Skeletal Muscle Oxidative Capacity in the Elderly

by

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B.A. University of Texas at Arlington, 2015

A Thesis Submitted in Partial Fulfillment

of the Requirements for the Degree of

MASTERS OF SCIENCE

In the Department of Kinesiology

College of Nursing and Health Innovation

May 2018

Supervisory Committee

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Abstract

Age is the greatest risk factor for chronic disease and is associated with a marked decline in functional capacity and quality of life, with loss of skeletal muscle function regarded as a key contributing factor. While the exact mechanism(s) for the loss of skeletal muscle function remains incompletely understood, age-related mitochondrial dysfunction is thought to play a major role. To explore this question further, we studied 15 independently-living seniors (age: 72 ± 5 yrs; m/f: 4/11; BMI: 27.6 ± 5.9) and 17 young volunteers (age: 25 ± 4 years; m/f: 8/9; BMI: 24.0 ± 3.3). Skeletal muscle oxidative function was measured in non-locomotor forearm muscle as the recovery kinetics of muscle oxygen consumption using near-infrared spectroscopy (NIRS). Aging was associated with a significant prolongation of the time constant of oxidative recovery following handgrip exercise (51.8 ± 5.4 s vs. 37.1 ± 2.1 s, p = 0.04, old vs. young, respectively), suggesting an overall reduction in mitochondrial function. To determine whether we could improve this age-related impairment in skeletal muscle oxidative capacity, we performed an exercise training study in eight elderly participants from the local Dallas-Fort Worth community (73 ± 7 yrs; m/f: 2/6; BMI: 25.9 ± 3.1 kg). Training was performed five days a week, at 30% of maximal voluntary contraction (MVC), for a total of 600, 900, 1200, and 1500 grips per day, respectively. Skeletal muscle oxidative capacity was assessed using the aforementioned NIRS protocol. In contrast to our hypothesis, neither grip strength (pre-training MVC: 24.5 ± 12.2 kg; post-training MVC = 26.1 ± 11.4 kg; p = 0.12) nor skeletal muscle oxidative capacity (pre-exercise training tau: 44.6 ± 14.1s; post-exercise training tau: 46.9 ± 11.3 s; p = 1.00) were significantly improved. These data conflict with several prior investigations showing an improvement in skeletal muscle oxidative capacity with exercise training in the elderly. That our participants were on average 10 years older than the majority of participants previously studied, with some history of cardiovascular risk factors, may suggest an upper limit to skeletal muscle adaptation. More work in this area is indeed needed.

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Acknowledgments

First and foremost, I want to thank each and every single research volunteer who has helped make this milestone possible. Without you — without your participation and support — we would not have been able to come to this very place of knowledge and understanding that we are at now.

I would also like to thank all of the faculty and staff in the Department of Kinesiology and the College of Nursing and Health Innovation for the support, encouragement, and guidance that they have provided over the years. For me and the many countless other students who have passed through this program, each conversation and encounter has helped shape our academic careers into what they are today. Specifically, I would like to dedicate my thanks to Dr. Barry McKeown, Dr. Mark Ricard, and Dr. Abu Yilla for always encouraging me to achieve my best, in the most gentle and supportive way possible. It was with your guidance that I learned to strive for excellence and appreciate the importance of innovation. This has helped me see the world in a way that is unhindered by barriers or limitations, where only possibilities and potential exist.

To the members of the Applied Physiology and Advancing Imaging Laboratory, thank you for all of the hard work you have put into making our work some of the most intriguing, innovative, and inspiring research conducted. Thank you for showing me the ropes, so to speak, and assisting me as I learned the ins-and-outs of the research world.

Of course, it goes without saying that I could not have achieved what I have today, or grown to be the person I am, without the support of my friends and family. Thank you for accepting me with open arms while always encouraging me to be my best. These 18 months would not have been the same without your love and kindness.

Finally, I would like to thank my faculty mentor, Dr. Michael D. Nelson for all of his guidance over the last 18 months. I am truly grateful to have had such an encouraging, supportive,

and passionate mentor as you. In my eyes, you are the truest form of what a scientist can be – innovative, creative, and revolutionary. It has been a true honor for me to be able to assist you as you navigate this world on a never-ending quest for the truth. Purely and simply, that is real science.

Dedication

I would like to dedicate my Master's thesis to my father Charlie Chung who, from a young age, instilled in me the value and importance of education.

Chapter 1: Introduction

The global population is aging at an unprecedented rate — such that by the year 2050, the population of those 65 years of age and older will have tripled and will comprise 15% of the total global population (Pew Research). This projection is alarming, as age is the greatest risk factor for chronic disease and associated with a marked decline in functional capacity and quality of life (Jakovljevic, 2017; Niccoli & Partridge, 2012; Torre et al., 2015). Strategies to avoid chronic disease, improve functional capacity, and optimize quality of life late in life are therefore critically important.

Loss of skeletal muscle function is a key contributing factor to the decline in functional capacity and quality of life in the elderly (S. V. Brooks & Faulkner, 1994; Buskirk & Hodgson, 1987; McCarter, 1990). Work from the Baltimore Longitudinal Study has clearly documented age related declines in "muscle quality", defined as a reduction in force production to limb cross-sectional area (Chiles Shaffer et al., 2017; Moore et al., 2014). Indeed, advancing aging is associated with several adverse skeletal muscle adaptations, which are summarized in **Table 1**.

Table 1. Overview of skeletal muscle adaptations associated with aging in humans.

Author Name	Subject Age (mean ± SD in years)	Measurements Made	Primary Findings
(A.-M. Joseph et al., 2012)	75±1	Bioenergetics	Key metabolic regulators significantly reduced (50%) with aging regardless of physical activity levels
(A. R. Coggan et al., 1993)	58–68 years of age	Morphology	Muscle CSA and volume lower in older men; metabolic response to exercise also impaired, even when corrected for muscle mass in both trained and untrained elderly group
(Conley, Jubrias, Cress, & Esselman, 2013)	68.8 ± 2.4	Bioenergetics	Aging linked to reduced power output per O_2 uptake

(Delmonico et al., 2009)	70-79 (baseline)	Morphology	Intramuscular fat significantly increased in both aging men and women regardless of changes in weight
(Hütter et al., 2007)	75.0 ± 5.7 (men); 75.0 ± 3.6 (women)	Biochemistry	Increased ROS production, presence of lipofuscin with aging
(Janssen, Heymsfield, & Ross, 2002)	70 ± 7 (men); 71 ± 7 (women)	Morphology	Prevalence of sarcopenia apparent between 30-60; sarcopenia is greater in women ≥ 60 years
(Larsson, Grimby, & Karlsson, 1979)	60-70 years of age	Morphology	Decreased proportion and atrophy of type II fibers; subsequent decrease in muscular strength
(Lexell, Taylor, & Sjöström, 1988)	70-83 years of age	Morphology	Post-mortem analysis of the vastus lateralis in men 15-83 years of age show a progressive decline in muscle mass caused by loss of muscle fibers, reduction in type II muscle fiber size
(Nilwik et al., 2013)	71 ± 1	Morphology	Quadriceps CSA significantly smaller in older men, type II muscle fiber size also reduced
(Roberts et al., 2018)	61-71 years of age	Morphology; Bioenergetics	Type II fiber distribution lower with aging, with potential age-sex interaction
(Short et al., 2005)	60-89 years of age	Bioenergetics	Mitochondrial ATP production rate reduced in healthy aging adults, linked to reduction of mitochondrial protein content and activity
(Trounce, Byrne, & Marzuki)	16-92 years of age	Biochemistry	Respiration rate and enzymatic activity is reduced with aging, suggesting a decline in mitochondrial oxidative capacity

Table 1 acronyms: ATP = adenosine tri-phosphate; CSA = cross-sectional area; ROS = reactive oxygen species;

Most notably, aging is associated with a reduction is skeletal muscle mass (Lexell, 1995; Park, Brown, Park, Cohn, & Chance, 1988), together with extramyocellular fat infiltration and diffuse myocellular fibrosis (Roberts et al., 2018) Moreover, aging causes a fiber-type shift, characterized by a reduction in type II (fast-twitch) muscle fibers (Kosek, Kim, Petrella, Cross, & Bamman, 2006; Lexell, 1995), and an increase in the proportion of type I (slow-oxidative) muscle fibers (Jakobsson, Borg, & Edström, 1990). Finally, and most pertinent to this thesis, aging is believed to impair skeletal muscle bioenergetics (Chabi et al., 2008; A. R. Coggan et al., 1993; Gouspillou et al., 2014; Hepple, 2014; Kerner, Turkaly, Minkler, & Hoppel, 2001; Marzetti et al., 2008; McCully, Fielding, Evans, Leigh, & Posner, 1993; Papa, 1996). Indeed, aging is associated with both structural (Seo et al., 2016) and enzymatic mitochondrial changes, which may compromise skeletal muscle oxidative capacity (Hütter et al., 2007; A.-M. Joseph et al., 2012; A. M. Joseph, Adhihetty, & Leeuwenburgh, 2016; Trounce et al.).

Measuring skeletal muscle oxidative capacity *in vivo*

Until recently, the assessment of mitochondrial function has been limited to invasive muscle biopsies and/or expensive and time-consuming magnetic resonance spectroscopy (MRS) techniques, which limit patient access. Functional evaluation of skeletal muscle oxidative metabolism has also been assessed by pulmonary oxygen uptake (Grassi et al. 1996; Rossiter et al. 1999); however, this assessment is indirect, and technically challenging. Recent advancements in near-infrared spectroscopy (NIRS), provide a robust, clinical platform to noninvasively assess muscle oxygen consumption/oxidative function across a wide range of muscles and disease states (McCully et al. 2011; Brizendine et al. 2013; Erickson et al. 2013; Ryan et al. 2013a,b,c, 2014a,b; Southern et al. 2014, 2015; Adami and Rossiter 2017). Accordingly, a standardized NIRS approach (Ryan, Brophy, Lin, Hickner, & Neufer, 2014; Ryan, Southern, Reynolds, & McCully, 2013) has

been validated and well-received by researchers and participants for its clinical utility, cost-effectiveness, ease of use, and non-invasiveness. This novel approach utilizes a series of rapid, supra-systolic arterial cuff occlusions to measure post-exercise muscle oxygen consumption recovery kinetics (i.e., mitochondrial function), which is analogous to the recovery of phosphocreatine using ^{31}P-MRS (Ryan et al. 2013c). In preparation for the experiments to be performed as part of this thesis, I established this novel technique in the Applied Physiology and Advanced Imaging Laboratory at the University of Texas at Arlington (**Appendix A**).

Is skeletal muscle oxidative capacity impaired with age?

Several studies have shown reduced skeletal muscle oxidative capacity with advanced age (A. Coggan et al., 1992; McCully et al., 1993; Petersen et al., 2003; Short et al., 2005; Tonkonogi et al., 2003). For example, Conley et al. (Conley, Jubrias, & Esselman, 2000) using phosphorus nuclear magnetic resonance NMR (^{31}P NMR) in the human vastus lateralis muscle found that compared to young subjects aging individuals had ~50% reduction in muscle oxidative capacity. These findings have since been corroborated with the same technique in the same muscle group (Short et al., 2005), and support the conclusion that advanced age leads to a reduction in muscle oxidative capacity. This conclusion has been challenged, however, by the Kent Laboratory at the University of Massachusetts, suggesting that the reduction in skeletal muscle oxidative capacity found with age in locomotor muscles is far more dependent on the physical activity level than age (Brierley, Johnson, James, & Turnbull, 1996; Kent-Braun & Ng, 2000; Lanza, Befroy, & Kent-Braun, 2005; Lanza, Larsen, & Kent-Braun, 2007; Larsen, Callahan, Foulis, & Kent-Braun, 2012; Rasmussen, Krustrup, Kjaer, & Rasmussen, 2003; Rimbert et al., 2004). This interpretation has also been reported by at least one other laboratory (Chilibeck et al., 1998). Taken together, it remains unclear what independent role, if any, aging has on skeletal muscle oxidative capacity.

Moreover, the vast majority of previous investigations in this area have focused on a relatively young population of seniors (50-65 years of age), who are surprisingly healthy (i.e. non-representative of general aging population in the United States). Accordingly, Aim 1 (Chapter 2) of this thesis sought to examine the influence of age on skeletal muscle oxidative capacity in non-locomotor muscle, and include an elderly population > 65 years of age with common cardiovascular risk factors. To avoid the limitations associated with muscle biopsies or ^{31}P NMR, we adopted the NIRS-based approach previously described.

Can skeletal muscle oxidative capacity be improved in the elderly?

While exercise training has been shown to improve muscular strength, increase the percentage of glycolytic muscle fibers, and increase overall muscle mass in the elderly (Frontera, Meredith, O'Reilly, Knuttgen, & Evans, 1988; Welle, Totterman, & Thornton, 1996), the effect of exercise training on skeletal muscle oxidative capacity remains equivocal.

Table 2 highlights the research conducted in aging individuals (defined as ≥ 60 years of age) examining the effects of exercise training in skeletal muscle and/or skeletal muscle oxidative capacity. From this summary, it is clear that the majority of studies have focused on muscular mass, muscular strength, and/or muscle fiber type shifts. To my knowledge, only five studies have assessed skeletal muscle oxidative capacity following exercise training in the elderly, producing mixed results (Bell et al., 2001; Irving et al., 2015 Murias et al., 2010; Southern et al., 2015). For example, Murias et al. (Murias, Kowalchuk, & Paterson, 2010) found that elderly men are able to see improvements in skeletal muscle oxidative capacity from endurance training in as little as three weeks. Bell et al. found nine weeks of single-leg endurance training improved VO_2 kinetics in the five elderly men studied (Bell et al., 2001). Irving et al. (Irving et al., 2015) found that eight weeks of either resistance, endurance, or combination training improved oxidative capacity, with

combined training showing the most benefit. Moreover, Southern et al. (Southern et al., 2015) found that four weeks of endurance training in the forearm was able to improve skeletal muscle oxidative capacity in relatively healthy elderly subjects, but not elderly subjects with heart failure. In contrast to the positive results mentioned however, Flack et al., who found that while 12 weeks of resistance training improved muscular strength, it did not improve skeletal muscle oxidative capacity. Taken together, several knowledge gaps remain. Similar to our critique in the previous section, the majority of work in this area has been conducted on a relatively younger population of seniors. Moreover, the majority of subjects in these prior investigations were men, raising the question about sex specific differences, Accordingly, Aim 2 (Chapter 3) of this thesis tested the hypothesis that exercise training would improve skeletal muscle oxidative capacity in elderly participants beyond the six decade of life. Care was taken to include a majority of female participants.

Table. 2. Overview of exercise training studies focusing on skeletal muscle and/or oxidative capacity in the elderly.

Author Name	Subject Age (mean ± SD in years)	Length of Training	Type of Training	Primary Findings
Skeletal muscle composition/strength				
(Andersson et al., 2017)	71 ± 1	8 weeks	RTG	8 weeks RTG increased muscular strength, type II fiber amount, and mitochondrial protein presence
(Bechshøft et al., 2017)	86.9 ± 3.2	12 weeks	RTG	12 weeks RTG in combination with protein supplementation increased quadriceps CSA 1.5±0.7 cm^2, muscular strength 10-15%

(N. Brooks et al., 2007)	66 ± 2	16 weeks	RTG	16-week RTG increased type I and II CSA in older Hispanic adults with type 2 diabetes
(Burich, Teljigović, Boyle, & Sjøgaard, 2015)	63.2 ± 4.7	12 weeks	ETG; CTG	MVC increased in both ETG and CTG groups, with the GTG see the most improvement
(Chen, Chung, Chen, Ho, & Wu, 2017)	65-75 years	8 weeks	ETG; RTG; CTG	8 weeks of exercise training regardless of modality increased muscular mass in older adults with sarcopenic obesity
(Conlon et al., 2017)	70.9 ± 5.1	22 weeks	RTG	Muscle CSA and peak power improved after 22-week RTG intervention
(Eckardt, 2016)	65-80	10 weeks	RTG	Lower-body RTG on both stable and unstable surfaces resulted in an improvement of muscular strength
(Englund, Sharp, Selsby, Ganesan, & Franke, 2017)	65.1 ± 6.7 (low intensity); 64.5 ± 2.4 (high intensity)	6 weeks	RTG (low, high intensity)	High-velocity RTG improved muscular strength more than that of low-velocity RTG, linked to increases in MyHC-IIa mRNA transcription
(Fiatarone et al., 1990)	90 ± 1	8 weeks	RTG	Muscular strength improved significantly (174% +/- 31%), associated with an increase in muscle area
(Fiatarone et al., 1994)	87.1 ± 0.6 (including controls)	10 weeks	RTG	RTG improved muscular strength and thigh muscle CSA
(Frontera et al., 1988)	60-72	12 weeks	RTG	12 weeks RTG increased total muscle area, with increases in both type I, II muscle fiber areas
(Hong, Kim, Kim, & Kong, 2017)	82.2 ± 5.6	12 weeks	RTG	Home-based RTG increased both total and lower limb muscle mass
(Ikenaga et al., 2017)	70.8 ± 4.0 (includes controls)	12 weeks	ETG	Low-intensity ETG reduced intermuscular adipose tissue and exercise tolerance
(Kosek et al., 2006)	60-75	16 weeks	RTG	Type II size increased with RTG, with potential age-sex interactions

(Liao et al., 2017)	67.3 ± 5.2	12 weeks	RTG	12 weeks RTG in older women significantly improved lean tissue mass and muscle quality (muscular strength:mass ratio)
(Melov, Tarnopolsky, Beckman, Felkey, & Hubbard, 2007)	70.5 ± 5.1	26 weeks	RTG	RTG significantly improved muscular strength and gene expression related to mitochondrial function
(Nilwik et al., 2013)	71 ± 1	6 months	RTG	Though muscle CSA significant smaller in older men, resistance training increased both CSA and fiber size (24 ± 8%, 100 ± 3%, respectively)
(Njemini et al., 2017)	68 ± 5	12 weeks	RTG (high; low)	Both elderly men and women increased in strength after 12 weeks of RTG, with women showing the greatest relative improvement due to lower baseline strength despite similar pre-training physical activity levels
(Osuka et al., 2017)	65-79	12 weeks	RTG; CTG	In combination with calcium supplementation, both RTG and CTG improved muscle mass, strength, and physical performance
(Petrella, Kim, Cross, Kosek, & Bamman, 2006)	63.7 ± 1	16 weeks	RTG	In young and elderly men and women, young men experienced two-fold greater hypertrophy than other age-sex groups, likely related to higher mechanogrowth factor and testosterone levels
(Santos et al., 2017)	69.6 ± 6.4	8 weeks	RTG	8 weeks of RTG in older women increased muscular strength without an increase in skeletal muscle mass
(Snijders et al., 2017)	71 ± 1	24 weeks	RTG	Significant increase in type II muscle fiber size but not change in type I or II muscle fiber capillarization after 24 weeks of RTG

(Solberg et al., 2013)	74.3 ± 4.6	13 weeks	ETG; RTG; FTG	Lean body mass improved with RTG, ETG, and FTG; FTG and STG both showed improvements in muscular strength
(Stec et al., 2016)	60-75 years	4 weeks	RTG	4 weeks of RTG produced heterogeneous responses in type II fiber size (no response, moderate, or extreme response)
(Torres et al., 2017)	60-90 years	16 weeks	RTG	16 weeks of RTG in combination with protein-rich diet improves lower limb muscle strength but not lean tissue mass

Skeletal muscle oxidative capacity

(Bell et al., 2001)	77 ± 7	9 weeks	ETG	9 weeks of ETG improved VO_2 kinetics in trained lower limbs of five elderly men and increased metabolic enzymatic activity, indicating improvement in O_2 utilization
(Flack et al., 2016)	64.8 ± 4.1	12 weeks	RTG	12 weeks of RTG increased muscular strength (34%) but did not increase metabolic enzymatic activity
(Irving et al., 2015)	70 ± 1 (both ET and RT)	8 weeks	ETG; RTG; CTG	CTG produces more robust improvements to oxidative capacity than ETG or RTG alone
(Murias et al., 2010)	68 ± 7	12 weeks	ETG	Improved local O_2 delivery to muscle VO_2 in both young and elderly men seen in as few as three weeks of ETG
(Southern et al., 2015)	61 ± 4.9 (both non- and exercise group)	4 weeks	ETG	4-week handgrip training program improved oxidative capacity by 50% in control group; no improvement found pre- and post-training in HF group

RTG = resistance training; ETG = endurance training; CTG = combined (endurance + resistance) training; FTG = functional training; HF = heart failure

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Chapter 2: Near-infrared spectroscopy detects age-related differences in skeletal muscle oxidative function: promising implications for geroscience

A version of this chapter has been published: S Chung, R Rosenberry, TE Ryan, M Munson, T Dombrowsky, S Park, A Nasirian, MJ Haykowsky, MD Nelson. Near-infrared spectroscopy detects age-related differences in skeletal muscle oxidative capacity: Promising implications for bedside diagnostics. *Physiological Reports*. 6(3), 2018.

Age is the greatest risk factor for chronic disease, and is associated with a marked decline in functional capacity and quality of life (Niccoli and Partridge 2012; Torre et al. 2015; Jakovljevic 2017). A key factor contributing to loss of function in aging is the decline in skeletal muscle function (Buskirk and Hodgson 1987; McCarter 1990; Brooks and Faulkner 1994). While the exact mechanism(s) remains incompletely understood, age-related mitochondrial dysfunction is thought to play a major role (Coggan et al. 1993; McCully et al. 1993; Papa 1996; Kerner et al. 2001; Chabi et al. 2008; Marzetti et al. 2008; Gouspillou et al. 2014; Hepple 2014).

Until recently, the assessment of mitochondrial function has been limited to invasive muscle biopsies and/or expensive and time consuming magnetic resonance spectroscopy (MRS) techniques, which limit patient access. Functional evaluation of skeletal muscle oxidative metabolism has also been assessed by pulmonary oxygen uptake (Grassi et al. 1996; Rossiter et al. 1999); however, this assessment is indirect, and technically challenging. Recent advancements in near-infrared spectroscopy (NIRS), however, have provided a robust, clinical platform to noninvasively assess muscle oxygen consumption/oxidative function across a wide range of muscles and disease states (McCully et al. 2011; Brizendine et al. 2013; Erickson et al. 2013; Ryan et al. 2013a,b,c, 2014a,b; Southern et al. 2014, 2015; Adami and Rossiter 2017). For a detailed review, readers are directed to (Grassi and Quaresima 2016; Willingham and McCully 2017). This novel approach utilizes a series of rapid, supra-systolic arterial cuff occlusions to measure postexercise muscle oxygen consumption recovery kinetics (i.e., mitochondrial function), which is analogous to the recovery of phosphocreatine using ^{31}P-MRS (Ryan et al. 2013c). Whether this approach can be used to assess age-related mitochondrial dysfunction remains untested. Addressing this fundamental question is critical to advance current clinical assessment practices and/or the design of large clinical trials focused on age-related diseases.

The purpose of this study was therefore to compare skeletal muscle oxidative function between two distinct age groups (<35 vs. >65 years) using the aforementioned NIRS approach. We hypothesized that aging would prolong postexercise muscle oxygen consumption recovery kinetics (i.e., mitochondrial function) compared to young individuals, demonstrating the clinical utility of this novel imaging approach.

Methods

Participants

A total of 39 participants between the ages of 20 and 80 years of age were recruited from the local Dallas-Fort Worth community. The participants were divided into two groups based on age: young (18–30 years) and elderly (60–85 years). None of the young participants had any history of cardiovascular, metabolic, or neurological disease. Some of the aging individuals had history of hypertension (n = 12) and hypercholesterolemia (n = 10); however, none had overt heart, metabolic, or neurological disease, or were current smokers. All subjects presented to the lab in a fasted state, having abstained from alcohol, caffeine, and vigorous exercise for at least 24 h. All subjects provided written informed consent before being enrolled to participate in this study. The study was approved by the Institutional Review Board at the University of Texas at Arlington, and conformed to the standards set by the latest version of the Declaration of Helsinki.

Activity level was self-reported. Subjects were asked if they performed regular exercise, as well as frequency, duration, and type. Activity level was then quantified by multiplying the frequency by duration and reported as minutes per week. The intensity of exercise was rated as follows: walking was defined as mild-to-moderate, whereas cycling or running was defined as moderate-to-vigorous.

Experimental protocol

Post-exercise muscle oxygen consumption (*skeletal muscle oxidative capacity*) was assessed as previously described (Ryan et al. 2013c, 2014a,b). Briefly, each subject was positioned in the supine position with their non-dominant hand extended to comfortably reach a handgrip dynamometer (Stoelting 56380; Stoelting Co., Wheat Lane, IL). A noninvasive dual-wavelength NIRS optode (OxiPlex TS, Model 95205; ISS, Champaign, IL) was placed longitudinally over the muscle belly of the flexor digitorum profundus, the main muscle responsible for handgrip exercise. This non-locomotor muscle was chosen to avoid influence of lower limb activity discrepancies likely found in these two populations (i.e., young vs. elderly). Oxyhemoglobin and deoxyhemoglobin concentration was measured using a single channel consisting of eight laser diodes emitting at wavelengths of 690 and 830 nm (four at each wavelength). The laser diodes and photomultiplier were contained in a light plastic probe consisting of two parallel rows of emitter fibers and one detector fiber bundle comprising source-detector separations of 2.0, 2.5, 3.0, and 3.5 cm for both wavelengths. The frequency modulation of laser intensity was 100 MHz. The NIRS optode was placed firmly against the skin, held in place with a Velcro strap, and then encased in a black cloth to block the entry of light near the optical sensor. A blood pressure cuff (Hokanson SC5, D. E. Hokanson Inc, Bellevue, WA) was placed on the upper arm of the exercising hand; the cuff was powered by a rapid cuff inflator (Hokanson E20).

As illustrated in Figure 1, following a brief baseline period, a 5 min arterial cuff occlusion was applied in order to establish each subject's desaturation reserve. After stabilization of oxygenated and deoxygenated hemoglobin, participants were instructed to perform a brief bout of isometric handgrip exercise (at 50% of each individual's maximal voluntary contraction, or MVC) until muscle oxygen saturation dropped by ~50% (~10–30 sec). After exercise cessation, the

following series of rapid cuff inflations were employed in order to form a skeletal muscle oxidative capacity recovery curve: 5 sec on/5 sec off for inflations #1–6, 7 sec on/7 sec off for inflations #7–10, 10 sec on/15 sec off for inflations #11–14, and 10 sec on/20 sec off for inflations #15–18. Once oxygenated and deoxygenated hemoglobin levels returned to baseline levels (typically between 2 and 5 min), the protocol was repeated and the average of at least two tests was reported.

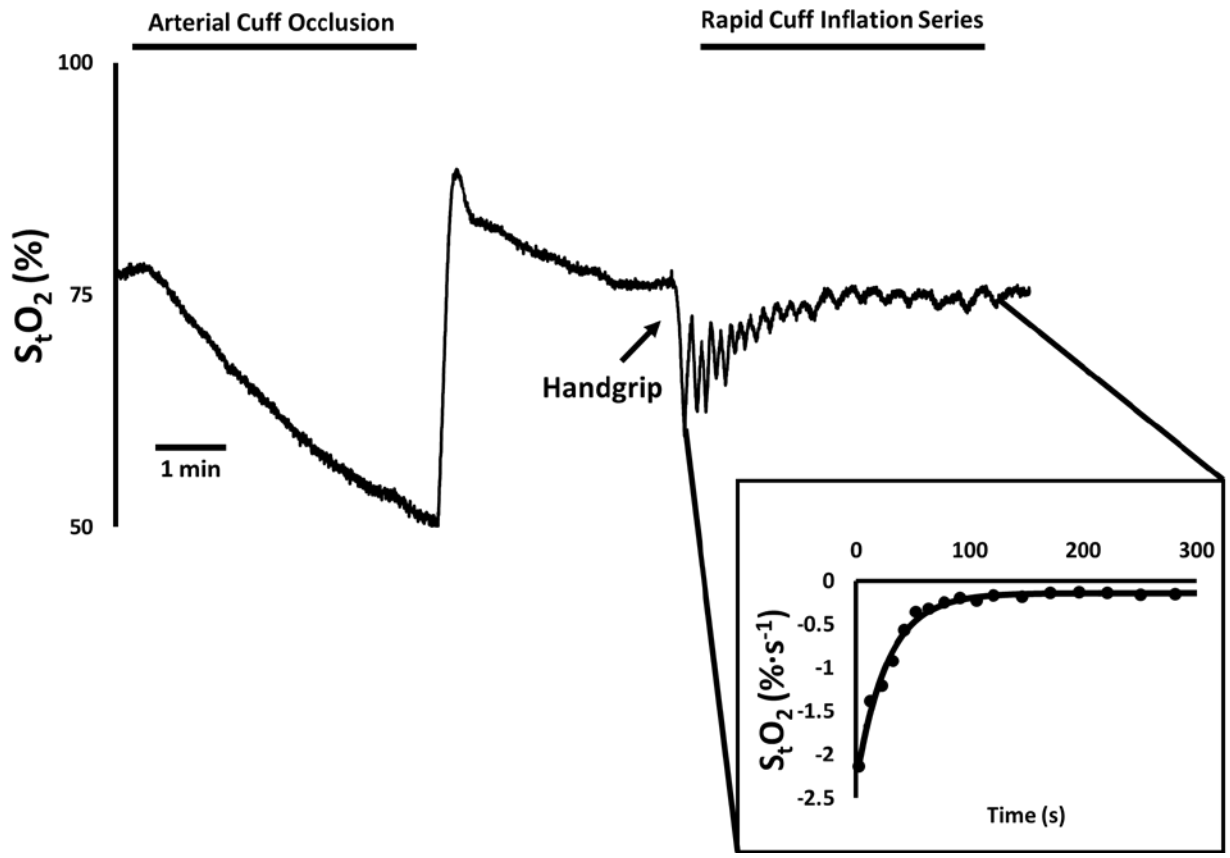

Figure 1. Representative data tracing from an individual subject showing a typical muscle oxygenation test. Oxygenated and deoxygenated hemoglobin were measured by near-infrared spectroscopy over the flexor digitorum profundus. Data are reported as tissue saturation (i.e., S_tO_2). Each test began with a brief baseline period, followed by a five minute cuff occlusion in order to establish the desaturation reserve. Subjects then performed a brief bout of isometric handgrip exercise at 50% of maximal voluntary contraction, followed by a series of rapid cuff occlusions. The slope of each post-exercise cuff occlusion was then measured, plotted against time, and fit to a monoexponential equation to calculate the muscle oxygen consumption recovery kinetics (as shown in the data insert).

Calculation of skeletal muscle oxidative capacity and its recovery rate

The calculation of skeletal muscle oxidative capacity was expressed by the slope of change in the tissue saturation (S_tO_2) signal (oxygenated hemoglobin [O_2Hb]/(oxygenated hemoglobin [O_2Hb] + deoxygenated hemoglobin [HHb]) × 100). The post-exercise recovery measurements of *skeletal muscle oxidative capacity* were fit to the following monoexponential curve, as previously described (Ryan et al. 2013c, 2014a,b) using commercially available software (OriginPro, OriginLab, Corp., Northampton, MA):

$$y = \text{End} - \Delta \text{ x } e^{-kt}$$

where "y" is the relative tissue saturation during cuff inflation, "End" represents the tissue saturation value immediately following the cessation of exercise; delta ("Δ") signifies the change in tissue saturation from rest to the end of exercise; "k" is the fitting rate constant; "t" is time. The initial rate of oxygen consumption was defined as the first measurement immediately after exercise cessation.

Statistical analyses

Statistical analysis was performed using SigmaPlot 13.0 (Systat Software, Inc. San Jose, CA). Data are expressed as a mean ± standard error unless otherwise specified. Normally distributed data were compared using a Student's t-test. If data were not normally distributed, a Mann–Whitney Rank Sum Test was used to compare group differences. Statistical analysis was not performed on activity level given the gross differences in sample size for the three predetermined exercise intensities.

Results

Of the 39 participants recruited, data analysis was possible in all but seven of the subjects. Data from five elderly participants and two young participants were excluded due to technical limitations (n = 3) or poor NIRS data quality (n = 4). Individual characteristics for the 32 who successfully completed the study are shown in Table 1. There were no adverse events or contraindications to testing.

Table 1. Subject characteristics

	Young	Old	P Value
n	13	14	---
Male/Female	8/5	3/11	---
Age (yrs)	25 ± 4	73 ± 4	<0.001
Height (cm)	172.3 ± 8.7	166.8 ± 8.9	0.112
Weight (kg)	74.8 ± 12.9	76.7 ± 15.4	0.736
BMI	25.1 ± 2.9	27.7 ± 6.1	0.170
MVC (kg)	38.6 ± 12.7	24.5 ± 6.0	<0.001
Activity Level			
Sedentary, min/week (n)	--- (1)	--- (0)	---
Mild to Moderate, min/week (n)	135 ± 106 (2)	182 ± 39 (11)	---
Moderate to Vigorous, min/week (n)	263 ± 234 (10)	435 ± 256 (3)	---
Medications			
β-blocker (n)	---	1	---
ACE-Inhibitor (n)	---	1	---
ARB (n)	---	3	---

Thyroid Hormone (n)	---	3	---
Statin (n)	---	3	---
NSAIDs/blood thinners (n)	---	5	---
Immunosuppressant (n)	---	1	---
α_2 agonist (n)	---	1	---

A typical NIRS recovery kinetics test is illustrated in Figure 1. Consistent with our hypothesis, the post-exercise muscle oxygen consumption recovery kinetics — analogous to the recovery of phosphocreatine using ^{31}P-MRS (Ryan et al. 2013c) — were significantly prolonged in the elderly participants compared to younger individuals (51.8 ± 5.4 sec vs. 37.1 ± 2.1 sec, p = 0.04; Fig. 2). The average r^2 value of the fit monoexponential equation was 0.95, with values ranging from 0.91 to 0.99.

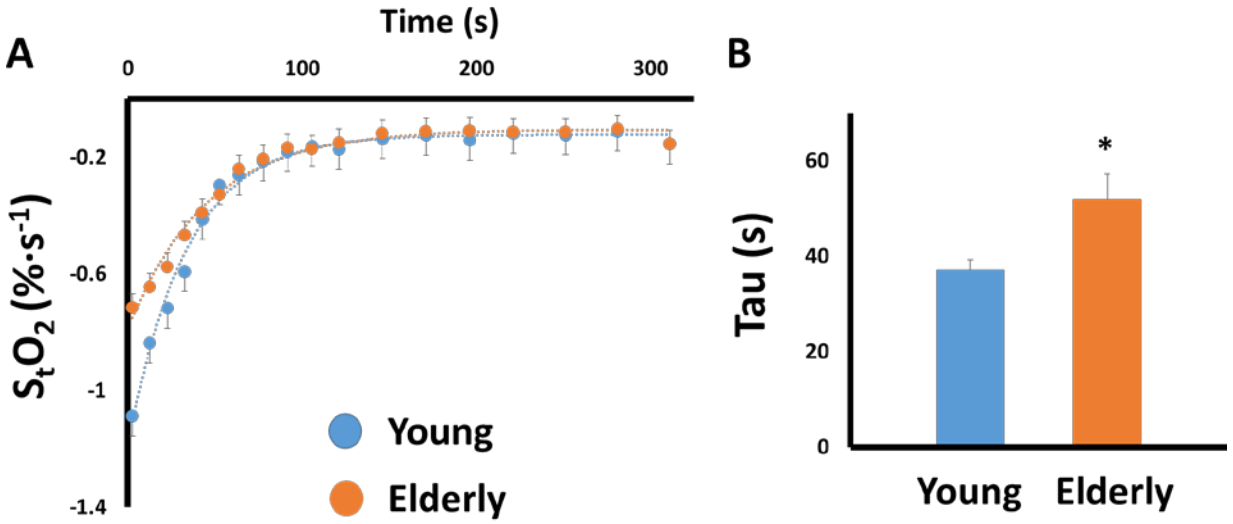

Figure 2. (A) Average post-exercise muscle oxygen consumption recovery data for young and elderly participants. Note the slower rate of recovery in the elderly compared to the young, summarized in panel (B).

Discussion

To the best of our knowledge, this is the first study to directly assess the effects of age on mitochondrial function using the rate constant for the recovery of skeletal muscle oxidative capacity measured with NIRS. The major novel finding of this study was that even in non-locomotor skeletal muscle, age significantly prolonged post-exercise muscle oxygen consumption recovery kinetics. This data highlights an age-related decline in muscle oxidative function and establishes the proof-of-concept for a new noninvasive, low-cost methodology for future clinical application in gerontology.

Only one other study utilizing this specific NIRS approach has measured muscle oxidative function in elderly subjects (Southern et al. 2015); however, this study did not directly assess age-related differences in muscle oxidative capacity (i.e., data were not compared to young control group). Instead, the elderly subjects studied in this prior investigation served as a healthy control group for a group of patients with heart failure with reduced ejection fraction. Moreover, the elderly participants included in this prior investigation were ~10 years younger than our elderly cohort. Despite modest differences in the type of forearm exercise performed and near-infrared technology used, combining these two data sets portrays a progressive age-related impairment in muscle oxidative function. Of note, our elderly participants appear closer to heart failure patients than young healthy controls or elderly participants ~10 years younger (**Table 2**).

Table 2. Comparison of the present muscle oxygen consumption recovery rate constant with previously published reports by the same technique in varying age groups

Reference/data set	Sample size (n)	Age of participants (years ± SD)	Tau (τ) (sec)
Present data (young)	17	25 ± 4	37
Southern et al. (2015) (elderly)	23	61 ± 5	38
Southern et al. (2015) (elderly + heart failure)	16	65 ± 7	46
Present data (elderly)	15	73 ± 4	52

That age was associated with a marked reduction in muscle oxidative function is consistent with previous studies using more established techniques (Coggan et al. 1993; McCully et al. 1993; Rooyackers et al. 1996; Conley et al. 2000; Short et al. 2005). For example, Conley et al. (2000) found aging to impair muscle oxidative function by ~50% compared to young subjects, which they attributed to both a decrease in mitochondrial volume and a lower oxidative function of the mitochondria. Unlike this prior investigation, however, the current data were obtained without the need for invasive muscle biopsies or expensive and time-consuming MRS techniques. Like Conley et al. (2000), we interpret the slower recovery rate of skeletal muscle oxidative capacity in our elderly participants to reflect an overall reduction in oxidative function.

The exact mechanism responsible for the observed age-related decline in skeletal muscle oxidative function is beyond the scope of the present investigation, but is likely multifactorial. Aging has indeed been associated with a marked reduction in mitochondrial-specific oxidative enzymes (Cooper et al. 1992; Boffoli et al. 1994; Rooyackers et al. 1996), mitochondrial DNA mutations (Cooper et al. 1992; Boffoli et al. 1994; Michikawa et al. 1999), oxidative damage by

reactive oxygen species (Papa 1996), reduced synthesis of mitochondrial proteins (Rooyackers et al. 1996), and increased ATP and/or oxidative cost during exercise (Ferri et al. 2007; Layec et al. 2014). However, several studies have challenged the concept that aging itself affects muscle bioenergetics (Kutsuzawa et al. 2001; Carlson et al. 2008; Tevald et al. 2014), and instead suggest that muscle oxidative function is far more dependent on the muscle group studied (Kent-Braun and Ng 2000; Lanza et al. 2005, 2007; Larsen et al. 2012) (locomotor vs. nonlocomotor) and/or the physical activity level of the individual (Brierley et al. 1996; Rasmussen et al. 2003; Rimbert et al. 2004; Larsen et al. 2012). That we specifically studied nonlocomotor muscle argues in favor of age-specific changes in mitochondrial/oxidative function, but cannot completely rule this possibility out. Likewise, our results contradict that of Kutsuzawa et al. (2001), who failed to find age-related differences in forearm phosphocreatine recovery kinetics using MRS. The elderly subjects studied herein however, were more than 10 years older (on average), with some history of cardiovascular risk factors. We therefore cannot rule out the possibility that age-related declines in skeletal muscle oxidative function may not simply be a continuous variable, and is likely influenced by a variety of factors (absolute age, cardiovascular risk, and activity level). Regardless of mechanism, however, this study highlights the simplicity, robustness, and clinical utility of this novel, noninvasive spectroscopic approach, and demonstrates its usefulness in gerontology.

While NIRS does not directly measure skeletal muscle oxidative function, it has previously been validated against both *in situ* muscle biopsy measurements (Ryan et al. 2014a) and *in vivo* MRS (Ryan et al. 2013c) with excellent agreement. We did encounter several instances where this NIRS-based approach was not successful. While half of these instances were attributed to nonbiological technical difficulties (i.e., patient compliance and instrument error), at least two instances appeared to be related to limb adiposity, which is known to affect NIRS (Ferrari et al.

2004). This will need to be accounted for if this technology is going to be incorporated into large clinical trials. Indeed, advancements in NIRS penetration depth, such as those previously described (Koga et al. 2015), may need to be considered. We also acknowledge that while our findings do not appear to be explained by the absence or presence of cardiovascular factors (i.e., hypertension or hypercholesterolemia) or specific medications used, we are in no way powered to fully test this relationship. Moreover, given the age and medical history of our elderly group, it is entirely possible that arterial stiffness may have influenced our results. Indeed, arterial stiffness can impact tissue hemoglobin oxygenation and therefore skeletal muscle oxidative recovery kinetics (Dipla et al. 2017). Because arterial stiffness was not measured in the present study, we cannot rule it out as a potential contributing factor.

In conclusion, we found that age was associated with a reduction in skeletal muscle oxidative function using a novel noninvasive and cost-effective spectroscopic approach. Taken together, we believe this NIRS-based approach holds great promise in gerontology (and clinical medicine in general) as a quantitative tool to assess skeletal muscle oxidative function at the bed side, in the clinic, and for the evaluation of therapeutic efficacy.

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Chapter 3: Exercise Training Does Not Improve Skeletal Muscle Oxidative Capacity in the Elderly

In addition to being the greatest risk factor for chronic disease, age is associated with a marked decline in quality of life and functional capacity (Jakovljevic, 2017; Niccoli & Partridge, 2012; Torre et al., 2015). Though the exact mechanism remains incompletely understood, skeletal muscle dysfunction plays a significant role (Brooks & Faulkner, 1994; Buskirk & Hodgson, 1987; McCarter, 1990). Together with skeletal muscle atrophy and adverse remodeling, aging also leads to mitochondrial dysfunction (Chabi et al., 2008; Coggan et al., 1993; Gouspillou et al., 2014; Hepple, 2014; Kerner, Turkaly, Minkler, & Hoppel, 2001; Marzetti et al., 2008; McCully, Fielding, Evans, Leigh, & Posner, 1993; Papa, 1996). Indeed, we and others have reported an age-related decline in oxidative capacity in both locomotor and non-locomotor skeletal muscle (Chung et al., 2018; Coggan et al., 1993; Conley, Jubrias, & Esselman, 2000; McCully et al., 1993). With the population of aging individuals increasing at unprecedented levels world-wide (Pew Research), strategies designed to attenuate age-related declines in skeletal muscle oxidative capacity are therefore desperately needed.

Exercise training later in life is indeed associated with positive improvements in muscular strength, muscle mass, and fiber-type distribution (Fiatarone et al., 1990; Frontera, Meredith, O'Reilly, Knuttgen, & Evans, 1988). It remains unclear however, whether exercise training can improve skeletal muscle oxidative capacity in aging individuals, as the limited amount of existing literature shows conflicting results (Flack et al., 2016; Irving et al., 2015; Southern et al., 2015). Exercise training early in life indeed promotes mitochondrial biogenesis through several well defined molecular pathways which appear to be equally responsive with advanced age (Moore et al., 2014). A limited number of studies exist however, investigating whether exercise training can improve skeletal muscle oxidative capacity, especially in elderly individuals beyond the sixth

decade of life. With the average age of life expectancy steadily increasing worldwide ("Global Health and Aging," 2011), this consideration has become even more important.

The purpose of this study was therefore to test the hypothesis that exercise training would improve skeletal muscle oxidative capacity in aging individuals beyond the sixth decade of life.

Methods

Participants

A total of 10 participants between the ages of 61-82 years of age were recruited from the local Dallas-Fort Worth community. None of the participants had overt cardiac, metabolic, or neurological disease. Some of the participants had history of hypertension (n = 3) and hypercholesterolemia (n = 4); however, at the time of the study, the hypertension and hypercholesterolemia was well controlled. General exclusion criteria included history of heart failure/heart attack/heart rhythm abnormality; history of fainting spells or seizures; pulmonary disease; known diabetes; current tobacco use; chronic infection; kidney disease; orthopedic or physical limitations that would prevent data collection or exercise training; or concurrent involvement in another exercise training program. All subjects provided written informed consent before being enrolled to participate. The study was approved by the Institutional Review Board at the University of Texas at Arlington, and conformed to the standards set by the latest version of the *Declaration of Helsinki*.

Activity level was self-reported. Participants were asked if they performed regular exercise, as well as the duration, frequency, and type of exercise. Activity level was then quantified by multiplying the duration by frequency, and was reported as minutes per week. The intensity of

exercise was qualified using the following criteria: walking was defined as mild-to-moderate, whereas running or cycling was defined as moderate-to-vigorous.

Experimental Protocol

As illustrated in **Figure 1**, participants attended the laboratory twice for baseline (pre-training) assessment of skeletal muscle oxidative capacity, to evaluate test-retest reliability and establish a true pre-training baseline. The two baseline visits occurred within 1-7 days of each other. Following the second baseline visit, subjects began a home-based four-week exercise-training program. To ensure compliance and evaluate training progression, subjects completed one of their weekly training sessions in the laboratory in the presence of an investigator. In addition, subjects tracked their training progress with a training logbook. Upon completion of the four-week exercise-training program, a final visit to the laboratory was completed to assess exercise training mediated changes in skeletal muscle oxidative capacity. The post-training assessment was completed within three days of their final exercise training session.

For each assessment visit, subjects presented to the lab in an overnight fasted state, having abstained from alcohol, caffeine, and vigorous exercise for at least 24 hours. Blood draws were performed at baseline to characterize the metabolic health of each participant.

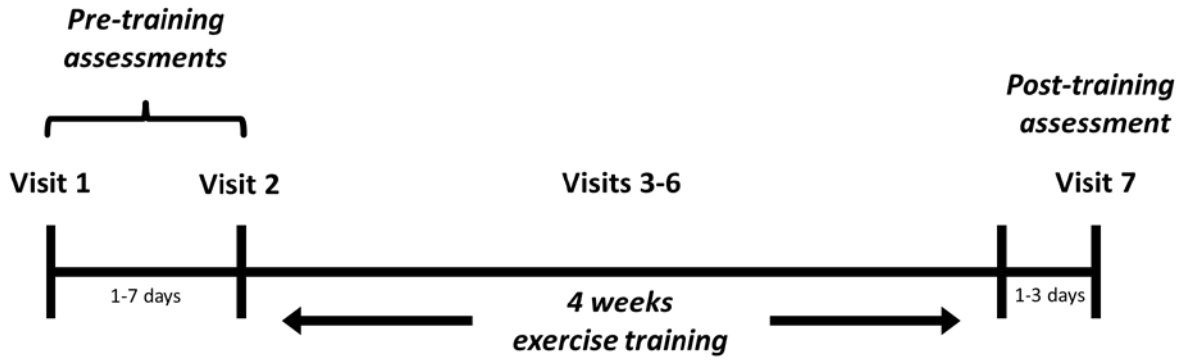

Figure 1. Participants completed two baseline pre-training assessments before undergoing four weeks of exercise training. Following exercise training, a post-training assessment was performed.

NIRS measurements of skeletal muscle oxidative capacity

Skeletal muscle oxidative capacity was assessed utilizing a non-invasive, well-validated near-infrared spectroscopy (NIRS) protocol (Rosenberry, Chung, & Nelson, 2018; Ryan, Brophy, Lin, Hickner, & Neufer, 2014; Ryan, Southern, Reynolds, & McCully, 2013). Each subject was placed in the supine position with their non-dominant hand extended to comfortably reach the handgrip dynamometer. A noninvasive dual-wavelength NIRS optode (OxiPlex TS, Model 95205; ISS, Champaign, IL) was placed longitudinally over the muscle belly of the flexor digitorum profundus — the main muscle responsible for handgrip exercise. We chose to assess non-locomotor forearm muscle because it is relatively superficial, and thus compatible with NIRS across a wide range of body type, and less influenced by locomotor activity level.

Oxygenated hemoglobin and deoxygenated hemoglobin concentration levels were measured using a single channel consisting of eight laser diodes emitting at wavelengths of 690 and 830 nm (four at each wavelength). The laser diodes and photomultiplier were contained in a light plastic probe consisting of two parallel rows of emitter fibers and one detector fiber bundle comprising source-detector separations of 2.0, 2.5, 3.0, and 3.5 cm for both wavelengths. The frequency modulation of laser intensity was 100 MHz. The NIRS optode was placed firmly against the skin, secured into place with a Velcro strap, and encased in a black cloth to block the entry of light near the optical sensor. A blood pressure cuff (Hokanson SC5, D. E. Hokanson Inc, Bellevue, WA) was placed on the upper arm of the exercising hand, with the cuff powered by a rapid cuff inflator (Hokanson E20).

Following a brief baseline period, a five minute arterial cuff occlusion was applied in order to establish each subject's desaturation reserve (**Figure 2**). After oxygenated and deoxygenated hemoglobin levels stabilized, participants were instructed to perform a brief bout of isometric

handgrip exercise at 50% of each individual's maximal voluntary contraction (MVC) until muscle oxygen saturation dropped by ~50% (~10–30 s). After exercise cessation, the following series of rapid cuff inflations were employed in order to form a skeletal muscle oxidative capacity recovery curve: 5 s on/5 s off for inflations #1–6, 7 s on/7 s off for inflations #7–10, 10 s on/15 s off for inflations #11–14, and 10 s on/20 s off for inflations #15–18. Once oxygenated and deoxygenated hemoglobin levels returned their respective baseline levels (~2-5 min), the protocol was repeated and the average of *at least* two tests was reported.

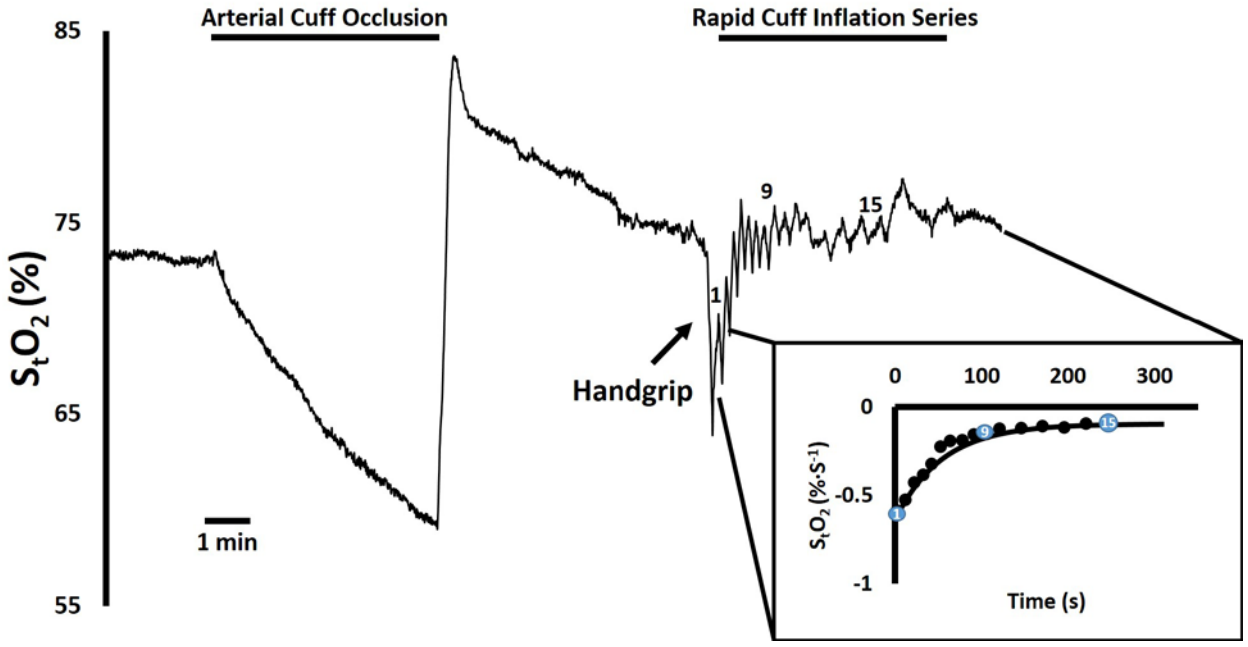

Figure 2. Representative tracing from one subject depicts a typical muscle oxygenation test obtained via NIRS. A NIRS optode placed over the flexor digitorum profundus measured oxygenated and deoxygenated hemoglobin levels, which were then used to calculate tissue saturation (S_tO_2). Each test began with a five minute cuff occlusion in order to establish the desaturation reserve. Subjects then performed a brief bout of isometric handgrip exercise (~10-30 s) at 50% of maximal voluntary contraction, which was then followed by a rapid cuff occlusion series.

Skeletal muscle oxidative capacity

Skeletal muscle oxidative capacity was defined as recovery rate of skeletal muscle oxygen consumption following a brief bout of handgrip exercise at 50% MVC. Data were expressed as the slope of change in the tissue saturation (S_tO_2) signal (oxygenated hemoglobin)/oxygenated hemoglobin [O_2Hb] + deoxygenated hemoglobin [HHb]) × 100). The post-exercise recovery measurements were fit to the following monoexponential curve, as previously described (Ryan et al., 2014; Ryan, Southern, Reynolds, et al., 2013) using commercially available software (OriginPro, OriginLab, Corp., Northampton, MA):

$$y = \text{End} - \Delta \text{ x } e^{-kt}$$

where "y" is the relative tissue saturation during cuff inflation, "End" represents the saturation value immediately following the cessation of exercise; delta ("Δ") signifies the change in saturation from rest to the end of exercise; "k" is the fitting rate constant; "t" is time.

Strength testing

Grip strength was assessed by having each participant complete a MVC with a handgrip dynamometer. For each assessment, subjects performed at least two MVCs, separated by at least 60 seconds, and the highest MVC was recorded. For the pre- and post-training assessments (visits 1, 2, and 7), MVC was performed using a Stoelting handgrip dynanometer (Stoelting 56380; Stoelting Co., Wheat Lane, IL), the same handgrip dynamometer used during the assessment of skeletal muscle oxidative capacity. During the exercise training weeks (visits 3-6), MVC was performed using a Trailite handgrip dynamometer — the handgrip dynamometer used at home for handgrip exercise training — in order to adjust the training intensity each week to ensure training progression.

Exercise training

Participants completed four weeks of handgrip exercise training. Handgrip training was performed five days a week (four days at home; one day in the laboratory) at 30% of each participant's MVC in accordance with previous exercise training studies utilizing the same NIRS protocol (Ryan, Southern, Brizendine, & McCully, 2013; Southern et al., 2015). For the first week of exercise training, participants performed 30 contractions, 20 times a day — for a total of 600 contractions each day. With each progressive week of exercise training, the number of contractions was gradually increased for a total of 600, 900, 1200, and 1500 grips per day, respectively (**Table 1**). MVC was reassessed at the beginning of each weekly laboratory visit and the participants' 30% MVC was adjusted accordingly. All exercise training was completed using a Trailite handgrip dynamometer (Trailite LSC100; LiteXpress GmbH, Ahaus, Germany). Exercise training compliance was verified using a weekly training log book.

Table 1. Exercise Training Program Overview

	# days per week	**% of MVC**	**Grip rate**	**# of contractions per bout**	**Rest period between bouts**	**Total # of bouts per day**	**Total grips per day**
Week 1	5	30%	1 per sec	**30**	1-2 min	20	**600**
Week 2	5	30%	1 per sec	**45**	1-2 min	20	**900**
Week 3	5	30%	1 per sec	**60**	1-2 min	20	**1200**
Week 4	5	30%	1 per sec	**75**	1-2 min	20	**1500**

Each participant underwent four weeks of handgrip exercise training at 30% of maximal voluntary contraction (MVC) in their non-dominant arm, five days a week. Four of the exercise days were completed at home, with one day of exercise completed in the laboratory. Each week,

MVC was reassessed and the total number of contractions was gradually increased, for a total of 600, 900, 1200, and 1500 grips per day of exercise, respectively.

Statistical analyses

Statistical analysis was performed using SigmaPlot 13.0 (Systat Software, Inc. San Jose, CA). Data are expressed as a mean ± standard error unless otherwise specified. To assess the day-to-day variability of measures, co-efficient of variation was calculated comparing baseline visit 1 and 2. The data collected on the second baseline visit (visit 2) was used to represent the pre-training measurements in comparison to the post-training measurements (visit 7). To compare the effect of exercise training on skeletal muscle oxidative capacity, normally distributed data were analyzed using a repeated measures one-way ANOVA. Normality was confirmed using a Shapiro-Wilk test and equal variance was confirmed using a Brown-Forsythe test. In the event that the data did not have equal variance, a Friedman test was used to analyze the data.

Results

Out of the 10 participants enrolled in the study, eight successfully completed the study in its entirety, including the four weeks of exercise training; the subject characteristics for the eight participants who successfully completed the study are shown in **Table 2**. Two participants were excluded during baseline testing upon identification of an abnormal heart rhythm. These individuals had no history of abnormal heart rhythm prior to enrollment and were referred to a cardiologist for further investigation.

The participants who successfully completed the exercise training were fully compliant with the exercise training protocol, as verified through the weekly exercise log books. There were no adverse events or contraindications to skeletal muscle oxidative capacity testing, or handgrip

exercise training in any of the participants. As expected, several participants reported muscle fatigue and soreness during the first week(s) of exercise training.

Table 2. Subject characteristics

	mean ± SD
n	8
Male/Female	2/6
Age (yrs)	73 ± 7
Height (cm)	163.9 ± 11.2
Weight (kg)	70.0 ± 11.6
BMI	25.9 ± 3.1
MVC, pre-training (kg)	26.0 ± 13.4
Metabolic Data	
Glucose, resting (mg/dL)	105 ± 16
Cholesterol, total (mg/dL)	171 ± 34
Triglycerides (mg/dL)	136 ± 60
HDL cholesterol (mg/dL)	54 ± 9
VLDL cholesterol (mg/dL)	27 ± 12
LDL cholesterol (mg/dL)	90 ± 23
Activity Level	
Sedentary, min/week (n)	--- (0)
Mild-to-moderate, min/week (n)	174 ± 68 (7)
Moderate-to-vigorous, min/week (n)	40 (1)

Medications

ACE-Inhibitor (n)	2
ARB (n)	1
Thyroid Hormone (n)	1
Statin (n)	3
NSAIDs/blood thinners (n)	3
Immunosuppressant (n)	1

ACE-inhibitor indicates an angiotensin-converting enzyme; ARB, angiotensin-receptor blocker; NSAID, non-steroidal anti-inflammatory drug. Blood collection was not possible in two subjects; thus metabolic data are reported for six of eight participants.

Test-retest reliability of skeletal muscle oxidative capacity

Our day-to-day reproducibility for skeletal muscle oxidative capacity, expressed as a coefficient of variation was 15%, or 6.6 seconds; comparable to *in vivo* magnetic resonance spectroscopy (MRS) and *in situ* muscle biopsy techniques.

Grip strength

On average, grip strength pre- and post-exercise training showed little change (24.5 ± 12.2 kg versus 26.1 ± 11.4 kg, respectively; p = 0.12; **Figure 3**). However, it is of interest to note that the two participants with the lowest beginning grip strength showed the greatest improvements in grip strength after the four-week exercise-training program (P1: 7 kg versus 13 kg, or 85.7% increase; P2: 13 kg versus 18 kg, or 38.5%).

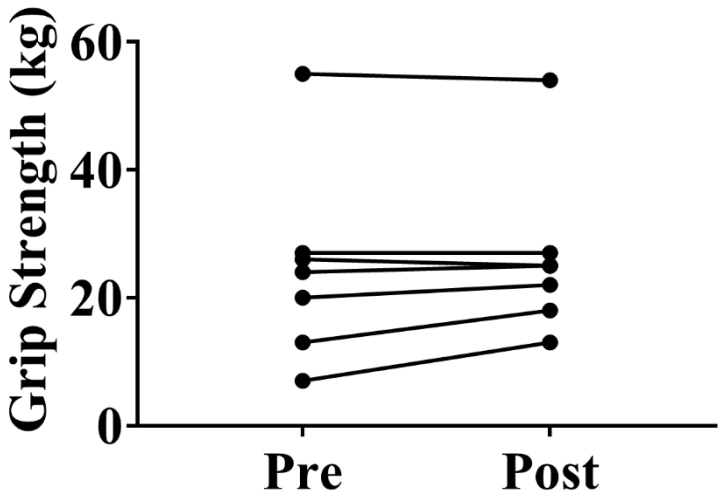

Figure 3. Overall, there was little change in grip strength before versus after four weeks of forearm exercise training (24.5 ± 12.2 versus 26.1 ± 11.4 kg, respectively; p = 0.12).

Skeletal muscle oxidative capacity

Representative skeletal muscle oxidative recovery curves from a single subject before and after exercise training are illustrated in **Figure 4A**. Consistent with these representative data, the "goodness of fit" for each subject's oxygen consumption recovery curve was also very good, with an average r^2 value of the fit monoexponential equation for all eight subjects was 0.97 (ranging from 0.94-0.99). **Figure 4B** illustrates the change in skeletal muscle oxidative capacity for each individual in response to exercise training. Overall, the response was quite variable, showing no appreciable change in skeletal muscle oxygen consumption recovery kinetics with four weeks of exercise training (44.6 ± 14.1 s versus 46.9 ± 11.3 s; p = 1.00). Individual data is reported for each individual in **Appendix C**.

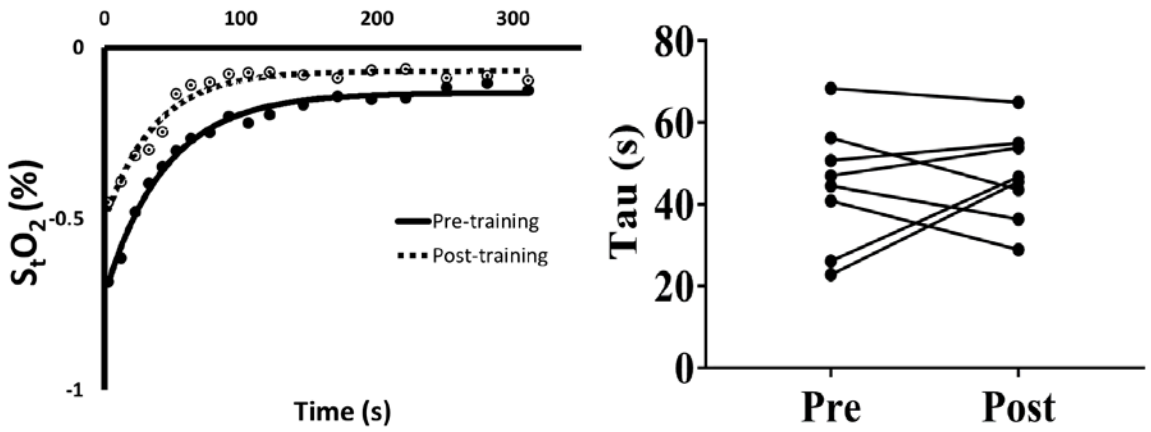

Figure 4. (**A**) Representative data tracing from an individual subject during a pre-exercise baseline assessment showing a typical muscle oxygenation test via NIRS over the flexor digitorum profundus. After subjects performed a brief bout of isometric handgrip exercise at 50% of maximal voluntary contraction, a series of 18 rapid cuff occlusions were applied. The slope of change in the tissue saturation (S_tO_2) signal during each cuff inflation was plotted, forming a monoexponential curve. (**B**) There was little difference in time to recovery, or tau, for participants before versus after four weeks of forearm exercise training (44.6 ± 14.1 s versus 46.9 ± 11.3 s, respectively; p = 1.00).

Discussion

Skeletal muscle oxidative capacity is reduced with age (Chung et al., 2018; Coggan et al., 1993; Conley et al., 2000; McCully et al., 1993). Whether exercise training can improve skeletal muscle oxidative capacity in aging individuals remains equivocal (Flack et al., 2016; Irving et al., 2015; Southern et al., 2015). We sought to address this major knowledge gap by training elderly individuals for four weeks. In contrast to our hypothesis, we observed no significant difference in skeletal muscle oxidative capacity.

It remains unclear why skeletal muscle oxidative capacity was not improved in this investigation. The exercise training program, intensity, and duration were identical to that previously performed by several other investigators, who showed a positive training adaptation (Ryan, Southern, Brizendine, et al., 2013; Southern et al., 2015). Specifically, Ryan, Southern, Brizendine, et al. were the first to report on the 4-week training program used in this investigation

(Ryan, Southern, Brizendine, et al., 2013). They observed a marked improvement in skeletal muscle oxidative capacity in a group of young, healthy volunteers. Of note, these investigators also used the same NIRS-based protocol for assessing skeletal muscle oxygen consumption recovery kinetics as was used in our investigation. In addition, Southern et al. have also reported positive oxidative skeletal muscle training adaptations in healthy elderly volunteers, also using the same training protocol and NIRS technique (Southern et al., 2015). These investigators did not observe similar positive training adaptations in patients with heart failure though, which they attributed to the health status of these participants. That we too did not observe a positive training adaptation in our elderly participants who were nearly a decade older than those in the Southern paper, and with greater cardiovascular risk factors, suggests a potential training threshold driven by age and/or health status.

If we accept that possibility that older individuals with cardiovascular risk factors have a different training threshold, then it is possible that increasing the training duration, exercise type, or a combination thereof is needed to impart positive training adaptations. For example, Irving et al. found that combining resistance and endurance exercise training for 8 weeks produced greater improvements in skeletal muscle oxidative capacity in elderly individuals, compared to either resistance-only or endurance-only (Irving et al., 2015). Likewise, Sparks et al. found that nine months of combined exercise training was more impactful in improving skeletal muscle metabolism in older individuals with type 2 diabetes than aerobic training or resistance training alone (Sparks et al., 2013).

Another possible explanation for our contrasting results, compared to previous training studies in gerontology, could be related to sex. Indeed, of the limited number of previous investigations exploring exercise training in the elderly, the majority of participants were male

(Flack et al., 2016; Frontera et al., 1988; Snijders et al., 2017; Southern et al., 2015). In contrast, our participants were predominantly female, and postmenopausal. While no study to date has directly compared the training adaptation for skeletal muscle oxidative capacity between pre- and post-menopausal women, there does appear to be a blunted training response for other end-point measurements such as muscular strength and type I muscle fiber cross-sectional area (Kosek, Kim, Petrella, Cross, & Bamman, 2006; Petrella, Kim, Cross, Kosek, & Bamman, 2006); however, these data are difficult to interpret because of the impact of age-sex interaction, as these studies included both young and older men and women by comparing each subset against their respective pre-training level(s). However, more work is admittedly needed in this area, as it remains unclear if training response differ between older men and women.

Accordingly, a genetic component may have contributed to our null findings. It has been previously shown that there is inter-individual variation in responses to exercise training to both endurance training and resistance training in regard to VO_{2max} (Bouchard et al. 1999; Pérusse et al, 2001) and strength and muscle mass (Wimore et al. 1998), respectively. However, a specific phenotypic model(s) has yet to be identified (Vellers et. al, 2018), in addition to the genetic component related specifically to aging and training adaptations. Further investigation into the genetic component of training adaptations is needed.

Another important consideration when interpreting our results, is the time between post-exercise training and post-training assessments. We purposely performed the post-training assessments within 24-48 hours after the final exercise training session to avoid deconditioning. While none of our participants reported delayed-onset muscle soreness (DOMS) or fatigue at the time of each post-training assessment, it remains possible that this short window of recovery may not have been sufficient to observe the maximal training response. Again, while this time window

was matched to that previously reported in younger participants (Ryan, Southern, Brizendine, et al., 2013; Southern et al., 2015) it is possible that a longer recovery window is needed in older participants, like those studied here.

In conclusion, we saw no significant change in grip strength or skeletal muscle oxidative capacity before and after four weeks of exercise training in those aged 60+ years. A longer exercise training program, with a variable training load, may provide better insight into whether or not handgrip training can successfully improve skeletal muscle oxidative capacity in those >60 years of age.

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Chapter 4: Discussion

Aging is associated with a wide range of functional limitations — many of which are related to decline in skeletal muscle function — ultimately reducing quality of living and limiting functional capacity in the elderly. Age-related skeletal muscle decline is often attributed to morphological changes which lower muscle mass and reduced overall tissue viability. Emerging evidence, including work presented in this thesis, suggests that aging is also associated with key biochemical and morphological changes which reduce skeletal muscle oxidative capacity. This is important because impaired skeletal muscle oxidative capacity can compromise one's capacity to perform exercise and activities of daily living, and also to recover from exercise and activities of daily living. With the aging population growing at an unprecedented rate, research in this area is critically needed to avoid such age-related adaptations, and/or reverse such changes if they already exist.

Assessment of skeletal muscle oxidative capacity has traditionally relied heavily on *in vivo* magnetic resonance spectroscopy (MRS) or *in situ* muscle biopsies; however, these methods are clinically inaccessible, costly, invasive, and/or reliant on skilled technicians. Recent advancements in near-infrared spectroscopy (NIRS) technology have made the assessment of skeletal muscle oxidative capacity much more cost-effective, non-invasive, and clinically accessible to more patient populations. In Chapter 2 (*Near-infrared spectroscopy detects age-related differences in skeletal muscle oxidative function: promising implications for geroscience*), we utilized this novel NIRS technique to show an age-related impairment in skeletal muscle oxidative capacity in non-locomotor muscle. These data help address a growing debate in the literature questioning whether advanced age independently affects skeletal muscle oxidative capacity, or if such impairments are merely related to age-related declines in physical activity level. That we found a marked

impairment in non-locomotor muscle in elderly participants compared to young healthy controls supports an age-related independent role.

Exercise training is known to improve skeletal muscle function and composition in the elderly, with the majority of improvements focused on muscular strength, muscular mass, and fiber type distribution. However, few studies have examined the impact of exercise training in regard to skeletal muscle oxidative capacity specifically. Moreover, these limited studies have produced conflicting findings, providing an unclear answer as to whether or not exercise training can positively influence skeletal muscle oxidative capacity. In Chapter 3, we addressed this knowledge gap by examining the impact of four weeks of exercise training on skeletal muscle oxidative capacity in non-locomotor muscle in elderly participants beyond the sixth decade of life. Our findings failed to show any improvement in either skeletal muscle oxidative capacity or muscle strength. We interpret our results to reflect an age/health status related training threshold. Indeed, our participants were ~10 years older than the majority of previous elderly participants studied. Moreover, we purposely recruited a population with expected cardiovascular risk factors in order to make our results more translatable to the general aging population.

Future studies are needed to control for potential sex-related differences between training adaptations between men and women. While we weighted our participant demographics towards the female sex, admittedly a balanced group design would be more impactful. Indeed, elderly men and women may experience a heterogeneous response to exercise training among a wide range of skeletal muscle-related variables, with older women showing a lesser response in type II muscle fiber size and overall hypertrophy than their age-matched male counterparts (Kosek et al., 2006; Petrella et al., 2006) Furthermore, Stec et al. (2016) discovered that it was predominantly elderly men that had a high response to resistance training, whereas the no-response and moderate-

response groups were balanced for sex. Thus, it is reasonable to say that a sex-related impact may be in play, and should be explored in future studies.

Additionally, the training stimulus may need to be altered between aging populations in order to avoid the potential of training-related thresholds related to health and/or age. As discussed in Chapter 1, Murias et al. (2010) discovered that young men adapted to endurance training quicker than that of their elderly counterparts, showing a clear age-related delay in the onset of training-related benefits. Additionally, Southern et al. (2015) showed an improvement in skeletal muscle oxidative capacity after four weeks of endurance training in cardiovascular risk-free elderly participants, whereas the heart failure group showed no such benefit. This suggests that training duration may need to be lengthened to account for age and/or health status. In future, it would be important to compare different training modalities (endurance vs. resistance vs. combination), and prolong the training stimulus.

Table of Appendices

Appendix A: A Methods Paper

A version of this appendix has been published: R Rosenberry, S Chung, MD Nelson. Skeletal muscle neurovascular coupling, oxidative capacity and microvascular function with near-infrared spectroscopy: ‘one stop shop’ clinical research assessment of cardioskeletal function. *J. Vis. Exp*. 132(e57317), 2018.

The hyperemic response to a brief period of tissue ischemia has emerged as a key non-invasive measure of (micro)vascular function. During occlusion of a conduit artery, downstream arterioles dilate in an effort to offset the ischemic insult. Upon release of the occlusion, the decreased vascular resistance results in hyperemia, the magnitude of which is dictated by one's ability to dilate the downstream microvasculature. While reactive hyperemia is a strong independent predictor of cardiovascular events (Huang et al., 2007; Suryapranata et al., 1994) and therefore a clinically significant endpoint, its functional significance to exercise tolerance and quality of life is less clear.

Indeed, dynamic exercise represents a major cardiovascular stress that demands a highly coordinated neurovascular response in order to match oxygen delivery to metabolic demand. For example, skeletal muscle blood flow can increase nearly 100-fold during isolated muscle contractions (Richardson et al., 1993), which would overwhelm the pumping capacity of the heart if such a hemodynamic response were extrapolated to whole-body exercise. Accordingly, to avoid severe hypotension, sympathetic (*i.e.*, vasoconstrictor) nervous activity increases to redistribute cardiac output away from inactive and visceral tissues and towards active skeletal muscle (Clifford & Hellsten, 2004). Sympathetic outflow is also directed to the exercising skeletal muscle (Hansen, Thomas, Jacobsen, & Victor, 1994); however, local metabolic signaling attenuates the vasoconstrictor response in order to ensure adequate tissue oxygen delivery (Fadel, Keller, Watanabe, Raven, & Thomas, 2004; Hansen, Thomas, Harris, Parsons, & Victor, 1996; Nelson et al., 2014; Nelson et al., 2015; Rosenmeier, Fritzlar, Dinenno, & Joyner, 2003; Thomas & Victor, 1998). Collectively, this process is termed functional sympatholysis (Remensnyder, Mitchell, & Sarnoff, 1962), and is imperative to the normal regulation of skeletal muscle blood flow during exercise. Since skeletal muscle blood flow is a key determinant of aerobic capacity — an

independent predictor of quality of life and cardiovascular disease morbidity and mortality (Kodama et al., 2009) — understanding the control of skeletal muscle blood flow and tissue oxygen delivery during exercise is of great clinical significance.

Oxygen delivery is only half of the Fick equation, however, with oxygen utilization satisfying the other half of the equation. Among the major determinates of oxygen utilization, mitochondrial oxidative phosphorylation plays an essential role in supplying adequate energy for cellular processes both at rest and during exercise. Indeed, impairments in muscle oxidative capacity can limit functional capacity and quality of life (Cabalzar et al., 2017; Tyni-Lenné, Gordon, Jansson, Bermann, & Sylvén, 1997; Westerblad, Place, & Yamada, 2010). Various measures are commonly used to provide an index of muscle oxidative capacity, including invasive muscle biopsies and expensive and time-consuming magnetic resonance spectroscopy (MRS) techniques.

Here, we propose a novel, non-invasive approach, using near-infrared spectroscopy (NIRS), to assess each of these three major clinical endpoints (reactive hyperemia, sympatholysis and muscle oxidative capacity) in a single clinic or laboratory visit. The major advantages of this approach are three-fold: First, this technique is easily portable, relatively low cost, and easy to perform. Current Doppler ultrasound approaches for measuring reactive hyperemia are highly operator-dependent — requiring extensive skill and training — and require sophisticated, high-cost, data acquisition hardware and post-processing software. Moreover, this could conceivably be introduced into the clinic and/or large clinical trials for bedside monitoring or testing therapeutic efficacy. Second, by virtue of the methodology, this technique focuses specifically on the skeletal muscle microvasculature, increasing the overall specificity of the technique. Alternative approaches using Doppler ultrasound focus entirely on upstream conduit vessels and infer changes

downstream, which can dampen the signal. Third, this technique is completely non-invasive. Skeletal muscle oxidative capacity is traditionally assessed with invasive and painful muscle biopsies, and functional sympatholysis may be assessed with intra-arterial injection of sympathomimetics and sympatholytics. This approach avoids these requirements all together.

PROTOCOL:

This protocol follows the guidelines of the institutional review board at the University of Texas at Arlington and conforms to the standards set by the latest version of the Declaration of Helsinki. Accordingly, written informed consent was (and should be) obtained prior to commencement of research procedures.

1. Instrumentation

Note: The following instrumentation description is based on the near-infrared (NIR) spectrometer and data acquisition system used in our lab (see *Table of Materials*). Thus, the instructions include steps that are necessary for the optimal function of these devices. These steps include the calibration of the NIR probe using the accompanying software and calibration phantom, and the application of a dark cloth to exclude ambient light. In the event that different data collection hardware and/or software are used, investigators should consult their own specific user manuals for calibration and ambient light considerations. **Figure 1** illustrates the experimental set-up and instrumentation described immediately below.

1.1. Instruct the subject to lie supine with their legs inside a lower body negative pressure (LBNP) chamber (**Figure 1A**), so that their belt line is approximately even with the opening to the LBNP box. For instructions on how to build a LBNP chamber, see *References* (Esch, Scott, & Warburton, 2007).

1.2. Place three electrocardiogram electrodes on the subject: two in an inferior, mid-clavicular location and one on the subject's left side medial to the iliac crest. This configuration provides the best results due to limited access to the lower limbs, instrumentation of the upper limbs, and arm movement during hand grip exercise.

1.3. Place a non-invasive blood pressure monitor module on the subject's dominant wrist. Place the finger blood pressure cuffs on each finger and connect them to the module (**Figure 1B**). Ensure the finger blood pressure cuffs are properly calibrated according to the user's manual accompanying your device.

1.4. Instruct the subject to grasp a hand grip dynamometer (HGD) with their non-dominant arm in a slightly abducted position. The arm should be comfortably positioned on a bedside table. The distance and angle of the HGD should be adjusted to allow for optimal grip strength with minimal arm movement (**Figure 1C**).

1.5. Secure the HGD to a bedside table.

1.6. Measure the maximum voluntary contraction (MVC) of the participant. Tell the participant that, when prompted, they must squeeze the HGD as hard as possible while only utilizing the muscles in the hand and forearm. Instruct the subject that they must refrain from recruiting their upper arm, chest, shoulder, or abdominal muscles when performing the maximum grip.

1.7. Repeat Step 1.6 three times, separated by at least 60 s. Record the maximum force achieved (best of 3). This maximum force will be used to calculate the exercise intensity for skeletal muscle oxidative capacity and neurovascular coupling (below).

1.8. Place a rapid-inflation cuff around the upper arm of the exercising hand. Connect the airline from the rapid inflation controller to the cuff.

1.9. Identify the flexor digitorum profundus. Use a skin marker to demarcate the borders of the palpable muscle.

1.10. Ensure that the NIR spectrometer is properly calibrated according to the user's manual included with your device. Clean the skin over which the NIR probe will be positioned with an alcohol prep wipe.

1.11. Place the NIR probe over the center of the belly of the muscle (flexor digitorum profundus) and affix it securely to the forearm.

1.12. Wrap the probe and forearm with dark cloth, minimizing interference from ambient light (**Figure 1C**, **Figure 1D**).

1.13. When ready to perform the functional sympatholysis portion of the study, seal the subject into the LBNP chamber.

2. Skeletal Muscle Oxidative Capacity

Note: A representative data tracing illustrating the experimental procedure for measuring skeletal muscle oxidative capacity is depicted in **Figure 2**. This experimental approach has previously been validated against *in vivo* phosphorus MRS (Ryan, Southern, Reynolds, et al., 2013) and *in situ* muscle respirometry (Ryan, Brophy, et al., 2014), and is gaining widespread acceptance (Adami & Rossiter, 2017).

2.1. Instrument the subject as indicated above (*Instrumentation*).

2.2. Instruct the subject to lie still for 2 min while monitoring deoxyhemoglobin (HHb) and oxyhemoglobin (HbO_2) via the NIR probe.

Note: This rest period allows the subject to recover from any movement artifact associated with the instrumentation process, and ensures stable baseline measurements. If after 2 min no significant fluctuations have occurred, the subject may be considered at a steady state, or resting baseline.

2.3. Prior to cuff occlusion, notify your subject that you will be inflating the cuff. Inflate the upper arm cuff at least 30 mmHg above systolic blood pressure for 5 min (*i.e.,* suprasystolic). Instruct the subject to keep their arm as still and relaxed as possible both during cuff inflation and following cuff deflation.

Note: This 5 min brachial artery cuff occlusion protocol closely reflects the currently accepted clinical standard for vascular occlusion tests(Corretti et al., 2002; Green, Jones, Thijssen, Cable, & Atkinson, 2011; Ryan, Erickson, Brizendine, Young, & McCully, 2012; Southern, Ryan, Reynolds, & McCully, 2014; Thijssen et al., 2011).

2.4. Record the initial/baseline value (prior to cuff occlusion) and the nadir value of tissue saturation (StO_2) during the cuff occlusion and determine the midpoint between these two values.

$$\mathrm{St_{O2}}\ (\%) = \frac{\mathrm{Hb_{O2}}}{\mathrm{Hb_{O2}} + \mathrm{HHb}} \times 100$$

2.5. Allow the subject to recover from the cuff occlusion and return to the resting baseline values. Once the subject has maintained a resting baseline for at least 1 full min, continue to the next step.

2.6. Instruct subject to squeeze and maintain an isometric hand grip at 50% of their MVC. Encourage the subject to maintain their isometric contraction until the tissue desaturates by 50%. Upon achieving this value, tell the subject to relax their hand and inform them that no more exercise or movement is needed.

2.7. Within 3-5 s following exercise cessation, administer the following rapid cuff occlusion series (one series = 1 inflation + 1 deflation), as previously established (Ryan, Southern, Reynolds, et al., 2013):

Series #1-6: 5 s on/5 s off

Series #7-10: 7 s on/10 s off

Series #11-14: 10 s on/15 s off

Series #15-18: 10 s on/20 s off

2.8. After completing the 18th inflation/deflation series, instruct the subject to rest, allowing tissue saturation to return to initial baseline values. After these values have remained consistent for at least 2 min, repeat steps 2.4 and 2.5.

2.9. Calculating Skeletal Muscle Oxidative Capacity

2.9.1. Calculate the slope of change in the StO_2 for each of the individual 18 cuff occlusions, forming the monoexponential recovery points illustrated in **Figure 2C**.

2.9.2. Fit the calculated data from 2.7 to the following monoexponential curve (Ryan, Brophy, et al., 2014; Ryan, Erickson, et al., 2014; Ryan, Southern, Reynolds, et al., 2013):

$$y = End - \Delta \times e^{-kt}$$

Note: 'y' is the relative muscle oxygen consumption rate ($m\dot{V}O_2$) during cuff inflation, 'End' represents the $m\dot{V}O_2$ immediately following the cessation of exercise; delta ('Δ') signifies the change in $m\dot{V}O_2$ from rest to the end of exercise; '*k*' is the fitting rate constant; 't' is time. Tau is calculated as 1/k.

3. Reactive Hyperemia

Note: A representative data tracing illustrating the experimental procedure for measuring reactive hyperemia is depicted in **Figure 3**.

3.1. With the subject lying supine and instrumented as described above (*Instrumentation*), instruct the subject to lie as still as possible.

3.2. Once the subject has achieved a consistent resting state, continue to record at least 1 min of baseline data and then rapidly inflate a blood pressure cuff on the upper arm to a suprasystolic pressure (30 mmHg above systolic blood pressure).

3.3. At the 5 min mark, rapidly deflate the cuff while recording the hyperemic response.

3.4. Continue recording for at least 3 min to capture the subject's recovery.

3.5. Calculating Reactive Hyperemia

Note: The NIRS parameters calculated are depicted in **Figure 3**.

3.5.1. Calculate baseline StO_2 as the average StO_2 over 1 full min prior to the onset of arterial cuff occlusion.

3.5.2. Determine the resting skeletal muscle metabolic rate as the desaturation rate (*i.e.*, average slope) during cuff occlusion (defined as Slope 1)*(Mayeur, Campard, Richard, & Teboul, 2011)*(McLay, Fontana, et al., 2016).

3.5.3. Calculate reactive hyperemia as follows:

a) the average upslope following cuff release (*i.e.*, reperfusion rate, defined as slope 2), calculated from the moment of cuff release through the linearly increasing phase of the rebound trace;

b) the highest StO_2 value reached after cuff release (denoted as StO_{2max});

c) the reactive hyperemia area under the curve (AUC); calculated from the time of cuff release to 1-, 2- and 3-min post cuff-occlusion (AUC 1-min, AUC 2-min, and AUC 3-min, respectively); and

d) the hyperemic reserve, calculated as the change in StO_2 above baseline and reported as a percent (%) change. This value is calculated as the highest saturation achieved during the post-occlusive rebound minus the average saturation calculated in step 3.5.1 (see above).

Note: Large differences in baseline data will greatly affect the interpretation of the hyperemic reserve.

4. Functional Sympatholysis

Note: A representative data tracing illustrating the experimental procedure for measuring functional sympatholysis is depicted in **Figure 4**.

4.1. Instrument the subject as indicated above (*Instrumentation*).

4.2. Ensure an airtight seal in the LBNP chamber.

4.3. With the subject lying still and at rest, collect 3 min of baseline data.

4.4. At the 3 min mark, turn on the vacuum. Adjust the vacuum so that the pressure inside the LBNP chamber is between -20 and -30 mmHg. Allow the vacuum to run for 2 min while monitoring the subject's response.

4.5. At the 5 min mark, turn off the vacuum and allow the subject to rest for 3 min.

4.6. At the 8 min mark, initiate the voice prompt guiding the subject through the rhythmic hand grip exercise (20% MVC).

4.7. Confirm that the subject is maintaining their squeeze throughout the entirety of each gripping phase and relaxing completely during between each repetition. Monitor their force output and confirm that they are achieving 20% MVC with each grip. Continue exercise until the 11 min mark.

4.8. At the 11 min mark, turn on the vacuum encouraging the subject to continue their rhythmic exercise. Allow the vacuum to run from 11-13 min, then turn it off.

4.9. Have the subject continue performing rhythmic hand grip exercise at 20% of their MVC for an additional 2 min. Upon exercise cessation, have the subject rest quietly and lie still.

4.10. <u>Calculating Functional Sympatholysis</u>

4.10.1. Normalize the change in oxyhemoglobin with LBNP to the total labile signal (TLS), determined during 5 min cuff occlusion:

$$\Delta Hb_{O2\ (rest)}(\%\ TLS) = \frac{\Delta Hb_{O2\ (baseline)} - \Delta Hb_{O2\ (rest+LBNP)}}{\Delta Hb_{O2\ (baseline)} - \Delta Hb_{O2\ (nadir)}} \times 100$$

$$\Delta Hb_{O2\ (exercise)}(\%\ TLS) = \frac{\Delta Hb_{O2\ (exercise)} - \Delta Hb_{O2\ (exercise+LBNP)}}{\Delta Hb_{O2\ (baseline)} - \Delta Hb_{O2\ (nadir)}} \times 100$$

4.10.2. Calculate each event as the final 20 min average of each event.

4.10.3. Calculate the exercise-induced attenuation of the oxyhemoglobin reduction:

$$\frac{\Delta Hb_{O2\ (rest)} - \Delta Hb_{O2\ (exercise)}}{\Delta Hb_{O2\ (rest)}} \times 100$$

REPRESENTATIVE RESULTS:

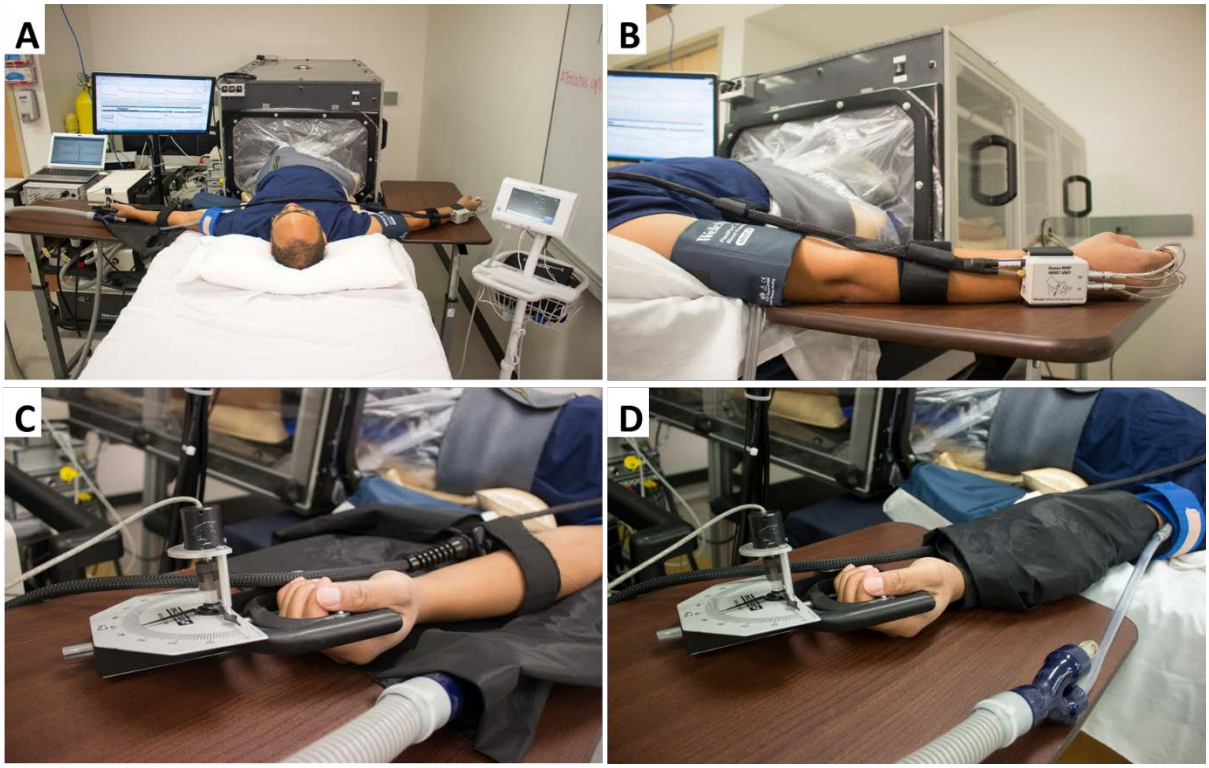

Figure 1. Experimental set-up and instrumentation. **(A)** Representative experimental set-up, with a typical subject lying supine on a bed with their legs inside the LBNP chamber and fully instrumented. **(B)** Dominant arm instrumented with a non-invasive beat-to-beat blood pressure device for beat-to-beat arterial blood pressure measurement, and a brachial artery blood pressure cuff for calibration and verification of the beat-to-beat system. **(C)** Instrumentation of the non-dominant arm. The hand is comfortably gripping a handgrip dynamometer (connected to data acquisition system), and the forearm muscle is instrumented with the near-infrared spectroscopy probe. **(D)** Once instrumented, the NIRS optodes are covered with a black vinyl cloth (to eliminate interference from ambient light). In addition, a rapid cuff inflation system is placed over the brachial artery.

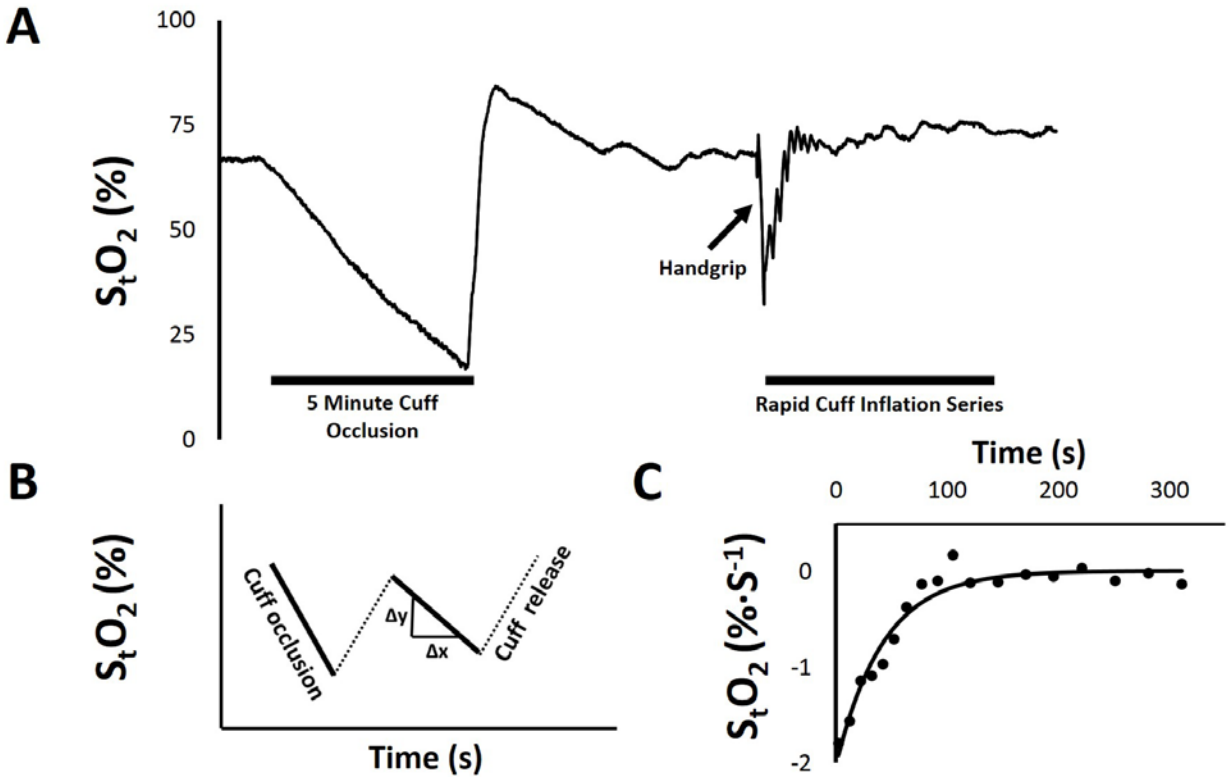

Figure 2. Skeletal muscle oxidative capacity protocol. **(A)** Raw data tracing from a representative subject measured via NIRS, showing tissue saturation (S_tO_2) over time. After establishing a stable baseline, the brachial artery of the non-dominant arm is occluded for five min in order to establish the subject's desaturation reserve (difference between baseline StO_2 and the nadir). After recovery from the occlusion, the subject is instructed to perform a 50% isometric handgrip, followed by 18 rapid cuff inflation series to assess muscle oxygen consumption recovery kinetics. **(B)** Data analysis is then performed offline by calculating the average slope of each cuff occlusion series following exercise; illustrated here using hypothetical cuff occlusion series data. **(C)** In order to calculate the recovery time constant of muscle oxygenation, the slope of each of the 18 rapid cuff occlusions (*i.e.*, post-exercise muscle oxygen consumption, $m\dot{V}O_2$) from **A** is plotted against time and fit to a monoexponential curve.

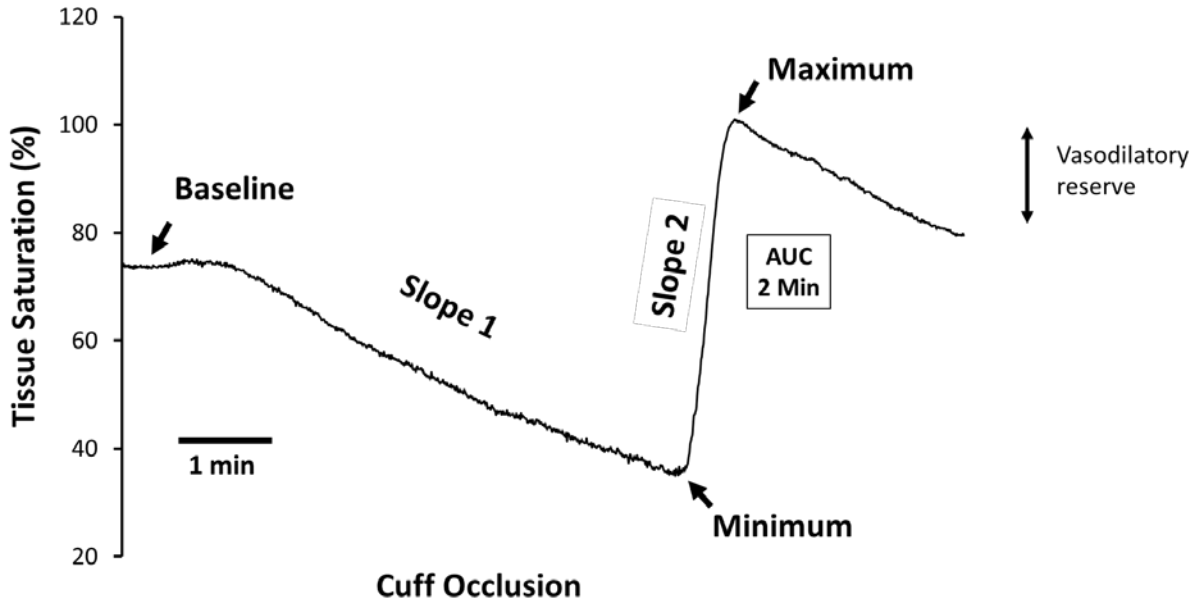

Figure 3. Reactive hyperemia experimental protocol. With the subject lying supine, record at least 1 min of baseline data, followed by 5 min of total arterial cuff occlusion, and at least 3 min of recovery following cuff release. Note the obvious overlap between the skeletal muscle oxidative capacity protocol (**Figure 2**) and this protocol. 'Baseline' defines the period of time prior to arterial cuff occlusion. 'Slope 1' defines the desaturation rate during cuff occlusion, and is regarded as a measure of resting skeletal muscle metabolic rate. The lowest StO_2 value obtained during ischemia is defined as 'StO_2 minimum', and is regarded as a measure of the ischemic stimulus to vasodilate. The tissue saturation reperfusion rate is denoted as 'Slope 2', and is an index of reactive hyperemia; as are StO_2 maximum, and the reactive hyperemia 'area under the curve' (AUC). To gain insight into the hyperemic reserve, the StO_2 maximum is expressed as a percent change from baseline.

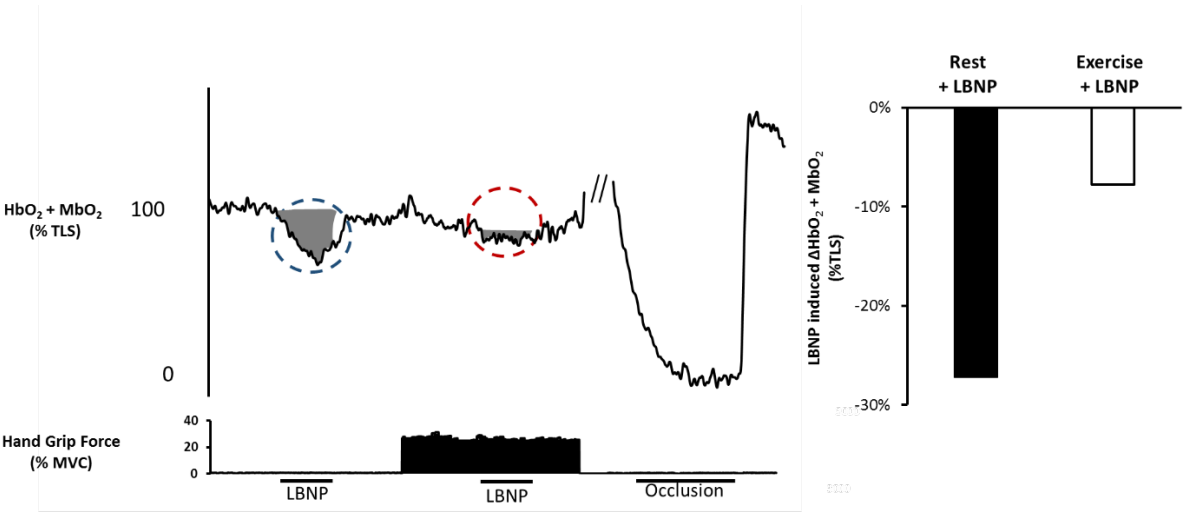

Figure 4. Functional sympatholysis experimental protocol. Left panel: Raw data tracing from a representative subject. With the subject lying supine in the LBNP chamber, allow 3 min of steady-state baseline data collection. Turn on LBNP to -20 mmHg for 2 min. Oxyhemoglobin/myoglobin should decrease in response to the reflex sympathetic vasoconstriction (blue circle, shaded area). Allow 2 min for recovery. Ask the subject to perform rhythmic handgrip exercise at 20% MVC (measured prior to data collection). After 3 min of rhythmic exercise, repeat -20 mmHg LBNP for 2 min while the subject continues to exercise, followed by 2 min of exercise without LBNP. The reduction in oxyhemoglobin/myoglobin should be significantly attenuated (red circle, shaded area). If not already performed, inflate a blood pressure cuff over the brachial artery of the exercising arm for 5 min to establish the subject's range of desaturation. Note that the shaded areas in the figure are only meant to highlight the changes in oxyhemoglobin/myoglobin; see protocol for details on how to analyze the outcome variables used to calculate sympatholysis. Right Panel: LBNP-induced change in oxyhemoglobin/myoglobin at rest and during handgrip exercise calculated from the data on the left.

Table 1. Summary of previously published reports across the health continuum using near-infrared spectroscopy to measure skeletal muscle oxidative capacity.

Reference/ Data Set	Study Population	Sample size (n)	Age of participants (years ± SD)	Tau (τ) (seconds)	Muscle group	Variable reported	Device
Brizendine et al. (2013)	Endurance Athletes	8	25 ± 3	19	Vastus lateralis	HB_{diff}/ total blood volume	Continuous wave (Oxymon MK III)
Ryan et al. (2014)	Young, healthy	21	26 ± 2	55	Vastus lateralis	HHb	Continuous wave (Oxymon MK III)
Southern et al. (2015)	Elderly	23	61 ± 5	63	Wrist flexor	HB_{diff}	Continuous wave (Oxymon MK III)
	Elderly + Heart Failure	16	65 ± 7	77	Wrist flexor	HB_{diff}	Continuous wave (Oxymon MK III)
Adami et al. (2017)	Smokers with normal spirometry	23	63 ± 7	80	Medial forearm	Tissue saturation index (TSI)	Continuous wave (Portamon)
	COPD Gold 2-4	16	64 ± 9	100	Medial forearm	Tissue saturation index (TSI)	Continuous wave (Portamon)
Erickson et. Al (2013)	Spinal cord injury	9	43 ± 11	143	Vastus lateralis	HBO_2 + myoglobin	Continuous wave (Oxymon MK III)

Table 2. Summary of previously published reports across the health continuum using near-infrared spectroscopy to measure reactive hyperemia.

Reference	Study Population	Muscle Group	Reported Outcomes	Outcome Value
Lacroix, *J Biomed Opt*, 2012	Healthy Males	Forearm	Peak Oxyhemoglobin	28.05 ± 3.15 μM
			Peak Total Hemoglobin	10.56 ± 1.80 μM
			Increase Rate to Peak HbO_2	0.75 ± 0.22 μM/s
			Increase Rate to Peak Total Hb	0.52 ± 0.16 μM/s
Kragelj, *Ann Biomed Eng*, 2001	Peripheral Vascular Disease	Forearm	Oxygen Consumption	0.68 ± 0.04 ml/min
			Time to Peak	153 ± 16 s
			Maximal Absolute Change in HbO_2	2.93 ± 0.22 μM/100ml
Suffoletto, *Resuscitation*, 2012	Post-Cardiac Arrest ICU Admittants	Thenar Eminence	Desaturation Rate	-5.6 ± 2 %/min
			Resaturation Rate	0.9 ± 0.6 %/sec
Dimopoulos, *Respir Care*, 2013	Pulmonary Artery Hypertension	Thenar Eminence	Baseline Saturation with 21% O_2	65.8 ± 14.9 %
			O2 Consumption Rate with 21% O_2	35.3 ± 9.1 %/min
			Reperfusion Rate with 21% O_2	535 ± 179 %/min
Doerschug, *Am J Physiol Heart Circ Physiol*, 2007	Organ Failure & Sepsis	Forearm	Baseline Saturation	84%
			Reoxygenation Rate	3.6 %/s
Mayeur, *Crit Care Med*, 2011	Septic Shock	Thenar Eminence	Baseline Saturation	80 ± 1.0 %
			Desaturation Slope	-9.8 ± 3.7 %/min
			Recovery Slope	2.3 ± 1.4 %/sec
McLay, *Exp Physiol*, 2016	Healthy Males	Tibialis Anterior	Baseline Saturation	71.3 ± 2.9 %
			Minimum Saturation	44.8 ± 8.6 %
			Desaturation Slope	-0.1 ± 0.03 %/s

			Recovery Slope	1.63 ± 0.5 %/s
			Peak Saturation	82.6 ± 2.3 %
McLay, Physiol Rep, 2016	Healthy Males	Tibialis Anterior	Baseline Saturation	71.1 ± 2.4 %
			Minimum Saturation	46.2 ± 7.5 %
			Peak Saturation	82.1 ± 1.4 %
			Recovery Slope	1.32 ± 0.38 %/s

Table 3. Summary of previously published reports across the health continuum using near-infrared spectroscopy, in combination with lower body negative pressure and handgrip exercise, to assess functional sympatholysis.

Reference	**Study Population**	**% Attenuation**
Nelson MD, *J. Physiol*, 2015	Healthy	-57
	Becker Muscular Dystrophy	-13
Vongpatanasin, *J. Physiol*, 2011	Healthy	-93
	Hypertension	-14
Fadel, *J. Physiol*, 2004	Pre-Menopause	-84
	Post-Menopause	-19
Sander, *PNAS*, 2000	Healthy	-74
	Duchenne Muscular Dystrophy	.+7
Nelson MD, *Neurology*, 2014	Healthy	-54
	Duchenne Muscular Dystrophy	-7
Price, *Hypertension*, 2013	Hypertension Pre-Treatment	-52
	Hypertension Post-Nebivolol Treatment	-97
Hansen, *J. Clin. Invest.*, 1996	Healthy Exercise at 20% MVC	-92
	Healthy Exercise at 30% MVC	-125

DISCUSSION

The methods described herein enable non-invasive, clinical evaluation of reactive hyperemia, neurovascular coupling, and skeletal muscle oxidative capacity in a single clinic or laboratory visit.

Critical Considerations

Although NIRS is relatively robust and easy to use, collection of these data require careful placement of the optodes directly over the muscle belly, secured tightly in place to avoid movement artifact, and covered with a black vinyl sheet in a dimly lit room to avoid interference of the near infrared from external light. In addition, obtaining good quality data relies heavily on clear communication between the tester and the subject, and the testing team. We, and others, have found that when performed with appropriate care and attention, NIRS is highly reproducible within a single study visit, and across multiple visits (McLay, Nederveen, Pogliaghi, Paterson, & Murias, 2016; Nelson et al., 2014; Nelson et al., 2015; Southern et al., 2014). Moreover, the physiological outcome variables reported herein (*i.e.,* skeletal muscle oxidative capacity, reactive hyperemia, and neurovascular coupling) are sensitive to experimental/clinical intervention, both within and between study visits (Nelson et al., 2014; Nelson et al., 2015; Ryan, Southern, Brizendine, et al., 2013; Southern et al., 2015).

There is currently limited consensus on the appropriate reporting of the NIRS outcome variables. For example, when measuring skeletal muscle oxidative capacity, investigators have fit the recovery kinetics of HbO_2 (Ryan, Brizendine, & McCully, 2013), HHb (Ryan, Brophy, et al., 2014), Hb_{diff} (Ryan, Southern, Brizendine, et al., 2013) and tissue O_2 saturation (present study and others (Adami, Cao, Porszasz, Casaburi, & Rossiter, 2017). Likewise, a similar spread in the outcome variables have also been reported for NIRS-based reactive hyperemia (Bopp, Townsend,

Warren, & Barstow, 2014; Kragelj, Jarm, Erjavec, Presern-Strukelj, & Miklavcic, 2001; Lacroix et al., 2012; Willingham, Southern, & McCully, 2016) Some of this discrepancy may relate to the type of NIRS device used. For example, frequency-domain devices (as used here) provide absolute quantification of HbO_2 and HHb, and thus may not be affected by acute changes in total Hb content (negating the need to correct the data). In contrast however, continuous-wave devices are greatly affected by acute changes in total hemoglobin, requiring data correction (Ryan et al., 2012).

Modifications and troubleshooting

One important and currently unavoidable limitation of NIRS is its limited penetration depth (~2 cm). Therefore, limb adiposity can significantly reduce — and even completely eliminate — the NIRS signal and should be considered when screening potential subjects. To control for this, investigators are encouraged to measure forearm skinfold thickness, and exclude participants with significant peripheral adiposity.

Any factor that can modulate vascular responsiveness, neurovascular coupling, and/or skeletal muscle oxidative capacity (*i.e.*, medication, genetic mutations, etc) will indeed affect the primary end-point measurements described herein. Investigators are therefore encouraged to take these factors into account when adapting this protocol and planning future experimentation.

For functional sympatholysis determination, investigators may wish to include a second resting LBNP challenge to ensure the signal is still present and that the differences observed during exercise-LBNP were not simply due to a loss of signal or measurement error. It is recommended to allow 3-5 min to allow the oxyhemoglobin signal to full recovery to baseline values before repeating the resting LBNP challenge.

Future applications or directions after mastering this technique

NIR spectroscopy uses laser light to assess the concentration of oxygenated and deoxygenated hemoglobin in tissue. During measurement of reactive hyperemia and functional sympatholysis, relative changes in these parameters are believed to represent changes in microvascular flow. Diffuse correlation spectroscopy (DCS) is an emerging near-infrared imaging approach which, in addition to evaluating the concentration of oxy- and deoxyhemoglobin, can also quantify microvascular perfusion (Gurley, Shang, & Yu, 2012). Given the obvious similarities between these two imaging approaches, incorporation of DCS into the proposed techniques would be virtually seamless and may provide additional insight into the quantification of microvascular function and perfusion.

Once this technique is mastered, application to clinical populations, such as those with heart failure, will provide important mechanistic insight into exercise intolerance and cardiovascular dysfunction.

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Appendix B: Published Viewpoint on measuring skeletal muscle oxidative capacity by near-infrared spectroscopy

This manuscript appear exactly as it was published: S Chung and MD Nelson. Commentary on Viewpoint: Principles, Insights and Potential Pitfalls of the Non-Invasive Determination of Muscle Oxidative Capacity by Near-Infrared Spectroscopy. *J Appl Physiol*. 124: 249-55, 2018.

In their viewpoint, Adami and Rossiter (1) eloquently explore the strengths and weaknesses of combining near-infrared spectroscopy (NIRS) with repetitive post-exercise arterial cuff occlusions, to measure skeletal muscle oxidative capacity in vivo. Before the advent of this technique, the assessment of mitochondrial function was limited to invasive muscle biopsies and/or expensive and time consuming magnetic resonance spectroscopy (MRS) techniques. Now, clinicians and researchers alike have a robust, high throughput clinical platform to non-invasively assess muscle oxidative capacity, across a wide range of muscles and disease states. That this approach is easily transportable and relatively low cost opens new possibilities for bedside medicine, clinical decision making, and incorporation into large multi-center clinical trials. However, this increased emphasis on clinical populations also emphasizes the need for future technology development to overcome the well-established limitations of NIRS with regards to limb adiposity (2). While Adami and Rossiter appropriately highlight the relatively large test-retest variability with this technique compared to the differences often observed between health and disease, it is important to emphasize that this variability is similar to that achieved with MRS (5) or muscle biopsies (4). This does highlight the importance of careful experimental/clinical design however. Indeed, over 40% of the investigations referenced in this viewpoint studied locomotor muscle groups, which are highly dependent on physical activity level (3). Targeting non-locomotor muscle groups should help limit within-group variability. With these considerations in mind, we believe NIRS-derived muscle oxidative capacity assessments hold great promise in clinical translational medicine.

References

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2. **Ferrari M, Mottola L, and Quaresima V**. Principles, Techniques, and Limitations of Near Infrared Spectroscopy. *Canadian Journal of Applied Physiology* 29: 463-487, 2004.

3. **Larsen RG, Callahan DM, Foulis SA, and Kent-Braun JA**. Age-related changes in oxidative capacity differ between locomotory muscles and are associated with physical activity behavior. *Applied physiology, nutrition, and metabolism = Physiologie appliquee, nutrition et metabolisme* 37: 88-99, 2012.

4. **Ryan TE, Brophy P, Lin CT, Hickner RC, and Neufer PD**. Assessment of in vivo skeletal muscle mitochondrial respiratory capacity in humans by near-infrared spectroscopy: a comparison with in situ measurements. *The Journal of physiology* 592: 3231-3241, 2014.

5. **Ryan TE, Southern WM, Reynolds MA, and McCully KK**. A cross-validation of near-infrared spectroscopy measurements of skeletal muscle oxidative capacity with phosphorus magnetic resonance spectroscopy. *Journal of applied physiology (Bethesda, Md : 1985)* 115: 1757-1766, 2013.

Appendix C: Supplemental Material for Chapter 3

Supplemental Table 1. Individual results following 4 weeks of handgrip exercise training

Subject #	Grip Strength (kg)	Pre	Post	Tau (s)	Pre	Post
1		20	22		50.68424	54.97526
2		55	54		68.39945	64.97726
3		27	27		46.97041	53.82131
4		7	13		40.84967	28.90173
5		13	18		22.78423	45.49591
6		24	25		44.48399	36.35042
7		26	25		56.21135	43.53505
8		24	25		26.11648	46.72897