body weight was measured twice a week for the duration of the experiment. B) Lean mass was measured by MRI, once a week. C) Body measured weekly by MRI D) Average hindlimb muscle mass measured as the sum of Gastrocnemius, Tibialis Anterior, Extensor Digitorum Longus, Soleus, and Plantaris on the day of the sacrifice. All data presented as means ± SEM. A repeated measures ANOVA was used to identify differences between groups over time (A-C). A one-way ANOVA was run to identify differences between groups (D). * $p<0.05$ compared to control. # $p<0.05$ compared to TRF-Immediate.

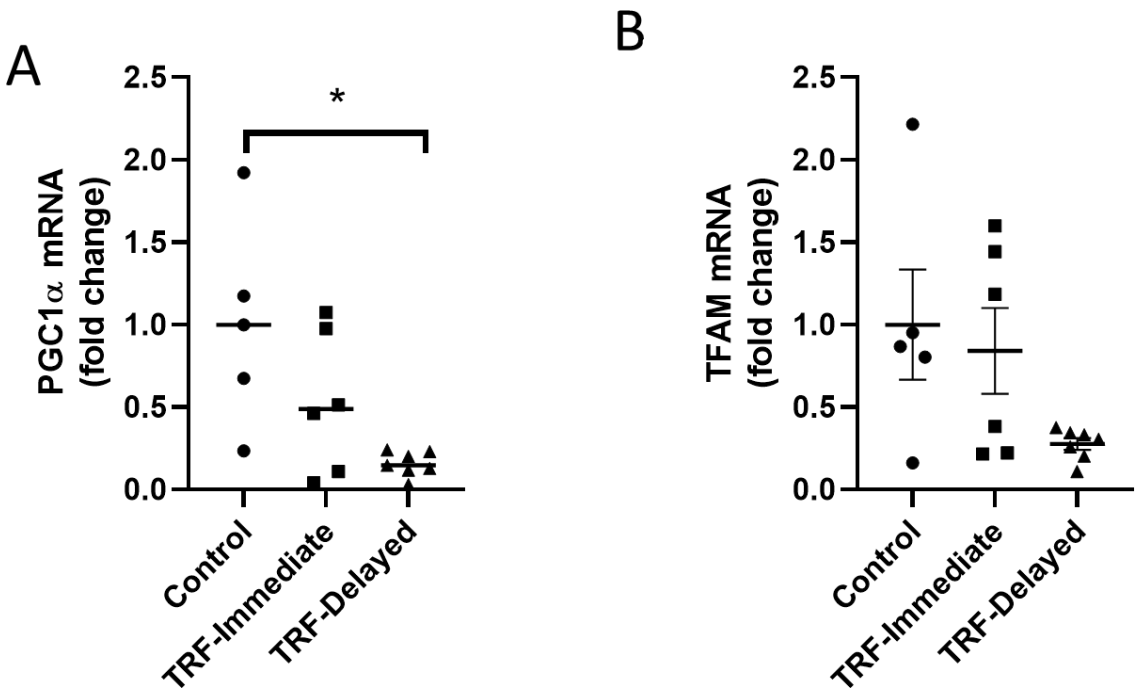

Figure 2: Transcriptional regulation of mitochondrial biogenesis. mRNA expression levels of A) PGC1α and B) TFAM were measured in the gastrocnemius muscle. All data are presented as means ± SEM. A one-way ANOVA was run to identify differences between groups. * $p<0.05$ compared to control

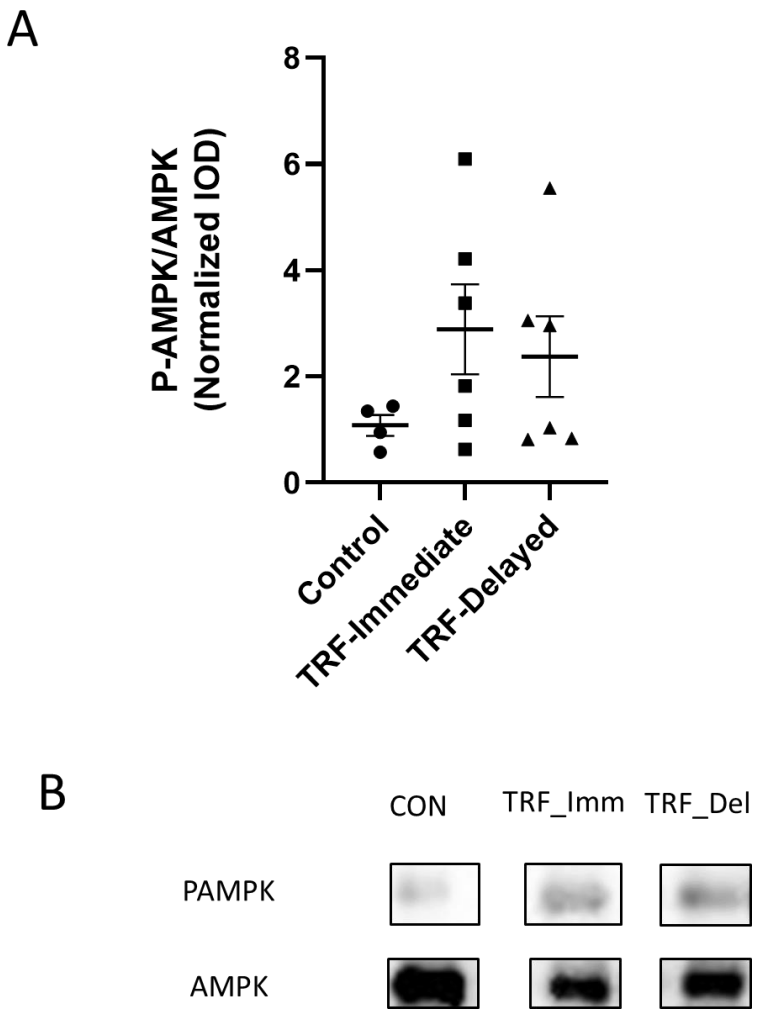

Figure 3: Marker of energy status in TRF mice after exercise. A) Protein quantification of P-AMPK (Thr172) normalized to total AMPK B) Representative blots. All data presented as means ± SEM. A one-way ANOVA, was run to identify differences between groups.

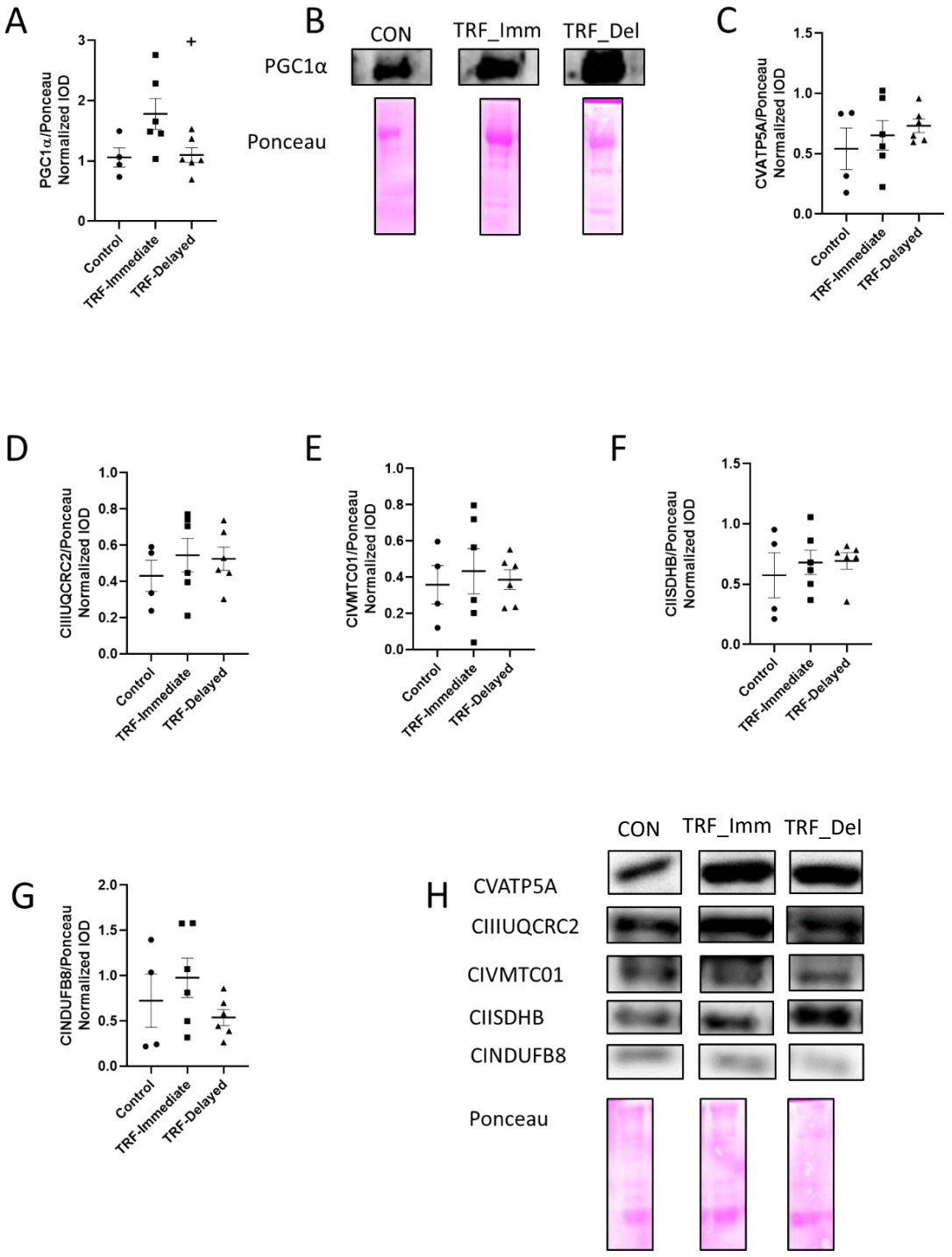

Figure 4: Skeletal muscle mitochondrial content and markers of biogenesis in TRF exercised mice. A) PGC1α protein quantification normalized to ponceau B) Representative blots of PGC1α C) CVATP5A normalized to ponceau D) CIIIUQCRC2 normalized to ponceau E) CIVMTC01 normalized to ponceau F) CIISDHB normalized to ponceau G) CINDUFB8 normalized to ponceau H) Representative blots All data are presented as means ± SEM. A one-way ANOVA, was run to identify differences between groups, + $p<0.05$.

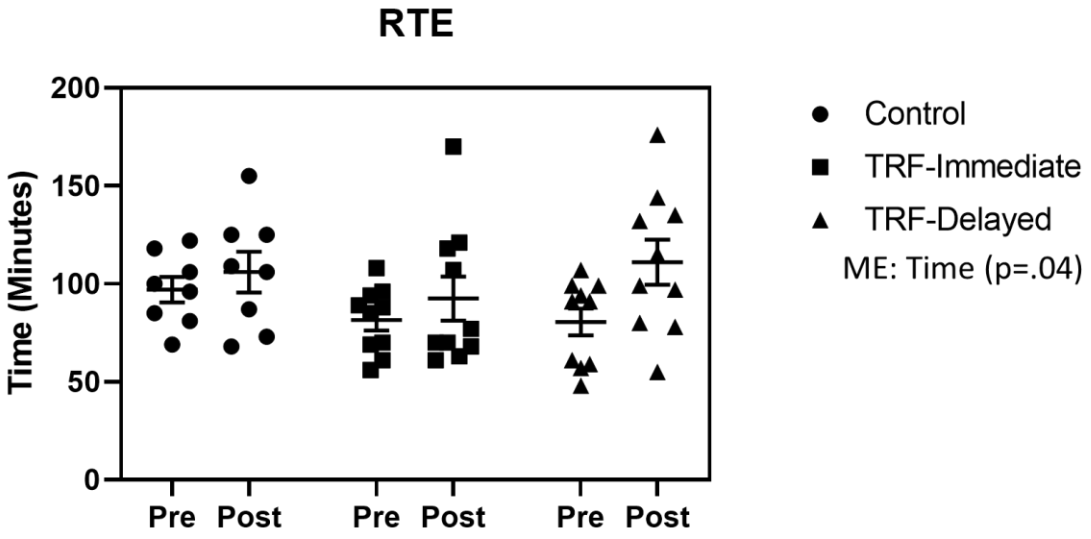

Figure 5: Run to exhaustion. Results from the run to exhaustion test run both prior to and post 8 weeks of training. All data are presented as means ± SEM. A Repeated measures ANOVA was run to identify differences between groups and time.

IACUC PROTOCOL ACTION FORM

To:	Richard J. Bloomer
From	Institutional Animal Care and Use Committee
Subject	Animal Research Protocol
Date	December 20, 2018

The institutional Animal Care and Use Committee (IACUC) has taken the following action concerning your Animal Research Protocol No. 833

833 Impact of time restricted feeding and exercise on body composition and associated measures in male C57BL/6 mice

☒ Your protocol is approved for the following period:

From: January 1, 2019 To: December 31, 2019

☐ Your protocol is not approved for the following reasons (see attached memo).

☐ Your protocol is renewed without changes for the following period:

From: _____ To: _____

☐ Your protocol is renewed with the changes described in your IACUC Animal Research Protocol Update/Amendment Memorandum dated _____ for the following period:

From: _____ To: _____

☐ Your protocol is not renewed and the animals have been properly disposed of as described in your IACUC Animal Research Protocol Update/Amendment Memorandum dated _____

Amy L. de Jongh Curry, PhD, Chair of the IACUC

Karyl K Buddington

Dr. Karyl Buddington, University Veterinarian and Director of the Animal Care Facilities