# BEET-ING MUSCLE DYSFUNCTION AND EXERCISE INTOLERANCE IN PULMONARY HYPERTENSION

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# DEDICATION

To Aubrey and Rhys, this one's for you.

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Gary Marshall Long

BEET-ING MUSCLE DYSFUNCTION AND EXERCISE INTOLERANCE IN PULMONARY HYPERTENSION

Background: Pulmonary Hypertension (PH) is a devastating disease characterized by pulmonary arterial remodeling, right ventricular dysfunction and ultimately right heart failure. Increased emphasis has been given to skeletal muscle dysfunction in PH, and to its implication in the severe exercise intolerance that is a hallmark of the condition. In this dissertation, skeletal muscle blood flow was measured via the microsphere technique at rest and during exercise (Aim 1), with an acute dose of dietary nitrate via beetroot juice (BRJ) gavage used to determine if supplementation could improve muscle blood flow and alter energetics (Aim 2). $VO_2$max, voluntary running and grip strength tests were used to determine the effect of disease on performance, and to test for an ergogenic effect of BRJ vs. placebo (PL) in healthy and PH rats (Aim 3). Methods: A prospective, randomized, counterbalanced, placebo-controlled trial was used to examine the aforementioned aims across four groups; PH rats (induced with monocrotaline, MCT, 60mg/kg, s.q., 4 weeks) supplemented with BRJ (MCT BRJ, n=9); PH rats supplemented with placebo (MCT PL, n=9); healthy control rats (vehicle, s.q.) supplemented with BRJ (CON BRJ, n=8); healthy control rats supplemented with placebo (CON PL, n=9). Results: Monocrotaline induced a severe PH phenotype evidenced by increased RV wall thickness, RV hypertrophy, RVSP and reduced cardiac output and stroke volume compared to controls ($p=<0.001$). MCT rats demonstrated lower muscle blood flow at rest, and more prominently during exercise compared to controls ($p=0.007$-$0.047$), regardless of supplementation. MCT rats displayed a greater reliance on anaerobic metabolism,

demonstrated by increased blood lactate accumulation (p=<0.001), and this was significantly related to reduced blood flow during exercise (r=-0.5879, p=0.001). BRJ supplementation resulted in increased plasma nitrate and nitrite compared to PL (p=<0.001), but at the skeletal muscle level, only nitrate was increased after BRJ. BRJ did not have a significant effect on blood flow, with no improvement during exercise shown vs. PL. Similarly, BRJ did not significantly improve exercise function in MCT or CON rats. Conclusion: MCT rats demonstrated a reduction in muscle blood flow, with BRJ supplementation not resulting in improved flow or exercise performance.

Andrew R. Coggan, Ph.D., Chair

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LIST OF ABBREVIATIONS

| | |
|---|---|
| ANOVA | Analysis of Variance |
| $BH_4$ | Tetrahydrobiopterin |
| BMPR | Bone Morphogenetic Protein Receptor |
| BRJ | Beetroot Juice |
| cGMP | Cyclic Guanosine Monophosphate |
| CPET | Cardiopulmonary Exercise Test |
| CSA | Cross Sectional Area |
| EDL | Extensor Digitorum Longus |
| eNOS | Endothelial Nitric Oxide Synthase |
| ERA | Endothelial Receptor Agonist |
| ET-1 | Endothelin-1 |
| GLUT-1 | Glucose Transporter 1 |
| HADH | 3-hydroxacyl-CoA dehydrogenase |
| HIIT | High Intensity Interval Training |
| HF | Heart Failure |
| HFrEF | Heart Failure with reduced ejection fraction |
| HFpEF | Heart Failure with preserved ejection fraction |
| HIF1α | Hypoxia Inducible Factor-1 |
| HPLC | High Performance Liquid Chromatography |
| iNOS | Inducible Nitric Oxide Synthase |
| LV | Left Ventricle |
| MCT | Monocrotaline |
| MIP | Mean Inspiratory Pressure |
| MEP | Mean Expiratory Pressure |
| MMP | Matrix Metalloproteinase |
| MuRF-1 | Muscle Ring Finger Protein 1 |
| nNOS | Neuronal Nitric Oxide Synthase |
| NO | Nitric Oxide |
| $NO_2^-$ | Nitrite |
| $NO_3^-$ | Nitrate |

| | |
|---|---|
| NOS | Nitric Oxide Synthase |
| NIRS | Near Infrared Spectroscopy |
| PAH | Pulmonary Arterial Hypertension |
| PDE 5 | Phosphodiesterase 5 |
| PDK | Pyruvate Dehydrogenase Kinase |
| PFK | Phosphofructokinase |
| PH | Pulmonary Hypertnension |
| PL | Placebo |
| RER | Respiratory Exchange Ratio |
| RV | Right Ventricle |
| RVSP | Right Ventricular Systolic Pressure |
| SERCA | Sarcoplasmic/Endoplasmic Reticulum Calcium ATPase |
| TBS | Tris Buffered Saline |
| $V_E$ | Volume of Expired Air |
| VEGF | Vascular Endothelial Growth Factor |
| XOR | Xanthine Oxidoreductase |

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## Chapter One: Introduction to the Study

### Introduction

Pulmonary Hypertension (PH) is a complex, progressive and devastating disease characterized by pulmonary vascular remodeling, right ventricular hypertrophy, skeletal muscle dysfunction and ultimately right heart failure (Elliot & Kiely, 2004). Defined clinically as a right ventricular systolic mean arterial pressure of >25mmHg, this broad disorder was first classified in 1973 by the World Health Organization (Kiely, Elliot, Sabroe, & Condliffe, 2013). In the subsequent years, advancing knowledge of PH etiologies has led to a refined understanding of the disease, now classified according to pathological characteristics and therapeutic approaches (Simonneau et al., 2014). *Table 1.1* summarizes the most recent classification of PH, outlining the five groups of disorders that are now widely accepted and adopted by experts worldwide.

| Group | Type | Associations |
|---|---|---|
| **I** | Pulmonary Arterial Hypertension (PAH) | • Idiopathic<br>• Heritable<br>• Drug and toxin induced<br>• Associated (HIV, portal HTN, congenital heart disease, schistosomiasis) |
| **II** | PH with left heart disease | • Systolic/diastolic dysfunction<br>• Valvular disease<br>• Cardiomyopathies |
| **III** | PH due to lung disease/hypoxia | • COPD<br>• Sleep disorder<br>• Exposure to high altitude |
| **IV** | Chronic thromboembolic PH (CTEPH) | • From chronic pulmonary embolism |
| **V** | Multifactorial PH | • Blood, metabolic and systemic disorders<br>• Chronic renal failure |

**Table 1.1:** PH classification (Simonneau et al., 2013)

Over the last three decades, significant attention has been given to the investigation of pulmonary arterial hypertension (PAH, Group 1), with significant strides made in understanding the development of, and potential treatment options in the disease (Hemnes & Humbert, 2017). PAH is recognized as a progressive disorder during which thrombosis, proliferation, inflammation and remodeling of the lung vasculature leads to a narrowing of pulmonary arteries and subsequent right ventricular overload, dilation, and ultimately myocardial failure (Galie, Palazzini, & Manes, 2010). Unfortunately, the symptoms of PAH are often subtle, non-specific and can easily be missed in a physical examination (Nauser & Stites, 2001), which may in part contribute to the 'silence' of the disease, and the poor prognosis once PAH has been diagnosed.

PAH is a relatively rare condition, with a prevalence of approximately 320 cases per 100,000 (Strange et al., 2012), and the current median survival for PAH patients is approximately 7 years from diagnosis (McGoon et al., 2013). This is an increase of close to 5 years over the last three decades, likely driven by an increase in available treatment options. The increasing mean age of patients at diagnosis, which has risen from $36 \pm 15$ years in the mid 1980's to $50 \pm 14$ and $65 \pm 15$ years would suggest greater prevalence in those of advancing age. While this shift may also represent an increased awareness of the condition (Rosenkranz, 2015), no cure exists, and patients suffer from a significantly decreased quality of life. Symptoms of PAH include fatigue, dyspnea, shortness of breath and severe exercise limitation (Barnes, Brown, Burns, & Williams, 2019). Indeed, this reduction in functional capacity has been used specifically to describe PAH symptoms by the New York Heart Associations and World Health Organization. Here, poorer physical capacity is directly linked to disease progression, with Group I patients begin described

as asymptomatic with normal activity, to Group IV who present with symptoms even at rest (Rubin, 2004).

**Background to the Problem**

The pathophysiology of PAH is complex, and as such, treatment is challenging (Bogaard, Abe, Vonk Noordegraaf, & Voelkel, 2009; Galie et al., 2014; Galie & Manes, 2013), with clinical decisions based on severity of disease, etiology, and response to drug therapy (Steiropoulos, Trakada, & Bouros, 2008). In a recent review, the most common strategies in battling PAH were outlined, including pharmacological and behavioral approaches that have shown promise in human and animal studies (Provencher & Granton, 2015). Endothelin receptor antagonists (ERA's) including Bosentan, Ambrisentan and Macicentan work by increasing vasodilation, and have shown promise in improving functional capacity, hemodynamics, and reduced morbidity and mortality when comparted to placebo. Additionally, the use of phosphodiesterase type 5 (PDE 5) inhibitors and guanylate cyclase stimulators that also increase vasodilation by targeting reduced nitric oxide bioavailability continue to show promising results. In this category, Sildenafil, Tadafil and Riociguat have been most extensively studied, with improved exercise tolerance, hemodynamics, and reduced clinical worsening all reported. Finally, the use of prostanoids and prostacyclin receptor agonists have been explored. Similar to PDE 5-based therapies, drugs such as Epoprostanol, Ilioprost and Selexipag are designed to manipulate the prostacyclin pathway and increase vasodilation. While this group of drugs has shown some promise, the challenge of delivery and inconsistency in efficacy warrants further research (Provencher & Granton, 2015).

Importantly, even under optimal pharmacological treatment, PAH patients still suffer from reduced quality of life and significant exercise limitation (Pandey et al., 2015). As a result, a growing body of work has considered the use of exercise interventions as an alternative therapeutic approach. Exercise is well known to improve outcomes in cardiopulmonary diseases such as left heart failure (Cattadori, Segurini, Picozzi, Padeletti, & Anza, 2018; Ding, 2017; Pearson, Mungovan, & Smart, 2017), Chronic Obstructive Pulmonary Disease (Blondeel, Demeyer, Janssens, & Troosters, 2019; Mohammed, Derom, Van Oosterwijck, Da Silva, & Calders, 2018) and hypertension (Boutcher & Boutcher, 2017; Moraes-Silva, Mostarda, Silva-Filho, & Irigoyen, 2017), however, understanding on the effectiveness of exercise as a tool in managing PAH is still in its infancy.

Individuals with PAH have significant exercise intolerance (Babu, Arena, et al., 2016; Neder et al., 2015). Patients consistently present with lower maximal aerobic capacity, reduced ventilatory threshold, lower muscle strength, as well as early-onset dyspnea and fatigue during exercise. This blunted exercise capacity, coupled with cardiopulmonary symptoms may be the main reason that exercise was initially contraindicated for PH patients (Desai & Channick, 2008). However, the increasing number of clinical trials demonstrating the benefits of exercise in PAH patients (Babu, Padmakumar, Maiya, Mohapatra, & Kamath, 2016; Buys, Avila, & Cornelissen, 2015; Pandey et al., 2015) has led to a push for its inclusion as a means of managing the disease. Additionally, there is a desire to more fully understand the pathophysiology of exercise intolerance, and how this might be overcome either through pharmacological and/or behavioral interventions.

Undoubtedly, exercise limitation in PAH is related to 'central' dysfunction. The failing right ventricle (RV) is unable to meet the cardiac output demands of exercise (Sun, Hansen, Oudiz, & Wasserman, 2001). However, a growing body of evidence suggests that peripheral dysfunction, specifically related to skeletal muscle abnormalities, are key contributors to exercise intolerance. A reduction in strength and power, shift toward anaerobic metabolism, atrophy, and greater percentage of type II muscle fibers have all been demonstrated in PAH patients (Batt, Ahmed, Correa, Bain, & Granton, 2014; de Man et al., 2011; Mainguy et al., 2010b; Malenfant, Potus, Mainguy, et al., 2015; Manders et al., 2012; Manders, Rain, et al., 2015; Manders, Ruiter, et al., 2015). Mechanistically, mitochondrial enzymes governing metabolism are altered (M. B. Brown et al., 2015; Malenfant, Potus, Fournier, et al., 2015), muscle fiber cross sectional area is reduced, atrophic signaling is upregulated (de Man et al., 2011) and calcium handling in myocytes is dysfunctional (Batt et al., 2014). While central and peripheral maladaptations both contribute to exercise intolerance, one area that remains understudied is the effect of skeletal muscle blood flow during exercise in PAH. It is not known if reductions in cardiac output may have downstream effects of reduced muscle blood flow, particularly during exercise, and this idea requires a full exploration in PAH (Figure 1.1). As the vast majority of cardiac output is directed toward working muscles during exercise (Hearon & Dinenno, 2016), *directly* measuring skeletal muscle blood flow during exercise would provide crucial mechanistic insight into exercise intolerance, and potentially elucidate a novel target for therapeutic intervention.

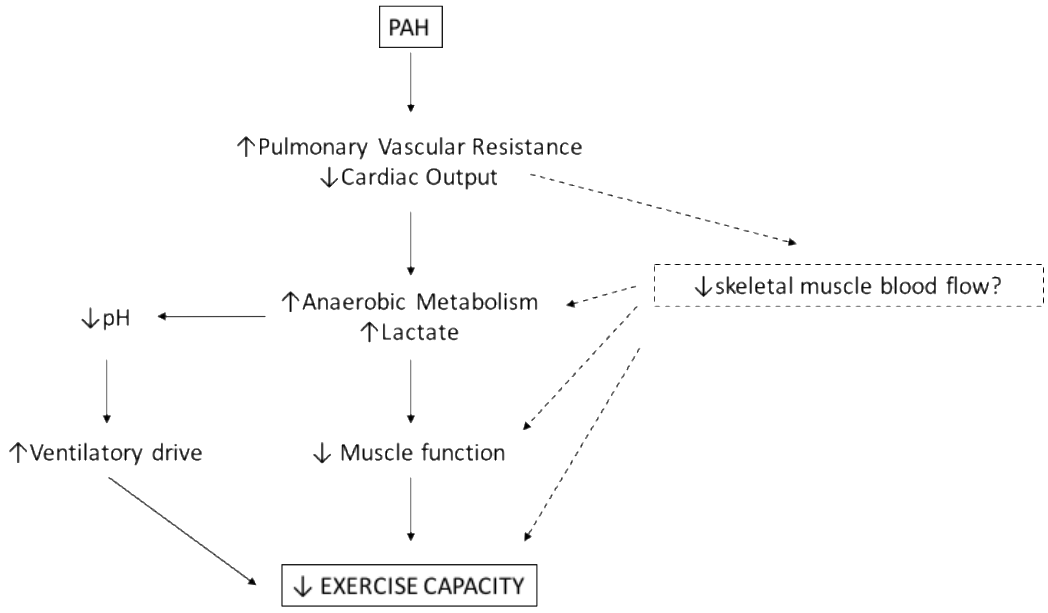



**Figure 1.1:** Exercise intolerance in PAH, adapted from Vescovo et al. (1998)

**Significance**

As mentioned, vascular dysfunction forms the basis of PAH development. As such, pharmacological treatments have centered on improving vasoreactivity by inducing vasodilation. Three main pathways are targeted as a means to combat vascular dysfunction (Figure 1.2). One such pathway is the nitric oxide (NO) pathway, which is made bioavailable via a group of enzymes known as nitric oxide synthases (NOS) acting on the substrate L-arginine, inducing vasodilation via the second messenger cyclic guanosine monophosphate (cGMP). It has been long established that NO acts as a potent vasodilator and therefore blood-flow modulator (Ignarro, 1989), however, it also directly affects skeletal muscle function via altered glucose and calcium signaling, altered mitochondrial respiration and contractile function. (Stamler & Meissner, 2001).

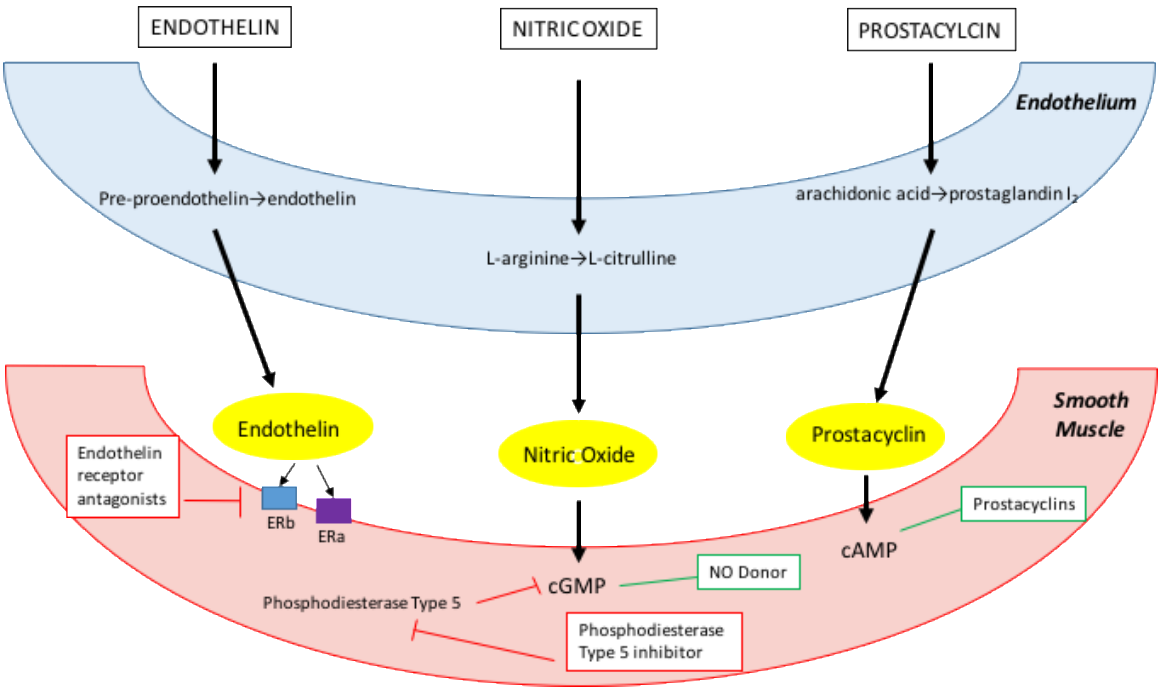



**Figure 1.2:** Pharmacological treatment in PAH, adapted from Schulze-Neick and Beghetti (2010)

Manipulation of this pathway is crucial, as it has been shown to be downregulated in PAH patients (Cua et al., 2011; Giaid & Saleh, 1995; Pullamsetti et al., 2005) and animal models (Alef et al., 2011; Goret, Tanguy, Guiraud, Dauzat, & Obert, 2008). While work continues on pharmacological treatment via the classic NO pathway, a promising alternative method of upregulating NO has recently been identified.

Nitrate ($NO_3^-$) and nitrite ($NO_2^-$) anions, initially thought to be a byproduct of NO metabolism, are now known to be potential donors of NO when reduced, particularly in hypoxic tissue (Alef et al., 2011; Friis, Steenholt, Lokke, & Hansen, 2017). Enzymatic reduction of $NO_3^-$/$NO_2^-$ is facilitated primarily by hemoglobin, myoglobin, and xanthine oxidoreductase (XOR), resulting in an increased bioavailability of NO outside the traditional L-arginine-NOS-NO pathway (Zuckerbraun et al., 2010). Importantly, a simple way of increasing circulating nitrate and nitrite is via the consumption of green

leafy, and root vegetables. Dietary nitrate is absorbed in the gastrointestinal tract, and converted to nitrite via facultative bacteria in the saliva (Gladwin et al., 2005; Lundberg et al., 2009). The subsequent circulating nitrite is then reduced to NO in an oxygen-independent manner, particularly in tissues undergoing metabolic stress (Ataya, Tzeng, & Zuckerbraun, 2011). This evidence points to a promising, alternative method of increasing NO bioavailability that could be harnessed in PAH via the administration of dietary nitrate. Indeed, the metabolic perturbation brought about by exercise may mean this pathway is ideally suited as a target for improving skeletal muscle function and combating exercise limitation (Woessner, McIlvenna, Ortiz de Zevallos, Neil, & Allen, 2018).

The majority of studies investigating the effectiveness of dietary nitrate as either a therapeutic option or as an ergogenic aid have involved the use of beetroot juice or associated beet-containing products. Beetroot contains high level of nitrates, and after administration has been shown to increase circulating levels of $NO_3^-$ and $NO_2^-$ (Mills, Khatri, Maskell, Odongerel, & Webb, 2017). A significant body of evidence has demonstrated that beet consumption can improve exercise performance in healthy and patient populations (A. M. Jones, 2014a). Interestingly, recent work has shown that beetroot juice (BRJ) may specifically increase exercise tolerance in individuals with cardiovascular disease (Coggan et al., 2018; Coggan, Leibowitz, Spearie, et al., 2015; Kenjale et al., 2011) , and in related animal models (Alef et al., 2011; Ataya et al., 2011; Ferguson et al., 2013; Ferguson et al., 2016; Zuckerbraun et al., 2010), with one trial specifically examining its effectiveness in PAH (Henrohn et al., 2018). While this work provides rationale for the investigation of dietary nitrate as an ergogenic aid in PAH, the

mechanisms related to its potentials effectiveness are somewhat speculative. Specifically, it is unknown whether BRJ could augment exercising blood flow in PAH, and subsequently improve exercise performance.

**Purpose**

Characterizing skeletal muscle blood flow at rest and during exercise in PAH is critical in developing a fuller understanding of exercise limitations in the disease. Similarly, targeting the aforementioned alternative $NO_3^-$ – $NO_2^-$ – NO pathway via the supplementation of BRJ provides a novel approach to potentially improve exercise performance. This research directly measured, for the first time, exercising skeletal muscle blood flow in an animal model of PAH, providing insights as to how this maladaptation may contribute to exercise intolerance. Additionally, the use of a readily available, non-pharmaceutical supplement with the potential to augment blood flow and improve exercise performance was investigated, providing new details as to the potential use of dietary nitrate as an ergogenic aid and therapeutic option in PAH. Results of the research will underscore the importance of reduced blood flow and skeletal muscle dysfunction in what has traditionally been considered a 'cardiopulmonary disease', while providing new mechanistic insight in to the nature of tissue adaptations beyond the heart and lungs. Finally, this work will add to the growing body of knowledge on the effectiveness of dietary nitrate as a strategy to improve exercise performance in disease states, while exploring the handling of dietary nitrate in multiple tissues, adding the mechanistic insight in this area (Figure 1.3).

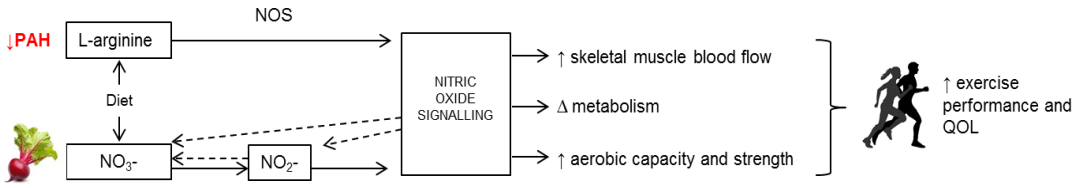



**Figure 1.3:** Hypothesis outline

1. Specific Aim 1: In a rat model of PAH, skeletal muscle blood flow will be characterized at rest and during moderate intensity exercise.

Hypothesis 1.1 – Skeletal muscle blood flow will be significantly reduced at rest and during exercise in a PAH rat model, when compared to healthy controls.

2. Specific Aim 2: To determine if an acute dose of dietary nitrate via beetroot juice (BRJ) supplementation improves exercising skeletal muscle blood flow and energetics in a rat model of PAH.

Hypothesis 2.1 – Animals supplemented with BRJ will demonstrate increased blood flow in comparison to those supplemented with placebo.

Hypothesis 2.2 - Animals supplemented with BRJ will demonstrate reduced reliance on anaerobic metabolism via lower blood lactate accumulation at rest and during exercise.

3. Specific Aim 3 – To determine if an acute dose of dietary nitrate will improve function by measuring the impact of BRJ on:

a. volitional running as assessed with 24-hour wheel monitoring in repeated trials

b. Maximal aerobic capacity ($VO_2max$) as assessed by maximal treadmill running test in repeated trials

c. Whole body muscle strength using a rat grip-strength test in repeated trials

Hypothesis 3.1 – BRJ supplementation will result in a significant increase in exercise function through improved $VO_2$max, strength and increased volitional running distance in both PAH and control rats, compared to those supplemented with placebo.

**Methodology**

The purpose of this investigation was to characterize skeletal muscle blood flow in a monocrotaline (MCT) model of PAH (Gomez-Arroyo et al., 2012), and subsequently employ a dietary supplement that may increase blood flow and improve exercise performance. A prospective, randomized, counterbalanced, placebo-controlled trial was used to examined the aforementioned aims across four groups; PAH rats supplemented with BRJ (MCT BRJ); PAH rats supplemented with placebo (MCT PL); healthy control rats supplemented with BRJ (CON BRJ); healthy control rats supplemented with placebo (CON PL). Counterbalanced functional exercise tests were carried out pre and post disease induction, with a final, terminal experiment examining blood flow during exercise under either BRJ or PL conditions. The independent variables in the study include health/disease status (MCT vs. CON) and dietary supplement administered (BRJ vs. PL). Dependent variables include direct and indirect measures of disease severity, skeletal muscle blood flow and capillarization, whole blood lactate, plasma and skeletal muscle $NO2^-/NO3^-$ post supplementation, and functional exercise performance in maximal aerobic testing, voluntary running distance, and grip strength. Quantitative analysis was used to determine the effect of disease status and supplementation on the dependent variables.

## Outline of Dissertation

The dissertation consists of five chapters. Chapter one outlines the problem and provides rationale for the investigation, as well as listing specific aims/hypothesis and briefly outlining the methodology. Chapter two contains a review of the relevant literature, including a description and pathophysiology of PAH, treatment options, benefits of exercise, importance of blood flow during exercise, and findings on the use of dietary nitrate as an intervention in health and disease. Chapter three describes the research design and methodological approach. Results are presented in chapter four. Implications of the results, potential future research questions and limitations are outlined in chapter five.

# Chapter Two: Literature Review

## Introduction

PAH is a rare disease with a varied and complex pathophysiology. Significant research in patients and animal models has provided new insights into the mechanisms of disease onset and progression, however, a cure remains elusive. Treatment options are expanding, yet as this stage pharmacological interventions have largely been limited to addressing symptom management. Interest in exercise as a means of limiting disease progression and improving quality of life is growing, and a convincing body of evidence points to the benefits of exercise in PAH. While patients display significant exercise intolerance, there is limited understanding into the mechanisms behind this problem. Specifically, the consequence of skeletal muscle dysfunction as it relates to exercise performance requires further work. Earlier studies have shown that metabolic and morphological changes play a role in limiting exercise capacity, however it is not known to what extent blood flow to exercising muscle is limited in the disease. The use of a well-established animal model of PAH will allow for a direct mechanistic investigation of muscle blood flow at rest and during exercise, and potentially elucidate a therapeutic target to improve exercise tolerance, and ultimately, quality of life for PAH patients. Finally, the evidence pertaining to the use of dietary nitrate as an ergogenic aid in health and disease will be considered, with specific reference to its potential benefits in modulating muscle blood flow and exercise tolerance in PAH.

## PAH Pathophysiology

In the late 1950's, after the advent of cardiac catheterization, PAH (also referred to as primary or idiopathic pulmonary hypertension) was recognized as a unique condition in which elevated pulmonary artery pressures were measured without the presence of coexisting cardiopulmonary diseases such as stenosis, septal defect, emphysema and pulmonary embolism. This early recognition was useful from a classification stand-point, however, it was still difficult to diagnose PAH as symptoms were often non-specific and common to other known cardiovascular diseases (Whitaker & Heath, 1959).

While the exact pathogenesis of PAH is still largely unknown, it is now widely accepted that endothelial dysfunction initiates the disease process (Toshner, Tajsic, & Morrell, 2010). Pulmonary arteries undergo remodeling via the proliferation of endothelial and smooth muscle cells (Humbert, 2010), leading to intimal and medial thickening and the development of plexiform lesions (Sutendra & Michelakis, 2014). These plexiform lesions have been described as a hallmark of PAH progression, (Abe et al., 2010) as they have been implicated in the vascular dysfunction described. Several pathways have been associated with the remodeling process. Matrix metalloproteinases (MMPs), which are linked to endothelial and smooth muscle cell proliferation and migration, have been shown to be upregulated in PAH patients (Lepetit et al., 2005). Additionally, elevated levels of endothelin-1 (ET-1) in the vascular endothelium and plasma have been recognized (Rosenblum, 2010). ET-1 has been shown to induce cell growth and downregulate apoptosis, and can be as much as 20-fold higher in patients compared to healthy controls (Stewart, Levy, Cernacek, & Langleben, 1991).

Prostacyclin, a mediator of endothelial cell function, is downregulated in PAH. This can lead to smooth muscle cell proliferation, platelet aggregation and vasoconstriction in small, medium and large pulmonary arteries (Tuder et al., 1999). Additionally, potassium channel dysfunction and/or downregulation may play a role in the vasoconstrictive nature of disease pathology (Newman et al., 2004). A further area that has been widely considered is endothelial dysfunction via altered growth factor signaling, primarily through the TGF-β superfamily. Patients with idiopathic and familial PAH have been shown to harbor a mutation in the gene that encodes bone morphogenetic protein receptor type II (BMPR-2) (Humbert, 2010). While the penetrance of this mutation is rather low (10-20%), patients without the mutation also demonstrate a reduced expression of BMPR-2 (Atkinson et al., 2002). This is important, as alterations in BMPR-2 signaling have been shown to increase cell proliferation and decrease apoptosis in vascular cells (Hemnes & Humbert, 2017). While altered BMPR-2 signaling seems to represent the most promising genetic basis for PAH pathogenesis, further studies are attempting to more fully understand the genetic mutations that may predispose one to the disease (de Jesus Perez, 2016).

### Right Ventricular Dysfunction

While pulmonary vascular remodeling is a central component of PAH, the subsequent increased pulmonary vascular resistance and increased strain on the RV is the hallmark of disease diagnosis (Bogaard, Abe, et al., 2009). Patient survival has been linked to RV function (Sandoval et al., 1994), and as such, there has been interest in understanding the mechanisms of right ventricular maladaptation. The RV is crescent

shaped, with a thinner wall and lower surface area in comparison to the left ventricle (LV). While this increases compliance, the RV is subject to dilation with increased pressure (Chin, Kim, & Rubin, 2005). Subsequent wall stress leads to increased oxygen demand, and the RV adapts by increasing muscle mass. This hypertrophy, thought to be driven by structural, neurohormonal and inflammatory alterations initially ensures the RV meets cardiac output demands (Bogaard, Abe, et al., 2009), however, sustained pressure increases can lead to RV failure (Archer, Fang, Ryan, & Piao, 2013). This 'transition' from compensatory to decompensatory hypertrophy is a critical component of PAH, particularly because RV function is the best predictor of clinical worsening in the disease (van de Veerdonk et al., 2011). In conjunction with the morphological and functional changes outlined, there is evidence of metabolic changes in the adapting RV. Initially a metabolic switch occurs, driven in part by an upregulation of pyruvate dehydrogenase kinase (PDK), leading to an increased reliance on glucose metabolism. While this shift decreases ATP production, it has been shown in experimental PAH models that it may also impair myocyte electrical contractile function (Piao, Fang, et al., 2010; Piao, Marsboom, & Archer, 2010). Interestingly, more recent work has demonstrated this metabolic shift may not continue through decompensation and RV failure. Instead, a downregulation of PDK and angiogenic factors via inhibition of hypoxia inducible factor-1 (HIF1$\alpha$) signaling leads to myocardial ischemia, and ultimately deterioration of the RV (Sutendra et al., 2013). In addition to the metabolic perturbation outlined, it has been demonstrated in patients and animal models that right heart failure may also be a consequence of myocardial fibrosis and reduced capillarity (Bogaard, Natarajan, et al.,

2009; Philip et al., 2019), adding to the complexity in describing the precise mechanisms by which RV function declines.

**Muscle Dysfunction**

Muscle dysfunction is being increasingly recognized as an important component of cardiopulmonary disease phenotype, including in left heart failure and COPD (Maltais et al., 2014; Pina et al., 2003), and this phenomenon has been receiving greater attention in PAH. In early work, Vescovo et al. (1998) demonstrated skeletal muscle dysfunction in a heart failure (HF) rat model. Specifically, it was noted that myosin heavy chain expression shifted toward faster isoforms, at the expense of slower-fiber phenotypes. Additionally, resting blood flow was statistically reduced in the soleus of the HF rats, but not in the extensor digitorum longus (EDL) muscle. Later work on animal models of PAH have led to a greater understanding of skeletal muscle abnormalities, as well as the identification of pathways central to these changes. In a severe PAH animal model, de Man et al. (2011) demonstrated a reduction in diaphragm muscle fiber cross sectional area when compared to controls. Interestingly, this difference was not seen in the EDL, leading the authors to propose a ‘diaphragm-specific’ atrophic phenotype. While this finding is important, it could be argued that only analyzing the EDL as a surrogate for skeletal muscle fiber loss may result in missed maladaptations elsewhere. Nevertheless, increased expression of the E3-ligases MAFbx and MuRF-1 were identified in PAH rat diaphragms, indicating that atrophy may be driven by muscle proteolytic activity. From a functional perspective, the same study noted a significantly reduced force production in skinned diaphragm fibers, which again was not seen in the EDL. The greatest strength of this work was to connect the animal model findings to that of PAH patients.

Maladaptations in the rat model were reflected in the diaphragm of patients, indicating a respiratory-muscle specific atrophy and reduction in force generating capacity. This work was later complimented by Ahn, Empinado, Al-Rajhi, Judge, and Ferreira (2013), who investigated diaphragm contractile function in a PAH mouse model. Here, PAH induction resulted a 25% decrease in cross sectional area (CSA) of diaphragm fibers, with a similar reduction in maximal and submaximal tetanic force production. Of greatest significance may the finding that shortening velocity and peak power of fibers were reduced by 40% and 63% respectively in the PAH model, variables that translate more closely to diaphragm action *in vivo* (Ahn et al., 2013). Interestingly, no differences in myosin heavy chain expression were noted, and it was speculated that in this case, reduction in muscle function may be due to either an upregulation of inflammatory cytokines, which has been previously demonstrated in PAH (Price et al., 2012), or via post-translational changes affecting myofibrillar function. The aforementioned findings on the diaphragm were further complimented by mechanistic work focusing on sarcomere action in a PAH rat model (Manders et al., 2012). Again, force-generating capacity was reduced when compared to controls, specifically in fast twitch fibers. In an attempt to understand this phenomenon, single muscle fibers were exposed to increasing calcium concentrations. It was noted that at submaximal activation levels, calcium sensitivity was reduced in PAH rats, leading to the idea that sarcomeric dysfunction plays a role in reduced force generating capacity. Finally, it has been shown that PAH patients have lower inspiratory and expiratory pressures when compared to healthy control subjects (Kabitz et al., 2008; Meyer et al., 2005), lending weight to the idea that respiratory muscle dysfunction translates directly into reduced ventilatory capacity.

While there is clear evidence supporting the idea of muscle dysfunction in the diaphragm of PAH patients and animal models, recent work has identified abnormalities in other muscle groups. It has been shown that forearm muscle strength is reduced in PAH patients, and that this reduction is closely linked to disease severity (Bauer et al., 2007). Similarly, fast twitch fibers in the vastus lateralis of PAH patients have lower contractile function when compared to controls, potentially due to a reduction of cross-bridges secondary to myosin protein loss (Manders, Ruiter, et al., 2015). This atrophic phenomenon was explored in greater detail in a wide-ranging mechanistic analysis of PAH patients quadriceps muscle dysfunction (Batt et al., 2014). Patients demonstrated a lower percentage of Type I fibers after biopsy, with a concurrent reduction in Type I cross sectional area. Phosphorylated-AKT and -p70S6 kinase signaling pathways, both recognized as important for muscle hypertrophy, were downregulated in PAH muscle. Additionally, mRNA associated with ubiquitin ligase pathways related to proteolysis of muscle, including muscle ring finger protein 1 (MuRF 1) and antrogin-1 were increased compared to controls, suggesting that both reduced hypertrophy and increased proteolysis may play a role in muscle CSA reduction.

Metabolic abnormalities, similar to those outlined in the RV, have been identified in skeletal muscle. Proteins involved in mitochondrial oxidative metabolism, including NADH, complex III cytochromes and complex V ATP synthase are decreased in the vastus lateralis of PAH patients. Conversely, proteins involved in pyruvate (non-oxidative) metabolism (lactoylglutathione lyase) and gluconeogenesis (fructose-1,6-biphosphatase) have been shown to be increased (Malenfant, Potus, Fournier, et al., 2015), adding weight to the idea of a ‘metabolic shift’ in the disease. These findings

supported earlier work showing an increased phosphofructokinase/3- hydroxyacyl-CoA-dehydrogenase (PFK/HADH) ratio in the quadriceps of PAH patients, again suggesting a greater reliance on energy metabolism via anaerobic means.

Finally, morphological changes, in addition to atrophy, have been noted in PAH. Specifically, it has been shown that microcirculation loss is present in the disease. CD31+ staining of the quadriceps muscle confirmed capillary rarefaction, driven in part by a reduction in miR-126 expression, as well as decreased activation of RAF and ERK pathways, both effectors of VEGF/MAP-dependent angiogenesis (Potus et al., 2014).

Figure 2.1 outlines the intrinsic and systemic factors that have been postulated as playing a role in skeletal muscle abnormalities outlined. While it is clear a myopathy exists in PAH patients, more work is required to fully understand the impact of muscle dysfunction in terms of disease development, quality of life, and importantly, if alternatives therapies may be directed in this area. Additionally, as muscle function is directly connected to exercise performance, further work must address the mechanistic basis of muscle function *during* whole-body exercise. This critical missing piece may help elucidate the importance of peripheral factors in exercise limitation, particularly due to the growing notion that exercise is a critical component in improving quality of life for PAH patients.

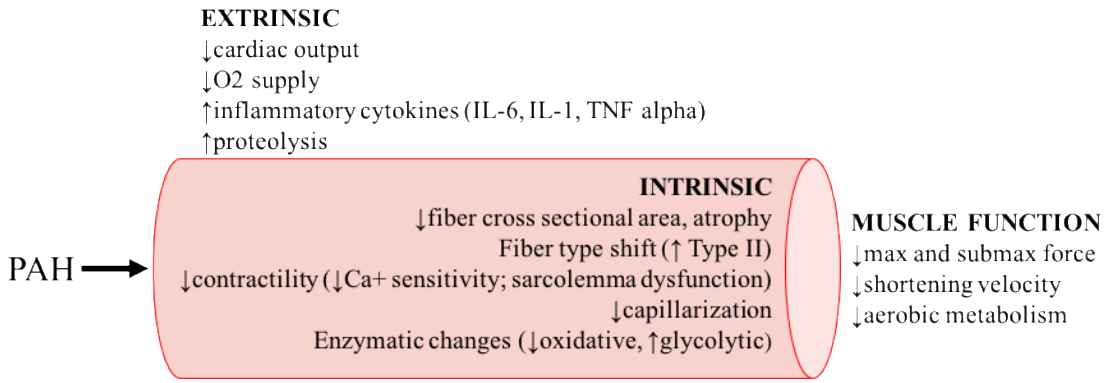



**Figure 2.1:** Extrinsic and intrinsic factors that contribute to muscle dysfunction in PAH. Adapted from Manders, Rain, et al. (2015)

## Pathophysiology of Exercise Intolerance in PAH

The central (heart/lung) and peripheral (muscle) abnormalities described both play an important role in the significant decline in exercise capacity (Sun et al., 2001). Understanding this phenomenon is crucial, as it has been shown that exercise intolerance is one of the primary factors limiting quality of life, as well as being closely related to clinical worsening in PAH (Tran, Lau, Celermajer, Davis, & Cordina, 2018). The increased use of exercise testing and rehabilitation programs has shed light on the mechanisms behind exercise intolerance, with a greater focus being placed on exercise as a therapeutic intervention. Connecting the dots between central and peripheral abnormalities in response to exercise is vital as it may provide further data on novel targeted therapies, and/or lead to a standardization of exercise interventions in the disease, which currently do not exist.

### Cardiac Dysfunction and Exercise Intolerance

As noted previously, maladaptive remodeling in the RV is characterized by initial hypertrophy as a consequence of increased afterload, progressing toward chamber dilation and eventually systolic dysfunction with reduced cardiac output. Additionally, it has been shown that myocyte orientation may limit the RV's ability to maintain stroke volume, in comparison to a more efficient contractile function in the LV. Several studies have questioned how the adapting RV may impact exercise performance. Using MRI and right heart catheterization to measure cardiac function and pulmonary pressures, Holverda et al. (2006) determined that PAH patients were unable to significantly increase stroke volume (SV) with the onset of exercise, in contrast to healthy control subjects. Additionally, right ventricular end diastolic volume increased in PAH, but crucially, left ventricular end diastolic volume decreased during exercise. This can be explained by a reduction in myocardial contractility, and the anatomical connection between the RV and LV. As RV diastolic filling increases in PAH, LV diastolic filling decreases. This again is critical, as a central factor in maintaining exercise tolerance is the ability to increase cardiac output to meet the increased metabolic demands of exercising muscle. These findings were mirrored by Sun et al. (2001), who retrospectively analyzed cardiac function data from cardiopulmonary exercise tests (CPET) in 53 PAH patients. While peak oxygen consumption and peak work rate were significantly lower in PAH patients, it was noted that maximal heart rate was also reduced, despite each test resulting in workloads above the ventilatory threshold. This is further supported the work of Provencher et al. (2006) who also described chronotropic incompetence in PAH patients. Interestingly, both reduced stroke volume and heart rate responses to exercise were

independently related to 6-minute walking distance, indicating that a heart rate increase likely cannot overcome the reduced stroke volume of PAH patients. Mechanistically, it has been proposed that a downregulation of myocardial β-adrenergic activity can in part explain the observed reduced maximal heart rate (Manders, Rain, et al., 2015; Provencher et al., 2006). RV hypertrophy, as a consequence of PAH, can lead to dyssynchrony and atrioventricular uncoupling (Badagliacca et al., 2017; Hill et al., 2012; Marcus et al., 2008; Spruijt et al., 2015). During exercise, it has been demonstrated that impaired contractility in the face of increased afterload can disrupt normal RV-arterial coupling, and this may be because contractility is already at maximal levels at rest. Specifically, PAH patients are unable to match contractility to afterload with the onset of exercise (Spruijt et al., 2015). Additionally, RV dyssynchrony (or uncoordinated contraction) (Tran et al., 2018) has been demonstrated in PAH, and it has recently been shown to be a predictor of reduced exercise capacity in the disease (Badagliacca et al., 2017).

In addition to mechanical abnormalities, previously described metabolic adaptations in the RV may also play a role in reduced exercise capacity. It stands to reason that with reduced capillarity (Bogaard, Abe, et al., 2009), and reduction in oxidative enzymes regulating energy metabolism (Piao, Marsboom, et al., 2010), the ability of the RV to adapt to the increased energy demands of exercise will be blunted. As the myocardium predominantly uses aerobic means to generate ATP, sufficient blood flow is necessary to ensure adequate energy supply. Reduced capillarization, in addition to the lower coronary flow present in PAH (van Wolferen et al., 2008) results in myocardial ischemia that may be exacerbated during exercise (Tran et al., 2018). It has been shown that RV adaptations may be more evident during exercise in PAH (Nootens,

Wolfkiel, Chomka, & Rich, 1995), and the outlined RV dysfunction undoubtedly plays a role in exercise intolerance. Indeed, a recent review determined that RV function and exercise capacity were strong predictors of survival in PAH (Humbert et al., 2010). Limiting exercise impairment to central deficiencies and the maladapted RV would miss the systemic nature of exercise responses, with pulmonary and skeletal muscle function also crucial in determining exercise capacity in PAH.

### Pulmonary Dysfunction and Exercise Intolerance

Pulmonary vascular remodeling as a result of endothelial dysfunction is a central component of PAH pathogenesis (Galie et al., 2010). In order to maintain the relative low-pressure system in the pulmonary circulation, the ability of pulmonary vessels to distend with the increased blood flow demands of exercise is crucial, particularly as this will in turn reduce RV wall stress. The maladapted pulmonary arteries, characterized by endothelial proliferation and vascular stiffness, are compromised in their ability to vasodilate to increase lung blood flow as needed with the onset of exercise. As such, an increase in pulmonary vascular resistance (PVR) is seen, leading to ventilation-perfusion mismatching. Underscoring the interconnectedness of exercise responses, this continued increased PVR reduces RV output, feeding forward to a lower LV preload, stoke volume and cardiac output (Waxman, 2012). Furthermore, early hyperventilation (increased rate of breathing) and dyspnea (shortness of breath) are common signs of reduced exercise capacity in PAH (Sun et al., 2001). In early work it was demonstrated that minute ventilation ($V_E$) at any given level of carbon dioxide production ($VCO_2$) is higher for PAH patients, and the increase with progressive exercise is more profound in the disease.

This would indicate ventilatory inefficiency, more so than ventilation limitation, as patients are able to achieve maximal ventilation levels similar to healthy control subjects (D'Alonzo et al., 1987). Indeed, two further mechanisms that may result in hyperventilation/dyspnea with exercise in PAH have been proposed. The lower cardiac output and reduced capacity for oxidative metabolism leads to a systemic reduction in pH, driven by increased lactate and hydrogen ion ($H^+$) accumulation (Sun et al., 2001). Additionally, the increased PVR during exercise increases right atrial pressures to the extent to which they may exceed those in the left atrium. As a result, venous blood may be shunted to the left atrium via a patent foramen ovale, and ultimately into the systemic circulation. This deoxygenated, acidic blood can subsequently lead to an increase in ventilation, driven primarily by systemic chemosensitivity (Sun, Hansen, Oudiz, & Wasserman, 2002). Interestingly, the incidence of left to right shunting may be high in PAH, with the aforementioned abnormalities being seen is as many as 45% of patients tested (Sun et al., 2002).

### Muscle Dysfunction and Exercise Intolerance

As outlined previously, there are multiple structural, mechanical and metabolic abnormalities identified in muscles of PAH animal models and patients. It has been demonstrated that exercise intolerance in PAH is only partially associated with altered central hemodynamics (i.e. reduced cardiac output), suggesting that peripheral mechanisms may also play a role. The onset of exercise and subsequent increased ventilation directs blood flow to diaphragm, which in trained individuals has been shown to increase by as much as 16% at high intensity workloads (Harms et al., 1998). This may

be particularly problematic in PAH as hyperventilation may lead to increased work of breathing, concomitant with a decreased overall cardiac output as previously described. While there are clearly diaphragmatic abnormalities in PAH, connecting respiratory muscle dysfunction specifically to exercise intolerance is challenging, due to the highly integrated response of the cardiovascular and muscular system to exercise stress. Breda et al. (2014) addressed this question by analyzing exercise capacity, hemodynamics and muscle function during a 6-minute walking and incremental exercise test in 16 PAH patients. As expected, patients had altered muscle fiber type, reduced force generating capacity and significantly lower maximal oxygen consumption ($VO_{2max}$). Importantly, maximum inspiratory pressure (MIP) and maximum expiratory pressure (MEP) were lower in the patient cohort, indicating compromised respiratory capacity during exercise. It was shown that MIP was negatively correlated with $VO_{2max}$, indicting for the first time that respiratory function can be directly connected to exercise intolerance in PAH. Crucially, this connection held true even when correcting for changes in cardiac output. These findings were complimented by a second study that demonstrated MIP/MEP were related to 6-minute walk distance, number of steps and physical activity scores in 33 PAH and chronic thromboembolic pulmonary hypertensive (CTEPH) patients (Aslan, Akinci, Yeldan, & Okumus, 2018). Finally, Saglam et al. (2015) analyzed physical activity history and 6-minute walking capacity of 31 PAH patients and connected exercise capacity to respiratory muscle function. It was determined that respiratory muscle function was lower in patients than controls, however, a novel finding was that this deficit can be used to delineate functional classes of PAH patients, as described in Chapter One.

Peripheral skeletal muscle abnormalities have also been shown to limit exercise performance. Bauer et al. (2007) demonstrated that forearm muscle strength, measured via handgrip dynamometry, was reduced in PAH patients, and that a significant correlation existed between grip strength and disease severity as measured by the 6-minute walk test. Similarly, it has been shown that quadriceps strength is positively correlated with $VO_{2max}$ in PAH patients (Mainguy et al., 2010b). In this study, not only was a connection made between muscle strength and exercise capacity, but an important metabolic link was observed. The oxidative capacity of muscle, as measured by levels of citrate synthase and 3-hydroxyacyl-CoA-dehydrogenase were reduced in PAH, and correlated with oxygen uptake ($VO_2$) at the onset of heavy exercise. Furthermore, the importance of altered muscle morphology was highlighted, with $VO_2$ also related to the number of capillaries per type I muscle fiber. This phenomenon has also been replicated in animal models of PAH. M. B. Brown et al. (2015) demonstrated a significant $VO_{2max}$ reduction in a rat model of PAH, and subsequent tissue analysis indicated greater reliance in non-oxidative metabolism via increase levels of glucose transporter 1 (GLUT-1) in both cardiac and skeletal muscle.

Several investigators have questioned how the interrelated concepts of oxygen delivery and metabolism may contribute to exercise intolerance in PAH. Potus et al. (2014) demonstrated that reduced muscle capillarity, measured via expression of CD31+ cells, was strongly correlated to $VO_{2max}$ in PAH patients, and went on to suggest microcirculation loss is likely related to exercise intolerance in the disease. These findings compliment the earlier work of Tolle, Waxman, and Systrom (2008), who determined that in mild PAH, systemic oxygen uptake/use abnormalities, potentially due

to capillary and/or mitochondrial dysfunction contribute to reduced exercise capacity. A further study by Malenfant, Potus, Mainguy, et al. (2015) more directly connected skeletal muscle microcirculation to a reduction in exercise performance. Quadriceps oxygenation was measured using near infrared spectroscopy (NIRS) during submaximal exercise both with and without supplemental oxygen. Interestingly, despite normal cardiac output responses to exercise PAH patients had lower skeletal muscle oxygenation that was not recovered with the addition of supplemental oxygen. While muscle blood flow as not directly measured, reduced capillarity was closely linked to diminished tissue oxygenation and exercise performance. PAH patients in this study had relatively high levels of functional capacity and it remains to be seen if exercise responses in a more severely afflicted cohort would be observed (Malenfant, Potus, Mainguy, et al., 2015).

It is clear that multiple factors play a role in limiting exercise in PAH (Figure 2.2 Adapted from (Tran et al., 2018). A fundamental understanding of disease pathophysiology would indicate a strong association between central limitation and exercise performance. However, a growing body of work is recognizing that PAH is a systemic disorder, and the hallmark exercise intolerance patients experience is clearly also due to muscle dysfunction. Further work is required to fully understand the mechanisms behind this association. Oxygen uptake, metabolic disturbances and contractility decrements have all been characterized, however, a missing piece of the puzzle is a direct measurement of muscle blood flow at rest and during exercise in PAH. Adequate muscle blood flow is an essential factor in maintaining exercise, hence it should be measured directly in the disease. As patients present with lower maximal oxygen consumption and anaerobic thresholds, combined with a reduced oxidative

metabolic machinery, it stands to reason that reduced blood flow and subsequent early onset of anaerobic metabolism may help elucidate an important limiting factor in exercise intolerance. It is well known that exercise training and/or ergogenic aids can improve muscle performance, providing rationale for their use in PAH management.

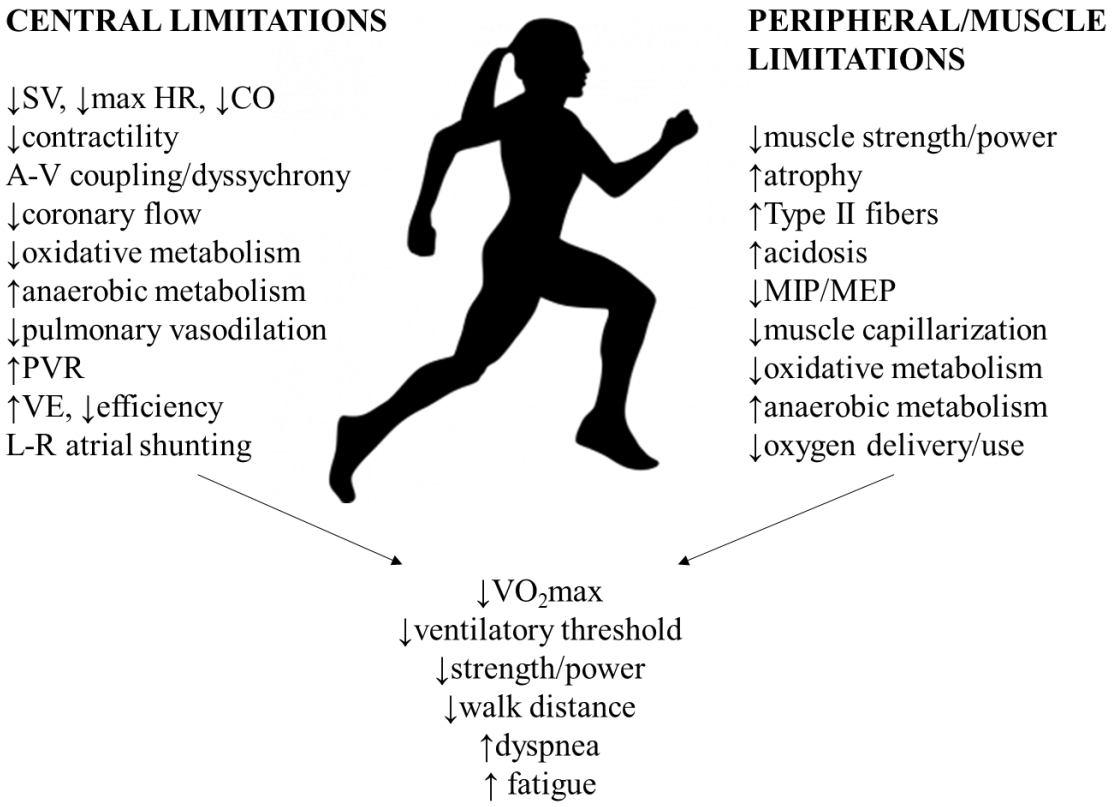



**Figure 2.2:** Central and peripheral determinants of exercise intolerance in PAH, adapted from Tran et al. (2018)

**Exercise as Therapy in PAH**

PAH management is a complex process that involves continual clinical observation, assessment of risk and response to drug therapy, and the exploration of novel strategies that may lead to improved quality of life (Galie et al., 2014; Madonna &

Cocco, 2015; Provencher & Granton, 2015; Rosenkranz, 2015). While it is outside the scope of the current project to review current pharmacologic approaches to PAH management, detailed algorithms for treating the disease have been published (Hemnes, 2014; Klinger et al., 2019; Provencher & Granton, 2015). Briefly, after referral to a specialized facility, vasoreactive testing is carried out, usually via the administration of inhaled NO. In vasoreactive patients, calcium-channel blockers are generally recommended, alongside close follow-up. In those patients that do not display acute vasoreactivity, PAH specific therapies as outlined in Chapter One are employed. In the most severe cases, intravenous epoprostinol is administered. Positive response to these drug interventions will mean continued therapy, however if they prove to be ineffective, lung transplantation is recommended (Provencher & Granton, 2015). While improvements in care have coincided with greater drug variety and availability, prognosis remains poor (McLaughlin, 2013). As such, the search for adjunctive therapies, including the use of exercise, has become a central theme in PAH management (Babu, Padmakumar, et al., 2016). As exercise intolerance is a common feature of disease progression (Babu, Arena, et al., 2016), there is rationale behind considering exercise training as a useful therapeutic tool. Due to the outlined pathophysiology and the inevitable exacerbation of cardiovascular stress brought about by exercise, it was initially contraindicated in this population (Desai & Channick, 2008). However, a strong body of evidence, including results from randomized clinical trials, now points to favorable outcomes for patients. Table 2.1 outlines the findings of exercise training interventions in PAH patients.

| Author/ Year | Participants | Functional Class | Intervention Description | Results |
|---|---|---|---|---|
| Saglam et al. (2015) | n=29, male and female | II and III | 6 weeks inspiratory muscle training, 30 mins per day | ↑MIP/MEP<br>↑expiratory volume<br>↑ 6-minute walk distance |
| Kabitz et al. (2013) | n=7, male and female | III and IV | 3 weeks in hospital, 12 weeks home-based. Cardiovascular, gait and respiratory training | ↑ 6-minute walk distance<br>↑ peak inspiratory and expiratory pressure |
| Weinstein et al. (2013) | n=24, female | I-IV | 10 weeks cardiovascular training, 3 x per week | ↑ 6-minute walk distance,<br>↑ physical activity<br>↓ fatigue |
| Ley et al. (2013) | n=20, male and female | II and III | In hospital 3 weeks cardiovascular/respiratory training | ↑ 6-minute walk distance<br>↑ pulmonary blood flow |
| Becker-Grunig et al. (2013) | n=20, male and female | II-IV | 3 weeks in hospital, 12 weeks home-based. Cardiovascular, gait, muscle and respiratory training | ↑ VO2max,<br>↑ 6-minute walk distance<br>↓ pain<br>↑ survival |
| Chan et al. (2013) | n=23, male and female | I-IV | 10 weeks cardiovascular training, 3 x per week | ↑ 6-minute walk distance<br>↑ quality of life |
| Nagel et al. (2012) | n=35, male and female | II-IV | 3 weeks in hospital, 12 weeks home-based. Cardiovascular, gait, muscle and respiratory training | ↑ VO2max,<br>↑ 6-minute walk distance<br>↑ quality of life<br>↑ survival |
| Grunig et al. (2012) | n=21, male and female | II-IV | 3 weeks in hospital, 12 weeks home-based. Cardiovascular, gait, muscle and respiratory training | ↑ 6-minute walk distance<br>↑ quality of life |
| Grunig et al. (2012) | n=183, male and female | II-IV | 3 weeks in hospital, 12 weeks home-based. Cardiovascular, gait, | ↑ 6-minute walk distance<br>↑ quality of life |

| | | | | |
|---|---|---|---|---|
| | | | muscle and respiratory training | |
| Grunig et al. (2011) | n=58, male and female | II-IV | 3 weeks in hospital, 12 weeks home-based. Cardiovascular, gait, muscle and respiratory training | ↑ VO2max, ↑ 6-minute walk distance ↑ quality of life |
| Fox et al. (2011) | n=22, male and female | II and III | 12 weeks in hospital and home-based. Cardiovascular and muscle training | ↑ VO2max, ↑ 6-minute walk distance |
| Mainguy et al. (2010) | n=5 | II and III | 12 weeks hospital based, cardiovascular and muscle training | ↑ 6-minute walk distance ↓ minute ventilation |
| de Man et al. (2009) | n=19, male and female | II and III | 12 weeks hospital based cardiovascular and quadriceps training | ↑ 6-minute walk distance ↑ quadriceps strength and endurance ↑ workload at anaerobic threshold |
| Shoemaker et al. (2009) | n=2 | | 6 weeks, 3 x per week cardiovascular | ↑ VO2max ↑ quality of life |
| Mereles et al. (2006) | n=30, male and female | II-IV | 3 weeks in hospital, 12 weeks home-based. Cardiovascular, gait, muscle and respiratory training | ↑ 6-minute walk distance ↑ quality of life |
| Uchi et al. (2005) | n=24, male and female | III and IV | 6 weeks, cardiovascular, gait, muscle and respiratory training | ↑ 6-minute walk distance ↓ resting heart rate ↑ muscle strength |

**Table 2.1:** Exercise training in PAH, adapted from Zafrir (2013) and Babu, Padmakumar, et al. (2016)

The majority of exercise interventions include some cardiovascular training component (cycling, walking, treadmill), with several also including resistance (body weight, dumbbells) and respiratory training. Fortunately, trials have encompassed various functional classes, age ranges, and durations of intervention. Based on the data presented, it is clear that exercise is beneficial in improving functional capacity and quality of life in PAH patients, adding weight to the idea of increased physical activity as adjunctive therapy (Babu, Padmakumar, et al., 2016). While the majority of those studies were not 'mechanistic' in design, studies using PAH animal models have shed greater light on the molecular changes brought about by exercise training (Nogueira-Ferreira et al., 2018).

M. B. Brown et al. (2017) determined that in a rat model of PAH induced via monocrotaline injection, 6 weeks of high intensity interval training (HIIT) reduced elevated pulmonary pressures, protected against RV hypertrophy, and improved cardiac function. At the skeletal muscle level, exercise trained rats had lower levels of GLUT-1, indicating a lower reliance on anaerobic metabolism and attenuation of the aforementioned glycolytic shift in the disease. Additionally, HIIT increased lung endothelial nitric oxide synthase (eNOS) expression. eNOS is recognized as a rate limiting enzyme in nitric oxide generation (Cocks et al., 2013), potentially increasing vasodilation in the lung, identified earlier as a barrier to exercise tolerance. Other animal studies have focused on the impact of exercise on the lung. Colombo et al. (2013) demonstrated that 3 weeks of treadmill running the same animal model increased lung hydrogen peroxide levels and vascular endothelial growth factor (VEGF), suggesting increased angiogenesis in response to training, concomitant with an improvement in RV function via echocardiography. Unsurprisingly, the majority of animal work has studied

the effects of exercise training on the RV. Treadmill running in PAH has been suggested to decrease RV hypertrophy via phosphorylation of glycogen synthase kinase (Colombo et al., 2013) and increase p-AKT protein expression, potentially upregulating anti-apoptotic signaling (Colombo et al., 2015). In a novel experimental design, Moreira-Goncalves et al. (2015) employed aerobic exercise either early or late in PAH induction. Both training groups improved cardiac function, explained in part by cardioprotective signaling via increased sarcoplasmic/endoplasmic reticulum calcium ATPase 2 (SERCA2a) and decreased ET-1 mRNA. Additionally, early exercise intervention resulted in lower inflammatory markers and reduced fibrosis in the RV.

From both a mechanistic and functional perspective, there is sufficient evidence to suggest regular exercise may be beneficial in PAH. RV, lung and to a lesser extent, skeletal muscle molecular mechanisms of disease have been shown to be positively modulated by exercise training (Nogueira-Ferreira et al., 2018). While this alone would provide rationale for careful but purposeful integration of exercise as part of a PAH management plan, the severe exercise intolerance inherent in the disease may limit patient's ability to reach a threshold of exercise needed to elicit the improvements necessary to improve quality of life (Manders, Rain, et al., 2015). With that said, novel strategies that might increase exercise tolerance in the short term may prove beneficial in bridging the gap to long term functional improvement.

## Nitric Oxide and Dietary Nitrate in PAH

As outlined in Chapter One, the main therapeutic avenues in PAH target endothelial dysfunction, primarily by ameliorating vasoreacitve dysfunction in the disease. Extensive work has been carried out on drug therapies that modulate nitric oxide, endothelin-1, and prostacyclin pathways, all recognized as key to altering the disrupted vasodilation/vasoconstriction balance present in PAH (Abman, 2009; Antoniu, 2006; Asaki et al., 2015; Barnes et al., 2019; Beltran-Gamez, Sandoval-Zarate, & Pulido, 2015; Burger, D'Albini, Raspa, & Pruett, 2016; Chaumais et al., 2015; Klinger, 2011). Nitric oxide is a key regulator of endothelial function, having been recognized as important in modulating vasoreactivity, smooth muscle cell proliferation, platelet aggregation and endothelial cell apoptosis (Ahanchi, Tsihlis, & Kibbe, 2007; Ataya et al., 2011), and as such, investigation on how to manipulate NO in PAH has garnered great interest. NO is endogenously generated via the metabolism of L-arginine by a family of nitric oxide synthases known as endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS) and ultimately induces vasodilation via the second messenger cGMP (Moncada & Higgs, 1993). Importantly, there is evidence to suggest this pathway is dysfunctional in PAH, potentially via increased levels of arginase, decreased NOS and/or eNOS uncoupling, in which superoxide ions are produced secondary to a decrease in tetrahydrobiopterin ($BH_4$) (Zuckerbraun, George, & Gladwin, 2011). As such, drug therapies that increase NO bioavailability via either a reduction in cGMP degradation (Sildenafil, Tadalafil), or more recently, through cGMP stimulation (Riociguat) have either been approved or are in advanced trials.

While work on the ‘classic’ L-arginine-NOS-NO pathway will undoubtedly continue, an alternative, NOS-independent mechanism of NO generation has recently been discovered (Lundberg et al., 2009; Lundberg, Weitzberg, & Gladwin, 2008). Once thought to be inert byproducts of NO production, circulating nitrate and nitrite ions are now known to be converted back to NO via anaerobic bacteria in the oral cavity, protonation in the stomach and reduction facilitated by hemoglobin, myoglobin and XOR (Zuckerbraun et al., 2010). Crucially, it has been demonstrated this alternative pathway is oxygen-independent, and the enzymatic reduction of nitrite to NO is upregulated in hypoxic conditions (Khatri, Mills, Maskell, Odongerel, & Webb, 2017). Thus, in the milieu of PAH, with disrupted NOS signaling and reduced oxygen delivery, manipulation of the nitrate-nitrite-NO pathway could be crucial in tackling disease progression and providing symptom relief. Fortunately, it has been shown that consumption of dietary nitrate can significantly elevate plasma nitrate and nitrite (Lundberg et al., 2009). As such, simple, non-pharmacological approaches, such as increased intake of nitrate-rich foods may be a useful tool in altering NO signaling and improving vascular function in PAH (Figure 2.3). Pre-clinical trials have employed dietary strategies and examined the therapeutic effects on animal models of vascular disease. Nitrate and nitrite in drinking water reduced RV hypertrophy, RV systolic blood pressure and vascular remodeling in hypoxia-induced PH mouse model (Baliga et al., 2012). Additionally, Alef et al. (2011) demonstrated that sodium nitrite in drinking water limited the development of intimal hyperplasia in a rat model of vascular injury.

Finally, although not strictly a dietary approach, nebulized nitrite has been shown to reverse RV hypertrophy in a PAH animal model, while also reducing smooth muscle cell proliferation *in vitro* (Zuckerbraun et al., 2010).

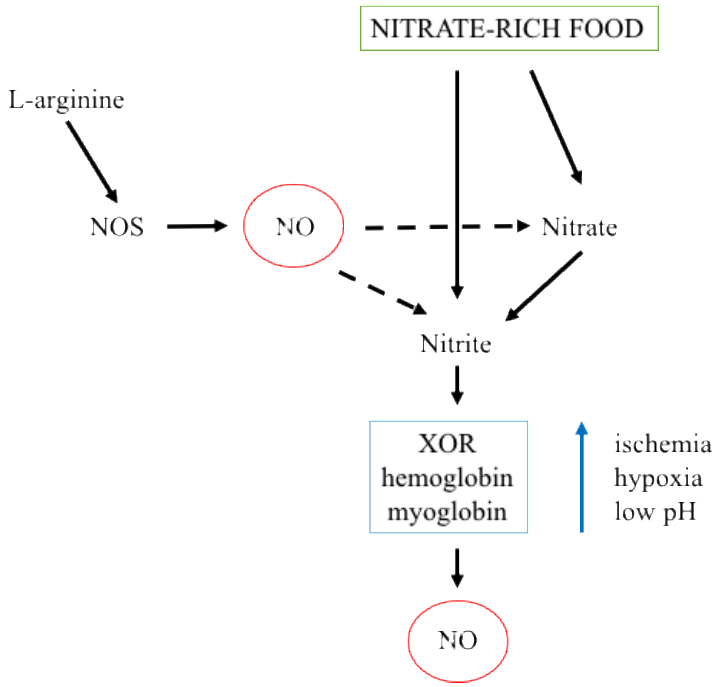



**Figure 2.3:** Pathways of NO generation, adapted from Ataya et al. (2011)

**Dietary Nitrate and Exercise**

While the potential disease mediating benefits of dietary nitrate warrant further research, there is a wealth of evidence pointing to its efficacy in improving exercise performance (Coggan & Peterson, 2018; A. M. Jones, 2014a, 2014b; A. M. Jones, Thompson, Wylie, & Vanhatalo, 2018). Acute and chronic supplementation of dietary nitrate has been shown to improve running and cycling performance in healthy subjects, however, these findings are not consistent and may depend on the initial training status of participants (A. M. Jones et al., 2018). In attempting to explain improvements, several mechanistic avenues have been explored. Dietary nitrate has been shown to reduce the

oxygen cost of exercise. In other words, after nitrate supplementation, oxygen consumption is significantly lower at the same absolute workload, and this has been confirmed during moderate and higher intensity exercise (Bailey et al., 2010; Bailey et al., 2009; Larsen, Weitzberg, Lundberg, & Ekblom, 2007; Vanhatalo et al., 2010). This improved economy of exercise may be due to several mechanisms, including the sparing of phosphocreatine and altered ATP cost of muscle power production. Additionally, it has been suggested that nitrate supplementation may directly affect mitochondrial action either indirectly via reduced respiratory stimulus, or directly by reducing proton leak across the mitochondrial membrane (Bailey et al., 2010; Larsen et al., 2011). Based on the above observations, the potential for dietary nitrate supplementation to improve exercise performance in PAH may be profound. It has been demonstrated that PAH patients have reduced skeletal muscle oxygen uptake and lower $VO_2max$ compared to healthy individuals, and as such, reducing the oxygen cost of exercise may prove beneficial in maintaining submaximal workloads in those that are profoundly exercise intolerant.

Dietary nitrate has been shown to increase blood flow in both humans and rats. Ferguson et al. (2013) directly measured skeletal muscle blood flow during exercise via radiolabeled microspheres, and determined that in rats, BRJ supplementation significantly increased flow, primarily in type II fibers. Casey, Treichler, Ganger, Schneider, and Ueda (2015) supported these findings by measuring forearm blood flow via Doppler imaging after BRJ ingestion in young and older adults. During rhythmic submaximal forearm contractions, muscle blood flow was increased via compensatory

vasodilation in response to hypoxia. As oxygen uptake is dependent in part on blood flow, it is suggested this mechanism may directly enhance exercise performance.

While early work on dietary nitrate and exercise considered its usefulness in longer-duration aerobic activity, recent evidence has shown the benefit of supplementation in shorter-duration, predominantly anaerobic efforts. It was recently demonstrated that 5 days of dosing with beetroot juice improved power output and intermittent cycling performance in team-sport athletes (Wylie et al., 2016). These findings were complimented by Thompson et al. (2016), who showed that 5 days of beetroot juice supplementation improved sprint-times and distance covered in a sports-specific high intensity running test. Again however, these findings are not consistent (A. M. Jones et al., 2018). Mechanistically, it has been postulated that dietary nitrate may upregulate muscle calcium-handling proteins and subsequently, muscle force production.

Coggan, Leibowitz, Kadkhodayan, et al. (2015) explored the potential benefits of dietary nitrate dosing on muscle performance. In a novel approach, a single dose of beetroot juice was administered, following which knee extensor function was measured via isokinetic dynamometry. Peak knee extensor power at the highest velocity was increased by 4% concomitant with a significant rise in breath NO post-BRJ supplementation. This work was mirrored by that of Rimer, Peterson, Coggan, and Martin (2016) who demonstrated that a single dose of dietary nitrate was sufficient to increase very short duration cycling sprints in comparison to controls. These findings are important, not least because a single dose approach would likely rule out potential changes in post-translational calcium-handling protein levels put forward as a mechanistic explanation for improved strength and power. To that end, Whitfield et al.

(2017) showed that despite an improvement in muscle power after one week of supplementation with beetroot juice, human muscle biopsies showed no increase in proteins associated with calcium-handling. Coggan and Peterson (2018) considered this phenomenon and postulated several mechanisms for increased contractile function after dietary nitrate ingestion. It was argued that nitrosylation of the ryanodine receptor on the sarcoplasmic reticulum may be forced open, with a subsequent increase in free calcium. An added downstream effect of this free calcium is to activate skeletal muscle myosin light chain kinase, which increases calcium sensitivity of contractile apparatus (Coggan & Peterson, 2018), potentially improving muscle strength and power.

**Dietary Nitrate and Exercise in Disease**

Several groups have sought to test the effectiveness of dietary nitrate as a means of improving exercise capacity in disease states, with one pilot study doing so in PAH patients (Henrohn et al., 2018). Ferguson et al. (2016) added to their previous work analyzing blood flow during exercise by supplementing a rat model of heart failure after myocardial infarction. As was seen in healthy animals, 5 days of nitrate supplementation in drinking water significantly elevated plasma nitrate and nitrite, and also increased skeletal muscle blood flow during exercise in comparison to those animals supplemented with placebo. As a result, this approach was recommended for further study as a novel treatment and ergogenic aid in cardiovascular diseases. Exercise capacity after nitrate ingestion has also been measured in patients with peripheral artery disease (Kenjale et al., 2011). Interestingly, a single dose of beetroot juice increased walking time by 17% in comparison to those given placebo treatment. Additionally, a trend toward reduction in

oxygen cost of exercise was seen at low intensities, similar to that noted previously in healthy subjects. Several studies have considered the effects of dietary nitrate on aerobic exercise performance in heart failure of various etiologies. Coggan et al. (2018) determined that a single dose of dietary nitrate significantly improved $VO_2$peak and time to exhaustion in patients with heart failure with reduced ejection fraction (HFrEF), concomitant with increases in plasma $NO_3^-$, $NO_2^-$ and breath NO, supporting previous work of Kerley et al. (2016) who demonstrated improved walking performance in patients with dilated cardiomyopathy after a single $NO_3^-$ ingestion. However, nine days of BRJ consumption did not improve exercise capacity in patients with HFrEF despite increases in plasma nitrite post supplementation (D. M. Hirai et al., 2017). In heart failure with preserved ejection fraction (HFpEF), it has been shown that a single dose of dietary NO3- improved VO2peak and total work performed during a maximal supine cycling exercise test (Zamani et al., 2015). In addition to aerobic exercise, the question as to muscle strength and power improvements after acute dietary nitrate supplementation has been addressed in patients with left heart failure (Coggan, Leibowitz, Spearie, et al., 2015). Similar to findings in healthy subjects, patients demonstrated a significant improvement in muscle power compared to those given placebo treatment. This is not to say that patient data is unequivocal. Two trials employing week-long dietary nitrate dosing strategy (via beetroot juice and sodium nitrate) in COPD patients elicited no improvements in walk distance, oxygen consumption and cycling endurance time, and as such, work exploring optimal dosing strategies, drug interactions and exercise test sensitivity is warranted (A. M. Jones et al., 2018).

When considering the aforementioned reasons behind exercise intolerance in PAH, and the potential mechanisms of action by which dietary nitrate may improve exercise performance (Figure 2.4), testing its viability in PAH patients is justified. One recent trial sought to determine the effectiveness of two weeks of BRJ supplementation on disease markers and exercise performance in 15 group I functional class PAH patients. It was noted that those treated with beetroot juice tended to have a higher peak power output/peak oxygen consumption ratio in the 6-minute walk test, as well as a tendency for improved right ventricular function via echocardiography. It was suggested that those who responded most favorably to the supplementation (via plasma nitrate/nitrite measures) showed the greatest exercise responses (Henrohn et al., 2018), adding a potential consideration when determining the effectiveness of dietary nitrate in populations with cardiovascular disease.

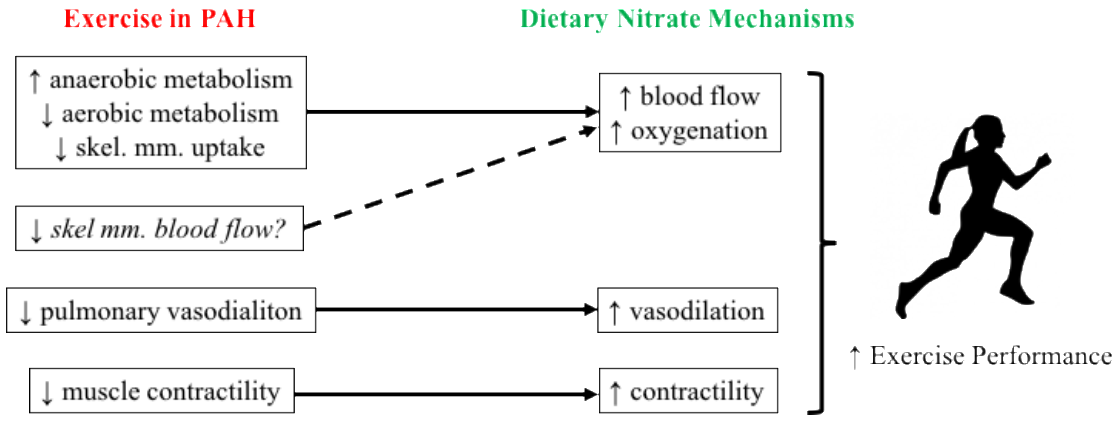



**Figure 2.4:** Potential mechanisms by which dietary nitrate may improve exercise performance in PAH.

## Summary

Review of the pertinent literature has described the complex an interrelated pathophysiology of PAH. The maladaptations described in the heart, lungs and skeletal muscle can be directly connected to the severe exercise intolerance suffered by PAH patients. It has been demonstrated that further mechanistic work on exercise pathophysiology is warranted, with specific emphasis on how skeletal muscle abnormalities may play a role in reduced exercise performance. To date, it has not been directly determined if skeletal muscle blood flow is reduced in PAH during exercise, and a full characterization of this phenomenon would be useful in developing a fuller understanding of the disease. Additionally, the search for alternative therapies has pushed exercise into the forefront of PAH management. As such, novel strategies to improve exercise tolerance are being considered. One such strategy is the supplementation of dietary nitrate, which has been shown to improve exercise performance in health and disease. Moreover, the proposed mechanisms of action seem to suggest that PAH may be an ideal condition in which to consider its effectiveness. Taken together, the data justifies the proposed work herein, with the ultimate aim of improving quality of life for PAH patients.

# Chapter Three: Methodology

## Introduction

This study aimed to characterize skeletal muscle blood flow at rest and during exercise in an animal model of PAH, and to interrogate the effectiveness of acute dietary nitrate supplementation in improving skeletal muscle blood flow and metabolism during exercise. Mechanistic analyses were complimented by functional testing to determine if dietary nitrate supplementation could impact exercise performance in PAH. The previous chapter outlined a review of the literature in this area, and provided rationale for the following experimental design.

## Research Design/Timeline

A randomized, between-animal experimental design provided the basis for characterizing skeletal muscle blood flow and determining the effectiveness of dietary nitrate supplementation in impacting skeletal muscle blood flow and exercise capacity in healthy and PAH afflicted animals. The study employed four groups of animals across three specific aims: Group 1 – PH + BRJ; Group 2 – PH + PL; Group 3 – CON + BRJ; Group 4 – CON + PL.

Using a counterbalanced, placebo-controlled approach, each rat carried out functional exercise testing (VO2max, 12-hour voluntary wheel run, grip-strength test) pre and post PAH induction under both BRJ and placebo supplementation, with control groups tested on the same timeline. After post-disease functional testing, blood flow analysis (via the administration of fluorescent microspheres) was carried out during exercise and at rest for all groups, under either BRJ or placebo conditions, followed by

invasive hemodynamics, euthanasia and tissue harvest. Tissues were processed to determine dietary nitrate dosing effectiveness, skeletal muscle blood flow and capillarization via immunofluorescent analysis. Study design and timeline are summarized in Figure 3.1.

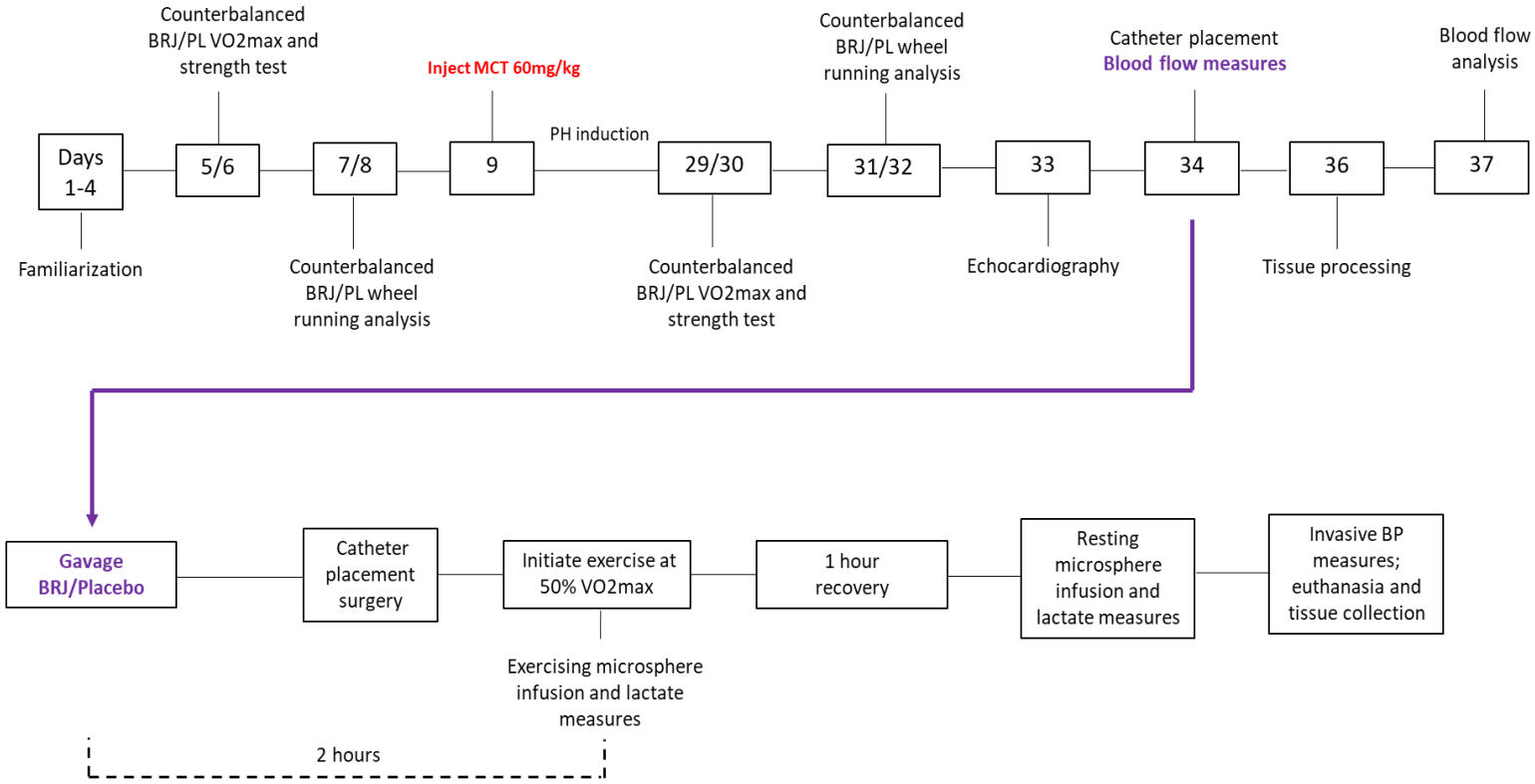



**Figure 3.1:** Research design/timeline

## Methods for Specific Aim 1

### Animal Experimental Model

Male Sprague Dawley rats (~200g) were randomly assigned into four groups as previously described. PAH rats were injected with monocrotaline (MCT – 60mg/kg, s.q, in sterile PBS vehicle, Sigma Aldrich). MCT is a plant-based toxic alkaloid that has been recognized as producing a pulmonary hypertensive phenotype in various species for half a century (Kay, Harris, & Heath, 1967). Once metabolized by mixed-function oxidases in the liver, MCT is known to induce vascular injury. While the exact mechanisms by which the initial vascular insult progresses to a PH phenotype are unknown, it is speculated to be a result of direct endothelial remodeling and/or accumulation of mononuclear inflammatory cells into the vascular adventitia, prior to RV hypertrophy and increased pulmonary arterial pressures (Stenmark, Meyrick, Galie, Mooi, & McMurtry, 2009). Dose response and timeline data have been published, with doses of 30-40mg/kg resulting in 'compensated' RV hypertrophy with increased RVSP and the maintenance of cardiac output. Higher doses of 60-80mg/kg has been shown to elevate pulmonary pressures to a greater extent, resulting increased RV wall stress, reduced ejection fraction, and ultimately pump failure at ~4wks post-MCT injection (Hessel, Steendijk, den Adel, Schutte, & van der Laarse, 2006). While the MCT model is well established and technically simple, it has limitations. It has been considered an inflammatory-driven model of disease, as opposed to alternative angioproliferative and vascular lesion-forming models such as that brought about by alternative methods, that may closer mimic PH phenotype in patients (Gomez-Arroyo et al., 2012). However, it continues to be used as a model of PH in a wide variety of animal studies, driven primarily by its reflection of

vascular injury and cardiac dysfunction as is seen in PAH patients. The timeline herein employed a timeframe of four weeks post MCT injection as the time point for terminal blood flow experiments, hence, a severe PH model is under investigation. Control rats were injected with s.q. vehicle solution (PBS). All animals were housed in pairs in the Indiana University animal facility and fed standard rat chow and water ad libitum. The rats were maintained at an ambient temperature of 21–24°C with a 12 h–12 h dark–light cycle.

### Power Analysis and Group Determination

Limited data exists regarding skeletal muscle blood flow at rest and during exercise in a PH rat model, however our lab has previously determined normative data for a major outcome variable in this study (%change in $VO_2$max pre-post disease, Aim 2), which can be used to determine group sizes as follows: mu 1=18, mu 2=3, sigma=11, alpha=0.5, desired power =0.8, calculated sample size = 9 each group. As such, the following groups were defined: Group 1 – PH + BRJ, n = 9; Group 2 – PH + PL, n = 9; Group 3 – CON + BRJ, n = 9 ; Group 4 – CON + PL, n = 9

### Exercise

#### Treadmill Familiarization

As running is a skill activity for rats, familiarization to the rodent motorized treadmill that permits metabolic measurements via indirect calorimetry (Columbus Instruments, Columbus, OH) was carried out for 4 days before exercise testing. The familiarization protocol involved 4 x 5 minute runs that gradually increased treadmill

speed (8m/min-12m/min) and incline (0 to 15 degrees). These inclines and speeds are similar to those experienced during initial maximal aerobic testing. Familiarization runs were kept at this short time-frame to minimize chronic training effects before baseline testing. A mild electric stimulus at the back of the treadmill chamber promoted the learning of running behavior, and the ability of the rat to run successfully was documented through this familiarization period. 'Cueing', including the use of verbal encouragement, treadmill lane taps and group-running were used to promote consistent running toward the front of the treadmill belt. If a rat contacted the stimulus three consecutive times without the ability to recover to the front of the treadmill belt, the familiarization session was terminated. Written notes were taken to document each rats' ability to run successfully, ensuring careful monitoring throughout.

### Maximal Oxygen Consumption ($VO_2$max) Testing

To measure exercising blood flow, all rats ran at 50% of their $VO_2$max as determined by their final $VO_2$max test on days 29/30 (Aim 1). In addition, $VO_2$max was used as a measure of functional capacity in pre and post disease testing, with either BRJ or placebo supplementation. (Aims 2 and 3). Gas analyzer calibrations were conducted before testing using standardized gas mixtures (Praxair, Indianapolis, IN, USA). Baseline $VO_2$ was monitored for 3-5 minutes until stabilization, and this was recorded as "resting $VO_2$". For determination of $VO_2$max, an incremental treadmill running protocol was employed, modified from (Kemi, Loennechen, Wisloff, & Ellingsen, 2002) using 3 minute stages as follows: 8 m/minute at 5 degrees (warm up), 8 m/minute at 15 degrees, 9.8 m/minute at 15 degrees, 11.6 m/minute at 15 degrees, etc., with the speed continuing

to increase by 1.8 m/minute every three minutes until test completion. $VO_2$ was measured at 20 second intervals throughout. The test was modified from Kemi et al. (2002), who suggest an incline of 25 degrees, for several reasons. First, it was determined that MCT rats' ability to run at such an incline was inconsistent, and the modified protocol resulted in a test that was more easily tolerated. Importantly, because blood flow measures were carried out at a known percentage of $VO_2$, it was important these conditions were replicated in terminal testing. Crucially, it is well established that acute responses to exercise in the rat, including muscle activation patterns are dependent on incline (Gillis & Biewener, 2002; Lynn, Talbot, & Morgan, 1998), and as such, treadmill incline would have to be the same between MCT and CON rats to carry out a meaningful comparison The test was terminated when $VO_2$ plateaued despite increasing workload, or if the rat was unable to maintain running after 3 consecutive electrical stimuli without recovery to the front of the treadmill belt. The highest $VO_2$ measured in the minute following test completion was recorded as $VO_2$max and expressed relative to body weight (ml/kg/hr). Respiratory exchange ratio (RER, $VO_2/VCO_2$) was recorded concomitantly during running. Following testing, rats were immediately removed from treadmill apparatus and placed back into their cage.

**Echocardiography**

To characterize disease phenotype, echocardiography was performed one day before terminal blood flow testing. Rats were anesthetized with 5% isoflurane in an induction box and then placed on the heated platform with a nose cone and maintained at 1-2%. The hair was clipped over the chest and fine hair removed using a depilatory

cream. The skin was cleaned with wet gauze squares. Ultrasonic gel was place over the chest for the echo procedure and an ultrasonic probe of approximately 6 cm by 8 cm was placed in contact with the gel. After the images are collected the chest was cleansed with gauze squares and the rat was placed into a heated cage to recover. All images were obtained by a blinded sonographer. LV/RV wall thicknesses, LV/RV mass, ejection fraction, stroke volume, RVSP and cardiac output (derived from RV outflow tract diameter and velocity time integral), which is expressed relative to body mass as cardiac index, were all reported by the sonographer.

**Blood Flow Measurement**

Blood flow to working skeletal muscle was measured using fluorescent microspheres injected during and after moderate intensity running (50% $VO_2$max) for PH and healthy control rats. This 'gold-standard' measure of blood flow was pioneered initially by Heymann, Payne, Hoffman, and Rudolph (1977) and Rudolph and Heymann (1967), who determined that injecting radio-labeled microspheres into the arterial circulation, and subsequently measuring radioactivity in specific harvested organs can result in extremely accurate measurements blood flow. This was further developed specifically for fluorescent-labeled spheres by Glenny, Bernard, and Brinkley (1993) Prinzen and Glenny (1994), driven in part by the increased legislation and cost of handling radioactive materials. This method relies on the fact that fluorescent microspheres injected via arterial catheters embed in the microcirculation of organs, and subsequent spectrometric measurements of those tissues accurately represents blood flow at the time of injection. Importantly, to determine flow most accurately, the 'reference

line' technique should be employed (Glenny et al., 1993). Here, measurement of regional perfusion is calculated as a fraction of total cardiac output, measured by capturing the fluorescence in blood sampled in a separate cannula at the time of microspheres injection in the arterial line (Ishise, Pegram, Yamamoto, Kitamura, & Frohlich, 1980). This method therefore requires animals to be cannulated with two separate lines, one being used to deliver microspheres, and the other used to collect reference line blood samples.

### Surgical Preparation

On the day of experimentation, rats were surgically prepared for blood flow analysis as outlined previously. Rats were anesthetized by inhaled isoflurane and then orotracheally intubated with a catheter (appx. 14 gauge). Rats were mechanically ventilated using a tidal volume of 6 ml/kg and a rate of 65-70 breaths/min. End expiratory pressures were at 3-4 $cmH_2O$ by a water overflow on the expiratory limb of the ventilator. Airway pressures were monitored continuously during surgeries. The rats were placed on a servo-controlled heated tray that maintains animal temperature at 37º C. Animals were shaved (total abdominal, thoracic surfaces), and the skin cleansed with Betadine and ETOH. The animal was fixed to the operating table with adhesive tape and covered with sterile drape with a hole allowing access to the surgical area. The animal was given carprofen (5mg/kg: 0.1-0.5ml,SQ) before surgery. The right carotid artery and caudal (tail) artery were cannulated via cutdowns through small skin incisions. Arterial and airway pressures were measured continuously. Normal saline boluses (10 ml/kg) were given at the beginning of the procedure to replace blood loss from surgery, blood sampling, and ongoing insensible losses during surgery. After placement, tubing was

tunneled subcutaneously between the scapulae, and tied in with approx. 10 inches of external line access for microsphere delivery and reference blood sampling in carotid and caudal lines. Incisions were closed using silk suture for muscle layers and monofilament suture for skin. Total time for surgery was approximately one hour. Following surgery, animals were placed back in the cage for recovery, and in preparation for exercising and resting blood flow measures.

## Resting and Exercising Microsphere Infusion

After a minimum of one-hour recovery post-surgery, fluorescent microspheres were prepared for infusion by sonicating for >5 mins and vigorously vortexing for > 1 minute. The caudal artery line was flushed with 0.5ml saline to encourage effective bleed-back of reference blood. Running was initiated at moderate intensity as determined by calculating 50% $VO_2$max from the animal's latest $VO_2$max test, using the Karvonen formula as follows:

$$50\%\ VO_2max = ([VO_2max - VO_2rest \times .5]) + VO_2rest$$

The calculated $VO_2$max was then referenced to the latest $VO_2$max testing data, and the stage at which this $VO_2$ was reached was used as the intensity for running. After 3 minutes of running at the predetermined intensity, fluorescent microspheres (n= approx. 400,000, Red, 580/620, Molecular Probes) were injected over 10 s into the carotid cannula. The line was subsequently flushed with 0.5ml heparinized saline to ensure all spheres were successfully injected. 10 s prior to microsphere infusion, collection of a reference blood sample from the caudal cannula was begun using a motorized syringe

withdrawal pump (Kent Scientific, CT, USA). Blood was collected for 60 seconds at a constant rate of 1 ml/min. Following exercise measures, the rat rested for 1 hour before determination of resting blood flow. At this time, a second microsphere infusion of a different color (n=approx. 400,000, Yellow-Green, 500/545, Molecular Probes) was performed in the same manner. It has previously been shown that rats familiarized to exercise may exhibit an anticipatory response to exercise that specifically increases skeletal muscle blood flow (Armstrong, Hayes, & Delp, 1989), hence exercising measures were carried out, and after a 1 hour rest period, resting measures were taken using the same protocol.

**Exercising and Resting Metabolism**

To determine the effects of PAH induction on resting and exercise metabolism and/or a potential link between blood flow and metabolism (Aim 1), as well as to investigate the effectiveness of BRJ in altering metabolic control (Aim 2), plasma lactate was measured using a small sample of arterial blood from the caudal line used for reference blood sampling. It is well established that non-oxidative means of ATP synthesis results in the accumulation of lactate and hydrogen ions in the blood, representing a switch from aerobic to anaerobic metabolism (Kenney, Wilmore, & Costill, 2012), hence its measurement can give an indication as to metabolic control and exercise capacity. Approximately 10 μl of blood was withdrawn after the reference blood sample and immediately transferred to a hand-held, portable lactate analyzer (Lactate Pro, Sports Resource Group, USA), previously used to measure blood lactate in exercising rats (Kato, Kurakane, Nishina, Park, & Chang, 2013; Lu et al., 1996). A small

drop of whole blood was placed on a lactate test strip (Sports Resource Group, USA), with results presented on the analyzer 13 seconds after initial contact.

### Invasive Hemodynamics

Immediately after resting blood flow and lactate measures, rats were anaesthetized by inhalation of isofluorane-$O_2$ mixture (5%), orotracheally intubated and mechanically ventilated under isofluorane maintenance (2%). The left carotid artery was cannulated with PE-50 tubing and the right internal jugular vein with a 2F Millar catheter (Millar Instruments, Houston, TX, USA). Surgery was performed on a servo-controlled heated tray that maintained animal temperature at 37°C. Recordings of pulmonary and systemic pressures were achieved within 30 minutes following transfer out of the treadmill chamber. Right ventricular systolic pressure (RVSP) and mean arterial pressure (MAP) were assessed in room air and recorded simultaneously.

### Tissue Processing

Immediately after hemodynamic measurements, rats were euthanized under anesthesia (5% isofluorance-$O_2$ mixture) via exsanguination and bilateral pneumonectomy as a secondary means of euthanasia which also allowed for immediate access to the diaphragm and heart muscles. Right ventricular hypertrophy was assessed by measuring the Fulton index [weight of the RV divided by weight of the left ventricle plus septum (S); RV/(LV + S)]. Sections of RV, LV, liver, kidney and muscle (diaphragm, biceps femoris, semitendinosus, extensor digitorum longus, tibialis anterior,

gastrocnemius, soleus, rectus femoris, vastus lateralis) were then snap frozen for further biochemical analysis. Muscle samples from the same muscle groups were collected from the contralateral leg for blood flow quantification. Muscles were weighed and placed in 5% ethanoic potassium hydroxide for 48 hours for tissue degradation. Samples were regularly vortexed to ensure full degradation. Reference blood collected during exercise and at rest were processed in the same fashion. After degradation, samples were reverse-filtered through 5μm polyamide mesh filters (Sterlitech, WA, USA). Each filter was carefully placed into a 1.5 ml microcentrifuge tube (Thermo Fisher, MA, USA) and 1 mL of cellosolve acetate (2-ethoxyethyl acetate, 98%, Sigma Aldrich) was added to each of the tubes to degrade microspheres and expose the fluorescence. At 1 h tubes were vortexed to ensure maximum exposure to cellosolve acetate. This was repeated at 2 h. 100 μL samples from each tube were then loaded in duplicate into a 96-well plate in preparation for spectroscopic analysis (Figure 3.2).

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **A** | **Blank** | **Blank** | | **Rec. Fem** | **Rec. Fem** | | **Vastus Lateralis** | **Vastus Lateralis** | | | **Ref blood exercise** | **Ref blood exercise** |
| **B** | | | | | | | | | | | | |
| **C** | **Left kidney** | **Left kidney** | | **Bic. Fem** | **Bic. Fem** | | **Soleus** | **Soleus** | | | **Ref blood rest** | **Ref blood rest** |
| **D** | | | | | | | | | | | | |
| **E** | **Right Kidney** | **Right Kidney** | | **EDL** | **EDL** | | **Semitend.** | **Semitend.** | | | | |
| **F** | | | | | | | | | | | | |
| **G** | **Gastroc** | **Gastroc** | | **Tib. Ant** | **Tib. Ant** | | **Diaphragm** | **Diaphragm** | | | | |
| **H** | | | | | | | | | | | | |

**Figure 3.2:** Plate set-up for spectroscopic analysis

### Blood Flow Quantification

All fluorescence measurements were made using the SpectraMax i3x microplate reader (Molecular Devices, CA). Red fluorescence (representing exercising flow) was measured using an excitation of 580 nm and an emission of 620 nm, and yellow-green (representing resting flow) at an excitation of 500 nm and an emission of 545 nm. Tissue blood flow was calculated using the reference blood sample with the following equation (Glenny et al., 1993; Vescovo et al., 1998).

$$\text{Flow (ml/min)} = \frac{(\text{fluorescence of sample}) \times (\text{reference line withdrawal rate})}{\text{fluorescence of reference blood}}$$

Blood flow units are calculated and presented as $ml \bullet min^{-1} \bullet g^{-1}$ of tissue by dividing calculated flow by the recorded muscle weight at time of harvest. Bilateral kidney flow was used as a measure of adequate microsphere mixing and subsequent uniform systemic blood flow *in vivo* (Ferguson et al., 2013), with a discrepancy of >20% in blood flow across right and left kidneys resulting in that animal being disregarded from blood flow analysis. Figure 3.3 depicts the microsphere/blood flow analysis protocol, adapted from Aref, Akans, and Allen (2017).

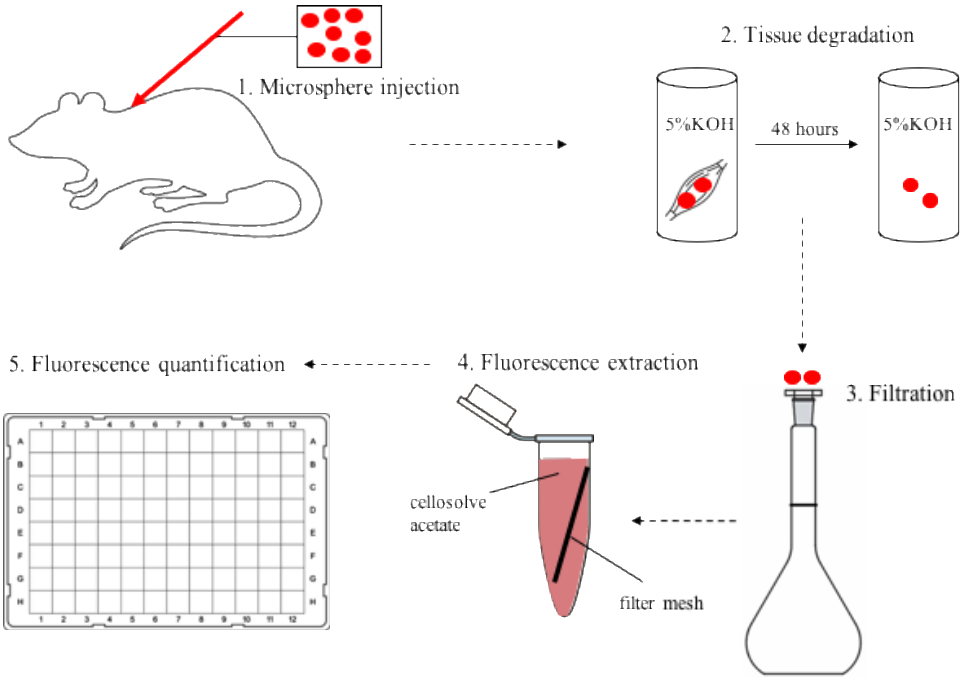



**Figure 3.3:** Microsphere/blood flow analysis protocol

### Capillarization

To determine if muscle capillarization differed between MCT and CON rats, immunofluorescent staining of frozen muscle sections was carried out. The midsection of frozen muscles was cut, embedded in OCT (Fisher Scientific, USA) and placed in -80°C in preparation for tissue sectioning. Muscles were sectioned at 8µm using a cryostat (Reichert-Jung Frigocut 2800) at -20°C and mounted on Superfrost Plus Microscope Slides (Fisher Scientific, USA). Slides were submerged in 2% paraformaldehyde for 10 minutes. They were subsequently air-dried and stored at –80°C in preparation for staining. After three washes in phosphor-buffered saline with Tween (PBS-T, Fisher Scientific, USA) sections were incubated for 48hrs at 4°C with an antibody cocktail

containing 1:500 dilution Wheat Germ Agglutinin (W6748 WGA-oregon green 488 conjugate 5mg, Thermo Fisher, USA), 1:75 dilution lectin (isolectin GS-IB4 alexaFluor 594 conjugate, Thermo Fisher, USA) in Tris Buffered Saline (TBS) vehicle (Thermo Fisher, USA). Negative controls were processed similarly, with antibodies replaced by incubation in TBS only. Slides were washed three times in PBS-T, after which coverslips containing DAPI ProLong Gold (Fisher Scientific, USA) for nuclei staining were mounted to slides and fixed using Biotium Covereslip Sealant (Biotium, CA, USA). Tissue sections were imaged using a Nikon Eclipse Ti2 inverted fluorescence microscope (NY, USA). Three different fields were imaged in each tissue section (9 per muscle sample). Green, red and blue images were taken at their optimal exposure times by a technician blinded to group assignments, and exported to ImageJ (NIH, USA) for further analysis.

## Methods for Specific Aim 2

### Dietary Nitrate Dosing

Both acute and chronic dietary nitrate dosing approaches have been employed in an attempt to investigate the effectiveness of supplementation on exercise performance (A. M. Jones et al., 2018). Depending on the outcome of interest, both acute (single dose) and chronic (multiple doses) have resulted in improved exercise performance, however, only a chronic dose has been used to specifically interrogate the effectiveness of BRJ in augmenting blood flow in rats (Ferguson et al., 2013). This poses a standardization problem in regards to dosing BRJ and subsequent exercise performance, as it cannot be known when the last intake of nitrate took place. As such, an acute, measured nitrate dose

may be required. A single dose of BRJ has been shown to improve exercise economy (Hoon et al., 2014; Vanhatalo et al., 2010; Wylie et al., 2013) and muscular power (Coggan, Leibowitz, Kadkhodayan, et al., 2015; Coggan, Leibowitz, Spearie, et al., 2015; Rimer et al., 2016) in healthy and patient populations, hence this dosing strategy warrants further research as a potential tool to alter blood flow and exercise performance in a PH animal model. Additionally, the translational value of a single dose of BRJ may be significant. If a patient with PAH were to gain an exercise advantage after ingestion of BRJ prior to an exercise bout, it may provide an alternative strategy to combat the exercise intolerance associated with the disease and improve likelihood of the patient achieving minimum threshold for positive exercise adaptations . It has been demonstrated that increasing exercise intensity, for both resistance training (Campos et al., 2002; Fry, 2004) and aerobic exercise (McNicol, O'Brien, Paton, & Knez, 2009; Seiler, Joranson, Olesen, & Hetlelid, 2013) is an important factor in mediating positive physiological adaptations, and as such, an increase in workload achieved during exercise may be critical for patients with PAH seeking to maximize the previously outlined benefits of exercise.

This strategy, while novel, poses a methodological problem in supplementing rats. While chronic dosing can be achieved by mixing BRJ in drinking water, there is no way to ensure an adequate acute dose prior to exercise if it were to be ingested *ad libitum*. To combat this problem, rats were gavaged with a measured dose of BRJ prior to functional exercise and blood flow testing, a common technique used to tests the pharmacokinetics of ingested compounds in rat models (A. P. Brown, Dinger, & Levine, 2000). Rats were anesthetized by inhaled isoflurane at 5% in a sealed chamber, before being quickly hung

on a specialized cage allowing access to the oral cavity. A soft-tipped hemostat was placed around the rat's tongue, moving it laterally for direct access to the esophagus. After the soft needle was inserted, the liquid supplement was slowly and consistently administered from a clear syringe until empty. The hemostats were then removed and the rat placed back in the cage. The total time of gavage was ~20 seconds, ensuring the rat did not wake during the procedure. Preliminary work was performed to determine time-course of plasma nitrate/nitrite increases after gavage. This data (see Appendix A) indicated that ideal time to dose before exercise was between 2-3 hours. This work allowed for optimization of supplementation timing prior to exercise, and was subsequently used prior to functional testing and blood flow analysis.

**Measurement of Plasma Nitrate/Nitrite**

An additional 300μl of blood was taken from the reference line during blood flow studies and processed for measurement of plasma nitrate/nitrite via High Performance Liquid Chromatography (HPLC, Eicom, ENO-30). This method involves post-column derivatization of nitrite with 2,3-diaminonaphthalene (DAN), forming compounds that are absorbed in the visible light range (Troutman, Gallardo, Brown, & Coggan, 2018), resulting in highly sensitive measures of nitrate and nitrite in biological samples. Upon withdrawal, whole blood was immediately centrifuged for 10 minutes at 10,000 rpm (Eppendorf, Hamburg, Germany), with the resulting plasma supernatant aliquoted into 1ml microcentrifuge tube (Thermo Fisher, MA, USA) and placed in -80°C in preparation for HPLC analysis. Plasma was thawed on wet ice, and mixed with equal parts HPLC-grade methanol (Fisher Scientific, USA) and centrifuged for 10 minutes at 10,000 rpm.

The supernatant was removed and aliquoted into 1ml microcentrifuge tubes. 10µl of the plasma/methanol solution was manually injected into the HPLC machine per user instructions. Chromatograms indicating 'peaks' of nitrate and nitrite in samples were then integrated and compared against known nitrate and nitrite standards to quantify levels in injected samples.

**Measurement of Muscle Nitrate/Nitrite**

A novel method using HPLC to quantify nitrate and nitrite levels in muscle samples has recently been developed (Troutman et al., 2018). This gives the advantage of comparing plasma and muscle nitrate/nitrite levels post supplementation in the same animal. Additionally, the effectiveness of dietary nitrate supplementation in increasing nitrate and nitrite in multiple tissues can be interrogated. This is crucial, as it has been shown that in rodents, skeletal muscle may be a major NO reserve via the outlined $NO_3$-$NO_2^-$-NO pathway (Piknova et al., 2015). Frozen muscle samples (~40mg) were pulverized at liquid $N_2$ temperature in a stainless steel tissue pulverizer (Bessman Tissue Pulverizer, Thermo Fischer Scientific). The muscle tissue was then transferred into a pre-weighed microcentrifuge tube containing 50µl ethanol, 0.5% triton X-100 and 0.1 mmol/l oxypurinol, a solution found to yield highest $NO_2^-$/$NO_3^-$ values and strongest reproducibility across samples (Troutman et al., 2018). After vortexing, samples were placed on ice for >30 min, reweighed to determine the amount of tissue added, then centrifuged. The supernatant was removed and aliquoted into 1ml microcentrifuge tubes, before HPLC analysis as was done for plasma samples.

## Methods for Specific Aim 3

To determine the effects of PAH induction on exercise capacity, as well as the effectiveness of acute dietary nitrate dosing in improving functional performance, three counterbalanced exercise tests were carried out pre and post disease and ~2 hours after BRJ or PL supplementation**.**

### Maximal Oxygen Consumption ($VO_2$max) Testing

As described under Aim 1.

### Voluntary Wheel Running

Voluntary wheel running is an exercise modality that has been used successfully in rats and mice, with animals displaying an inherent drive to run when given access to activity wheels. Rats voluntarily ran on an activity wheel (Lafayette Instruments, Model 80850S Scurry Rat Activity Wheel); with wheel revolutions monitored by 86115 Scurry Sensor/Counter, 86130 Interface and 86165 Scurry Software, connected to a computer interface for complete data collection (3 second sample rate), analysis and charting. Voluntary running distance (meters) and maximum speed achieved was measured over the 12-hour dark cycle, with gavage taking place 2-3 hours prior to the dark cycle beginning.

### Grip Strength

Voluntary grip strength was measured using specialized apparatus for the rat (Harvard Apparatus, MA, USA). Rats were held from the base of the tail and gently lowered until all four paws grasped the grid of testing apparatus. From a horizontal

position, the tail was gently and steadily pulled back until the grip was voluntarily released. The maximal force achieved by the animal was recorded (grams), with each animal will performing 3 repetitions, with a minimum of 1-minute rest between each trial (Ling, Authier, Balayssac, Eschalier, & Coudore, 2007).

**Statistical analysis**

Statistical analyses were carried out using GraphPad Prism 7.0 (San Diego, CA, USA). Data are presented as means ± SE. Differences at α level of 0.05 ($P < 0.05$) were considered statistically significant. In addition to analyzing blood flow measured within each harvested skeletal muscle, a 'compiled blood flow' variable was also created by averaging resting flow and separate exercising flow in individual muscles for each rat. Analysis of variance (ANOVA) was carried out by group assignment for the following measurements: resting and exercising blood flow, Fulton Index, stroke volume, cardiac output, cardiac index, RVSP, and plasma and skeletal muscle nitrate and nitrite. ANOVA by group assignment with repeated measures was performed for the following within-rat serial measurements: blood flow rest and exercise, blood lactate rest and exercise, body mass pre-MCT and 3 wks post-MCT, , $VO_2$max pre-MCT and 3 wks post-MCT, $VO_2$max BRJ trial and placebo trial, grip strength pre-MCT and 3 wks post-MCT, grip strength BRJ trial and placebo trial, voluntary running distance pre-MCT and 3 wks post-MCT, and running distance BRJ trial and placebo trial. Post-hoc analysis to determine individual group differences was performed where appropriate using Tukey's multiple comparison model. Pearson product correlations were used to determine relationships between blood flow, plasma/muscle nitrate and metabolic/disease measures.

# Chapter Four: Results

## Animal Attrition and Final Numbers

A total of n=57 rats were used during data collection, which began 2/13/18 and ended 5/14/19. n=7 rats died during gavage procedures (2 in pre-testing, 5 post-testing), n=4 rats died of natural causes post MCT injection. n=46 rats carried out terminal blood flow testing as outlined, however, n=9 did not demonstrate sufficient kidney blood flow to include the data in blood flow analysis. Final groups for terminal testing were: CON+BRJ n=8, CON+PL n=9, MCT+PL n=9, MCT+BRJ, n=9.

## Aim 1

### PH Phenotype

For phenotype characterization, MCT (n=11-21) rats were compared to CON (n=16-22) regardless of the final experimental condition (i.e. acute dosing of either BRJ or PL for blood flow analysis). Four weeks after MCT injection, rats showed a PH phenotype (Figure 4.1) as demonstrated by an increased Fulton Index (0.65 ± 0.043 vs. 0.29 ± 0.011, p=<0.001), larger RV thickness (2.25mm ± 0.13 vs. 1.63mm ± 0.12, p=0.001) and increased RVSP (52.27mmHg ± 4.7 vs. 24.25mmHg ± 1.59, p= p=<0.001). Echocardiographic measurement indicated a significantly reduced stroke volume (218.3uL ± 13.18 vs. 264.3uL ± 10.82, p=0.011), cardiac output (67.46ml/min ± 4.333 vs. 87.43ml/min ± 3.588, p=0.001), cardiac index (0.19ml/min/kg ± 0.01 vs. 0.22ml/min/kg ± 0.01, p=0.008). Significantly reduced pulmonary acceleration time (PAT, 26.14 ± 1.296 vs. 32.39 ± 1.232, p=0.01), pulmonary artery velocity time interval (VTI, 39.62 ± 2.601 vs. 49.67 ± 2.018, p=0.04), and significantly increased total

pulmonary resistance index (TPRi 178.4 ± 33.4 vs. 114.4 ± 9.96, p=0.04) compared to CON where also identified (Table 4.1).

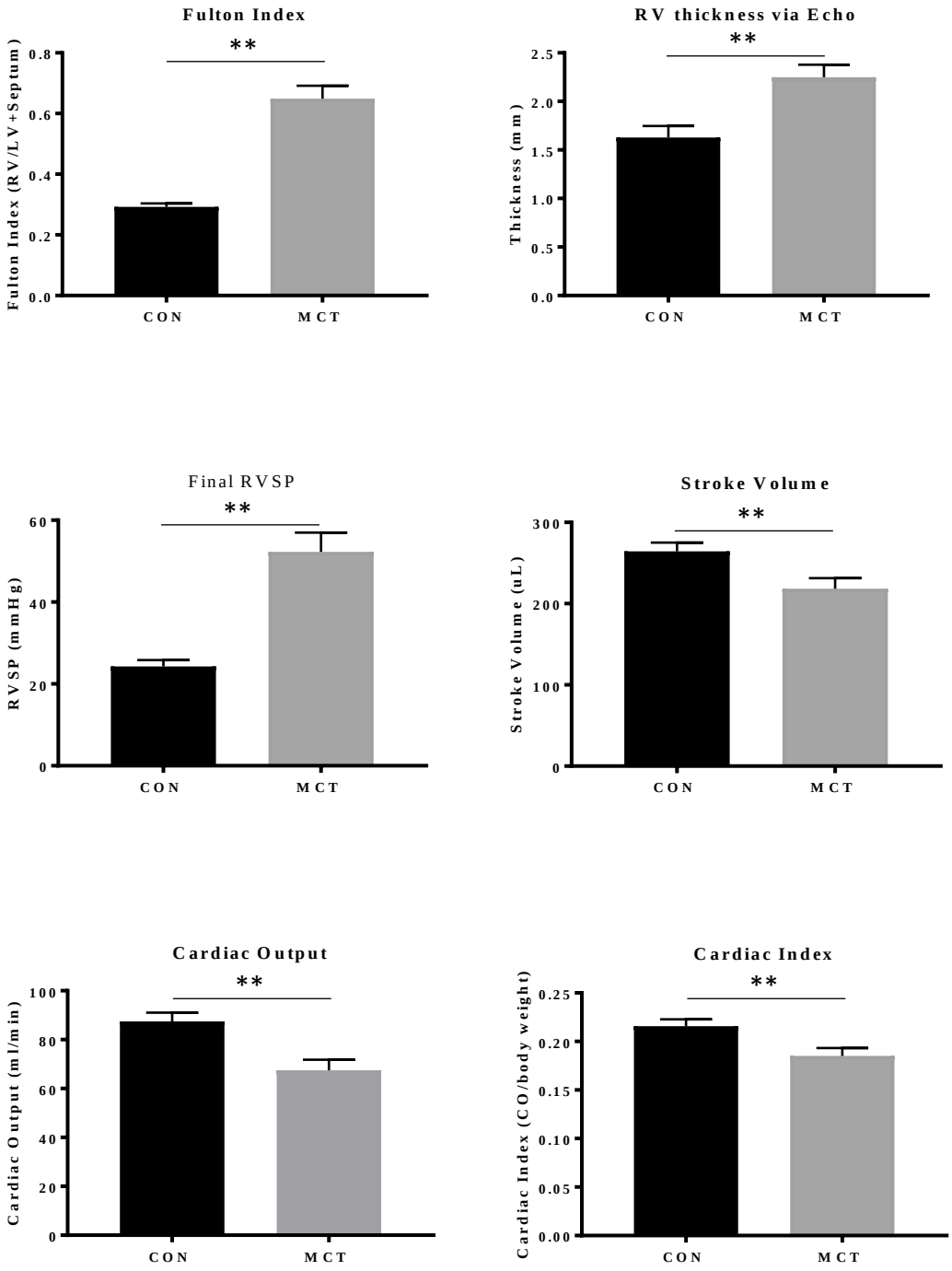



**Figure 4.1:** PH phenotype

| | CON (n=14-18) | MCT (n=16-17) |
|---|---|---|
| Pulm. Acceleration Time (PAT) | 32.39 ± 1.232 | 26.14 ± 1.296 |
| Pulm. Artery VTI | 49.67 ± 2.018 | 39.62 ± 2.601 |
| TPRi (RVSP/CI) | 114.4 ± 9.96 | 178.4 ± 33.4 |

**Table 4.1:** Echocardiographic parameters between CON and MCT

Body mass (Figure 4.2) was not different between groups pre MCT/vehicle injection (215.2g ± 8.3, n=18 vs. 232.3g ± 7.5, n=21), however, at harvest MCT rats were significantly lighter than their CON counterparts (331.8g ± 11.6, n=15 vs. 400.3g ± 9.4, n=17).

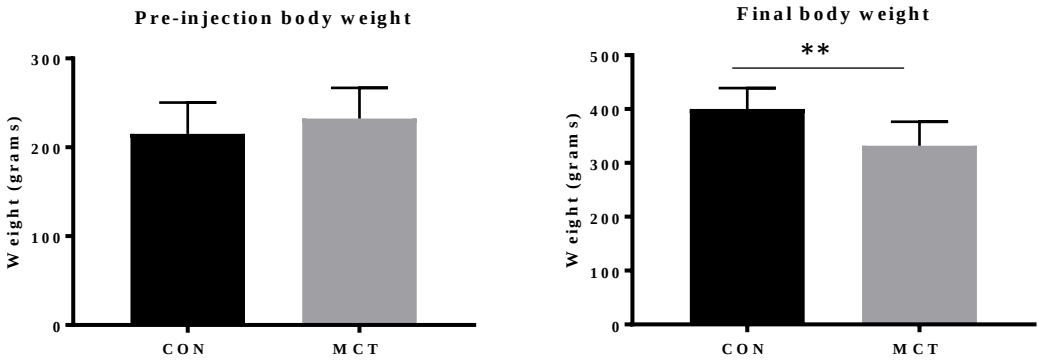



**Figure 4.2:** Body mass pre-injection and at harvest

**Skeletal Muscle Blood Flow**

The fluorescent microsphere technique was used to directly measure blood flow at rest and during moderate intensity exercise. Briefly, a known quantity (400,000) of red (exercise) and yellow (rest) microspheres were injected into the aortic arch, during which time reference blood was drawn from the caudal artery. Subsequent tissue degradation and spectrographic measurement of individual muscles reliably determined tissue blood flow in each condition. For the purpose of testing hypothesis 1 (that MCT blood flow is reduced) analysis will initially show a comparison of all MCT (n=13-17) against all CON

(n=12-15) regardless of whether a single dose of BRJ or PL was administered in the terminal experiments, since subsequent analyses (to be detailed in Aim 2) indicated no effect of BRJ. For completeness, data showing a comparison between MCT (n=6-8) and CON (n=6-8) receiving only PL in their terminal experiment are also included in the following analyses.

**Resting Flow**

Table 4.2 compares resting blood flow in muscle tissue between CON and MCT rats. Blood flow in the gastrocnemius (p=0.04) and tibialis anterior (p=0.009) were significantly lower in MCT, with flow significantly increased (p=>0.001) in the diaphragm of the same group (Figure 4.3).

| Muscle | Resting Blood Flow ($ml/min^{-1}/g^{-1}$) CON (n=13-17) | MCT (n=12-15) | p-value |
|---|---|---|---|
| ***Gastrocnemius*** | ***0.352 ± 0.05*** | ***0.228 ± 0.03**** | ***0.04*** |
| Rectus femoris | 0.405 ± 0.07 | 0.303 ± 0.05 | 0.22 |
| Biceps femoris | 0.177 ± 0.025 | 0.14 ± 0.02 | 0.28 |
| EDL | 0.276 ± 0.054 | 0.207 ± 0.04 | 0.33 |
| ***Tibialis anterior*** | ***0.329 ± 0.05*** | ***0.178 ± 0.02**** | ***0.009*** |
| Vastus lateralis | 0.214 ± 0.03 | 0.157 ± 0.02 | 0.11 |
| Soleus | 0.897 ± 0.20 | 0.883 ± 0.10 | 0.95 |
| Semitendinosus | 0.349 ± 0.08 | 0.255 ± 0.054 | 0.34 |
| ***Diaphragm*** | ***1.114 ± 0.064*** | ***2.09 ± 0.22**** | ***<0.001*** |

**Table 4.2** Resting muscle blood flow

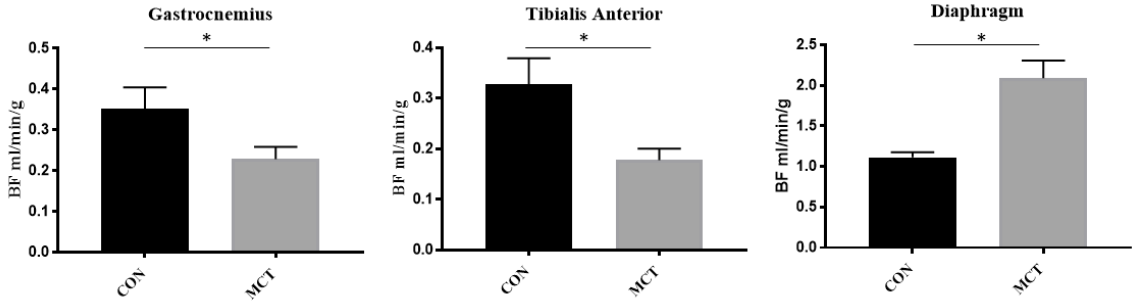


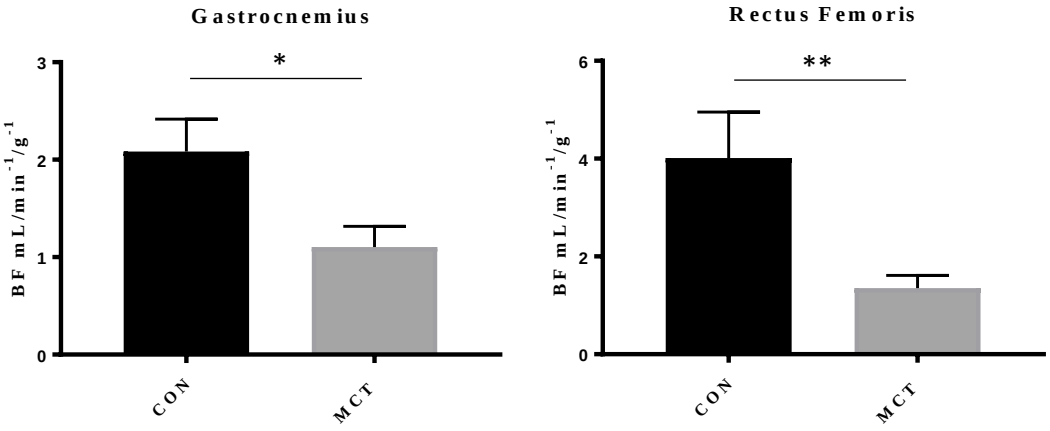

**Figure 4.3:** Resting blood flow in gastrocnemius, tibialis anterior and diaphragm

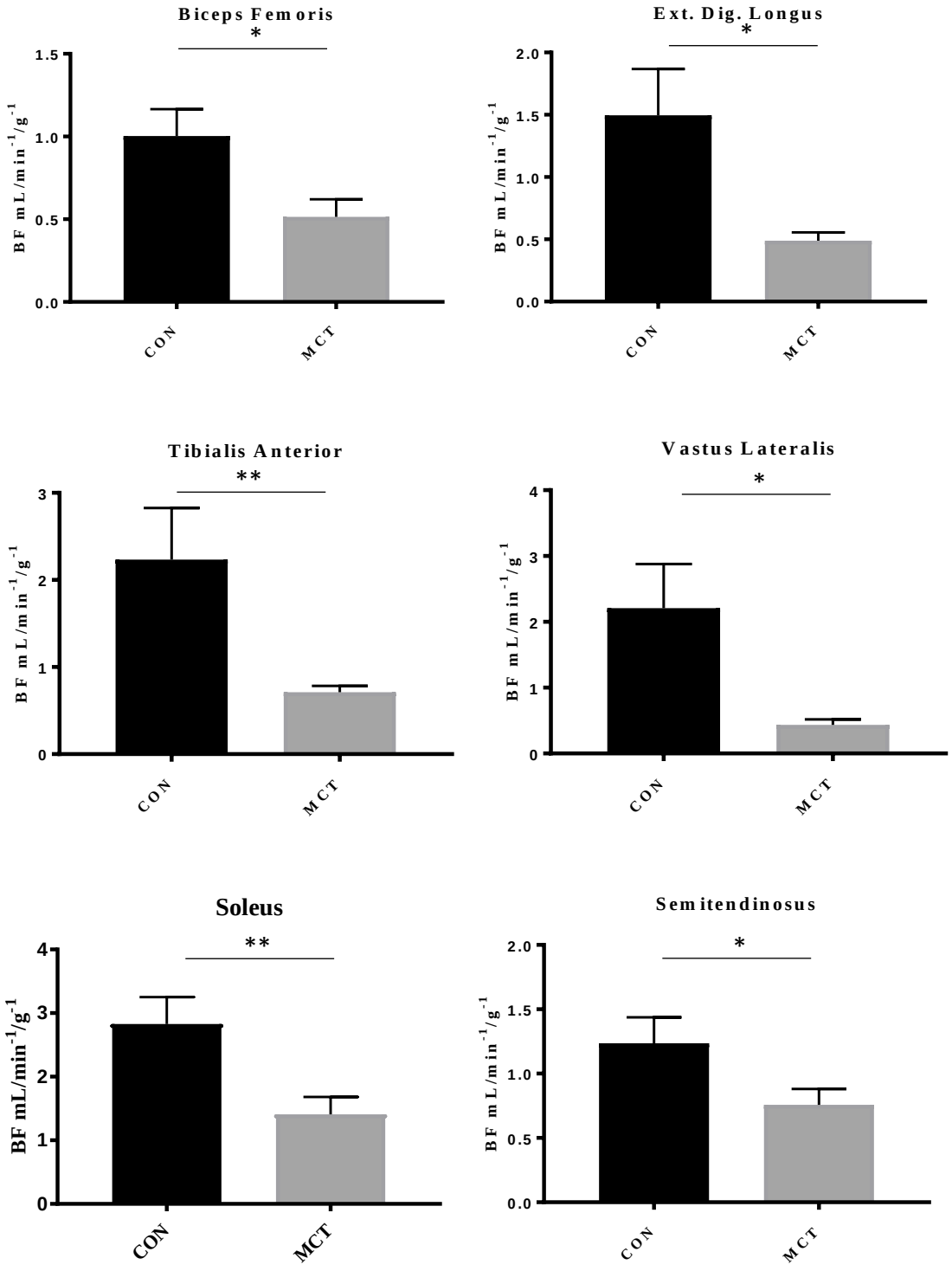
**Exercising Flow**

Table 4.3 compares blood flow during moderate intensity exercise in muscle tissue between CON and MCT rats. Blood flow in all muscles was significantly lower in MCT vs. CON (Figure 4.4), other than diaphragm, which tended to be increased in MCT vs. CON (p=0.074).

| Muscle | Exercising Blood Flow (ml/min$^{-1}$/g$^{-1}$) CON (n=16-17) | MCT (n=12-16) | p-value |
|---|---|---|---|
| ***Gastrocnemius*** | ***2.084 ± 0.33*** | ***1.105 ± 0.21**** | ***0.015*** |
| ***Rectus femoris*** | ***4.013 ± 0.94*** | ***1.354 ± 0.26**** | ***0.009*** |
| ***Biceps femoris*** | ***1.003 ± 0.16*** | ***0.515 ± 0.11**** | ***0.016*** |
| ***EDL*** | ***1.495 ± 0.37*** | ***0.4888 ± 0.06**** | ***0.012*** |
| ***Tibialis anterior*** | ***2.235 ± 0.59*** | ***0.7105 ± 0.073**** | ***0.007*** |
| ***Vastus lateralis*** | ***2.208 ± 0.67*** | ***0.4336 ± 0.085**** | ***0.014*** |
| ***Soleus*** | ***2.828 ± 0.42*** | ***1.409 ± 0.27**** | ***0.007*** |
| ***Semitendinosus*** | ***1.235 ± 0.20*** | ***0.7569 ± 0.12**** | ***0.047*** |
| Diaphragm | 1.597 ± 0.20 | 2.248 ± 0.26 | 0.074 |

**Table 4.3:** Exercising muscle blood flow




Biceps Femoris
*
BF mL/min-1/g-1
1.5
1.0
0.5
0.0
CON
MCT
Ext. Dig. Longus
*
BF mL/min-1/g-1
2.0
1.5
1.0
0.5
0.0
CON
MCT
Tibialis Anterior
**
BF mL/min-1/g-1
3
2
1
0
CON
MCT
Vastus Lateralis
*
BF mL/min-1/g-1
4
3
2
1
0
CON
MCT
Soleus
**
BF mL/min-1/g-1
4
3
2
1
0
CON
MCT
Semitendinosus
*
BF mL/min-1/g-1
2.0
1.5
1.0
0.5
0.0
CON
MCT

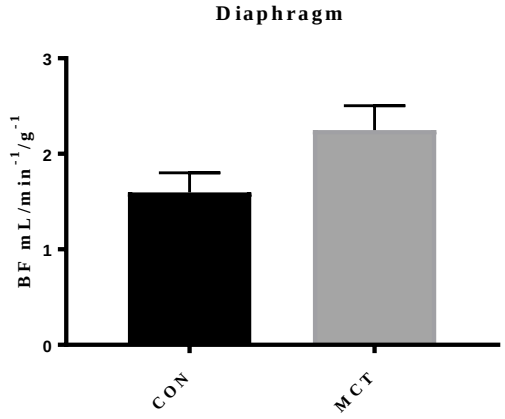


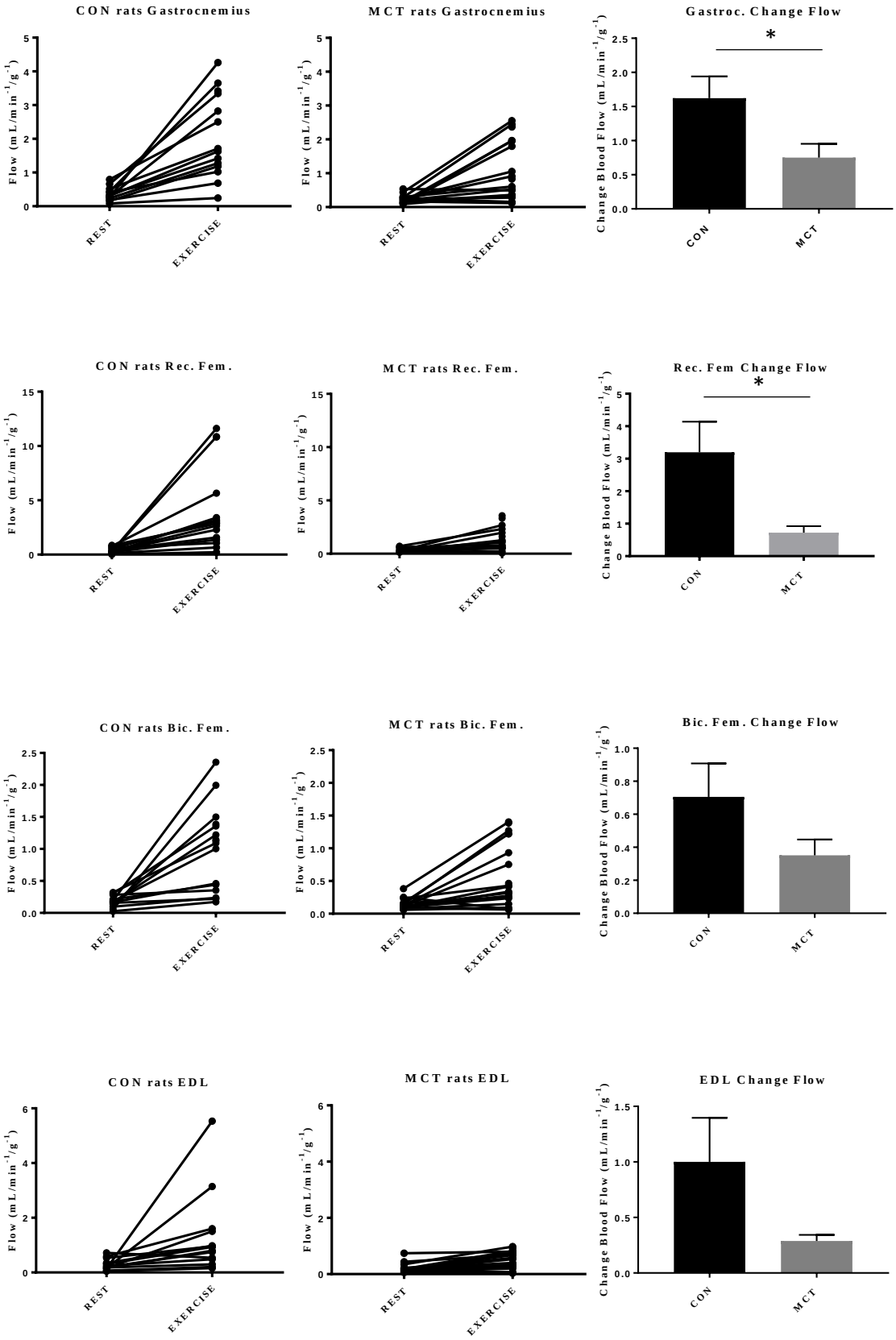

**Figure 4.4:** Exercising blood flow in all muscles

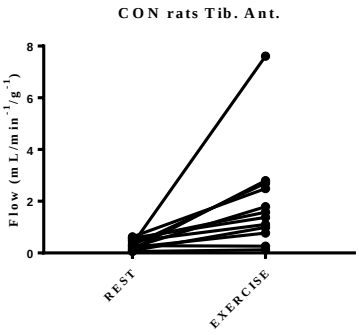
**Change in Blood Flow from Rest to Exercise**

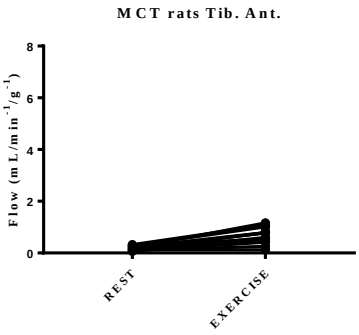
Table 4.4 compares change in blood flow from rest to exercise in MCT and CON rats. Blood flow from rest to exercise conditions was significantly greater in CON rats in the gastrocnemius, rectus femoris, tibialis anterior, vastus lateralis and soleus muscle groups, with a tendency for greater change in biceps femoris (p=0.09) and EDL (p=0.053). Figure 4.5 highlights how blood flow changes from rest to exercise for MCT and CON rats In this figure, absolute blood flow at rest and exercise (where each line represents one rat) is depicted, and to the right the bar graph represents group mean±SE of absolute change in blood flow from rest to exercise calculated for each rat.

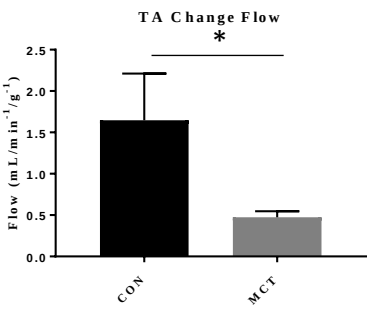
| Muscle | Change in Blood Flow ($ml/min^{-1}/g^{-1}$) CON (n=12-18) | MCT (n=11-14) | p-value |
|---|---|---|---|
| ***Gastrocnemius*** | ***1.62 ± 0.32*** | ***0.750 ± 0.20**** | ***0.0230*** |
| ***Rectus femoris*** | ***3.19 ± 0.94*** | ***0.723 ± 0.19**** | ***0.0104*** |
| Biceps femoris | 0.706 ± 0.20 | 0.351 ± 0.096 | 0.0948 |
| EDL | 1 ± 0.396 | 0.288 ± 0.056 | 0.0530 |
| ***Tibialis anterior*** | ***1.65 ± 0.57*** | ***0.47 ± 0.074**** | ***0.0363*** |
| ***Vastus lateralis*** | ***1.56 ± 0.51*** | ***0.132 ± 0.053**** | ***0.0178*** |
| ***Soleus*** | ***1.79 ± 0.47*** | ***0.454 ± 0.29**** | ***0.0194*** |
| Semitendinosus | 0.893 ± 0.21 | 0.498 ± 0.13 | 0.1115 |
| Diaphragm | 0.40 ± 0.164 | 0.175 ± 0.25 | 0.5061 |

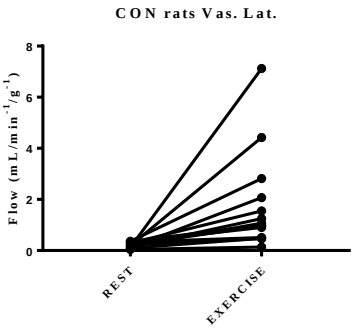
**Table 4.4:** Change in blood flow from rest to exercise

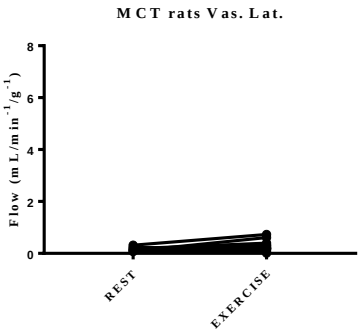

CON rats Gastrocnemius
MCT rats Gastrocnemius
Gastroc. Change Flow
CON rats Rec. Fem.
MCT rats Rec. Fem.
Rec. Fem Change Flow
CON rats Bic. Fem.
MCT rats Bic. Fem.
Bic. Fem. Change Flow
CON rats EDL
MCT rats EDL
EDL Change Flow
Flow (mL/min-1/g-1)
Change Blood Flow (mL/min-1/g-1)
REST
EXERCISE
CON
MCT
*

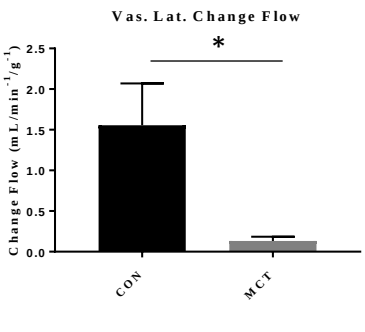

CON rats Tib. Ant.
Flow (mL/min-1/g-1)
REST
EXERCISE

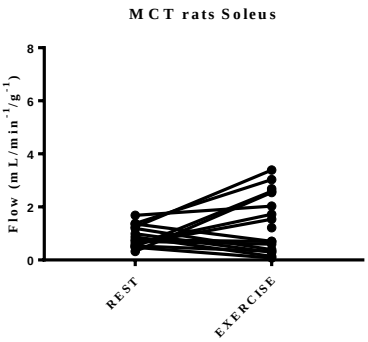

MCT rats Tib. Ant.
Flow (mL/min-1/g-1)
REST
EXERCISE

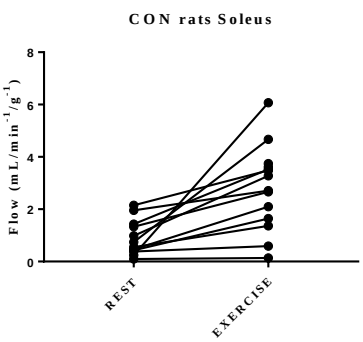

TA Change Flow
*
Flow (mL/min-1/g-1)
CON
MCT

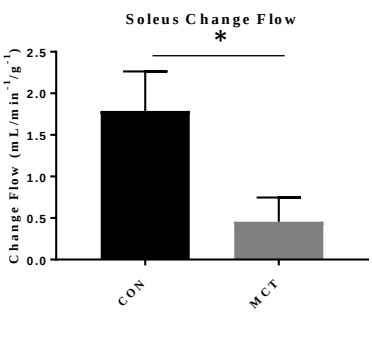

CON rats Vas. Lat.
Flow (mL/min-1/g-1)
REST
EXERCISE

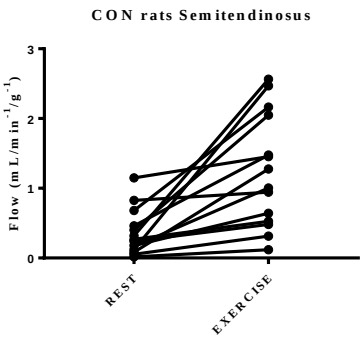

MCT rats Vas. Lat.
Flow (mL/min-1/g-1)
REST
EXERCISE

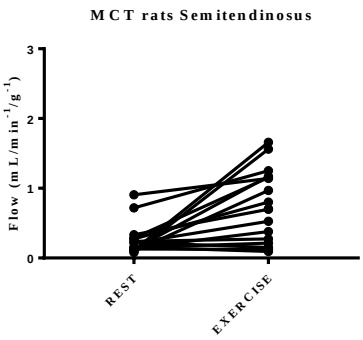

Vas. Lat. Change Flow
*
Change Flow (mL/min-1/g-1)
CON
MCT

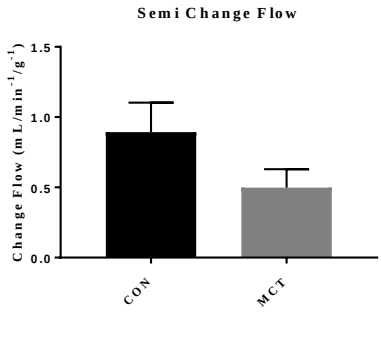

MCT rats Soleus
Flow (mL/min-1/g-1)
REST
EXERCISE


CON rats Soleus
Flow (mL/min-1/g-1)
REST
EXERCISE


Soleus Change Flow
*
Change Flow (mL/min-1/g-1)
CON
MCT


CON rats Semitendinosus
Flow (mL/min-1/g-1)
REST
EXERCISE


MCT rats Semitendinosus
Flow (mL/min-1/g-1)
REST
EXERCISE


Semi Change Flow
Change Flow (mL/min-1/g-1)
CON
MCT

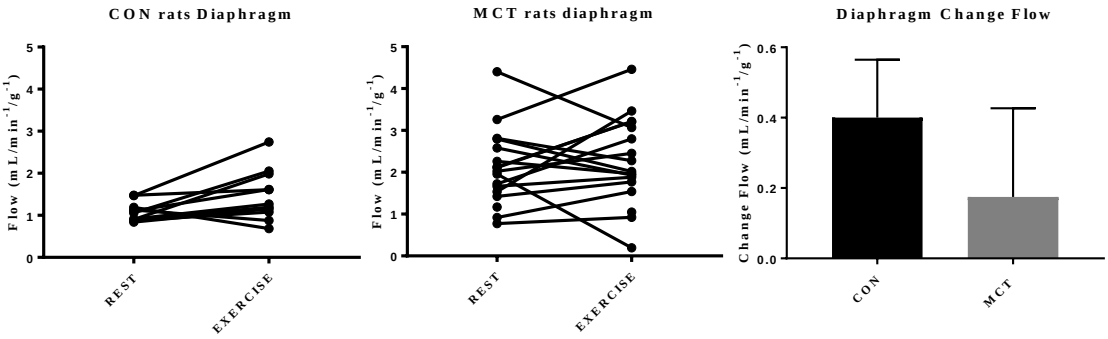



**Figure 4.5:** Change in blood flow from rest to exercise

### Complied Blood Flow

In addition to presenting data for individual skeletal muscle groups, it is helpful to look at compiled skeletal muscle blood flow values as well. Compiled flow was calculated as an average of blood flow across all skeletal muscle groups for each animal, with one value representing a 'global skeletal muscle blood flow' (Ferguson et al., 2016). This was done for both the resting and exercise condition for each animal. Using the compiled skeletal muscle blood flow value, blood flow at rest was not different at rest between MCT and CON rats (0.33 ± 0.037 vs. 0.42 ± 0.051 mL/min$^{-1}$/g$^{-1}$, p=0.18) but was significantly reduced during exercise in MCT (0.86 ± 0.13 vs. 2.28 ± 0.45, p=0.004, Figure 4.6). When expressed as percent change from rest to exercise, compiled skeletal muscle blood flow tended to be reduced in MCT rats vs CON (165.1 ± 45.39 vs. 590.9 ± 232.2, p=0.07). When expressed as absolute change from rest to exercise (Figure 4.7), compiled skeletal muscle blood flow was significantly attenuated in MCT compared to CON (0.498 ± 0.12 vs. 1.67 ± 0.44, p=0.008).

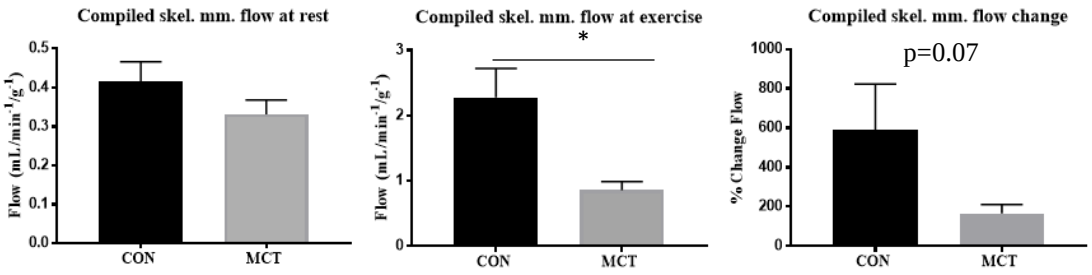



**Figure 4.6:** Compiled blood flow from rest to exercise with % change

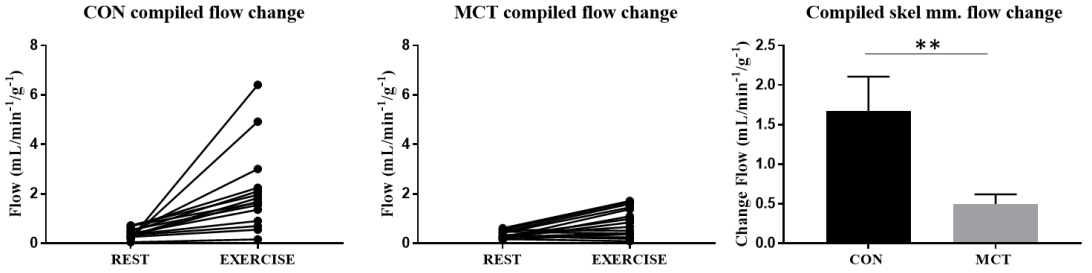



**Figure 4.7:** Compiled blood flow from rest to exercise with absolute change

### Resting and Exercise Metabolism

Whole blood lactate was significantly increased both at rest (5.424mmol/L ± 1.03 vs. 2.04mmol/L ± 0.15, p=0.007) and during exercise (11.47mmol/L ± 0.89 vs. 4.92mmol/L ± 0.55, p=<0.001) for MCT vs. CON (Figure 4.8). Change in lactate from rest to exercise (Figure 4.9) was significantly increased in MCT compared to CON (6.59mmol/L ± 0.77, n=21 vs. 2.88mmol/L ± 0.54, n=16, p=<0.001).

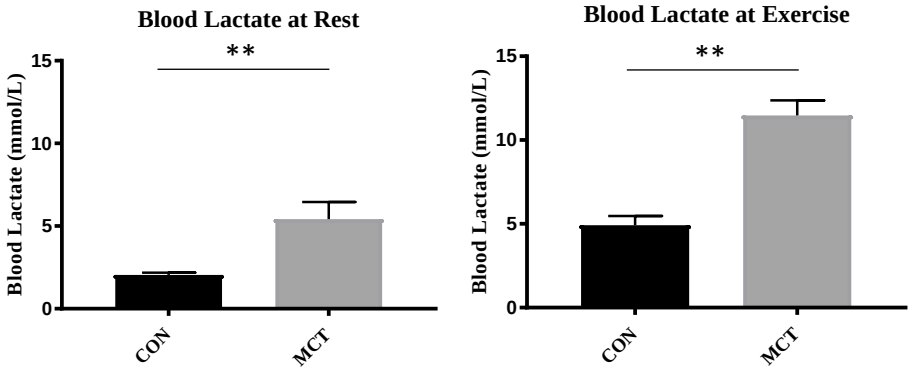



**Figure 4.8:** Blood lactate at rest and exercise

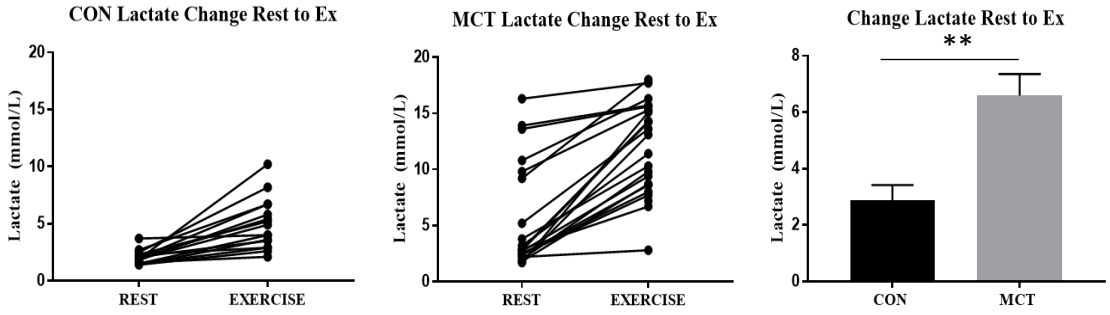



**Figure 4.9:** Change in blood lactate from rest to exercise

### Associations Between Blood Flow and Other Measures

Compiled skeletal muscle blood flow, as described previously, was used to represent global limb muscle blood flow in MCT and CON rats, and used in a correlation analysis to explore the relationships between blood flow and other variables of interest, including exercising metabolism and classic PAH disease measures.

### Blood Flow and Metabolism

Blood flow was not related to whole blood lactate at rest (r=-0.2006, p=.31) but was significantly associated during exercise for all rats (r=-0.5879, p=0.001), and this relationship was strengthened in a sub-group analysis of MCT rats only (r=-0.672, p=0.003, Figure 4.10)

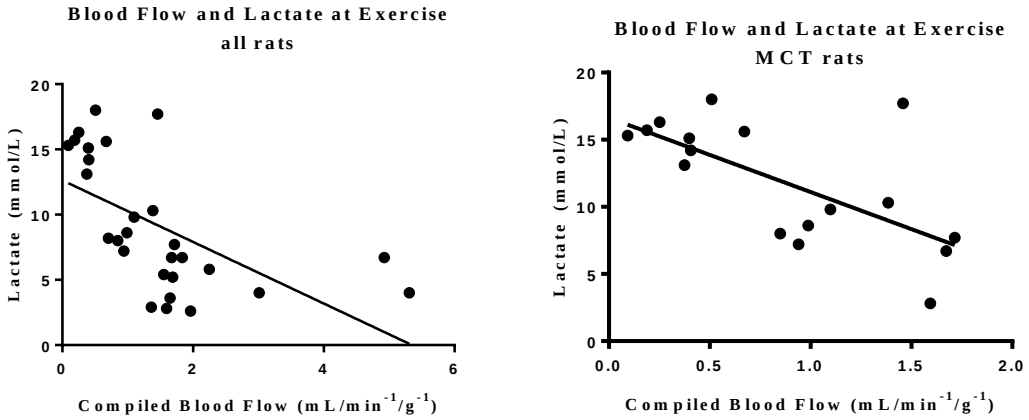



**Figure 4.10:** Blood flow and exercising lactate

### Blood Flow and Disease Measures

Compiled blood flow at rest was not related to disease measures and cardiac function in either MCT or CON. Importantly, compiled blood flow during exercise *was* significantly related to many disease indicators (Figure 4.11) including Fulton Index (r=-

0.42, p=0.03), RV weight (r=-0.47, p=0.01), resting cardiac output (r=0.47, p=0.02) and cardiac index (r=0.46, p=0.035). There were no significant associations detected for compiled blood flow and stroke volume and RV thickness as measured by echocardiography, however in each instance a tendency toward significance was reported (p=0.08). In a separate analysis considering only MCT rats, there were no significant associations between compiled exercising skeletal muscle blood flow and the aforementioned disease measures.

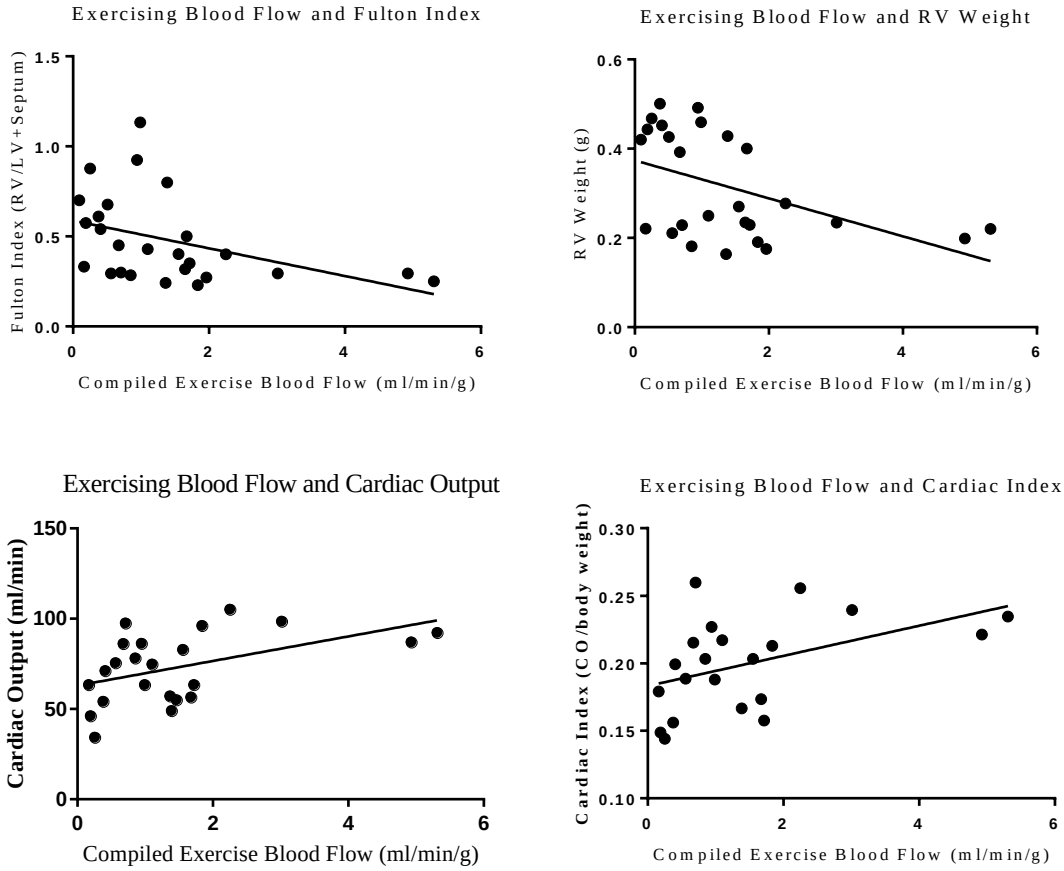



**Figure 4.11:** Exercising blood flow and disease measures

**Resting and Exercise Skeletal Muscle Blood Flow Examined for Just PL Rats**

The analysis above focused on comparing blood flow between MCT and CON and utilized all rats regardless of BRJ or PL dosing in the terminal experiment. While it will be shown that BRJ had limited effect on blood flow (Aim 2), for completeness, the following analysis will look at blood flow in only those animals that received PL. As a result, rat numbers are reduced from 12-16 to 6-8 per group, likely under powering statistical analysis and limiting the ability to detect differences between MCT and CON. For example, when examined for resting blood flow, there were no significant differences between MCT and CON, other than for diaphragm, which was significantly higher in MCT (Table 4.5). When examined during exercise (Table 4.6), blood flow for MCT vs. CON was only significantly lower in the EDL (p=0.043), and tended to be less in rectus femoris (p=0.089), biceps femoris (p=0.055), tibialis anterior (p=0.07) and semitendinosus (p=0.092) for MCT vs. CON.

| Muscle | Resting Blood Flow ($ml/min^{-1}/g^{-1}$) CON+PL (n=6-8) | PAH+PL (n=6-8) | p-value |
|---|---|---|---|
| Gastrocnemius | 0.29 ± 0.06 | 0.25 ± 0.056 | 0.67 |
| Rectus Femoris | 0.38 ± 0.093 | 0.39 ± 0.09 | *0.96* |
| Biceps Femoris | 0.15 ± 0.04 | 0.084 ± 0.01 | 0.12 |
| EDL | 0.25 ± 0.068 | 0.27 ± 0.09 | 0.87 |
| Tibialis anterior | 0.29 ± 0.06 | 0.18 ± 0.04 | *0.13* |
| Vastus lateralis | 0.17 ± 0.03 | 0.14 ± 0.02 | *0.55* |
| Soleus | 0.91 ± 0.37 | 0.88 ± 0.16 | *0.93* |
| Semitendinosus | 0.37 ± 0.15 | 0.16 ± 0.02 | 0.17 |
| ***Diaphragm*** | ***1.12 ± 0.099*** | ***2.11 ± 0.22**** | ***0.002*** |

**Table 4.5:** Resting blood flow in PL rats

| Muscle | Exercising Blood Flow (ml/min$^{-1}$/g$^{-1}$) CON+PL(n=5-8) | PAH+PL (n=7-8) | p-value |
|---|---|---|---|
| Gastrocnemius | 2.83 ± 1.03 | 0.94 ± 0.31 | 0.10 |
| Rectus Femoris | 4.28 ± 1.56 | 1.34 ± 0.41 | 0.089 |
| Biceps Femoris | 1.12 ± 0.29 | 0.46 ± 0.13 | 0.055 |
| EDL | ***1.94 ± 0.61*** | ***0.44 ± 0.12*** | ***0.043*** |
| Tibialis anterior | 2.38 ± 0.95 | 0.64 ± 0.13 | 0.07 |
| Vastus lateralis | 2.06 ± 0.86 | 0.51 ± 0.15 | 0.1 |
| Soleus | 2.80 ± 0.72 | 1.41 ± 0.48 | 0.12 |
| Semitendinosus | 1.6 ± 0.52 | 0.6 ± 0.2 | 0.092 |
| Diaphragm | 1.58 ± 0.2 | 2.34 ± 0.48 | 0.26 |

**Table 4.6:** Exercising blood flow in PL rats

**Skeletal Muscle Capillarization**

To further characterize skeletal muscle in PAH, capillarization was analyzed in a type I (soleus) and type II (EDL) muscles. An antibody cocktail of Wheat Germ Agglutinin, lectin and DAPI stained muscle cross sections green (cell membrane), red (vasculature) and blue (nuclei) respectively. Resulting yellow structures are identified as capillaries, and are expressed as capillaries per myocyte in each field (Figure 4.12). Scale bars on each image indicate a length of 100um. There were no significant differences in capillarization in either EDL (p=0.12) or soleus (p=0.85) muscles between MCT and CON rats (Table 4.7)

| Muscle | CON (capillaries per myocyte) | MCT (capillaries per myocyte) |
|---|---|---|
| EDL | 0.96 ± 0.03 | 1.1 ± 0.05 |
| Soleus | 1.42 ± 0.12 | 1.45 ± 0.06 |

**Table 4.7:** Capillaries per myocyte in EDL and soleus

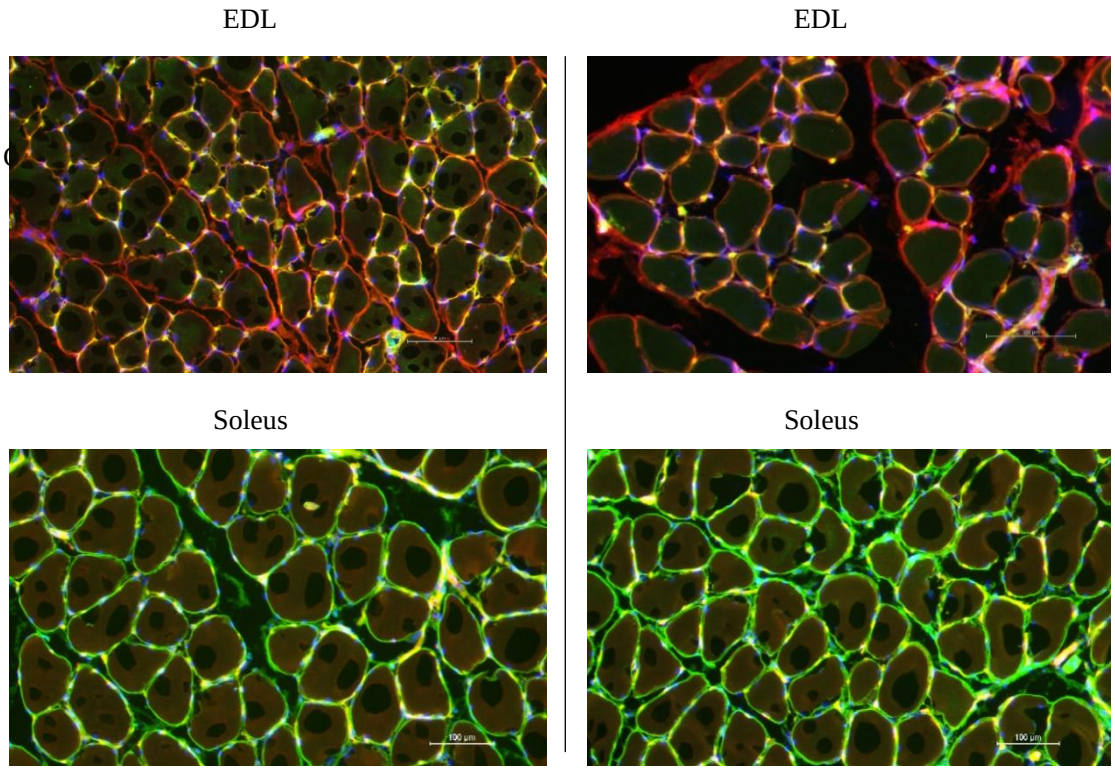



**Figure 4.12:** Capillaries (yellow), myocyte membrane (green), vasculature (red), nuclei (blue) in muscle sections

## Aim 2

### Plasma Nitrate and Nitrite after BRJ and PL Supplementation

In order to determine the effect of dietary nitrate in improving blood flow during exercise, it was important to test the efficacy of a single dose of BRJ in increasing bioavailability of nitrate and nitrite in vivo. If NO is to be upregulated via the $NO_3^-$-$NO_2^-$-NO pathway as outlined, an increase in nitrate and nitrite levels after supplementation would be an important finding. Plasma samples were analyzed for $NO_3^-$ and $NO_2^-$ via High Performance Liquid Chromatography (HPLC), and integrated against known nitrate/nitrite standards, providing reliable measures of each. The following analysis will first consider the effect of BRJ and PL on plasma $NO_3^-$/$NO_2^-$, before moving to measurement in the tissue of interest (skeletal muscle). As results are expressed for BRJ/PL across MCT and CON rats, it is also possible to interrogate any potential effect of MCT on plasma/muscle levels of nitrate and nitrite post gavage. Figure 4.13 shows plasma nitrate and nitrite levels after BRJ and PL supplementation in each group. Plasma nitrate was significantly elevated in rats that received BRJ when compared to rats that received PL for CON (555.1 ± 56.28umol/L, n=10 vs. 27.62umol/L ± 3.68, n=9, p=<0.001) and MCT (756.4 ± 118.3umol/L , n=9 vs. 63.36umol/L ± 22.46, p=<0.001). The effect that the BRJ dosing had on elevating plasma nitrate was not different between MCT and CON rats (p=.13). Similarly, plasma nitrite was significantly elevated in rats that received BRJ when compared to rats that received PL for CON (0.81 ± 0.12umol/L, n=11 vs. 0.33 ± 0.039umol/L, n=8) and MCT (0.63 ± 0.079umol/L, n=9 vs. 0.24 ± 0.045umol/L, n=6), and there was no difference in the plasma nitrite-elevating effect of BRJ when compared between CON and MCT groups (p=0.25).

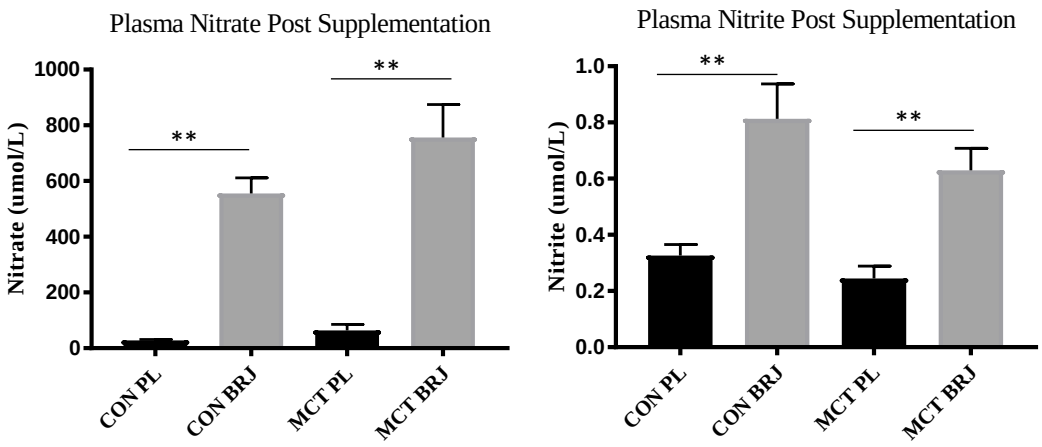



**Figure 4.13:** Plasma nitrate and nitrite after BRJ or PL supplementation

### Skeletal Muscle Nitrate and Nitrite after BRJ and PL Supplementation

#### Soleus

Previous studies have looked at plasma nitrate and nitrite as a means of determining supplementation effectiveness, however, interrogating bioavailability at the skeletal muscle level is an essential step, made possible by recent advances in measurement of each in muscle tissue. Briefly, frozen muscle tissue was pulverized and combined with methanol, 0.5% triton X-100 and oxypurinol before centrifugation. Supernatant was then injected in the HPLC machine and $NO_3^-/NO_2^-$ measured. An increase in muscle nitrate and nitrite after acute BRJ supplementation would allow for a tissue-specific interrogation of dose effectiveness, as well as providing new data on nitrate/nitrite storage and activity in a type I (soleus) and type II (vastus lateralis) muscles. Figure 4.14 shows soleus nitrate and nitrite levels after BRJ or PL supplementation in each group. Soleus nitrate was significantly higher in rats that received BRJ vs. PL in CON (336.3pmol/mg ± 73.27, n=6 vs. 159pmol/mg ± 27.35, n=6,

p=0.046) and MCT rats (282.8pmol/mg ± 31.95, n=5 vs. 121pmol/mg ± 24.07, n=4, p=0.006). Soleus nitrite was not different across groups (CON, p=0.88 and MCT, p=.13). There was also no difference in the effect of BRJ on soleus nitrate (p=0.55) or nitrite (p=0.39) levels between CON and MCT.

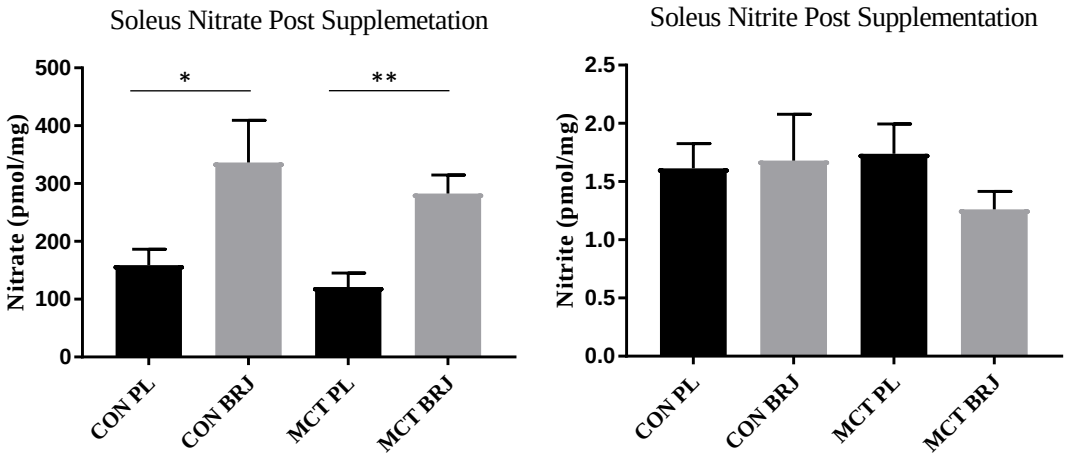



**Figure 4.14:** Soleus nitrate and nitrite after BRJ or PL supplementation

### Vastus Lateralis

Figure 4.15 shows vastus lateralis nitrate and nitrite levels after BRJ or PL supplementation in each group. Vastus lateralis nitrate was significantly higher in rats that received BRJ vs. PL in CON (165.7pmol/mg ± 24.19, n=9 vs. 159pmol/mg ± 79.08 ± 15.72, n=10, p=0.07) and MCT rats (173.6pmol/mg ± 39.45, n=7 vs. 79.38pmol/mg ± 13.57, n=9 p=0.025). However, similar to what was observed for the soleus, nitrite was not different across groups (CON, p=0.26 and MCT, p=.66), and there was also no difference in the effect of BRJ on vastus lateralis nitrate (p=0.86) or nitrite (p=0.51) levels between CON and MCT.

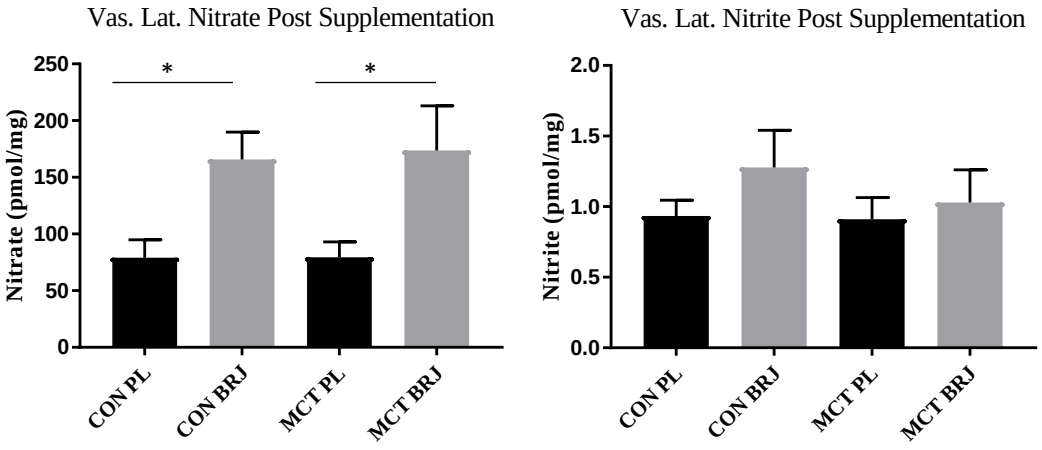



**Figure 4.15:** Vastus lateralis nitrate and nitrite after BRJ or PL supplementation

### Dietary Nitrate Supplementation and Blood Flow

In CON rats at rest, there were no significant differences in muscle blood flow for rats that received BRJ vs. PL (Table 4.8), however, blood flow tended to be greater in the gastrocnemius (p=0.09) and vastus lateralis (p=0.052) for rats that received BRJ vs. PL. There were no significant differences in exercising blood flow in CON rats, when rats that received PL are compared to rats that received BRJ (Table 4.9).

| Muscle | Resting Blood Flow (ml/min/g) CON+PL (n=6-8) | CON+BRJ (n=6-8) | p-value |
|---|---|---|---|
| Gastrocnemius | 0.29 ± 0.06 | 0.57 ± 0.15 | 0.09 |
| Rectus femoris | 0.38 ± 0.09 | 0.69 ± 0.27 | 0.27 |
| Biceps femoris | 0.15 ± 0.04 | 0.27 ± 0.07 | 0.16 |
| EDL | 0.25 ± 0.07 | 0.46 ± 0.18 | 0.32 |
| Tibialis anterior | 0.29 ± 0.06 | 0.42 ± 0.08 | 0.26 |
| Vastus lateralis | 0.17 ± 0.03 | 0.35 ± 0.08 | 0.052 |
| Soleus | 0.91 ± 0.37 | 0.89 ± 0.18 | 0.95 |
| Semitendinosus | 0.37 ± 0.15 | 0.35 ± 0.06 | 0.87 |
| Diaphragm | 1.11 ± 0.09 | 1.11 ± 0.09 | 0.93 |

**Table 4.8:** Resting blood flow in CON rats with either BRJ or PL supplementation

| Muscle | Exercising Blood Flow (ml/min/g) CON+PL (n=5-8) | CON+BRJ (n=7-8) | p-value |
|---|---|---|---|
| Gastrocnemius | 2.83 ± 1.03 | 2.26 ± 0.44 | 0.64 |
| Rectus femoris | 4.28 ± 1.56 | 3.75 ± 1.15 | 0.79 |
| Biceps femoris | 1.12 ± 0.29 | 0.89 ± 0.17 | 0.50 |
| EDL | 1.94 ± 0.61 | 1.05 ± 0.40 | 0.24 |
| Tibialis anterior | 2.38 ± 0.95 | 1.87 ± 0.66 | 0.67 |
| Vastus lateralis | 2.06 ± 0.86 | 2.36 ± 1.09 | 0.83 |
| Soleus | 2.8 ± 0.72 | 2.85 ± 0.52 | 0.95 |
| Semitendinosus | 1.6 ± 0.52 | 1.31 ± 0.29 | 0.63 |
| Diaphragm | 1.58 ± 0.2 | 1.61 ± 0.33 | 0.96 |

**Table 4.9:** Exercising blood flow in CON rats with either BRJ or PL supplementation

In MCT rats at rest (Table 4.10), blood flow in the biceps femoris was significantly elevated in rats that received BRJ vs. PL, and tended to be greater in the rectus femoris (p=0.09) and semitendinosus (p=0.09). There were no significant differences in exercising blood flow in MCT rats that received BRJ vs. PL (Table 4.11).

| Muscle | Resting Blood Flow (ml/min/g) MCT+PL (n=6-9) | MCT+BRJ (n=6-9) | p-value |
|---|---|---|---|
| Gastrocnemius | 0.25 ± 0.06 | 0.20 ± 0.03 | 0.43 |
| Rectus femoris | 0.39 ± 0.09 | 0.23 ± 0.03 | 0.09 |
| ***Biceps femoris*** | ***0.08 ± 0.01*** | ***0.18 ± 0.03**** | ***0.03*** |
| EDL | 0.27 ± 0.09 | 0.15 ± 0.02 | 0.19 |
| Tibialis anterior | 0.18 ± 0.04 | 0.18 ± 0.03 | 0.93 |
| Vastus lateralis | 0.14 ± 0.02 | 0.22 ± 0.05 | 0.26 |
| Soleus | 0.88 ± 0.16 | 0.89 ± 0.15 | 0.95 |
| Semitendinosus | 0.16 ± 0.02 | 0.34 ± 0.09 | 0.09 |
| Diaphragm | 2.1 ± 0.22 | 2.07 ± 0.38 | 0.93 |

**Table 4.10:** Resting blood flow in MCT rats with either BRJ or PL supplementation

| Muscle | Exercising Blood Flow (ml/min/g) MCT+PL (n=7-8) | MCT+BRJ (n=8-9) | p-value |
|---|---|---|---|
| Gastrocnemius | 0.94 ± 0.31 | 1.25 ± 0.3 | 0.48 |
| Rectus femoris | 1.34 ± 0.41 | 1.37 ± 0.36 | 0.96 |
| Biceps femoris | 0.46 ± 0.13 | 0.57 ± 0.17 | 0.62 |
| EDL | 0.44 ± 0.12 | 0.52 ± 0.08 | 0.57 |
| Tibialis anterior | 0.64 ± 0.13 | 0.77 ± 0.08 | 0.41 |
| Vastus lateralis | 0.51 ± 0.15 | 0.35 ± 0.08 | 0.36 |
| Soleus | 1.41 ± 0.48 | 1.41 ± 0.31 | 0.99 |
| Semitendinosus | 0.6 ± 0.2 | 0.9 ± 0.15 | 0.23 |
| Diaphragm | 2.34 ± 0.48 | 2.2 ± 0.25 | 0.74 |

**Table 4.11:** Exercising blood flow in MCT rats with either BRJ or PL supplementation

### Dietary Nitrate Supplementation and Metabolism

There were no significant differences in blood lactate concentration at rest or during exercise between rats that received BRJ vs. PL in either MCT or CON rats (Figure 4.16).

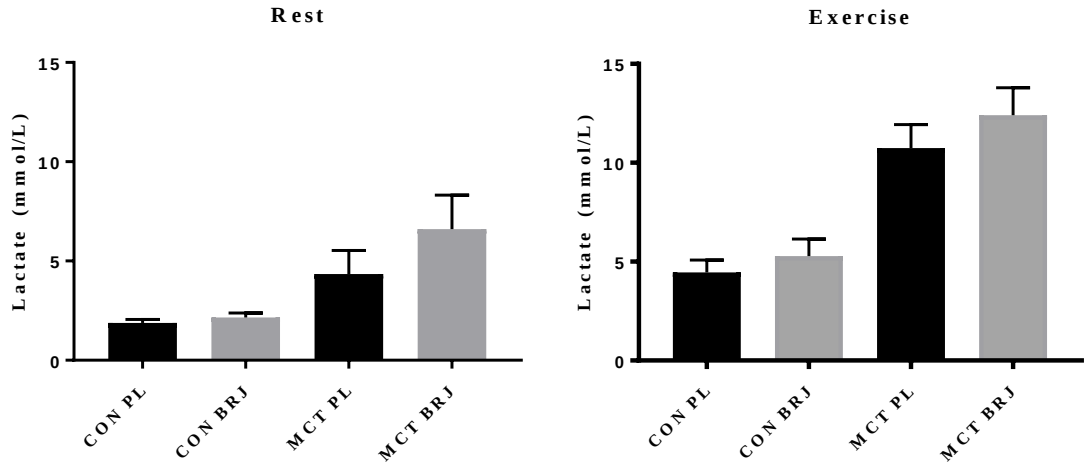



**Figure 4.16:** Blood lactate at rest and exercise after BRJ or PL supplementation

### Plasma $NO_3^-/NO_2^-$ and Blood Flow/Metabolism

While no significant correlations existed between plasma nitrate/nitrite and compiled muscle blood flow at either rest or exercise under each dosing strategy, BRJ supplemented rats demonstrated a significant negative association between plasma nitrite ($NO_2^-$) and exercising plasma lactate (r=-0.5694, p=0.017, Figure 4.17) and a tendency toward significance for resting lactate (r=-0.4179, p=0.095).

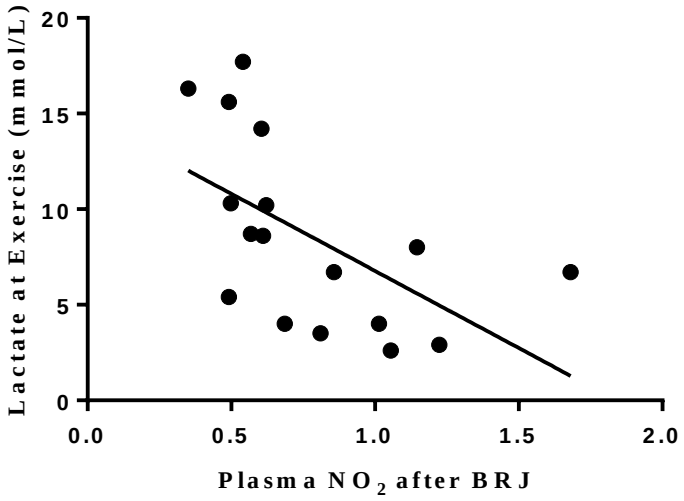



**Figure 4.17:** Plasma nitrite and exercising lactate after BRJ supplementation

### Skeletal Muscle $NO_3^-/NO_2^-$ and Blood Flow/Metabolism

Neither soleus nitrate nor nitrite were significantly associated with compiled resting or exercising skeletal muscle blood flow. Additionally, soleus nitrate and nitrite were not significantly related to blood flow specific to the soleus muscle at rest or exercise in either the BRJ or PL condition. A significant association between soleus $NO_3^-$ and lactate at exercise was present in animals supplemented with PL in the terminal

experiment (r=-.73, p=0.04), however, no further associations were reported for the soleus muscle and blood flow or metabolism. In the vastus lateralis, there were no significant associations between muscle nitrite ($NO_2^-$) and blood flow at rest or exercise (including specific to the vastus lateralis) or blood lactate in either BRJ or PL conditions. Similarly, vastus latetralis nitrate ($NO_3$) was not associated with blood flow or metabolism measures at rest or during exercise after either BRJ or PL supplementation.

**Aim 3**

In order to test the hypothesis that BRJ supplementation can improve functional exercise performance, pre and post MCT testing was carried out for maximal aerobic capacity, voluntary wheel running distance and grip strength under both BRJ and PL supplementation. Each exercise test was carried out in a counterbalanced fashion, with each rat (MCT and CON) performing under repeated trials of BRJ and PL, separated by 24hrs. Therefore, the following results are based on repeated measures statistical testing, allowing for a within-rat comparison of BRJ effectiveness in altering functional performance. Data for the BRJ condition will be graphically presented as striped bars.

**Functional Testing – $VO_2$max**

Repeated treadmill testing of VO2max was carried out pre and post injection using a graded exercise protocol to exhaustion. Running took place between 2-3 hours post supplementation, ensuring optimal timing in accordance with measured increases in plasma nitrate/nitrite (Appendix A). Post MCT induction, $VO_2$max was significantly reduced compared to CON in both PL (3190 ± 245.5 ml/kg/hr, n=14 vs. 4308 ml/kg/hr ± 110.5, n=18, p=<0.001) and BRJ (3074 ml/kg/hr ± 302.5, n=16 vs. 4162 ml/kg/hr ± 192.2, n=16, p=0.005) conditions (Figure 4.18). In both pre and post MCT/vehicle testing, repeated measures $VO_2$max was not significantly improved by BRJ supplementation in comparison to PL for either MCT or CON rats. In final testing, $VO_2$max was decreased an average of 193.7 ml/kg/hr by BRJ (-5.1%) for CON rats (n=16), and increased 139.4ml/kg/hr (3.9%) in MCT (n=11) (Figure 4.19).

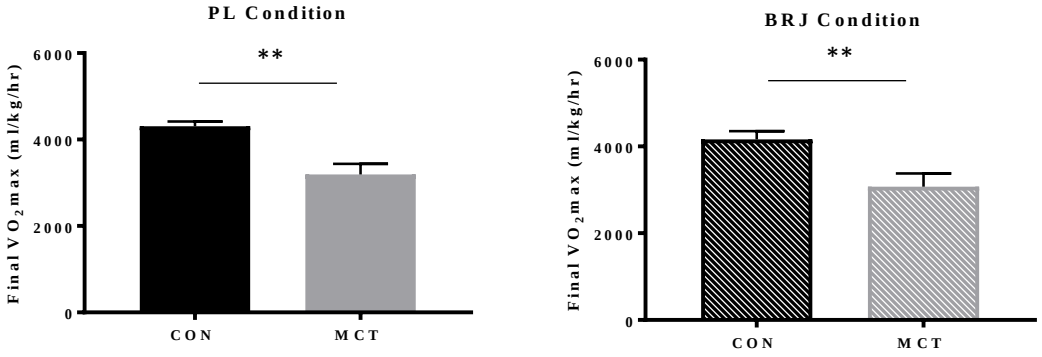



**Figure 4.18:** Final $VO_2$max under BRJ and PL conditions

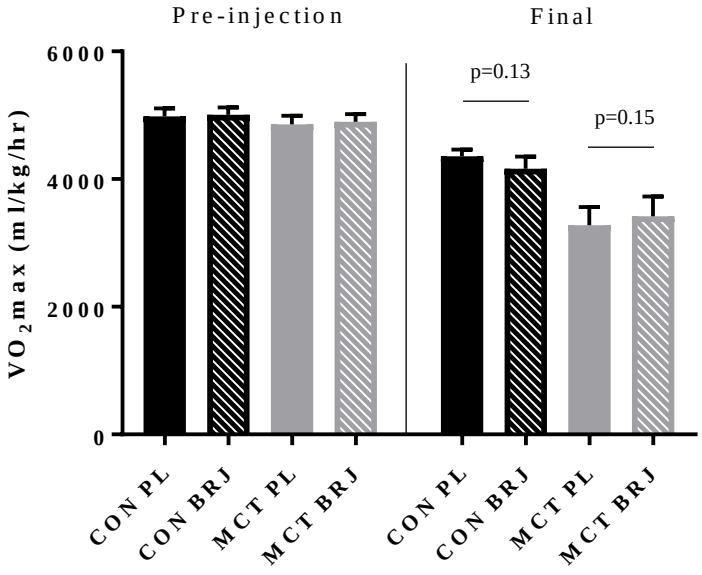



**Figure 4.19:** VO2max under BRJ and PL conditions in pre-injection and final testing

Time to exhaustion during $VO_2$max testing was significantly different between MCT and CON in final testing under both PL (n=13-18, 10.68 min ± 1.857 vs. 18.08 min ± 1.554, p=0.004) and BRJ (n=13-14, 12.07 min ± 2.052 vs. 20.27 min ± 1.341, p=0.003) conditions (Figure 4.20). In both pre-injection and final testing, time to exhaustion was not improved by BRJ supplementation in comparison to PL for either MCT or CON rats (Figure 4.21). In final testing, time to exhaustion was decreased an average of 0.58mins by BRJ for CON rats (n=13), and increased 1.9mins in MCT (n=11).

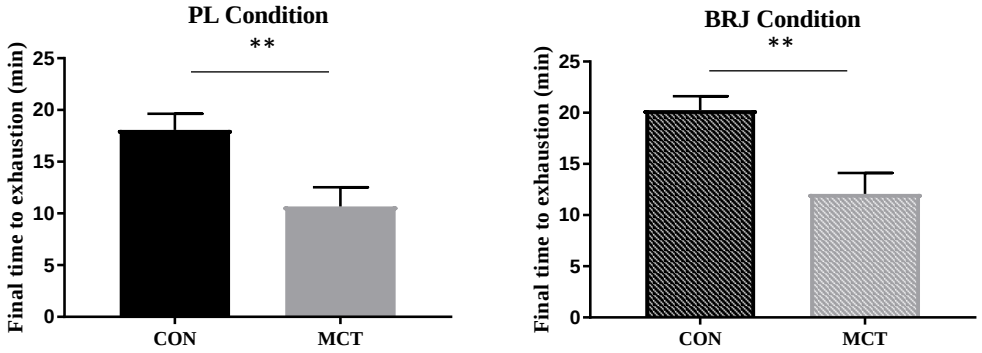



**Figure 4.20:** Final time to exhaustion under BRJ and PL conditions

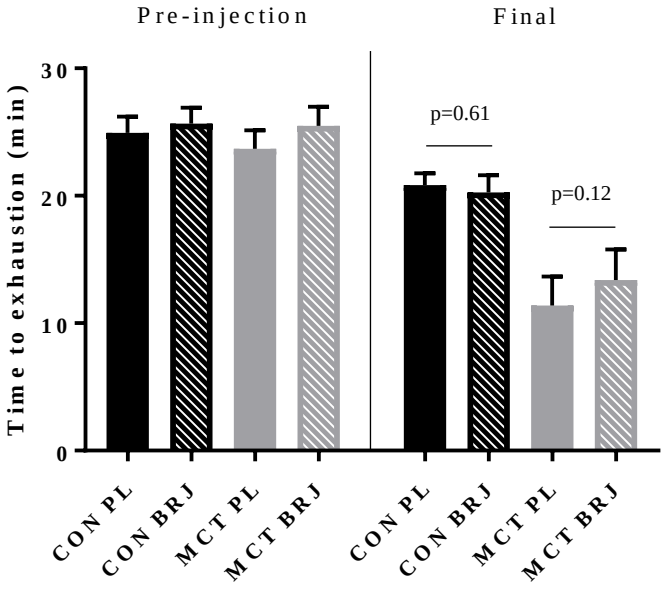



**Figure 4.21:** Time to exhaustion under BRJ and PL conditions in pre-injection and final testing

**RER at 50% $VO_2$max**

Respiratory exchange ratio (RER) considers the relative amounts of oxygen consumed and carbon dioxide exhaled, and can be used as a metabolic measure and/or indicator of substrate use during exercise. Measuring RER at 50% $VO_2$max (the same intensity of terminal blood flow experiments) may give an indication as to altered metabolism at the same relative exercise intensity under either BRJ or PL conditions in a within-animal design. In final testing, RER at moderate exercise intensity (50% $VO_2$max) was not significantly different between the BRJ and PL conditions in either CON (n=13, p=0.41) or MCT (n=7, p=0.17) (Figure 4.22).

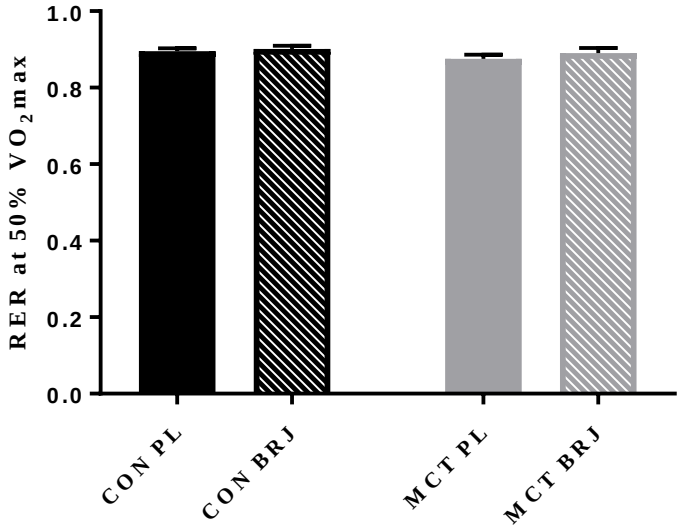



**Figure 4.22:** RER at 50% VO2max

**Functional Testing – Grip Strength**

Repeated grip strength tests were carried out 24hr apart in both pre-injection and final testing using the specialized rodent dynamometer in a counterbalanced design. Grip tests were completed 2-3hrs post gavage to ensure optimal supplement timing, with an average of three trials calculated. In final testing, grip strength was not different between MCT (n=13-14) and CON (n=17) under PL or BRJ conditions (Figure 4.23).

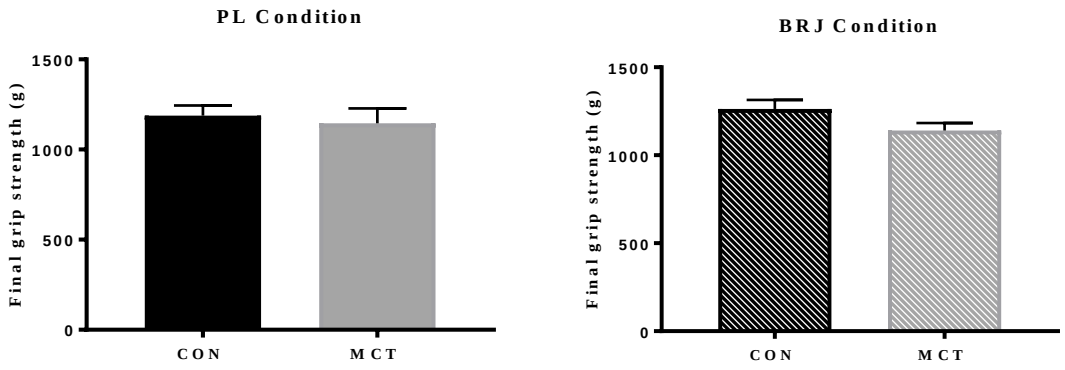



**Figure 4.23:** Final grip strength under BRJ and PL conditions

In both pre-injection and final testing, grip strength was not improved by BRJ supplementation in comparison to PL for MCT, but tended to be increased for CON rats ($p=0.07$, Figure 4.24). In final testing, grip strength was increased an average of 128.6g (14.19%) by BRJ for CON rats (n=15), and decreased 110.2g (-4.6%) in MCT (n=12).

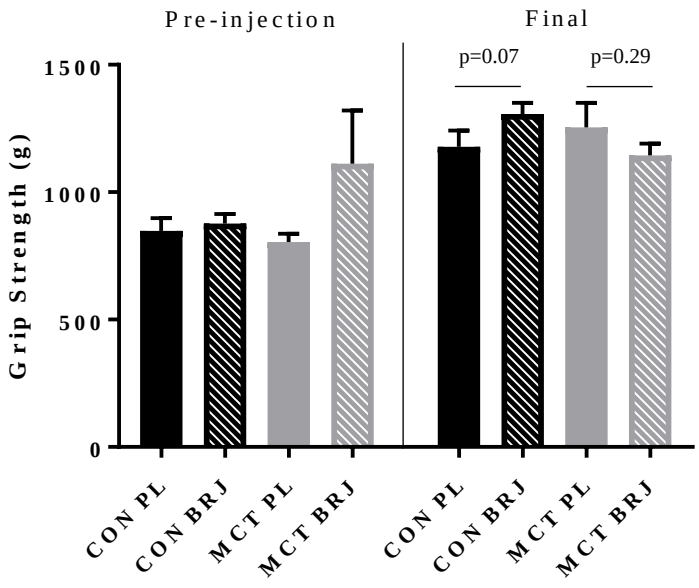



**Figure 4.24:** Grip strength under BRJ and PL conditions in pre-injection and final testing

### Functional Testing – Voluntary Wheel Running

Voluntary 12-hour wheel running was carried out pre and post injection under BRJ and PL conditions for each rat in a counterbalanced fashion. As the vast majority of running takes place in the dark hours, supplementation of BRJ and PL was timed 2-3 hours prior to the initiation of darkness, ensuring consistency in dosing and subsequent exercise measurement. In pre-injection testing, there were no differences in running distance between groups under either the BRJ (1230 m ± 300.1, n=16 vs. 1245 m ± 144.4, n=15, p=0.97) or PL conditions (989 m ± 318.4, n=16 vs. 1477 m ± 180.9, n=15, p=0.20) In final testing, voluntary wheel running distance tended to be lower in MCT vs. CON (Figure 4.25), in both the PL (p=0.08, n=12-16) and BRJ condition (p=0.12, n=11-16). In both pre-injection and final testing, voluntary wheel running distance was not

improved by BRJ supplementation in comparison to PL for either MCT or CON rats (Figure 4.26), with BRJ supplementation decreased running distance 172.5m for CON (n=15) and 145.3m for MCT (n=9).

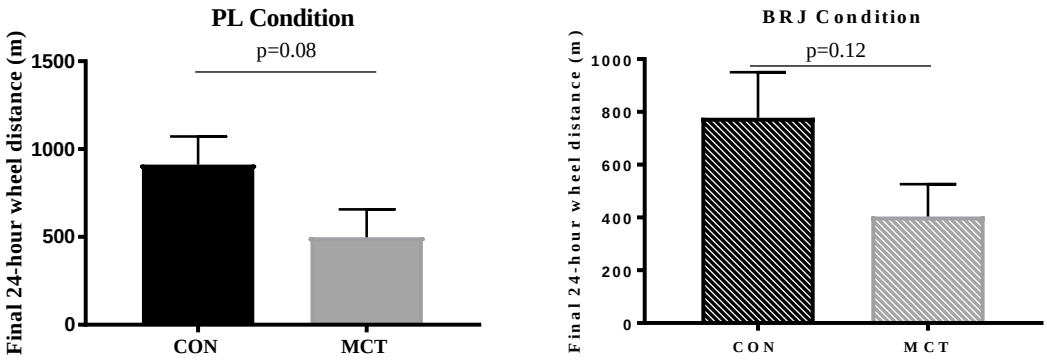



**Figure 4.25:** Final voluntary running distance under BRJ and PL conditions

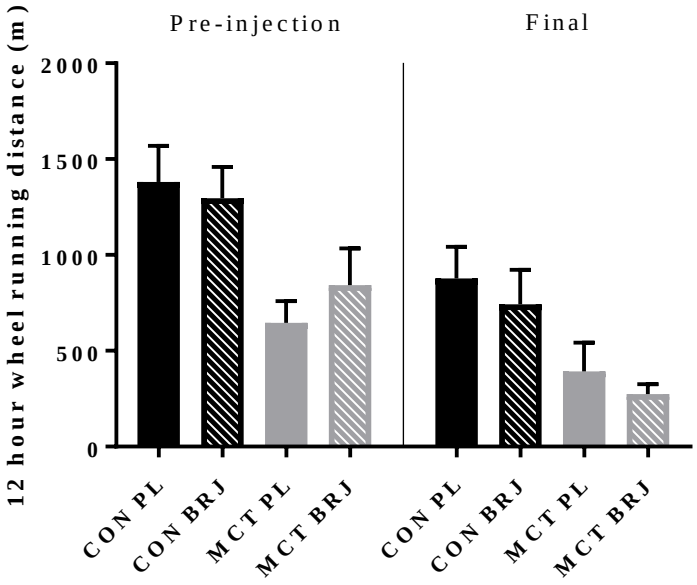



**Figure 4.26:** Voluntary running distance under BRJ and PL conditions in pre-injection and final testing.

In final testing, wheel running speed was not significantly different between MCT and CON in the PL condition (19.27 m/min ± 1.504, n=16 vs. 19 m/min ± 1.996, n=12, p=0.91) however, it tended to be *higher* in MCT after BRJ supplementation compared to CON under the same condition (21.24 m/min ± 0.8208, n=16 vs. 18.88 m/min ± 0.8087, n=11, p=0.06). In both pre-injection and final and post MCT/vehicle testing, repeated measures voluntary wheel running speed was not improved by BRJ supplementation in comparison to PL for either MCT or CON rats (Figure 4.27).

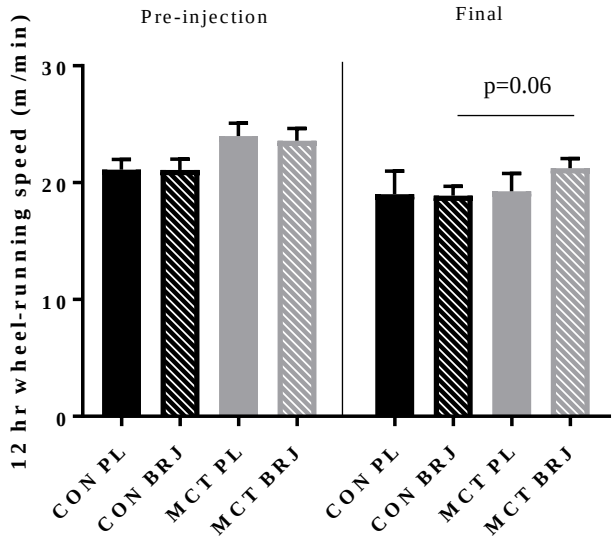



**Figure 4.27:** Voluntary running speed under BRJ and PL conditions in pre-injection and final testing

# Chapter Five: Discussion

## Summary

This study sought to characterize skeletal muscle blood flow at rest and during exercise in an experimental model of PH, with a specific emphasis on reduced blood flow as a contributor to exercise intolerance in the disease. Additionally, the use of dietary nitrate as a potential mechanism to augment blood flow and improve functional capacity was investigated. This analysis has added to the body of knowledge in PH, most notably by identifying a pathological hemodynamic response to exercise in a reliable animal model of the condition. While it is clear further mechanistic insight into this problem is warranted, a novel target to potentially improve exercise capacity in PAH has been identified. Although dietary nitrate was not an effective mediator of blood flow and exercise performance (in either MCT or CON rats), mechanistic analysis has provided a potentially novel explanation for its lack of efficacy under these conditions. The preliminary work of measuring muscle nitrate and nitrite should be complemented by further study into the handling, transport and activity of these ions in tissues of interest. Expanding this investigation to their downstream target is a logical approach to further understand the conditions under which dietary nitrate may produce an ergogenic effect at the skeletal muscle level in health and disease.

**PH Phenotype**

A single subcutaneous injection of MCT, as used in this study, has been demonstrated to produce a progressive pulmonary hypertensive phenotype (Stenmark et al., 2009). While the exact mechanisms by which this occurs is still under investigation (Gomez-Arroyo et al., 2012), it is well known that increasing doses of MCT reliably leads to more severe affliction. Importantly, the dose can be administered to 'mimic' either compensatory hypertrophy, or increased to illicit characteristics of RV failure (Handoko et al., 2009). This is important, as it has previously been described that a transition from functional to detrimental RV hypertrophy is critical in determining survival in the disease. Several studies have specifically tested an increasing MCT dose and the subsequent genetic, morphological and functional changes in the RV (Buermans et al., 2005; Handoko et al., 2009; Hessel et al., 2006), with one study specifically measuring cardiac parameters at bi-weekly intervals after MCT injection (Sutendra et al., 2013). Here, RV failure was shown via a loss of pump function, manifested by a significant reduction in cardiac output at 6 weeks post 60mg/kg MCT administration. Our results would suggest a similar phenotype, with significant reductions in stroke volume (~18%), cardiac output (~23%) and cardiac index (~14%) in MCT vs. CON animals all demonstrated. The decline in RV function presented herein mirrors that of Hessel et al. (2006), who reported a ~26% reduction in cardiac output 4 weeks after an 80mg/kg MCT dose, further supporting a severe phenotype and a progression toward heart failure in our model.

MCT rats presented with significant RV morphological changes. Direct measures (RV weight and Fulton Index) indicated a significant increase in RV hypertrophy in MCT

rats, supporting data from similar studies using a higher-dose strategy as employed here (Hessel et al., 2006; J. E. Jones et al., 2002; Schermuly et al., 2005; Schermuly et al., 2004). Additionally, echocardiographic measures demonstrated a ~28% increase in RV size in MCT rats compared to CON. These findings supports previous data that established a progressive increase in RV size post MCT injection, with a difference between treated and control rats apparent approximately 4 weeks post disease induction (Hessel et al., 2006; J. E. Jones et al., 2002; Kim, Kim, Chan, Kim, & Sohn, 2018). As noted at the outset, a direct measurement of RV pressures are required to diagnose PAH in clinical populations, with an increased pulmonary mean arterial pressure of >25mmHg considered the criterion standard for disease presence (Galie et al., 2016). An increase in RVSP has been reported in the MCT model (Gomez-Arroyo et al., 2012), and this data is supported here. A mean RVSP of 52mmHg in MCT rats is similar to that recorded by other investigators (Handoko et al., 2009; Sutendra et al., 2013), and represented a 116% increase over CON. However, pressures of >75mmHg have been reported in rats after 60mg/kg MCT dose (Benoist, Stones, Drinkhill, Bernus, & White, 2011; Klein et al., 2008), indicating that greater doses may illicit adaptations in the RV beyond those described here.

Further to the aforementioned RV maladaptations of the MCT model, an additional indication of the progression toward heart failure are the body weight alterations noted after injection. Here, we describe no significant differences at injection, with a progression toward significantly lower body weights in the MCT group at 4 weeks. This supports the work of Hessel et al. (2006) who noted a significant retardation of weight gain in high dose MCT rats compared to controls, which, importantly, may

mimic the cachexic effects of heart failure in patient populations. Finally, a reduction in exercise capacity was apparent in MCT rats. Aerobic capacity ($VO_2max$) was significantly lower ($p=<0.05$) in MCT rats compared to controls, with voluntary wheel-running distance also tending to be lower ($p=0.08$), representing a 26% and 45% decline respectively. This reduction in functional capacity supports previous work done in pulmonary hypertensive animal models (M. B. Brown et al., 2015; M. B. Brown et al., 2017; Frump et al., 2015; Lahm et al., 2016), and in patient populations (Badagliacca et al., 2017; Breda et al., 2014; Malenfant, Potus, Mainguy, et al., 2015; Potus et al., 2014; Provencher et al., 2006; Spruijt et al., 2015; Sun et al., 2001; Tolle et al., 2008), who have consistently demonstrated lower walking distances and aerobic capacity when compared to healthy controls. Taken together, the clear decrements in cardiac function, morphological adaptations in the RV, blunted weight gain and decreased exercise performance would suggest a robust pulmonary hypertensive animal model, with a severe phenotype characteristic of progression toward heart failure.

**Aim 1**

Hypothesis 1.1 – Skeletal muscle blood flow will be significantly reduced at rest and during exercise in a PAH rat model, when compared to healthy controls.

The most significant finding in this study is the reduction in skeletal muscle blood flow at rest, and more prominently during exercise, in a pulmonary hypertensive animal model. The microsphere method of blood flow analysis has also allowed us to confirm the persistence of reduced flow while accounting for blunted cardiac output in MCT rats.

To the author's knowledge, this is the first study to demonstrate such a deficiency, and adds to the understanding of skeletal muscle dysfunction and exercise intolerance in PAH. Regardless of supplementation, significant reductions in flow were demonstrated at rest in 2 of the 8 hind limb muscles analyzed (tibialis anterior and gastrocnemius). Crucially, during moderate intensity exercise, blood flow was significantly lower in each of the skeletal muscles sampled. Additionally, it was shown that the increase in muscle blood flow from rest to exercise was attenuated in 5 of the 8 skeletal muscles sampled in MCT rats (gastrocnemius, tibialis anterior, rectus femoris, vastus lateralis, soleus), providing further mechanistic insight into the blunted exercise response in PAH. In a sub-group analysis of rats supplemented with PL, exercising blood flow was significantly reduced in the EDL ($p=0.043$), with a tendency for reduced flow in the rectus femoris, biceps femoris, tiblialis anterior, and semitendinosus ($p=0.05$ to $0.92$).

Vescovo et al. (1998) highlighted a reduction in skeletal muscle blood flow at rest in a heart failure rat model, however, this discrepancy was demonstrated in the soleus muscle, contrary to our findings. Interestingly, we have shown a tendency for reduced flow at rest in the EDL ($p=0.054$), but no significant difference was seen in the Vescovo study. While this is the first investigation to directly measure exercising skeletal muscle blood flow in a pulmonary hypertensive animal model, our findings can be linked to previous work in this area. Others have shown that circulatory maladaptations at the skeletal muscle level contribute to a reduced exercise performance in PAH (Dimopoulos et al., 2013; Malenfant, Potus, Mainguy, et al., 2015; Potus et al., 2014; Tolle et al., 2008). Microcirculatory loss, reduced oxygen saturation/extraction and slower hyperemia responses have all been suggested as peripheral mechanisms restricting exercise tolerance

in PAH, and a directly-measured blood flow reduction may now be considered as a corollary limitation. It is well established that at exercise, oxygen delivery via increase blood flow is increased to meet the rising metabolic demands of skeletal muscle (Poole, Hirai, Copp, & Musch, 2012; Sarelius & Pohl, 2010). It stands to reason that a reduction in blood flow to working muscle may predicate downstream effects including reduced oxygen supply (Malenfant, Potus, Mainguy, et al., 2015) and extraction (Tolle et al., 2008). Interestingly, while central limitations are apparent during exercise in PAH, it has been argued that peripheral abnormalities such as those highlighted may be greater contributors to the early onset of fatigue present in the disease, primarily because exercise training has improved functional capacity without significant changes in central hemodynamics (L. Chan et al., 2013; Fox et al., 2011; Mereles et al., 2006).

Blood flow limitations during exercise have been characterized to a greater extent in left heart failure, and the data tends to support the findings in this study. In early work, Drexler, Faude, Hoing, and Just (1987) demonstrated a significant reduction in blood flow to the red portion of the gastrocnemius during maximal exercise in an infarct model of left heart failure. These findings were supported by later work that showed greater reductions in muscle blood flow at rest and during exercise in a similar model, with rats more severely afflicted demonstrating the most profound decrements in blood flow to exercising muscles (Musch & Terrell, 1992). Importantly, this was the first study to show that a more severe heart failure phenotype predicated a greater reduction in blood flow. These early works have since been supported by a plethora of studies investigating exercises responses in heart failure, comprehensively reviewed by Poole et al. (2012). It has been shown that central limitations in heart failure only partially explain a reduction

in blood flow to working muscles in LHF. While a reduced cardiac output may decrease the speed and magnitude of blood supply to skeletal muscle at the onset of exercise, systemic vasoconstriction, vascular stiffness, endothelial dysfunction, inflammatory markers and reduced NO bioavailability are all postulated as factors culminating in compromised exercising blood flow in the disease (Poole et al., 2012). A further exploration of these factors may be crucial in understanding the deficits shown in this study, as each of the aforementioned abnormalities have also been identified in PAH (Humbert, 2010; Price et al., 2012; Rosenblum, 2010; Toshner et al., 2010; Zuckerbraun et al., 2011).

One further mechanistic explanation for a reduction in muscle blood flow could be a reduction in capillarization in PAH. Potus et al. (2014) described significant angiogenic deficiencies in the quadriceps muscles of PAH patients, and were able to directly connect this microcirculatory loss to exercise intolerance. A downregulation of the angiogneic signaling miR-126 was apparent in PAH muscle, and correlated with a reduction in exercise capacity. Interestingly, exercise training had no effect on miR-216 expression, and as such, it is argued that physical inactivity is not sufficient to explain muscle-specific maladaptations that contribute to exercise intolerance in the disease. In line with these findings, reduced capillarization was demonstrated in the RV of an MCT rat model, secondary to downregulation of the HIF-1 pathway (Sutendra et al., 2013). This would indicate a microcirculatory myopathy that extends to both the myocardium and peripheral muscle in PAH. While reduced skeletal muscle capillarity could provide an explanation for reduced blood flow, we did not see a significant difference in capillary numbers between MCT and CON rats. Investigating capillary numbers in the EDL and

soleus allowed for potential discrepancies between primarily Type I and Type II muscle fibers (Armstrong & Phelps, 1984) , however, in each case MCT did not induce rarefaction at the myocyte level. This seems to contrast to the findings of Sutendra et al. (2013) who showed reduced capillarity *in the RV* after MCT injection, however, this study measured capillary number at 6 weeks post injection, as opposed to 4 weeks used herein. As such, it may be the case that more time is needed post-PH induction to see such changes at the skeletal muscle level. Nevertheless, this finding is important as it suggests capillary loss may not be an explanation for the observed reduction in blood flow, but may also provide insight as to how closely the MCT model might mimic skeletal muscle dysfunction in PAH. As such, mechanistic investigations aimed at explaining reduced muscle blood flow during exercise in this model should shift toward microcirculatory *function*, rather than morphology. While adequate capillary density may exist to meet the increased oxygen demand of working muscle, endothelial dysfunction as a consequence of disease may limit vasoreactivity and subsequently reduce blood flow at the muscle level. Hence, as noted previously, further investigation is this area is warranted.

We have demonstrated increased blood lactate accumulation at rest and during exercise in a PH rat model. Lactate and hydrogen ions accumulate in the blood as a result of several interrelated mechanisms, including a reliance on anaerobic glycolysis, conversion of pyruvate to lactate (primarily in Type II muscle fibers), and reduced systemic clearance via lactate oxidation. It is well established that lactate rises with increasing exercise intensity, and contributes to the onset of fatigue (McArdle, Katch, & Katch, 2015). Previous work in PAH has shown an early accumulation of lactate relative

to exercise intensity (Neder et al., 2015), indicating an ineffective switch from aerobic to anaerobic means of ATP production. Several mechanisms may explain this phenomenon in PAH, including blunted cardiac response to exercise (Holverda et al., 2006; Manders, Rain, et al., 2015; Sun et al., 2001; Waxman, 2012) and muscle adaptations that compromise oxidative metabolism (Batt et al., 2014; M. B. Brown et al., 2015; de Man et al., 2011; Mainguy et al., 2010b; Malenfant, Potus, Fournier, et al., 2015). We have added to this understanding by identifying reduced skeletal muscle blood flow as a contributor to increased lactate accumulation during exercise. It stands to reason that an increased oxygen demand that cannot be met by increased flow would result in an overreliance on anaerobic metabolism, and to the authors knowledge, this is the first evidence to directly support this phenomenon in a PH model. Indeed, the significant inverse relationship between blood flow and lactate accumulation was strengthened in the MCT rats specifically, indicating the importance of adequate blood flow in combating metabolic disturbance in the disease.

Skeletal muscle blood flow was significantly correlated with disease measures, including RV hypertrophy and cardiac output. It should be noted while exercising blood flow was associated with cardiac output measured at rest, it may the case that an exercise stimulus is required to elucidate the connection between central and peripheral dysfunction in this model. Prior evidence has shown that exercise stress (such as that carried out in an incremental exercise test) may be required to identify physiological abnormalities that are central to disease progression, and may indeed have an important diagnostic role in the clinical setting (Farina et al., 2018) Indeed, the presence of increased RV pressures during exercise have been suggested as a precursor to resting PH

(Medarov, Jogani, Sun, & Judson, 2017), however, the variable nature of responses to exercise in healthy individuals has made defining an 'exercise pulmonary hypertension' an ongoing challenge (Provencher, Chemla, & Herve, 2011) However, it is clear the integrated nature of the exercise response can shed light on a host of clinically relevant factors, including the metabolic, ventilatory and cardiovascular abnormalities present in the disease (Neder et al., 2015), and ultimately determine the severity of disease and patient prognosis (Paolillo et al., 2012). The findings herein support this idea, with blood flow specifically measured at moderate intensity exercise having a stronger predictive power of disease status than that measured at rest.

A novel finding in this study is the elevated blood flow to the diaphragm in MCT rats at rest, and the tendency for increased flow during exercise ($p=0.07$), opposing the hemodynamic pattern seen in skeletal muscles. There is significant evidence pointing to respiratory muscle dysfunction in PAH, including increased proteolytic activity leading to diaphragm atrophy (de Man et al., 2011), sarcomeric abnormalities culminating in reduced force production in humans (Manders et al., 2012) and atrophy-driven power reductions in an MCT rat model (Ahn et al., 2013). These maladaptations have important clinical repercussions, as respiratory muscle weakness can exacerbate the feeling of breathlessness and fatigue often seen in patient populations (Ahn et al., 2013), while also providing some rationale for the compromised ventilatory response to exercise previously identified (Sun et al., 2001). In chronic left heart failure, respiratory muscle dysfunction has been linked to an increased cost of breathing (Cross, Sabapathy, Beck, Morris, & Johnson, 2012; Olson et al., 2010). That is, energy demands of the respiratory muscles are heightened at the same level of external work, leading to increased shunting of

cardiac output away from skeletal muscle to sustain ventilatory function (de Man et al., 2011). Hyperventilation has been demonstrated in PAH patients at rest (Naeije, 2005), and although this is the first study to highlight an increase in blood flow to the diaphragm in a PAH model, it supports the idea of increased oxygen demand and subsequent redirection of cardiac output to the respiratory muscles in the disease. Furthermore, while not statistically significant, there was a tendency for diaphragmatic flow to be increased during exercise in MCT rats, and this supports previous work in left heart failure (Miller, Smith, Hemauer, & Dempsey, 2007; Olson et al., 2010). Indeed, it is argued this abnormal hemodynamic response may contribute to the decreased exercise tolerance seen in that condition (Smith et al., 2018). It seems logical to 'connect the dots' and propose this as a potential mechanism explaining reduced blood flow to skeletal muscles as reported here. During exercise, blood flow is redistributed to those tissues with an increased metabolic demand (McArdle et al., 2015), however, the elevated oxygen requirements of respiratory muscles, as proposed in PAH, may limit the cardiac output available for locomotor muscles, explaining at least in part the reduced skeletal muscle blood flow reported here.

**Aim 2**

Hypothesis 2.1 – Animals supplemented with BRJ will demonstrate increased blood flow in comparison to those supplemented with placebo.

Hypothesis 2.2 - Animals supplemented with BRJ will demonstrate reduced reliance on anaerobic metabolism via lower blood lactate accumulation at rest and during exercise.

Given the importance of muscle blood flow as a mediator of exercise performance, and its reported reduction in a PAH animal model *(Aim 1),* the study of therapeutic interventions that have been demonstrated to increase blood flow during exercise is warranted. To do so, an understanding of the mechanisms by which blood flow is controlled at the onset of exercise is critical. An expansive review by Sarelius and Pohl (2010) identified mediators intrinsic and extrinsic to muscle tissue that are central to the redistribution of blood flow during exercise, with specific emphasis on the importance of signaling pathways implicated in vasodilation. The review highlights the complexity of the vasodilatory response to exercise, identifying metabolic markers such as increased lactate/adenosine, mechanical shear stress, acetylcholine, and endothelium-derived factors such as NO and prostacyclin as having a synergistic effect of rapidly increasing flow to working muscle. Indeed, NO has been comprehensively studied, and is well characterized as an endothelium-derived vasorelaxing agent and powerful mediator of blood flow (Palmer, Ferrige, & Moncada, 1987). NO is made bioavailable as the substrate L-arginine is acted on by a group of nitric oxide synthases, ultimately upregulating the second messenger cGMP (Moncada & Higgs, 1993). There is evidence this pathway is compromised in PAH (Zuckerbraun et al., 2011) and as such, drug interventions have focused heavily on its manipulation as a treatment option in the disease. Early evidence that NO plays a significant role in muscle blood flow in the rat (T. Hirai, Visneski, Kearns, Zelis, & Musch, 1994) has led to more recent work demonstrating that increasing NO bioavailability via dietary nitrate intervention can improve skeletal muscle blood flow in healthy rats (Ferguson et al., 2014) and chronic heart failure models (Ferguson et al., 2016). The use of dietary nitrate is somewhat

unique in that it has been shown to increase NO in an oxygen-independent manner, outside the traditional L-arginine pathway that is compromised in PAH (Khatri et al., 2017). Taken together, this would point to a promising therapeutic approach to improve muscle blood flow and combat the apparent hemodynamic abnormalities identified in *Aim 1* of this study. The mechanisms by which dietary nitrate may have a therapeutic effect, specifically at the skeletal muscle level, are still under investigation, however, supplementation has been shown to increase plasma nitrate and nitrite (Lundberg et al., 2009), which can theoretically increase NO bioavailability via enzymatic reduction of nitrite in vivo. Interestingly, dietary nitrate has been shown to mediate PH progression in animal models via altered NO signaling and a reduction of pathologic vascular remodeling (Alef et al., 2011; Baliga et al., 2012), however, this is the first study to test its efficacy in manipulating blood flow in the disease.

In this study, an acute dietary nitrate dosing strategy was employed. This is a novel approach in rodent models, likely driven by difficulty in administering known quantities of a dietary supplement to the intact animal. The potential advantages of the acute dose are two-fold; it has previously been shown that a single dose of dietary nitrate can increase NO bioavailability and improve exercise performance (Coggan, Leibowitz, Kadkhodayan, et al., 2015; Coggan, Leibowitz, Spearie, et al., 2015; Coggan & Peterson, 2018; Kenjale et al., 2011; Lansley et al., 2011; Murphy, Eliot, Heuertz, & Weiss, 2012; Rimer et al., 2016), and from a translational perspective, the simplicity of a one-off, pre-exercise drink that can potentially increase blood flow would likely be an attractive option for patients interested in increasing their exercise tolerance.

Contrary to the findings of Ferguson et al. (2016) in the left heart failure rat model, we did not see a significant effect of BRJ in augmenting muscle blood flow, particularly during exercise. In MCT rats supplemented with BRJ, blood flow to the rectus femoris was significantly elevated at rest compared to those given placebo ($p=0.03$), with a tendency for improvement in the rectus femoris and semitendinosus muscles ($p=0.09$), however, no differences were seen during exercise. A similar effect was seen in CON rats, with BRJ supplementation tending to increase blood flow to the gastrocnemius (p-0.09) and vastus lateralis ($p=0.052$) at rest, with no apparent improvements during running. In the aforementioned left heart failure study, blood flow was increased in nine different muscle sections of the ankle extensors, knee extensors and knee flexors during exercise after dietary nitrate intervention, with no differences reported at rest (Ferguson et al., 2016). Similarly, Casey et al. (2015) showed that in older healthy individuals, an acute dose of dietary nitrate increased vasodilation and blood flow during subsequent forearm exercise, again seeming to contradict the findings in this study.

In exploring the factors that may have resulted in limited efficacy of BRJ in augmenting exercising blood flow, it would be tempting to point to the acute administration of nitrate in comparison to the chronic dosing strategy used by Ferguson et al. (2016). However, we have shown that a single dose of BRJ significantly increases plasma nitrate and nitrite in a similar fashion reported in that study, and in others that employed a longer term dietary nitrate intervention (Alef et al., 2011; Bailey et al., 2010; Bailey et al., 2009; Baliga et al., 2012; Beijers et al., 2018; Friis et al., 2017; Henrohn et al., 2018; Larsen et al., 2011; Smith et al., 2018). As plasma nitrate/nitrite levels are

consistently used to demonstrate the effectiveness of BRJ dosing, reporting fold increases of ~12-20 and ~2.5 in nitrate and nitrite respectively across MCT and CON groups after BRJ gavage goes some way to indicate the efficacy of the acute dosing strategy employed here. However, as the rationale for using BRJ to improve blood flow involves increasing NO as a precursor to vasodilation and increased flow *in skeletal muscle*, an important next step was to measure nitrate and nitrite in the tissue of interest. Indeed, the importance of NO metabolism in skeletal muscle has received increased attention in recent years, most notably through the work of Barbora Piknova. This work in rodents revealed that skeletal muscle acts as a large nitrate reservoir capable of adding to whole body nitrite and NO levels (Piknova et al., 2015), and importantly, that reduction of nitrite to NO likely plays a functional role in increasing skeletal muscle blood flow during exercise stress (Piknova, Park, Kwan Jeff Lam, & Schechter, 2016). As it is now known muscle nitrate and nitrite levels change at the onset of exercise (Piknova et al., 2016) and that their levels can be altered through dietary intervention (Gilliard et al., 2018), direct measurement in the tissue of interest may help provide an understanding as to the action of the NO pathway we have attempted to manipulate in this study. Fortunately, a reliable method using high performance liquid chromatography (HPLC) for measuring muscle nitrate/nitrite has recently been developed (Troutman et al., 2018), and allows for the precise measurement of each in rodent tissue samples.

In support of previous work (Piknova et al., 2015), we show that the rat skeletal muscle contains higher levels of nitrate and nitrite in comparison to plasma, confirming its role as a significant storage site for each in vivo. In both the soleus and vastus lateralis muscles, nitrate levels were significantly increased in the BRJ supplemented groups

compared to PL, however, there were no differences in muscle nitrite in either tissue. This is contrary to the findings in plasma, where both nitrate and nitrite were significantly elevated in BRJ rats. Indeed, this finding held true for both MCT and CON rats, with MCT seemingly not effecting dietary nitrate handling after gavage. It can be speculated that no differences in nitrite, despite elevated nitrate levels in muscle indicates a lack of reduction in the $NO_3^-$-$NO_2^-$-NO pathway, and therefore, no significant hyperemic effect. One would expect an increase in nitrite (and potentially NO), as a consequence of reduced nitrate, however, the findings here would call this into question. This would seem to coincide with the finding that BRJ was not effective in reducing lactate levels during exercise. In both MCT and CON rats, lactate was not significantly different after BRJ or PL gavage. While it was demonstrated that plasma nitrite was negatively correlated to exercising lactate, this was not the case with nitrate, nor was it related to blood flow either in individual muscles or as a compiled blood flow measure. In relation to the findings in *Aim 1*, if BRJ is unable to increase blood flow during exercise, the reliance on anaerobic metabolism may not be attenuated, and thus higher blood lactate accumulation will occur. As noted at the outset, the proposed mechanisms by which nitrate/nitrite are reduced to NO involve reductase enzymes including xanthine oxidoreductase, however, it is not known if this action may is compromised in the milieu of skeletal muscle dysfunction present in PAH. To further illuminate this suggestion, a logical next step would be to measure cGMP in tissue samples. An increase in cGMP would provide a more direct measure of NO bioavailability, and deliver greater mechanistic insight into the effect of supplementation on this pathway during exercise. Granted, as vasodilation in muscle is achieved via a variety of intrinsic and extrinsic

mechanisms (Sarelius & Pohl, 2010) altered NO metabolism as a result of nitrate supplementation may only provide part of the story, However, prior work connecting the two, specifically in terms of blood flow mediation (Casey et al., 2015; Ferguson et al., 2013; Ferguson et al., 2016; Piknova et al., 2016), would suggest further work is warranted.

Interestingly, previous findings have indicated a potential 'fiber type' effect of BRJ supplementation and subsequent exercise performance. This idea has originated from two separate lines of evidence, including the demonstration that blood flow and vascular conductance may be improved specifically in rat Type II muscle fibers after BRJ ingestion (Ferguson et al., 2013), and in humans, initial work indicates BRJ may be effective in improving shorter duration, high intensity efforts that would require Type II fiber recruitment (Coggan, Leibowitz, Kadkhodayan, et al., 2015; Coggan, Leibowitz, Spearie, et al., 2015; Rimer et al., 2016; Thompson et al., 2016; Wylie et al., 2016). We show that in rat muscle, nitrate/nitrite levels are higher in the soleus than vastus lateralis, which may be considered surprising as the former is almost entirely Type I in composition, and the latter Type II (Armstrong & Phelps, 1984). It has been postulated that neuronal NOS (NOS1, nNOS) is active primarily in type II fibers in the rat, and therefore exercises of higher intensity may lead to greater NO production via this well-established pathway (Copp et al., 2013). Based on the findings herein, it could be hypothesized that the greater levels of nitrate/nitrite in Type I fibers may be a compensatory mechanism due to a reduced NOS1 function in oxidative muscle. Whether Type I fibers rely more heavily on other NO production mechanisms (such as the $NO_3^-$-

$NO_2^-$-NO pathway) remains to be seen, but it may in part explain the discrepancy seen in muscle nitrate/nitrite levels reported here.

**Aim 3**

Hypothesis 3.1 – BRJ supplementation will result in a significant increase in exercise function through improved $VO_2$max, strength and increased volitional running distance in both PAH and control rats, compared to those supplemented with placebo.

As noted in *Aim 1*, exercise function was reduced in MCT rats. $VO_2$max was significantly lower in both the BRJ and PL condition in MCT compared to CON ($p=<0.01$). There was a trend toward reduced grip strength in MCT under BRJ supplementation ($p=0.08$) and a decline in voluntary running performance in the PL trial ($p=0.08$) and to a lesser extent after BRJ gavage ($p=0.12$) when compared to CON. In a similar finding to *Aim 2*, repeated measures analysis determined BRJ had limited effectiveness in increasing exercise performance in MCT or CON rats in either pre-injection or final testing. To the author's knowledge, this is the first study to test the effectiveness of BRJ on maximal aerobic capacity and voluntary running in an animal model, and while supplementation did increase $VO_2$max and time to exhaustion in absolute terms, this change was not robust enough to reach statistical significance. There has been significant work on dietary nitrate as an ergogenic aid in healthy individuals, athletes, and patient populations, with previous studies determined that acute dosing of BRJ reduced the oxygen cost of exercise at submaximal intensities (Bailey et al., 2009; Larsen et al., 2007; Vanhatalo et al., 2010). Mechanistically, it has been postulated that

lower oxygen cost of exercise (reduced ATP requirement at a given work output) may be a consequence of an improved aerobic metabolic efficiency, demonstrated via reduced intramuscular metabolites ADP and phosphate, as well as a sparing of muscle phosphocreatine during exercise post-supplementation (A. M. Jones, 2014a). Additionally, Larsen et al. (2011) reported improved mitochondrial function after three days of dietary nitrate supplementation, explained via reduced expression of adenine nucleotide translocase (ANT), which was subsequently correlated to recued $VO_2$ consumption during submaximal exercise. While we did not directly measures mitochondrial function, we did not see altered evidence of altered exercise metabolism (as measured via RER response at incremental workloads during submaximal exercise) post BRJ consumption in either MCT or CON rats. As such, a direct interrogation of mitochondrial function may elucidate an improved exercise economy in the PAH rat. With that said, there is evidence that mitochondrial function is altered in PAH (S. Y. Chan & Rubin, 2017; Piao, Marsboom, et al., 2010; Yu & Chan, 2017), therefore, the possibility of improved exercise economy via this mechanism is uncertain. Importantly, improved exercise economy should be considered distinct from exercise *capacity*. While a reduced $VO_2$ at a given exercise intensity may theoretically increase submaximal exercise tolerance, there is equivocal evidence that nitrate supplementation improves exercise capacity. (A. M. Jones et al., 2018). Indeed, the effectiveness of BRJ in improving exercise performance in running/cycling tasks is variable, and seems to depend on the training status and dose-duration in healthy subjects (McMahon, Leveritt, & Pavey, 2017).

Improvements in exercise performance after an acute dose of BRJ have been shown, but not consistently. In a recent review Zafeiridis (2014) showed that exercise performance, measured via cycling and running time trials may be improved, however, the evidence that $VO_2$max may be improved is limited. Indeed, one study showed that an acute dose of nitrate (via sodium nitrate in drinking water) *reduced* maximal oxygen consumption in trained cyclists (Bescos et al., 2011), further highlighting the inconsistency in results to date. What may be more important is a consideration of dietary nitrate and its potential ergogenic effects in clinical populations. Of greatest relevance to this study, BRJ has been shown to increase peak walking time and delay the onset of claudication in peripheral artery disease patients, representing a clinically relevant improvement in exercise performance (Kenjale et al., 2011). Interestingly, the improvement in walking performance was not associated with an increase in maximal aerobic capacity. It is postulated that in this instance, the demonstrated reduction in tissue deoxygenation may indicate increased muscle perfusion post BRJ supplementation, however, this was not measured directly. Similarly, dietary nitrate has been shown to improve aerobic exercise capacity in COPD patients (Berry et al., 2015), with a single dose of BRJ extending submaximal exercise time and reducing blood pressure compared to placebo. However the aforementioned inconsistency is highlighted by a more recent study indicating that 6 days of BRJ supplementation did not improve walking distance in the same condition, despite significant elevations in plasma nitrite levels (Friis et al., 2017). Several studies have determined that dietary nitrate may be effective in improving exercise tolerance in left heart failure. A single dose of dietary nitrate was shown to increase $VO_2$max and total work performed on a supine cycle exercise-test, despite no

improvement in exercise efficiency (Zamani et al., 2015). This is in slight contrast to the work of Eggebeen et al. (2016) who demonstrated that a single dose of BRJ was ineffective in increasing $VO_2$max or submaximal cycling endurance in heart failure patients, but that a longer term (6 day) strategy was required to elicit positive changes in submaximal performance. However, the discrepancy in outcomes may have been due to dosage levels, which were higher in the previous study. Furthermore, it has been shown that dietary nitrate improves $VO_2$peak and time to fatigue in heart failure patients with reduced ejection fraction (Coggan et al., 2018), and one study has considered the use of BRJ as an ergogenic aid in PAH. After 7 days of dietary nitrate consumption PAH patients tended to improve their power output at a given submaximal $VO_2$, however, a significant difference was only observed in a sub-group of patients that were classed as dose 'responders'. Additionally, $VO_2$max and 6 minute walking distance were unaltered following supplementation (Henrohn et al., 2018). Initially, it would seem our results oppose those that have been demonstrated in patient populations, however, there are factors which must be taken into consideration when contextualizing our findings. Most notably, the predominant conclusion in aerobic exercise points to an improvement in submaximal exercise performance, however, we did not perform a steady-state submaximal test. As such, we were not able to determine the effectiveness of BRJ in augmenting performance from this perspective. Granted, voluntary wheel running is submaximal in activity, however, by definition it is carried out at a self-selected and variable pace. While it is possible to examine responses to exercise at submaximal intensities during a $VO_2$max test, the fact that it is externally controlled and not subject to optimal self-pacing (as would usually be carried out during an endurance task), may limit

our ability to accurately assess true ‘performance’ parameters from a translational perspective (Noakes, 2008). However, the equivocal nature of findings in both healthy and patient populations to date adds weight to the idea that regardless of testing protocol, BRJ may have been ineffective at improving functional capacity in this model.

The duration of BRJ dosing must also be taken into consideration. Of interest are the results of Eggebeen et al. (2016), who demonstrated a distinct difference in responses to exercise in HF patients, arguing that a longer nitrate intervention may be required to elicit mechanistic changes that account for improved performance. A. M. Jones et al. (2018) notes that acute dosing may be effective in improving exercise economy, but that greater doses (>8.5mmol) may be necessary to see changes in functional performance. Additionally, some of the potential mechanisms by which dietary nitrate may impact aerobic performance, including mitochondrial protein changes, blood flow modulation and calcium handling (A. M. Jones, 2014a), which may require a sustained dosing period to be sufficiently altered at the cellular level. Taken together, while we have not demonstrated a significant effect of an acute dose of BRJ on improving aerobic exercise function, this can be considered supportive of pervious work in this area. The inconsistencies outlined would suggest a need to optimize dosing strategies based on the outcome of interest. The findings have added to the body of literature, most notably by demonstrating a single dose of BRJ may not be sufficient to bring about changes in maximal exercise capacity or voluntary running distance in a PAH model.

The work indicating dietary nitrate supplementation may target type II, fast twitch muscle fibers (Ferguson et al., 2013) led to the hypothesis that it may be efficacious in improving performance in higher intensity, short duration activities that would be

considered ‘anaerobic’ in nature. This was a novel concept, primary because much of the rationale for using BRJ to improve performance centered on increasing NO with the downstream effect of increasing vasodilation leading to greater oxygen delivery to working muscle. However, such high intensity activity is less depended on oxygen availability to replenish ATP stores, and as such, would require interventions that directly modulate muscle contractile function. With that said, and increasing body of work has pointed to the benefits of dietary nitrate and anaerobic performance in healthy individuals, indicting the effectiveness of supplementation in improving repeated sprint performance, supramaximal cycling, and knee extensor power in healthy individuals (Aucouturier, Boissiere, Pawlak-Chaouch, Cuvelier, & Gamelin, 2015; Coggan, Leibowitz, Kadkhodayan, et al., 2015; Thompson et al., 2016; Wylie et al., 2016) and in clinical populations (Coggan, Leibowitz, Spearie, et al., 2015). The data in the present study would seem to contrast that found previously, in that we did not find an effect of BRJ in increasing grip strength in the PAH rat. However, as is the case in aerobic activity, results seem to be mediated by the duration of nitrate dosing. It may be that acute dosing improves contractile function via a direct effect on the sarcoplasmic reticulum and enhanced contractile sensitivity (Coggan & Peterson, 2018), however, we did not see this represented via improved force production. It has been argued that longer term supplementation may lead to protein changes in muscle contractile apparatus, resulting in improved force production (Hernandez et al., 2012), however, this idea has been challenged with evidence that in human muscle, an improvement in force post-BRJ ingestion is not related to changes in muscle protein expression (Whitfield et al., 2017). While the mechanisms of action are still under investigation, we may not have seen an

effect due to the nature of the grip-strength test. While it allows for the calculation of maximal force production, the test does not allow for rate of force production, and therefore measures of muscle power at various velocities, and an interrogation of these may lead to a more robust finding on the modulation of muscle contractile function in a PAH model.

**Conclusion**

We have shown that in a reliable pulmonary hypertensive animal model, blood flow is significantly reduced at rest, and to a greater extent, during moderate intensity exercise. These findings can be linked to previous work in this area, most notably that tissue oxygen supply and extraction are reduced in PAH patients. As such, a direct investigation of skeletal muscle blood flow, as carried out here, provides a novel description of peripheral maladaptations and exercise responses in the disease. It has also been shown that this reduced blood flow is not a result of capillary rarefaction at the skeletal muscle level, highlighting the potential importance of microcirculatory function as imperative in modulating blood flow. Importantly, a direct association between exercising blood flow and lactate accumulation was identified, adding a mechanistic explanation for the previously observed abnormal metabolic response to exercise in PAH. A link between exercising blood flow and traditional disease measures has also been highlighted, emphasizing the importance of exercise stress in understanding the adaptation to right heart functional and morphological changes that are central to PAH pathology.

Contrary to previous investigations, we did not see an effect of BRJ in augmenting exercising blood flow in either MCT or CON rats. Tendencies for improved flow at rest were seen in both groups, however, only in the rectus femoris muscle group were significant differences present. Findings herein did not show that BRJ mediated exercising blood flow either in health or disease. Interestingly, despite no improvements in blood flow, an acute dose of BRJ was sufficient to increase plasma nitrate and nitrite in caparison with longer term dosing strategies in both humans and animal models. A potential explanation for the limited effectiveness of dietary nitrate was proposed following the analysis of muscle nitrate and nitrite levels in both type I and type II muscle groups. We show that a primary type I muscle stores more nitrate/nitrite than type II, however, the lack of nitrite increases in muscle post-BRJ supplementation may indicate a compromised $NO_3^-$-$NO_2^-$-NO reduction pathway and therefore explain a lack of efficacy in dietary nitrate altering blood flow. Finally, acute dosing of BRJ did not significantly improve functional exercise capacity as measured by $VO_2$max, voluntary wheel running, and grip strength. Given the equivocal nature of findings on dietary nitrate supplementation and exercise performance, this work adds to that body of knowledge by specifically testing its usefulness in a PAH model. Finally, results support the notion that an optimization of dosing strategies dependent on the desired outcome measures and patient characteristics may be required.

**Limitations**

The use of the monocrotaline model of pulmonary hypertension has limitations. While it has been employed as a model in PH research for decades, its validity as a representative pre-clinical model has been called into question. Most notably, the obstructive plexiform lesions found in the lungs of PAH patients do not seem to develop in this model (Gomez-Arroyo et al., 2012). As such, the mechanisms by which pulmonary pressures are altered and hemodynamic abnormalities arise may not be adequately replicated. Additionally, it has been shown that the induction of pulmonary hypertension via MCT may have systemic effects. This includes evidence of liver toxicity and lymphocytic myocarditis (Gomez-Arroyo et al., 2012; Stenmark et al., 2009), which are not consistently reported in PAH patients. Nevertheless, the RV hypertrophy and increased RV pressures are advantages that have resulted in the MCT model being a mainstay PH researchers, but as with all animal models, the extent to which they fully reflect disease pathophysiology in patients should be taken into consideration.

The acute dosing approach employed in this study was technically challenging, and also limited the amount of dietary nitrate that could be administered pre-exercise. Similar studies in rodents have traditionally involved the mixing of beetroot juice in drinking water over several days, with rats consuming the product *ad libitum*. While this is a methodologically simpler approach, the longer dosing strategy may allow for greater overall nitrate intake in the day(s) leading up to exercise. The gavage technique is limited by the amount of liquid that can be delivered at one time, hence our acute dose may have resulted in fewer mmol per kg body weight compared to previous investigations. For example, (Ferguson et al., 2013) reported a dose of 1mmol/kg/day in drinking water,

however, to achieve this dose via gavage would involve the administration of ~10ml of beetroot juice. Preliminary work determined rats could not consistently retain this amount of fluid via gavage, with instances of regurgitation common. Hence, we employed a lower dosing strategy of 0.5mmol/kg, which was more easily tolerated. With that said, we were able to achieve significant elevations in plasma nitrate/nitrite as previous groups using the longer term technique, highlighting at the very least, the gavage technique as suitable for increasing circulating nitrate/nitrite in treated animals.

Given the variability in blood flow measures, a within-rat design, as opposed to the between-rat comparison used here may have led to more conclusive findings. This would involve a more rigorous, and likely precarious methodology with each rat being gavaged twice and completing two run bouts following catheter placement surgery. Indeed, preliminary work indicated that both MCT and CON rats struggled to complete the second run bout when this approach was attempted. Another source of variability may lie in the florescent microsphere method itself. Blood flow quantification requires the adequate mixing of beads pre-injection, degradation of tissue, filtration, bead capture, the dissolving of dye and accurate pipetting before plate analysis. Each of these have the potential for microsphere (and therefore fluorescence) loss. Moreover, during bead injection, it is vital catheters are accurately placed in the aortic arch. If not, systemic flow may not be accurately captured. There were instances whereby blood flow data were compromised due to unequal left to right blood flow, and in such circumstances, the blood flow data was removed from subsequent analysis. However, continued work on optimizing the method proved useful in mitigating this problem.

**Future Directions**

Undoubtedly, follow up studies should be carried out in other PAH models. Our lab has used the sugen-hypoxia and MCT-pneumonectomy models, both of which have been shown to produce a pulmonary hypertensive phenotype. Moreover, the sugen-hypoxia model has been postulated as a more accurate model, with evidence that its mechanisms more closely related to heart and lung dysfunction seen in patient populations. With that said, the downstream effects, including that in skeletal muscle, require greater exploration, and investigating the blood flow response to exercise in this model would provide a fuller picture from a preclinical perspective.

Further mechanistic analysis that may help explain the reduction in blood flow in PAH is warranted, particularly as this study suggested skeletal muscle capillary rarefaction may not be a determining factor. To rigorously assess this suggestion, a more thorough investigation of capillaization of skeletal muscle could be carried out. For example, a recent study measured capillarization via eATPase staining in rats that had undergone 12 weeks of wheel running (Beleza et al., 2019), and this alternative method of quantification may result in different findings. Moreover, interrogating known angiogenic and/or rarefactory pathways may elucidate the role of capillary changes at the muscle level. Potus et al. (2014) probed micro-RNAs and the VEGF pathway (a known mediator of angiogenesis) as a means of explaining the previously measured capillary rarefaction in muscle of PAH patients. Thus, measuring whole muscle protein via immunblotting, or alternatively, using RT-PCR to target relevant gene expression may be an appropriate next step in determining this activity in the MCT model. Lastly, the reduction in cardiac output and elevated respiratory muscle flow after MCT injection can

be connected to reduced peripheral perfusion, however, an interrogation into arterial function may highlight further maladaptations and potential therapeutic targets. As noted, in left heart failure there is evidence of arterial dysfunction leading to reduced blood flow, and future studies should attempt to understand how this may play a role in PAH.

The extent to which training might improve muscle blood flow should be investigated. Exercise has long been known as an angiogenic stimulus (Bloor, 2005), and given this description of reduced flow to skeletal muscle, pursuing this type of investigation would likely have significant translational value. Ideally, the identification of therapeutic targets should be followed up with interventions to address the problem, and given previous work (including in our lab) that exercise training has benefits specific to skeletal muscle, determining if blood flow improvements are possible would be a meaningful line of enquiry. Moreover, given the strong evidence that exercise training is beneficial in PAH, such a study would likely induce positive changes beyond those being interrogated at the skeletal muscle level.

Ultimately, the use of dietary nitrate in improving exercise tolerance in PAH patients should be investigated. While one study has begun this work, further exploration of potential ergogenic effects should be pursued. Specifically, the benefits demonstrated in left heart failure after BRJ supplementation, both in producing improvements in aerobic performance and strength/power would provide rationale for similar testing in PAH. To date, no study in patients has examined the effects of either acute or chronic dosing on strength and power parameters, which, given the clear deficiency reported in patients, merits further research. It is likely this work would involve the development of optimized dosing protocols, as has been suggested in healthy populations. The potential

benefits of using a readily available, non-pharmacological product to improve exercise performance would warrant a fuller investigation in this condition.

# Appendices

## Appendix A: Dietary Nitrate Dose Response

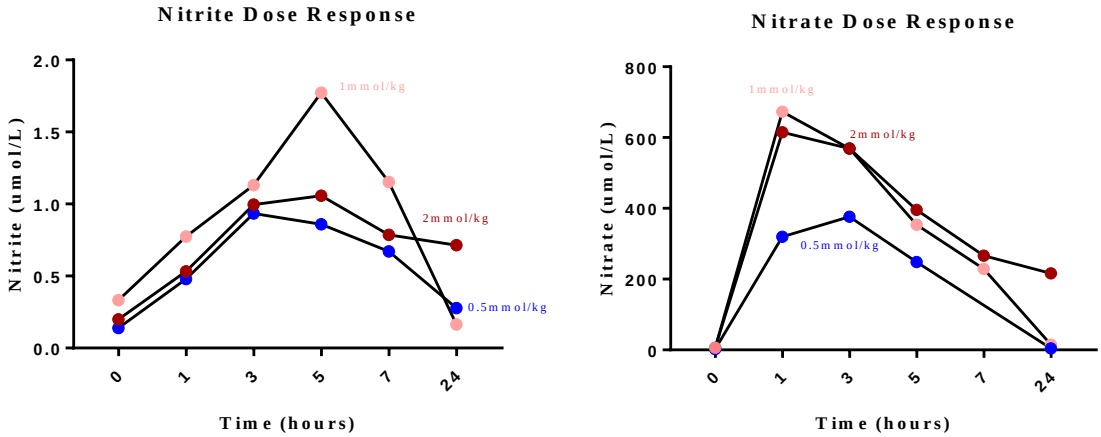



**Figure A5.1:** Nitrate and nitrite dose responses

Figure A5.1 represents plasma nitrate/nitrite responses to 3 different doses of dietary nitrate supplement, achieved via serial blood-draws at specific time-points post gavage (1mmol/kg – pink; 0.5mmol/kg – blue; 2mmol/kg – maroon, n=3). Greatest nitrate responses are seen between 2-3hrs post gavage, with a similar effect seen in nitrite at the 0.5mmol/kg dosing level. At either 0.5 or 1mmol/kg, initial increases in nitrate and nitrite have returned to baseline at 24 hours. Both nitrate and nitrite remain elevated at 24hours at the highest dosing level.

**Appendix B: Blood Flow Experiment Recording Sheet**

**Rat number _____ MCT / CON _____**

**Rat weight _____**

Calculation for gavage dose:

Calculation for microsphere number:

Running intensity:

Comments from Catheter placement surgery:

_____

_____

_____

_____

Description of blood flow experiment (Gary to complete):

_____

_____

_____

_____

_____

_____

| Tissue | Weight / Volume |
|---|---|
| Ref blood 1 | ml |
| Ref blood 2 | ml |
| L. kidney | g |
| R. Kidney | g |
| Gast | g |
| RF | g |
| BF | g |
| EDL | g |
| TA | g |
| VL | g |
| Sol | g |
| Semi | g |
| Diap | g |

**Hemodynamics**

| Time | BP | RVP | Airway Pressure | Temp | Lactate | Comments |
|---|---|---|---|---|---|---|
| | | | | | | |
| | | | | | | |
| | | | | | | |
| | | | | | | |
| | | | | | | |

LV + S weight (g) _____ Right heart weight (g) _____ Fulton _____

Tissues placed in box labeled

_____

**Additional notes:**

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# Curriculum Vitae

**Gary Marshall Long**

## Education

PhD, Health and Rehabilitation Science '19 Indiana University, Indianapolis, Indiana

- Concentration - Exercise Physiology, Minor - Human Anatomy
- Dissertation: *Beet-ing Muscle Dysfunction and Exercise Intolerance in Pulmonary Hypertension*

Master of Science, Exercise Science '08 University of Dayton, Ohio

- Graduated with a 3.97 cumulative GPA
- Thesis: *Statistically Profiling Performance of Division I Collegiate Soccer Athletes Using a Battery of Field Tests*

Bachelor of Science, Sports Coaching '06 University of Wales Institute Cardiff, UK

- Graduated with honor
- Dissertation: *Sport and Politics in Northern Ireland*

## Publications

Brown MB, Kempf A, Collins CM, **Long GM**, Owens M, Gupta S, Hellman Y, Wong V, Farber M, Lahm T. A Prescribed Walking Regimen Plus Arginine Supplementation Improves Function and Quality of Life for Patients with Pulmonary Arterial Hypertension: a Pilot Study. Pulmonary Circulation, 2017; 8(1) 1-12.

Brown MB, Neves E, **Long GM,** Graber J, Gladish B, Wiseman A, Owens M, Fisher AJ, Presson RG, Petrache I, Kline J, Lahm T**.** High Intensity Interval Training but not Continuous Training Reverses Right Ventricular Hypertrophy and Dysfunction in a Rat Model of Pulmonary Hypertension. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 2016; 312, 197-210.

## Abstracts/Presentations

**Long GM**, Frump A, Troutman A, Mailand M, Ellis KA, Fisher A, Avin K, Coggan A, Lahm T, Brown MB.
Exercising Skeletal Muscle Blood Flow is Diminished in a Rat Model of Pulmonary Arterial Hypertension.
*American College of Sports Medicine (ACSM) National Conference May 19*

Brown MB, Frump A, **Long GM**, Troutman A, Fisher A, Presson R, Lahm T.
Differential Effects of Three Distinct Training Approaches in a Rat Model of Severe Pulmonary Hypertension.
*American College of Sports Medicine (ACSM) National Conference June 18*

Troutman A, Blessinger J, Kurzhal T, Studebaker S, **Long GM**, Coggan A, Brown MB. Plasma Nitrate and Nitrite Following Exercise Training in a Rat Model of Pulmonary Hypertension.
*Midwest American College of Sports Medicine Annual Conference* *Nov 17*

Brown MB, Frump A**, Long GM**, Fisher AJ, Presson R, Lahm T. Six Weeks of Treadmill Training with a High Intensity Interval or Mild Continuous Exercise Approach Increases Cardiac Output in a Sugen-Hypoxia Rat Model of Pulmonary Arterial Hypertension.
*American Thoracic Society National Conference* *May 17*

Brown, MB, **Long GM,** Frump A, Wiseman A, Owens M, Blessinger J, Kurzhal T, Studebaker S, Jay K, Troutman A, Lahm T. High Intensity Interval Training in a Rat Model of Severe, Angioproliferative Pulmonary Arterial Hypertension.
*American College of Sports Medicine (ACSM) National Conference* *May 17*

**Long GM,** Frump A, Fisher AJ, Lahm T, Brown MB. Six Weeks of Treadmill Training with a High Intensity Interval or Mild Continuous Exercise Approach Increases Cardiac Output in a Sugen-Hypoxia Rat Model of Pulmonary Arterial Hypertension.
*Invited presentation – Indiana Physiological Society Annual Meeting* *Jan 17*

**Long GM**, Neves E, Kline J, Brown MB. Exercise Responses Indicate Right Ventricular Failure in a Novel Rat Model of CTEPH.
*Midwest American College of Sports Medicine Annual Conference* *Nov 16*

Brown MB, Neves E, **Long GM**, Kline J. A High Intensity Interval Training Protocol is not better than Customary Continuous Exercise for a Rat Model of Chronic Thromboembolic Pulmonary Hypertension.
*American Heart Association Annual Conference* *Nov 16*

Brown MB, Neves E, **Long GM**, Novack R, Fisher AJ, Presson R, Petrache I, Kline J, Lahm T. High Intensity Interval Training is Superior to Continuous Training in a Pulmonary Hypertension Rat Model.
*American College of Sports Medicine Annual Meeting* *June 16*

**Long GM**, Neves E, Kline J, Brown MB. Novel telemetric recording of exercise hemodynamics over disease development in three different rat models of pulmonary vascular disease.
*Invited presentation – Indiana Physiological Society Annual Meeting* *Jan 16*

**Long GM**, Neves E, Novack R, Fisher AJ, Presson R, Petrache I, Kline J, Lahm T, Brown MB. Pulmonary and Systemic Pressure Responses to High Intensity Interval vs. Continuous Mild Exercise Recorded via Implantable Telemetry in a Rat Model of Pulmonary Arterial Hypertension and Pulmonary Embolism.
*Indiana Clinical and Translational Sciences Institute* *Sep 15*
*IUPUI SHRS Interdisciplinary Research and Continuing Education Conference* *Nov 15*

**Long GM.** The Emergency Treatment and Active Labor Act – EMTALA
*Invited Presentation to IUPUI Physician's Assistant Program* *June 15*

## Research Awards

President's Cup Winner (National Graduate Student Award)
*American College of Sports Medicine* *May, 17*

Presidents Cup Representative Award
*Midwest American College of Sports Medicine* *May, 17*

Best Oral Presentation
*Indiana Physiological Society Annual Meeting* *Jan, 16*

## Professional Experience

University of Indianapolis, Indianapolis, IN *Aug 18 – Aug 19*
***Associate Adjunct Professor of Kinesiology***

- Preparing and delivering classes in Exercise Science
- Student advising, monitoring and reporting student academic progress
- Committee duties as assigned by department/administration

St. Francis Soccer Club, Indianapolis, IN *Feb 17 – March 19*
***Director of Coaching***

- Recruiting and evaluating youth soccer players
- Development of age appropriate training curriculum
- Preparation and delivery of practice sessions
- Recruitment, hiring, training and development of staff coaches
- Budget management and club-wide fundraising

Indiana University, Indianapolis, IN *July 15 – May 19*
***Research Assistant/PhD Candidate***

- Researcher in exercise physiology/pulmonary disease
- Specific focus in optimizing exercise interventions for patients with pulmonary hypertension and pulmonary embolism
- Grant writing, application and maintenance of IACUC/IRB approval, data collection and management, writing for publication
- Specific research skills include immunohistochemistry, exercise testing (human and animal), telemetry implantation and analysis, tissue microscopy, animal surgery and harvesting

Franklin College, Franklin, IN *Sep 13 - June 15*

***Tenure Track Instructor of Exercise Science (left to pursue full-time PhD study)***

- Preparing and delivering classes in exercise physiology, anatomy and physiology, biomechanics, exercise testing and prescription, strength and conditioning methods and principles of nutrition
- Development of new Exercise Science major and associated curriculum
- Monitoring and reporting student academic progress
- Committee duties as assigned by department/administration

Franklin College, Franklin, IN *Aug 10 – Jul 13*

***Adjunct Instructor in Health Science***

- Teaching health and wellness classes to undergraduate students
- Designing, implementing and managing new course curriculum

Franklin College, Franklin, IN *May 10 – June 15*

***Assistant Soccer Coach***

- Coaching, recruiting, budget management

Franklin College, Franklin, IN *May 10 – June 15*

***Strength and Conditioning Coach – Men's Soccer***

- Designing and implementing strength and conditioning programs
- Teaching technique and pre-post health/fitness evaluation

North Central High School, Indianapolis, IN *Feb 10 - May 10*

***Volunteer Teacher/ Coach in Strength, Conditioning and Health***

- Developing and teaching programs for students at the $9^{th}$-$12^{th}$ grade level

**Teaching Assignments**

KINS 190 Introduction to Kinesiology, 2019
KINS 249 Basic Sport and Community Nutrition 2019
KINS 102 Advanced Wellness 2019
KINS 268 Stress Management in Human Health, 2018
KINS 230 Fundamentals of Officiating, 2018
EXE 372 Exercise Physiology, 2012-2015
EXE 380 Biomechanics, 2012-2015
BIO 110/115 Anatomy and Physiology, 2013-2015
EXE 244 Strength and Conditioning Methods, 2013-2015
EXE 375 Exercise Prescription, 2013-2015
EXE 208 Principles of Nutrition, 2010-2012
EXE 119 Concepts of Wellness, 2010-2012

**Professional Membership**

| | |
|---|---|
| American Heart Association (AHA) | *2016-Present* |
| American College of Sports Medicine (ACSM) | *2013-Present* |

**Professional Development**

| | |
|---|---|
| ACSM Certified Personal Trainer | *Aug, 13* |
| American Red Cross First Aid and Adult CPR/AED certified | *May, 13* |
| USSF National 'D' License | *Nov, 10* |