DELINEATING FACTORS THAT IMPACT MUSCULOSKELETAL CHARACTERISTICS THROUGHOUT THE LIFETIME IN HORSES

A Dissertation

by

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ABSTRACT

Research regarding factors that impact musculoskeletal characteristics of horses is sparse. Therefore, we conducted four experiments to assess potential effects of breed, exercise training, complexed trace mineral supplementation, transportation stress, age and thyroid stimulating hormone concentration on a) mitochondrial density and function, b) oxidative stress and antioxidant status and c) muscle fiber type and size in various age groups of horses. Relative to weanling Standardbred and Thoroughbred horses, weanling Quarter Horses had lower integrated maximum oxidative and electron transport system capacity ($P \leq 0.02$), and the three breeds differed with regard to contribution of mitochondrial complexes to oxidative capacity. In a study of yearling Quarter Horses, transportation stress elevated serum creatine kinase activity ($P < 0.0001$; a marker of muscle damage), and muscle malondialdehyde concentration ($P \leq 0.009$; a marker of oxidative stress). However, complexed trace mineral supplementation increased muscle antioxidant activity, as measured by glutathione peroxidase activity ($P \leq 0.02$), in yearling Quarter Horses. Compared to young horses, aged horses exhibited higher citrate synthase (CS) activity ($P = 0.04$), but lower cytochrome *c* oxidase (CCO) activity ($P = 0.04$), and a lower percentage of type IIx muscle fibers ($P = 0.03$). Exercise training increased CS activity ($P \leq 0.003$), CCO activity ($P = 0.04$), and maximum oxidative capacity ($P \leq 0.006$) in young and aged horses, and increased the percentage of type IIx fibers in aged horses ($P < 0.0001$). In an *ex vivo* study of equine skeletal muscle in mature and aged horses, 10 mIU/mL of TSH increased maximum oxidative capacity ($P \leq 0.007$). Together, these data provide a wealth of information regarding factors that impact musculoskeletal characteristics in throughout the lifetime of horses.

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NOMENCLATURE

SB	Standardbred
THB	Thoroughbred
QH	Quarter Horse
CS	Citrate Synthase
CCO	Cytochrome *c* Oxidase
P_{CI}	Oxidative Capacity with Complex I Substrates
P_{CI+II}	Oxidative Capacity with Complex I&II Substrates
ETS	Electron Transport System
E_{CI+II}	ETS Capacity with Complex I&II Substrates
E_{CII}	ETS Capacity with Complex II
FCR	Fractional Control Ratio
ROS	Reactive Oxygen Species
GM	Gluteus Medius
TB	Triceps Brachii
Cu	Copper
Zn	Zinc
Mn	Manganese
Co	Cobalt
SOD	Superoxide Dismutase
SDHA	Succinate Dehydrogenase A
HPRT1	Hypoxanthine Phosphoribosyltransferase 1
B2M	Beta-2-Microglobulin

SOD1	Cu-Zn Superoxide Dismutase
SOD2	Mn Superoxide Dismutase
GPx	Glutathione Peroxidase
CK	Creatine Kinase
MDA	Malondialdehyde
MyHC	Myosin Heavy Chain

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1. INTRODUCTION AND LITERATURE REVIEW

1.1. Introduction

Ample research has been conducted investigating factors that impact musculoskeletal health throughout the lifespan in species such as humans and rodents. Similar research on musculoskeletal health in horses is comparatively sparse. However, horses have the potential to be an excellent model for human aging if changes in musculoskeletal health across the lifespan are similar. The objectives of this research are to 1) investigate factors such as breed, sex, transportation stress, exercise training and dietary complexed trace mineral supplementation, which may impact musculoskeletal health in horses, 2) to better understand how musculoskeletal health declines with age in horses and 3) to examine the effect of thyroid stimulating hormone (TSH) dose on equine skeletal muscle *in vitro*. Together, this research will provide a wealth of basic information on muscle health across the lifespan of horses, as well as factors that may improve muscle health, such as exercise, organic trace mineral supplementation, and elevated concentrations of systemic TSH.

1.2. Breed Differences in Skeletal Muscle Characteristics

Medication regulations in equine performance horses have changed drastically in the past decade. Recently, the proposed Horseracing Integrity Act of 2017 (HR 2651) has been met with controversy, as it proposes to require a "uniform anti-doping and medication control program to be developed and enforced by an independent Horseracing Anti-doping and Medication Control Authority," that will govern medication rules for racing Standardbreds, Thoroughbreds, and Quarter Horses. The proposed governing board is to consist of a myriad of experts including horse owners, racetrack executives, veterinarians, and jockeys, but does not specify representation for each racing breed.

Therefore, many owners and associations are concerned that breed-specific regulations may be overlooked.

Expertise in the demands of each racing discipline may be critical because muscle characteristics of the breeds are dissimilar. Adult horses have three muscle fiber types delineated by the myosin heavy chain isotype they contain: 1) type I muscle fibers, which have a slow twitch motor unit, high oxidative capacity, and moderate glycolytic capacity, 2) type IIa muscle fibers, which have a fast, fatigue resistant motor unit, moderate to high oxidative capacity and high glycolytic capacity, and 3) type IIx muscle fibers, characterized by a fast, fatigable motor unit, low oxidative capacity, and high glycolytic capacity. These muscle fiber types are all necessary for optimal performance of horses, but the importance of each varies depending on breed and use of the horse. Type I and type IIa fibers are critical for longer bouts of exercise, such as Thoroughbred and Standardbred races, which require oxidative metabolism to sustain their work load. Conversely, type IIx fibers are extremely important for Quarter Horse racing, where explosive power is required, but exercise duration is shorter. Different muscle fiber types are recruited for different modes of training, with type IIa fibers being increasingly recruited for aerobic training, and hybrid type IIa/IIx and type IIx fibers being increasingly recruited for anaerobic, high intensity training (Leisson et al., 2008).

Table 1.1 Characteristics of muscle fiber types of the horse.

	Muscle Fiber Type		
	Type I	**Type IIa**	**Type IIx**
Motor Unit	Slow	Fast, Fatigue-Resistant	Fast, Fatigable
Oxidative Capacity	High	Moderate-High	Low
Glycolytic Capacity	Moderate	High	High
Succinate Dehydrogenase	Strongly positive (++)	Positive (+)	Negative (-)

Some medications have been restricted or banned across breeds and disciplines because of purported performance enhancing abilities, despite a sparsity of evidence demonstrating that such performance enhancement actually occurs across different racing disciplines. Breed differences in training methods and muscle characteristics may make a treatment performance enhancing in some breeds while limiting performance in other breeds. For example, clenbuterol is a clinically valuable, FDA approved therapeutic β-2 agonist used for the treatment of inflammatory airway disease. After 8 wk of clenbuterol administration, both trained and untrained Standardbreds showed an increase in percentage of whole-body fat free mass and a decrease in percentage of fat mass (Kearns et al., 2001). Additionally, chronic clenbuterol use in Standardbreds was shown to increase percentages of type IIx muscle fibers while decreasing the percentage of type IIa fibers (Beekley et al., 2003). While the switch to faster twitch, non-oxidative type IIx fibers may be beneficial in racing breeds that compete for shorter distances and rely more on glycolytic than oxidative metabolism, the decrease in oxidative, type IIa fibers would likely be detrimental to performance in racing disciplines that require oxidative metabolism to support longer bouts of exercise. As predicted by changes in proportions of

muscle fiber types, clenbuterol administration actually impairs aerobic performance in Standardbreds (Kearns and Mckeever, 2002).

While differences in trained horses of varying breeds have been described, there is also ample research that suggests Standardbreds, Quarter Horses and Thoroughbreds have dissimilar muscle characteristics from a young age. Standardbreds have been shown to have more fast, oxidative/glycolytic and slow, oxidative fibers, and less fast, glycolytic fibers compared to Quarter Horses from birth through one yr of age (Bechtel and Kline, 1987). Additionally, Standardbreds were shown to have higher lactate dehydrogenase (LDH; marker of glycolytic activity) and citrate synthase (CS; marker of mitochondrial number) activities than Quarter Horses from birth through one yr of age. The changes in these enzymatic activities were the most prominent over the first 6 mo of life, and were not the same between breeds, likely because of selection differences between breeds for varying types of exercise and therefore dissimilar metabolic profiles (Kline and Bechtel, 1990). Similar to Standardbreds, in a study of horses that received no forced exercise for at least one year, Thoroughbreds tended to have more type I and IIa fibers, and less IIx fibers, as assessed by succinate dehydrogenase activity staining, than Quarter Horses (Stull and Albert, 1980). It was proposed that individuals with high percentages of type I fibers in their muscle are best suited for longer bouts of exercise training and competition and those with more fast twitch, IIx fibers are better suited for shorter sprint type activities (Stull and Albert, 1980). To support this idea, Quarter Horses that were successful runners were shown to have a higher percentage of fast twitch, non-oxidative fibers, and a lower percentage of slow twitch, oxidative fibers (Wood et al., 1988). Conversely, excellent performers in endurance competitions had a higher percentage of type I and type IIa fibers and a lower percentage of type IIx fibers than moderate performers (Rivero et al., 1993).

Taking all of the data together, there is evidence that inherent differences in skeletal muscle characteristics related to performance exist between racing breeds. Furthermore, the inherent differences that are present from an early age and that persist through the racing career suggest that different racing breeds require very specific muscle phenotypes for optimal performance. Therefore, therapeutic drug use, such as clenbuterol, which alters skeletal muscle fiber profile by increasing the percentage of type IIx fibers, may be performance enhancing in some breeds such as the Quarter Horse, that is selected and trained in a manner that increases the percentage of type IIx muscle fibers. However, breeds such as Standardbreds and Thoroughbreds, that are selected and trained in a manner that increases the percentage of type IIa fibers, may not benefit from increasing type IIx fibers because their performance demands rely more heavily on oxidative metabolism that cannot be conducted efficiently by type IIx fibers.

The available research assessing muscle fiber type differences between breeds is confounded by varying means of assessing muscle fiber type, such as myosin ATPase activity, succinate dehydrogenase staining, and immunohistochemical evaluation of myosin heavy chain isotypes. Furthermore, much of the available literature comparing breeds is decades old and employs outdated methodology. Arguably, one of the most important differences between muscle fiber types for performance is the oxidative capacity of the muscle, where higher oxidative capacity can support longer bouts of exercise. Until recently, oxidative capacity was commonly estimated by measuring enzymatic activities related to mitochondrial number and function, such as citrate synthase and cytochrome *c* oxidase. However, new technologies such as high-resolution respirometry (HRR) have made it possible to measure oxidative capacity more directly.

Mitochondria are the powerhouses of animal cells. In the presence of oxygen, these sub-cellular structures convert sugar into energy by transferring electrons from substrate molecules to acceptor molecules. This transfer of electrons occurs across 4 main complexes situated within the inner mitochondrial membrane, better known as the electron transport system. With oxygen serving as the final acceptor molecule, energy is ultimately produced. Without oxygen, sugar is converted into energy by glycolysis, or anaerobic metabolism, a type of energy production that is less efficient, and produces lactate as a by-product, which may lead to enhanced lactic acid production. Glycolytic muscle fibers rely more heavily on this anaerobic metabolism for energy, whereas oxidative fibers rely more heavily on aerobic, mitochondrial based energy production. The HRR technique is able to directly measure the ability of the cells to create energy, or work, utilizing fuel and oxygen. Further, HRR allows the determination of the contribution of each of the 4 complexes of electron transport to total energy production, as well as the overall efficiency of energy production.

1.3. Complexed Trace Mineral Supplementation, Antioxidant Status and Oxidative Stress

Young equine athletes experience several stressors as they begin training and competing. For example, acute exercise bouts and trailer transportation between competitions or training facilities cause an increase in mitochondrial production of reactive oxygen species (**ROS**), which can be deleterious to skeletal muscle health if not properly sequestered by antioxidant enzymes.

Biologically relative ROS include superoxide, hydrogen peroxide, singlet oxygen, lipid peroxides and hydroxyl radicals, which are generated at many sites, including the electron transport system, the endoplasmic reticulum, NADPH oxidase enzymes (Nox), dual oxidase enzymes (Duox), and various other sources (Dickinson and Chang, 2011). While ROS production has long been considered a deleterious consequence of oxidative metabolism,

research over the past few decades has shown that ROS are important biological molecules that can signal anabolism following exercise (Morales-Alamo and Calbet, 2016). However, in the absence of means to properly sequester ROS, oxidative stress can occur and damage proteins, lipids and DNA.

Oxidative stress is kept in check by endogenous antioxidants (e.g., glutathione, coenzyme Q, and melatonin), as well as exogenous antioxidants such as vitamins C and E and resveratrol, and antioxidant enzyme defense systems such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx) (Pisoschi and Pop, 2015). Superoxide dismutase, catalase and GPx serve as "first line of defense" against ROS. Superoxide dismutase converts superoxide radicals to hydrogen peroxide, and catalase and GPx ultimately convert hydrogen peroxide to water (Pisoschi and Pop, 2015). Three forms of SOD are present in animal tissues; SOD1 is present in the cytosol and contains copper and zinc, SOD2 is present in the mitochondrial matrix and contains manganese, and SOD3 is extracellular (Pisoschi and Pop, 2015). The functional role of copper, zinc, and manganese in the above-mentioned enzymes is to serve as an electron acceptor for the free radicals associated with ROS (Azadmanesh and Borgstahl, 2018). Similarly, GPx contains selenium to achieve the same end. Complexed, or "organic" trace minerals are trace minerals bound to proteins, amino acids, carbohydrates or lipids (Świątkiewicz et al., 2014). Complexed trace minerals have been studied in many species including pigs and chickens (Lee et al., 2001), cows (Nocek et al., 2006) and horses (Wagner et al., 2011). The advantages of complexed trace mineral supplementation over inorganic sources are often attributed to be improved bioavailability of these minerals when they are complexed to organic molecules (Świątkiewicz et al., 2014).

Since trace minerals are integral components of antioxidant enzymes, and complexed trace minerals are thought to have improved bioavailability, several studies have investigated the effects of complexed trace mineral supplementation on antioxidant status and oxidative stress. Dietary complexed trace mineral supplementation has been shown to reduce oxidative stress in food animal models. Dietary proteinate complexed-Zn supplementation increased GPx and SOD activities in the spleen of barrows (She et al., 2017). Additionally, biocomplexed minerals Fe, Mn, Zn, and Cu enhanced SOD and GPx activities in the liver of grower-finisher pigs (Liu et al., 2016) and decreased plasma malondialdehyde (MDA), a measure of oxidative stress, in broilers (Echeverry et al., 2016).

In horses, research on the effects of dietary organic trace mineral supplementation is relatively sparse. One study in horses showed that Cu-Lys may be better absorbed than $CuSO_4$ (Wagner et al., 2011), but mineral intakes in the study exceeded requirements, and mineral balances were negative, making it difficult to draw conclusions about the efficacy of complexed mineral supplementation. In the same group of horses, Cu-Lys and Zn-Met supplementation did not seem superior to $CuSO_4$ and $ZnSO_4$ in improving erythrocyte SOD activity during exercise and immediately after recovery in horses (Wagner et al., 2010). It should be noted that exercise itself did not induce a change in SOD activity in that study, suggesting the exercise did not significantly increase systemic oxidative stress (Wagner et al., 2010). Additionally, in both experiments, supplemented mineral intakes of Cu-Lys and Zn-Met were higher than $CuSO_4$ and $ZnSO_4$, making interpretation of the results difficult.

Therefore, research in other species suggests that complexed trace mineral supplementation may improve bioavailability of trace minerals and improve antioxidant status.

However, more research is needed to determine the efficacy of complexed trace minerals on improving antioxidant status in horses.

1.4. Systemic and Muscular Adaptations to Aging in Horses

Horses over 16 yr of are generally considered to be “aged,” but the onset of classic characteristics of aging in horses such as loss of muscle mass and function, decline in immune function, and frailty have not been well studied. Commonly reported conditions in aged horses such as pituitary pars intermedia dysfunction, dental abnormalities and increased risk of tendon and ligament damage (Ralston and Harris, 2013) have the potential to impact skeletal muscle, through alterations in metabolism, nutrient availability, and mobility. While there is a great deal of research on healthy aging in human skeletal muscle, very little is known about how to support the muscle health and well-being of our aging equine companions.

In humans and rodents, aging is accompanied by a loss of muscle mass and function, and research in other species indicates this is likely due to mitochondrial dysfunction, elevated ROS production, impaired mitophagy (Chabi et al., 2008; Hepple, 2014), chronic inflammation (Beyer et al., 2012), and deficiencies in satellite cell, or resident muscle stem cell, number and function (Conboy and Rando, 2005; Shefer et al., 2006). Further, research in humans shows declines in whole muscle functionality with age due to the concurrent reduction in fiber size and in the percentage of fibers expressing single myosin isoforms (Deschenes, 2004).

Previous work in horses has reported similar adaptations to aging as those seen in humans: decreased skeletal muscle mitochondrial number (Li et al., 2016), elevated muscular and circulating levels of inflammatory cytokines (McFarlane and Holbrook, 2008), and decreased muscle mass (Hintz, 1995). A number of interventions have been utilized to improve these parameters individually, including dietary antioxidant supplementation and anti-

inflammatory pharmaceuticals, but these do not address the collective global alterations associated with aging.

In aged humans and other models, exercise training has been shown to result in improvements in skeletal muscle health. Exercise training has been shown to improve parameters that altered in aged individuals and are deleterious to muscle health such as increased systemic and muscular inflammation (Woods et al., 2012), oxidative stress (Radák et al., 2002) and catabolic signaling and apoptotic signaling (Pasini et al., 2012). Additionally, exercise has been shown to improve parameters that are impaired with aging such as mitochondrial characteristics (Short et al., 2003) and anabolic signaling (Pasini et al., 2012). However, exercise in aged humans often does not result in full rescue of biological parameters, such as antioxidant status (Rousseau et al., 2006), insulin sensitivity (Short et al., 2003), anabolic signaling, and muscle protein synthesis (Cuthbertson et al., 2005).

In horses, few studies have been conducted to examine the effects of exercise training in aged horses. However, the available literature does suggest that exercise in aged horses improves parameters such as maximal aerobic capacity, maximal oxygen pulse, and citrate synthase activity (Betros et al., 2002; Kim et al., 2005b). Few studies in horses have examined the effects of exercise and compared them to adaptations in young or mature horses undergoing the same training to determine if exercise can rescue age-related impairments.

Low intensity exercise has the potential to improve antioxidant enzyme activity, decrease chronic inflammation, increase satellite cell number, and increase lean muscle mass. However, the effect of exercise on these parameters in aged horses has not been closely studied.

1.5. Thyroid Stimulating Hormone: Extreme Longevity and Extrathyroidal Action

Research has shown that certain populations of individuals over 100 years old (centenarians) have significantly higher circulating thyroid stimulating hormone (TSH) concentrations than younger controls in the same population, or age-matched control populations of thyroid disease-free individuals; however, these individuals maintain normal circulating thyroid hormone (TH) concentrations (Atzmon et al., 2009). The role of increased TSH in extreme longevity has not yet been determined, but it has been hypothesized that TSH plays a signaling role outside of the thyroid (Jansen et al., 2015). While there is not a wealth of research on the extrathyroidal effects of TSH, several studies have shown that TSH receptors (TSHR) are expressed in various tissues other than the thyroid, including skeletal muscle (Williams, 2011; Moon et al., 2016). Interestingly, in skin, TSH stimulates mitochondrial biogenesis (Poeggeler et al., 2010) and function (Vidali et al., 2014). Additionally, TSH improved insulin sensitivity in mouse myotubes *in vitro* (Moon et al., 2016). Taken together, these studies suggest that TSH could play an integral role in regulating peripheral organ metabolism of extremely aged individuals. A possible mechanism by which TSH increases the lifespan could be stimulation of mitochondrial biogenesis in peripheral tissues, leading to an improvement in metabolic efficiency. Considering that muscle comprises approximately 40 to 50% of body weight in humans (Janssen et al., 2000), improvements in metabolic efficiency of this system could have ample impacts on overall health in aged individuals. There is a no research on changes in TSHR in skeletal muscle with age or with other factors that influence metabolic efficiency, such as exercise. However, research does show that factors that influence metabolism, such as exercise training, cause changes in TSH and TH production, as well as changes in thyroid hormone

receptor (THR) mRNA expression and protein levels in mouse skeletal muscle (Lesmana et al., 2016). Therefore, basic information on changes in TSHR number and localization with age and exercise may act as an extremely informative basis to begin exploring the mechanism by which increased TSH extends the lifespan.

Much of the exploratory research investigating the effects of TSH in peripheral organs has been performed in mouse models, which may not be an ideal representation of human aging and longevity. Compared to humans, mice have a considerably shorter lifespan, differences in physiological changes with aging, and dissimilarities in muscle physiology, such as the presence of type IIb muscle fibers, which are not present in the majority of large mammals. As an alternative to rodents, horses have been suggested as a superior model for human aging, as horses are the longest living domestic animal and share many physiological similarities with humans. Unfortunately, there is little research investigating the physiological changes associated with aging in horses. Further, there is currently no literature in horses on extrathyroidal expression of TSHR, or effects of TSH on skeletal muscle metabolism in horses.

2. DIFFERENTIAL SKELETAL MUSCLE CHARACTERISTICS OF WEANLING RACING-BRED HORSES*

2.1. Synopsis

Responses of equine skeletal muscle characteristics to growth and training have been shown to differ between breeds. These differential responses may arise in part because muscle fiber type and mitochondrial density differ between breeds, even in untrained racing-bred horses. However, it is not known when these breed-specific differences manifest. To test the hypothesis that weanling Standardbreds (SB) and Thoroughbreds (THB) would have higher mitochondrial indices than Quarter Horses (QH), gluteus medius samples were collected from SB (mean ± SD; 6.2 ± 1.0 mo; n = 10), THB (6.1 ± 0.5 mo; n = 12), and QH (7.4 ± 0.6 mo; n = 10). Citrate synthase (CS) and cytochrome *c* oxidase (CCO) activities were assessed as markers of mitochondrial density and function, respectively. Mitochondrial oxidative (P) and electron transport system (E) capacities were assessed by high-resolution respirometry (HRR). Data for CCO and HRR are expressed as integrated (per mg protein and per mg tissue wet weight, respectively) and intrinsic (per unit CS). Data were analyzed using PROC MIXED in SAS v 9.4 with breed as a fixed effect. Mitochondrial density (CS) was higher for SB and THB than QH ($P \leq 0.0007$). Mitochondrial function (integrated and intrinsic CCO) was higher in THB and QH than SB ($P \leq 0.01$). Integrated CCO was also higher in THB than QH ($P < 0.0001$). However, SB had higher integrated maximum P (P_{CI+II}) and E (E_{CI+II}) than QH ($P \leq 0.02$) and greater integrated and intrinsic complex II-supported E (E_{CII}) than both QH and THB ($P \leq 0.02$), while THB exhibited higher integrated P with complex I substrates (P_{CI}) than SB and QH ($P \leq 0.003$)

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and higher integrated P_{CI+II} and E_{CI+II} than QH ($P \leq 0.02$). In agreement, THB and QH had higher contribution of complex I (CI) to max E than SB ($P \leq 0.001$), while SB had higher contribution of CII than QH and THB ($P \leq 0.002$). Despite having higher mitochondrial density than QH and THB, SB showed lower indices of mitochondrial function and differences in contribution of complexes to oxidative and electron transport system capacities. Breed differences in mitochondrial parameters are present early in life and should be considered when developing feeding, training, medication, and management practices.

Introduction

Skeletal muscle fiber type composition and energetic profile of both mature trained and untrained racing-bred horses differ between breeds (Hodgson et al., 2014), and appear to diverge early in life (Bechtel and Kline, 1987; Kline and Bechtel, 1990). Stull and Albert (1980) proposed that these differences arise due to selection and adaptation for varying lengths and intensities of training and competition. Differences in adaptation to exercise have been demonstrated, and successfully raced Quarter Horses (Wood et al., 1988) and endurance horses (Rivero et al., 1993) have markedly distinctive muscle fiber type populations. While all muscle fiber types are necessary for optimal performance, oxidative fibers and oxidative capacity are critical for longer bouts of exercise, such as Standardbred (Essèn-Gustavsson and Lindholm, 1985) and perhaps Thoroughbred (Lindholm et al., 1983) races. Conversely, fast twitch, non-oxidative fibers are extremely important for Quarter Horse racing (Wood et al., 1988), where explosive power is required, but exercise duration is shorter.

Previous studies established disparities in metabolic enzymes and muscle fiber type in mature horses of different breeds and disciplines, but there is no research comparing mitochondrial characteristics and oxidative capacity between racing breeds from a young age.

Therefore, the aim of this study was to test the hypothesis that weanling Standardbreds (SB) and Thoroughbreds (THB) would have higher mitochondrial indices than Quarter Horses (QH).

2.3. Methods

2.3.1. Horses

This study was reviewed and approved by the Texas A&M Institutional Animal Care and Use Committee (2016-0294). Samples were collected from weanling racing-bred Thoroughbreds (THB; mean ± SD; 6.1 ± 0.5 mo; range 6 to 7 mo; 9 colts, 3 fillies), Quarter Horses (QH; 7.4 ± 0.6 mo; range 7 to 8 mo; 9 colts, 1 filly) and Standardbreds (SB; 6.2 ± 1.0 mo; range 5 to 8 mo; 9 colts, 1 filly) from farms in Kentucky and Texas with owner consent. Thoroughbreds were housed in pastures and brought to stalls to receive morning and evening concentrate meals. Quarter Horses were housed and fed concentrate in groups in pastures. Four Standardbreds were being housed and fed in stalls at the time of collection, and the remainder were housed and fed concentrate in groups in the pasture. No horses had received forced exercise prior to sampling.

2.3.2. Sample Collection and Analysis

Muscle samples were collected from the gluteus medius muscle using a 14-gauge biopsy needle (SuperCore; Argon Medical Devices Inc., Frisco, TX) as previously described (White et al., 2016). Citrate synthase (CS) and cytochrome *c* oxidase (CCO) activities were analyzed as markers of mitochondrial density and function, respectively (Larsen et al., 2012). For measurement of CS and CCO activities, frozen skeletal muscle samples were prepared, and activities were measured as previously described (Spinazzi et al., 2012; Li et al., 2016). Enzymatic activities were normalized to homogenate supernatant protein content, determined using the Bradford Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA). Cytochrome *c* oxidase activity is presented on an integrated (per mg protein) and intrinsic (per unit CS) basis.

For high-resolution respirometry (HRR), muscle fibers were collected, prepared and permeabilized as previously described (Li et al., 2016) and then analyzed within 24 h of collection. Oxygen flux and respiratory states were determined by HRR with the following substrate-uncoupler-inhibitor titration protocol modified from a previously described protocol for equine skeletal muscle (Li et al., 2016): (1) pyruvate (5 mM) and malate (2 mM) to support electron flow through complex I (CI) of the electron transport system (ETS) (LEAK respiration); 2) adenosine diphosphate (ADP; 2.5 mM) to stimulate respiration (OXPHOS, P_{CI}); 3) cytochrome *c* (cyt c; 10 μM) to assess outer mitochondrial membrane integrity (samples with responses to cyt c greater than 15% were excluded); 4) glutamate (10 mM) as an additional CI substrate and succinate (10 mM) to support convergent electron flow through complex II (CII) of the ETS (P_{CI+II}); 5) uncoupler carbonyl cyanide 3-chlorophenylhydrazone (CCCP; 0.5 μM steps) to assess maximum ETS capacity (E_{CI+II}); 6) rotenone (0.5 uM), an inhibitor of complex I, to measure maximal ETS capacity of complex II (E_{CII}); 7) antimycin A (2.5 μM), an inhibitor of complex III, to measure residual oxygen flux (ROX) independent of the ETS. Sample flux control ratio (FCR) for each complex was calculated by dividing the flux in each complex by the sample's E_{CI+II} flux.

2.3.3. Statistical Analysis

Differences in enzyme activities and mitochondrial respiration measurements between breeds were analyzed using the MIXED procedure of SAS (Version 9.4, SAS Institute Inc., Cary, NC). Data were tested for normality by Shapiro-Wilk, Kolmogorov-Smirnov, Cramer-von Mises and Anderson-Darling tests and then log-transformed prior to analysis if not normally distributed. All log transformed data were normally distributed. Intrinsic CCO and HRR, and FCR data were not transformed as they were already normalized to CS and E_{CI+II} flux,

respectively. Breed was included in the model as a fixed effect. The QH and SB groups only contained one filly each, so sex was not included in the model. Additionally, because THB contained no 8-mo-old horses, and QH contained no 5- or 6-mo-old horses, age was not included in the model. However, within breed, none of the measured variables correlated with days of age ($P > 0.05$; PROC CORR in SAS 9.4; data not shown). All data are expressed as mean ± SEM. Significance was considered at $P \leq 0.05$, and trends were acknowledged at $P \leq 0.10$.

2.4. Results

Citrate synthase activity was used as a marker of mitochondrial density (Larsen et al., 2012). Standardbreds and THB had higher CS activity than QH ($P \leq 0.0007$). Citrate synthase activity did not differ between SB and THB. Cytochrome *c* oxidase activity was used as a marker of mitochondrial function (Larsen et al., 2012). Integrated CCO activity was higher in THB than QH ($P < 0.0001$) and SB ($P < 0.0001$) and was also higher in QH than SB ($P = 0.01$). Intrinsic CCO was lower in SB than QH and THB ($P < 0.0001$; Table 2.1) but did not differ between QH and THB.

Table 2.1. Citrate synthase (CS) and integrated (per mg protein) and intrinsic (per unit CS) cytochrome *c* oxidase (CCO) activities in the gluteus medius of weanling racing-bred Quarter Horses (QH; n = 10), Thoroughbreds (THB; n = 12) and Standardbreds (SB; n = 10). Reprinted with permission from "Rapid Communication: Differential skeletal muscle mitochondrial characteristics of weanling racing-bred horses" by Latham, C.M., C.K. Fenger and S.H. White. J. Anim. Sci. doi: 10.1093/jas/skz203, Copyright 2019 by The American Society of Animal Science.

Enzyme	QH	THB	SB	SEM	*P*-Value
CS Activity, nmol • min^{-1} • mg $protein^{-1}$	9.7 [a]	16.0 [b]	16.2 [b]	1.2	0.0006
Integrated CCO Activity, nmol • min^{-1} • mg $protein^{-1}$	15.3 [b]	25.8 [c]	9.0 [a]	1.7	<0.0001
Intrinsic CCO Activity, nmol • min^{-1} • unit CS^{-1}	1.6 [b]	1.7 [b]	0.6 [a]	0.01	<0.0001

[a-c] Within a row, breeds lacking common letters differ ($P \leq 0.05$)

Mitochondrial respiratory capacities were determined by HRR. Thoroughbreds had higher integrated ($P = 0.002$; Fig. 2.1A) and intrinsic ($P = 0.02$; Fig. 2.1B) LEAK than QH. Additionally, THB tended to have higher integrated ($P = 0.07$) and intrinsic LEAK ($P = 0.07$) compared to SB. Integrated and intrinsic LEAK did not differ between SB and QH.

Integrated P_{CI} was higher for THB than SB and QH ($P \leq 0.003$; Fig. 2.1A) but did not differ between SB and QH. Intrinsic P_{CI} was not different between breeds (Fig. 2.1B). Integrated P_{CI+II} was lower for QH than THB and SB ($P \leq 0.01$; Fig. 2.1A) but did not differ between SB and THB. A trend for an effect of breed ($P = 0.1$) for intrinsic P_{CI+II} suggested that SB was higher than THB ($P = 0.03$), while QH did not differ from SB or THB (Fig. 2.1B). Integrated E_{CI+II} was lower for QH than THB and SB ($P \leq 0.02$; Fig. 2.1A) but did not differ between THB and SB. However, a trend for a main effect of breed ($P = 0.07$) on intrinsic E_{CI+II} showed that SB was higher than THB ($P = 0.02$), while QH did not differ from SB or THB (Fig. 2.1B).

Integrated (Fig 2.1A) and intrinsic (Fig. 2.1B) E_{CII} were higher for SB than QH ($P \leq 0.005$) and THB ($P \leq 0.02$), and integrated E_{CII} was higher for THB than QH ($P = 0.04$).

Thoroughbreds had higher FCR for LEAK than QH and SB ($P \leq 0.01$) but LEAK FCR did not differ between SB and QH (Fig. 2.1C). The FCR for P_{CI} was higher for THB and QH than SB ($P \leq 0.001$) but did not differ between THB and QH (Fig. 2.1C). A trend for an overall effect of breed ($P = 0.09$) indicated that the FCR for P_{CI+II} was lower for QH than THB ($P =0.03$) and tended to be lower for QH than SB ($P = 0.1$) but did not differ between THB and SB. The FCR for E_{CII} was higher in SB than QH and THB ($P \leq 0.002$; Fig. 2.1C) but did not differ between QH and THB.

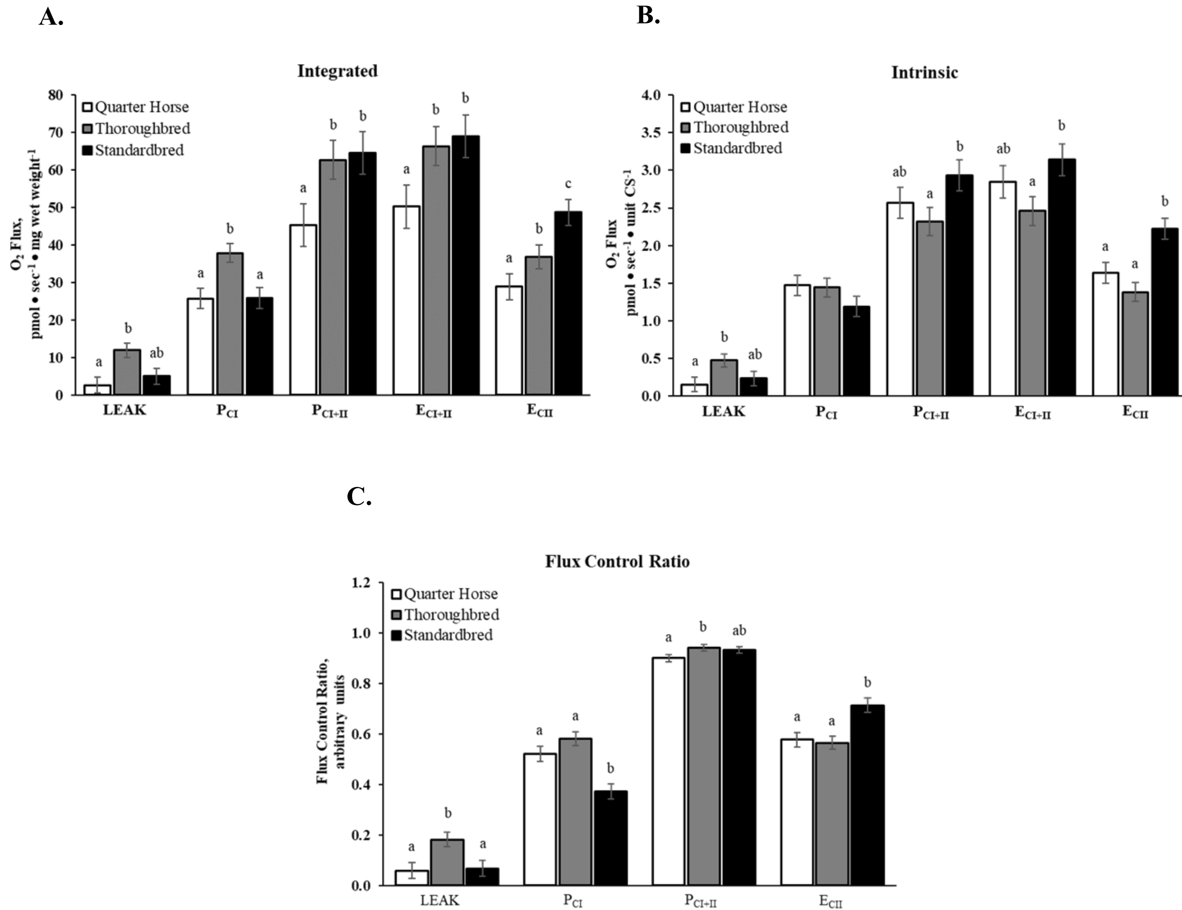


Figure 2.1. Mitochondrial respiration of permeabilized fibers from the gluteus medius of weanling racing-bred Quarter Horses (QH; n = 10), Thoroughbreds (THB; n = 12) and Standardbreds (SB; n = 10) via high-resolution respirometry.Mass specific O_2 flux (A; pmol • sec^{-1} • mg wet $weight^{-1}$), O_2 flux normalized to CS activity (B; pmol • sec^{-1} • unit CS^{-1}), and O_2 flux normalized to maximum electron transport system (ETS) capacity (C; arbitrary units) with LEAK respiration (LEAK), oxidative phosphorylation (OXPHOS) capacity of complex I (P_{CI}), OXPHOS capacity of complex I and II (P_{CI+II}), maximum ETS capacity (E_{CI+II}), and maximum ETS capacity of complex II (E_{CII}). ^{a,b} Within a variable, bars lacking common letters differ (*P* ≤ 0.05). Reprinted with permission from "Rapid Communication: Differential skeletal muscle mitochondrial characteristics of weanling racing-bred horses" by Latham, C.M., C.K. Fenger and S.H. White. J. Anim. Sci. doi: 10.1093/jas/skz203, Copyright 2019 by The American Society of Animal Science.

2.5. Discussion

Oxidative capacity of skeletal muscle depends primarily on mitochondrial density and the capacities of five main protein complexes in the mitochondria, which are responsible for electron transport and, ultimately, adenosine triphosphate (ATP) production. The present study demonstrates that racing-bred QH, THB and SB differ in their skeletal muscle mitochondrial density and capacities as early as 6 mo of age. This is unsurprising, as individuals have been selected within each breed based on excellence at unique race distances (1/4 mile for QH, longer distances for THB and SB) that have disparate oxidative respiration requirements. Young and mature untrained QH have previously been shown to have a lower percentage of slow twitch, oxidative muscle fibers and lower CS activity when compared to SB and THB (Stull and Albert, 1980; Bechtel and Kline, 1987; Kline and Bechtel, 1990). In agreement, we found CS activity and several indices of integrated oxidative capacity to be lower in QH weanlings than THB and SB. Given that successful racing QH have a higher percentage of fast twitch, non-oxidative fibers (Wood et al., 1988) and rely more heavily on anaerobic metabolism for work and competition, our findings suggest that these metabolic characteristics have been selected for and are present from a young age. The current study did not investigate muscle fiber type, but the lower mitochondrial markers suggest a lower presence of oxidative type I fibers in QH even as weanlings.

Reduction of NADH at complex I (CI) and succinate at complex II (CII), and the subsequent transfer of electrons through the ETS to oxygen as the terminal electron acceptor ultimately results in ATP synthesis. However, insufficient concentrations of adenosine diphosphate (ADP), damaged cellular membranes, or the presence of uncoupling proteins may lead to ETS activity uncoupling from ATP synthesis. Instead, protons “leak” across the inner

mitochondrial membrane, consuming cellular oxygen and as such, potentially mitigating reactive oxygen species (ROS)-induced oxidative damage (Brand, 2000). Increased CI activity has been associated with increased mitochondrial ROS production (St-Pierre et al., 2002) that may be related to anabolic signaling and adaptation following exercise (Seifert et al., 2012). The elevated LEAK respiration exhibited by THB in the current study may be related to THB also exhibiting higher integrated capacity with CI substrates; greater proton leak could alleviate CI-linked ROS production.

Standardbreds showed the lowest FCR for CI respiration, along with the highest integrated and intrinsic ETS capacity with CII, and the highest FCR for CII. Furthermore, SB had higher intrinsic maximum oxidative and electron transport system capacities than THB. Complex II is a source of reserve respiratory capacity that promotes cell survival during times of stress, such as when energy demands exceed supply (Pfleger et al., 2015). Therefore, it is possible that because SB typically undergo longer bouts of exercise during both training and competition, they have been selected to achieve a higher reserve respiratory capacity to maintain energy production during prolonged bouts of physical activity.

Despite having comparable integrated maximum oxidative and ETS capacity with THB, SB showed lower indices of mitochondrial function as evidenced by lower CCO activity. To our knowledge, this is the first study to examine breed differences in CCO activity in weanling horses. While CCO activity did not increase with growth in 18-mo-old QH (White et al., 2017a), it is possible that CCO activity increases with growth of younger horses, in concert with observed increases in percentages of oxidative fibers and other enzymes for oxidative metabolism (Yamano et al., 2005). Therefore, the observed lower CCO activity of weanling SB may increase with age.

The present study provides some insight into one of the mechanisms by which medications, training programs, and other management factors may lead to differential outcomes in different breeds of racing horses. An applicable example is the use of clenbuterol in performance horses. In SB, clenbuterol treatment increased fat free mass (Kearns et al., 2001) but impaired aerobic performance (Kearns and Mckeever, 2002). Similarly, clenbuterol failed to improve aerobic performance in THB (Rose and Evans, 1987). The current study demonstrates a greater capacity for aerobic metabolism from a young age in SB and THB as compared to QH. This finding supports the idea that clenbuterol, which acts as a repartitioning agent (Kearns et al., 2001) by increasing the percentage of myosin heavy chain (MyHC) type IIx (fast twitch, non-oxidative) in skeletal muscle (Beekley et al., 2003) would be unlikely to enhance performance in breeds that rely heavily on oxidative fibers. The effects of clenbuterol treatment on performance measures in QH have not been specifically examined. However, an increase in the percentage of MyHC IIx may benefit racing QH, as successfully raced QH rely more heavily on this anaerobic fiber type (Wood et al., 1988).

As with any study, this experiment has its limitations. Citrate synthase activity was used as a marker of mitochondrial density, as it has been shown to have the strongest association with mitochondrial content as measured by electron microscopy in healthy young (24 ± 0.9 yr) human males, when compared to cardiolipin content, mitochondrial DNA content, mitochondrial complex I through IV protein content, and mitochondrial complex I through IV activities (Larsen et al., 2012). It should be noted that CS activity has not been validated as a marker of mitochondrial content in the horse. Therefore, deduced differences in mitochondrial density and intrinsic variables (normalized to CS activity) in the present study should be interpreted with caution. In the same previous validation study (Larsen et al., 2012), cytochrome *c* oxidase

activity was shown to be most closely correlated with maximum oxidative capacity, an indicator of mitochondrial function. In the present study, CCO activity did not show the same relationship to OXPHOS capacity, as SB exhibited the highest intrinsic maximum oxidative capacity, and the lowest intrinsic CCO activity. It is possible that the differences arose because the horses in the present study were still growing. Future research in horses should address the applicability of CS and CCO activities as markers of mitochondrial density and function in both growing and mature horses and explore other markers that may be better correlated to mitochondrial number and OXPHOS capacity.

In this study, we found that SB and THB weanlings had higher CS activity and integrated maximum oxidative capacity compared to QH, as well as differences in contribution of complexes to oxidative and electron transport system capacities between breeds. Breed differences in mitochondrial parameters appear to be present early in life, but their changes during growth and training programs are not well studied and remain poorly understood. Inherent breed differences likely impact responses to feeding, training, medications, and other management strategies. The impact of interventions on different breeds during growth, training, and competition must be further examined in order to construct informed regulation and management practices that maximize welfare of racing horses of all breeds.

3. COMPLEXED TRACE MINERAL SUPPLEMENTATION ALTERS ANTIOXIDANT ACTIVITIES AND EXPRESSION IN RESPONSE TO TRAILER STRESS IN YEARLING HORSES

3.1. Synopsis

Upon entering a training program, young equine athletes experience oxidative stress that may lead to compromised muscle health if oxidant radicals are not properly sequestered by antioxidants. Dietary complexed trace minerals have been shown to increase antioxidant capacity in food animal models. To test the hypothesis that complexed trace mineral supplementation would similarly increase antioxidant capacity and decrease muscle oxidative stress and damage in young horses entering an exercise training program, Quarter Horses (mean ± SD; 9.7 ± 0.7 mo) balanced by age, gender, and BW were assigned to receive complexed (CTM; n = 8) or inorganic (INORG; n = 8) trace minerals. Blood and muscle samples were collected before (wk0) and after 8 (wk8) and 12 wk (wk12) of light exercise training. At each wk, tissue from the gluteus medius (GM) and triceps brachii (TB) muscles and blood were collected before, and 1 (1hPT) and 24 h (24hPT) after a 1.5-h trailer stressor; blood was also collected 0 (0hPT) and 6 h (6hPT) post-trailering. Pre-trailering muscle samples were evaluated for oxidative capacity by high resolution respirometry and muscle samples surrounding trailering were evaluated for citrate synthase (CS), cytochrome *c* oxidase (CCO), glutathione peroxidase (GPx) and superoxide dismutase (SOD) activities, *SOD1* and *SOD2* expression, and malondialdehyde (MDA) concentration; whole blood was evaluated for SOD and GPx activities and serum was evaluated for creatine kinase (CK) activity. Data were analyzed using PROC MIXED in SAS 9.4 with repeated measures. Diet, time, trailering, and all interactions were fixed effects and horse within diet was a random effect. Serum CK was lower at wk8 and 12 than wk0 ($P < 0.001$) for

all horses. Muscle GPx activity was higher for CTM than INORG ($P < 0.0001$) throughout the study. Activity of SOD in the GM tended to be lower for CTM than INORG at wk0 ($P = 0.07$) but increased over time for CTM ($P = 0.0008$). Following all trailer stressors, serum CK increased at 0hPT ($P < 0.0001$) and remained elevated through 24hPT. In response to the trailer stressor at wk8 and 12, MDA increased ($P \leq 0.009$) and CS and CCO activities decreased ($P \leq 0.03$) in the GM. Whole blood GPx activity increased by 0hPT ($P = 0.04$) after all trailer stressors in CTM only. Expression of *SOD2* increased 24 h after each trailer stressor in both muscle groups for INORG ($P \leq 0.0001$) but did not change in response to trailering in the TB for CTM at wk8 or 12. Supplementation with CTM to young horses in combination with 12 wk exercise training resulted in higher muscle GPx activity compared to horses receiving inorganic minerals. Dietary CTM may be useful for improving antioxidant capacity and maintaining muscle health in young equine athletes.

3.2. Introduction

Young equine athletes experience several stressors as they begin training and competing. For example, acute exercise bouts and trailer transportation between competitions or training facilities cause an increase in mitochondrial production of reactive oxygen species (**ROS**), which can be deleterious to skeletal muscle health if not properly sequestered by antioxidant enzymes. Dietary complexed trace mineral supplementation has been shown to reduce oxidative stress in food animal models. Dietary proteinate complexed-Zn supplementation increased glutathione peroxidase (**GPx**) and superoxide dismutase (**SOD**) activities in the spleen of barrows (She et al., 2017). Additionally, biocomplexed minerals Fe, Mn, Zn, and Cu enhanced SOD and GPx activities in the liver of grower-finisher pigs (Liu et al., 2016) and decreased plasma malondialdehyde (**MDA**), a measure of oxidative stress, in broilers (Echeverry et al., 2016).

In horses, research on the effects of dietary organic trace mineral supplementation is relatively sparse. One study in horses showed that Cu-Lys may be better absorbed than $CuSO_4$ (Wagner et al., 2011). However, in the same group of horses, Cu-Lys and Zn-Met supplementation did not seem superior to $CuSO_4$ and $ZnSO_4$ in improving erythrocyte SOD activity during exercise and immediately after recovery in horses (Wagner et al., 2010). It should be noted that exercise itself did not induce a change in SOD activity in that study, suggesting the exercise did not significantly increase systemic oxidative stress (Wagner et al., 2010). Additionally, supplemented mineral intakes of Cu-Lys and Zn-Met were higher than $CuSO_4$ and $ZnSO_4$, making interpretation of the results difficult.

Our objective was to test the hypothesis that complexed trace mineral supplementation would increase systemic and muscle antioxidant gene expression and enzyme activities, and decrease oxidative stress and muscle damage in response to trailering stress in yearling Quarter Horses enrolled in a submaximal exercise training program.

3.3. Methods

3.3.1. Horses

This study was reviewed and approved by the Texas A&M Institutional Animal Care and Use Committee (IACUC 2016-0294). Sixteen yearling Quarter Horses (7 fillies and 9 colts) with a mean age of 9.7 mo (SD 0.7) and BW of 295 kg (SEM 5) were used in this study. Horses were housed in paddocks by sex (0.53 and 0.72 ha for colts and fillies, respectively) at the Texas A&M University Freeman Equestrian Center in College Station, TX. This study commenced in January and was completed in April.

3.3.2. Dietary Treatments

Horses were balanced by age, gender, and BW and randomly assigned to receive custom concentrates containing either complexed (**CTM**; Zn-Met, Mn-Met, Cu-Lys, and Co-glucoheptonate; n = 8) or inorganic (**INORG**; $CuSO_4$, $ZnSO_4$, $MnSO_4$ and $CoCO_3$; n = 8) supplemental trace minerals. Horses were enrolled in a separate but related study prior to the beginning of this study (unpublished data). Therefore, all horses had received the experimental treatments for 12 wk prior to the current experiment. To facilitate trailer transportation and sample collection, horses were further divided into eight pairs each with equal representation of dietary treatments. Dietary treatments were initiated in one pair of horses per day over two wk with four consecutive days of diet initiation and trailering each wk. Horses were allocated to separate pens by sex and had *ad libitum* access to Coastal bermudagrass hay. Hay intake per horse per day was estimated by the following formula:

$$\frac{Total\ hay\ offered\ in\ the\ pen\ per\ day\ (kg) - total\ hay\ refused\ in\ the\ pen\ per\ day\ (kg)}{Number\ of\ horses\ in\ the\ pen}$$

Concentrate was offered at 1.25% BW/d (DM basis). Horses received grain meals individually in stalls (3.7 × 3.7 m) split equally into two meals fed at 0700 h and 1700 h. Refusals were monitored and recorded daily to calculate actual concentrate intake. Total daily intake of estimated hay intake plus actual concentrate intake was 2.0% BW/d (DM basis). Diets were formulated to maintain a BCS of 5 to 6 according to the Henneke body condition scoring system (Henneke et al., 1983), and to meet all requirements of growing horses in light exercise (NRC, 2007). Throughout the study, BW of horses was recorded weekly using a livestock scale

accurate to 1 kg (Cardinal Scales, Webb City, Missouri) and heart girth, body length, hip height, and wither height measurements were collected at wk 0, 8 and 12 to assess growth.

All feeds were analyzed prior to beginning the study. Concentrates were analyzed by Equi-Analytical Laboratories (Ithaca, NY), and hay was analyzed by Elk River Forage Lab (Elk River, MN) using standard analytical methods. The nutrient composition of dietary ingredients is presented in Table 3.1.

Table 3.1. Nutrient composition of bermudagrass hay, inorganic (INORG) concentrate and complexed trace mineral (CTM) concentrate.

Nutrient[1]	Bermudagrass Hay	INORG	CTM
DE, Mcal/kg	1.97	2.72	2.78
Fat, %	2.3	7.7	7.6
CP, %	12.5	19.3	20.0
NDF, %	66.0	40.0	36.9
ADF, %	35.5	25.9	24.5
Ca, %	0.38	0.94	1.20
P, %	0.26	0.92	1.05
Se, ppm	0.39	0.73	0.97
Zn, ppm	29	142	210
Mn, ppm	187	157	212
Cu, ppm	8	50	58
Co, ppm	0.00	6.14	8.04

[1] Values presented on a 100% DM basis.

3.3.3. Exercise

Horses had received no forced exercise prior to the beginning of this study. Beginning at wk0, horses were enrolled in a 12-wk submaximal exercise program. Exercise was designed to achieve light work as defined by the NRC (NRC, 2007), and consisted of 12 min of walking, 15 min of trotting, and 3 min of cantering in a free-stall exerciser (30 min total/d) 5 d/wk. Each gait

was performed in both directions each day, and horses alternated starting the exercise bout clockwise or counterclockwise each day. The speed of each gait was progressively increased throughout the study to ensure that all horses remained in the intended gait. Gait speeds started at 1.1 m/s for the walk, 2.5 m/s at the trot and 5.0 m/s at the canter at the beginning of training and were increased to 1.2 m/s at the walk, 3.0 m/s at the trot and 5.4 m/s at the canter by the end of the 12 wk exercise training program.

3.3.4. Trailer Stressor

Before beginning exercise training (wk0), and after 8 (wk8) and 12 wk (wk12) of exercise training, horses were trailered for 1.5 h to assess the effects of trailer stress on antioxidant and mitochondrial activities and markers of oxidative stress and muscle damage. Trailering was performed on the same route by the same driver for all trailering sessions.

3.3.5. Sample Collection

Muscle and blood samples were collected surrounding each 1.5-hour trailer stressor at wk 0, 8 and 12. Muscle samples were collected before trailering (PreT), and 1 (1hPT) and 24 h (24hPT) after trailering for analysis of muscle MDA concentration, GPx, SOD, CS and CCO activities, and *SOD* gene expression; oxidative capacity was also analyzed PreT. Muscle tissue samples were collected from the gluteus medius (GM) and triceps brachii (TB) using a tissue collection procedure as previously described (White et al., 2016). Briefly, horses were sedated with detomidine hydrochloride (Dormosedan; Zoetis, Parsippany-Troy Hills, NJ) prior to beginning tissue collection procedures. The collection areas were clipped, scrubbed with a 7.5% povidone-iodine solution, and then scrubbed with a 70% ethanol solution. The tissue collection sites were desensitized with 0.5 mL of 2% lidocaine (Vetone, Boise, ID) and a 14-gauge needle was used to create the initial puncture through the skin. Tissue was collected using a 14-gauge,

9-cm biopsy needle (SuperCore; Argon Medical Devices Inc., Frisco, TX) inserted to a depth of 3.5 cm. The tissue collection site altered between left and right muscle groups at each sampling interval. Samples obtained from the same side of the horse were obtained approximately 2 cm from the previous insertion site. At each sampling interval, approximately 75 mg (wet weight) of tissue was placed into 1 mL of RNALater (Invitrogen, Waltham, MA) and stored at -20° C until gene expression analysis was performed. An additional 300 mg (wet weight) of muscle tissue was flash frozen in liquid nitrogen and stored at -80° C until enzymatic activity analyses were performed. For high-resolution respirometry (HRR), muscle fibers were collected, prepared and permeabilized as previously described (Li et al., 2016) and then analyzed within 24 h of collection. Flash frozen muscle was cryopulverized into a fine powder (Spectrum™ Bessman Tissue Pulverizer; Thermo Fisher Scientific, Waltham, MA) for evaluation for SOD, GPx, CS and CCO activities and MDA concentration. Blood samples were collected before trailering (PreT), directly after (0hPT), and 1, 6 and 24 h after trailering (1hPT, 6hPT and 24hPT, respectively) for analysis of serum CK activity and whole blood GPx and SOD activities. At each sampling interval, approximately 15 mL of blood was collected into evacuated containers (Vacutainer; Becton, Dickinson and Co., Franklin Lakes, NJ), containing either no anticoagulant for harvesting of serum or sodium heparin for harvesting of whole blood. Serum samples remained at 25° C and plasma samples were placed on ice for approximately 1 h prior to processing. Samples were then centrifuged at 3,000 × *g* for 10 min at 4° C, and serum and whole blood were harvested and stored at -80° C until analysis.

3.3.6. Malondialdehyde Concentration

Muscle samples were evaluated for MDA concentration per manufacturer instructions using a commercially available kit (Northwest Life Science Specialties, LLC, Vancouver, WA)

as previously described (White and Warren, 2017). Previously cryopulverized muscle powder was diluted 1 mg tissue (wet weight) to 10 uL assay buffer provided in the kit and sonicated 3 times for 3 sec each on ice, and then centrifuged at 11,000 × *g* for 10 min at 4° C. Homogenate supernatants were collected and stored at -80° C until analysis. Samples were analyzed in triplicate with an intra-assay CV of 3.6% and an inter-assay CV of 1.4%. Samples for analysis of MDA concentration were within the standard range of the assay (0.1 to 10 μM). Muscle sample total protein was quantified using the Coomassie Protein Assay kit (Thermo Fisher Scientific), and MDA concentration was normalized to MDA homogenate supernatant total protein concentration.

3.3.7. Enzyme Activities

Serum samples were analyzed for creatine kinase (CK) activity as a marker of muscle damage (Siciliano et al., 1995) using a commercially available kit, following manufacturer instructions (CK Liqui-UV; EKF Diagnostics, Boerne, TX). Samples were analyzed in triplicate with an intra-assay CV of 3.9% and an inter-assay CV of 9.8%. All samples were within the linear range of the assay (1 to 1200 U/L).

Whole blood and muscle samples were analyzed for SOD and GPx activities as measures of antioxidant status using commercially available kits (Cayman Chemical Company, Ann Arbor, MI). Muscle tissue that had been previously cryopulverized and stored at -80º C was diluted 1 mg tissue (wet weight) to 40 uL extraction buffer (1mM EGTA, 210 mM mannitol, 70mM sucrose, pH 7.2), and 1 mg tissue (wet weight) to 80 uL extraction buffer for GPx and SOD analysis, respectively. Diluted samples were sonicated using a sonic dismembrator (Fisher Scientific, Hampton, NH) 3 times for 3 sec each on ice, and then centrifuged at 10,000 × *g* for 15 min at 4º C. Homogenate supernatants were collected and stored at -80º C until analysis.

Activity of GPx was evaluated in whole blood diluted 1:34 (v/v), and SOD activity was evaluated in whole blood diluted 1:150 (v/v) with assay buffer provided in the respective kits to ensure sample concentration was within the standard curve of assays. Whole blood and muscle samples were analyzed per manufacturer instructions in triplicate for GPx activity and in duplicate for SOD activity. Intra-assay CV was 4.5, 3.5, 4.8, and 3.6% and inter-assay CV was 10.3, 12.1, 9.3, and 10.1% for muscle GPx, muscle SOD, blood GPx, and blood SOD activities, respectively. Samples for analysis of GPx and SOD activities were within the dynamic range of the assays (50 to 344 nmol • min^{-1} • mL^{-1} for GPx, 0.005 to 0.05 U/mL for SOD). Muscle and blood sample total protein was quantified using the Coomassie Protein Assay kit (Thermo Fisher Scientific), and all antioxidant enzyme activities were normalized to homogenate supernatant total protein concentration.

Whole muscle citrate synthase (CS) and cytochrome *c* oxidase (CCO) activities were analyzed as markers of mitochondrial density and function, respectively (Larsen et al., 2012). For measurement of CS and CCO activities, frozen skeletal muscle samples were prepared, and activities were measured as previously described (Spinazzi et al., 2012; Li et al., 2016). Whole muscle samples were analyzed in duplicate for CS and CCO activities. Intra-assay CV was 2.3 and 1.8% and inter-assay CV was 10.2, and 8.0% for CS and CCO activities, respectively. Enzymatic activities were normalized to homogenate supernatant protein content, determined using the Bradford Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA). Cytochrome *c* oxidase activity is presented on an integrated (per mg protein) and intrinsic (per unit CS) basis.

3.3.8. High Resolution Respirometry

For high-resolution respirometry (HRR), muscle fibers were collected, prepared and permeabilized as previously described (Li et al., 2016) and then analyzed within 24 h of

collection. Oxygen flux and respiratory states were determined by HRR with the following substrate-uncoupler-inhibitor titration protocol modified from a previously described protocol for equine skeletal muscle (Li et al., 2016): (1) pyruvate (5 mM) and malate (2 mM) to support electron flow through complex I (CI) of the electron transport system (ETS) (LEAK respiration); 2) adenosine diphosphate (ADP; 2.5 mM) to stimulate respiration (OXPHOS, P_{CI}); 3) cytochrome *c* (cyt c; 10 μM) to assess outer mitochondrial membrane integrity (samples with responses to cyt c greater than 15% were excluded); 4) glutamate (10 mM) as an additional CI substrate and succinate (10 mM) to support convergent electron flow through complex II (CII) of the ETS (P_{CI+II}); 5) uncoupler carbonyl cyanide 3-chlorophenylhydrazone (CCCP; 0.5 μM steps) to assess maximum ETS capacity (E_{CI+II}); 6) rotenone (0.5 uM), an inhibitor of complex I, to measure maximal ETS capacity of complex II (E_{CII}); 7) antimycin A (2.5 μM), an inhibitor of complex III, to measure residual oxygen flux (ROX) independent of the ETS. Sample flux control ratio (FCR) for each complex was calculated by dividing the flux in each complex by the sample's E_{CI+II} flux.

3.3.9. Gene Expression

Muscle was examined for expression of antioxidant enzymes *Cu-Zn superoxide dismutase* (*SOD1*) and *Mn superoxide dismutase* (*SOD2*). Briefly, approximately 75 mg (wet weight) tissue was homogenized in TRIzol (Invitrogen, Carlsbad, CA) using a bead homogenizer (Fisher Scientific) 4 times for 30 sec intervals. 1-Bromo-3-cholorpropane was used to extract RNA from samples followed by isolation on a spin column according to manufacturer instruction (Purelink RNA Mini-kit; Invitrogen). Samples were treated with RNase-free DNase (Invitrogen) on the column during RNA extraction to remove any DNA contamination. Spectrophotometry was used to assess RNA concentration and optical density (OD) ratios on a 16-well plate (Biotek

Synergy H1, Winooski, VT). In all cases, RNA yield was greater than 15 ng/uL and $OD_{260/280}$ was greater than 1.5. Samples were reverse transcribed using the Superscript II Reverse Transcription kit (Invitrogen). Two hundred ng RNA was reverse transcribed to cDNA for the majority of the samples. However, 37 samples had a low (<25 ng/uL) RNA yield; for those samples, 120 ng RNA was reverse transcribed. The cDNA from reverse transcription was amplified with SYBR Green PCR master mix (Applied Biosystems, Foster City, CA) and the appropriate forward and reverse primers (Table 3.2). Thermal cycling parameters included a denaturation step of 95° C for 5 min followed by 40 cycles at 15 sec at 95° C and 1 minute at 60° C. Efficiency of PCR for each primer set was determined using a standard curve of pooled cDNA (Gonzalez et al., 2008). A Cycle of Quantification (**Cq**) value was determined for each sample via PCR. Data for each gene were normalized using the geomean of succinate dehydrogenase A (SDHA), hypoxanthine phosphoribosyltransferase 1 (HPRT1), and beta-2 microglobulin (B2M) as reference genes to account for total cDNA in each sample. The resulting ΔCq was calculated by subtracting $Cq_{geomean}$ from $Cq_{gene\ of\ interest}$. In figures, gene expression in represented as $40 - \Delta Cq$, in which 40 is the total number of cycles ran. Fold changes in gene expression are also reported and are calculated using the formula $2^{-\Delta\Delta Cq}$, in which $-\Delta\Delta Cq$ is the $\Delta Cq_{time\ of\ interest} - \Delta Cq_{PreT}$ (before trailer stressor) for each horse (Livak and Schmittgen, 2001).

Table 3.2 Sequence of equine-specific PCR primers used for measurement of SDHA, HPRT1, B2M, SOD1 and SOD2 mRNA content.

Gene	Primers 5'-3' (Forward, Reverse)	Slope[1]	Accession Number
SDHA	AAGTCGATGCAAAGTCATGCT	-3.129	DQ402987
	ACCATTCCTCTGTCAAACGTCT		
HPRT1	TCATGGAGTAATTATGGACAGGACT	-3.245	XM_023634464
	TCATAATCCAGCAGGTCAGCAA		
B2M	TTCTATCTTCTGGTCCATACTGACTT	-3.142	NM_001082502
	GAGGTCTCGATCCCACTTAACTAT		
SOD1	AGATAATACACAAGGCTGTACCACT	-3.158	NM_001081826
	ATTGCCCAGGTCTCCAACAT		
SOD2	AGCCTGCACTCAAGTTCAAT	-3.168	NM_001082517
	TCGAAGGAACCAAAGTCACGTT		

[1] Slope of standard curve of primer sequence used for RT-PCR analysis.

3.3.10. Statistical Analysis

Differences in serum CK activity, muscle MDA concentration, muscle and blood SOD and GPx activities, muscle CS and CCO activities, mitochondrial respiration measurements and muscle *SOD1* and *SOD2* expression were analyzed using the MIXED procedure of SAS 9.4 (SAS Institute, Inc, Cary, NC) with repeated measures. The response of the GM was analyzed separately from the TB. Additionally, the responses to diet and exercise over time (wk 0, 8, and 12 pre-trailer) were analyzed separately from responses to trailering at each wk. Data were tested for normality and then log-transformed before analysis if not normally distributed. For pre-trailer responses to diet and exercise, diet (CTM and INORG), time (wk0, wk8 and wk12), and the

interaction of diet × time were included in the model as fixed effects, and horse within diet was a random effect. For responses to trailering, the model included diet, time, trailering (Pre-T, 1hPT and 24hPT for muscle; PreT, 0hPT, 1hPT, 6hPT and 24hPT for blood), and all interactions as fixed effects, and horse within diet as a random effect. In cases where either wk0 or PreT values were significantly different between treatments, the covariate was tested. If the covariate was not significant, it was removed from the model. All data are expressed as means ± SEM. Significance was considered at $P \leq 0.05$, and trends were acknowledged at $P \leq 0.10$.

3.4. Results

3.4.1. Responses to Diet and Exercise Training

3.4.1.1. Growth

Body weight, heart girth, wither height and hip height increased by wk8 ($P < 0.0001$; Table 3.3), and body length increased by wk12 ($P < 0.0001$; Table 3.3). Body condition score decreased from 6.1 at wk0 to 5.6 at wk8 ($P < 0.0001$; Table 3.3) but was not different between wk8 and 12.

Table 3.3 Body weight, body condition score, heart girth, body length, wither height, and hip height in yearlings (n = 16) before exercise training (wk0) and following 8 (wk8) and 12 wk of exercise training (wk12). Due to lack of effect of diet, dietary treatments have been combined.

					P-Value		
Variable	wk0	wk8	wk12	SEM	Diet	Time	Diet × Time
Body Weight, kg	294.8	313.3*	331.8*	5.2	0.771	<0.0001	0.901
Body Condition Score	6.1	5.6*	5.5*	0.1	0.356	<0.0001	0.962
Heart girth, cm	148.8	154.5*	156.4*	1.3	0.555	<0.0001	0.637
Body Length, cm	145.0	146.1	149.7*	1.0	0.893	<0.0001	0.345
Wither Height, cm	51.8	52.8*	53.4*	0.2	0.926	<0.0001	0.623
Hip Height, cm	54.3	55.5*	56.0*	0.3	0.721	<0.0001	0.567

*Within a row, mean differs from wk0 ($P \leq 0.05$).

3.4.1.2. Citrate Synthase and Cytochrome *c* Oxidase Activities

Citrate synthase activity in the GM increased from wk 0 to 8 ($P = 0.007$; Fig 3.1A) and remained higher at wk 12 than 0 ($P = 0.001$) but was not affected by diet. In the TB, a trend for an effect of time ($P = 0.083$) suggested that CS activity was higher at wk 12 than 0 ($P = 0.028$; Fig. 3.1B) but was not affected by diet.

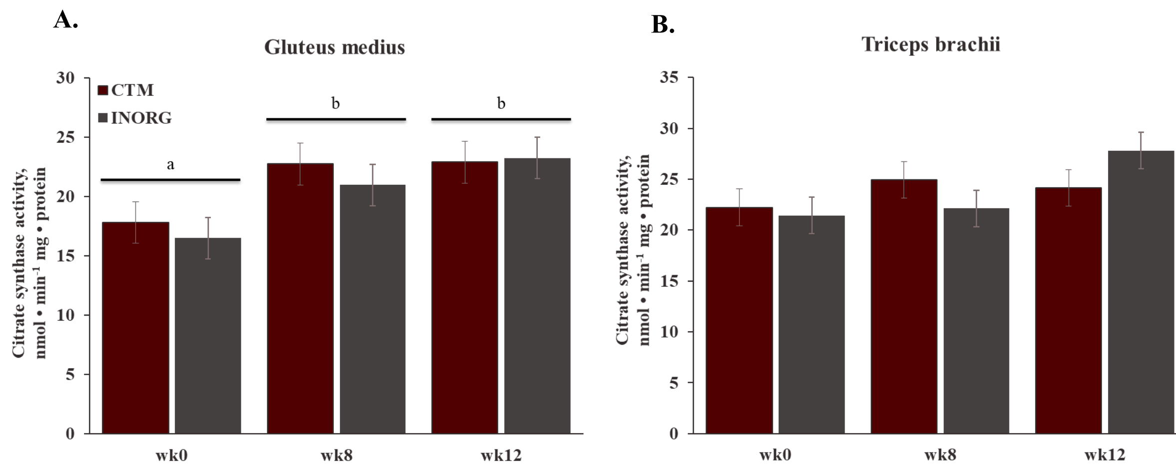


Figure 3.1. Citrate synthase (CS) activity in the gluteus medius (A) and triceps brachii (B) at 0, 8 and 12 wk of submaximal exercise training (wk0, wk8 and wk12) in yearling horses supplemented with either complexed trace minerals (CTM; n = 8) or inorganic trace minerals (INORG; n = 8). Overall effect of diet (*P* = 0.628; *P* = 0.747), time (*P* = 0.029; *P* = 0.085) and diet × time (*P* = 0.754; *P* = 0.236) for GM and TB, respectively. [a,b] Within a dietary treatment, time points lacking common letters differ ($P \leq 0.05$). Lines across bars indicate a main effect of time for both dietary treatments.

In the GM, integrated CCO activity increased at wk 8 (*P* = 0.044; Fig. 3.2A) and remained higher at wk 12 than 0 (*P* = 0.012) but was not affected by diet. Integrated CCO activity was not affected by diet, time, or their interaction (Fig 3.2B). Intrinsic CCO activity in the GM (Fig. 3.2C) and TB (Fig. 3.2D) were not affected by diet, time, or their interaction.

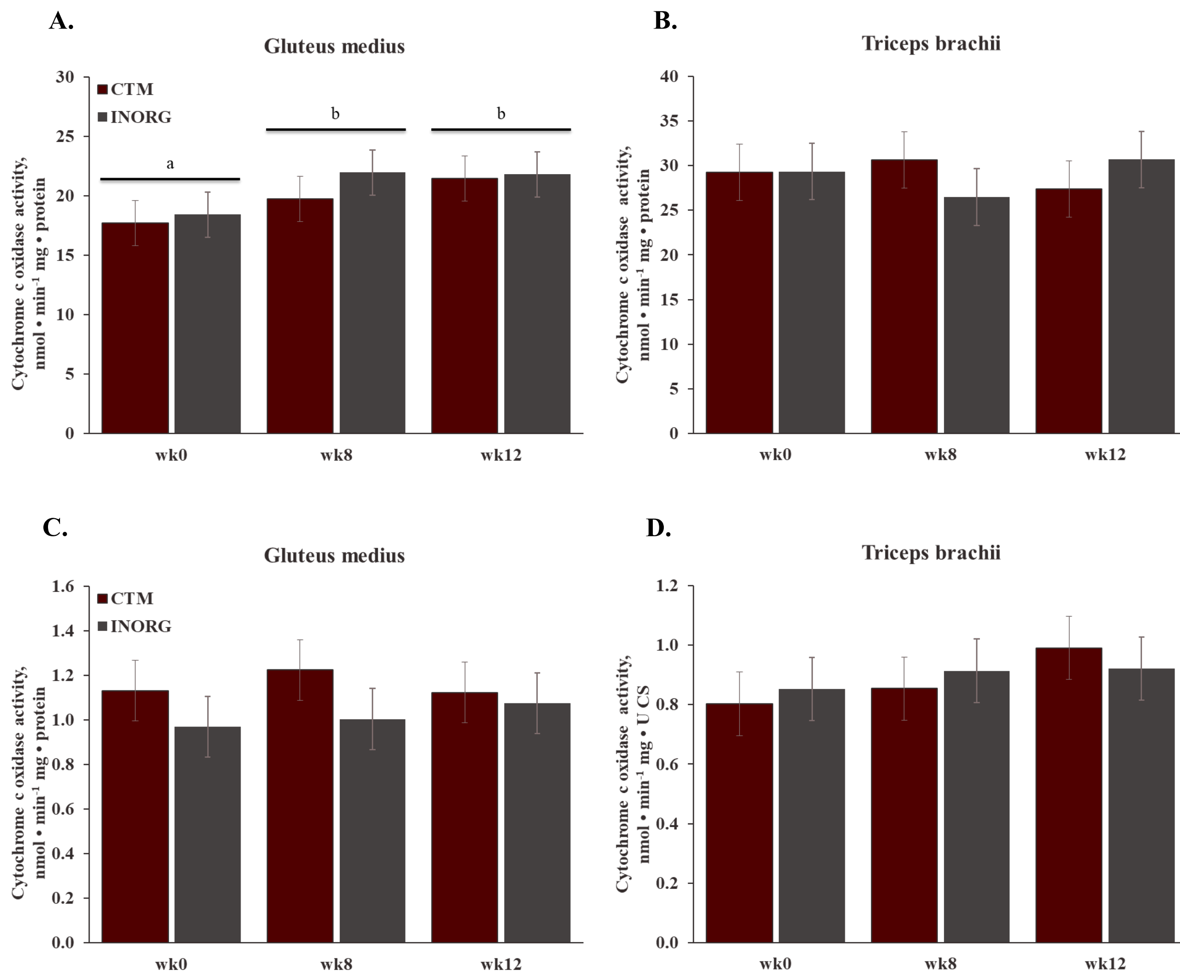


Figure 3.2. Integrated (A and B) and intrinsic (C and D) cytochrome *c* oxidase (CCO) activity in the gluteus medius (A and C) and triceps brachii (B and D) at 0, 8 and 12 wk of submaximal exercise training (wk0, wk8 and wk12) in yearling horses supplemented with either complexed trace minerals (CTM; n = 8) or inorganic trace minerals (INORG; n = 8). Overall effect of diet ($P = 0.628$; $P = 0.747$; $P = 0.376$; $P = 0.901$), time ($P = 0.029$; $P = 0.085$; $P = 0.794$; $P = 0.403$) and diet × time ($P = 0.754$; $P = 0.236$; $P = 0.671$; $P = 0.746$) for GM integrated CCO, TB integrated CCO, GM intrinsic CCO and TB intrinsic CCO, respectively. [a,b] Time points lacking common letters differ ($P \leq 0.05$). Lines across bars indicate a main effect of time for both dietary treatments.

3.4.1.3. Oxidative Capacity

Integrated LEAK in the GM was higher for INORG than CTM throughout training ($P = 0.031$; Fig. 3.3A), decreased from wk 0 to 8 ($P < 0.0001$) and remained lower at wk 12 than 0 (P

< 0.0001) for both diets. Integrated LEAK in the TB was not different between diets and decreased from wk 0 to 8 ($P < 0.0001$; Fig. 3.3B) and remained lower at wk 12 than 0 ($P < 0.0001$) for both diets. In both the GM (Fig. 3.3C) and the TB (Fig. 3.3D), intrinsic LEAK decreased from wk 0 to 8 ($P \leq 0.006$) and remained lower at wk 12 than 0 ($P \leq 0.003$) for both diets.

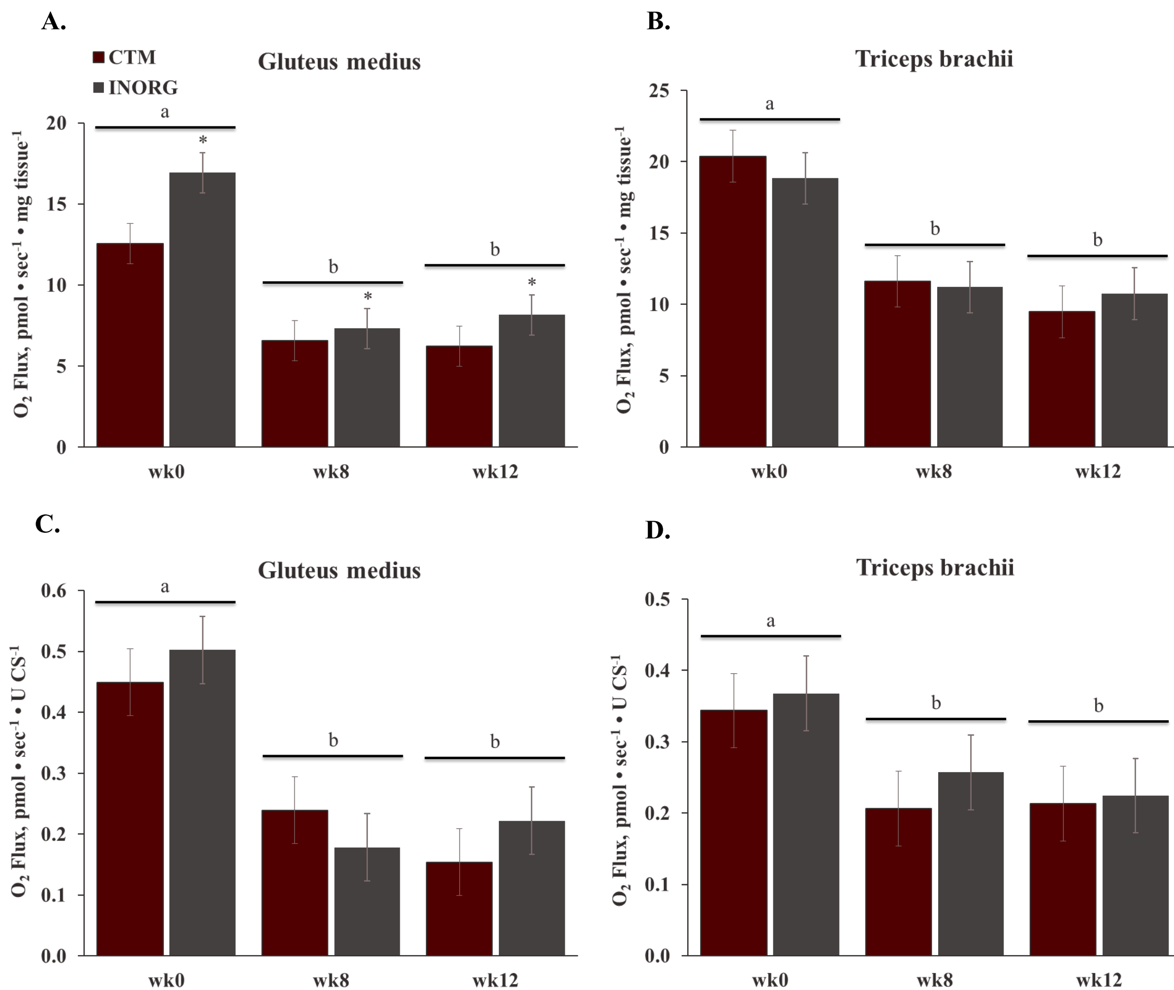


Figure 3.3. Integrated (A and B) and intrinsic (C and D) LEAK respiration in the gluteus medius (A and C) and triceps brachii (B and D) at 0, 8 and 12 wk of submaximal exercise training (wk0, wk8 and wk12) in yearling horses supplemented with either complexed trace minerals (CTM; n = 8) or inorganic trace minerals (INORG; n = 8). Overall effect of diet ($P = 0.031$; $P = 0.887$; $P = 0.667$; $P = 0.619$), time ($P < 0.0001$; $P < 0.0001$; $P < 0.0001$; $P < 0.0001$) and diet × time ($P = 0.358$; $P = 0.726$; $P = 0.441$; $P = 0.891$) for GM integrated LEAK, TB integrated LEAK, GM intrinsic LEAK and TB intrinsic LEAK, respectively. [a,b] Time points lacking common letters differ ($P \leq 0.05$). [*] Within time point, CTM differs from INORG ($P \leq 0.05$). Lines across bars indicate a main effect of time for both dietary treatments.

Integrated P_{CI} in the GM was higher for INORG than CTM throughout training ($P = 0.042$; Fig. 3.4A). Integrated P_{CI} tended to increase from wk 8 to 12 ($P = 0.097$) and was higher at wk 12 than 0 ($P = 0.009$) for both diets. A trend for a diet × time interaction ($P = 0.055$)

indicated that integrated P_{CI} in the TB was higher for CTM than INORG at wk 0 (*P* = 0.033; Fig. 3.4B) but increased at wk 8 for INORG (*P* = 0.023), leading to no difference between diets at wk 8 and 12. In the GM, intrinsic P_{CI} was not affected by diet, time or their interaction (Fig. 3.4C). In the TB, a trend for diet × time interaction (*P* = 0.100) suggested that INORG P_{CI} tended to be higher than CTM at wk 8 (*P* = 0.073; Fig. 3.4D), and that INORG P_{CI} increased from wk 0 to 8 (*P* = 0.015) but was not different from wk 0 or CTM at wk 12.

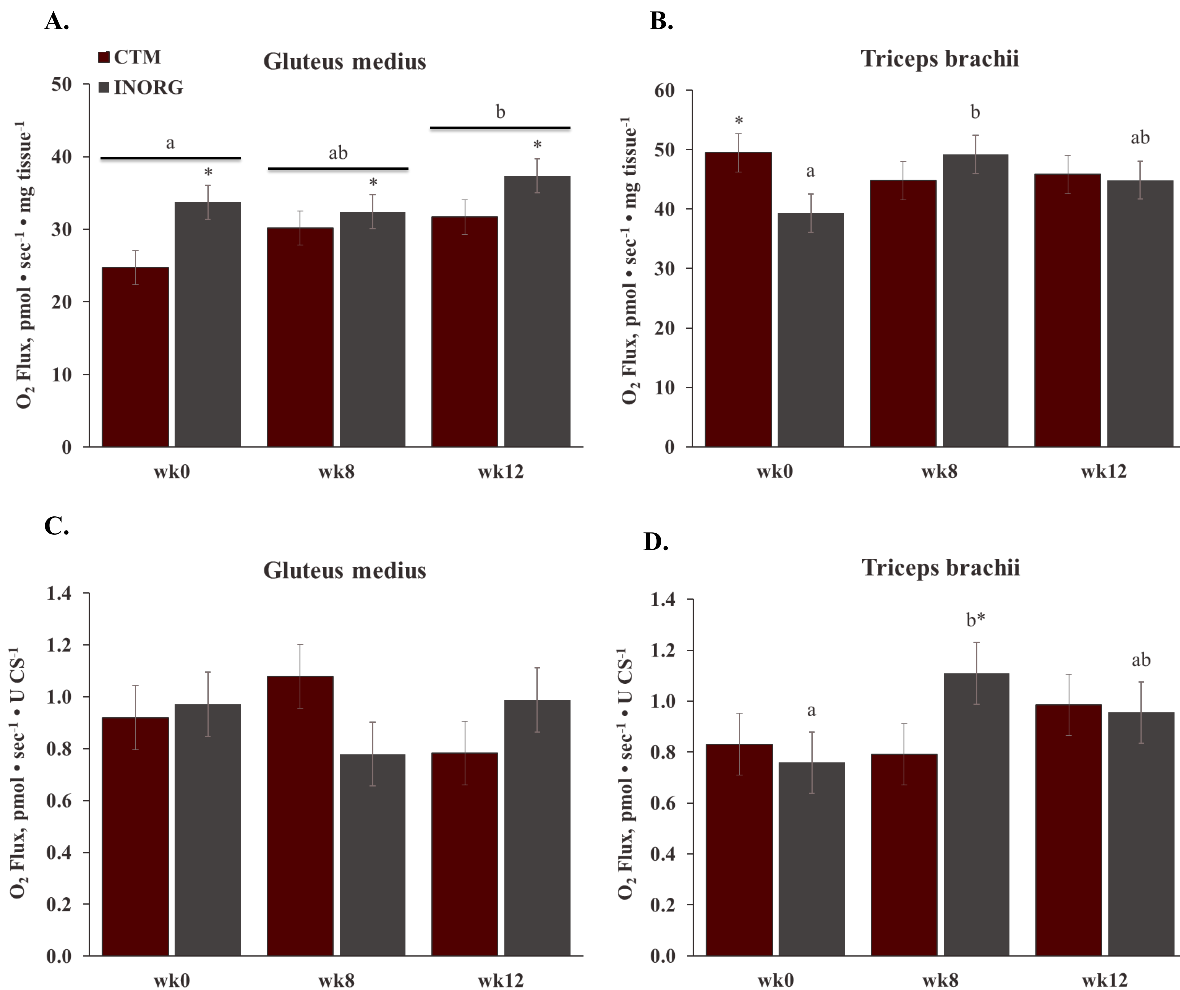


Figure 3.4. Integrated (A and B) and intrinsic (C and D) oxidative phosphorylation capacity with complex I substrates (P_{CI}) in the gluteus medius (A and C) and triceps brachii (B and D) at 0, 8 and 12 wk of submaximal exercise training (wk0, wk8 and wk12) in yearling horses supplemented with either complexed trace minerals (CTM; n = 8) or inorganic trace minerals (INORG; n = 8). Overall effect of diet (*P* = 0.042; *P* = 0.472; *P* = 0.888; *P* = 0.588), time (*P* = 0.028; *P* = 0.665; *P* = 0.883; *P* = 0.153) and diet × time (*P* = 0.214; *P* = 0.055; *P* = 0.135; *P* = 0.100) for GM integrated P_{CI}, TB integrated P_{CI}, GM intrinsic P_{CI} and TB intrinsic P_{CI}, respectively. [a,b] Time points lacking common letters differ ($P \leq 0.05$). [*] Within time point, CTM differs from INORG ($P \leq 0.05$). Lines across bars indicate a main effect of time for both dietary treatments.

Integrated P_{CI+II} in the GM tended to be lower for CTM than INORG throughout training ($P = 0.077$; Fig. 3.5A). Integrated P_{CI+II} in the GM increased from wk 8 to 12 ($P = 0.007$) and was higher at wk 12 than 0 ($P < 0.0001$). Integrated P_{CI+II} in the TB and intrinsic P_{CI+II} in the GM

were not affected by diet, time, or their interaction (Fig. 3.5B and C). Intrinsic P_{CI+II} in the TB increased from wk 0 to 8 ($P = 0.038$; Fig. 3.5D) and remained higher at wk 12 than 0 ($P = 0.003$) but was not affected by diet or diet × time interaction.

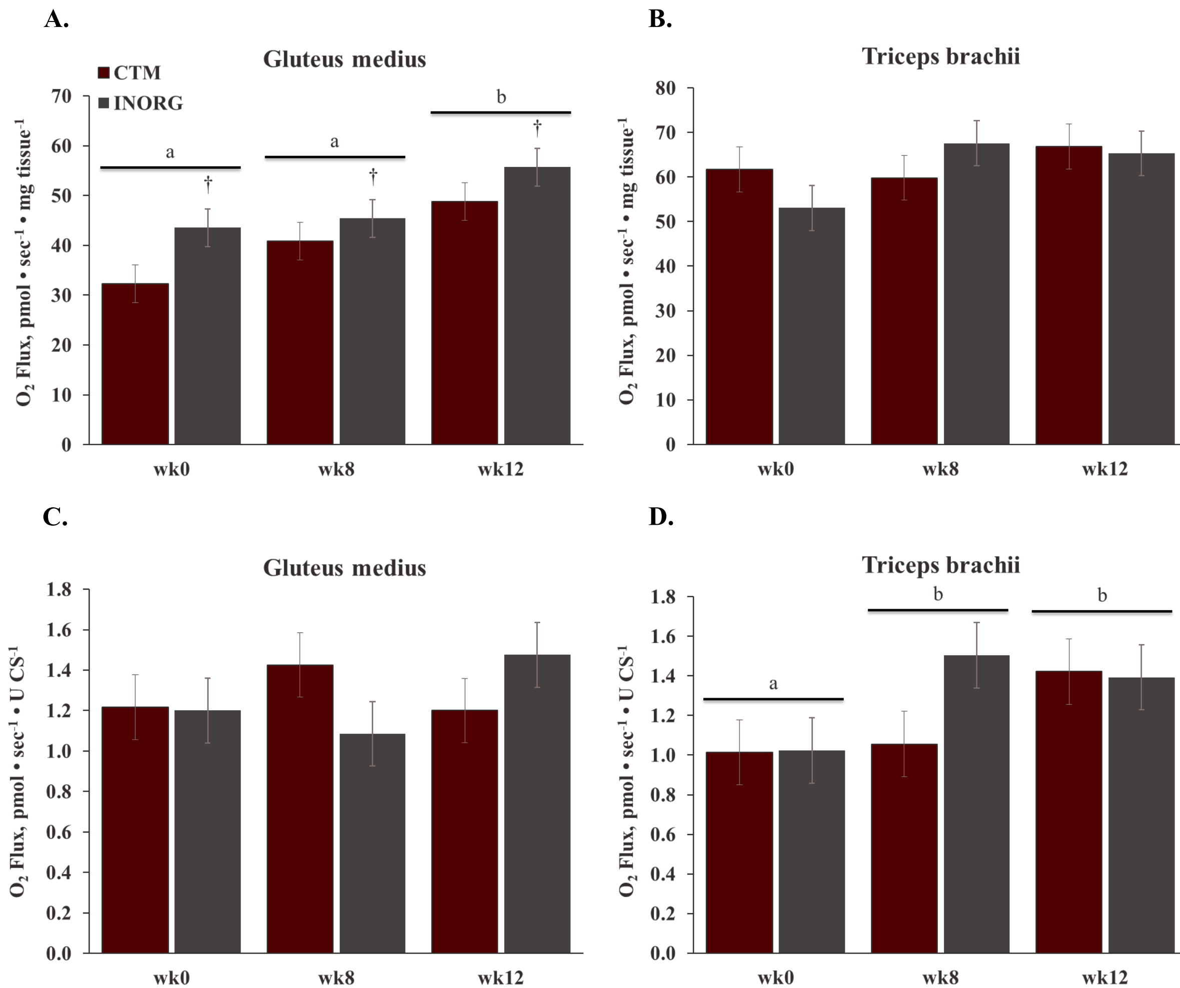


Figure 3.5. Integrated (A-B) and intrinsic (C-D) oxidative phosphorylation capacity with complex I and II substrates (P_{CI+II}) in the gluteus medius (A and C) and triceps brachii (B and D) at 0, 8 and 12 wk of submaximal exercise training (wk0, wk8 and wk12) in yearling horses supplemented with either complexed trace minerals (CTM; n = 8) or inorganic trace minerals (INORG; n = 8). Overall effect of diet (*P* = 0.077; *P* = 0.875; *P* = 0.846; *P* = 0.457), time (*P* = 0.0003; *P* = 0.148; *P* = 0.699; *P* = 0.010) and diet × time (*P* = 0.557; *P* = 0.199; *P* = 0.152; *P* = 0.104) for GM integrated P_{CI+II}, TB integrated P_{CI+II}, GM intrinsic P_{CI+II} and TB intrinsic P_{CI+II}, respectively. [a,b] Time points lacking common letters differ ($P \leq 0.05$). [*]Within time point, CTM differs from INORG ($P \leq 0.05$). [†]Within time point, CTM differs from INORG ($P \leq 0.05$). Lines across bars indicate a main effect of time for both dietary treatments.

Integrated E_{CI+II} in the GM tended to be lower for CTM than INORG throughout training ($P = 0.056$; Fig. 3.6A). Integrated E_{CI+II} in the GM increased from wk 8 to 12 ($P = 0.012$) and was higher at wk 12 than 0 ($P = 0.0008$). Integrated E_{CI+II} in the TB and intrinsic E_{CI+II} in the GM were not affected by diet, time, or their interaction (Fig. 3.6B and C). Intrinsic E_{CI+II} in the TB tended to increase from wk 0 to 8 ($P = 0.067$; Fig. 3.6D) and was higher at wk 12 than 0 ($P = 0.011$) but was not affected by diet or diet × time interaction.

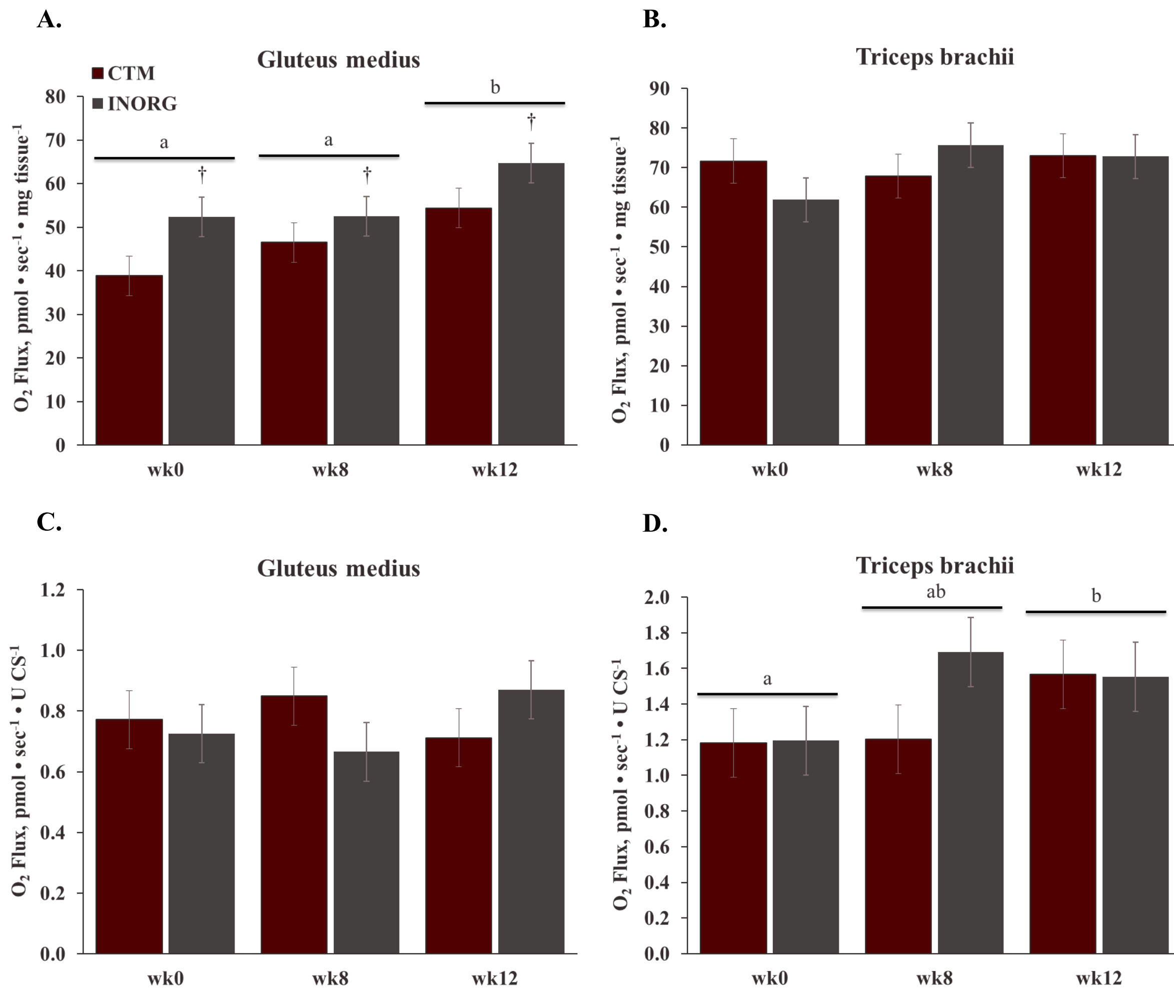


Figure 3.6. Integrated (A and B) and intrinsic (C and D) electron transport system capacity with complex I and II (E_{CI+II}) in the gluteus medius (A and C) and triceps brachii (B and D) at 0, 8 and 12 wk of submaximal exercise training (wk0, wk8 and wk12) in yearling horses supplemented with either complexed trace minerals (CTM; n = 8) or inorganic trace minerals (INORG; n = 8). Overall effect of diet (*P* = 0.077; *P* = 0.875; *P* = 0.846; *P* = 0.457), time (*P* = 0.0003; *P* = 0.148; *P* = 0.699; *P* = 0.010) and diet × time (*P* = 0.557; *P* = 0.199; *P* = 0.152; *P* = 0.104) for GM integrated E_{CI+II}, TB integrated E_{CI+II}, GM intrinsic E_{CI+II} and TB intrinsic E_{CI+II}, respectively. [a,b] Time points lacking common letters differ ($P \leq 0.05$). [*] Within time point, CTM differs from INORG ($P \leq 0.05$). [†]Within time point, CTM differs from INORG ($P \leq 0.10$). Lines across bars indicate a main effect of time for both dietary treatments.

Integrated E_{CII} in the GM tended to be lower for CTM than INORG throughout training (*P* = 0.095; Fig. 3.7A). Integrated E_{CII} in the GM increased from wk 8 to 12 (*P* = 0.021)

and was higher at wk 12 than 0 (P = 0.0008). Integrated E_{CII} in the TB and intrinsic E_{CII} in the GM were not affected by diet, time, or their interaction (Fig. 3.7B and C). Intrinsic E_{CII} in the TB tended to increase by 8 (P = 0.046; Fig. 3.7D) and remained higher at wk 12 than 0 (P = 0.004) but was not affected by diet or diet × time interaction.

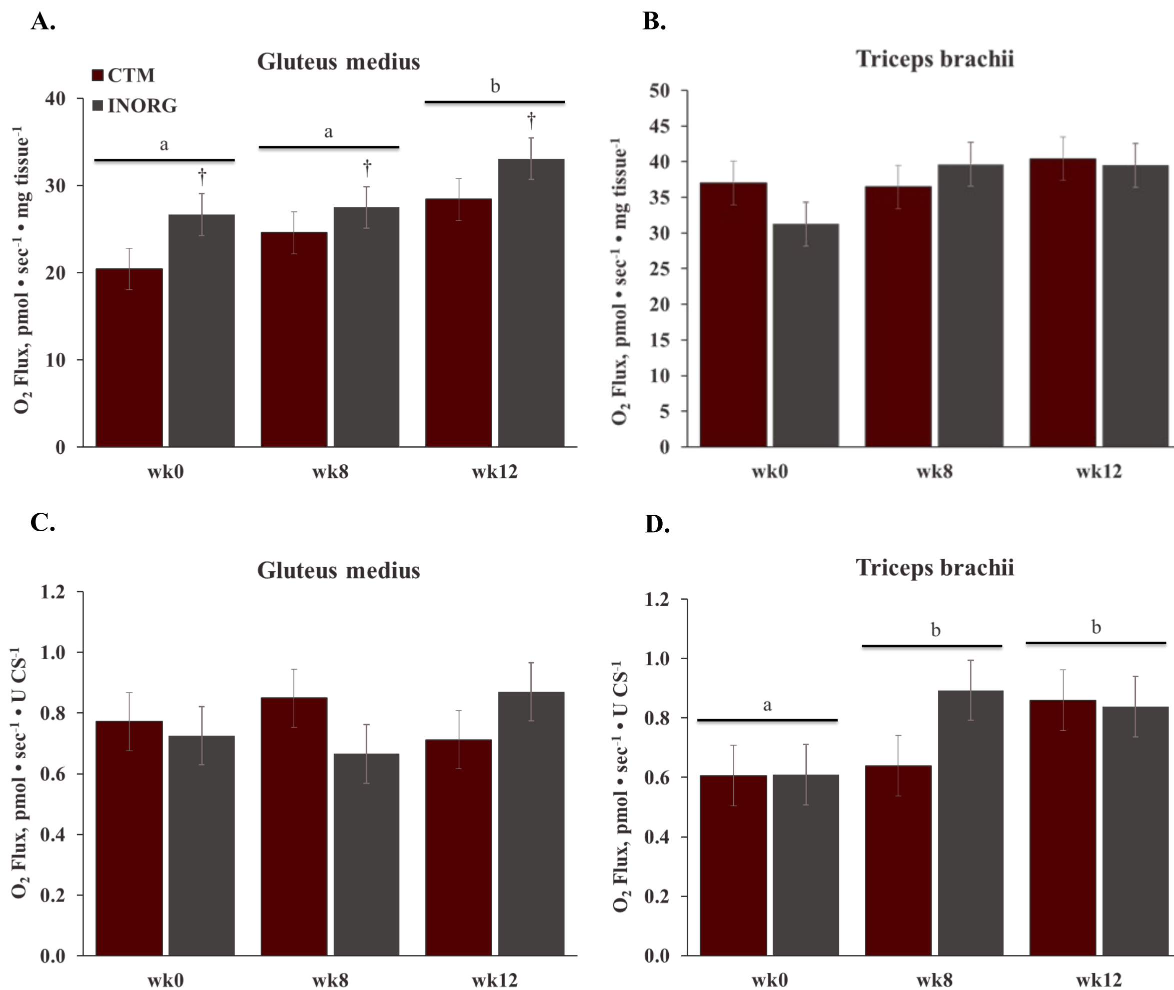


Figure 3.7.Integrated (A and B) and intrinsic (C and D) electron transport system capacity with complex II (E_{CII}) in the gluteus medius (A and C) and triceps brachii (B and D) at 0, 8 and 12 wk of submaximal exercise training (wk0, wk8 and wk12) in yearling horses supplemented with either complexed trace minerals (CTM; n = 8) or inorganic trace minerals (INORG; n = 8). Overall effect of diet (*P* = 0.095; *P* = 0.687; *P* = 0.786; *P* = 0.500), time (*P* = 0.0003; *P* = 0.131; *P* = 0.883; *P* = 0.012) and diet × time (*P* = 0.687; *P* = 0.303; *P* = 0.187; *P* = 0.153) for GM integrated E_{CII}, TB integrated E_{CII}, GM intrinsic E_{CII} and TB intrinsic E_{CII}, respectively. [a,b] Time points lacking common letters differ ($P \leq 0.05$). [†]Within time point, CTM differs from INORG ($P \leq 0.10$). Lines across bars indicate a main effect of time for both dietary treatments.

In the GM, the FCR for LEAK (Fig. 3.8A), PCI (Fig. 3.8B), PCI+II (Fig. 3.8C), and ECII (Fig. 3.8D) were not affected by diet, time or their interaction.

In the TB, the FCR for LEAK increased at wk 8 ($P = 0.016$; Fig. 3.9A) but was not different from wk 0 or 8 at wk 12 and was not affected by diet. The FCR for P_{CI} in the TB was not affected by diet, time or their interaction (Fig. 3.9B). A trend for an effect of time ($P = 0.062$) on P_{CI+II} FCR in the TB indicated that it was higher at wk 12 than 0 ($P = 0.022$; Fig. 3.9C) but was not different between diets. The FCR for E_{CII} in the TB tended to increase at wk 8 ($P = 0.096$) and was higher than wk 0 by wk 12 ($P = 0.009$; Fig. 3.9D) but was not affected by diet.

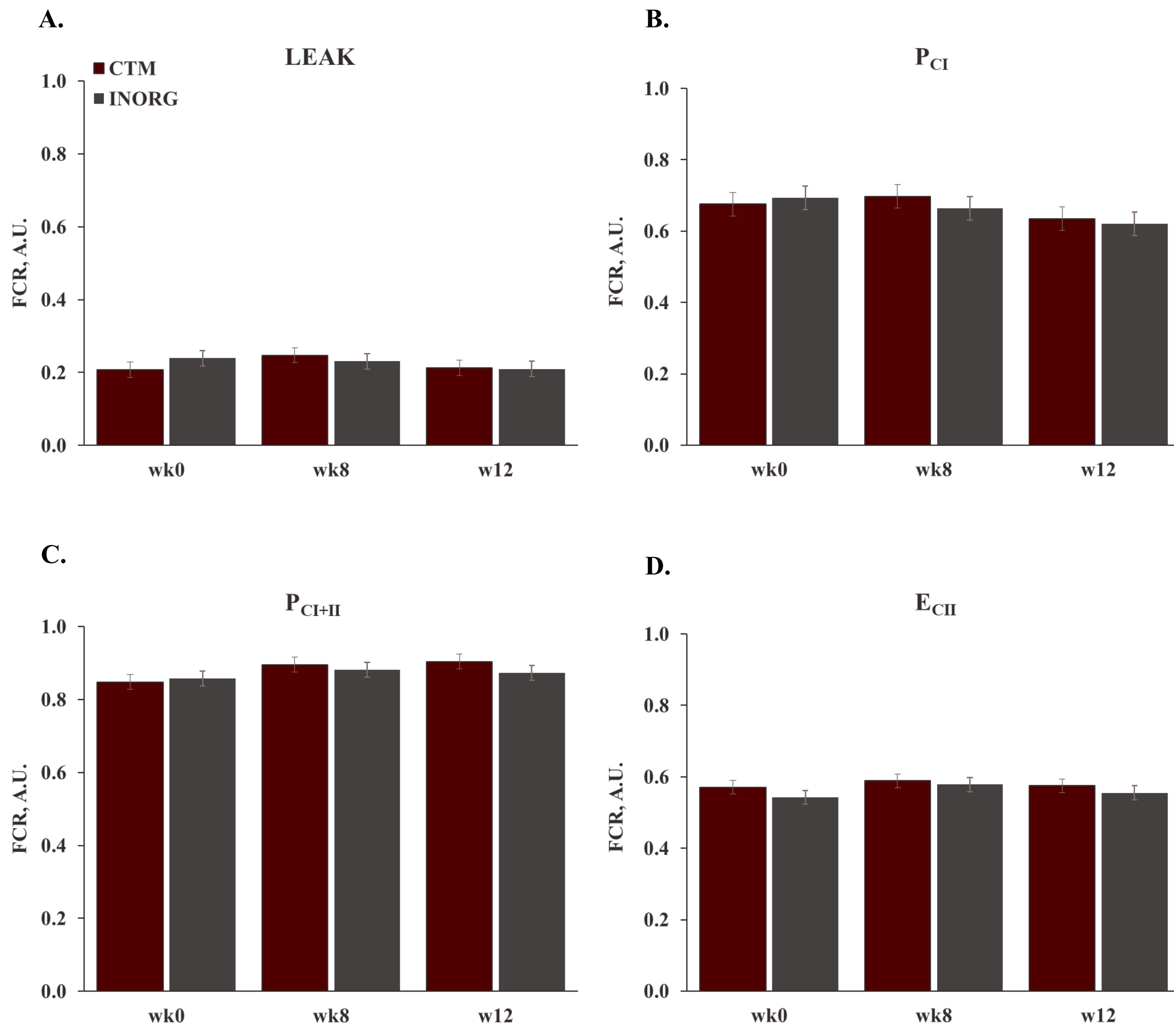


Figure 3.8. Fractional control ratio (FCR) for LEAK (A), oxidative phosphorylation with complex I substrates (P_{CI}; B), oxidative phosphorylation with complex I and II substrates (P_{CI+II}; C) and electron transport system capacity with complex II (E_{CII}; D) in the gluteus medius at 0, 8 and 12 wk of submaximal exercise training (wk0, wk8 and wk12) in yearling horses supplemented with either complexed trace minerals (CTM; n = 8) or inorganic trace minerals (INORG; n = 8). Overall effect of diet (*P* = 0.830; *P* = 0.747; *P* = 0.544; *P* = 0.224), time (*P* = 0.353; *P* = 0.118; *P* = 0.103; *P* = 0.390) and diet × time (*P* = 0.433; *P* = 0.685; *P* = 0.559; *P* = 0.897) for LEAK, P_{CI}, P_{CI+II} and E_{CII}, respectively.

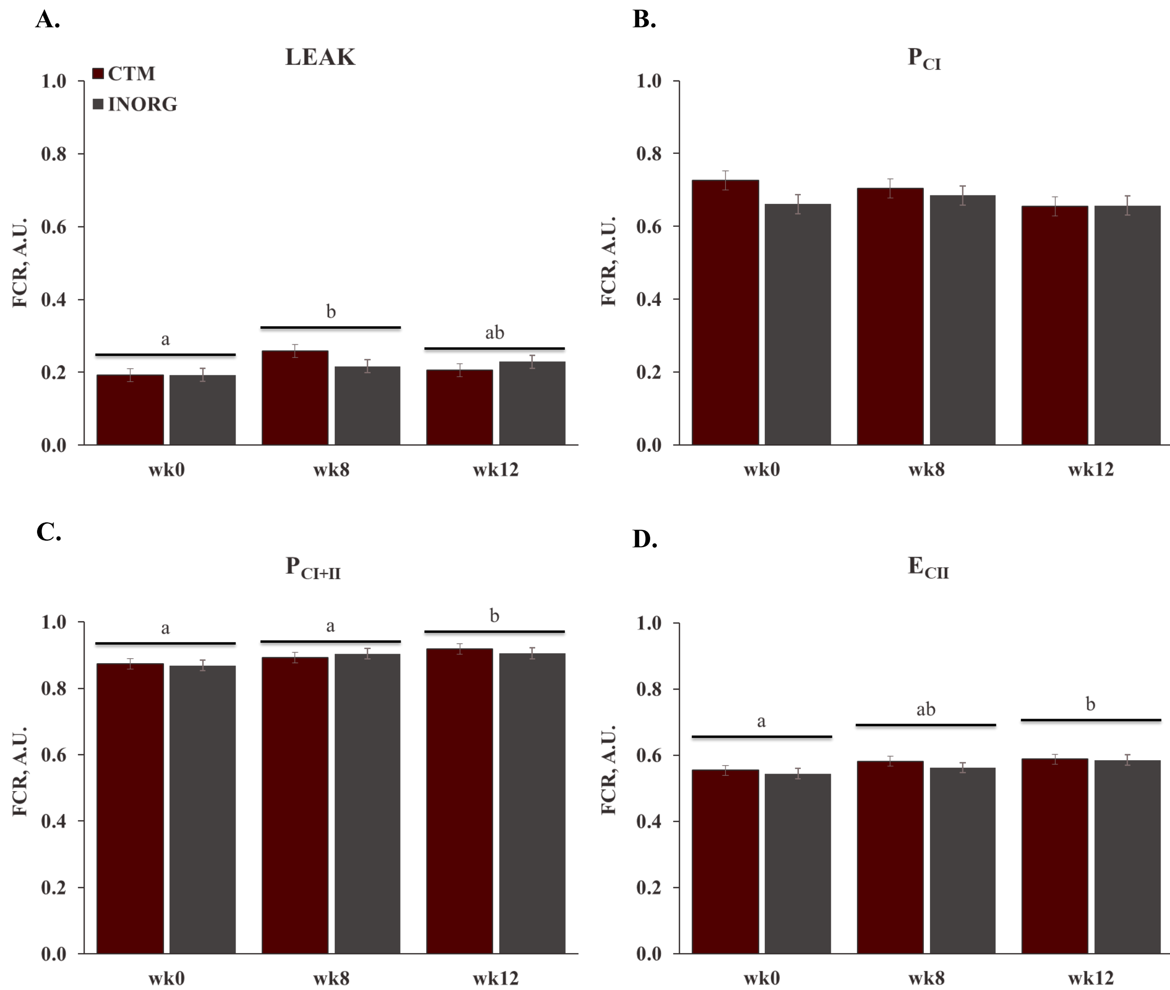


Figure 3.9. Fractional control ratio (FCR) for LEAK (A), oxidative phosphorylation with complex I substrates (P_{CI}; B), oxidative phosphorylation with complex I and II substrates (P_{CI+II}; C) and electron transport system capacity with complex II (E_{CII}; D) in the triceps brachii at 0, 8 and 12 wk of submaximal exercise training (wk0, wk8 and wk12) in yearling horses supplemented with either complexed trace minerals (CTM; n = 8) or inorganic trace minerals (INORG; n = 8). Overall effect of diet (*P* = 0.830; *P* = 0.747; *P* = 0.544; *P* = 0.224), time (*P* = 0.353; *P* = 0.118; *P* = 0.103; *P* = 0.390) and diet × time (*P* = 0.433; *P* = 0.685; *P* = 0.559; *P* = 0.897) for LEAK, P_{CI}, P_{CI+II} and E_{CII}, respectively. [a,b] Time points lacking common letters differ (*P* ≤ 0.05). Lines across bars indicate a main effect of time for both dietary treatments.

3.4.1.4. Oxidative Stress and Muscle Damage

The concentration of MDA was not affected by diet or any diet interactions in either muscle group. In the GM, PreT MDA decreased from wk 0 to 8 (*P* = 0.001) and remained lower

than wk 0 at wk 12 (*P* = 0.03; Table 3.4). In the TB, MDA concentration was not affected by time (Table 3.4).

Resting serum CK activity was lower for CTM than INORG after 6 wk supplementation (prior to the start of the current trial; data not shown). However, PreT serum CK activity tended to be higher for CTM than INORG (*P* = 0.07; Table 3.4) throughout the present study. Pre-trailer serum CK activity decreased from wk 0 to 8 (*P* = 0.03) and remained lower than wk 0 at wk 12 (*P* = 0.01) for both treatment groups.

Table 3.4 Pre-trailer muscle malondialdehyde (MDA) concentration in the gluteus medius (GM) and triceps brachii (TB) and serum creatine kinase (CK) activity at 0, 8 and 12 wk of submaximal exercise training (wk0, wk8 and wk12) in yearling horses supplemented with either complexed trace minerals (CTM; n = 8) or inorganic trace minerals (INORG; n = 8).

						P-Value		
Variable	Diet	wk0	wk8	wk12	SEM	Diet	Time	Diet × Time
GM MDA concentration, nmol/mg protein	CTM	0.052	0.041*	0.050*	0.005	0.314	0.004	0.219
	INORG	0.058	0.035*	0.040*				
TB MDA concentration, nmol/mg protein	CTM	0.067	0.072	0.076	0.009	0.574	0.780	0.275
	INORG	0.085	0.079	0.065				
Serum CK activity, units/L	CTM	84.92	78.60*	74.65*	6.91	0.070	0.022	0.440
	INORG	76.68	59.67*	58.50*				

*Within a row, mean differs from wk0 ($P \leq 0.05$).

3.4.1.5. Antioxidant Activities

Overall, GPx activity was higher for CTM than INORG in both muscle groups ($P \leq 0.02$) but was not affected by time in either muscle group (Table 3.5). A trend for an effect of the diet × time interaction on PreT SOD activity ($P = 0.09$) suggested that CTM increased at wk 12 in the GM ($P = 0.02$; Table 3.5), while SOD activity was unaffected by time in INORG. In the TB, a trend was noted for an effect of time ($P = 0.09$), whereby SOD activity increased from wk 0 to 12 in both dietary treatment groups ($P = 0.04$). Neither PreT whole blood GPx nor SOD activities were affected by time, diet, or their interaction (Table 3.5).

Table 3.5. Pre-trailer muscle glutathione peroxidase (GPx) and superoxide dismutase (SOD) activities in the gluteus medius (GM) and triceps brachii (TB), and whole blood GPx and SOD activities at 0, 8 and 12 wk of submaximal exercise training (wk0, wk8 and wk12) in yearling horses supplemented with either complexed trace minerals (CTM; n = 8) or inorganic trace minerals (INORG; n = 8).

							P-Value		
Variable	Muscle	Diet	wk0	wk8	wk12	SEM	Diet	Time	Diet × Time
Muscle GPx activity, nmol • min^{-1}• mg protein^{-1}	GM	CTM	12.72[a]	11.40[a]	8.64[a]	1.49	0.018	0.193	0.591
		INORG	7.92[b]	6.44[b]	5.73[b]				
	TB	CTM	17.65[a]	20.18[a]	18.12[a]	2.19	0.0001	0.723	0.926
		INORG	9.06[b]	9.88[b]	10.31[b]				
Muscle SOD activity, units • mg protein^{-1}	GM	CTM	16.26	19.74	23.74*	2.68	0.349	0.248	0.093
		INORG	21.10	24.91	19.99				
	TB	CTM	22.46	25.93	30.22*	3.66	0.536	0.095	0.721
		INORG	23.30	30.54	29.94*				
Whole blood GPx activity, nmol • min^{-1}• mg protein^{-1}	-	CTM	21.20	17.08	23.09	3.58	0.179	0.502	0.728
	-	INORG	24.77	23.33	25.43				
Whole blood SOD activity, units/mg protein	-	CTM	0.21	0.22	0.23	0.03	0.412	0.713	0.498
	-	INORG	0.18	0.21	0.18				

*Within a row, mean differs from wk0 ($P \leq 0.05$).

[a,b] Within a column, differing letters indicate CTM differs from INORG within the specified variable ($P \leq 0.05$).

3.4.1.6. Muscle Antioxidant Gene Expression

Pre-trailer *SOD1* expression in the GM was lower at wk 8 than 0 (*P* = 0.01) and 12 (*P* = 0.03; Table 3.6) but was not affected by diet or the interaction of diet and time. Pre-trailer *SOD1* expression in the TB was unaffected by diet, time, or their interaction. Pre-trailer *SOD2* expression in the GM did not change from wk 0 to 8, but increased from wk 8 to 12 (*P* = 0.0007), resulting in higher expression at wk 12 than 0 (*P* = 0.03; Table 3.6) for both treatment groups. In the TB, PreT *SOD2* expression decreased from wk 0 to 8 (*P* = 0.02) and tended to increase from wk 8 to 12 (*P* = 0.07; Table 3.6) for both treatments. Pre-trailer *SOD2* expression was not affected by diet or the interaction of diet and time in either muscle group.

Table 3.6. Pre-trailer muscle superoxide dismutase 1 (SOD1) and superoxide dismutase 2 (SOD2) expression in the gluteus medius (GM) and triceps brachii (TB) at 0, 8 and 12 wk of submaximal exercise training (wk0, wk8 and wk12) in yearling horses (n = 16). Due to lack of effect of diet, dietary treatments have been combined.

						P-Value		
Variable	Muscle	wk0	wk8	wk12	SEM	Diet	Time	Diet × Time
SOD1, 40-ΔCq	GM	39.90	39.61*	39.86#	0.09	0.309	0.026	0.418
	TB	39.75	39.70	39.71	0.07	0.191	0.829	0.922
SOD2, 40-ΔCq	GM	39.31	39.15	39.67*#	0.09	0.443	0.002	0.450
	TB	39.74	39.48*	39.66	0.08	0.599	0.047	0.815

*Within a row, mean differs from wk0 ($P \leq 0.05$).
#Within a row, mean differs from wk8 ($P \leq 0.05$).

3.4.2. Response to Trailer Stressors

3.4.2.1. Citrate Synthase and Cytochrome *c* Oxidase Activity

In the GM, CS activity in response to trailer stress did not differ between groups and did not change in response to the trailer stressor at wk 0. In response to the wk 8 trailer stressor, CS activity decreased at 24hPT in the GM ($P = 0.032$; Fig. 3.10A). In response to the wk 12 trailer stressor, CS activity in the GM decreased at 1hPT ($P = 0.042$) and remained lower than PreT at 24hPT ($P = 0.002$). In the TB, CS activity was not affected by time during any of the trailer stressors (Fig. 3.10B).

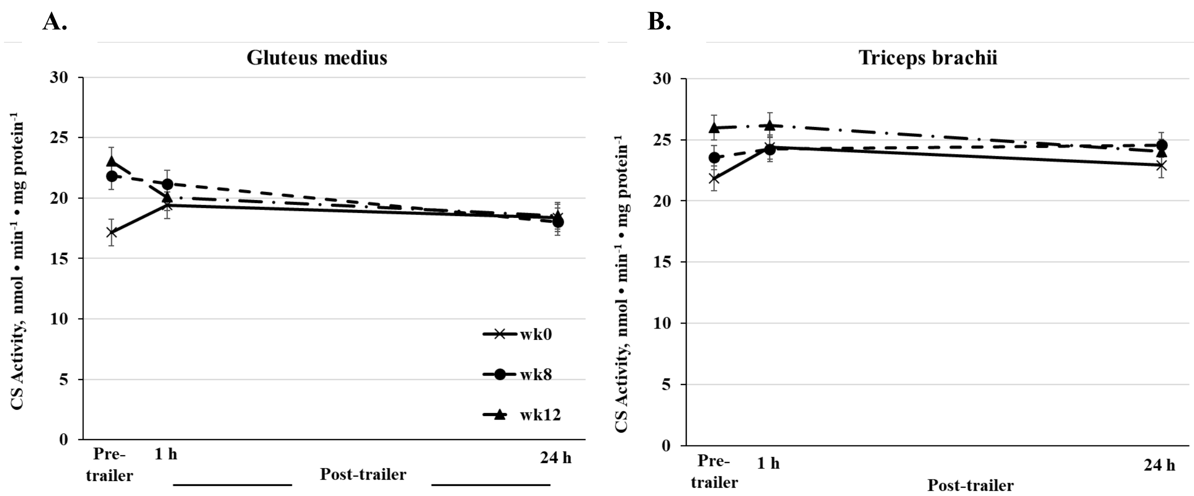


Figure 3.10 Citrate synthase (CS) activity in the gluteus medius (A) and triceps brachii (B) before (Pre-Trailer), and 1 (1 h) and 24 h (24 h) after a 1.5-h trailer stressor. Trailer stressors occurred at 0, 8, and 12 wk of submaximal exercise training (wk0, wk8, and wk12, respectively) in yearling horses (n = 16). Due to lack of effect of diet, dietary treatments have been combined. Overall effect of time ($P = 0.014$; $P = 0.046$), trailering ($P = 0.012$; $P = 0.389$) and time × trailering ($P = 0.027$; $P = 0.534$) for GM and TB, respectively.

At wk 0 in the GM, integrated CCO activity decreased at 24hPT ($P = 0.002$; Fig 3.11) and was lower than PreT ($P = 0.011$) but was not different between diets. At wk 8, integrated

CCO activity in the GM decreased at 24hPT ($P = 0.012$) and tended to be lower at 24hPT than PreT ($P = 0.056$). At wk 12, integrated CCO activity in the GM decreased at 1hPT ($P = 0.003$) and tended to remain lower at 24hPT than PreT ($P = 0.070$). In the TB, integrated CCO activity decreased at 24hPT ($P = 0.017$; Fig. 3.11B) and was lower at 24hPT than PreT ($P = 0.002$) in response to all trailer stressors. Overall, the decrease in integrated CCO in response to trailering was greater at wk 8 than 0 for CTM ($P = 0.010$) but was not different between wk 0 and 12. INORG response of integrated CCO to trailering did not differ between trailer stressors.

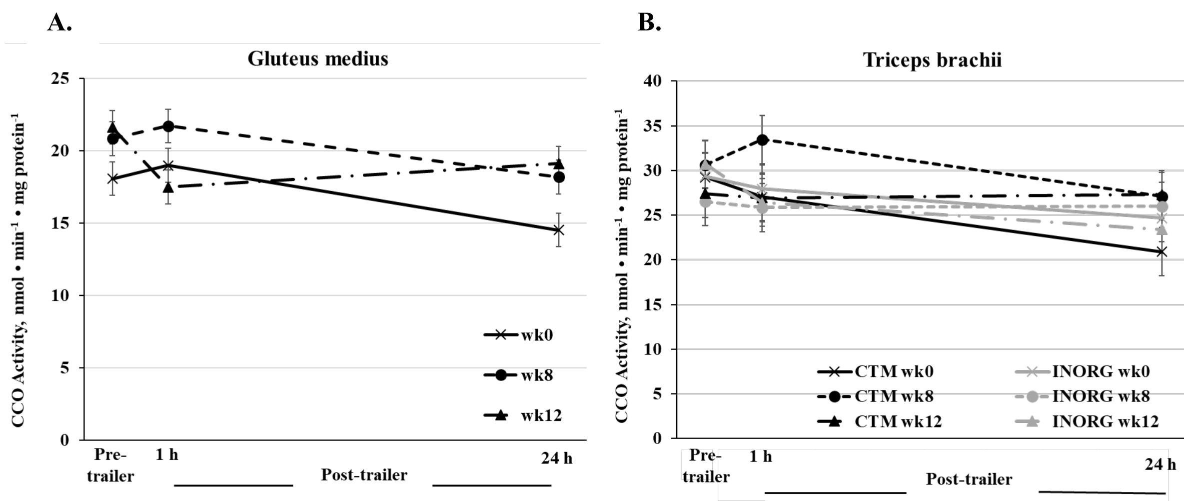


Figure 3.11. Integrated cytochrome *c* oxidase (CCO) activity in the gluteus medius (A) and triceps brachii (B) before (Pre-Trailer), and 1 (1 h) and 24 h (24 h) after a 1.5-h trailer stressor. Trailer stressors occurred at 0, 8, and 12 wk of submaximal exercise training (wk0, wk8, and wk12, respectively) in yearling horses supplemented with either complexed trace minerals (CTM; n = 8) or inorganic trace minerals (INORG; n = 8). Due to lack of effect of diet, dietary treatments have been combined for integrated CCO in the GM. Overall effect of diet ($P = 0.771$; $P = 0.678$), time ($P = 0.0006$; $P = 0.373$), trailering ($P = 0.001$; $P = 0.005$), diet × time ($P = 0.427$; $P = 0.064$), diet × trailering ($P = 0.152$; $P = 0.637$), time × trailering ($P = 0.011$; $P = 0.526$), and diet × time × trailering ($P = 0.317$; $P = 0.311$) for GM and TB, respectively.

During the wk 0 trailer stressor, intrinsic CCO activity in the GM increased at 24hPT ($P = 0.018$; Fig. 3.12A). Intrinsic CCO activity in the GM did not change in response to the trailer

stressor at wk 8. Intrinsic CCO activity decreased from 1hPT to 24hPT in response to the trailer stressor at wk 12 ($P = 0.050$) but was not different between PreT and 24hPT. In the TB, intrinsic CCO activity increased at 24hPT ($P = 0.045$; Fig. 3.12B) during all trailer stressors. A trend for diet × time × trailering interaction suggested that CTM intrinsic CCO in the TB increased at 24hPT in response to the wk 0 ($P = 0.055$) and wk 8 ($P = 0.015$) and did not change in response to the wk 12 trailer stressor, while INORG did not change in response to any of the trailer stressors.

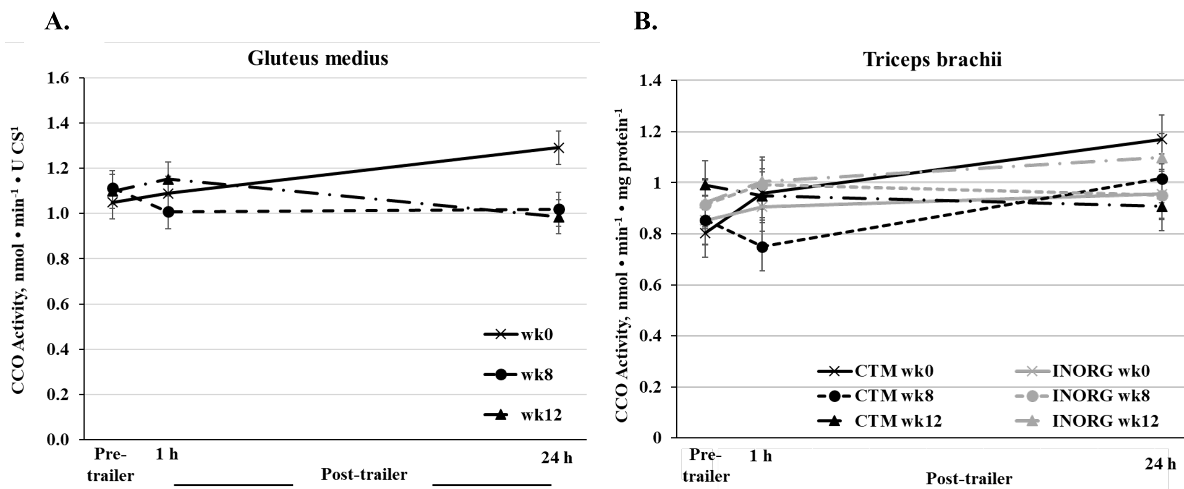


Figure 3.12. Intrinsic cytochrome *c* oxidase (CCO) activity in the gluteus medius (A) and triceps brachii (B) before (Pre-Trailer), and 1 (1 h) and 24 h (24 h) after a 1.5-h trailer stressor. Trailer stressors occurred at 0, 8, and 12 wk of submaximal exercise training (wk0, wk8, and wk12, respectively) in yearling horses supplemented with either complexed trace minerals (CTM; n = 8) or inorganic trace minerals (INORG; n = 8). Due to lack of effect of diet, dietary treatments have been combined for the GM. Overall effect of time ($P = 0.014$; $P = 0.046$), trailering ($P = 0.012$; $P = 0.389$) and time × trailering ($P = 0.027$; $P = 0.534$) for GM and TB, respectively.

3.4.2.2. Oxidative Stress and Muscle Damage

Concentration of GM MDA did not change in response to the trailer stressor at wk 0 but increased 24 h post-trailering ($P = 0.009$) at wk 8 and increased at 1 h post-trailering ($P = 0.004$) at wk 12 (Fig. 3.13A). In the TB, MDA concentration was not affected by trailering (Fig. 3.13B).

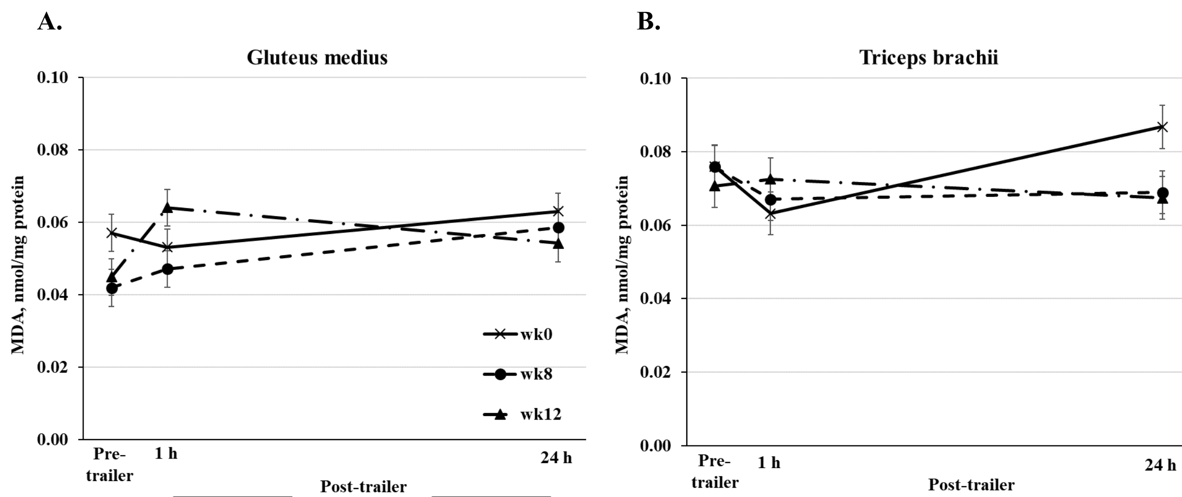


Figure 3.13. Muscle malondialdehyde (MDA) concentration in the gluteus medius (A) and triceps brachii (B) before (Pre-Trailer), and 1 (1 h) and 24 h (24 h) after a 1.5-h trailer stressor. Trailer stressors occurred at 0, 8, and 12 wk of submaximal exercise training (wk0, wk8, and wk12, respectively) in yearling horses (n = 16). Due to lack of effect of diet, dietary treatments have been combined. Overall effect of time ($P = 0.021$; $P = 0.516$), trailering ($P = 0.029$; $P = 0.214$) and time × trailering ($P = 0.036$; $P = 0.159$) for GM and TB, respectively.

Following all trailer stressors, serum CK activity increased at 0hPT ($P < 0.0001$) and remained elevated through 24hPT ($P < 0.0001$; Fig. 3.14) for both treatments. At wk 8, CTM had higher CK activity than INORG throughout to the trailer stressor ($P = 0.01$), but no other effects of diet on CK activity response to trailering were noted.

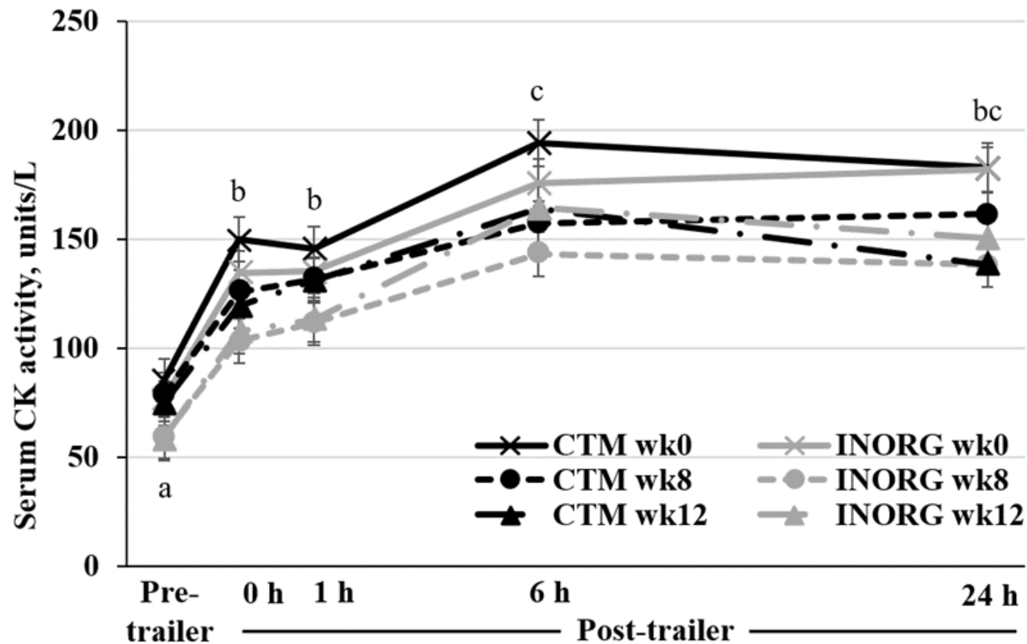


Figure 3.14. Serum creatine kinase (CK) activity before (Pre-Trailer), and 0, 1, 6, and 24 h after (0 h, 1 h, 6 h, and 24 h, respectively) a 1.5-h trailer stressor. Trailer stressors occurred at 0, 8, and 12 wk of submaximal exercise training (wk0, wk8, and wk12, respectively) in yearling horses supplemented with either complexed trace minerals (CTM; n = 8) or inorganic trace minerals (INORG; n = 8). Overall effect of diet ($P = 0.093$), time ($P < 0.0001$), trailering ($P < 0.0001$), diet × time ($P = 0.092$), diet × trailering ($P = 0.284$), time × trailering ($P = 0.794$), and diet × time × trailering ($P = 0.931$). [a,b,c] Time points lacking common letters differ ($P \leq 0.05$).

3.4.2.3. Antioxidant Activities

Overall, GPx activity was higher for CTM than INORG in both muscle groups throughout all three trailer stressors ($P < 0.0001$; Fig. 3.15). In response to trailering, GPx activity in the GM increased from PreT to 1hPT ($P = 0.02$) and remained higher 24 h post-trailering ($P = 0.01$) than PreT (Fig. 3.15A); responses to trailering in the GM did not differ between wk 0, 8, and 12. In the TB, GPx activity was not affected by trailering (Fig. 3.15B).

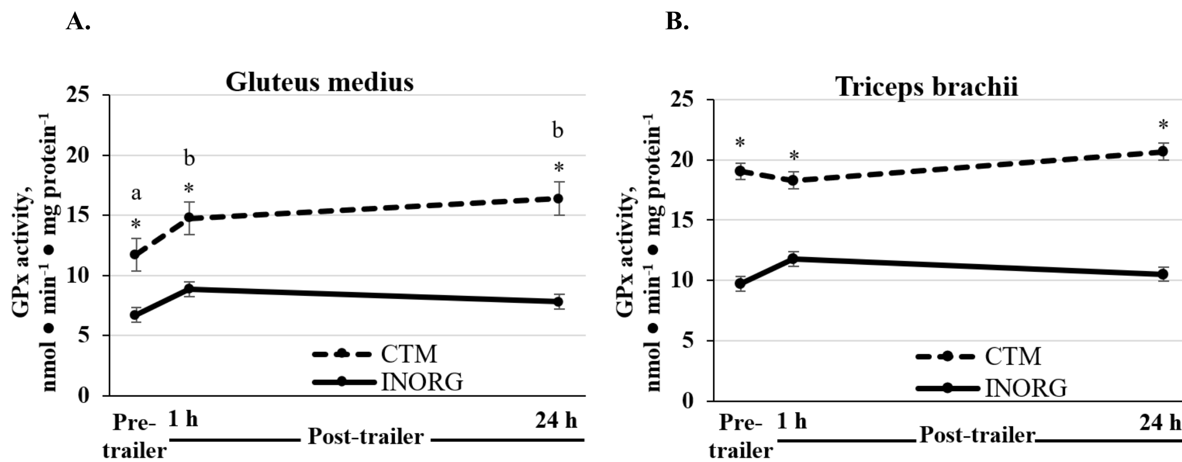


Figure 3.15. Muscle glutathione peroxidase (GPx) activity in the gluteus medius (A) and triceps brachii (B) before trailering (Pre-Trailer), and 1 (1 h) and 24 h (24 h) after a 1.5-h trailer stressor in yearling horses supplemented with complexed trace minerals (CTM; n = 8) or inorganic trace minerals (INORG, n = 8). Due to lack of effect of time, weeks have been combined. Overall effect of diet ($P < 0.0001$; $P < 0.0001$), trailering ($P = 0.018$; $P = 0.659$), and diet × trailering ($P = 0.244$; $P = 0.348$) for GM and TB, respectively. [a,b] Time points lacking common letters differ ($P \leq 0.05$). * Within time point, CTM differs from INORG ($P \leq 0.05$).

Superoxide dismutase activity in the GM tended to be higher for INORG than CTM at wk 0 throughout the trailer stressor ($P = 0.07$) and was higher for INORG than CTM at wk 8 throughout the trailer stressor ($P = 0.02$; Fig. 3.16A). In the GM of INORG horses, SOD activity during the trailer stressor tended to increase from wk 0 to 8 ($P = 0.1$) but was not different between wk 0 and 12 (Fig. 3.16A). Conversely, GM SOD activity during the trailer stressor in CTM horses was higher at wk 12 than 0 ($P = 0.0008$) and 8 ($P = 0.03$; Fig. 3.16A). Superoxide dismutase activity in the TB was lower 24 h post-trailering compared to PreT ($P = 0.03$; Fig. 3.16B) following each trailer stressor, but GM SOD activity was not affected by trailering.

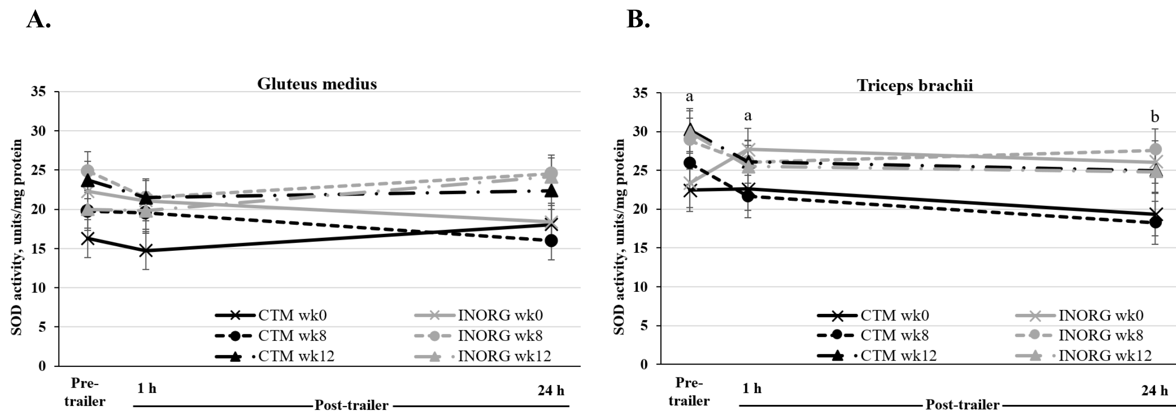


Figure 3.16. Muscle superoxide dismutase (SOD) activity in the gluteus medius (A) and triceps brachii (B) before trailering (Pre-Trailer), and 0, 1, 6, and 24 h after (0 h, 1 h, 6 h, and 24 h, respectively) after a 1.5-h trailer stressor. Trailer stressors occurred at 0, 8, and 12 wk of submaximal exercise training (wk0, wk8, and wk12, respectively) in yearling horses supplemented with complexed trace minerals (CTM; n = 8) or inorganic trace minerals (INORG; n = 8). Overall effect of diet (*P* = 0.130; *P* = 0.118), time (*P* = 0.023; *P* = 0.078), trailering (*P* = 0.527; *P* = 0.097), diet × time (*P* = 0.029; *P* = 0.118), diet × trailering (*P* = 0.858; *P* = 0.385), time × trailering (*P* = 0.822; *P* = 0.419), and diet × time × trailering (*P* = 0.226; *P* = 0.889) for GM and TB, respectively.

Activity of GPx in whole blood of INORG horses did not change following the trailer stressors. Conversely, CTM blood GPx activity increased from PreT to 0hPT (*P* = 0.04) and continued increasing to 6hPT (*P* = 0.005 compared to PreT; Fig. 3.17A). Blood SOD activity was not affected by treatment, trailering, or their interaction (Fig. 3.17B).

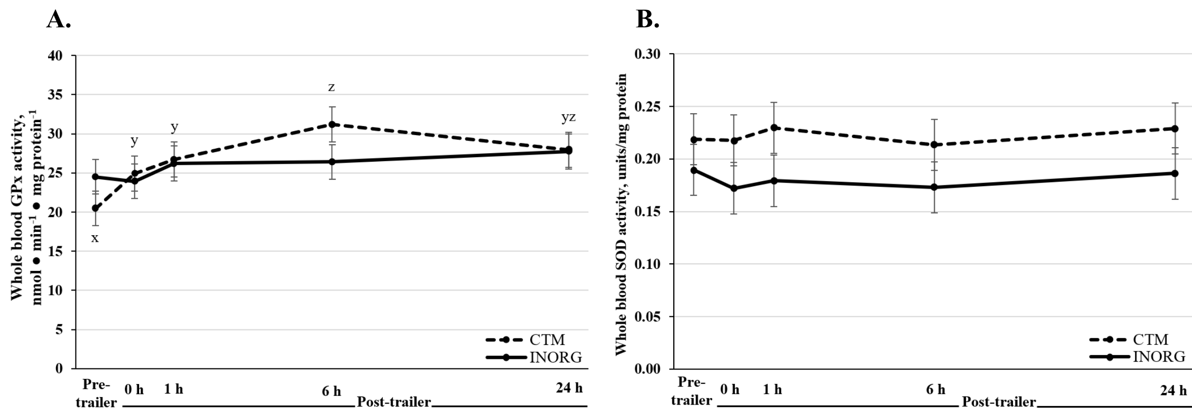


Figure 3.17.Whole blood glutathione peroxidase (GPx; A) and superoxide dismutase (SOD; B) activities before (Pre-Trailer), and 0, 1, 6, and 24 h after (0 h, 1 h, 6 h, and 24 h, respectively) a 1.5-h trailer stressor in horses supplemented either complexed trace minerals (CTM; n = 8) or inorganic trace minerals (INORG; n = 8). Due to lack of effect of time, weeks have been combined. Overall effect of diet (*P* = 0.686; *P* = 0.211), trailering (*P* = 0.0002; *P* = 0.564), and diet × trailering (*P* = 0.095; *P* = 0.880) for GPx and SOD activities, respectively. [a,b,c] Time points lacking common letters differ for CTM (*P* ≤ 0.05).

3.4.2.4. Muscle Antioxidant Gene Expression

Superoxide Dismutase 1 expression in the GM throughout trailering decreased from wk0 to 8 for CTM (*P* = 0.01) but tended to increase from wk 8 to 12 (*P* = 0.06) and was not different between wk 0 and 12 (Fig. 3.18A). In the GM of INORG, *SOD1* expression throughout trailering increased from wk 0 to 12 (*P* = 0.03; Fig. 3.18A). Together, these changes in *SOD1* expression led to lower expression throughout trailering for CTM than INORG in the GM at wk 8 (*P* = 0.008), and a trend for lower expression throughout trailering for CTM than INORG at wk12 (*P* = 0.07; Fig. 3.18A). For both treatments, *SOD1* expression in the GM decreased from PreT to 24hPT at wk 0 (*P* = 0.03), increased from PreT to 1hPT at wk 8 (*P* = 0.007; Fig. 3.18A) and was not affected by the trailer stressor at wk 12. Expression of *SOD1* in the TB was not affected by trailering or treatment by trailering interaction (Fig. 3.18B).

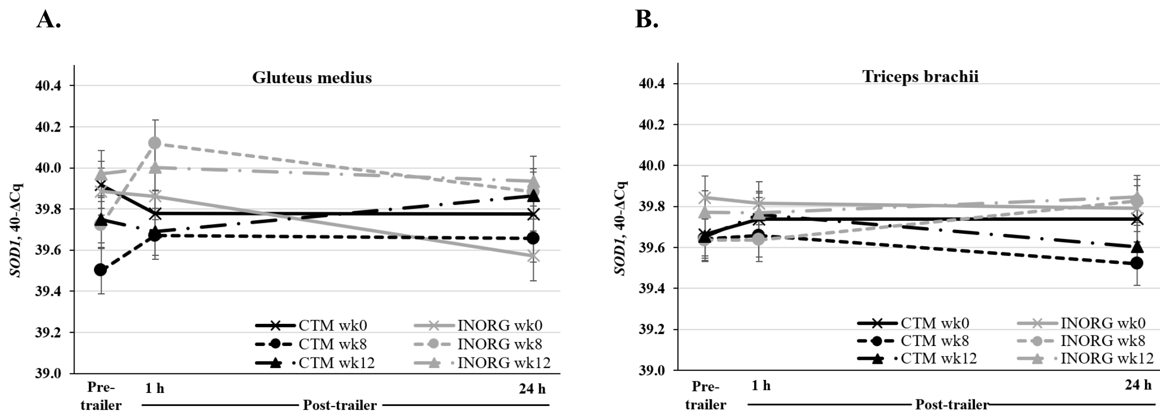


Figure 3.18.Muscle *superoxide dismutase 1* (*SOD1*) expression in the gluteus medius (A) and triceps brachii (B) before (Pre-Trailer), and 1 (1 h) and 24 h (24 h) after a 1.5-h trailer stressor. Trailer stressors occurred at 0, 8, and 12 wk of submaximal exercise training (wk0, wk8, and wk12, respectively) in yearling horses supplemented with complexed trace minerals (CTM; n = 8) or inorganic trace minerals (INORG; n = 8). Overall effect of diet ($P = 0.103$; $P = 0.199$), time ($P = 0.180$; $P = 0.118$), trailering ($P = 0.424$; $P = 0.874$), diet × time ($P = 0.014$; $P = 0.961$), diet × trailering ($P = 0.119$; $P = 0.273$), time × trailering ($P = 0.032$; $P = 0.992$), and diet × time × trailering ($P = 0.975$; $P = 0.557$) for GM and TB, respectively.

Superoxide dismutase 2 expression in the GM increased at 24hPT following all trailer stressors ($P < 0.0001$; Fig. 3.19A). At wk 12, *SOD2* expression in the GM tended to decrease from PreT to 1hPT ($P = 0.08$). The increase in *SOD2* expression at 24hPT in the GM was lower at wk 12 than 0 ($P = 0.03$) or wk8 ($P = 0.01$; Fig. 3.19A), resulting in a 3.3-fold increase at wk 0, a 3.8-fold increase at wk 8, and a 2.5-fold increase at wk 12 (Table 3.7). At wk 0, *SOD2* expression in the TB increased 3.4-fold (Table 3.7) by 24hPT for both treatments ($P \leq 0.0001$; Fig. 3.19B), and CTM *SOD2* expression was higher than INORG at 24hPT ($P = 0.05$). While INORG *SOD2* expression in the TB increased at wk 8 and wk 12 24hPT ($P \leq 0.03$) following the trailer stressor, CTM *SOD2* expression did not change in response to trailering at wk 8, and only tended to increase at wk 12 24hPT ($P = 0.06$; Fig. 3.19B). These differing responses to trailering

between treatments led to lower *SOD2* expression for CTM than INORG at wk 8 24hPT (*P* = 0.0002), but no difference between treatments at wk 12 24hPT (Fig. 3.19B).

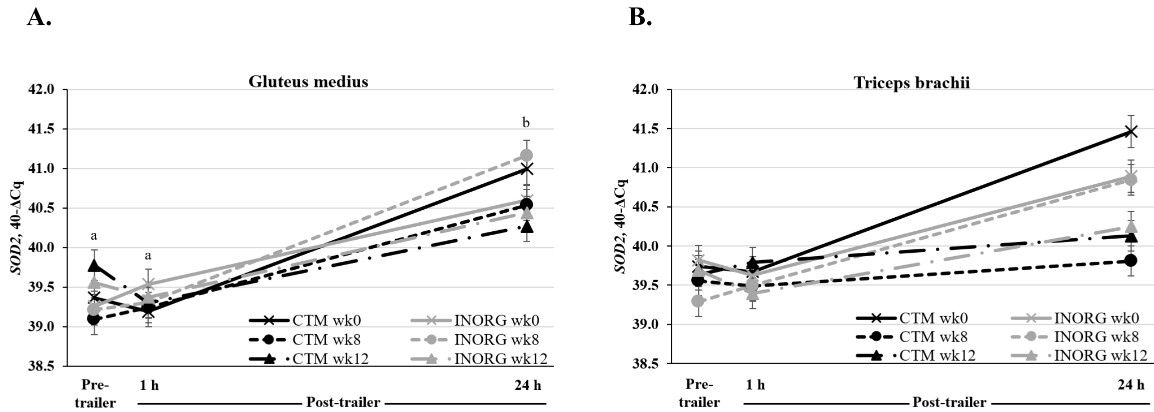


Figure 3.19. Muscle *superoxide dismutase 2* (*SOD2*) expression in the gluteus medius (A) and triceps brachii (B) before (Pre-Trailer), and 1 (1 h) and 24 h (24 h) after a 1.5-h trailer stressor. Trailer stressors occurred at 0, 8, and 12 wk of submaximal exercise training (wk0, wk8, and wk12, respectively) in yearling horses supplemented with complexed trace minerals (CTM; n = 8) or inorganic trace minerals (INORG; n = 8). Overall effect of diet (*P* = 0.507; *P* = 0.993), time (*P* = 0.507; *P* < 0.0001), trailering (*P* < 0.0001; *P* < 0.0001), diet × time (*P* = 0.295; *P* = 0.107), diet × trailering (*P* = 0.516; *P* = 0.279), time × trailering (*P* = 0.005; *P* = 0.004) and diet × time × trailering (*P* = 0.200; *P* = 0.004) for GM and TB, respectively. [a,b] Time points lacking common letters differ (*P* ≤ 0.05).

Table 3.7. Fold changes in gene expression (mean ± SEM) of *Cu-Zn superoxide dismutase* (*SOD1*) and *Mn superoxide dismutase* (*SOD2*) in the gluteus medius (GM) and triceps brachii (TB) from young Quarter Horses (n = 16) before (wk 0) and after 8 and 12 wk of exercise training. Fold-changes indicate changes from before a 1.5-h trailer stressor to 1 (1hPT) and 24 (24hPT) after trailering across treatments.

Gene	Week	Muscle	1hPT	24hPT
SOD1	0	GM	0.98 ± 0.07	0.91 ± 0.09
		TB	1.03 ± 0.04	1.04 ± 0.06
	8	GM	1.31 ± 0.13	1.16 ± 0.08
		TB	1.04 ± 0.08	1.07 ± 0.09
	12	GM	1.01 ± 0.06	1.06 ± 0.06
		TB	1.09 ± 0.09	1.05 ± 0.07
SOD2	0	GM	1.07 ± 0.07	3.30 ± 0.50
		TB	0.94 ± 0.06	3.38 ± 0.79
	8	GM	1.20 ± 0.14	3.85 ± 0.59
		TB	1.17 ± 0.15	2.51 ± 0.57
	12	GM	0.83 ± 0.07	1.90 ± 0.35
		TB	1.03 ± 0.10	1.69 ± 0.30

3.5. Discussion

In the present study, we aimed to examine the effects of complexed trace mineral supplementation and trailering on oxidative stress and antioxidant enzyme activity and expression in growing horses undergoing a submaximal exercise training program. Compared to inorganic trace minerals, complexed trace mineral supplementation resulted in higher muscle GPx activity at rest and an increased muscular and systemic GPx response to trailer stress, and similar CS and CCO activities, and oxidative capacity, but lower indices of integrated oxidative capacity. Twelve wk of exercise training in growing horses resulted in decreased resting MDA

concentration and altered antioxidant activity and expression responses to trailering. Trailering resulted in increased markers of oxidative stress and muscle damage, and alterations in muscle and systemic antioxidant enzyme activity and expression.

Previous studies have demonstrated the effectiveness of certain complexed trace minerals on increasing antioxidant enzyme activity and decreasing indices of oxidative stress in food animal models (Echeverry et al., 2016; Liu et al., 2016). The decrease in oxidative stress has generally been attributed to increased antioxidant enzyme activity, and no studies have examined the effects of complexed trace mineral supplementation on oxidative capacity to determine if improved mitochondrial efficiency contributes to the reduction in oxidative stress. In the present study, INORG horses exhibited higher integrated LEAK and P_{CI} in the GM compared to CTM. Previous studies have indicated that elevated P_{CI} activity results in excess reactive oxygen species (ROS) production (St-Pierre et al., 2002). Therefore, in this respect, complexed trace mineral supplementation may have decreased the overall ROS production associated with oxidative metabolism; the lack of difference in MDA concentration, a marker of oxidative stress, between dietary treatment groups would suggest otherwise. The disparity between results for increased complex I capacity and MDA concentration may lie in the increased integrated LEAK respiration observed in INORG horses. LEAK respiration is a dissipative component of respiration not available for performing biochemical work. LEAK respiration results from proton "leak" across the inner mitochondrial membrane, which has been proposed to mitigate ROS induced damage (Brand, 2000). Therefore, elevated LEAK respiration in INORG horses in the present study may have alleviated excess ROS production associated with elevated complex I activity, and circumvented increases in indices of oxidative stress.

Muscle SOD activity was similar amongst all horses through 6 wk of dietary treatments (prior study; data presented in Supplemental Table 2). However, at the beginning of the current study but after horses had been on experimental diets for 12 wk (wk 0 in the current study), GM SOD activity throughout the trailer stressor tended to be higher for INORG than CTM, whereas GPx activity was higher for CTM than INORG in both muscle groups at all sampling intervals. These results are in contrast to previously published literature in food animals showing an increase in SOD activity in various organs following complexed trace mineral supplementation (Liu et al., 2016; She et al., 2017). A similar inverse relationship between SOD and GPx expression and activity has been shown in horses supplemented with dietary antioxidants (de Moffarts et al., 2005; White et al., 2016), which has been suggested to indicate a preference for use of the GPx over the SOD antioxidant system in horses with more favorable antioxidant or selenium (Se) status. The link between CTM supplementation and increased GPx activity in the present study is not clear, as GPx is a selenoenzyme, and Se was not one of the complexed trace minerals supplemented. However, research has shown an increase in GPx activity in the serum and liver of rats following Zn supplementation (Galażyn-Sidorczuk et al., 2012). The increased GPx activity purportedly resulted from increased Se availability in tissues in response to Zn supplementation. Additionally, Cu deficiency has been shown to reduce GPx activity in rats despite maintenance of normal dietary and tissue Se levels, suggesting a Se-independent influence of Cu on GPx activity (Prohaska et al., 1992). Therefore, bioavailability of dietary trace minerals in the CTM group may have influenced GPx activity in a Se-dependent or independent manner. More research is needed to determine the effects of individual complexed trace minerals on GPx activity.

Muscle MDA concentration was used as a marker of oxidative stress. Pre-trailering MDA concentration in the GM and serum creatine kinase activity were lower at wk 8 and 12 than wk 0 but increased in response to the trailer stressor at wk 8 and 12. These results are contrary to recent literature in horses which shows an increase in resting MDA concentration in the GM with exercise training (Smarsh and Williams, 2016; White and Warren, 2017). However, our results are similar to research in horses showing a decrease in resting plasma MDA concentration with training (Avellini et al., 1999), and research in rodent models showing a decrease in resting MDA concentration of primarily fast-twitch muscles (like the GM), but not predominantly slow twitch muscles (like the TB) following exercise training (Cunningham et al., 2005). Our results may differ from other equine studies because exercise intensity was not increased throughout the duration of the study so horses had a longer period of acclimation to the exercise training intensity.

Trailer transportation for 1.5 h caused a decrease in CS activity, a marker of mitochondrial density, at wk 8 and 12. These results are similar to previously published literature showing a trend for a decrease in CS activity after moderate exercise in horses (Kinnunen et al., 2005), but contrary to human literature showing an increase in CS activity following acute exercise bouts (Tonkonogi et al., 1997; Leek et al., 2001). Changes in CS activity and MDA concentration in response to trailer stressors before and after exercise training were similar in that no change was found at wk 0, but decreases in CS activity and increases in MDA concentration were observed at wk 8 and 12. Rather than reflecting a stronger response to oxidative stress and a more marked decrease in CS enzymatic activity after exercise training, it is possible that higher PreT concentrations of MDA and lower PreT CS activity at wk 0 masked responses to transportation stress at wk 0. In addition to a decrease in CS activity, a decrease in

CCO activity was observed in both muscle groups at all trailer stressors. Similar reductions in CCO activity and abundance after acute exercise have been demonstrated in humans (Green et al., 2008; Stepto et al., 2012) and have been proposed to result from mitochondrial remodeling, or oxidative damage (Stepto et al., 2012). Together, the changes in CS and CCO activities led to an increase in intrinsic (per mitochondrial density) CCO activity in the GM following the trailer stressor at wk 0 and in the TB following all trailer stressors.

Serum creatine kinase was used as a marker of muscle damage, and followed a similar pattern to muscle MDA, our marker of oxidative damage. Creatine kinase is an enzyme found primarily in muscle, and its activity in the serum has been shown to increase in response to strenuous exercise as a result of altered muscle cell membrane permeability (Anderson, 1975) or muscle damage (Lindholm, 1987). While serum CK activity of horses in the present study remained within normal reference ranges, horses exhibited an increase in serum CK activity in response to trailer stress at each week. This was expected based on previous literature (Codazza et al., 1974; Tateo et al., 2012) and suggested that 1.5 h of trailering caused sufficient stress to induce muscle perturbation. It should be noted that the increase in serum CK activity following the trailer stressors at wk 8 and 12 were lower than at wk 0, which is in agreement with previous literature showing that exercise training attenuates the rise in serum CK activity following an exercise test (Siciliano et al., 1995). While trailer stress did not cause a change in muscle MDA concentration at wk0 for either muscle group, the trailer stressor at wk 0 did cause alterations in antioxidant enzyme activity and expression indicative of alterations in redox homeostasis.

At each week, whole blood GPx activity of CTM horses increased immediately after trailering and again 6 h post-trailering, mirroring the rise in serum CK activity, while INORG blood GPx activity was not affected by the trailer stressor. These results for the CTM group are

contrary to studies that have shown either no change (Kruljc et al., 2014) or a decrease in systemic GPx activity in horses following transportation (Niedzwiedz et al., 2012). These differences may have arisen due to differences in length of transportation or the age of horses in the studies. Alternatively, the observed increase in systemic GPx response to trailering may have been a result of the combination of complexed trace mineral supplementation and exercise training. Complexed trace mineral supplementation has been shown to increase GPx activity in various organs of food animals (Liu et al., 2016; She et al., 2017). A combination of training and antioxidant supplementation has been shown to result in increased systemic antioxidant activity, as well as protection from disruption in oxidant/antioxidant equilibrium with training in horses (Avellini et al., 1999; de Moffarts et al., 2005). Therefore, taken together with results from previous literature, our results suggest that complexed trace mineral supplementation improved systemic antioxidant response to transportation stress.

For both treatments, the wk0 trailer stressor induced a decrease in *SOD1* gene expression and increase in *SOD2* expression at 24hPT in the GM. In the TB, the wk0 trailer stressor led to a decrease in SOD activity at 24hPT that was accompanied by an increase in *SOD2* expression. Since ROS and inflammatory cytokines are known to regulate transcription of *SOD2* (Kim et al., 2005a; Ji, 2007), the increase in *SOD2* expression at 24hPT further suggests that redox balance of the horses may have been affected by the trailer stressor. As such, we would have expected an increase in oxidative stress. An alternative marker to MDA, such as protein carbonyls or lipid hydroperoxides, or different sampling intervals may have revealed a more prevalent oxidative stress response. Conversely, an increase in inflammatory cytokine signaling following trailering could have also been responsible for the observed increase in *SOD2* expression. The decrease noted in *SOD1* expression is contrary to previously reported literature which showed an increase

in expression 24 h after a 2-h submaximal exercise test in horses (White et al., 2016). It is possible that the horses in the present study responded differently because they were young and naïve to trailering and exercise, whereas the horses used in the aforementioned study were mature. To support this idea, *SOD1* expression increased in response to trailering at wk 8 and 12 of the current study after horses had been exposed to exercise and trailering stress. Week 0 trailering also increased GPx activity at 1hPT in the GM. The increase in GPx following the trailer stressor is in agreement with previous literature in horses that showed an increase in GPx activity following a prolonged submaximal exercise test (White and Warren, 2017). It is interesting to note that although CTM had significantly higher resting GPx activity compared to INORG, CTM still exhibited an increase in activity in response to trailering. This may result in part from the fact that GPx can be activated by signals other than increased cellular oxidant concentration. For example, GPx is activated by nuclear factor (NF) κβ (Zhou et al., 2001). Activation of NFκβ after acute exercise has often been attributed to increased ROS production during exercise, but may also be influenced by pro-inflammatory cytokines and other intermediates independently of exercise-induced ROS (Ji et al., 2004). Therefore, it is possible that increases in stress signals independent of ROS caused activation of NFκβ and therefore an increase in GPx activity in response to trailering.

In the GM at wk 8, SOD activity was significantly higher for INORG than CTM throughout the trailer stressor. Furthermore, PreT *SOD1* expression in the GM was lower at wk8 than wk0 for both treatments. It is worth noting that several of the horses became ill with a respiratory infection at approximately 3 wk exercise training, and symptomatic horses were not exercised. All horses were healthy and resumed exercise by wk 6. Nonetheless, a combination of the gap in exercise training and illness may have impacted SOD activity and *SOD1* expression at

wk8. It is not clear why SOD activity and expression in the GM of CTM seem to have been more affected by the illness than INORG horses, as horses from each treatment seemed to have been similarly affected by the illness in terms of symptom severity and duration, and there were no statistical outliers in the dataset for SOD activity or expression at wk8. However, the difference in change over time between treatments may have been influenced by the fact that GM SOD activity tended to be lower for CTM horses at wk 0.

For both dietary treatments, the wk 8 trailer stressor led to an increase in *SOD1* expression at 1hPT and an increase in *SOD2* expression at 24hPT in the GM. The rise in *SOD1* expression at 1hPT is in contrast to the decrease at 24hPT observed during wk 0. However, it does precede the observed rise in MDA at 24hPT, suggesting that the increase in expression may be a redox mediated event. Several redox signals may have led to induction of *SOD1* expression at 1hPT, including redox activation of Nrf2 (Done and Traustadottir, 2016), or NFκβ (Ji et al., 2004) and subsequent induction of *SOD1* expression (Milani et al., 2011).

At wk 12, PreT MDA concentration in the GM remained lower than wk 0, but increased 1 h after the trailer stressor, returning to PreT levels by 24hPT. Taken together with the wk 8 response of GM MDA to trailer stress, our results show that 12 wk exercise training brought about an earlier rise and decline in GM MDA concentration when compared to 8 wk exercise training. In the GM, CTM SOD activity throughout the trailer stressor was higher than at wk 0 or 8, abolishing treatment differences at wk 12. Interestingly, GM *SOD1* expression throughout the trailer stressor tended to remain lower for CTM than INORG, likely resulting from a trend for an increase in CTM horses throughout the trailer stressor at wk 12 but a significant increase in INORG at wk 12. Nonetheless, the decrement in *SOD1* expression was not significant enough to translate to a decrement in SOD activity, likely because exercise training induced increases in

SOD activity are primarily due to increases in SOD2 activity and expression (Ji, 2007). These results again suggest that CTM horses favor the GPx system for antioxidant defense over the SOD system. Growing horses supplemented with dietary complexed trace minerals showed an increase in GM SOD activity with 12 wk exercise training, whereas INORG GM SOD activity was not different between wk 0 and 12. Therefore, 12 wk exercise training in growing horses in addition to CTM supplementation ultimately led to similar GM SOD activities between treatments, but higher sustained GPx activity for CTM. For both treatments, the wk 12 trailer stressor led to an increase in *SOD2* expression at 24hPT that was lower than the increase observed at wk 0 or 8, and no change in *SOD1* expression in response to trailer stress in the GM. Taken together, these results suggest that the redox response to trailering in the GM was less prominent after 12 wk exercise training in growing horses. In the TB, the wk 12 trailer stressor led to an increase in *SOD2* expression at 24hPT for INORG, but only a trend for an increase for CTM. The increase in *SOD2* expression in the TB at 24hPT was lower at wk 12 than 0 for both treatments, suggesting that the redox response to trailering in the TB was also attenuated by 12 wk exercise training. It is important to note that while we did observe changes in response to trailering after 12 wk exercise training, it is possible that growth could also have contributed to the attenuated redox response. More research is needed to determine the individual impacts of growth and training on skeletal muscle redox response to trailer stress in young horses.

Although responses in the GM were not directly compared to those in the TB in the present study, it is worth noting that the responses differed considerably between muscle groups. In horses, the GM is a propulsive muscle, and the TB is a stabilization muscle. As such, the GM is composed of a greater percentage of fast-twitch, non-oxidative fibers when compared to the TB (Snow and Guy, 1980). Additionally, the TB has been found to have higher indices of

mitochondrial density (White et al., 2017b). In the present study, malondialdehyde concentration, GPx activity, and *SOD1* expression changed in response to training and trailering in the GM, but not the TB. While statistical comparisons between muscle groups were not made, the concentration of MDA and the activities of SOD and GPx were numerically higher in the TB than the GM. These data suggest that in addition to having a lower percentage of fast-twitch, non-oxidative fibers and higher mitochondrial density, the TB also has a higher antioxidant capacity and is less affected by stressors such as trailering when compared to the GM.

In the present study of yearling horses, trailering caused an increase in CK activity and muscle MDA concentration, and changes in antioxidant defense systems indicative of alterations in redox homeostasis. Horses showed decreased systemic markers of muscle damage and resting MDA concentrations in the GM, increased resting expression of *SOD1* and *SOD2*, and attenuated redox induced alterations in *SOD* expression associated with trailer stress through 12 wk of submaximal exercise training. Supplementation with complexed trace minerals increased systemic GPx response to trailer stress and muscle GPx activity at rest and in response to trailer stress, ultimately leading to preferential utilization of the GPx system over the SOD system in response to oxidative stress in young untrained horses. After 12 wk exercise training, CTM supplementation resulted in similar systemic and muscle SOD activity between treatments but maintained significantly higher muscle GPx activity. Therefore, complexed trace minerals may be a useful tool for mitigating oxidative stress and maintaining muscle health in young equine athletes.

4. SKELETAL MUSCLE ADAPTATIONS TO EXERCISE TRAINING IN AGED HORSES

4.1. Synopsis

Low intensity exercise has been shown to increase mitochondrial density, function and oxidative capacity, decrease the prevalence of hybrid fibers, and increase lean muscle mass in aged humans. The effects of low intensity exercise have not been closely studied in aged horses. Effects of age and exercise training on muscle fiber type and size, mitochondrial number (citrate synthase activity; CS), function (cytochrome *c* oxidase activity; CCO), and oxidative capacity (P) by high-resolution respirometry (HRR) were evaluated in skeletal muscle from aged (n=9; 22 ± 4.5 yr) and yearling (n = 8; 9.7±0.7 mo) horses. Muscle was collected at wk 0, 8, and 12 of exercise for aged and young horses for analysis of CS, CCO and oxidative capacity, and at wk 0 for young horses and wk 0, 4, 8 and 12 for analysis for muscle fiber type and size. Data was analyzed using PROC MIXED in SAS 9.4 with age, time, muscle, and all interactions as fixed effects. Aged horses had a greater percentage of type I/IIa and IIa/x hybrid fibers than young horses in the GM ($P \leq 0.07$), but the percentage of type I/IIa and IIa/x hybrid fibers in the GM decreased by wk 4 ($P \leq 0.002$) in aged horses. Mitochondrial density and function increased due to training, as evidenced by higher CS ($P = 0.003$) and integrated (per mg protein) CCO activities ($P = 0.011$) at wk 12 than 0. Activity of CS was higher in aged than young TB ($P = 0.029$), but intrinsic (per unit CS; $P = 0.001$) CCO activity was lower, and integrated CCO activity tended to be lower ($P = 0.07$) in both muscle groups of aged horses. Integrated P with complex I substrates (P_{CI}) increased by wk 8 in aged horses ($P < 0.003$), but did not change in young horses. Integrated P_{CI} plus complex II substrates (P_{CI+II}) and complex II electron transport

capacity (E_{CII}) increased by wk 8 in both age groups ($P < 0.0005$). Maximum electron transport capacity (E_{CI+II}) increased by wk 8 in aged horses ($P = 0.0001$), but not until wk 12 for young horses ($P = 0.01$). Overall, intrinsic oxidative and electron transport system capacities were higher in aged than young horses ($P < 0.02$), and HRR measures were higher in the TB than the GM ($P < 0.0001$). Exercise improved all mitochondrial measures in both young and aged horses; however, aged horses showed impaired mitochondrial function and differences in adaptation to exercise training.

4.2. Introduction

Previous work in horses has reported similar adaptations to aging as those seen in humans: decreased skeletal muscle mitochondrial number (Li et al., 2016), elevated muscular and circulating levels of inflammatory cytokines (McFarlane and Holbrook, 2008), and decreased muscle mass (Hintz, 1995). However, other parameters such as the prevalence of hybrid fibers, have yet to be examined in horses. A number of interventions have been utilized to improve age-related alterations in skeletal muscle parameters individually, including dietary antioxidant supplementation and anti-inflammatory pharmaceuticals, but these do not address the collective global alterations associated with aging.

Low intensity exercise has the potential to improve mitochondrial density, function and oxidative capacity, decrease the prevalence of hybrid fibers, and increase lean muscle mass. However, the effect of exercise on these parameters in aged horses has not been closely studied. The objective of this study was to characterize the effects of age and exercise training on mitochondrial density, function, and oxidative capacity. It was hypothesized that aged horses would have lower mitochondrial density, function and oxidative capacity, and alterations in muscle fiber type when compared to young horses.

4.3. Methods

4.3.1. Horses

This study was reviewed and approved by the Texas A&M Institutional Animal Care and Use Committee (IACUC 2016-0294). Ten aged (9 mares and 1 gelding; 22 ± 4.5 yr) and eight yearling (5 colts and 3 fillies; 9.7 ± 0.7 mo) Quarter Horses were used in this study. Horses were housed in paddocks devoid of fresh grass at the Texas A&M University Freeman Equestrian Center in College Station, TX.

4.3.2. Diets

Horses were allocated to separate pens by age and had *ad libitum* access to Coastal bermudagrass hay. Hay intake per horse per day was estimated by the following formula:

$$\frac{Total\ hay\ offered\ in\ the\ pen\ per\ day\ (kg) - total\ hay\ refused\ in\ the\ pen\ per\ day\ (kg)}{Number\ of\ horses\ in\ the\ pen}$$

Concentrate was offered to aged horses at 0.5% BW/d (DM basis) and to young horses at 1.25% BW/d (DM basis). Horses received grain meals individually in stalls (3.7 × 3.7 m) split equally into two meals fed at 0700 h and 1700 h. Refusals were monitored and recorded daily to calculate actual concentrate intake. Diets were formulated to maintain a BCS of 5 to 6 according to the Henneke body condition scoring system (Henneke et al., 1983), and to meet all requirements of mature or growing horses in light exercise (NRC, 2007). Throughout the study, BW of horses was recorded weekly using a livestock scale accurate to 1 kg (Cardinal Scales, Webb City, Missouri).

4.3.3. Exercise

Horses had received no forced exercise for at least 6 mo prior to the beginning of this study. Beginning at wk 0, horses were enrolled in a 12-wk submaximal exercise program.

Exercise was designed to achieve light work as defined by the NRC (NRC, 2007), and consisted of 12 min of walking, 15 min of trotting, and 3 min of cantering in a free-stall exerciser (30 min total/d) 5 d/wk. Each gait was performed in both directions each day, and horses alternated starting the exercise bout clockwise or counterclockwise each day. For aged horses, the walk was performed at a speed of 1.2 m/s, the trot at 2.5 m/s and the canter at 5.2 m/s. Due to changes in stride length with growth, the speed of each gait for young horses was progressively increased throughout the study to ensure that all horses remained in the intended gait. Gait speeds for young horses started at 1.1 m/s for the walk, 2.5 m/s at the trot and 5.0 m/s at the canter at the beginning of training and were increased to 1.2 m/s at the walk, 3.0 m/s at the trot and 5.4 m/s at the canter by the end of the 12-wk exercise training program.

4.3.4. Sample Collection

Muscle samples were collected at wk 0, 8 and 12, for analysis of CS, CCO and oxidative capacity, and at wk 0, 4, 8 and 12 for aged horses and at wk0 for young horses for analysis of muscle fiber type. Muscle tissue samples were collected from the gluteus medius (GM) and triceps brachii (TB) using a tissue collection procedure as previously described (White et al., 2016). Briefly, horses were sedated with detomidine hydrochloride (Dormosedan; Zoetis, Parsippany-Troy Hills, NJ) prior to beginning tissue collection procedures. The collection areas were clipped, scrubbed with a 7.5% povidone-iodine solution, and then scrubbed with a 70% ethanol solution. The tissue collection sites were desensitized with 0.5 mL of 2% lidocaine (Vetone, Boise, ID) and a 14-gauge needle was used to create the initial puncture through the skin. Tissue was collected using a 14-gauge, 9-cm biopsy needle (SuperCore; Argon Medical Devices Inc., Frisco, TX) inserted to a depth of 5 cm in aged horses and 3.5 cm in young horses. The tissue collection site altered between left and right muscle groups at each sampling interval.

Samples obtained from the same side of the horse were obtained approximately 2 cm from the previous insertion site. At each sampling interval, approximately 300 mg (wet weight) of muscle tissue was flash frozen in liquid nitrogen and stored at -80° C until analyses were performed. For muscle fiber type analysis, approximately 400 mg tissue was embedded in OCT compound (Fisher Scientific, Hampton, NH, USA), frozen in liquid nitrogen-cooled isopentane and stored at -80º C until analysis. For high-resolution respirometry (HRR), muscle fibers were collected into ice cold biopsy preservation medium (BIOPS) and stored on ice until analysis. Flash frozen muscle was cryopulverized into a fine powder (Spectrum™ Bessman Tissue Pulverizer; Thermo Fisher Scientific, Waltham, MA) for evaluation for CS and CCO activities.

4.3.5. Enzyme Activities

Citrate synthase (CS) and cytochrome *c* oxidase (CCO) activities were analyzed as markers of mitochondrial density and function, respectively (Larsen et al., 2012). For measurement of CS and CCO activities, frozen skeletal muscle samples were prepared, and activities were measured as previously described (Spinazzi et al., 2012; Li et al., 2016). Enzymatic activities were normalized to homogenate supernatant protein content, determined using the Bradford Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA). Cytochrome *c* oxidase activity is presented on an integrated (per mg protein) and intrinsic (per unit CS) basis.

4.3.6. High Resolution Respirometry

For high-resolution respirometry (HRR), muscle fibers were collected, prepared and permeabilized as previously described (Li et al., 2016) and then analyzed within 24 h of collection. Oxygen flux and respiratory states were determined by HRR with the following substrate-uncoupler-inhibitor titration protocol modified from a previously described protocol for equine skeletal muscle (Li et al., 2016): (1) pyruvate (5 mM) and malate (2 mM) to support

electron flow through complex I (CI) of the electron transport system (ETS) (LEAK respiration); 2) adenosine diphosphate (ADP; 2.5 mM) to stimulate respiration (OXPHOS, P_{CI}); 3) cytochrome *c* (cyt c; 10 μM) to assess outer mitochondrial membrane integrity (samples with responses to cyt c greater than 15% were excluded); 4) glutamate (10 mM) as an additional CI substrate and succinate (10 mM) to support convergent electron flow through complex II (CII) of the ETS (P_{CI+II}); 5) uncoupler carbonyl cyanide 3-chlorophenylhydrazone (CCCP; 0.5 μM steps) to assess maximum ETS capacity (E_{CI+II}); 6) rotenone (0.5 uM), an inhibitor of complex I, to measure maximal ETS capacity of complex II (E_{CII}); 7) antimycin A (2.5 μM), an inhibitor of complex III, to measure residual oxygen flux (ROX) independent of the ETS. Sample flux control ratio (FCR) for each complex was calculated by dividing the flux in each complex by the sample's E_{CI+II} flux.

4.3.7. Muscle Fiber Type

For this and subsequent studies, antibodies for myosin heavy chain (MyHC) type I (BA-D5, Developmental Studies Hybridoma Bank, University of Iowa) and type IIx (6H1; Developmental Studies Hybridoma Bank) that are commonly used for concurrent staining of muscle fiber type in human and mouse models were validated for use in horses. Briefly, muscle tissue samples were cut into 7 μm sections using a cryostat (Leica Biosystems, Wetzlar, Germany), air dried onto glass slides (Fisher Scientific), then stored at -20º C until analysis. Before analysis, sections were separated with a hydrophobic pen (Vector Laboratories, Burlingame, CA) and rehydrated with 0.01 M phosphate buffered saline (PBS; Fisher Scientific). For primary antibody validation, skeletal muscle samples from the GM and TB of one colt, one mare, and one gelding were used to determine fiber type; two sections were incubated in previously validated primary antibodies for myosin heavy chain Type I (MAB1628, Millpore,

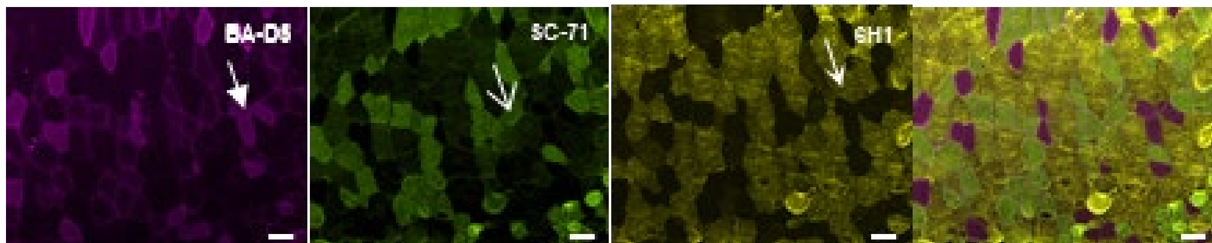
Darmstadt, Germany) and myosin heavy chain Type II muscle fibers (MHCf, Leica Biosystems, Wetzlar, Germany) (Tulloch et al., 2011) diluted 1:50 in phosphate buffered saline (PBS). A second section was incubated in BA-D5 (1:100), SC-71, a previously validated antibody for MyHC type IIa (Rivero et al., 1996) (1:100; Developmental Studies Hybridoma Bank), and 6H1 (undiluted supernatant). A third section was stained for succinate dehydrogenase activity using previously validated methods (Blanco et al., 1988) and then in the proposed primary antibodies and SC-71. Sections were then incubated in fluorescent secondary antibody (Alexa Fluor, Thermo Scientific, Rockford, IL, USA) diluted in PBS. Finally, sections were imaged with the appropriate fluorescent filter for immunohistochemistry, and in brightfield for SDH staining (Nikon Instruments, Melville, NY, USA). A minimum of 50 muscle fibers were compared for each sample. Myosin heavy chain type I primary antibodies (BA-D5 and MAB1628) identified the same fibers for all fibers counted. To confirm that the 6H1 antibody labels type IIx fibers and not type IIa fibers, sections were incubated in a previously validated antibody for type IIa fibers (SC-71), as well as the proposed primary antibodies. Together, 6H1 and SC-71 (Fig. 4.1A) labeled the same fibers as MHCf (Fig. 4.1B). Additionally, SDH staining labeled type I and IIa fibers but not type IIx fibers (Fig. 4.1C). Therefore, the proposed antibodies, in combination with SC-71 allow for multiple immunofluorescent labeling of fiber types on a single section without the use of direct labeled antibodies.

The antibodies BA-D5, SC-71 and 6H1 were used as described for validation for analysis of muscle fiber type of aged and young horses. The number of oxidative fibers in a sample was calculated by adding the number of type I, IIa, I/IIa and IIa/x fibers in the sample. The percentage of type I, IIa, IIx, I/IIa, IIa/x and oxidative fibers in each sample was calculated by the following equation:

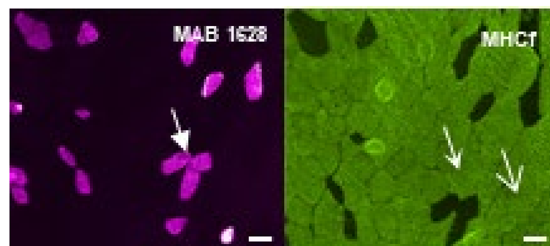
$$\left(\frac{Number\ of\ Muscle\ Fibers\ of\ a\ Given\ Type}{Total\ Number\ of\ Fibers\ Counted}\right) \times 100$$

Fiber type-specific minimum feret diameter was analyzed as an indicator of muscle fiber size using ImageJ (National Institutes of Health, Bethesda, MD).

A.



B.



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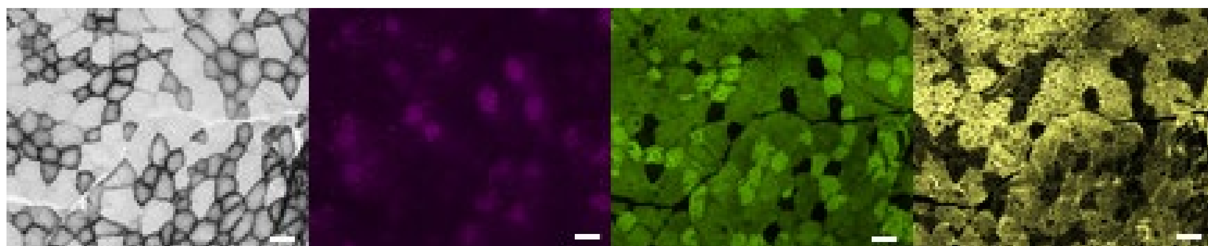

Figure 4.1. Representative images of fluorescent staining of muscle fiber type using the proposed antibodies (A), previously validated antibodies (B), and SDH and fluorescent staining (C) in skeletal muscle samples from the GM and TB of one mature mare. Scale bar = 100 µm.

4.3.8. Statistical Analysis

Differences in body weight, body condition score, enzyme activities, mitochondrial respiration measurements and muscle fiber type were analyzed using the MIXED procedure of SAS (Version 9.4, SAS Institute Inc., Cary, NC) with repeated measures. Data were log-transformed prior to analysis if not normally distributed. Intrinsic CCO and HRR, FCR and muscle fiber type percentage data were not transformed as they were already normalized to CS, E_{CI+II} flux and total fibers, respectively. Age (aged or young), time (wk 0, 8 or 12), muscle group (GM or TB) and all interactions were included in the model as fixed effects for analysis of CS, CCO, and HRR data. Age (aged or young), muscle group (GM or TB), and age × muscle group were included as fixed effects in the model for pre-training (wk 0) muscle fiber type. Time (wk 0, 4, 8 or 12), muscle group and time × muscle group were included in the model as fixed effects for analysis of aged horse muscle fiber type and size in response to training. Significance was considered at $P \leq 0.05$, and trends were acknowledged at $P \leq 0.10$.

4.4. Results

4.4.1. Body Weight and Body Condition Score

Body weight was higher for aged than young horses ($P < 0.0001$; Fig. 4.2A) and increased with growth in young horses from wk 0 to 8 ($P = 0.0003$; Fig. 4.2A) and from wk 8 to 12 ($P = 0.0001$). Body condition score was lower for aged than young horses at wk 0 ($P = 0.0004$; Fig. 4.2B), was not different between age groups at wk 8 and was lower in aged than young horses at wk 12 ($P = 0.038$). Aged horse body condition score tended to decrease by wk 8 ($P = 0.055$; Fig. 4.2B); young horse body condition score decreased by wk 8 ($P <0.0001$) and remained lower than wk 0 at wk 12 ($P < 0.0001$).

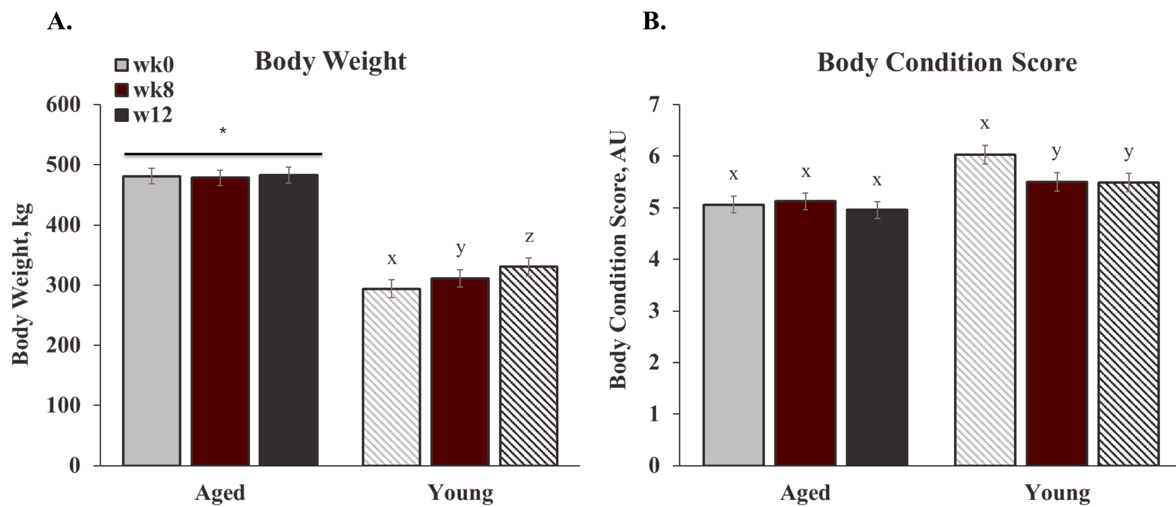

Figure 4.2. Body weight (A) and body condition score (B) of aged (n = 10), and young (n = 8) Quarter Horses before exercise training (wk0) and after 8 (wk8) and 12 (wk12) wk exercise training. Overall effect of age (*P* < 0.0001; *P* = 0.016), time (*P* < 0.0001; *P* < 0.0001) and age × time (*P* < 0.0001; *P* < 0.0001) for body weight and body condition score, respectively. [x,y] Time points lacking common letters differ (*P* ≤ 0.05).

4.4.2. Mitochondrial Density, Function and Capacity

Citrate synthase activity increased by wk 8 (*P* = 0.001) and remained elevated above wk 0 at wk 12 (*P* = 0.003; Fig. 4.3) in the TB, but was not affected by time in the GM. A trend for an effect of age × time (*P* = 0.063) suggested that CS activity increased for aged horses by wk 12 (*P* = 0.0008), while CS activity was not affected by time in young horses. Citrate synthase activity was higher in the TB than the GM for aged (*P* < 0.0001), but not for young horses, and was higher for aged than young horses in the TB (*P* = 0.004).

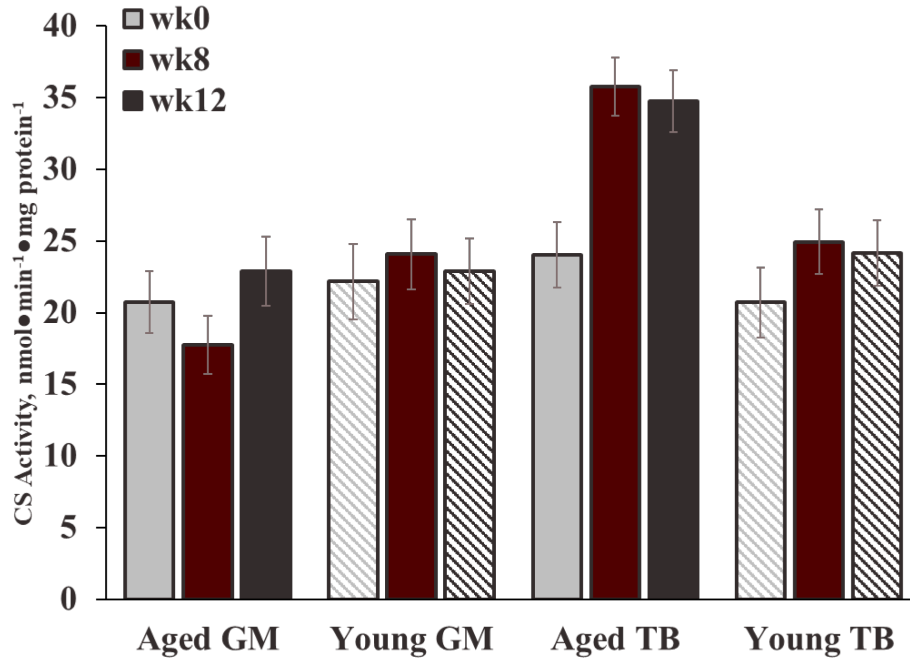

Figure 4.3. Citrate synthase (CS) activity in the gluteus medius (GM) and triceps brachii (TB) of aged (n = 10) and young (n = 8) Quarter Horses before exercise training (wk0) and after 8 (wk8) and 12 (wk12) wk exercise training. Overall effect of age (*P* = 0.263), time (*P* = 0.010), muscle (*P* = 0.0008), age × time (*P* = 0.063), age × muscle (*P* = 0.0007), time × muscle (*P* = 0.017) and age × time × muscle (*P* = 0.112).

Integrated CCO activity was lower for aged than young horses (*P* = 0.040), and higher in the TB than the GM (*P* < 0.0001; Fig. 4.4A). A trend for an effect of time (*P* = 0.090) indicated that integrated CCO activity tended to be higher at wk 8 than 0 (*P* = 0.080) and increased by wk 12 (*P* = 0.044) in both age groups. Intrinsic CCO activity was lower in aged than young horses (*P* = 0.018; Fig. 4.4B) but was not affected by time and was not different between muscle groups.

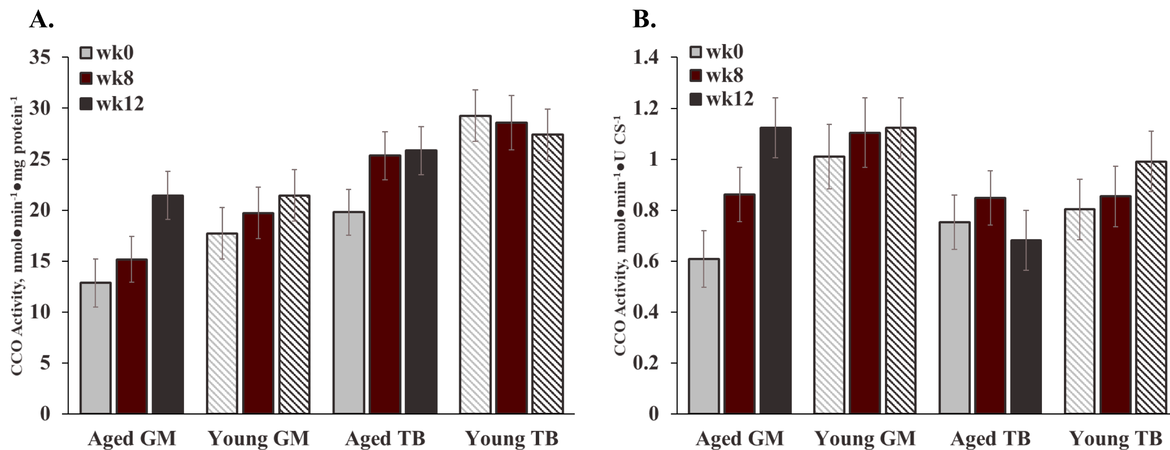

Figure 4.4. Integrated (A) and intrinsic (B) cytochrome c oxidase (CCO) activity in the gluteus medius (GM) and triceps brachii (TB) of aged (n = 10) and young (n = 8) Quarter Horses before exercise training (wk0) and after 8 (wk8) and 12 (wk12) wk exercise training. Overall effect of age ($P = 0.038$; $P = 0.017$), time ($P = 0.090$; $P = 0.132$), muscle ($P < 0.0001$; $P = 0.928$), age × time ($P = 0.262$; $P = 0.421$), age × muscle ($P = 0.210$; $P = 0.135$), time × muscle ($P = 0.377$; P = 0.575) and age × time × muscle ($P = 0.959$; $P = 0.995$) for integrated and intrinsic CCO activity, respectively.

Integrated LEAK was higher for young than aged horses in the TB ($P = 0.0003$; Fig. 4.5A). Integrated LEAK respiration decreased by wk 8 for young horses ($P < 0.0001$) and remained lower at wk 12 ($P < 0.0001$) but did not change over time in aged horses. Intrinsic LEAK decreased from wk 0 to 8 ($P = 0.012$; Fig. 4.5B) and tended to decrease further from wk 8 to 12 ($P = 0.083$). A trend for an effect of age × time (P = 0.060) suggested that aged horse intrinsic LEAK tended to decrease at wk 8 ($P = 0.082$) and tended to remain lower at wk 12 ($P = 0.056$), while young horse LEAK decreased at wk 8 ($P = 0.002$) and remained lower at wk 12 ($P = 0.002$).

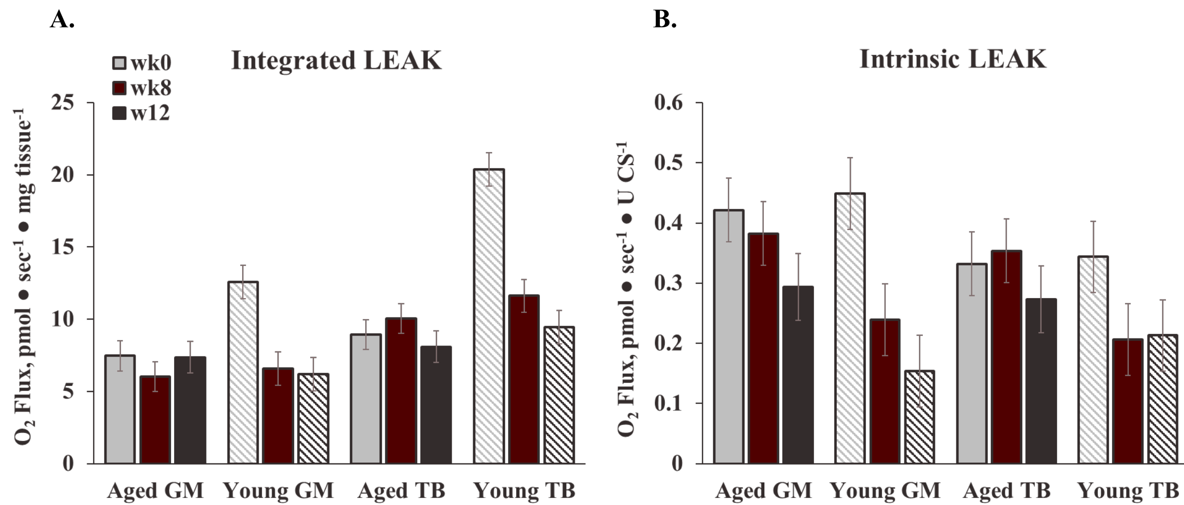

Figure 4.5. Integrated (A) and intrinsic (B) LEAK respiration in the gluteus medius (GM) and triceps brachii (TB) of aged (n = 10) and young (n = 8) Quarter Horses before exercise training (wk0) and after 8 (wk8) and 12 (wk12) wk exercise training. Overall effect of age (P = 0.002; P = 0.148), time (P < 0.0001; P = 0.0001), muscle (P < 0.0001; P = 0.216), age × time (P < 0.0001; P = 0.060), age × muscle (P = 0.011; P = 0.727), time × muscle (P = 0.129; P = 0.254) and age × time × muscle (P = 0.167; P = 0.755) for integrated and intrinsic LEAK respiration, respectively.

Integrated P_{CI} was lower for aged than young horses (P = 0.042) and was higher in the TB than the GM (P <0.0001; Fig. 4.6A). Integrated P_{CI} increased by wk 8 (P = 0.014) and tended to remain higher at wk 12 than 8 (P = 0.056). A trend for an effect of age × time suggested that aged horses had lower integrated P_{CI} than young horses at wk 0 (P = 0.005), but that aged horse integrated P_{CI} increased by wk 12 (P = 0.027), leading to no difference between age groups at wk 8 and 12. A trend for an effect of age × muscle group indicated that integrated P_{CI} was lower for aged than young in the TB (P = 0.010), but not in the GM. Intrinsic P_{CI} tended to be higher for aged than young horses overall (P = 0.054; Fig. 4.6B). Intrinsic P_{CI} increased from wk 0 to 8 (P = 0.044) and tended to decrease from wk 8 to 12 (P = 0.060) but was not different between muscle groups (Fig. 4.6B).

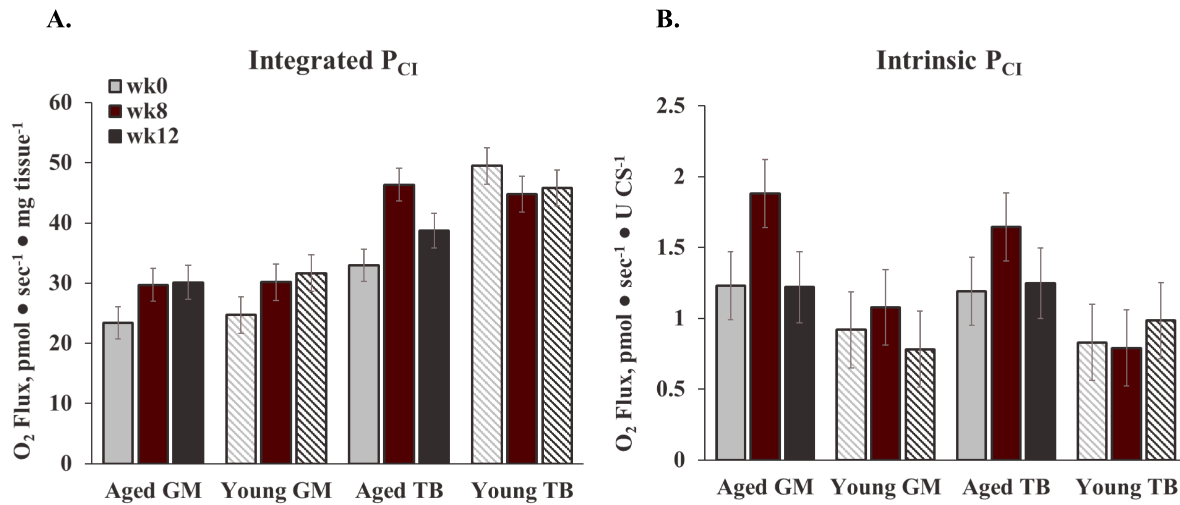

Figure 4.6. Integrated (A) and intrinsic (B) oxidative phosphorylation capacity with complex I substrates (P_{CI}) in the gluteus medius (GM) and triceps brachii (TB) of aged (n = 10) and young (n = 8) Quarter Horses before exercise training (wk0) and after 8 (wk8) and 12 (wk12) wk exercise training. Overall effect of age (*P* = 0.180; *P* = 0.054), time (*P* = 0.0003; *P* = 0.079), muscle (*P* < 0.0001; *P* = 0.565), age × time (*P* = 0.104; *P* = 0.177), age × muscle (*P* = 0.241; *P* = 0.918), time × muscle (*P* = 0.568; *P* = 0.450) and age × time × muscle (*P* = 0.213; *P* = 0.909) for integrated and intrinsic P_{CI}, respectively.

Integrated P_{CI+II} was not different between age groups but increased from wk 0 to 8 (*P* = 0.006; Fig. 4.7A) and remained higher at wk 12 than 0 (*P* = 0.001). Integrated P_{CI+II} was higher in the TB than the GM (*P* < 0.0001). Intrinsic P_{CI+II} tended to be higher for aged than young horses (*P* = 0.051; Fig 4.7B), but was not affected by time, muscle group, or any interactions.

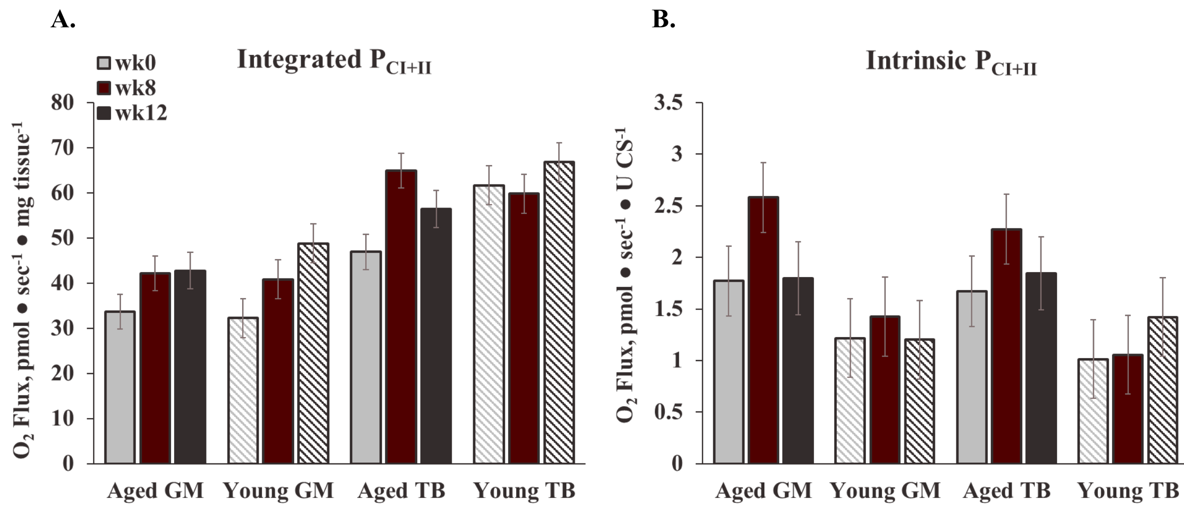

Figure 4.7. Integrated (A) and intrinsic (B) oxidative phosphorylation capacity with complex I and II substrates (P_{CI+II}) in the gluteus medius (GM) and triceps brachii (TB) of aged (n = 10) and young (n = 8) Quarter Horses before exercise training (wk0) and after 8 (wk8) and 12 (wk12) wk exercise training. Overall effect of age (*P* = 0.180; *P* = 0.051), time (*P* = 0.0003; *P* = 0.120), muscle (*P* < 0.0001; *P* = 0.473), age × time (*P* = 0.104; *P* = 0.201), age × muscle (*P* = 0.241; *P* = 0.992), time × muscle (*P* = 0.568; *P* = 0.503) and age × time × muscle (*P* = 0.213; *P* = 0.931) for integrated and intrinsic P_{CI+II}, respectively.

Integrated maximum ETS (E_{CI+II}) tended to be lower for aged than young horses at wk 0 (*P* = 0.062; Fig. 4.8A) but was not different between age groups at wk 8 or 12. Integrated E_{CI+II} increased by wk 8 in aged horses (*P* = 0.0002), remained higher at wk 12 than 0 (*P* = 0.02), and tended to increase in young horses by wk 12 (*P* = 0.075). Integrated E_{CI+II} was higher in the TB than the GM (*P* <0.0001). Intrinsic E_{CI+II} was higher for aged than young horses (*P* = 0.043; Fig. 4.8B) but did not differ between muscle groups and did not change over time.

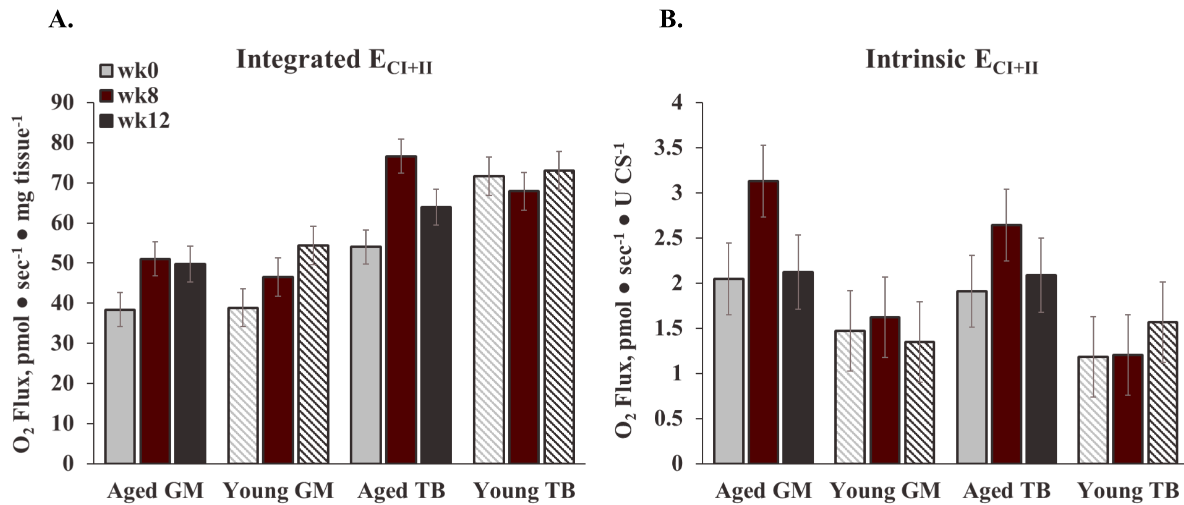

Figure 4.8. Integrated (A) and intrinsic (B) electron transport system capacity with complex I and II substrates (E_{CI+II}) in the gluteus medius (GM) and triceps brachii (TB) of aged (n = 10) and young (n = 8) Quarter Horses before exercise training (wk0) and after 8 (wk8) and 12 (wk12) wk exercise training. Overall effect of age (*P* = 0.325; *P* = 0.043), time (*P* = 0.004; *P* = 0.101), muscle (*P* < 0.0001; *P* = 0.330), age × time (*P* = 0.033; *P* = 0.141), age × muscle (*P* = 0.268; *P* = 0.881), time × muscle (*P* = 0.400; *P* = 0.514) and age × time × muscle (*P* = 0.229; *P* = 0.908) for integrated and intrinsic E_{CI+II}, respectively.

Integrated E_{CII} increased by wk 8 (*P* = 0.004; Fig 4.9A) and remained higher at wk 12 than 0 (*P* = 0.0003). Integrated E_{CII} was higher in the TB than the GM (*P* <0.0001) but was not affected by age or any interactions. Intrinsic E_{CII} was higher in aged than young horses (*P* = 0.041; Fig 4.9B) but was not affected by time, muscle group or any interactions.

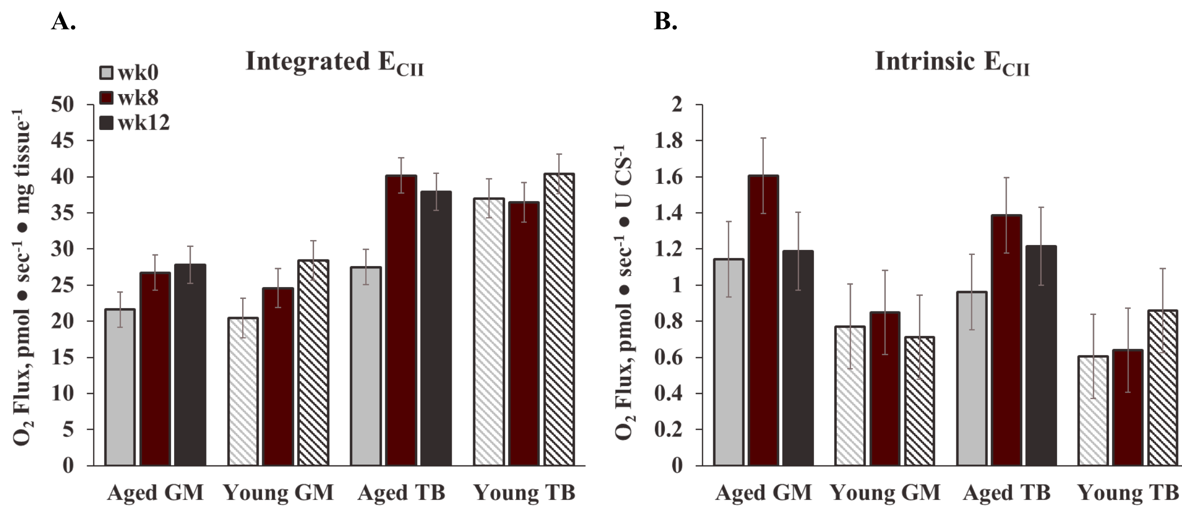

Figure 4.9. Integrated (A) and intrinsic (B) electron transport system capacity with complex II (E_{CII}) in the gluteus medius (GM) and triceps brachii (TB) of aged (n = 10) and young (n = 8) Quarter Horses before exercise training (wk0) and after 8 (wk8) and 12 (wk12) wk exercise training. Overall effect of age (*P* = 0.632; *P* = 0.041), time (*P* = 0.0009; *P* = 0.120), muscle (*P* < 0.0001; *P* = 0.313), age × time (*P* = 0.129; *P* = 0.215), age × muscle (*P* = 0.212; *P* = 0.805), time × muscle (*P* = 0.882; *P* = 0.395) and age × time × muscle (*P* = 0.202; *P* = 0.967) for integrated and intrinsic E_{CII}, respectively.

The FCR for LEAK was higher in aged than young horses at wk 0 (*P* = 0.010; Fig. 4.10A), lower for aged than young horses at wk 8 (*P* = 0.027) and not different between age groups at wk 12. The FCR for LEAK increased in young horses at wk 8 (*P* = 0.0009; Fig. 4.9A) and then decreased from wk 8 to 12 (*P* = 0.029). The FCR for LEAK decreased at wk 8 in aged horses (*P* = 0.018) but was not different from wk 0 or 8 at wk 12. The FCR for LEAK was not different between muscle groups. The FCR for P_{CI} was lower for aged than young horses (*P* = 0.023; Fig. 4.10B) but was not different between muscle groups and was not affected by training or any interactions. The FCR for P_{CI+II} was not different between age groups at wk 0 but was higher for young horses than aged horses at wk 8 (*P* = 0.012; Fig. 4.10C) and tended to remain higher for young horses than aged horses at wk 12 (*P* = 0.079). The FCR for P_{CI+II} increased at wk 8 for young horses (*P* = 0.017) and remained higher at wk 12 than 0 (*P* = 0.0006). The FCR

for P_{CI+II} decreased from wk 0 to 8 for aged horses (P = 0.036) and increased from wk 8 to 12 (P = 0.022). The FCR for P_{CI+II} was not different between muscle groups. A trend for an age × time interaction (P = 0.061; Fig. 4.10D) indicated that the FCR for E_{CII} was not different between age groups at wk 0 or 8, but was higher for aged horses than young horses at wk 12 (P = 0.032). The age × time interaction suggested that FCR for E_{CII} increased for aged horses at wk 12 (P = 0.002) but did not change over time in young horses. The FCR for E_{CII} was higher in the GM than the TB at wk 0 (P = 0.030) but was not different between muscle groups at wk 8 or 12. The FCR for E_{CII} increased from wk 8 to 12 in the TB (P = 0.013) but did not change over time in the GM.

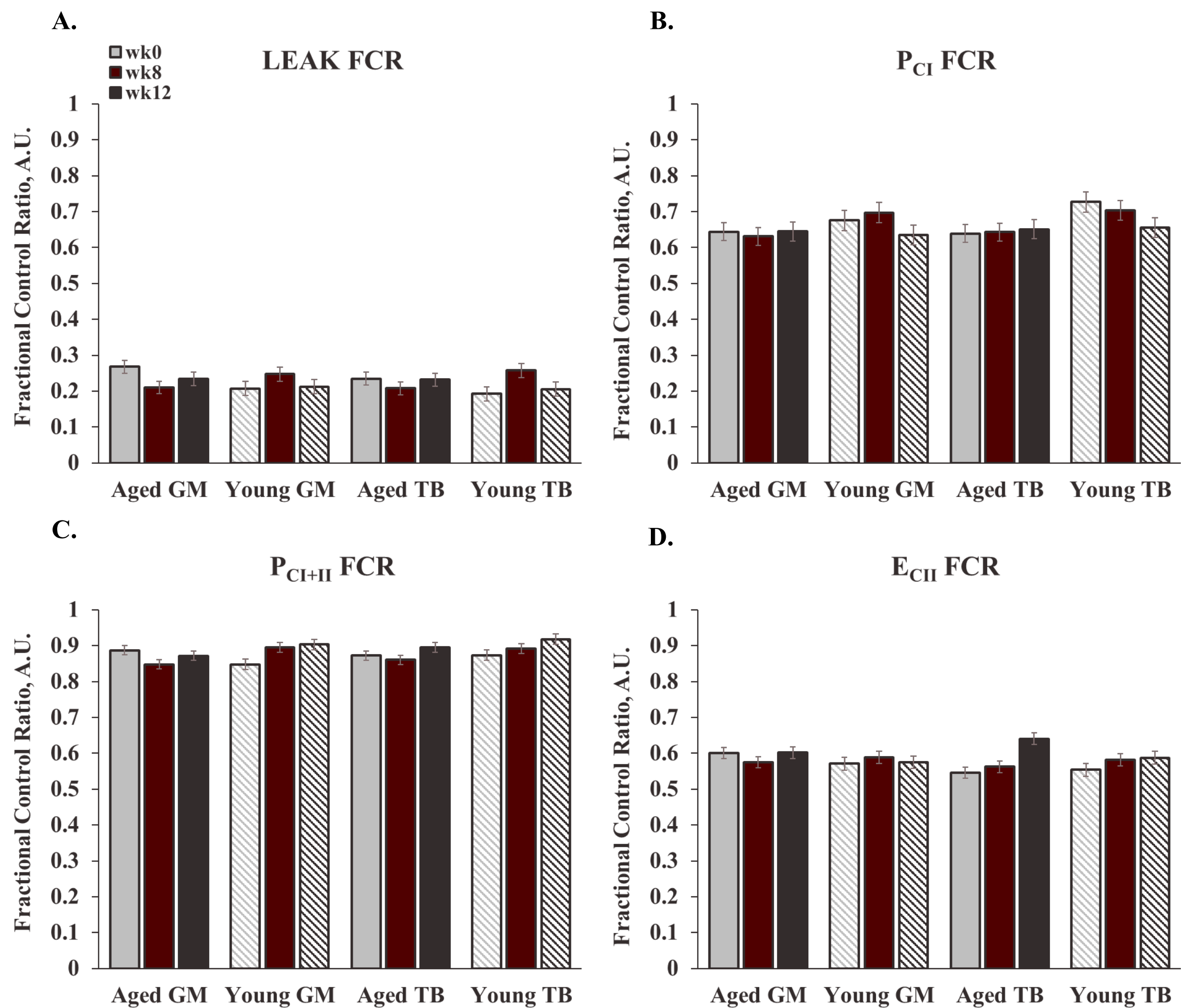

Figure 4.10.Fractional control ratio (FCR) for LEAK respiration (A), oxidative phosphorylation capacity with complex I substrates (P_{CI}; B) oxidative phosphorylation capacity with complex I and II substrates (P_{CI+II}; C) and electron transport system capacity with complex II (E_{CII}; D) in the gluteus medius (GM) and triceps brachii (TB) of aged (n = 10) and young (n = 8) Quarter Horses before exercise training (wk0) and after 8 (wk8) and 12 (wk12) wk exercise training. Overall effect of age (*P* = 0.387; *P* = 0.021; *P* = 0.161; *P* = 0.333), time (*P* = 0.756; *P* = 0.363; *P* = 0.011; *P* = 0.018), muscle (*P* = 0.462; *P* = 0.338; *P* = 0.193; *P* = 0.473), age × time (*P* = 0.002; *P* = 0.162; *P* = 0.005; *P* = 0.060), age × muscle (*P* = 0.676; *P* = 0.491; *P* = 0.729; *P* = 0.742), time × muscle (*P* = 0.540; *P* = 0.926; *P* = 0.697; *P* = 0.035) and age × time × muscle (*P* = 0.900; *P* = 0.694; *P* = 0.237; *P* = 0.378) for LEAK, P_{CI}, P_{CI+II} and E_{CII}, respectively.

4.4.3. Muscle Fiber Type

4.4.3.1. Comparison Between Aged and Young Horses Before Training

Both age groups tended to have a greater percentage of type I fibers in the TB than the GM ($P = 0.085$; Fig 4.11B). Aged horses tended to have a lower percentage of type IIa fibers than young horses in the GM ($P = 0.096$; Fig 4.11C) but aged had a greater percentage of type IIa fibers in the TB than the GM ($P = 0.0006$). Aged horses had a lower percentage of type IIx fibers than young horses ($P = 0.034$; Fig 4.11D) but the percentage of type IIx fibers did not differ between muscle groups.

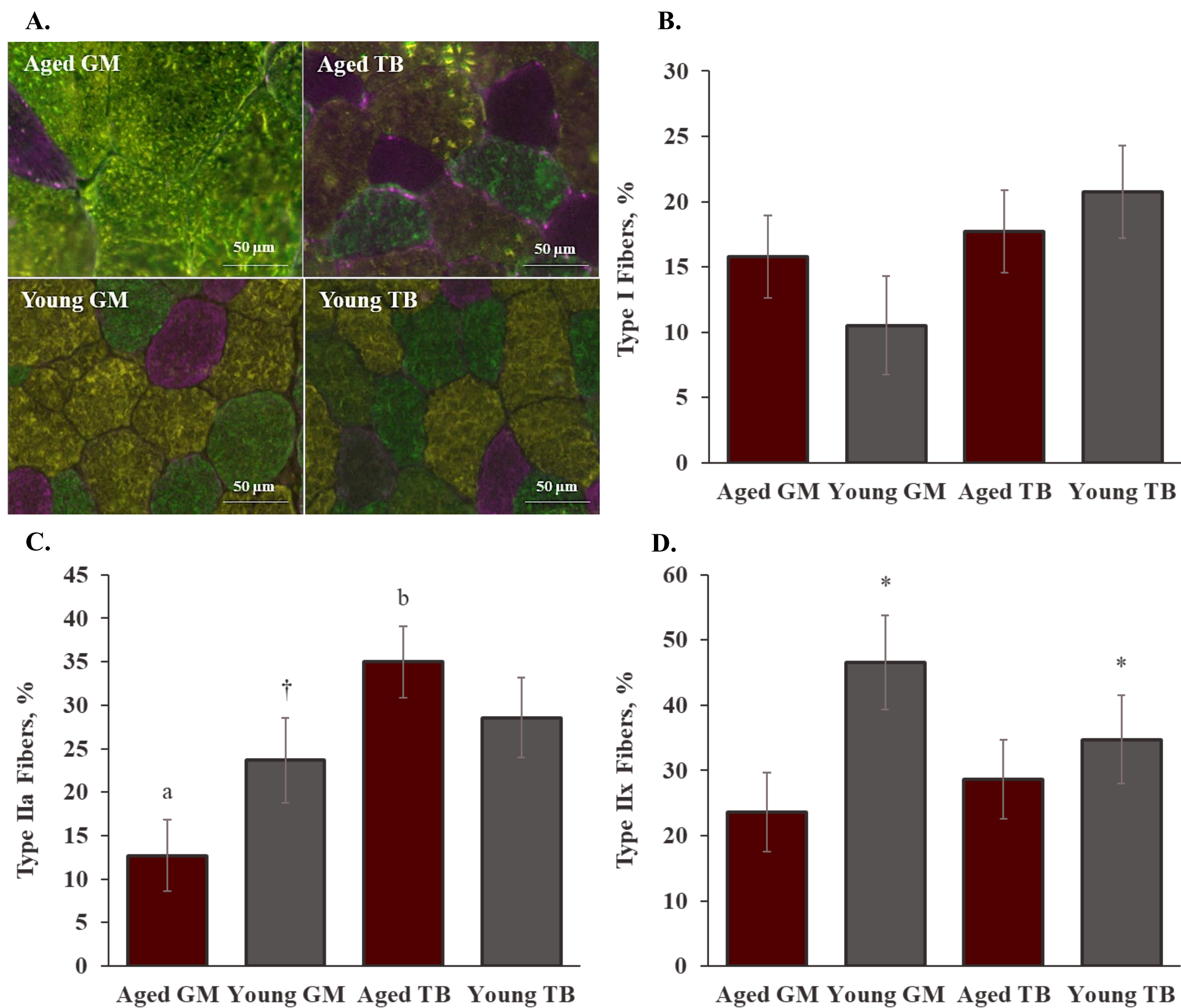

Figure 4.11. Representative images of muscle fiber type (A) with type I fibers (pink), type IIa fibers (green) and type IIx fibers (yellow), percentage of type I (B), type IIa (C) and type IIx (D) muscle fibers in the gluteus medius (GM) and triceps brachii (TB) of aged (n = 10) and young (n = 8) Quarter Horses before exercise training. Overall effect of age (*P* = 0.747; *P* = 0.612; *P* =0.034), muscle (*P* = 0.085; *P* = 0.004; *P* = 0.611) and age × muscle (*P* = 0.236; *P* = 0.236; *P* = 208) for type I, type IIa and type IIx fibers, respectively. [a,b] Within an age group GM differs from TB ($P \leq 0.05$). [*] Within time point, aged differs from young ($P \leq 0.05$), [†] Within time point, aged differs from young ($P \leq 0.10$).

Aged horses tended to have a higher percentage of type I/IIa hybrid fibers than young horses (*P* = 0.074; Fig. 4.12A) but the percentage of type I/IIa fibers did not differ between muscle groups. Aged horses had a greater percentage of type IIa/x hybrid fibers in the GM than the TB (*P* = 0.001; Fig. 4.12B) and had a greater percentage of type IIa/x fibers than young in the

GM (P = 0.014). The percentage of type IIa/x fibers did not differ between muscle groups for young horses. Aged horses had a greater percentage of oxidative fibers than young horses (P = 0.040; Fig. 4.12C), but the percentage of oxidative fibers did not differ between muscle groups.

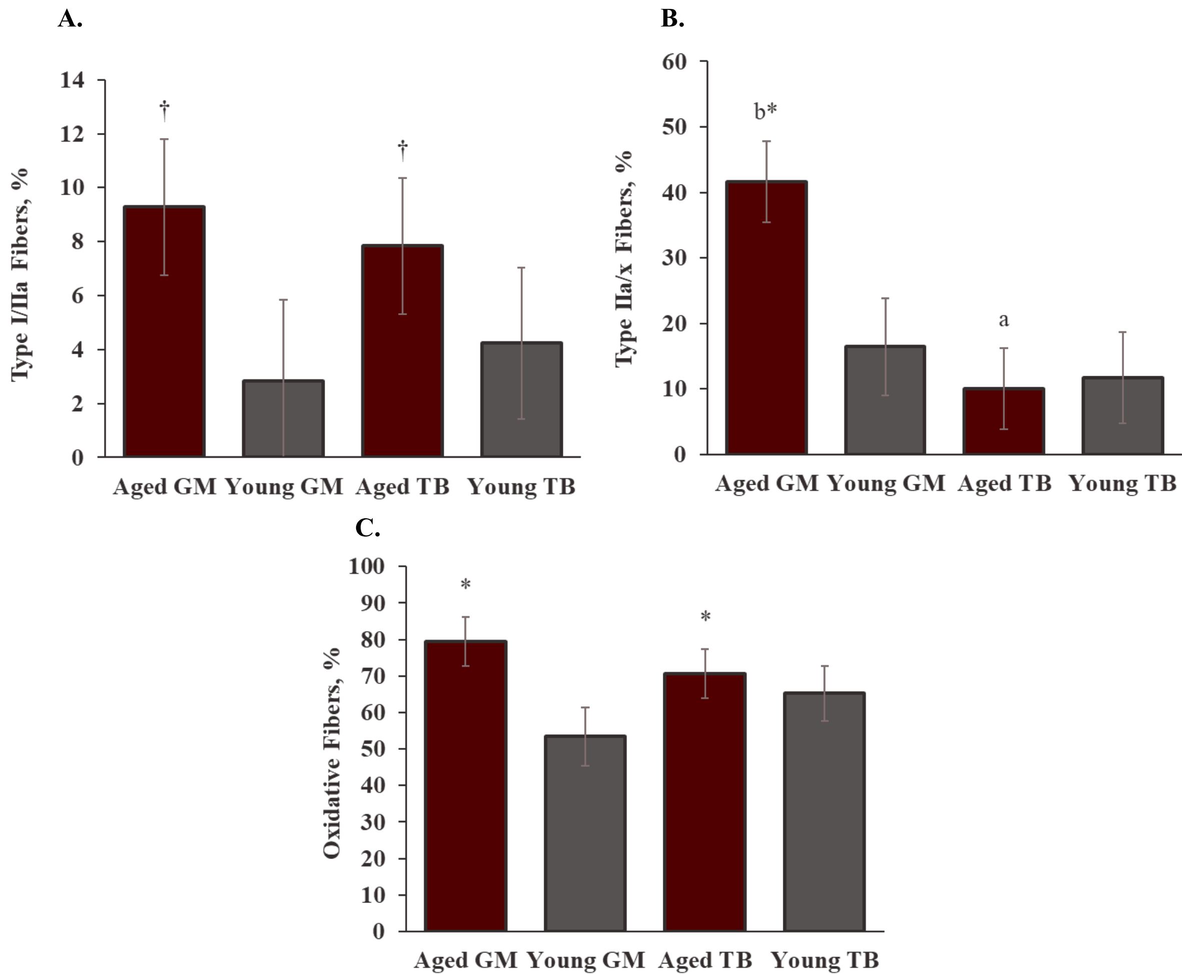

Figure 4.12.Percentage of type I/IIa (A), type IIa/x (B) and oxidative (C) muscle fibers in the gluteus medius (GM) and triceps brachii (TB) of aged (n = 10) and young (n = 8) Quarter Horses before exercise training. Overall effect of age (P = 0.074; P = 0.089; P =0.040), muscle (P = 0.995; P = 0.011; P = 0.838) and age × muscle (P = 0.604; P = 0.053; P = 0.166) for type I, type IIa and type IIx fibers, respectively. ^{a,b} Within an age group GM differs from TB ($P \leq 0.05$). * Within time point, aged differs from young ($P \leq 0.05$), † Within time point, aged differs from young ($P \leq 0.10$).

Type I fiber minimum feret was not different between age or muscle groups (Fig. 4.13A). Aged horses had a larger type IIa fiber minimum feret diameter than young horses ($P = 0.007$; Fig. 4.13B) and the minimum feret for type IIa fibers was larger in the TB than the GM for both age groups ($P = 0.017$). The minimum feret for type IIx fibers tended to be larger in the TB than the GM ($P = 0.065$; Fig. 4.13C) for both age groups.

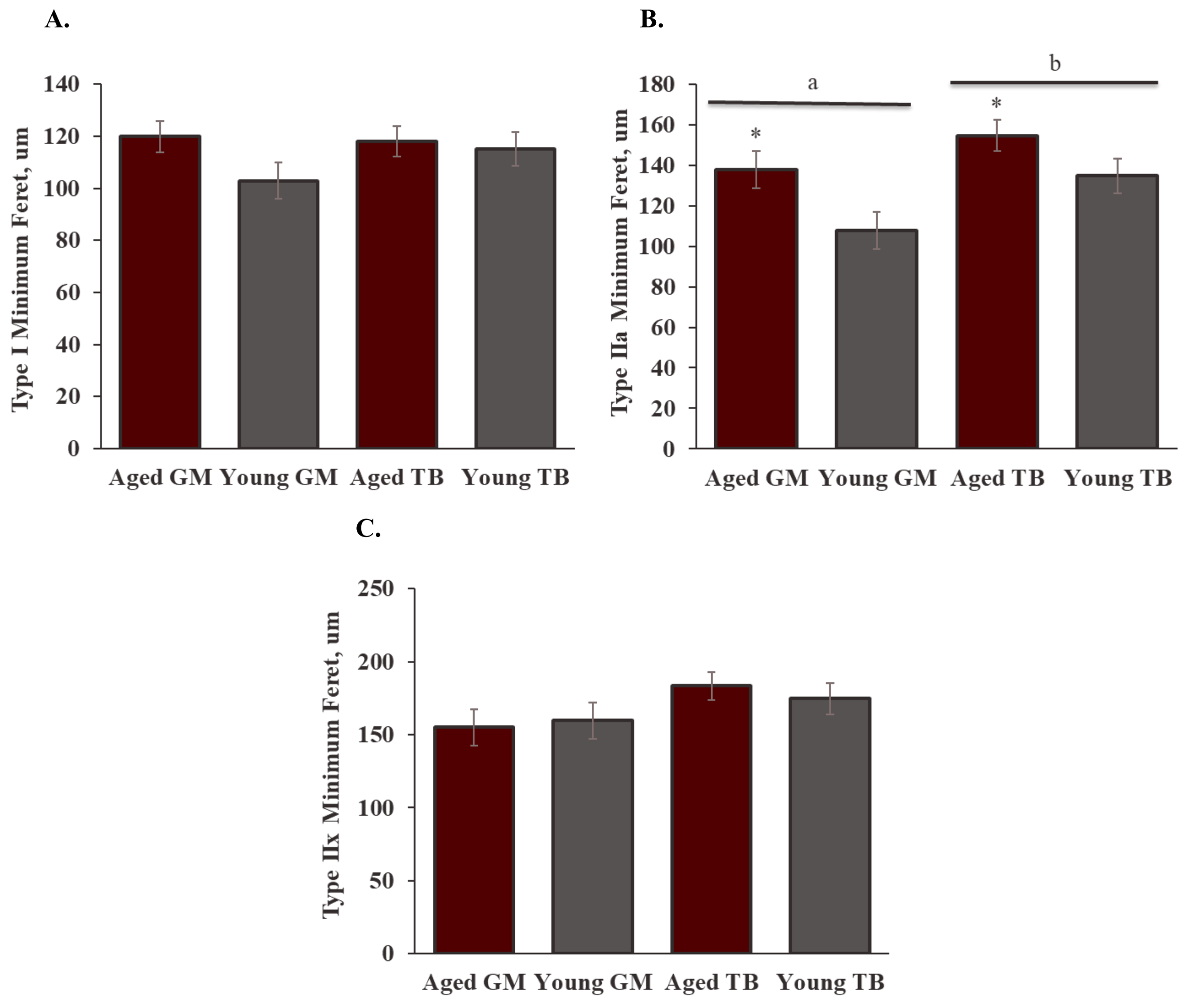

Figure 4.13. Minimum feret diameter of type I (A), type IIa (B) and type IIx (C) muscle fibers in the gluteus medius (GM) and triceps brachii (TB) of aged (n = 10) and young (n = 8) Quarter Horses before exercise training. Overall effect of age (P = 0.127; P = 0.007; P =0.857), muscle (P = 0.411; P = 0.017; P = 0.065) and age × muscle (P = 0.272; P = 0.570; P = 0.561) for type I, type IIa and type IIx fibers, respectively. [a,b] Within an age group GM differs from TB ($P \leq 0.05$). * Within time point, aged differs from young ($P \leq 0.05$).

The minimum feret for type I/IIa and type IIa/x fibers was larger in aged than young horses ($P \leq 0.022$; Fig. 4.14 A and B).

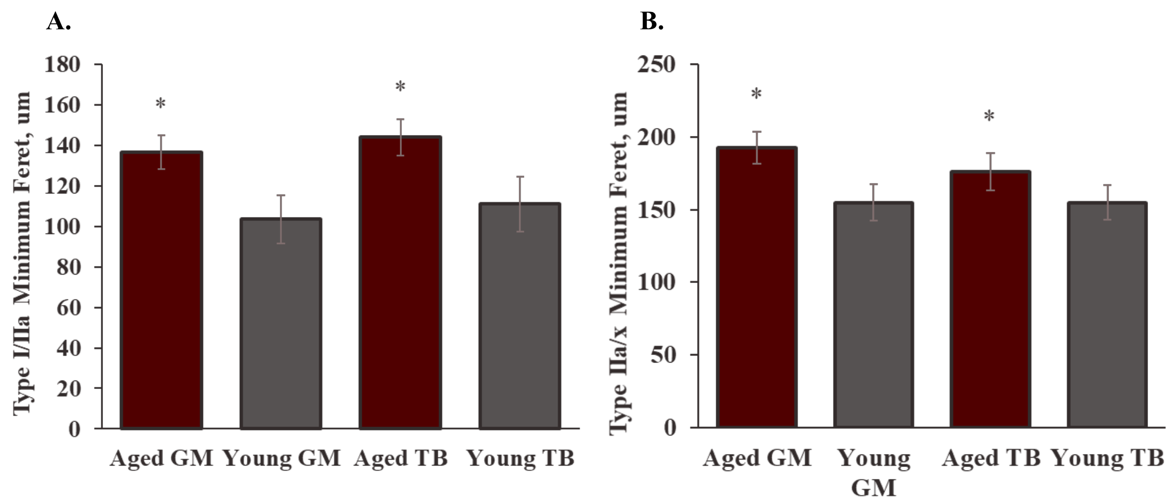

Figure 4.14. Minimum feret diameter of type I/IIa (A) and type IIa/x (B) muscle fibers in the gluteus medius (GM) and triceps brachii (TB) of aged (n = 10) and young (n = 8) Quarter Horses before exercise training. Overall effect of age ($P = 0.007$; $P = 0.022$), muscle ($P = 0.493$; $P = 0.501$) and age × muscle ($P = 0.995$; $P = 0.502$) for type I/IIa and type IIa/x fibers, respectively. * Within muscle group, aged differs from young ($P \leq 0.05$).

4.4.3.2. Changes in Aged Horses with Training

In aged horses the percentage of type I and type IIa fibers was greater in the TB than the GM throughout training ($P \leq 0.002$; Fig 4.15B and C) but did not change with training in either muscle group. The percentage of type IIx fibers was not different between muscle groups at wk 0, but the percentage of type IIx fibers in the GM increased by wk 4 ($P < 0.0001$; Fig. 4.15D) and the percentage of type IIx fibers was higher in the GM than the TB from wk 4 to 12 ($P < 0.003$).

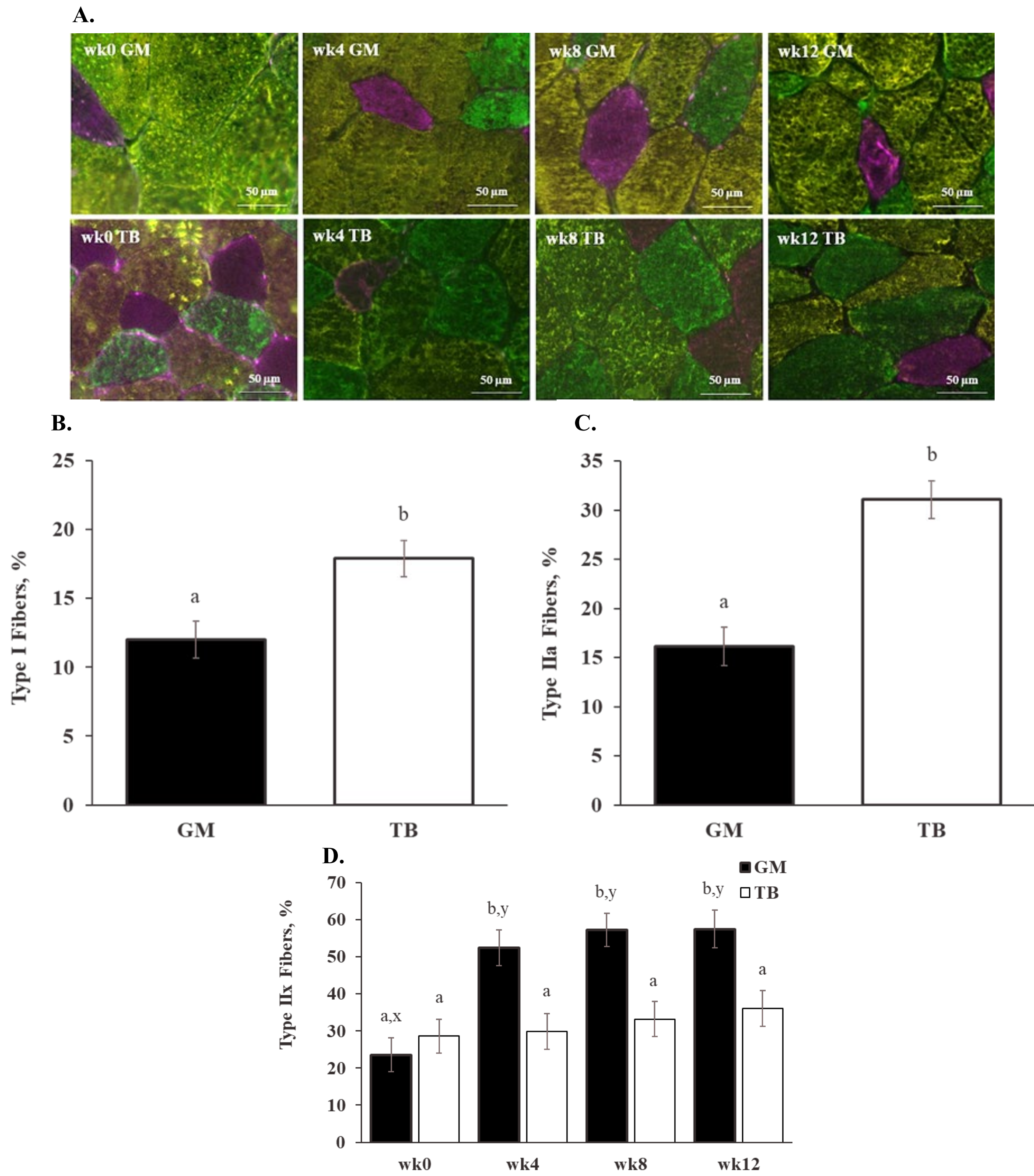

Figure 4.15. Representative images of muscle fiber type (A) with type I fibers (pink), type IIa fibers (green) and type IIx fibers (yellow), and percentage of type I (B) and type IIa (C) and type IIx (D) muscle fibers in the gluteus medius (GM) and triceps brachii (TB) of aged Quarter Horses (n = 10) before exercise training (wk0) and after 4 (wk4), 8 (wk8) and 12 (wk12) wk exercise training, and representative images of muscle fiber type (D). Due to lack of an effect of time, time points have been combined for type I and type IIa fibers. Overall effect of time ($P = 0.626$; $P = 0.687$; $P < 0.0001$), muscle ($P = 0.002$; $P < 0.0001$; $P < 0.0001$) and time × muscle ($P = 0.137$; $P = 0.474$; $P = 0.005$) for percentage of type I, type IIa, and type IIx fibers, respectively. [a,b] Within time point GM differs from TB ($P \leq 0.05$). [x,y] Within a muscle group, time points lacking common letters differ ($P \leq 0.05$).

The percentage of type I/IIa fibers decreased by wk 4 (P <0.0001; Fig. 4.16A) and remained lower at wk 8 and 12 than 0 (P <0.0001) but was not different between muscle groups throughout training. The percentage of type IIa/x fibers in the GM decreased by wk 4 ($P = 0.002$; Fig. 4.16B) and remained lower at wk 8 and 12 than 0 ($P < 0.0004$), while the percentage of type IIa/x fibers in the TB did not change over time. Together, this led to a higher percentage of type IIa/x fibers in the GM than the TB at wk 0 ($P < 0.0001$) but no difference between muscle groups at wk 4, 8 or 12. The percentage of oxidative fibers in the GM decreased by wk 4 ($P = 0.003$; Fig. 4.16C) and remained lower at wk 8 and 12 than 0 ($P < 0.0002$). There was no difference in the percentage of oxidative fibers between muscle groups at wk 0, but the percentage of oxidative fibers was higher in the TB than the GM at wk 4, 8 and 12 ($P \leq 0.041$).

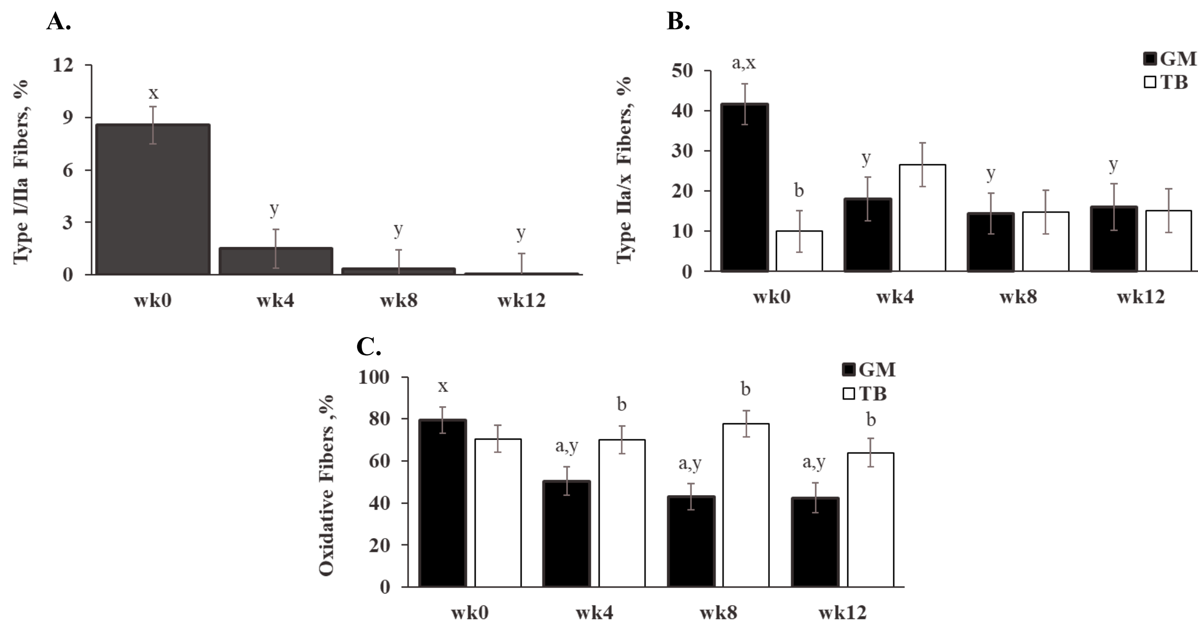

Figure 4.16. Percentage of type I/IIa (A) and type IIa/x (B) and oxidative (C) muscle fibers in the gluteus medius (GM) and triceps brachii (TB) of aged Quarter Horses (n = 10) before exercise training (wk0) and after 4 (wk4), 8 (wk8) and 12 (wk12) wk exercise training. Due to lack of an effect of muscle group, muscles have been combined for type I/IIa fibers. Overall effect of time ($P < 0.0001$; $P = 0.110$; $P = 0.010$), muscle ($P = 0.337$; $P = 0.123$; $P = 0.0006$) and time × muscle ($P = 0.896$; $P = 0.001$; $P = 0.010$) for percentage of type I, type IIa, and type IIx fibers, respectively. [a,b] Within time point GM differs from TB ($P \leq 0.05$). [x,y] Within a muscle group, time points lacking common letters differ ($P \leq 0.05$).

Minimum feret diameter of type I fibers was not different between muscle groups throughout training and was not affected by time (Fig. 4.17A). The minimum feret diameter of type IIa fibers was greater in the TB than the GM ($P = 0.007$; Fig 4.17B) but was not affected by time or the interaction of time and muscle group. Type IIx minimum feret diameter increased at wk 4 ($P = 0.010$; Fig. 4.17C), but was not different from wk 0 at wk 8 or 12. Minimum feret diameter of type IIx was not different between muscle groups.

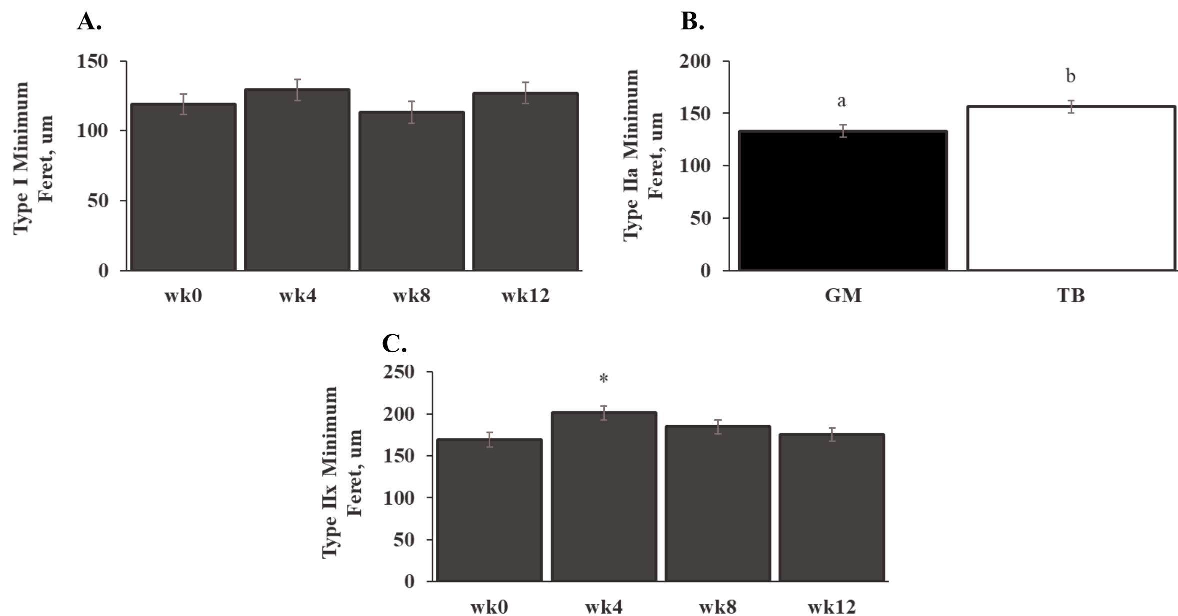

Figure 4.17. Minimum feret diameter of type I (A) and type IIa (B) and type IIx (C) muscle fibers in the gluteus medius (GM) and triceps brachii (TB) of aged Quarter Horses (n = 10) before exercise training (wk0) and after 4 (wk4), 8 (wk8) and 12 (wk12) wk exercise training. Due to lack of an effect of muscle group in type I and type IIx muscle fibers, muscles have been combined. Due to lack of an effect of time, time points have been combined for type IIa fibers. Overall effect of time ($P = 0.431$; $P = 0.328$; $P = 0.053$), muscle ($P = 0.890$; $P = 0.007$; $P = 0.782$) and time × muscle ($P = 0.986$; $P = 0.896$; $P = 0.162$) for percentage of type I, type IIa, and type IIx fibers, respectively. ^{a,b} Within time point GM differs from TB ($P \leq 0.05$). ^{x,y} Within a muscle group, time points lacking common letters differ ($P \leq 0.05$).

Type I/IIa minimum feret diameter was not different between muscle groups and did not change over time (Fig. 4.18A). However, due to the low percentage of type I/IIa fibers in samples before exercise training, and the decrease in the percentage of type I/IIa fibers with exercise training, type I/IIa minimum feret diameter at wk 12 was not able to be estimated. The minimum feret diameter of type IIa/x fibers tended to decrease at from wk 8 to 12 ($P = 0.056$; Fig. 4.18B) and was lower at wk 12 than 0 ($P = 0.005$).

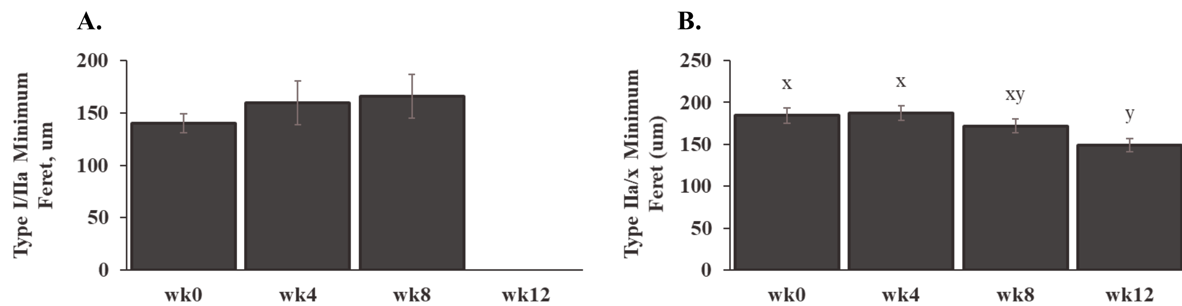

Figure 4.18. Minimum feret diameter of type I/IIa (A) and type IIa/x (B) muscle fibers in the gluteus medius (GM) and triceps brachii (TB) of aged Quarter Horses (n = 10) before (wk0) and after 4 (wk4), 8 (wk8) and 12 (wk12) wk exercise training. Due to lack of an effect of muscle group, muscles have been combined. Overall effect of time ($P = 0.628$; $P = 0.010$), muscle ($P = 0.108$; $P = 0.245$) and time × muscle ($P = 0.159$; $P = 0.253$) for type I/IIa and type IIa/x fibers, respectively. [a,b] Within time point GM differs from TB ($P \leq 0.05$). [x,y] Within a muscle group, time points lacking common letters differ ($P \leq 0.05$).

4.5. Discussion

Overall, aged horses in the present study exhibited higher mitochondrial density in the TB, but lower integrated and intrinsic CCO activity, lower integrated LEAK, P_{CI}, and E_{CI+II}, and higher intrinsic P_{CI}, P_{CI+II}, E_{CI+II} and E_{CII} than young horses. Mitochondrial density increased in the TB for aged horses with exercise. Integrated mitochondrial function and capacity and intrinsic P_{CI} increased, while LEAK respiration decreased for both age groups with 12 wk exercise training.

Aged horses in the present study exhibited higher CS activity in the TB than young horses. These results are contradictory to previous research that has shown lower CS activity in the TB of aged horses compared to young horses (Li et al., 2016). Citrate synthase activity has been shown to increase during the first year of life in horses (Kline and Bechtel, 1990). In the present study, young horses had a mean age of 9.7 mo, whereas in the aforementioned study, the young horses were older (1.8 ± 0.1 yr). Therefore, it is possible that mitochondrial density was still increasing in the young horses during the present study, masking differences that would

typically be seen in comparison to more mature horses. Despite having higher mitochondrial density in the TB, aged horses had lower CCO activity, a marker of mitochondrial function on an integrated and intrinsic basis. Decreased CCO activity with age has been well documented in humans (Müller-Höcker, 1990; Rooyackers et al., 1996) and more recently in horses (Li et al., 2016).

The differences in mitochondrial density between age groups observed in this study are mirrored by differences in muscle fiber type between age groups. Aged horses exhibited similar CS activity in the GM to young horses, which was accompanied by a lower percentage of type IIa fibers, but a larger type IIa minimum feret diameter and a higher total percentage of oxidative fibers. Citrate synthase activity in the TB was higher for aged than young horses and was reflected by a greater total percentage of oxidative fibers and a greater percentage and minimum feret diameter of type IIa fibers in the TB. Aged horses exhibited a decreased percentage of type IIx fibers compared to young horses, which is similar to previous research showing a lower percentage of MyHC IIx in aged compared to young horses (Li et al., 2016) and humans (Verdijk et al., 2007). The reduction in percentage and size of type II fibers in aged individuals has been associated with decreased satellite cell abundance around type II fibers, and therefore a reduced ability to regenerate damaged fibers (Verdijk et al., 2007). Because type IIx fibers typically have the largest cross-sectional area and the fastest twitch speed, loss of these fibers contributes greatly to loss of muscle mass and contractile function associated with sarcopenia during aging.

In addition to having a lower percentage of type IIx fibers, aged horses in the present study tended to have a greater percentage of type I/IIa fibers than young horses, and had a greater percentage of type IIa/x fibers than young horses in the GM. An increase in the percentage of hybrid fibers in aged subjects has been demonstrated in humans (Deschenes, 2004). An increase

in the presence of hybrid fibers has been noted in both aging and disuse atrophy (Canepari et al., 2010), and may represent damaged muscle fibers (Adams, 2006).

In the present study the size of type IIa fibers was larger in aged horses, and the size of type IIx fibers tended to be larger in aged compared to young horses. This is in contrast to literature in humans that shows a reduction in type II fiber size with aging (Deschenes, 2004). Greater fiber size in aged horses in the present study likely reflects the fact that young horses were still growing and had not reached their adult muscle fiber size. Therefore, comparisons of fiber size between aged and young horses in the present study may not fully reflect the change that may be observed from mature to aged groups.

Overall, intrinsic P_{CI}, P_{CI+II} and E_{CI+II} were higher in aged than young horses. These results are similar to previous research in aged horses indicating elevated intrinsic P_{CI} and E_{CI+II} in aged horses (Li et al., 2016). However, in the previous study, integrated mitochondrial capacity was not different between age groups, whereas in the present study integrated P_{CI} and E_{CI+II} were lower for aged than young horses. In the previous study, the authors noted that they may not have observed decrements in integrated or intrinsic oxidative capacity because the horses may not have reached the age at which mitochondrial capacity becomes impaired, but instead horses may have been in more of a transitionary state (Li et al., 2016). The differences in integrated P_{CI} and E_{CI+II} may have arisen because the aged horses in the present study were older (18 to 30 yr) than the horses in the previous study (17 to 25 yr) and may have had more advanced decrements in mitochondrial capacity due to more advanced age.

While integrated P_{CI} and E_{CI+II} were lower for aged than young horses before exercise training, they increased with training in aged horses, bridging the gap in capacity between age groups by wk 12. In this respect, exercise appears to have aided in correcting the deficiency in

capacity for aged horses for these complexes. However, aged horses showed a lag in improvements in mitochondrial function for other complexes, and failed to recover fully in response to exercise training in other complexes. Integrated CCO activity increased by wk 8 in both age groups, in agreement with previous studies showing improvements in CCO activity with exercise training in humans (Lanza and Nair, 2008), but integrated and intrinsic CCO activity remained lower for aged than young horses overall. Aged horses also exhibited a lag in the decrease of intrinsic LEAK respiration with exercise training compared to young horses. LEAK respiration is a dissipative component for respiration associated with proton slip and leak and electron leak. LEAK respiration is therefore not available for performing biochemical work. A decrease in LEAK respiration has been demonstrated with exercise training in humans (Fernström et al., 2004), and the lag in decrease in LEAK respiration in the present study indicates latency to improve mitochondrial efficiency with exercise training in aged horses. Similarly, the FCR for P_{CI+II}, which is an indicator of efficiency of ATP production, was comparable between age groups at wk 0, but was lower in aged than young horses at wk 8 and tended to remain lower at wk 12. These differences between age groups arose because of an increase in the FCR for P_{CI+II} at wk 8 in young horses, but a decrease in aged horses at wk 8 and an increase at wk 12. The latency in ability to improve efficiency of ATP production with training in aged horses suggests that aged horses may take longer to adapt metabolically to exercise training to meet energy demands.

Furthermore, aged horses showed differing changes on the reliance of complex I and II over time in response to exercise training. In the present study, intrinsic P_{CI} increased from wk 0 to 8 but tended to decrease from wk 8 to 12. The decrease in intrinsic P_{CI} at wk 12 may result from a shift to reliance on complex II during adaptation to training. While, the FCR for P_{CI} did

not change over time in either age group, a trend for age × time interaction showed that the FCR for P_{CI+II} increased at wk 8 and remained higher at wk 12 in young horses and, the FCR for P_{CI+II} decreased at wk 8 in aged horses, and increased at wk 12. Complex II links the tricarboxylic acid cycle to the ETS, and therefore influences rate of substrate entry into the ETS. Increasing capacity for substrate entry into the ETS contributes to increasing reserve respiratory capacity, which is important in cases where energy demands exceed supply (Pfleger et al., 2015). Therefore, an increase in complex II capacity and preference with exercise results in the ability to avoid 'ATP crisis' during exercise bouts. The lag in increase of variables associated with improved complex II capacity in aged horses in the present study suggests that aged horses may exhibit a reduced ability to increase substrate entry into the ETS with exercise training, and therefore may not adapt to the energy demands of exercise as readily as younger horses.

Although aged horses exhibited a lower percentage of type IIx fibers and a higher percentage of type I/IIa and IIa/x fibers when compared to young horses before training, the percentage of type IIx fibers in the GM increased, and the percentage of type IIa/x fibers in the GM decreased by wk 4 of exercise training in aged horses. These results are in agreement with human literature indicating that training results in a decrease in the percentage of hybrid fibers (Williamson et al., 2001). Research in humans (Williamson et al., 2001) and horses (Essèn-Gustavsson and Lindholm, 1985) typically demonstrates an increased percentage of type IIa fibers and a decrease in the percentage of type IIx fibers with training, regardless of intensity. It is possible that the percentage of type IIx fibers increased in aged horses with training in the present study because they started out with a decrement in type IIx fibers compared to young horses. All horses used in the present study were Quarter Horses, which have been shown to have a higher percentage of type IIx fibers compared to other breeds, such as Standardbreds

(Bechtel and Kline, 1987). Therefore, the increase in the percentage of type IIx fibers with training in aged horses in the present study may not precisely represent the changes with training in aged horses of all breeds.

The present study demonstrates deficits in mitochondrial function and oxidative capacity and altered fiber type in aged compared to young horses that are similar to the deficits and differences observed in aged humans. Exercise training in both age groups resulted in improvements in many indicators of mitochondrial function and density and resulted in a decrease in percentage of hybrid fibers in aged horses, as has been demonstrated in humans. Taken together, these results suggest that exercise could be an extremely useful tool for improving the health and welfare of aged horses. Additionally, the phenotypic similarities of aged horses and aged humans show that aged horses can be a useful model for aging in human skeletal muscle.

5. THYROID STIMULATING HORMONE INCREASES MITOCHONDRIAL OXIDATIVE CAPACITY IN AN *IN VITRO* STUDY OF EQUINE SKELETAL MUSCLE

5.1. Synopsis

Thyroid-stimulating hormone (TSH) has been shown to increase mitochondrial number and function in human epidermis (Poeggeler et al., 2010). Extrathyroidal action of TSH has not been examined in horses. Therefore, we aimed to test the hypothesis that TSH would increase mitochondrial density and activity in muscle from mature (n = 3; 11 ± 4.6 yr) and aged (n = 4; 19 ± 3.6 yr) Quarter horses (4 mares, 3 geldings). Triceps brachii muscle samples (600 mg) were placed into chilled PBS for immediate transport to the laboratory. Tissue from each horse was separated into 8 wells (75 mg/well) containing 2 mL incubation media (DMEM, 5% fetal bovine serum, 10% horse serum, 50 ug/mL gentamycin) with 2 replicates of 4 doses of bovine pituitary TSH (Millipore Sigma): 0 (CON), 2.5 (T2.5), 10 (T10) or 20 (T20) mIU TSH/mL. Alamar Blue (10%) was added to one replicate for each TSH concentration to assess metabolic rate; the second replicate was used for analysis of oxidative capacity (P_{CI+II}) via high-resolution respirometry and mitochondrial number (citrate synthase activity; CS) and function (cytochrome *c* oxidase; CCO) by colorimetry. Samples were incubated for 18 h at 37ºC, 5% CO_2. For each horse, values were normalized to CON and data were analyzed using PROC MIXED (SAS v9.4) with TSH dose, age, sex and all interactions as fixed effects. Citrate synthase activity response to TSH was lower for aged than mature ($P = 0.004$) and lower for mares than geldings ($P = 0.0008$). Geldings has higher CS activity in response to T2.5, T10, and T20 compared to CON ($P \leq 0.10$). Integrated (per mg protein) P_{CI+II} was higher for T10 than CON, T2.5 and T20 ($P = 0.012$). Thus, TSH *in vitro* elicited increases in CS activity and oxidative capacity in muscle samples, however, responses differed between age and sex groups.

5.2. Introduction

Research has shown that certain populations of individuals over 100 years old (centenarians) have significantly higher circulating thyroid stimulating hormone (TSH) concentrations than younger controls in the same population, or age-matched control populations of thyroid disease-free individuals. The role of increased TSH in extreme longevity has not yet been determined, but it has been hypothesized that TSH plays a signaling role outside of the thyroid (Jansen et al., 2015).

In the skin, TSH stimulates mitochondrial biogenesis (Poeggeler et al., 2010) and function (Vidali et al., 2014). Additionally, TSH improved insulin sensitivity in mouse myotubes *in vitro* (Moon et al., 2016). These studies suggest that TSH could play an integral role in regulating peripheral organ metabolism of extremely aged individuals. A possible mechanism by which TSH increases the lifespan could be stimulation of mitochondrial biogenesis in peripheral tissues, leading to an improvement in metabolic efficiency. However, the role of elevated circulating TSH concentration in lifespan extension remains elusive.

The objective of this study was to determine whether TSH alters mitochondria in equine skeletal muscle *in vitro*. It was hypothesized that TSH would increase mitochondrial density and capacity in mature and aged horse skeletal muscle.

5.3. Methods

5.3.1. Sample Collection

Mature (n = 3; 11 ± 5 yr) and aged (n = 4; 19 ± 4 yr) Quarter horses (4 mares, 3 geldings) that had received no forced exercise for at least 6 mo prior to sample collection were used. Muscle tissue samples were collected from the triceps brachii (TB) using a tissue collection procedure as previously described (White et al., 2016). Briefly, horses were sedated with

detomidine hydrochloride (Dormosedan; Zoetis, Parsippany-Troy Hills, NJ) prior to beginning tissue collection procedures. The collection areas were clipped, scrubbed with a 7.5% povidone-iodine solution, and then scrubbed with a 70% ethanol solution. The tissue collection sites were desensitized with 0.5 mL of 2% lidocaine (Vetone, Boise, ID) and a 14-gauge needle was used to create the initial puncture through the skin. Tissue was collected using a 14-gauge, 9-cm biopsy needle (SuperCore; Argon Medical Devices Inc., Frisco, TX) inserted to a depth of 5 cm. Approximately 600 mg (wet weight) of muscle tissue was collected into ice-cold, sterilized PBS (Fisher Scientific) for immediate transport to the laboratory.

5.3.2. Incubation in Thyroid Stimulating Hormone

Each sample was rinsed with sterile PBS and separated into eight wells of a 24-well tissue culture plate (Corning Life Sciences, Tewksbury, MA) containing 2 mL incubation medium (IM; Dulbecco's Modified Eagle Medium, 5% fetal bovine serum, 10% horse serum, 50 ug/mL gentamycin; all from Thermo Fisher Scientific) with 2 replicates of 4 concentrations of bovine pituitary TSH (Millipore Sigma): 0 (CON), 2.5 (T2.5), 10 (T10) or 20 (T20) mIU TSH/mL. Alamar blue was added to one replicate of each TSH concentration and one blank well containing no sample (Thermo Fisher Scientific) for assessment of metabolic rate. The second replicate of each TSH concentration was used to assess mitochondrial density, function and oxidative capacity. Samples were incubated for 18 h at 37º C, 5% CO_2. Following incubation, approximately 50 mg (wet weight) of muscle tissue was flash frozen in liquid nitrogen and stored at -80° C until enzymatic activity analyses were performed. For high-resolution respirometry, muscle fibers were collected into ice cold mitochondrial preservation solution (BIOPS) and analyzed within 24 h following the end of TSH incubation. Flash frozen muscle was cryopulverized into a fine powder (Spectrum™ Bessman Tissue Pulverizer; Thermo Fisher Scientific, Waltham, MA) as described previously for evaluation of CS and CCO activities.

5.3.3. Metabolic Rate and Enzyme Activities

For measurement of metabolic rate, sample media in wells containing Alamar blue was collected and analyzed as previously described (Renquist et al., 2013). Citrate synthase (CS) and cytochrome *c* oxidase (CCO) activities were measured as markers of mitochondrial density and function, respectively (Larsen et al., 2012). For measurement of CS and CCO activities, frozen skeletal muscle samples were prepared, and activities were measured as previously described (Spinazzi et al., 2012; Li et al., 2016). Enzymatic activities were normalized to protein content, determined using the Bradford Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA). Cytochrome *c* oxidase activity is presented on an integrated (per mg protein) and intrinsic (per unit CS) basis.

5.3.4. High Resolution Respirometry

For high-resolution respirometry (HRR), muscle fibers were prepared and permeabilized as previously described (Li et al., 2016) and then analyzed within 24 h of collection. Oxygen flux and respiratory states were determined by HRR with the following substrate-uncoupler-inhibitor titration protocol modified from a previously described protocol for equine skeletal muscle (Li et al., 2016): (1) pyruvate (5 mM), malate (2 mM) and adenosine diphosphate (ADP; 2.5 mM) to support electron flow through complex I of the electron transport system; 2) cytochrome *c* (cyt c; 10 μM) to assess outer mitochondrial membrane integrity (samples with responses to cyt c greater than 15% were re-analyzed); 3) glutamate (10 mM) as an additional CI substrate and succinate (10 mM) to support convergent electron flow through complex II of the ETS (P_{CI+II}); 7) antimycin A (2.5 μM), an inhibitor of complex III, to measure residual oxygen flux (ROX) independent of the ETS.

5.3.5. Statistical Analysis

Data for metabolic rate, enzyme activities and mitochondrial respiration measurements for T2.5, T10, and T20 for each sample were normalized to CON using the following equation:

$$\frac{Value\ of\ Variable\ for\ TSH\ Concentration\ of\ Interest}{Value\ of\ Variable\ for\ CON}$$

Data were analyzed using the MIXED procedure of SAS (Version 9.4, SAS Institute Inc., Cary, NC). Data were log-transformed prior to analysis if not normally distributed. TSH dose, age, sex, and all interactions were included in the model as fixed effects. All data are expressed as mean ± SEM. Significance was considered at $P \leq 0.05$, and trends were acknowledged at $P \leq 0.10$.

5.4. Results

Citrate synthase activity in response to TSH was lower for aged than mature horses ($P = 0.0018$; Fig. 5.1) and was lower for mares than geldings ($P = 0.0004$). A trend for an age × sex interaction ($P = 0.100$) suggested that aged geldings had lower CS activity than mature geldings in response to TSH ($P = 0.003$), but aged mares only tended to have lower CS activity than mature mares in response to TSH ($P = 0.107$). A trend for an effect of TSH dose × sex ($P = 0.059$) indicated that CS activity was higher for geldings at T2.5 ($P = 0.024$) and T10 ($P = 0.007$) compared to T0, and tended to be higher at T20 compared to T0 ($P = 0.099$). Activity of CS did not differ between TSH concentrations in mares.

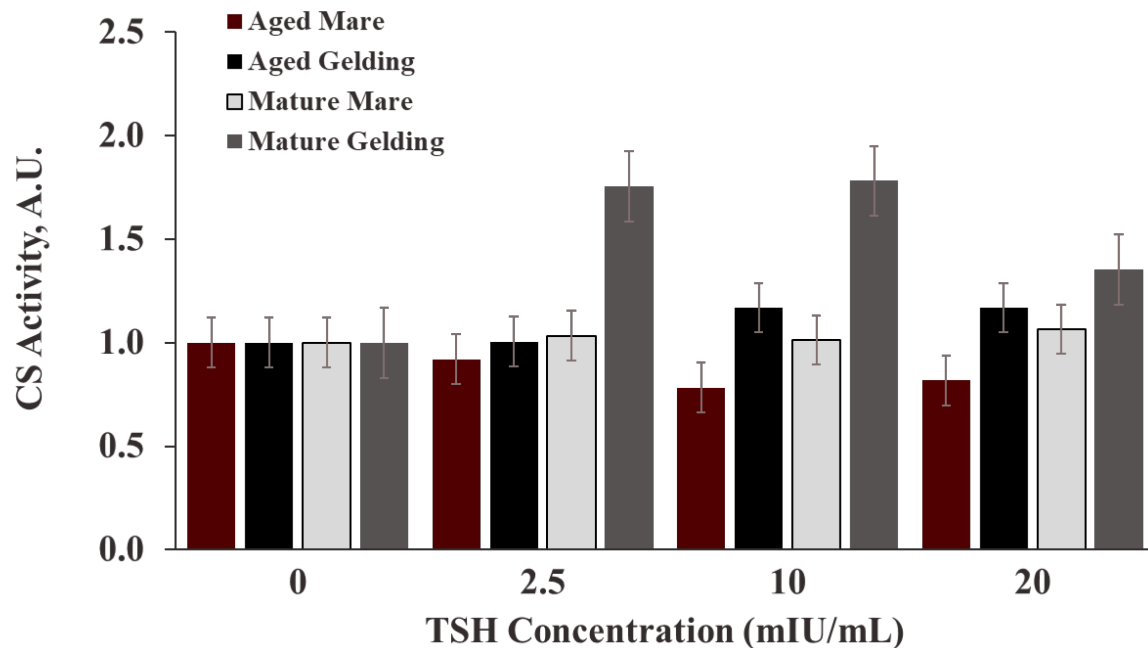

Figure 5.1. Citrate synthase (CS) activity in skeletal muscle samples collected from the triceps brachii of mature (n = 3) and aged (n = 4) Quarter Horses incubated in 0, 2.5, 10 and 20 mIU/mL TSH for 18 h. Variable responses to elevated concentrations of TSH are presented relative to the control well for each sample (0 mIU/mL). Overall effect of TSH (*P* = 0.224), age (*P* = 0.002), sex (*P* = 0.0004), TSH × age (*P* = 0.126), TSH × sex (*P* = 0.059), age × sex (*P* = 0.100) and TSH × age × sex (*P* = 0.257).

Integrated CCO activity response to TSH was highest in mature geldings compared to all other groups ($P \leq 0.039$; Fig 2.5A), but was not different between TSH concentrations ($P >$ 0.05). Intrinsic CCO activity (Fig. 5.2B) and metabolic rate (Fig. 5.2C) were not affected by TSH concentration, age, or any interactions.

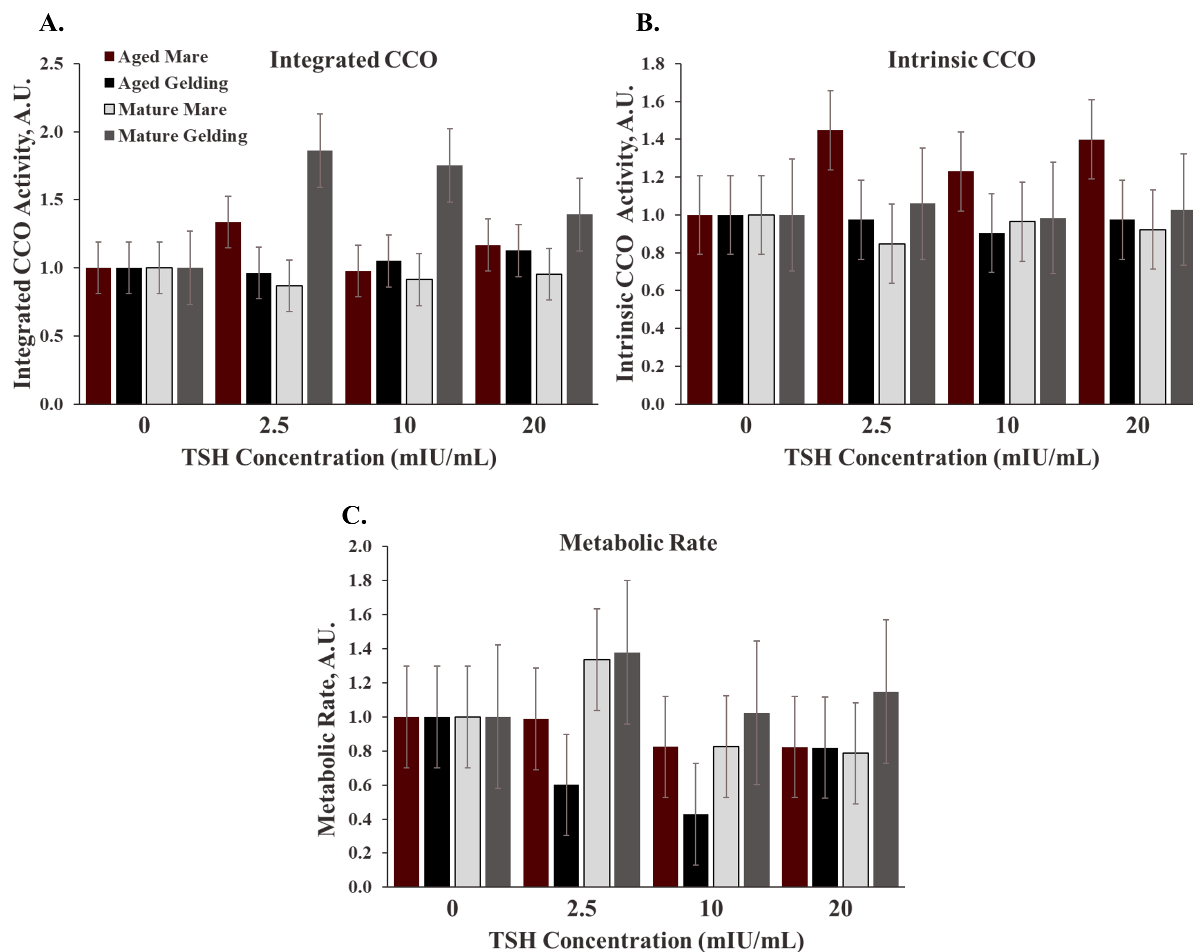

Figure 5.2.Integrated (A) and intrinsic (B) cytochrome *c* oxidase (CCO) activities and metabolic rate (C) in skeletal muscle samples collected from the triceps brachii of Quarter Horses (n = 7) incubated in 0, 2.5, 10 and 20 mIU/mL TSH for 18 h. Variable responses to elevated concentrations of TSH are presented relative to the control well for each sample (0 mIU/mL). Overall effect of TSH ($P = 0.416$; $P = 0.940$; $P = 0.619$), age ($P = 0.211$; $P = 0.251$; $P = 0.157$), sex ($P = 0.042$; $P = 0.361$; $P = 0.892$), TSH × age ($P = 0.675$; $P = 0.860$; $P = 0.677$), TSH × sex ($P = 0.506$; $P = 0.957$; $P = 0.888$), age × sex ($P = 0.010$; $P = 0.120$; $P = 0.319$) and TSH × age × sex ($P = 0.200$; $P = 0.755$; $P = 0.934$) for integrated CCO activity, intrinsic CCO activity and metabolic rate by Alamar Blue, respectively.

Integrated P_{CI+II} was higher for T10 than CON, T2.5 and T20 ($P \leq 0.007$; Fig. 5.3A). A trend for TSH × sex interaction suggested that P_{CI+II} was higher for T10 than CON, T2.5 and T20 ($P \leq 0.004$) for geldings, but not for mares. Intrinsic P_{CI+II} was not affected by TSH, age, sex, or any interactions (Fig. 5.3B).

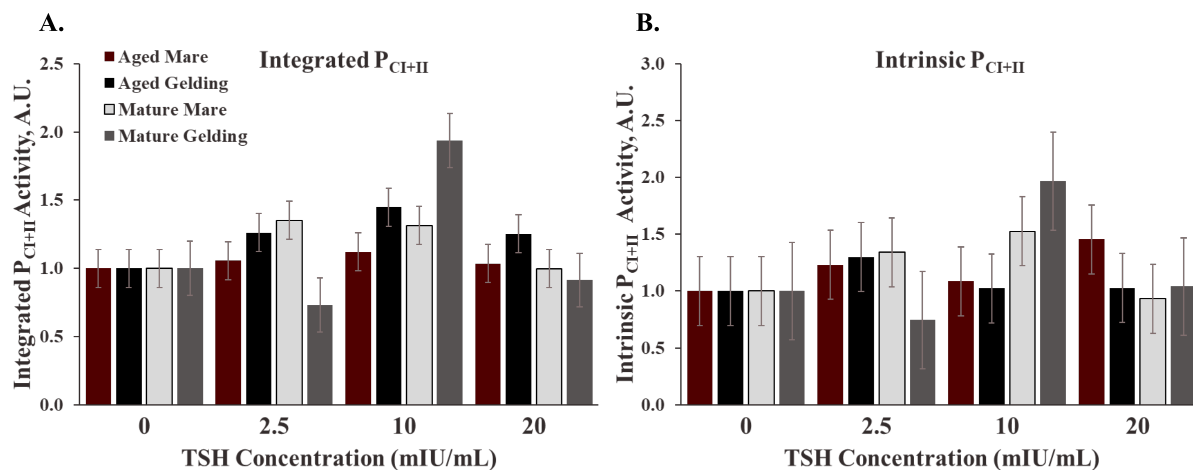

Figure 5.3. Integrated (A) and intrinsic (B) oxidative capacity with complex I and II substrates (P_{CI+II}) in skeletal muscle samples collected from the triceps brachii of Quarter Horses (n = 7) incubated in 0, 2.5, 10 and 20 mIU/mL TSH for 18 h. Variable responses to elevated concentrations of TSH are presented relative to the control well for each sample (0 mIU/mL). Overall effect of TSH ($P = 0.005$; $P = 0.431$), age ($P = 0.916$; $P = 0.754$), sex ($P = 0.304$; $P = 0.735$), TSH × age ($P = 0.133$; $P = 0.224$), TSH × sex ($P = 0.055$; $P = 0.798$), age × sex ($P = 0.206$; $P = 0.787$) and TSH × age × sex ($P = 0.125$; $P = 0.580$) for integrated and intrinsic P_{CI+II}, respectively.

5.5. Discussion

TSH concentration increased CS activity and P_{CI+II} in the present study, while CCO activity and metabolic rate remained unaffected. However, improvements in CS and CCO activities with TSH administration differed between age and sex groups.

Previous research in organ cultured full thickness human skin from women showed an increase in CS activity after 12 h incubation in 10 mIU/mL TSH (Poeggeler et al., 2010). In the present study, a trend for TSH × sex interaction indicated an increase in CS activity at 2.5 and 10 mIU/mL and a trend for an increase at 20 mIU/mL, but only in geldings. Additionally, response of CS activity to TSH was lower for mares than geldings overall. These data suggest an increase in mitochondrial density that is specific to males, while previous research indicated that TSH does in fact increase mitochondrial density in female skin. These differences could have arisen due to tissue or species differences in mitochondrial response to TSH. Conversely, the

differences in the present study could reflect a small sample size for each sex within age groups. The observed decrement in CS response to TSH in aged horses likely results from decreased muscle plasticity in aged horses, which has been documented extensively in this dissertation. Interestingly, while this research supports the idea that improving mitochondrial density and efficiency may be a mechanism by which elevated circulating TSH concentration contributes to extended lifespan in centenarians, the advantages of increased TSH concentration seem to benefit younger, mature horses more than aged horses. Previous research examining TSH concentration in families enriched for familial longevity suggests that elevated TSH may occur earlier in life (Jansen et al., 2015). Therefore, increased TSH concentration at a younger age may serve to increase mitochondrial density while muscle is more plastic, as was observed in the present study, rather than increase mitochondrial density at more advanced ages, when muscle is less responsive to adaptation.

While TSH increased CS activity in some groups of horses in the present study, no dose of TSH studied was sufficient to increase integrated CCO activity above that of CON. These results are contrary to a previous study in human skin that showed increased CCO activity in samples treated with 10 mIU/mL TSH. Again, it is likely that a low number of samples in each age group contributed to the observed results, as integrated CCO response to TSH did show differences between ages and sexes, with the response being highest in mature geldings.

A dose response of TSH concentration was observed for TSH on maximum oxidative capacity whereby 10 mIU/mL TSH resulted in an increase in oxidative capacity, while 2.5 and 20 mIU/mL TSH did not affect oxidative capacity. The lack of effect of TSH concentration on intrinsic oxidative capacity (per mitochondrial density) suggests that the observed increase in oxidative capacity was due to an increase in mitochondrial density. The optimal TSH concentration to elicit mitochondrial adaptations found in the current study is in contrast to

previous literature in human hair follicles, which showed an increase in *mitochondrially-encoded cytochrome c 1* expression in female human hair follicles with 100, but not 10 mIU/mL TSH (Bodó et al., 2009). However, research in cultured rat myotubes has shown a numerically similar pattern for insulin receptor 1 abundance peaking at 10 mIU/mL (Moon et al., 2016), suggesting that the dose response observed may be specific to skeletal muscle.

One limitation of the present study is that it does not confirm the presence of TSH receptors in equine skeletal muscle. However, the presence of TSH receptors has been documented in several extrathyroidal tissues, such as the skin, kidney, immune cells and bone marrow (Williams, 2011) and skeletal muscle (Ohn et al., 2013) of other species. Research on the presence and distribution of TSH receptors in skeletal muscle is sparse, and to date there has been no research examining whether the effects of TSH in skeletal muscle are receptor mediated. Therefore, the results in the current study are promising, but require verification of TSH receptor expression in equine skeletal muscle before firm conclusions can be drawn.

In sum, TSH increased indices of mitochondrial density in the present study similarly to what has been reported in human skin, and also increased integrated maximum oxidative capacity. These results suggest that TSH may stimulate mitochondrial biogenesis in skeletal muscle. However, the improvements in the present study were dose-, age-, and sex-dependent. More research should be conducted to determine if the disparities between age and sex groups result from a difference in receptor presence, dose response, or other factors.

6. CONCLUSIONS

Potential effects of breed, exercise training, complexed trace mineral supplementation, transportation stress, age and thyroid stimulating hormone concentration on a) mitochondrial density and function, b) oxidative stress and antioxidant status and c) muscle fiber type and size in various age groups of horses were studied in the presented experiments. The resulting data provide insight into breed and age differences in muscle mitochondrial characteristics, and the effects of complexed trace mineral supplementation, exercise, transportation stress and TSH administration on skeletal muscle characteristics of horses of various ages. The findings here are novel in many respects and can be applied across various research models, as well as to the care and management of horses.

It was hypothesized that Quarter Horses would have lower mitochondrial indices than Standardbreds and Thoroughbreds. To an extent, the hypothesis can be accepted, as Quarter Horses had lower CS activity and integrated maximum oxidative and electron transport system capacities. However, CCO activity in Quarter Horses was comparable to Thoroughbreds, and Quarter Horses had similar intrinsic maximum oxidative and electron transport system capacities as Standardbreds. The observed breed differences in weanling racing-bred horses serve to extend the current literature which thoroughly documents differences in skeletal muscle fiber type in young and mature, trained and untrained Standardbreds, Thoroughbreds and Quarter Horses. However, our data demonstrate that the differences between breeds are likely more intricate than a simple difference in muscle fiber type and mitochondrial density, but rather a broad physiological difference that reflects adaptation to different lengths and intensities of exercise, and therefore substrate preference.

Likewise, our research regarding transportation stress, exercise training and complexed trace mineral supplementation corroborated previous literature indicating that transportation results in increased markers of muscle damage and oxidative stress, but that complexed trace mineral supplementation increases antioxidant enzyme activity and exercise training improves the response to oxidative insult. It was hypothesized that complexed trace mineral supplementation would increase systemic and muscle antioxidant gene expression and enzyme activities, and decrease oxidative stress and muscle damage in response to trailering stress. This study is the first to show that complexed trace mineral supplementation improves antioxidant enzyme activity in horses, and is also the first to show that previously observed improvements in oxidative stress with complexed trace mineral supplementation may result not only from an improvement in antioxidant enzyme activity, but also from changes in mitochondrial characteristics. Our results indicate that complexed trace mineral supplementation may be a useful tool for maintaining skeletal muscle health of young equine athletes. However, contrary to our hypothesis, complexed trace mineral supplementation did not increase antioxidant gene expression or alter oxidative stress and muscle damage in response to trailer stress.

It was hypothesized that aged horses would have lower mitochondrial density, function and oxidative capacity, and alterations in muscle fiber type when compared to young horses. Contrary to our hypothesis, intrinsic oxidative capacity was higher in aged horses. In agreement with our hypothesis and previous research in aged humans and horses, our data indicated that aged horses had impaired mitochondrial function and alterations in myosin heavy chain expression. However, our research is the first to show that, like aged humans, exercise training may not fully rescue decrements in oxidative capacity caused by aging. The similarities between aged humans and horses in the presented study suggest that horses could be an excellent model for human aging and exercise training. Additionally, they show that exercise training in aged

horses improves many indices of mitochondrial function and capacity that are impaired with aging and may be used to improve the health and wellbeing of aged horses.

Lastly, it was hypothesized that TSH would increase mitochondrial density and capacity in mature and aged horse skeletal muscle. The observed effects of TSH on mitochondrial density and maximum oxidative capacity agree with our hypothesis and corroborates previous studies which show similar improvements in human skin. However, we are the first to show that these effects are present in skeletal muscle, and the first to show any effect in horses. Effects of TSH on mitochondrial density in skeletal muscle could be the link between elevated TSH concentration and extreme longevity. While our research suggested that the effect of TSH may be age, sex and dose-dependent, it also indicates that horses are a potential model for studying these effects further. With horses as a model for the mechanisms by which elevated serum TSH relates to extreme longevity, there is a wealth of progress that can be made on research that has the potential to improve the lifespan and healthspan of both humans and horses.

Together, this research provides new insights into skeletal muscle physiology that extend across ages and species. The information obtained presents many interventions that can improve the health of horses, and that should be considered when designing feeding, training, and management programs for horses. Future research should be conducted to explore the full potential of these findings as they relate to humans, horses, and other species.

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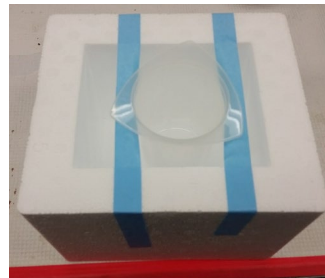
APPENDIX

6.1. Cryopulverization

Keep all samples in liquid nitrogen while processing. Do not allow to thaw

1. Pour liquid nitrogen into Nalgene container and in small clear cup
    a. Liquid nitrogen doesn't evaporate as quickly from white cup if place cup in Styrofoam
    b. The Styrofoam cooler can also be filled with liquid nitrogen so that when samples are powdered, they can be transferred to the outside cooler (prevents having to sift through so many tubes each time you are looking for your next sample)



2. Place BioPulverizer (Spectrum™ Bessman Tissue Pulverizer; Thermo Fisher Scientific, Waltham, MA) and a spatula in the Nalgene container with liquid nitrogen to cool
    a. Place up to 5 sample vials, 5 labeled 2-mL cryovials for muscle powder storage, and spatula in white cup

3. Record empty cryovial weight (for powdered muscle) in excel file for the given set of samples, tare, and return to clear cup
4. Once BioPulverizer is sufficiently cold (3-4 min), remove from Nalgene container and place on counter (use blue cryo gloves)
5. Remove pestle from BioPulverizer and use COLD spatula to transfer muscle tissue from vial to BioPulverizer, place spatula back in liquid nitrogen
6. Insert pestle and slam with hammer 3-4 times, turn pestle 1-2 rotations, and repeat 3-5 times

7. Transfer powdered muscle into tared, labeled cryovial. Re-weigh the tube with the powder. Record weight in excel file for the given set of samples
    a. Note: If the powdered muscle is in big chunks after powdering, break them up into smaller pieces before transferring into the cryovial. This will make it much easier when you go to take portions out for homogenization.
8. Return to liquid nitrogen until return to -80°C freezer for storage

6.2. Citrate synthase and Cytochrome *c* Oxidase Tissue Homogenization

Adapted from Spinazzi et al. (2012)

Before Beginning Homogenization

- You will need to label 3 tubes for each sample with either sample name, date and project, or a sample number

1. A "trash" tube (these will be thrown away after sonication and centrifugation)
2. A 40-fold (40X) tube (this is where you will put the supernatant you pull off after centrifugation.

3. An 80-fold (80X) tube (this is where you will put your diluted sample that you use to run assays)

- Ensure that you have enough sucrose homogenization buffer to homogenize the number of samples you want to run (the amount you need to make sucrose homogenization buffer with detergent (see below) plus what you need to dilute to 80X samples (50 μL/sample)
    - Sucrose homogenization buffer: 20 mM Tris, 40 mM KCl, 2 mM EGTA, 250 mM sucrose, pH 7.4 (in silver fridge)
- Make sucrose homogenization buffer with detergent to be used (made fresh daily)
    - Dilute 1 part 5% detergent to 100 parts sucrose buffer
    - The "Solution Maker" spreadsheet has a calculator for how much 5% detergent to make based on the number of samples you will be putting into solution
        - The detergent is n-Dodecyl β-D-maltoside; Sigma D4641; in white -20 º C freezer

To prepare muscle homogenate

1. Add 10-15 mg powdered (cryopulverized, see cryopulverization protocol) muscle to each microvial, recording exact tissue weight in the excel file for the sample set
2. Add the volume of sucrose homogenization buffer **with detergent** required to obtain a 40-fold dilution (will be calculated in spreadsheet when you enter sample weight)
    a. Example: 10 mg muscle powder x 40 = 400 μL sucrose homogenization buffer with detergent
3. Sonicate (F60 Sonic Dismembrator, Fisher Scientific, Waltham, MA) each tube 3 times for 3 seconds each
    a. Keep on ice while sonicating

   b. Clean sonicator probe in between samples by rinsing with ddH_2O and patting dry with a kimwipe
4. Centrifuge microvials for 3 min at 11,000 x *g* at 0°C
5. Collect supernatant (40-fold dilution; 40X)
6. Make 80-fold (80X) dilution of sample
   a. Example: 50 µL of 40X + 50 µL of sucrose buffer **without** detergent = 100 µL total
7. Can store 40X and 80X homogenates at -80°C until analysis
8. Use 80-fold dilution for assay

6.3. Citrate Synthase Activity

Adapted from Spinazzi et al. (2012)

To prepare muscle homogenate See CS and CCO Tissue Homogenization

Before beginning assay

1. Prepare 1 mM DTNB (Called Ellman's Reagent, in silver fridge)
   a. Dissolve 7.9 mg DTNB in 20 mL of 100 mM Tris (pH 8.0, in fridge)
   b. Prepare fresh daily
2. Turn on microplate reader, make sure pathlength correction ("L") is activated in your protocol, and preheat to 37°C
3. Prepare 10 mM oxaloacetic acid (OAA, in silver fridge)
   a. Dissolve 6.6 mg OAA in 5 mL distilled H_2O
   b. Prepare fresh daily
4. Prepare reaction mix (See "Solution Maker" spreadsheet for calculation based on the number of samples you will run)
   a. Per well:
      i. 76 µL ddH_2O

ii. 125 µL Tris (200 mM, pH 8.0, in fridge) with Triton-X (, 0.2% (vol/vol))

1. To make, dissolve 1.21 g of Tris in 40 mL ddH_2O, adjust to pH 8.0 with HCl, add 0.1 mL of Triton-X (in cabinet "IHC Supplies") and adjust the volume to 50 mL; can be stored at 4°C for up to 2 mo

iii. 25 µL DTNB (1 mM from step 1)

iv. 7.5 µL Acetyl CoA (in -80º C freezer, 10 mM)

1. To make dissolve 100 mg Acetyl CoA disodium salt (in white -20ºC freezer by the door) in 12.35 mL ddH_2O, 50 mg Acetyl CoA in 6.175 mL ddH_2O, or 25 mg Acetyl CoA in 3.0875 mL ddH_2O
2. Store at -80°C in 200 µL aliquots for several months
3. **Once thawed, use same day**

5. Add 4 µL sample to each well
6. Add 233.5 µL reaction mix (from step 4) to each well
7. Read baseline activity at 412 nm for 2 min
8. Start reaction by adding 12.5 µL OAA (10 mM from step 3) to each well using multichannel pipette one column at a time
    - Add OAA, pop bubbles quickly, read immediately
9. Monitor increase in absorbance at 412 nm for 3 min

To calculate activity

1. Calculate slope (change in absorbance over time) for baseline reading and activity reading for each sample
2. Subtract baseline slope from activity slope
3. CS activity (nmol/min/mg protein) =

$$\frac{(\Delta Absorbance/\min \times 1000) \times Total\ volume\ (0.250)}{\in (13.6) \times L\ (cm) \times Sample\ volume\ (0.004) \times total\ protein\ in\ sample\ (\frac{mg}{mL})}$$

6.4. Cytochrome *c* Oxidase Activity

Adapted from Spinazzi et al. (2012)

To prepare muscle homogenate See CS and CCO Tissue Homogenization

Before beginning assay

1. Turn on microplate reader, make sure pathlength correction is activated in your protocol, and preheat to 37°C
2. Prepare reaction mix (See "Solution Maker" spreadsheet for calculation based on the number of samples you will run)
    a. Per well:
        i. 115.7 µL distilled H_2O
        ii. 137.75 µL potassium phosphate buffer (100 mM, pH 7.0)
            1. Titrate 100 mM potassium phosphate dibasic (solid is in cabinet "chemicals") with 100 mM potassium phosphate monobasic (solid is in cabinet "chemicals") up to a pH of 7.0; see "Solution Maker" sheet for suggestions on starting amounts; can store potassium phosphate buffer at 4°C for up to 2 mo
        iii. 16.53 µL reduced cytochrome *c*
            1. Dissolve 12.5 mg oxidized cytochrome *c* (in white -20º C freezer by the door) in 1 mL of 20 mM potassium phosphate buffer (20 mM, dilute from 100 mM, see "Solution Maker")
            2. Reduce cytochrome *c* solution with a few grains of dithionite (enough to fill the end of a pipette tip)
                - **Just before use**
                - Vortex thoroughly (will change color from brown to orange-pink

To perform assay

1. Add 270 µL of reaction mix (from step 2) to each well of a background plate
    a. Read baseline activity of background plate at 550 nm for 10 min
    a. Keep background plate in thermomixer at 37°C after baseline read
2. Add 5 µL sample to each well in separate sample plate
    a. Transfer 245 µL reaction mix from background plate to each well of sample plate using multichannel pipette one column at a time
    b. Monitor decrease in absorbance at 550 nm for 3 min

To calculate activity

1. Calculate slope (change in absorbance over time) for baseline reading and activity reading for each sample
2. Subtract baseline slope from activity slope
3. CCO Activity (nmol/min/mg protein)=

$$\frac{(\Delta Absorbance/\min \times 1000)\ \times Total\ volume\ (0.250)}{\in (18.5) \times L\ (cm) \times Sample\ volume\ (0.005) \times total\ protein\ in\ sample\ \left(\frac{mg}{mL}\right)}$$

6.5. High Resolution Respirometry

General Notes

When washing, we only need to wash in 100% EtOH if we used uncoupler, rotenone, or antimycin A in the chambers.

Calibrations Before Beginning Experiments

Volume Calibration:

If volume calibration has not been conducted, or if you are unpacking the machine, conduct a volume calibration

1. Ensure the inside of the chambers are completely dry
2. Add 2 mL ddH_2O
3. Loosen the screw on the white ring around the stopper, and push the stopper in just until you can see the water coming out of the top of the stopper
    - Note: If you push too hard, it will squirt out the top, and you will have to remove all of the water, dry the chambers completely (usually by adding 100% EtOH and then taking it out and letting it sit to dry) and then re-add the 2 mL H_2O and start again
4. Tighten the screw around the white ring

Washing Before Beginning Experiments and Calibration

1. If you just set up the machine
    a. Soak 5 min in 70% EtOH
    b. Rinse 3x in 70% EtOH
    c. Soak 5 minutes water
    d. Rinse 3x water
2. If you are starting from storage in 70% EtOH
    a. Remove liquid from chambers
    b. Soak 5 min in water
    c. Rinse 3x in water

Background Calibration

Conducted the day before the beginning of an experiment

1. Add 2.2 mL Mir06 (don't need creatine unless you're going to run samples immediately after calibration) in each chamber
    a. 6 mL Mir05
    b. 15 uL catalase
2. Close chambers all the way, remove excess Mir06 from the top of the stoppers, and then open chambers to the width of the spacer
3. Open DatLab and load the 37º C setup
4. Create a Background Calibration file (save in "O2K Calib" → "BKGRD CALIB" Folder as "year-month-day BKGRD CALIB")
5. In the "Edit experiment" box, Experimental Code is BKGRD CAL. Unit is "Unit". Concentration and amount for both chambers are 0. Medium is Mir06. Chamber volume is 2.00. All other fields are left empty.
6. Allow flux to stabilize, and then mark a 3-5 minute span as R1 (100% Air Saturation)
    a. Mark R1 **in the O_2 concentration**. Not the O_2 flux per V. The box for the mark you make should be blue.
    b. O_2 concentration should be 170 uM-190 µM
        i. If the concentration is <168 or >210, there is a problem
            1. Empty chamber, look at sensors
                a. If there are bubbles on the sensors, change the membrane
                b. If the cathode or anode look dirty, clean the sensors and replace the membrane

c. If that does not fix the problem, leave the machine on overnight with spinners on in 70% EtOH.

7. Close the chambers
8. Add H_2O_2 to bring O_2 concentration to 650-700 µM in each chamber
    a. Add 204 µL 50% wt/v H_2O_2 to 14.796 mL ddH_2O to make 200 mM H_2O_2
    b. Add 340 µL 30% H_2O_2 to 14.66 mL ddH_2O to make 200 mM H_2O_2
    c. If O_2 concentration keeps dropping, it is likely because of bubbles from the H_2O_2 in the chambers. Use F11 (chamber A) and F12 (chamber B) to stop stirrers in chambers. Allow bubbles to float to hole at top of the chamber, and then restart stirrers. You may have to do this a few times. If it does not work, wait for O_2 concentration to stabilize, and then add more H_2O_2 in smaller increments (2-5 µL at a time).
9. Allow flux to stabilize
    a. Mark stable flux as J1 **in the O_2 flux per V**. The box for the mark you make should be red.
10. Open chamber and allow O_2 to drop to ~475 (target O_2 is 450) and close chamber
11. Allow flux to stabilize
    a. Mark stable flux as J2 **in the O_2 flux per V**. The box for the mark you make should be red.
12. Open chamber and allow O_2 to drop to ~375 (target O_2 is 350) and close chamber
13. Allow flux to stabilize
    a. Mark stable flux as J3 **in the O_2 flux per V**. The box for the mark you make should be red.

14. Open chamber and allow O_2 to drop to ~275 (target O_2 is 250) and close chamber
15. Allow flux to stabilize
    a. Mark stable flux as J4 **in the O_2 flux per V**. The box for the mark you make should be red.
    b. Note: Before moving to the next step, consider that you DO NOT add dithionite to bring O_2 to zero if you are planning to run samples without washing and adding new medium first. You can always add the zero powder and get your zero reading after you are done running your samples.
16. Add a spatula tip full of dithionite (Called "Zero Powder", in the POS service kit) to ~1mL ddH_2O, and add 100µL to the chamber.
    a. Note: It is extremely important that the dithionite powder does not get wet. If you get your spatula wet (even just a little bit) putting the dithionite into solution, dry the spatula VERY THOROUGHLY before you go back to add more to the solution. Never leave the dithionite jar open while you are working, and always work from small jars of dithionite. Even moisture from the air can degrade the quality of dithionite, and it will have to be replaced.
    b. If 100 µL does not bring the O_2 concentration to 0 (or close to it; < 20), add more dithionite to your solution and try another 100 µL
    c. If that doesn't work, there's something wrong with your sensors. See sensor check instructions in number 6-1 of this protocol.
17. Mark R0 **in the O_2 concentration**. Not the O2 flux per V. The box for the mark you make should be blue.

18. Click “File” → “Save and Disconnect”, and then click “no” when prompted whether or not you want to close the Datlab file.
19. Go to “Calibration” tab and open the “A: Oxygen, O2” window. Click the drop down box for R1 and select your R1 value, repeat for the R0 box. Click “Calibrate and copy to clipboard”.
20. Open the air calibration file for the O2K you are using and paste the data into the next row in the “O2-Calibration A Template” sheet.
21. Repeat steps 19-20 for chamber B.
22. Go to “Flux/Slope” tab and open the “A O2 Slope” window. Check the box for background correction.
    a. Click “Calibrate BG” and check your R^2 in the box that pops up. It must be >0.98.
        i. If it’s not, you can click “Show Graph”, to determine if there is one point that is messing up your R^2. If so, it can be dropped by unchecking its box in the “O_2 Background correction” window (previous window).
23. Click “OK” to exit the calibration dialogue box when you have finished reviewing you curve.
24. Repeat steps 22-23 for chamber B
25. Go to “Marks” → “Statistics”, ensure that chamber A is selected in the top left corner, and click “Copy to clipboard” in the bottom right corner
26. Open the background calibration file for the O2K you are using and paste the data into the next available chamber A section in the “Paste” box in column J in the “Template_O2-background_high” sheet.
27. Repeat steps 25-26 for chamber B

28. Wash

    a. Remove liquid from chambers

    b. Soak 5 min in water

    c. Rinse 3x in water

    d. Soak 5 min in 70% EtOH

    e. Rinse 3x in 70% EtOH

    f. Store in 70% EtOH

Running Samples

1. Wash: (Assuming you're starting in 70% EtOH)

    a. Soak 5 min in water

    b. Rinse 3x in water

2. Air calibration

    a. This is done before sample collection, and the oxygraphy stabilizes during collection so that when you get back, all you have to do is mark R1 and move on.

    b. Add Mir 06Cr (make enough in the beginning of the day for the number of samples you plan to run)

        i. 2.2 mL in each chamber

Number of Samples	2	4	6	8	10	12
Component	5mL	10 mL	15 mL	20 mL	25 mL	30 mL
Mir05 (mL)	5	10	15	20	25	30
Creatine (mg)	15	30	45	60	75	90
Catalase (µL)	12.5	25	37.5	50	62.5	75

*1 mL Mir05 + 3 mg Creatine + 2.5 µL Catalase

3. Close the chambers all the way, remove excess liquid from the top of the stoppers, and then open chambers the width of the spacer

4. Open DatLab and load the 37 C setup
5. Create an Air Calibration file (save file in “O2K Calib” → “AIR CALIB” Folder as “year-month-day AIR CALIB”)
6. In the “Edit experiment” box, Experimental Code is AIR CAL. Unit is “Unit”. Concentration and amount for both chambers are 0. Medium is Mir06Cr. Chamber volume is 2.00. All other fields are left empty.
7. Allow flux to stabilize, and then mark a 3-5 minute span as R1 (100% Air Saturation)
8. Mark R1 **in the O2 concentration**. Not the O2 flux per V. The box for the mark you make should be blue.
    - ii. O2 concentration should be 170 uM-190 μM
    - iii. If the concentration is <168 or >210, there is a problem
        - i. Empty chamber, look at sensors
            1. If there are bubbles on the sensors, change the membrane
            2. If the cathode or anode look dirty, clean the sensors and replace the membrane
        - ii. If that does not fix the problem, leave the machine on overnight with spinners on in 70% EtOH.
9. Click “File” → “Save and Disconnect”, and then click “no” when prompted whether or not you want to close the Datlab file.
10. Go to “Calibration” tab and open the “A: Oxygen, O2” window. Click the drop down box for R1 and select your R1 value. Click “Calibrate and copy to clipboard”.
11. Open the air calibration file for the O2K you are using and paste the data into the next row in the “O2-Calibration A Template” sheet.

12. Repeat steps 10-11 for chamber B.

Prepare Samples

1. Label 1 saponin tube and 1 Mir05 tube for each sample
    a. 980uL BIOPS in saponin tube
    b. ~1mL Mir05 in Mir05 tube
2. Put a few drops of BIOPS in a culture plate on ice and tweeze apart sample with tweezers
    a. Remove connective tissue and sample should look loose and wavy. Don't over tweeze, or you can damage the sample and increase the cyt *c* response. Always push the fibers apart, do not pull them apart.
3. Put sample in its saponin tube and repeat with as many samples as you plan to do on this run
4. After all the samples for this run are in saponin tubes, add 20 uL saponin to each tube and put on a rocker in the fridge for 30 min
    a. Saponin concentrate: 200 μL BIOPS / mg saponin (x mg saponin * 200 = μL BIOPS)
    b. Write down in your lab notebook the time you put all samples in saponin on the rocker
5. Now is a good time to take substrates out of the freezer to thaw on ice
6. After 30 minutes, remove samples from saponin tubes and put them in Mir05 tubes
7. Rock samples in Mir05 tubes in the fridge for 10 minutes
    a. Write down in your lab notebook the time you put all samples in Mir05 on the rocker
    b. Now is a good time to start getting the O_2 in the chambers up (See "Running Samples"

Running Samples

1. Add 10 uL H_2O_2 to each chamber

2. Open DatLab and load the 37 C setup
3. Create a Sample file (Presumably you have a folder that you save your data in. Name files as year-month-day chamber-1-sample I.D. _chamber-2-sample I.D.)
4. In the "Edit experiment" box,
    a. Experimental Code is chamber-1-sample I.D. _chamber-2 sample-I.D.
    b. Protocol is PERM MUSC FIBERS
    c. Sample Type is PERM MUSC FIBERS
    d. Cohort is the name of the study the samples are from
    e. Sample Code is the sample name that is in each chamber
    f. Sample number is the nth sample that has been run for this cohort
    g. Subsample number is the nth time this sample has been run
    h. Unit is "mg"
    i. Type the weight of each sample in the "Amount" box, and it will calculate concentration for you (you can leave it blank until after you weigh and add samples, and then come back to the box by "Experiment' → "Edit" or F3)
    j. Medium is Mir06Cr
    k. Chamber volume is 2.00
5. Bring O_2 concentration in each chamber to 650 µM with H_2O_2
6. When O_2 is at the appropriate concentration in the chambers, weigh samples according to tissue type (table below) from the Mir05 tubes onto either side of a small weigh boat
    a. Label the sides of the weigh boat A and B so that you can tell them apart
    b. Dab the liquid off the tissue gently before you weigh it
    c. Record the weight of the sample and be sure to put it in the O2K file

d. Sample should be kept in open air as little as possible, so be quick with weighing

Sample Type	Target Weight (Range)
Quarter Horse Glut/Tri	3 mg (2.5-3.5 mg)
Thoroughbred Glut	1.8 mg (1.7-1.9mg)
Cow LD/Trap	6.5 mg (6-7 mg)

7. Open chamber and quickly add sample to chamber with tweezers
    a. Be careful not to grip the sample too tightly with your tweezers to avoid damaging the sample or having it stick to your tweezers
    b. Be careful not to slam the chambers shut (which will cause bubbles)
8. Add Pyruvate (5 μL) and Malate (5 μL)
9. If O_2 is lower than 550, add H_2O_2 to bring the O2 up to 550-600 μM
10. Allow flux to stabilize and mark a 3-5 min span in O2 flux per mass
    a. The protocol driver will automatically label the mark “1PM”
    b. Flux for LEAK (1PM) is typically around 10-20 pmol/sec • mg, if it is far above that, check to be sure that you have entered the tissue weight
11. Add 10 μL ADP, allow flux to stabilize, and mark a 3-5 min span in O2 flux per mass
    a. The protocol driver will automatically label the mark “2D”
    b. The flux for P_{CI} (2D) should be higher than LEAK, but the overall increase from LEAK flux differs between breeds/species
12. Add 5 μL cytochrome *c*, allow flux to stabilize, and mark a 2-3 min span in O2 flux per mass
    a. The protocol driver will automatically label the mark “2D(c)”
    b. Go to “Marks” → “Statistics” and calculate the percent increase in O2 slope neg from 2D to 2D(c) [(2D(c) – 2D) / 2D]

c. If 2D(c) flux is >15% higher than 2D, sample is not usable because outer mitochondrial membrane integrity is compromised

d. Note: 15% is an absolute upper limit. If the majority of samples from a project/species are running with much lower cyt *c* responses, you may want to make a lower limit, such as 10%

13. Add 10 µL glutamate allow flux to stabilize, and mark a 3-5 min span in O2 flux per mass

    a. The protocol driver will automatically label the mark “3G”

    b. The flux for P_{CI} with glutamate (3G) may be higher than 2D(c), but often is not much higher

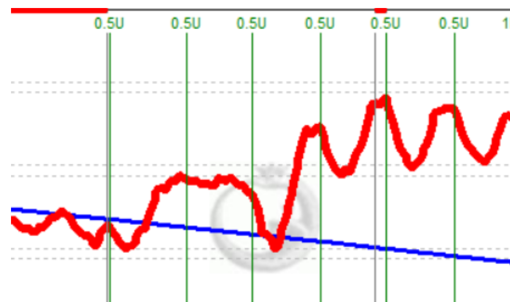
14. Add 20 µL succinate allow flux to stabilize, and mark a 3-5 min span in O2 flux per mass

    a. The protocol driver will automatically label the mark “4S”

    b. The flux for P_{CI+II} (4S) is typically much higher than 3G, but the overall increase from 3G to 4S differs between breeds/species

15. Titrate uncoupler (CCCP) in 0.5 µL increments

    a. Addition of CCCP typically results in a decrease, followed by an increase in flux (see below)



b. After each CCCP titration, mark the highest part of the peak (this will not be a long mark, and is typically only a few seconds to capture the highest part of the peak)

c. When peaks after titrations appear to decline, check the peak values ("Marks" → "Statistics"), retain the highest peak value mark, and delete the rest of the peak marks (ensuring that the peak mark is labeled "5U")

d. Note: Adding too much uncoupler can inhibit complex II activity, so be careful not to add too many CCCP titrations after peaks begin to lower.

16. Add 1 µL rotenone, allow flux to stabilize, and mark a 3-5 min span in O2 flux per mass
    a. The protocol driver will automatically label the mark "6ROT"
    b. The flux for E_{CII} (6ROT) is lower than 5U, but the overall decrease from 5U to 6ROT differs between breeds/species
17. Add 1 µL antimycin A, allow flux to stabilize, and mark a 3-5 min span in O2 flux per mass
    a. The protocol driver will automatically label the mark "7AMA"
    b. The flux for ROX (7AMA) is typically close to zero (0-15). Make sure to give the chamber plenty of time to stabilize before marking 7AMA, as all values in the run are corrected for this mark, and over-estimating it will change all of the respiratory state values for the sample.
18. Click "File" → "Save and Disconnect", and then click "no" when prompted whether or not you want to close the Datlab file.
19. Go to "Marks" → "Statistics", ensure that chamber A is selected in the top left corner, and click "Copy to clipboard" in the bottom right corner
20. Open the Datlab analysis file for the project you are running and paste the data into the next available empty yellow cell in the "Data" sheet.
21. Repeat steps 19-20 for chamber B

After Finishing the Protocol

1. Add zero powder to zero if background calibration was done the same day
    a. See instructions in “Background Calibration” step 16 of this protocol
2. Remove liquid from chambers
3. Soak 5 min in water
4. Rinse 3x in water
5. Soak 5 min in 70% EtOH
6. Rinse 3x in 70% EtOH
7. Soak 30 min in 100% EtOH
    a. 100% EtOH soaks are used if you use any reagents solubilized in 100% EtOH (i.e. uncoupler, rotenone and antimycin A)
8. If done for the day, replace 100% EtOH with 70% EtOH
9. If moving on to another sample batch, soak 5 min in water, rinse 3x water, add Mir06Cr

6.6. Muscle Malondialdehyde Concentration

This procedure describes a modification of the method outlined in the NWLSS™ Malondialdehyde Assay kit to enable reading on a microplate reader.

<u>*Supplies Needed*</u>

- NWLSS™ Malondialdehyde Assay kit (Northwest Life Science Specialties, LLC, Vancouver, WA; Product #NWK-MDA01)
- Muscle sample homogenate in MDA Buffer (see below)
- 2-mL Eppendorf microcentrifuge vials
- Plastic, 96-well flat-bottomed plates
- Microplate reader with spectral reading capability
- Adjustable pipettes (30-200 μL) and pipette tips

- 25-gauge needles to pop bubbles in wells (if necessary)

Preparing Muscle Homogenate

1. Add 30-50 mg powdered (cryopulverized) muscle to each microvial, recording exact tissue weight
    i. The amount needed will depend on how much supernatant you get back when you centrifuge it. If the pellet is small and you get plenty of supernatant, use 30-40 mg, if the pellet is large and not much supernatant is collected, use 40-50 mg.
2. Add the volume of MDA Assay Buffer (in kit) required to obtain a 10-fold or 15-fold dilution (typically we use 10-fold but if that doesn't run well you can try 15-fold.)
    i. Example: 40 mg muscle powder x 10 = 400 μL Assay Buffer
3. Sonicate (F60 Sonic Dismembrator, Fisher Scientific, Waltham, MA) each tube 3 times for 3 seconds each
    i. Keep on ice while sonicating
    ii. Clean sonicator probe in between samples by rinsing with dH_2O
4. Centrifuge microvials for 10 min at 11,000 x *g* at 0°C
    i. Original protocol says 3 minutes but when we have a very large pellet after 3 minutes we adjust centrifugation to 10 minutes to try to get more.
5. Collect supernatant (15-fold dilution)
    a. Can store at -80°C until analysis
6. Use 10-fold or 15-fold supernatant for assay (whichever one you made)

Performing Assay

7. To each 2-mL Eppendorf microvial, add:
    a. 6 μL BHT (duplicate); 9 μL (triplicate)

b. 150 µL calibrator or muscle sample (duplicate); 225 µL (triplicate)

c. 150 µL Acid Reagent (in kit)(duplicate) ; 225 µL (triplicate)

d. 150 µL TBA Reagent (in kit) (duplicate); 225 µL (triplicate)

i. Add 10.5 mL DD H2O, insert stirrer bar, mix until dissolved (~10 min)

ii. Store at RT. DO NOT REFRIGERATE. Stable for at least 1 week at RT

8. Vortex vigorously (5 count)

9. Incubate 60 min at 60°C on plate/tube warmer next to the liquid nitrogen tank

10. Centrifuge at 10,000 × *g* for 3 min at room temp

a. Color development is stable for at least 1 hour

11. Transfer 200 µL of each calibrator and sample (in duplicate or triplicate) to each well in the plate (make sure not to disturb the pellet in the bottom)

12. Perform spectral scan from 400-700 nm in plate reader every 1 nm

13. Make sure to only select the wells you want to read in the protocol plate layout. Otherwise it will scan every well regardless of whether you have marked a sample in the plate layout

a. Scanning the entire plate takes a long time, for this purpose it is better to pipette in order across rows, because you can select portions of rows but not portions of columns

b. Procedure → Double Click on the Read →Top right corner says "Full Plate" → click on the button and highlight the columns in which you have samples

14. Perform 3rd derivative analysis at http://www.nwlifescience.com/sg/

6.7. Serum Creatine Kinase Activity

This procedure is a modification of the protocol outlined in the CK, Liqui-UV® kit (Stanbio Laboratory) to enable reading on a microplate reader.

Supplies Needed

- CK, Liqui-UV® (Kit #2910; Stanbio Laboratory, Boerne, TX) – **Store kit at 2-8°C**
- Ser-T-Fy® 1 Level 1 Control Serum (Cat. #G427; Stanbio Laboratory)
- Undiluted serum samples
- Plastic 96-well, flat-bottomed plates
- Microplate reader with pathlength correction function
- Adjustable pipette (to 12.5 µL)
- Multichannel pipette (250 µL capacity)
- Appropriate pipette tips
- 25 gauge needles to pop bubbles in wells (if necessary)

Procedure

1. Prepare CK Working Reagent
    a. Mix 1 part Enzyme Reagent to 4 parts Buffer Reagent
    b. Working Reagent is stable for 3 wk at 2-8°C or 2 d at room temp
    c. Calculator for working reagent is in "Solution Maker" excel workbook
2. Prepare Control Serum
    a. Add 5 mL dd H2O to the lyophilized powder and allow to sit for 10 minutes at RT
    b. After ten minutes, gently invert 3 times and swirl to mix
        i. DO NOT SHAKE OR VORTEX
        ii. Avoid foaming
    c. Control serum should be stored on ice or in the refrigerator away from light.
        i. Reconstituted Control serum is stable for at least 7 days when kept at 4-8º C away from light

3. Add 12.5 µL Control Serum or sample serum to each well (run samples at least in duplicate)
    a. Control Sample should be run at 2 levels:
        i. Undiluted, and diluted 1 part control sample with 1 part ddH2O (1:2)
        ii. Undiluted serum should have a mean of approximately 116 U/L with a range of 81-151 U/L
        iii. Diluted should run half of that
4. Warm Working Reagent to 37°C for 4 min using a water bath
5. Add 250 µL warmed Working Reagent to each well and mix gently (mixing is already in the microplate protocol)
6. Read absorbance every min for 5 min at 340 nm at 37°C
    a. Be sure to activate the "pathlength correction" option on the reader
7. Determine average absorbance/min (ΔA/min)
    a. Use either the "slope" calculation in Excel, or create a scatterplot of points, fit a linear trendline, and then obtain the slope from the trendline. Graph both options to see which is more accurate (higher R-value)
    b. If the ΔA/ min is > 0.345, dilute 1 part sample with 9 parts saline (1:9) and re-assay, multiply by 10 to correct output for dilution
    c. Expected CV within plate is 0.7-2%, expected CV between plates is 1-2.1%
8. Calculate CK Activity (U/L) =

$$\frac{(\Delta A/\min) \times 0.2625}{0.00622 \frac{\mu mol}{cm} \times pathlength\ (cm) \times 0.0125}$$

Where,

$\Delta A/min$ = slope

0.2625 = total volume/well

0.00622 µmol/cm = NADP extinction coefficient

pathlength (cm) = pathlength for well calculated by plate reader

0.0125 = volume of sample/well

6.8. SOD and GPx Muscle Homogenization

Before Beginning Homogenization

- You will need to label 3 tubes for each sample with either sample name, date and project, or a sample number
    1. A 20-fold (20X) tube (this is where you will homogenize and sonicate)
    2. A "trash" tube (these will be thrown away after centrifugation)
    3. An 80-fold (80X) tube (this is where you will put the supernatant that you use to run assays)

Homogenization

1. Add 12-17 mg powdered (cryopulverized, see cryopulverization protocol) muscle to each tube, recording exact tissue weight in the excel file for the sample set
2. Add the volume of Extraction Buffer required to obtain a 20-fold dilution (20X; will be calculated in spreadsheet when you enter sample weight)
    a. Extraction Buffer: 0.1 M KH_2PO_4, 1 mM EDTA, pH 7.2
    b. Example: 10 mg powder × 20 = 200 mL extraction buffer

3. Mix on a vortex with blue Styrofoam tube attachment at the highest vortex setting for 15 min at 4º C
4. Sonicate (F60 Sonic Dismembrator, Fisher Scientific, Waltham, MA) each tube 3 times for 3 seconds each, cleaning the probe in between samples
    a. Keep on ice while sonicating
    b. Clean sonicator probe in between samples by rinsing with ddH_2O and patting dry with a kimwipe
5. Make 80-fold (80X) dilution of sample
    a. Example: 70 µL of 20-fold sample + 210 µL of Extraction Buffer = 280 µL total
    b. Keep samples on ice
6. Centrifuge 80X for 2 min at 14,000 x *g* at 0°C
7. Collect 80X supernatant
    a. Freeze 20X dilution and 80X supernatant in -80°C

6.9. Superoxide Dismutase Activity

This method follows the procedure outlined in the Superoxide Dismutase Assay Kit (Item #706002; Cayman Chemical).

Supplies Needed

- Superoxide Dismutase Assay Kit (Item #706002; Cayman Chemical) *Store kit at -20°C*
- Plasma, whole blood or muscle homogenate sample
- 2-mL Eppendorf microcentrifuge vials (for dilutions)
- Plastic, 96-well flat-bottomed plates

- Microplate reader
- Adjustable pipettes (10-200 μL)
- Multichannel pipette (20-200 μL capacity)
- Appropriate pipette tips
- 25-gauge needles to pop bubbles in wells (if necessary)

Before Beginning Procedure

1. Prepare SOD Assay Buffer
    a. Dilute 3 mL assay buffer (Item #706001) with 27 mL HPLC-grade H_2O
    b. Store at 4°C, stable for 2 months
2. Prepare SOD Sample Buffer
    a. Dilute 2 mL sample buffer concentration (Item #706003) with 18 mL HPLC- grade H_2O
    b. Store at 4°C, stable for 6 months
3. Sample preparation – WHOLE BLOOD
    a. Dilute sample 1:1000 with SOD Sample Buffer (from step 2)
4. Sample preparation – MUSCLE
    a. See SOD and GPx Muscle Homogenization
    b. Dilute 80X 1:5 with SOD sample buffer (from step 2)

Performing Assay (in duplicate or triplicate)

5. Prepare SOD Standards:
    a. Label 2 mL Eppendorf tubes A-G
    b. Dilute 20 μL SOD Standard (Item #706005) with 1.98 mL SOD Sample Buffer

(from step 2) to create SOD Stock

6. Aliquot the SOD Stock and SOD Sample Buffer (from step 2) as described below

Tube	SOD Stock (µL)	Sample Buffer (µL)	Final SOD Activity (U/mL)
A	0	1000	0
B	20	980	0.005
C	40	960	0.010
D	80	920	0.020
E	120	880	0.030
F	160	840	0.040
G	200	800	0.050

7. Dilute 50 µL Radical Detector (Item #706004) with 19.95 mL diluted Assay Buffer (from step 1)
   a. Cover in foil
   b. Stable for 2 h at RT
8. Pipette Plate
   a. Standard wells
      i. 200 µL diluted Radical Detector (from step 8)
      ii. 10 µL of each standard (A-G)
   b. Sample wells
      i. 200 µL diluted Radical Detector (from step 8)
      ii. 10 µL sample
9. Add 20 µL diluted Xanthine Oxidase to all wells
   a. Dilute 50 µL Xanthine Oxidase (Item #706006) with 1.95 mL diluted SOD Sample Buffer (from step 2)
   b. Stable for 1 hour

c. Use multichannel pipette

10. Gently agitate plate (on mechanical rocker) for a couple seconds to mix

11. Cover plate with plate cover and incubate at RT on plate rocker for 20 min

12. Read absorbance at 450 nm
13. Calculating Results
    a. Calculate average absorbances of each standard and sample (mean of duplicate or triplicate wells)
    b. Divide Standard A's absorbance by itself and divide Standard A's absorbance by all other standards and samples to yield the linearized rate (LR)
        i. Example: LR for Std A = Abs Std A/Abs Std A; LR for Std B = Abs Std A/Abs Std B
    c. Plot the LR as a function of final SOD Activity
    d. Fit a linear trendline to the graph and obtain the slope and y-intercept

14. SOD Activity (U/mL) =

$$\left[\left(\frac{sample\ LR - y\ intercept}{slope}\right) \times \frac{0.23\ mL}{0.010\ mL}\right] \times sample\ dilution$$

Where,

Sample LR = linearized rate (step 14b)

0.23 mL = total volume/well

0.01 mL = sample volume/well

sample dilution = 5 for either plasma or muscle

6.10. GPx Activity

This procedure follows that provided in the Glutathione Peroxidase Assay Kit (Cayman Chemical).

Supplies Needed

- Glutathione Peroxidase Assay Kit (Item #703102; Cayman Chemical) *Store kit at -20° C*
- Whole blood or muscle homogenate samples
- 2-mL Eppendorf microcentrifuge vials (for dilutions)
- Plastic, flat-bottomed 96-well plates
- Microplate reader
- Adjustable pipettes (20-100 µL)
- Multichannel pipette (20-120 µL capacity)
- Pipette tips
- 25-gauge needles to pop bubbles in wells (if necessary)

Before Beginning Procedure

1. Prepare GPx Assay Buffer
    a. Dilute 3 mL of assay buffer (Item #703110) with 27 mL HPLC-grade H_2O
    b. Store at 4°C, stable for 6 months
2. Prepare GPx Sample Buffer
    a. Dilute 2 mL of sample buffer concentration (Item #703112) with 18 mL HPLC-grade H_2O
    b. Store at 4°C, stable for 1 month

3. Reconstitute co-substrate mixture
    a. Add 2 mL HPLC-grade H_2O to Co-Substrate vial (Item #703116)
        a. Stable for 2 days after reconstitution, refrigerate when not using
4. Sample preparation – WHOLE BLOOD
    a. Dilute sample 1:34 with GPx Sample Buffer (from step 2)
    b. Example: 6 uL sample in 198 uL sample buffer
5. Sample Preparation – MUSCLE
    a. See SOD and GPx Muscle Homogenization
    b. Use 80X dilution for assay

Performing Assay (in triplicate)

6. Pipette onto 96-well plate
    a. Background wells
        i. 120 µL Assay Buffer
        ii. 50 µL co-substrate mixture (from step 4a)
    b. Positive control wells
        1. Aliquot GPx control into 10 uL aliquots upon thawing the first time.
        ii. 100 µL Assay Buffer
        iii. 50 µL co-substrate mixture (from step 4a)
        iv. 20 µL diluted GPx Control
            1. Dilute 10 µL bovine erythrocyte GPx (Item #703114) with 490 µL diluted Sample Buffer (from step 2) to create GPx Control, stable for 4 hours
    c. Sample wells

i. 100 µL Assay Buffer

ii. 50 µL co-substrate mixture (from step 5a)

iii. 20 µL sample

7. Add 20 µL Cumene Hydroperoxide (Item #703118) to all wells

    a. Use multichannel pipette

8. Gently agitate plate (on mechanical plate rocker) for a couple seconds to mix

9. Read absorbance every min for 6 min at 340 nm at 25º C

10. Calculate Results

    a. Determine average absorbance/min (ΔA/min) of all background, control, and sample wells

        i. Either using "slope" calculation in Excel or creating a scatterplot of points, fitting a linear trendline, and using the slope (have to graph to see which is more accurate)

        ii. SLOPE IS NEGATIVE. Use absolute value to obtain a positive value

    b. Subtract ΔA/min of background wells from all positive controls and samples

    c. GPx Activity (nmol/min/mL) =

$$\frac{\left(\frac{\Delta A}{\min}\right) \times 0.19\, mL}{0.00373\, \mu M^{-1} \times 0.02\, mL}$$

Where,

ΔA/min = average absorbance/min for sample – average absorbance/min for background wells

0.19 mL = total volume/well

$0.00373\ \mu M^{-1}$ = NADPH extinction coefficient ($0.00622\ \mu M^{-1} cm^{-1}$) corrected for a pathlength of 0.6 cm

0.02 mL = sample volume/well

6.11. qRT-PCR

For the duration of this protocol, handle samples to prevent RNAse contamination and maximize RNA yield by:

1. Using sterile, disposable and individually wrapped plasticware
2. Une ONLY sterile, disposable RNAse free pipette tips and microcentrifuge tubes
3. Wear gloves when handling reagents and samples and change them frequently, particularly as the RNA isolation protocol progresses from crude extracts to more purified material (e.g. from Wash Buffer I to Wash Buffer II)
4. Always use proper microbiological aseptic techniques
5. Use RNAse zap to remove RNAse from work surfaces and non-disposable items such as centrifuges, pipettes and bead mills that will be used during homogenization and purification

Collection

1. Ensure that tissue sections are ≤0.5cm in any single dimension
    a. If they are not, cut them into smaller pieces using sterile tools
2. Collect an adequate amount of sample for RNA isolation
    a. ~ 50 mg of GOOD tissue is usually enough to give adequate RNA yields. Collect more than enough (~300 mg) in case sample is of lower quality and does not homogenize as well.
3. Sample preservation
    a. RNAlater: Place the fresh tissue in ~1mL of RNA later

i. Store RNAlater solution at 4C overnight to allow the RNAlater to thoroughly penetrate the tissue
ii. After the overnight incubation at 4C, remove excess RNAlater solution and store at -20º C or -80º C until analysis
1. RNAlater will freeze at -80º C
2. RNAlater will not freeze at -20º C, but crystals may form. This will not affect the quality of subsequent RNA isolation
3. Samples should be thawed on ice

b. Whole muscle: Remove any excess blood from the sample by rinsing quickly in PBS, flash freeze the muscle in liquid nitrogen and store at -80º C

Reagent Preparation

4. Prepare Wash Buffer II with ethanol
   a. Add 60 mL of 96-100% **molecular biology grade** EtOH directly to the bottle for the 50 preparation kit
   b. Add 300 mL of 96-100% **molecular biology grade** EtOH directly to the bottle for the 250 preparation kit
   c. Check the box on the Wash Buffer II label to indicate the EtOH was added
   d. Store at RT
5. Resuspend PureLink DNAse by dissolving in 550 µL RNAse free $H2O$
   i. Can be stored at 4º C for up to 6 wk
   ii. For long term storage, prepare aliquots and store at -20º C
   iii. Avoid repeated freeze-thawing
6. Prepare 80 µL PureLink DNAse working solution per sample in a clean RNAse free microcentrifuge tube
   a. 8 µL 10x DNAse I reaction buffer
   b. 10 µL resuspended DNAse
   c. 62 µL RNAse free $H2O$

Number of Samples	10x DNAse Rxn Buffer (uL)	Resuspended DNAse (uL)	RNAse Free H2O (uL)	Total Volume (uL)
2	26	20	124	170
4	32	40	248	320
8	64	80	496	640
12	96	120	744	960

Homogenization and Isolation

Adapted from Tissue isolation protocol 4330252 Applied Biosystems, PureLink RNA Mini Kit Guide and recommendations for RNAlater and RNA isolation from Thermo

1. Label two 2mL skirtless round bottom tube with beads (~3 per tube if you are loading them yourself) for each sample to be homogenized
    a. This assumes you're homogenizing > ~50 mg tissue. If you don't have ~50 mg tissue, only one bead beater tube is necessary
2. Prepare a fresh amount of working TRIzol containing 1% 2-mercaptoethanol
    a. Prepare 1 mL of working TRIzol per tube by adding 10 µL 2-mercaptoethanol to each mL of TRIzol
3. If TRIzol was stored at RT, chill working TRIzol on wet ice in bead homogenization tubes for at least 30 min prior to addition of sample
    a. Bead beater systems generate heat from friction of the bead in the tube. Exposure of the lysate to temperatures above 37º C will cause a significant decrease in yield.
4. Sample processing
    a. RNAlater: Thaw samples in RNAlater on wet ice

i. Retrieve ~50-100 mg tissue (estimate, do not take the time to weigh or RNA degradation may start) from RNAlater with sterile forceps, quickly and thoroughly blot away excess RNAlater with a kimwipe

1. DO NOT RINSE RNAlater solution off samples before using. RNAse inactivation is reversible.

b. Whole muscle: Retrieve ~50-100 (estimate, do not take the time to weigh or RNA degradation may start) mg FROZEN whole muscle and place directly into cryopulverizer

i. DO NOT allow sample to thaw at all, or RNAse activity will resume

5. Cryopulverize muscle and split the powder between two bead beater tubes for >50 mg muscle tissue, or put it all into one tube for ≤ 50 mg muscle tissue
6. Place the tissue samples in the bead homogenizer and macerate for 30 sec, 4 times, with 30 sec of rest at RT between bead beatings
   a. DO NOT place the homogenate back on ice after the last homogenization
7. Incubate the homogenate at RT 10 min to allow nucleoprotein complexes to dissociate completely, and then transfer back to ice until all of the other samples in the batch are processed
8. Transfer the homogenate to a 1.5 mL RNAse free tube and centrifuge at 14,000 × *g* for 10 minutes at 4º C to remove cell debris
9. Transfer the supernatant to a clean RNAse free tube containing 200 µL BCP (1-bromo-3-chloropropane)
10. Invert tubes for ~1 min
    a. Vortexing may increase DNA contamination of sample

11. Incubate at RT for 3 min
12. Centrifuge at 14,000 × *g* for 10 min at 4º C
    a. After centrifugation, the mixture separates into a lower red phenol-BCP phase, an interphase, and a colorless upper aqueous phase which contains the RNA. The volume of the aqueous upper phase is typically ~620 µL
13. Transfer the colorless upper phase containing RNA to a fresh RNAse free tube containing 400 µL TRIzol and 200 µL BCP
14. Invert tubes for ~1 min
15. Incubate at RT for 3 min
16. Centrifuge at 14,000 × *g* for 10 min at 4º C
17. Transfer 750 µL of the aqueous phase to a tube containing 70% EtOH
    a. Make 70% EtOH with molecular biology grade EtOH and RNAse free H_2O
18. Pipette to mix thoroughly and disperse any visible precipitate that may have formed after adding EtOH
19. Transfer the 500 µL of homogenate and 70% EtOH to the spin cartridge with the collection tube
20. Centrifuge at 12,000 × *g* for 15 sec at RT
21. Discard the flow through and re-insert the cartridge into the same collection tube
    a. Repeat steps 19-20 until the entire sample is processed. You will need to centrifuge 3 times for one volume of homogenate and 6 times for 2 volumes
22. Add 350 µL Wash buffer I to the spin cartridge containing bound RNA and centrifuge at 12,000 × *g* for 15 sec at RT

23. Discard the flow through and add 80 µL DNAse working solution directly onto the surface of the spin cartridge membrane
24. Incubate at RT for 15 min
25. Add 350 µL Wash Buffer I to the spin cartridge and centrifuge at 12,000 × *g* for 15 sec at RT
26. Discard the flow through AND the collection tube, and insert the collection cartridge into a NEW collection tube
27. Add 500 µL Wash Buffer II with EtOH and centrifuge at 12,000 × *g* for 15 sec
28. Discard the flow through and reinsert the spin cartridge into THE SAME collection tube
29. Repeat Steps 27-28 twice (3 wash buffer II washes total)
    a. If you are concerned about running low on WBII, 80% RNAse free EtOH can be used in place of the last WBII wash
30. Centrifuge the spin cartridge at 14,000 × *g* for 3 minutes to dry the membrane bound RNA, then discard the collection tube and insert the spin cartridge into a RECOVERY TUBE
31. Add 50-70 µL RNAse free H_2O (warmed to 37º C) to the center of the spin cartridge (depending on what volume of RNA you need)
32. Incubate at RT 5 min
33. Centrifuge the spin cartridge and recovery tube 3 min at 14,000 × *g* at RT
34. Remove the eluent from the tube and reapply it to the top of the spin filter
35. Incubate at RT 5 min
36. Centrifuge the spin cartridge and recovery tube 3 min at 14,000 × *g* at RT (this gives you your first elution)

37. Place spin cartridge into a NEW collection tube and add an additional 30-100 ºL RNAse free H_2O to the center of the spin cartridge
38. Incubate at RT 5 min
39. Centrifuge the spin cartridge and recovery tube 3 minutes at 14,000 × *g* at RT (this gives you your second elution)

RNA Quantification

The main advantage of micro-volume analysis of RNA is that nucleic acid samples do not have to be diluted to be accurately quantified, as optical density of the measurement is reduced by the same factor as the pathlength reduction.

1. Clean the upper and lower optical surfaces of the Take3 plate by pipetting 2 µL of clean RNAse free H2O onto the lower optical surface, closing the lever arm, and then opening and wiping off both optical surfaces with a kimwipe
    a. If dust is left on the plate, canned air can be used to remove it
2. Open Gen 5, select “Read Now” and select the Nucleic Acid Quantification
3. Sample type is “RNA230”
    a. The extinction coefficient of RNA is 40 ng-cm/µL
    b. The normalization wavelengths are 280 nm (protein) and 230 nm (salt, GITC, EtOH, etc.)
4. Open the plate arm and pipette 2 µL of blank (RNAse free H2O) into each well that will be used for sample analysis
    a. Lower the plate lid gently to avoid splashing the samples and click “Read”
5. Provided that all the wells are highlighted green after the BLK read, approve the BLK read (button on the right side of the screen)

6. Open the arm of the plate, use a kimwipe to remove the BLK, and pipette 2 μL purified RNA sample into each microspot
    a. Flick RNA samples to mix. DO NOT vortex.
7. Read samples in "RNA230", and then export data
8. After each Take3 run, wipe the plate clean with a kimwipe, wipe with a kimwipe with 70% EtOH and then pipette 2 μL nuclease free water onto each spot used, close and then open plate arm, wipe with a kimwipe, and proceed to next run
9. After all samples have been processed, use the "End of Batch" button to the right of the screen to export a summary
10. Save quantitation data and check the ratios and yields for each sample to ensure that:
    a. The 260/280 ratio to ensure that they are ≥1.8 (~2 is preferable)
        i. Values less than 1.8 may indicate the presence of protein, phenol, or other contaminants
    b. The 260/230 ratio to ensure they are ≥1 (~2 is preferable)
        i. Values less than 1.5 may indicate contamination with EtOH, GITC, or phenol
    c. Compare first elution concentration to second elution concentration. Second elution should be ~1/4 or less of the first elution.
11. Take a 5 μL aliquot of isolated RNA to reserve for bioanalysis
12. Split the remainder of the isolated RNA into PCR tubes (one tube for each transcript to be analyzed)

Reverse Transcription: SuperScript II

1. Determine the maximum amount of RNA that you can put into RT

a. The maximum volume of RNA you can put into RT is 8 µL
b. Ideally, you would like to put 1 µg of RNA into RT, which would be approximately equal to 8 µL of 125 ng/µL RNA
c. If some of your samples are below 125 ng/µL, you need to calculate the maximum amount of RNA you can put into RT for your lowest yield sample (8 µL*x ng/µL= y ng RNA into RT
d. Then do the calculations for what volume of RNA to add to RT for each sample to achieve the same amount of RNA into each RT reaction (y ng RNA into RT/ concentration of sample of interest; round to one decimal place)
e. For standards, you want to put as much RNA into RT as you can for standard 1 (aim for 1.5 µg)

2. Mix and briefly centrifuge Components of the kit before use
3. For each sample, combine the following in a Lo-Bind tube (labeled cDNA)
    a. n µL diluted RNA
    b. 1 µL oligo dT primer
    c. RNAse free water to 9 µL
    d. 11 uL of the 2x reaction mix
    e. See "Solution Maker" excel file for calculations

2x Rxn mix (per sample)	
Component	Amount (uL)
10 mM dNTP mix	1
10x RT buffer	2
25 mM MgCl2	4
0.1 M DTT	2
RNAse OUT	1
Superscript II	1

4. After adding 11 µL of the 2X reaction mix to each RNA/primer mix, mix gently and collect by centrifugation
5. Incubate at 42º C for 50 min
6. Terminate the reaction at 70º C for 15 min. Chill on ice.
7. Collect the reaction by brief centrifugation
8. Dilute samples and standard 1 1:5 by adding 80 µL RNAse free H_2O, flick to mix, and centrifuge to collect
9. Store at -20º C

Standards

1. Retrieve Standard 1
2. Create a standard curve by diluting 1:5 with H_2O
3. Flick to mix and centrifuge to collect after each dilution
4. Repeat for remaining standards:

Standard #	Dilution Factor	Dilutions	Value
Std 1	Pool (diluted 1:5)	Diluted After RT	1
Std 2	1:5	20 µL Std 1 + 80 µL H_2O	0.2
Std 3	1:25	20 µL Std 2 + 80 µL H_2O	0.04
Std 4	1:125	20 µL Std 3 + 80 µL H_2O	0.008
Std 5	1:625	20 µL Std 4 + 80 µL H_2O	0.0016
Std 6	1:3125	20 µL Std 5 + 80 µL H_2O	0.00032
Std 7	1:15625	20 µL Std 6 + 80 µL H_2O	0.000064
Std 8	1:78125	20 µL Std 7 + 80 µL H_2O	0.0000128

Primers

1. To make stock solution:
    a. Dilute to 10 µM
        i. See "Solution Maker" excel file for calculations
        ii. In most cases you will dilute a 100 µM solution 1:10
        iii. 9 µL of H_2O + 1 µL concentrated primer
    b. Flick to mix
    c. Centrifuge to collect

SYBR Green Master Mix (per gene)

1. Create Master Mix for each gene (See "Solution Maker" excel file for calculations)

Component	**Volume (µL)**
SYBR Green (add last)	10
Forward Primer (**diluted**)	0.6
Reverse Primer (**diluted**)	0.6
H_2O	4.8
TOTAL	**16**

*Make enough for 10% more than your number of samples

2. Add 16 µL SYBR Green Master Mix to each well
3. Add 4µL standard/sample to appropriate well
4. Check to make sure there are no bubbles in the bottom of wells
    a. If there are, spin the plate briefly and re-check
    b. Bubbles on the top of the liquid are OK, as they will pop as the temperature increases during PCR
5. Cover with plate cover and heat seal
6. Perform PCR on thermal cycler
    a. Use the "CML Sybr" Protocol on the 384 well thermal cycler
        i. 95º C denaturation step, 5 min
        ii. 40 cycles of 15 sec at 95º C followed by 1 min at 60º C

6.12. Cutting Muscle using the Cryostat

Before you begin

Go to the Cardoso lab and sign up for the time you want to use the cryostat. During heavy use times (e.g. before ASAS abstracts are due) you may have to reserve the cryostat several days in advance.

List of Items to Bring to the Cryostat:

1. Dry ice in a cooler + Samples
2. Gloves
3. Razors for cutting mounted sample
4. Cryostat blade(s) (Leica, Low Profile)
5. Light Microscope
6. OCT gel
7. Tweezers
8. Freeze spray
9. Kim wipes
10. Practice slides
11. Sample slides
12. Pencil or fine point sharpie
13. Timer
14. Box for drying slides

Important Things to Remember:

- NEVER move the angle of the stand outside of its normal range. On the left side of the stand where you change the angle, there is a grey mark on the base and numbers from 0-10º on the stand. The grey mark MUST stay within the range on the stand, or the block can hit the stand. If the machine ever makes a grinding noise while you are cutting, check the angle on the stand to make sure you are within normal ranges and you are not hitting the stand with the block.
- NEVER turn the dial for changing sectioning width lower than 0 or higher than 60. If you want to go from 20 to 7 move the dial counter clockwise from 20 to 7. If you want to go from 7 to 20 move the dial counterclockwise from 7 to 20. Do not move the dial clockwise from 7 to 20 or counterclockwise from 20 to 7. This will break the mechanism and you will not be able to advance the stage.
- NEVER touch the samples with your fingers. Only touch them with COLD tweezers. Touching the sample with anything that is warmer than -20º C will cause it to thaw.

When the sample re-freezes, it will freeze fracture and become unusable. It's safe to assume that WE CAN NEVER GET THAT SAMPLE BACK.

- Always keep your samples on dry ice when you are not working with them or warming them to -20 º C in the cryostat
- Always make sure the wheel for sectioning the tissue is locked when your hands are in the cryostat.
- Remember that the sectioning blade is very sharp. When your hands get cold in the cryostat, you may not even feel it if you cut yourself on the blade, so be very careful not to touch it.
- After you section a sample, remember to bring the wheel back to the top. If you don't the next section will be a bit off.
- To save time, you may want to bring your next sample to -20 when you begin to cut a sample (remember, it takes at least 20 minutes to come to temperature).
- To save time, you may want to mount your next sample (assuming you have 2 mounting wheels) before you begin cutting your current sample. However, do not bring more samples to temperature or mount more samples than you actually plan to do in a day. This wastes time and compromises sample integrity.
- Remember, you have to wait 1 hour for samples to dry before you can store them in the freezer, so you will have to quit an hour before you have to leave the lab, unless there is someone else to put the samples away for you.

Setting Up

1. As soon as you get to the lab, put your first two samples in the cryostat to allow them to come to temperature (-20º C)
    a. Set a timer for 20 min
2. Put your razor blades, tweezers and cryostat blades in the cryostat to allow them to come to temperature.
3. Unlock the cryostat functions by holding the lock button (looks like a key) until the colon between the numbers on the time of day appears and does not blink.
4. Turn on the light in the cryostat by pressing the light bulb button
5. Sign in on the sign in sheet with your name, the date and time, and Dr. White's name as the PI
    a. Note: You may have already signed UP to use the cryostat, but you must actually sign IN when you go to use the cryostat
6. Set out your light microscope and plug it in
7. Take out one practice slide and label it "practice"
8. Place plexiglass on top of the -80º C freezer to prevent air from blowing directly into the cryostat.
9. Take out as many duplicates of your sample slides as you will need (3-5), and label them appropriately
10. Take your first sample out of the tube and determine which direction the fibers are going, and which way you would like to place it.

    a. Note: you probably want to use the side with the least OCT on top and the largest number of tissue punches aligned evenly to prevent having to cut through a lot of sample/OCT before you reach a usable section.

11. Put a small amount of OCT on the first pedestal, quickly place your sample in the OCT, and use the tweezers to hold the sample in place while you use the freeze spray to solidify the OCT.
12. Build OCT up around the sample all the way to the top by alternately adding OCT and freeze spraying it.

Cutting the Tissue

1. Place your blade in the stand and use the lever on the left side to secure it.
2. Place the pedestal with your sample into the holder and secure it by tightening the knob on the left side of the holder.
3. Use the arrow buttons on the left side of the cryostat to position the sample to where it is nearly touching the cryostat blade
    a. Note that if you get it too close and cut a large piece of the sample off on the first revolution, it may cause the sample to break. This will waste sample and you will have to remount the remaining tissue.
4. Use the knob on the inside of the cryostat to adjust the cutting width to 20µm
    a. NOTE: DO NOT move the dial across the 0-60 range. go clockwise to decrease the width or counterclockwise to increase the width.
5. Unlock the handle on the right side of the cryostat, and section the sample until there is enough sample sectioned to analyze (usually about 3-4 biopsy needles wide)

6. When you have enough sample being sectioned, correct the cutting width to 7 µm, cut 3 sections, and then flip down the plate on the left side of the sectioning stand and cut a section
7. With brushes in hand, flip open the plate and smooth the section before it curls
    a. Note: Some samples curl very quickly, so make sure you are ready the brush it as soon as you lift the plate.
8. After the sample is smooth and stable, position the practice slide over the sample and allow it to “jump” onto the slide
    a. Note: You may have to get very close to the section to get it onto the slide but DO NOT touch the slide to the stand, as you may smear the sample
    b. Note: if the sample does jump onto the slide, you can use your finger to warm the back of the slide
9. Take the sample to the light microscope and bring it into focus. Check for
    a. Fibers in cross section
    b. Freeze fracture
    c. Adequate number of fibers (At least 50 in cross section)
10. If the sample is in cross section, use the “sample” slides to collect the appropriate number of replicates of the sample
    a. Note: It has been suggested that after you cut a section, the plate should be lifted before the sample is brought back up in order to avoid hitting the sample with the plate.

11. If the sample is not in cross section, use the knobs on the left side of the stand to adjust the angle of the stand, and re-section using the "practice" slides until the sample is in cross
    a. NOTE: The range must be within 0-10º. Do not change the angle outside of that range or you may hit the stage with the block and damage the machine.
12. If the sample is freeze fractured, give up and go complain to your PI that we need competent people to process OCT samples.

Removing Samples from the Pedestal and Cleaning Up

1. After you have finished your last sample, set the timer in the cryostat box for 1 hr
2. To remove samples from the pedestal, use the razor blades to cut excess mounting OCT off the sides, and then dislodge the sample from the pedestal.
    a. Note: Even after the sample has been removed from the pedestal, excess OCT may have to be removed to fit it back into the tube. When removing excess OCT, be careful not to cut off parts of the sample. If you do cut off parts of the sample (no matter how little), place them back in the tube.
3. Place sample tubes back on dry ice
4. Clean the inside of the cryostat using big brushes
    a. All of the excess OCT/sections should be swept into the tray below the blade, and the tray can be quickly dumped in the trash
        i. Note: If you take too long, the OCT in the tray will melt, making it very difficult to clean. In the event that happens, you must clean it. DO NOT leave it for other people to deal with.

5. **TAKE THE PLEXIGLASS OFF THE TOP OF THE -80º C freezer!!!!!!!!!!!**
6. Use your cryostat list to collect all the items you brought into the lab
7. Sign out on the cryostat log
8. Place the samples back on dry ice and transport back to our lab and store at -80º C immediately
9. Allow the sections to dry for 1 hr (you should already have a timer running for that) and then store in a slide box at -20º C

6.13. Muscle Fiber Type

1. Take muscle sections out of freezer, make sure the slides are dry and draw a circle around the sections with a PAP pen (Vector cat#H-4000)
    a. Draw the circles as close to the section as possible, preferably around each individual section. DO NOT get PAP circle on the sample, as that part of the sample will not stain.
2. Rehydrate sections with PBS 2 times for 5 min
    a. Pipette 1X PBS to cover each section and incubate 5 min rocking at RT
    b. Shake the PBS off the sections and re-cover each section with 1X PBS, incubate for 5 min rocking at RT
3. While sections are rehydrating, make 1º the antibody mixture (amounts below are per slide, see the “Solution Maker” excel file for calculation)
    a. 225 µL 6H1-s (IgM; DSHB) +
    b. 2.25 µL BA.D5-c (IgG2b; DSHB) +
    c. 2.25 µL SC.71-c (IgG1; DSHB)

4. Incubate the sections in 1º antibody mixture for 90 min rocking at RT or overnight at 4º C
    a. Note: Do not rock the samples overnight at 4º C, as it may lead to evaporation of the antibody solution, which will compromise the staining
5. Wash 3 times for 5 min in 1X PBS
    a. Pipette 1X PBS to cover each section and incubate 5 min rocking at RT
    b. Shake the PBS off the sections and re-cover each section with 1X PBS, incubate for 5 min rocking at RT
    c. Shake the PBS off the sections and re-cover each section with 1X PBS, incubate for 5 min rocking at RT
6. While sections are being washed, make 2º antibody mixture (amounts below are per slide, see the "Solution Maker" excel file for calculation)
    a. NOTE: Secondary antibody mixtures must be made and incubated IN THE DARK. Exposure to fluorescent light quenches the signal. Turn off the light in the back of the lab when you start making your 2º antibody mixture and do not turn it back on until your slides and the secondary antibodies are safely in a closed box where they will not be exposed to fluorescent light. Make 2º antibody mixture in the brown tubes in the tube cabinet.
    b. 500 uL 1X PBS +
    c. 2 uL Alexa Fluor (AF) 555 (IgM; 1:250; Invitrogen cat # A21426)
    d. 2 uL AF 647 (IgG2b; 1:250; Invitrogen cat # A21242)
    e. 1 uL AF 488 (IgG1; Invitrogen cat # A21121)
7. Incubate the sections in 2º antibody for 60 min rocking at RT

8. Wash 3 times for 5 min in 1X PBS
    a. Pipette 1X PBS to cover each section and incubate 5 min rocking at RT
    b. Shake the PBS off the sections and re-cover each section with 1X PBS, incubate for 5 min rocking at RT
    c. Shake the PBS off the sections and re-cover each section with 1X PBS, incubate for 5 min rocking at RT
9. Add 1-2 drops Vectashield mounting media (Vector cat # H-100) to the slide and carefully slowly place a cover slip over the slide, allowing the mounting media to create a film under the slide. If bubbles form over the samples, remove the cover slip and try again.
10. Place the slides in a box with a kimwipe in the bottom (to prevent getting a bunch of mounting media in the bottom of the box) and place the box in the fridge. Be careful not to disrupt the cover slip when placing the slides in the box.
    a. Boxes should be labeled (put a piece of tape on them) with your initials and the date they were stained.