EFFECTS OF DECREASED DIETARY VITAMIN E PLUS A PROPRIETARY ANTIOXIDANT BLEND ON MITOCHONDRIA IN YOUNG PERFORMANCE HORSES

A Thesis

by

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ABSTRACT

Mitochondrial health is pertinent to optimizing athletic performance and is maintained in part by dietary antioxidants such as selenium (Se) and vitamin E (vitE). Mitochondrial adaptations to elevated dietary Se coupled with decreased vitE have not yet been determined. Young Quarter Horses (mean ± SEM; 17.6 ± 0.2 mo) were used to test the hypothesis that horses receiving a proprietary antioxidant blend containing Se yeast (EconomasE, Alltech, Inc., Nicholasville, KY) would have improved mitochondrial characteristics compared to horses receiving Se and vitE at current requirements, regardless of reduced vitE intake. Horses were balanced by age, sex, BW, and farm of origin and randomly assigned to one of three custom-formulated concentrates fed at 1% BW (DM basis): 1) 100 IU vitE/kg DM and 0.1 mg Se/kg DM (CON, n = 6), 2) no added vitE plus EconomasE to provide 0.1 mg Se/kg DM (ESe1, n = 6), or 3) no added vitE plus EconomasE to provide 0.3 mg Se/kg DM (ESe3, n=6). Tissue was collected from the gluteus medius at wk 0 and 12 of dietary treatment and evaluated for mitochondrial enzyme activities by kinetic, colorimetry and mitochondrial capacities by high-resolution respirometry. Data were analyzed using PROC MIXED in SAS v9.4 with repeated measures (time) and fixed effects of time, diet, and time × diet; horse(diet) served as a random effect. Mitochondrial number (citrate synthase activity; CS), function (cytochrome *c* oxidase activity; CCO), and integrated (per mg tissue) oxidative (P) and electron transport (E) capacities increased from wk 0 to 12 in all horses ($P \leq 0.05$). Intrinsic (relative to CS) CCO activity and P and E capacities, decreased from wk 0 to 12 ($P \leq 0.02$). Horses in CON had higher integrated P with complex I and II substrates (P_{CI+II}), E_{CI+II}, and E_{CII} than ESe1 throughout the study ($P \leq 0.03$); integrated E_{CII} was also higher in CON than ESe3 ($P = 0.03$). Results from the current

study suggest that feeding EconomasE to provide 0.3mg Se/kg DM may prevent adverse effects of removing 100 IU dietary vitE/kg DM on mitochondria in young horses.

DEDICATION

I dedicate this thesis to my husband, John. Thanks for your endless support.

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CHAPTER I

INTRODUCTION

Mitochondria are an essential component of proper energy production during exercise. Maintenance of healthy muscle energetics is critical to prevent fatigue and potential injury, especially during times of elevated stress such as growth or exercise (Brooks et al., 2005). A by-product of normal metabolism, reactive oxygen species (ROS) are produced by mitochondria, and ROS production increases during exercise (Brooks et al., 2005). Reactive oxygen species function as signaling molecules for muscle adaptations (Powers et al., 2011). However, the exercise-induced increase in ROS can lead to oxidative damage of cellular components if not regulated by antioxidants, which are primarily provided through the diet. Exercise training has also been shown to combat the production of ROS (Hodgson et al., 2014). Studies in rat skeletal muscle showed a decrease in mitochondrial uncoupling after endurance training (Zoladz et al., 2016). The improved mitochondrial coupling enhances the efficiency of energy production and decreases the opportunity for free electrons to become damaging ROS intermediates (Bratic and Larsson, 2013).

Among others, dietary selenium (Se) and vitamin E (vitE) are potent antioxidants necessary to mitigate elevated ROS production during exercise (White et al., 2016; Brigelius-Flohé and Traber, 1999), which is essential for maintaining mitochondrial health. However, high levels of antioxidants have been shown to limit cellular adaptations to training (Peterneli and Coombes, 2011). Interestingly, previous research noted elevated mitochondrial density in the gluteus medius muscle of 2-year-old horses receiving organic Se at 0.3 mg Se/kg DM (as Sel-Plex, Alltech Inc., Nicholasville, KY) compared to horses receiving 0.1 mg Se/kg DM, regardless of whether or not they were enrolled in a submaximal exercise program (White et al.,

2017). The current NRC (2007) requirement for Se is 0.1mg/kg DM, but little research has been conducted to determine the optimal level of Se for growing or exercising horses. Additionally, vitE deficiency in humans has been shown to cause muscle weakness and changes in the immune system (Rizvi et al., 2014). To date, the effects of decreased dietary vitE in conjunction with elevated organic dietary Se on mitochondrial function has not been addressed. There is also limited research on the combined effects of growth and exercise which is essential due to the relatively young age that horses often enter a training program.

Due to the importance of Se as an antioxidant, the objective of this study was to determine the effects of supplementation of a proprietary antioxidant blend containing Se yeast on skeletal muscle mitochondria in 2-year-old Quarter Horses enrolled in a submaximal exercise training program. We hypothesized that increased levels of the Se yeast antioxidant blend would enhance skeletal muscle mitochondrial oxidative capacity.

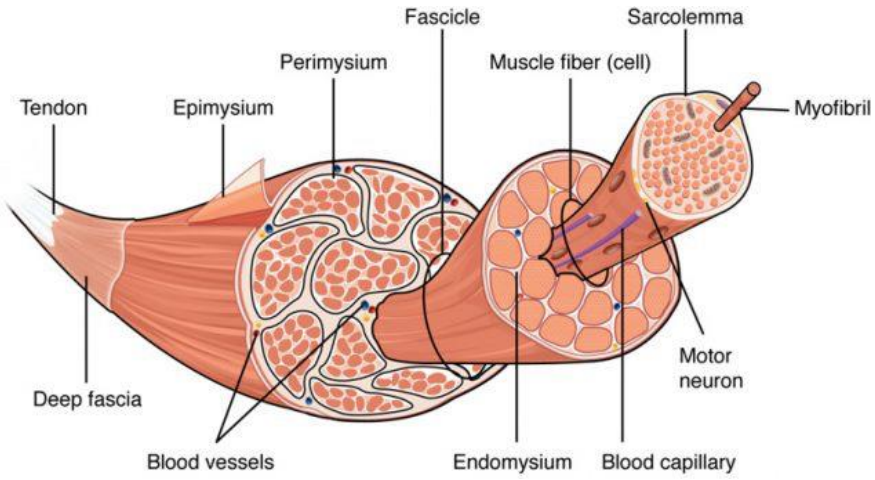
CHAPTER II

REVIEW OF THE LITERATURE

Introduction to Skeletal Muscle

Skeletal muscle makes up approximately 55% of the body weight of the horse, which is a greater percentage than in most mammals (Hodgson et al., 2014). Horses have over 700 muscles making up the deep and superficial musculature (Hodgson et al., 2014). Skeletal muscle has several functions: movement, stabilization of joints, support, storage of fuels, and insulation (Brooks et al., 2005). Skeletal muscle is a complex structure. A loose layer of connective tissue surrounds the muscle, groups of muscle fibers, and individual muscle fibers; these tissues are known as the epimysium, perimysium, and endomysium, respectively (Hodgson et al., 2014). The muscle fiber is made up of groups of myofibrils which are composed of thick and thin myofilaments (Brooks et al., 2005). The thick and thin myofilaments are known as myosin and actin, respectively, and repeating units of these myofilaments, or sarcomeres, make up the main contractile unit of muscle (Hodgson et al., 2014). Movement is created by actin-myosin crossbridge cycling which is powered by the hydrolysis of adenosine triphosphate (ATP; Egan and Zierath, 2013). Figure 1A and 1B depict the anatomy of a muscle and the actin myosin cross-bridge cycle respectively.

A



B

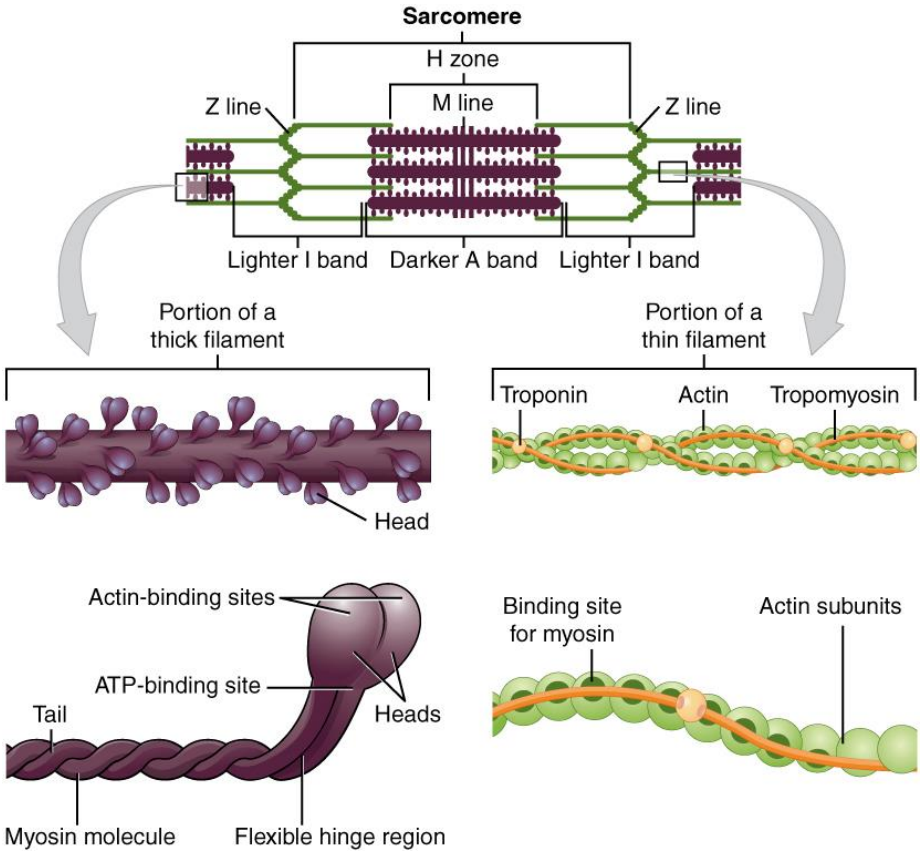


Figure 1. (A) The layers of a muscle from the outermost layer, the epimysium, to the myofibril. Reprinted from Biga et al. (2019). (B) The top image represents a sarcomere, the main contractile unit of a muscle fiber. The left and right image depict myosin and actin respectively. Reprinted from Biga et al. (2019).

Oxidative phosphorylation takes place in the mitochondria and drives the production of ATP (Egan and Zierath, 2013). Skeletal muscle contains many mitochondria. However, certain fiber types have more mitochondria than others (Hodgson et al., 2014). Myosin heavy chains (MyHC) are the major protein making up the thick filaments and the MyHC isoform is the main determinant of fiber type (Serrano et al., 2000). The MyHC isoform changes during development (Serrano et al., 2000). In adult rodent skeletal muscle, four MyHC isoforms exist: type I, type IIa, type IIx, and type IIb (Bar and Pette, 1988; Schiaffino et al., 1989), though in larger mammals such as humans and horses, type IIb is not expressed (Goldspink 1998; Rivero et al., 1999). Type I fibers are the most oxidative, mitochondrial dense, and resistant to fatigue (Brooks et al., 2005). They are also smallest in cross-sectional area, have the slowest contractile speed, and are the least glycolytic (Brooks et al., 2005). Type IIx fibers are the least oxidative, mitochondrial dense, and resistant to fatigue. They are also the largest in cross-sectional area, have the fastest contractile speed, and are the most glycolytic (Brooks et al., 2005). Type IIa fibers fall between type I and type IIx fibers when considering the above characteristics (Brooks et al., 2005). The percentage of each fiber type varies with sampling depth. More superficial muscles will have a greater percentage of type IIx fibers and a smaller percentage of type I fibers when compared to deeper muscles (Hodgson et al., 2014). The varying compositions of muscles at different depths creates a need for a consistent sampling depth and caution when comparing results across studies.

Mitochondria

Mitochondria are known as the powerhouse of the cell, supplying the main source of adenosine triphosphate (ATP). Adenosine triphosphate provides energy the cell needs for many biological functions, including muscle fiber contraction (Kuhlbrandt, 2015). The ATP is

produced from adenosine diphosphate (ADP) and phosphate ions via ATP synthase (complex V of the electron transport system) in the mitochondria (Kuhlbrandt, 2015). Mitochondria also produce NADH, are an integral part of the stress response and cell signaling, and regulate apoptosis (Bratic and Larsson, 2013). Mitochondria are semi-autonomous with their own DNA code and are equipped with their own protein translation system (Gray et al., 1998). Mitochondria are highly complex and dynamic organelles constantly changing their morphology and function (Picard et al., 2011). Mitochondria contain both an outer and inner membrane. In the inner membrane cristae, oxidative phosphorylation and electron transport take place via a set of 5 protein complexes that produce an electrochemical gradient (Bratic and Larsson, 2013). The complexes are as follows: complex I (NADH dehydrogenase), complex II (succinate dehydrogenase), complex III (cytochrome *c* reductase), complex IV (cytochrome *c* oxidase), and complex V (ATP synthase; Kuhlbrandt, 2015). Complexes I, III, and IV are responsible for the inner mitochondrial membrane proton gradient which drives the production of ATP (Bratic and Larsson, 2013). Even though mitochondrial isolation techniques and electron microscopy have helped to discover an extensive array of information, much is still unknown about the intricacies of mitochondrial function. High-resolution respirometry is a novel technique that presents an innovative approach to analyzing mitochondria *in situ,* allowing the mitochondria to interact with the sarcoplasmic reticulum as they would *in vivo* (Picard et al., 2011). Being able to properly measure mitochondrial function is critical to studies involving cellular metabolism.

Introduction to Antioxidants and Reactive Oxygen Species

Several antioxidants work together in a complementary system to protect the cell from reactive oxygen species (ROS), which are primarily produced via the electron transport system during exercise (Niki et al., 1995). Antioxidant defense systems utilize enzymatic and

nonenzymatic defense; the enzymes are responsible for detoxification of peroxides and protection of the cell, while the nonenzymatic defense mechanisms work to directly remove ROS (Borek et al., 1986; Ghazi et al., 2012). Some of the key players in the antioxidant network are selenium (Se)-dependent glutathione peroxidase (GPx), superoxide dismutase (SOD), vitamin E (vitE), vitamin C, and catalase (Finkley and Holbrook, 2000). Superoxide dismutase converts superoxide radicals to hydrogen peroxide, GPx converts hydrogen peroxide to water and glutathione disulfide (Bratic and Larsson, 2013), and catalase converts hydrogen peroxide to water and oxygen (Jenkins, 1988). Additionally, Se and vitamin C have been shown to enhance the antioxidant properties of vitE, which works in the lipid bilayer to prevent the production of reactive oxygen species (Chan, 1993; Ghazi et al., 2012). Antioxidant systems have three layers of defense: prevent ROS from forming, remove ROS that have formed, and repair any damage caused by ROS (Niki, 2014). The speed at which antioxidant genes are activated depends on the stressor (Ji, 2008). For example, an antioxidant will be immediately activated if there is an infection but will be activated slower in the case of aging. Lipid peroxidation is one of the main consequences of free radicals not quenched by the antioxidant defense system, along with protein and nucleic acid damage (Davis, 1987; Kagan, 1988; Breen and Murphy, 1995). The above effects can result in tissue damage, especially in skeletal muscle due to relatively low levels of antioxidants when compared with other tissues (Valberg et al., 1993). Skeletal muscle has fewer antioxidant enzymes due to its lower oxygen consumption at rest (Jenkins, 1993). Oxidative damage can also contribute to aging, cancer, and various other diseases (Harman and Piette, 1966). It is important to note, however, that while potentially damaging in large quantities, ROS play a key role in cellular adaptations to training and maintaining fundamental biological processes (Peterneli and Coombes, 2011).

Skeletal muscle antioxidant systems are known to upregulate in response to exercise, because exercise increases the need for antioxidants due to the increase in oxygen uptake by the muscle, approximately 5% of which ends up as free radicals (Davies et al., 1982). It is also widely accepted that antioxidant defense systems adapt with training. However, it is important to understand that other physiological adaptations such as mitogenesis (Baar, 2004) and fiber type switching (Lin, 2002) occur with exercise training and contribute to changes in the antioxidant defense network. These changes in response to antioxidant supplementation have not been widely studied, though analysis of these changes presents a unique approach to determining the mechanism by which specific skeletal muscle adaptations occur, such as increased mitochondrial density, function, and oxidative capacity.

Selenium

Selenium (Se) is a powerful micromineral that functions as an antioxidant (NRC, 2007). Selenium works as an antioxidant in the form of GPx, the most abundant peroxidase in the mitochondria (Rotruck et al., 1973), and helps to control the thyroid hormone (Hotz et al., 1997). Selenium is linked to the thyroid hormone by deiodinase, which depends on Se, and consequently effects protein synthesis and growth (Geor et al., 2013). Several other selenoproteins including thioredoxin reductases, methionine sulfoxide reductase B, and selenoprotein P and W serve important antioxidant and protective functions (Geor et al., 2013).

As a dietary additive, Se is present in either an organic or inorganic form depending on the feedstuff. Organic Se is present in the form of selenocysteine and selenomethionine, while inorganic Se is commonly in the form of sodium selenite and sodium selenate (NRC, 2007). Organic and inorganic forms of Se are not metabolized the same (Surai, 2006). Organic Se, most commonly selenomethionine, is absorbed via active transfusion and incorporated into tissue

proteins in place of methionine, while inorganic Se is absorbed by passive diffusion and only small amounts are incorporated into tissues (Wolffram, 1999). The absorption rate of Se is approximately 59% of intake, which is high when compared to other trace elements (Pagan et al., 1999; Geor et al., 2013). Muscle, bone, and skin are the major Se stores in the body (Stowe, 1967). There is contradicting evidence regarding the bioavailability of different forms of Se. Studies have found no difference in the bioavailability of organic versus inorganic Se (Podoll et al., 1992; Richardson et al., 2006). However, other studies have found a difference in the bioavailability of varying forms of Se (Pagan et al., 1999, Janicki et al., 2001). Pagan et al. (1999) noted a trend with the apparent absorption of organic Se being greater than that of inorganic Se. Similarly, Janicki et al. (2001) found higher serum Se concentrations in horses supplemented with organic Se compared to the inorganic form. However, a study by White et al. (2016), found an elevated level of the inorganic form of Se, 0.3 mg/kg DM sodium selenite, increased muscle GPx activity and decreased lipid peroxidation 6 hours post-exercise. Serum Se also significantly increased in the group fed 3 times the NRC (2007) Se requirement after 36 days of supplementation when compared to the control group fed Se at requirements (NRC, 2007; White et al., 2016). The differences in these results could be in part due to the weak correlation between Se intake and Se blood concentrations, potentially because of the interaction with other minerals (Hayes et al., 1987). Another explanation for the varied results between studies may be due to levels of Se supplementation, variables measured, or the length of time of the study.

Selenium concentrations in plants is highly influenced by Se concentration in the soil as well as the pH (NRC, 2007). Plants with deep roots, which often deepen during drought conditions, accumulate more Se (Finley, 2005). Selenium absorption is more efficient in

nonruminants than ruminants and therefore Se toxicity presents a concern in horses (Wright and Bell, 1966, Rogers et al., 1990). Ingestion of high levels of Se can cause blind staggers, hair loss, hoof issues, and death (Rosenfeld and Beath 1964; Fan and Kizer, 1990; Desta et al., 2011). Selenium deficiency is also an issue with Se deficiency diseases in livestock being confirmed in 46 states (Edmonson et al., 1993). Selenium deficiency is often characterized by weakness, impaired movement, and respiratory issues (Dill and Rebhun, 1985). Selenium deficiency has been linked to white muscle disease in horses (Lofstedt, 1997). The Se content of horse feeds is not restricted by the Food and Drug Administration (FDA), but commercial feedstuffs commonly contain between 0.01 and 0.03 mg/kg DM (Ullrey, 1992; NRC, 2007). The NRC (2007) sets the recommendation at 0.1 mg/kg DM and states the upper daily Se limit to be 2 mg/kg DM. However, Janicki et al. (2001) found that foals from mares receiving Se slightly above the NRC (2007) requirements had increased influenza antibodies. Additionally, a study in broiler chickens found that elevated dietary Se supplementation significantly increased skeletal muscle GPx activity (Ghazi, 2012). The above studies indicate a potential benefit of increased Se supplementation.

The NRC (2007) does not currently set a Se requirement for growing or exercising horses. A study in untrained Thoroughbreds found that horses receiving 0.3 mg Se/kg DM for 34 d showed a decrease in red blood cell GPx activity from 0 to 24 hours after a submaximal exercise test and an increase in skeletal muscle GPx activity 6 hours post-exercise compared to horses receiving 0.1 mg Se/kg DM (White et al., 2016). Another study by White et al. (2017) found that Quarter Horse yearlings receiving 0.3 mg Se/kg DM had higher citrate synthase (CS) activity, a marker of mitochondrial number, in the gluteal muscle after a 120-minute submaximal exercise bout than horses receiving 0.1 mg Se/kg DM. Future studies are needed to determine the

optimum levels of dietary Se for horses in various intensities of work, growth, and horses growing and exercising simultaneously. Horses typically enter training programs at a young age when substantial growth is occurring, yet little research has focused on the combined effects of growth and exercise.

Vitamin E

Vitamin E (tocochromanols) is a collective group of fat-soluble antioxidants (Niki and Traber, 2012). Vitamin E is an essential dietary nutrient, as it cannot be synthesized by the body of the horse or human (NRC, 2007; Niki and Traber, 2012). It has eight natural forms, with alpha-tocopherol and gamma-tocopherol making up the greatest proportions (Zingg, 2007). Alpha-tocopherol has received the most attention due to its accumulation in non-hepatic tissues, being found most abundantly in cellular and mitochondrial membranes where ROS production is elevated (Rizvi et al., 2014). Alpha-tocopherol inhibits the production of new ROS while gamma-tocopherol neutralizes ROS already present (Rizvi et al., 2014). Reactive oxygen species are mitigated by vitE through the donation of a hydrogen atom by vitE that attaches to lipid peroxyl radicals to form a more stable peroxide (Pryor, 2001).

Vitamin E is primarily found in vegetable oils but is also present in certain nuts, seeds, fruits, and vegetables (Ball, 2005). In order for proper absorption and utilization of vitE, other nutrients must be present, such as vitamin C, vitamin B_3, and Se (Combs et al., 1975; Halpner et al., 1998; Lauridsen and Jensen, 2005; Rizvi et al., 2014). It is also important to consider that the optimum level of vitE intake is relative to the intake of polyunsaturated fatty acids (Geor et al., 2013). It is recommended than an additional 5 to 7 mg vitE/kg $BW^{0.75}$ per day be added to the diet of a horse receiving the maximum recommended intake of supplemental fats, 1 g supplemental fat/kg BW/day (Geor et al., 2013). The majority of vitE is stored in adipose tissue

(Roneus et al., 1986) and vitE has been shown to be essential for skeletal muscle homeostasis due to its role in lipid peroxidation (Howard et al., 2011). It also acts to prevent diseases such as cancer and Alzheimer's, reduce inflammation, and enhance the immune system (Rizvi et al., 2014).

Vitamin E is present in plants, but the levels of vitE drop as the plant matures, is stored, or is processed (NRC, 2007). Levels of naturally occurring vitE vary widely in horse feeds (Lynch, 1996b). Due to instability of vitE and the varied concentration in feeds, ester forms are typically added to feeds in the form of all-rac-α-tocopheryl or RRR-α-tocopheryl (Gassman and Ulbricht, 1979).

While a vitE deficiency is rare in humans, livestock may face issues with intaking sufficient levels of vitE, especially if engaged in exercise training. Work in humans and rodents has shown that exercise decreases vitE levels in the blood (Bowles et al., 1991; Meydani et al., 1993). Studies in horses engaged in exercise training indicated that between 80 to 300 IU/kg DM is needed to maintain blood and skeletal muscle vitE concentrations (Saastamoinen and Juusela, 1993; Siciliano et al., 1997). In rodents and zebra fish, vitE-deficient diets have been shown to negatively impact mitochondrial capacity and function (Mabalirajan et al., 2009; Navarro et al., 2011; McDougall et al., 2017). In contrast, several studies in exercised horses have shown no negative effects of vitE-deficient diets on skeletal muscle integrity (McMeniman and Hintz, 1992; Siciliano et al., 1997). While differences in muscle integrity were not detected, Siciliano et al. (1997) did find that greater than 80 IU vitE/kg DM is needed to maintain vitE blood and skeletal muscle concentrations. Additionally, a study in 3-year-old race horses found that 20 μg/kg DM per day of Se and 40 mg/kg DM of vitE per day may not be sufficient for horses undergoing high intensity or chronic exercise (Avellini et al., 1999). Studies in humans have

found that vitE supplementation reduced levels of MDA present after exercise (Dillard et al., 1978; Sumida et al., 1989). Vitamin E deficiency can also worsen preexisting muscular disorders (Amelink et al., 1991). White muscle disease and equine motor neuron disease have been linked to vitE deficiency in horses (Schougaard et al., 1972; Divers, 1994). Vitamin E toxicity should also be considered as it can lead to impaired bone mineralization. However, vitE toxicity is rare in humans and horses (Geor et al., 2013). The NRC (2007) sets the upper safe limit at 1000 mg/kg DM.

Costs of manufacturing vitE to incorporate into feedstuffs is rising due a shortage of citral, a precursor in manufacturing, and Chinese environmental protection measures. Due to the costs and environmental impact of vitE, EconomasE™ (a proprietary antioxidant blend; Alltech Inc., Nicholasville, KY) has been developed in an attempt to best utilize the vitE that is present in feedstuffs and provide a similar cellular response. Currently, the maintenance vitE recommendation for horses is 50 IU/kg DM and the recommendation of growing or exercising horses is 80 IU/kg DM (NRC, 2007). Very few studies concerning the vitE requirements of growing horses have been conducted.

Biomarkers

Biomarkers are any substances or processes that provide an objective measure which can predict the likelihood of biological functions and properties (World Health Organization, et al., 2001; Strimbu and Tavel, 2010). Mitochondria are essential to maintaining skeletal muscle health as they are responsible for supplying ATP to the muscle via oxidative phosphorylation (OXPHOS) and the electron transport system (ETS; Li et al., 2016). Markers of mitochondrial function can be used to measure mitochondrial density, overall mitochondrial function, uncoupled OXPHOS and ETS, and individual mitochondrial complexes (Joseph et al., 2012,

Larsen et al., 2012; Jacobs et al., 2013). Markers of the mitochondrial function are essential to diagnosing and treating diseases and disorders, as well as understanding the effects of exercise and aging. Several biomarkers of mitochondrial OXPHOS and ETS are commonly used. However, this wide array of biomarkers produces varied results. Among these are CS and cytochrome *c* oxidase (CCO) activities (Spinazzi et al., 2012). Larsen et al. (2012) tested several commonly used mitochondrial biomarkers and found CS to be highly correlated with mitochondrial number determined by electron microscopy and CCO to have a strong correlation to mitochondrial oxidative phosphorylation capacity. These assays are widely used due to the relatively simple protocol, small amount of sample needed, commonly used laboratory equipment, and ability to freeze samples for later use (Spinazzi et al., 2012). Skeletal muscle is the most commonly used tissue due to its high metabolic rates and ease of collection (Spinazzi et al., 2012). A novel approach that allows for several highly specific mitochondrial biomarkers to be tested is high-resolution respirometry (HRR; Li et al., 2016). High-resolution respirometry is a sensitive test that can provide a better understanding of mitochondrial respiration and pathology by isolating the mitochondrial complexes and uncoupling OXPHOS and ETS to allow for separate evaluation in a stepwise protocol (Pesta and Gnaiger, 2012). However, the HRR protocol requires equipment and expertise not available in most laboratories and fresh tissue must be analyzed within 24 hours of collection.

Citrate Synthase

Citrate synthase plays a key role in the tricarboxylic (TCA) cycle within the mitochondria by catalyzing the reaction of acetyl-coenzyme A with oxaloacetate which results in the formation of citrate (Weigand and Remington, 1986). Citrate synthase has a high specificity to oxaloacetate and acetyl-coenzyme A providing it protection from denaturation by other substances present

(Weigand and Remington, 1986). Due to citrate synthase being a prominent mitochondrial enzyme, it has been validated by Larsen et al. (2012) as a biological marker of mitochondrial density. Citrate synthase is an important biomarker because not only does it give a measure of mitochondrial number, it provides a means to calculate intrinsic properties of the mitochondria. Intrinsic properties allow for a measure of individual mitochondrial characteristics as opposed to integrated (relative to wet weight) characteristics which may be skewed by the relative numbers of mitochondria. For example, a study in humans found that maximal mitochondrial respiration in the vastus lateralis increased after 4 weeks of sprint interval training but CS was unchanged throughout the study (Granata et al., 2015). Mitochondrial content is also known to differ across muscle groups. A study in young (1.8 ± 0.1 yr) and aged (17 to 25 yr) Quarter Horses found that CS activity in the triceps brachii was 2 times greater than in the gluteus medius regardless of age (Li et al., 2016). This finding was further corroborated by analysis of MyHC isoforms in the gluteus and triceps that found that the triceps has a greater amount of type I fibers and fewer type IIx fibers when compared to the gluteus (van den Hoven et al., 1985; Li et al., 2016). Another study in sedentary men found greater CS activity in the gastrocnemius compared to the vastus lateralis (Houmard et al., 1998).

Cytochrome *c* Oxidase

Cytochrome *c* oxidase is an important mitochondrial enzyme that catalyzes the reaction of oxygen to water and is coupled with proton pumping (Yoshikawa et al., 1998). It is a multi-subunit, membrane-bound terminal enzyme in the electron transport system (Rousseau and Han, 2002). Cytochrome *c* oxidase activity is a strong indicator of OXPHOS capacity (Larsen et al., 2012). It gives a measure of mitochondrial complex IV activity, which is determined by the oxidation of cytochrome *c* from the flow of electrons through cytochrome oxidase (Gianni et al.,

2004). Cytochrome *c* oxidase catalyzes the oxidation of cytochrome *c* and the reduction of oxygen (Hu and Wang 2016). These reactions are closely tied to energy production and interference with cytochrome *c* oxidase activity leads to build up of ROS (Ogbi et al., 2004). Activity of CCO varies between muscle groups. The triceps has been shown to have lower intrinsic CCO activity when compared to gluteus in the horse (Li et al., 2016; White et al., 2017).

High-Resolution Respirometry

High-resolution respirometry is an innovative technique that allows for evaluation of subcellular and cellular bioenergetics that are beyond the scope of the above-mentioned spectrophotometric assays (Kuznetsov et al., 2008; Gnaiger 2009; Pesta and Gnaiger, 2012). Performing this analysis in permeabilized, intact muscle fibers allows for evaluation of individual and combined mitochondrial complexes, as well as preserves the interaction of mitochondria with other cellular organelles (Kuznetsov et al., 2008; Gnaiger 2009; Pesta and Gnaiger, 2012). This provides an advantage over mitochondria isolation techniques where a large tissue sample is also required. Cytochrome *c* is used in HRR to test for outer mitochondrial membrane integrity because cytochrome *c* cannot travel through the intact membrane (White et al., 2017). Results obtained from the assay are further normalized to CS activity (marker of mitochondrial number) to determine individual mitochondrial quality (Pesta and Gnaiger, 2012). High-resolution respirometry has been shown to be a pertinent tool in the diagnosis of neuromuscular disorders in humans (Rustin et al. 1994; Kunz et al., 1993). A study in children with varying neuromuscular disorders found that HRR was able to detect mitochondrial defects and decreased oxidation rates in diseased children (Sperl et al., 1997). A study in aged horses found that mitochondrial respiratory capacity, measured by HRR, remained similar to young

horses, but aged horses showed a decline in mitochondrial number (CS activity) and function (CCO activity; Li et al., 2016).

Skeletal Muscle Adaptations to Exercise and Training

Skeletal muscle is dynamic and has repeatedly been shown to change with exercise. Endurance and strength training result in different muscular adaptations. However, strength training is difficult to isolate in horses. Aerobic exercise training results in a greater percentage of type I and IIa and a decline in the amount of type IIx fibers (Hodgson et al., 2014). High-intensity anaerobic exercise results in a greater percentage of IIa fibers at the expense of IIx fibers (Hodgson et al., 2014). A study in 4-year-old Andalusian horses found that the percentage of type IIx fibers significantly decreased after 3 and 8 months of training, while the percentage of type IIa fibers significantly increased when compared to pretraining values (Serrano et al., 2000). Exercise training has also been shown to increase the recruitment of type IIa fibers in aged individuals, slowing the shift to type I fibers that occurs with age (Kim et al., 1985).

The majority of the studies focused on the adaptations of skeletal muscle to exercise are conducted over a relatively short period of time, possibly not long enough for full training adaptations to take place. Muscle plasticity is dependent on the half-life of myofibrillar proteins and therefore turnover of slow MyHC isoforms is likely slower than fast MyHC isoforms (Williams and Neufer, 2011; Serrano et al., 2000). Future studies investigating the changes in skeletal muscle over a longer duration of time and at varied intensities are needed to better understand the mechanisms by which changes in the skeletal muscle occur.

Lipid peroxidation products, a marker of oxidative damage, have been shown to increase after exercise (Brooks et al., 2005). Lipid peroxidation occurs when ROS degrade the polyunsaturated fats in the lipid membranes of cells (Mylonas and Kouretas, 1999). A study in

old Quarter Horse mares between 23 and 30 years of age found that there was evidence of a five-fold increase in muscle damage, determined by ultrasound, in the semimembranosus after an exercise test but there was a reduction in damage after 10 weeks of exercise training for approximately 20 minutes per exercise bout 3 days per week on the treadmill (Kim et al., 2005). Avellini et al. (1999) found that horses trained for 70 days had lower plasma malondialdehyde (MDA) concentrations, a marker of lipid peroxidation, following an exercise bout than untrained horses. In contrast, White et al. (2017) found that muscle MDA concentration was not affected by prior training. Exercise also causes mitochondrial damage. A study in human marathon runners reported mitochondrial damage after intense exercise as measured my electron microscopy (Hikida et al., 1983). Skeletal muscle oxidative damage is more likely to occur after an intense exercise bout in untrained subjects, because the amount of oxidative damage is greater than the antioxidant defense system can handle (Witt et al., 1992). The differences in these results may be due to the intensity of the exercise bout, level of training, and muscle group sampled.

The quantity of antioxidant enzymes has been linked to the oxidative capacity of skeletal muscle when compared among various muscles (Jenkins, 1988). It has also been shown that exercise training induces changes that leave the muscle better equipped to handle free radicals (Salminen and Vihko, 1983; Armstrong and Phelps, 1984). A study in rodents found that concentrations of superoxide dismutase, catalase, and glutathione peroxidase (GPx) were greatest in more highly oxidative muscles (Laughlin et al., 1990). Sedentary and exercised rats had greater concentrations of the above enzymes in soleus (slow twitch oxidative) and red gastrocnemius (fast twitch oxidative) when compared to the white gastrocnemius (fast twitch glycolytic; Laughlin et al., 1990). This is expected because mitochondria, which are in greater

concentration in oxidative fibers, are the primary producers of free radicals during exercise. Other skeletal muscle antioxidant enzymes correlate with exercise training. Muscle GPx activity tends to be greater after exercise training (Kanter et al., 1985; Laughlin et al., 1990). Succinate dehydrogenase, an enzyme that functions as a part of the Kreb's cycle and electron transport system, activity was shown to increase in type IIa and IIx fibers after 3 and 8 months of training in horses (Serrano et al., 2000).

Mitochondrial density and size increase in response to aerobic exercise training (Morgan et al., 1971, Booth and Baldwin, 2011). Half-lives of mitochondrial proteins are approximately one week (Booth, 1977) and mitochondrial density has been shown to increase between 50 to 100 percent after 6 weeks of exercise training (Hood, 2001). A study in rats determined that respiratory capacity of the gastrocnemius muscle, determined by oxygen uptake in whole muscle homogenates, CS activity, and CCO activity increased in relation to the length of time of the daily training sessions (Fitts et al., 1975). Mitochondrial markers were also positively correlated with the length of time the rats could run before exhaustion (Fitts et al., 1975). A study in horses found that after 3 and 8 months of training, CS activity increased significantly (Serrano et al., 2000). In this same study, intramuscular glycogen and triglyceride levels significantly increased after training (Serrano et al., 2000). Increased glycogen and triglyceride levels are advantageous to the horse because the horse has more substrate available to utilize for energy. Mitochondrial volume increased more with endurance style exercise than strength training and has been shown to increase throughout at least 34 weeks of training in horses (Tyler et al., 1998). Increases in mitochondrial volume parallel increases in VO_{2max} (Tyler et al., 1998). The parallel increase in VO_{2max} and mitochondrial volume supports the idea that mitochondria play a primary role in energy production and oxygen utilization.

Conclusion

In conclusion, skeletal muscle health in the horse is of vital importance due to the ever-increasing demands being placed on performance horses. Skeletal muscle health is maintained in large part by mitochondria. Mitochondria are responsible for the production of energy via ATP and the regulation of ROS. Antioxidants are known to be a key component in the regulation of ROS. Selenium is a powerful antioxidant that is a main player in the mitigation of ROS. However, the optimum levels of Se in the equine diet of growing or exercising horses has yet to be established. Furthermore, the potential to replace vitE with increased levels of organic Se should be considered due to the high costs of vitE and environmental effects of vitE production. High-resolution respirometry presents a novel approach to evaluating mitochondria in various models. This innovative technology can be of use to veterinarians or horse owners for diagnosis and treatment of muscular disorders, or to a trainer seeking to compete at the highest level.

CHAPTER III

MATERIALS AND METHODS

Horses

Eighteen Quarter Horses (9 fillies and 9 colts) entering their two-year-old year (mean ± SEM; 17.6 ± 0.2 mo) with starting BW of 365 ± 9 kg were used in this trial. Horses originated from two sources: 9 were born and raised at Texas A&M University Dick Freeman Arena (College Station, TX) and 9 were leased from Birdsong Farms (Hearne, TX). Prior to the beginning of the trial, horses from Birdsong Farms were relocated to Freeman Arena, where all horses remained throughout the 12-wk trial. For at least 6 mo prior to the initiation of the study, all horses were maintained on a diet consisting of ≤ 0.1 mg Se/kg DM. Four d/wk, horses were housed in stalls from 0700 to 1700; otherwise, all horses were housed by sex on approximately 0.5-hectare dry lots. All care, procedures, and handling of animals was reviewed and approved by the Institutional Animal Care and Use Committee at Texas A&M University (AUP 2016-0294).

Dietary Treatments

Horses were balanced by age, sex, BW, and farm of origin, and randomly assigned to receive one of three custom-formulated concentrate grains (McCauley Bros, Inc., Versailles, KY) for 12 wk: 1) 100 IU vitE/kg DM and 0.1 mg Se/kg DM (**CON**, n = 6), 2) no added vitE plus EconomasE™ (Alltech, Inc., Nicholasville, KY) to provide 0.1mg Se/kg DM (**ESe1**, n = 6), or 3) no added vitE plus EconomasE™ to provide 0.3mg Se/kg DM (**ESe3**, n = 6). Diets were designed to meet or slightly exceed requirements for growing horses in light work (NRC, 2007). Nutrient analysis was performed on all feedstuffs before the initiation of the trial by Equi-

Analytical (Ithaca, NY). The nutrient composition of each concentrate is presented in Table 1. Due to potential variations between laboratories, feedstuffs were analyzed for Se content by three independent laboratories [Equi-Analytical, Eurofins (Desoto, TX), and New Jersey Feed Lab (Trenton, NJ)], and vitE was determined by Eurofins and New Jersey Feed Lab using standard wet-chemistry analytical methods.

Table 1. Nutrient composition of custom-formulated treatment concentrates offered to 2-year-old horses.

Nutrient[1]	CON[2]	ESe1[2]	ESe3[2]
DE, Mcal/kg	3.10	1.45	3.23
CP, %	13.70	13.70	13.30
ADF, %	11.20	11.80	10.80
NDF, %	25.20	20.30	18.80
Starch, %	33.80	32.40	33.60
Crude Fat, %	5.30	5.30	5.10
Ca, %	1.38	1.47	1.58
P, %	1.10	1.08	1.13
Mg, %	0.37	0.37	0.37
K, %	1.10	1.11	1.11
Na, %	0.28	0.30	0.32
Cl, %	0.51	0.52	0.51
S, %	0.25	0.26	0.26
Fe, ppm	330	342	383
Zn, ppm	109	111	101
Cu, ppm	36	35	38
Mn, ppm	120	129	118
Co, ppm	0.33	0.50	0.40
Se, ppm	0.34	0.32	0.89
VitE, mg/100g	23.6	4.1	3.3

[1] Values presented on a 100% DM basis.
[2] CON = control diet, 0.1 mg Se/kg DM + 100 IU vitamin E/kg DM; ESe1 = EconomasE™ to provide 0.1 mg Se/kg DM + no added vitamin E; ESe3 = EconomasE™ to provide 0.3 mg Se/kg DM + no added vitamin E.

During the study, horses were hand walked into 3.2 x 3.2 m stalls twice daily to individually receive their concentrate grain meal. Concentrate grain was offered at 1% BW (DM basis), split equally into AM and PM meals at 0700 and 1700, respectively. Any refused feed was weighed and recorded daily, and subtracted from the total grain offered to obtain accurate grain intake values for each horse. All horses received *ad libitum* access to Coastal bermudagrass hay. Hay was supplied in the form of a round bale when horses were group housed, and offered individually to horses when stalled. Individual hay intake was calculated from intake while in stalls combined with estimated intake while group-housed. Group-housed estimates were obtained from disappearance of known quantities in each dry lot and were assumed to be equal among all horses in each group. This resulted in an average hay intake of 1.5% BW (DM basis) for each horse. Throughout the study, BW was monitored every two wk using a livestock scale accurate to 1 kg (Cardinal Scales, Webb City, Missouri). Body condition score was also evaluated by two investigators using the 1 to 9 scale described by Henneke et al. (1983) on the same day BW was measured. Rations were adjusted every two wk based on changes in BW due to growth and to maintain a BCS of 5 to 6.

Submaximal Exercise Training

Beginning after wk 0 collections, horses received submaximal exercise 4 d/wk (the same days horses were stalled) as part of Texas A&M University's Equine Behavior and Training course. Undergraduate students enrolled in the course were each assigned to one horse. Students worked with their horse for 1 h/d, 4 d/wk in a low to moderate intensity program designed to mimic a typical training regime of a young western pleasure horse. The first 4 wk of the course consisted of basic ground work at the walk, trot, and canter. During the remaining 8 wk, riding

was slowly incorporated, increasing in intensity over the remainder of the course. By the end of the 12 wk, horses were walking, trotting, and cantering under saddle.

Sample Collection

Whole blood, plasma, and serum samples were collected at wk 0 and 12 of the study. Sampling occurred at 0600 prior to receiving any concentrate. Blood was collected via venipuncture from the jugular vein. Approximately 30 mL of blood was collected from each horse into evacuated tubes (Vacutainer; Becton, Dickson and Co., Franklin Lanes, NJ) containing either no anticoagulant for serum collection or in tubes containing sodium heparin for whole blood and plasma collection. Serum and plasma were isolated within 2 hr of collection, aliquoted, and stored at -80°C.

Muscle samples were collected at wk 0 and 12 from the middle gluteus muscle (GM), alternating from the left to right side from wk 0 to wk 12, respectively. A standardized sample site on the GM was located on the croup by tracing approximately one-third of the distance along an imaginary line between the tuber coxae and the tailhead (Bechtel and Kline, 1987). A 3 × 3 cm square of hair was surgically clipped on the corresponding side of the GM. The clipped area was thoroughly cleaned with 7.5% povidone-iodine, then rinsed with 70% alcohol. Horses were sedated by intravenous administration of approximately 0.2 mL of detomidine hydrochloride (Dormosedan; Zoetis Services LLC, Parsippany, NJ) and 0.1 mL of Lidocaine (Sparhawk Laboratories, Inc., Lenexa, KS) was administered to the sterile area subcutaneously. A 14-gauge needle was used to create the initial puncture through the skin. Tissue was collected using a 14-gauge needle (SuperCore™ Semi-Automatic Biopsy Instrument, Argon Medical Devices, Frisco, TX) by insertion of the biopsy needle to a standardized depth of 5 cm into the GM at a 90° angle to obtain tissue samples. The sample puncture site created only needed manual pressure with

sterile gauze to close the wound. Four muscle samples (approximately 50 mg each) were collected for each horse at each sampling period. One sample was immediately placed in a 1.5 mL microcentrifuge tube with 1 mL ice-cold relaxing solution (BIOPS; 10 mM Ca-EGTA buffer, 0.1μM free calcium, 20 mM imidazole, 20 mM taurine, 50mM K-MES, 0.5 mM dithiothreitol, 6.56mMMgCl, 5.77mM ATP, and 15mM phosphocreatine; pH7.1) and placed on ice or stored at 4°C for same day (within 24 h post-collection) analysis of oxidative capacity by high resolution respirometry (HRR). The remaining three samples were placed in cryovials and immediately flash frozen in liquid nitrogen for analysis of citrate synthase (CS) and cytochrome *c* oxidase (CCO) activities; samples were stored at -80°C until analysis. Before analysis, frozen muscle tissue was cryopulverized into a fine powder using a cryopulverizer (Spectrum™ Bessman Tissue Pulverizer; Spectrum Laboratories, Inc., Rancho Dominguez, CA).

Serum Se

Serum was analyzed for Se concentration by Alltech Inc. (Nicholasville, KY). Samples were weighed at approximately 0.5 g (0.5 mL) into 55 mL Teflon digestion tubes and digested using MARS6 microwave using 10 mL optima grade nitric acid plus 10 mL deionized water and 2 mL 30% hydrogen peroxide. Samples were brought to a final volume of 50 mL with deionized water, and ran via Agilent Technologies ICP-MS in hydrogen mode.

Muscle Mitochondrial Enzymes

Mitochondrial enzyme activity measurements of CS and CCO were measured by preparing homogenates of skeletal muscle samples and running kinetic, colorimetric analyses previously described in horse skeletal muscle (White et al., 2017). Skeletal muscle samples were cryopulverized into a powder, homogenized in sucrose homogenization buffer (n-Dodecyl ®-D-maltoside; Sigma D4641), sonicated (F60 Sonic Dismembrator, Fisher Scientific, Waltham, MA)

and centrifuged at 11,000 × *g* for 3 min. The supernatant was stored at -80°C until analysis. Enzymatic activities were measured using a microplate reader (Synergy HT, BioTek Instruments, 237 Winooski, VT, USA). An 80-fold dilution of the muscle homogenate was used for analysis of CS and a 40-fold dilution was used for CCO. Eighty-fold diluted muscle homogenate (4 μL) was added to a 96-well plate in duplicate, followed by 233.5 μL of the reaction mix (76 μL distilled H_2O, 125 μL Tris with Triton-X, and 25 μL DTNB) per well. Baseline activity was read at 412 nm for 2 min, then 12.5 μL of oxaloacetic acid (OAA) was added one column at a time to ensure accurate measurement timelines, and the increase in absorbance was read at 412 nm for 3 min. Citrate synthase activity was determined measuring the linear rate of reaction of free CoA-SH with DTNB at 412 nm at 37°C (Spinazzi et al., 2012)., with an intra-assay CV of 2.9% and an inter-assay CV of 4.2%. Forty-fold diluted muscle homogenate (5 μL) was added to each well of a 96-well plate in duplicate for CCO analysis. The reaction mix (270 μL; 115.7 μL distilled H_2O, 137.75 μL potassium phosphate buffer, and 16.53 μL reduced cytochrome *c*) was added to a separate plate and the baseline activity of the background plate was read at 550 nm for 10 min. After the baseline reading, the background plate was kept on a plate warmer at 37°C and 245 μL of the warm reaction mix from the background plate was transferred to the sample plate, and absorbance was monitored at 550 nm for 3 min one column at a time. Cytochrome *c* oxidase activity was determined by measuring the linear rate of oxidation of fully reduced cytochrome *c* at 550 nm (Spinazzi et al., 2012), with an intra-assay CV of 4.3% and an inter-assay CV of 2.3%. Enzyme activities in muscle homogenates were normalized to total protein (integrated activity) determined using the Bradford Protein Assay Kit (Thermo Scientific, Rockford, IL). Activity of CCO was further

normalized to mitochondrial content (intrinsic activity) by normalizing to CS activity as a marker of total mitochondria in the sample (Larsen et al., 2012).

High Resolution Respirometry

Immediately after collection, muscle samples to be used for HRR were placed in ice-cold BIOPS as described by White et al. (2017) and held on ice until processing (within 24 h of collection). Fat and connective tissue were removed from the samples, and tissue was chemically permeabilized in a 2% saponin solution on a plate rocker (Nutator™, TCS Scientific, New Hope, PA) at 4°C for 30 min. Muscle fibers were then rinsed with 1 mL of MiR05 (110 mM sucrose, 60 mM potassium lactobionate, 0.5 mM EGTA, 3mM $MgCl_2 \cdot 6H_2O$, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 1g/L BSA, pH 7.1) on the plate rocker at 4°C for 10 min. Permeabilized fibers were immediately used for HRR. Permeabilized fibers (approximately 3 mg wet weight) were added to each respirometer chamber of the Oxygraph-2k (O2k; Oroboros, Innsbruck, Austraia) containing 2 mL of MiR06 (MiR05 + 5 μL 280 U/mL catalase) and 20 mM creatine and maintained at 37°C. Throughout the substrate-uncoupler-inhibitor titration (SUIT) protocol, hyperoxic O_2 concentrations (200-500 μM O_2) were maintained by titration of H_2O_2 (100 mM). The following SUIT protocol previously validated for use in the horse (Latham et al., 2019) was modified slightly and utilized in this trial: pyruvate + malate (both at 2 mM; LEAK respiration), adenosine diphosphate (ADP; 2.5 mM; OXPHOS, P_{CI}), cytochrome *c*, a measure of mitochondrial membrane integrity (cyt *c;*10 μM), glutamate (10mM, P_{CI}), succinate (10 mM; P_{CI+CII}), 0.5 μM titrations of uncoupler carbonyl cyanide m-chlorophenyl hydrazon (CCCP; E_{CI+II}), and antimycin A, and inhibitor of complex III (2.5 μM; ROX). Samples with a greater than 15% response to cytochrome *c* were excluded from the data set and re-analyzed due to

indication of mitochondrial membrane damage. Throughout each step of the protocol, O_2 flux was stable for approximately 3 to 5 min before the addition of the next solution to the chamber.

Statistical Analysis

Data were analyzed using the MIXED procedure of SAS (Version 9.4, SAS Institute Inc., Cary, NC) with repeated measures (time) and fixed effects of time, diet, and time × diet; horse(diet) served as a random effect. Data were tested for normality and log-transformed prior to analysis if not normally distributed. All data are expressed as least squares means ± SEM. Significance was declared at $P \leq 0.05$, and trends declared at $P \leq 0.10$.

CHAPTER IV

RESULTS

Overall, serum selenium concentrations were higher at wk 12 than 0 ($P = 0.02$) but there was no overall effect of treatment or the treatment × time interaction (Table 2).

Table 2. Serum selenium concentrations of two-year-old Quarter Horses before (wk 0) and after 12 weeks (wk 12) of submaximal exercise and dietary treatment.

					P – value		
Variable	**Treatment[1]**	**wk 0**	**wk 12**	**SEM**	**Time**	**Trt**	**Time × Trt**
Serum Se, μg/L	**CON**	202.57	205.74	5.05	0.02	0.67	0.41
	ESe1	190.08	205.39				
	ESe3	191.54	209.66				

[1] CON = control diet, 0.1 mg Se/kg DM + 100 IU vitamin E/kg DM; ESe1 = EconomasE™ to provide 0.1 mg Se/kg DM + no added vitamin E; ESe3 = EconomasE™ to provide 0.3 mg Se/kg DM + no added vitamin E.

Citrate synthase activity, a marker of mitochondrial number, increased from wk 0 to 12 in all horses ($P = 0.0001$), but was not affected by diet or the interaction of diet and time (Fig. 2A). Similarly, integrated CCO activity, a measure of mitochondrial function, increased from wk 0 to 12 ($P = 0.002$) but was unaffected by diet and the diet × time interaction (Fig. 2B). However, intrinsic CCO activity decreased from wk 0 to 12 ($P = 0.001$) but was not different between dietary treatments (Fig. 2C).

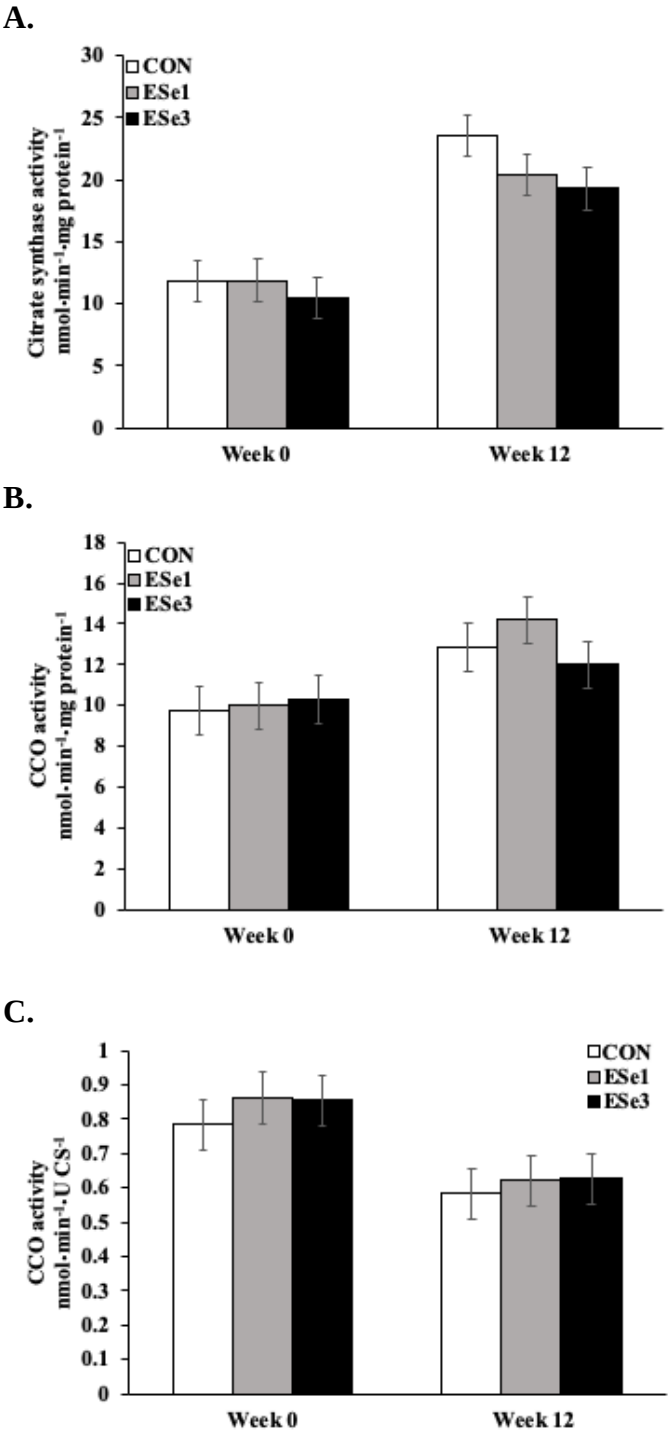


Figure 2. (A) Citrate synthase (CS), (B) integrated (per mg protein) cytochrome *c* oxidase (CCO), and (C) intrinsic (per U CS) CCO activities in the gluteus medius of two-year-old Quarter Horses before (week 0) and after (week 12) 12 wk of undergoing submaximal exercise training and receiving 1 of 3 custom-formulated concentrates: 1) 0.1 mg Se/kg DM plus 100 IU vitamin E/kg DM (CON), 2) 0.1 mg Se/kg DM plus no added vitamin E (ESe1), or 3) 0.3 mg Se/kg DM plus no added vitamin E (ESe3). Overall effects of dietary treatment ($P = 0.337$, $P = 0.761$, $P = 0.703$), time ($P < 0.0001$, $P = 0.002$, $P = 0.001$), and treatment × time ($P = 0.586$, $P = 0.472$, $P = 0.954$) for panels A, B, and C, respectively.

Integrated (per mg tissue) oxidative (P) and electron transport (E) capacities increased from wk 0 to 12 in all horses ($P \leq 0.05$), while intrinsic (per U CS) P and E decreased from wk 0 to 12 ($P \leq 0.02$; Fig. 3 and 4). Integrated P_{CI+II} ($P = 0.03$; Fig. 3B) and E_{CI+II} ($P = 0.03$; Fig. 4B) were greater for CON than ESe1, but there was no difference between ESe3 and CON nor ESe3 and ESe1 for integrated P_{CI+II} and E_{CI+II}. Integrated E_{CII} was also higher in CON than ESe1 and ESe3 ($P \leq 0.03$; Fig. 4D).

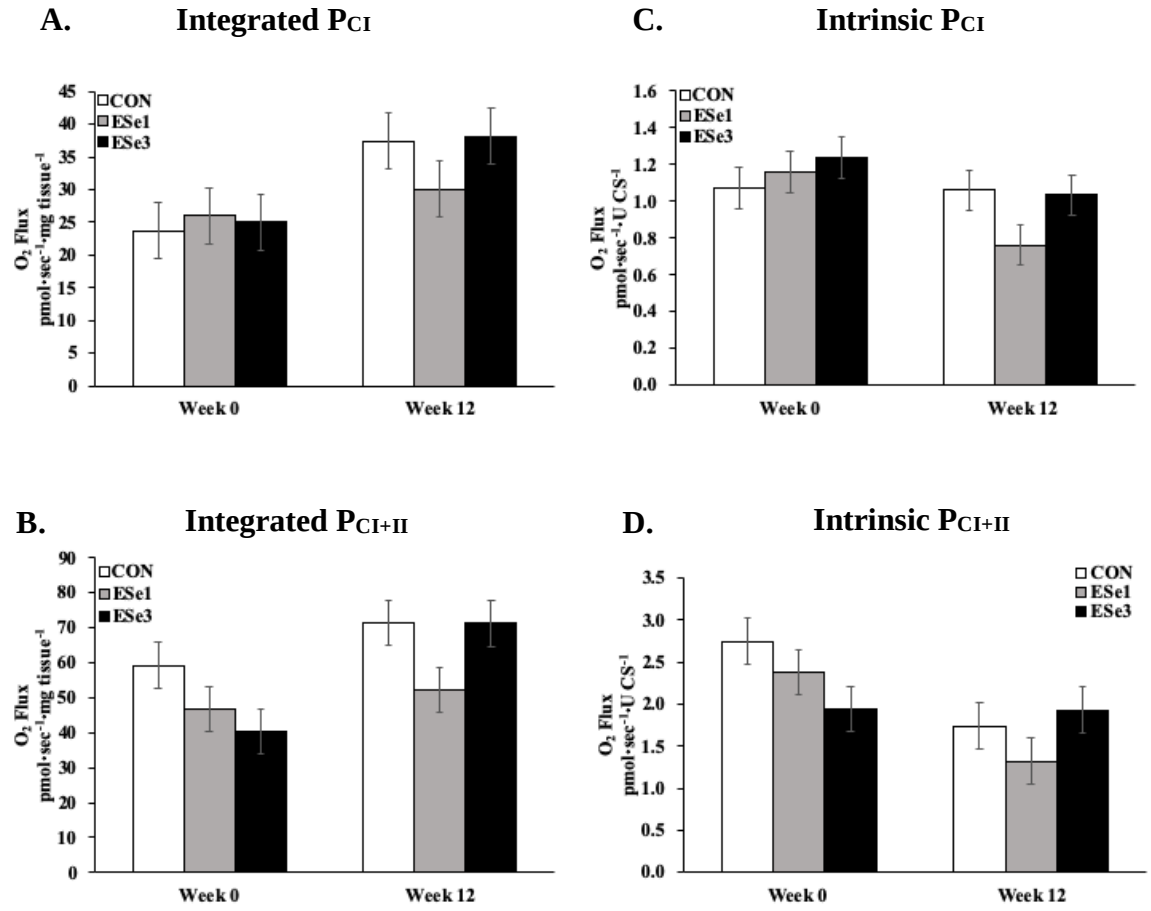


Figure 3. (A) Integrated (relative to tissue wet weight) oxidative phosphorylation (P) with complex I substrates (P_{CI}), (B) integrated P with complex I and II substrates (P_{CI+II}), (C) intrinsic (relative to citrate synthase activity; CS) P_{CI}, and (D) intrinsic P_{CI+II} in the gluteus medius of two-year-old Quarter Horses before (week 0) and after (week 12) 12 wk of undergoing submaximal exercise training and receiving 1 of 3 custom-formulated concentrates: 1) 0.1 mg Se/kg DM plus 100 IU vitamin E/kg DM (CON), 2) 0.1 mg Se/kg DM plus no added vitamin E (ESe1), or 3) 0.3 mg Se/kg DM plus no added vitamin E (ESe3). Overall effects of dietary treatment ($P = 0.723$, $P = 0.082$, $P = 0.434$, $P = 0.342$), time ($P = 0.009$, $P = 0.008$, $P = 0.016$, $P = 0.012$), and treatment × time ($P = 0.441$, $P = 0.142$, $P = 0.146$, $P = 0.164$) for panels A, B, C, and D, respectively.

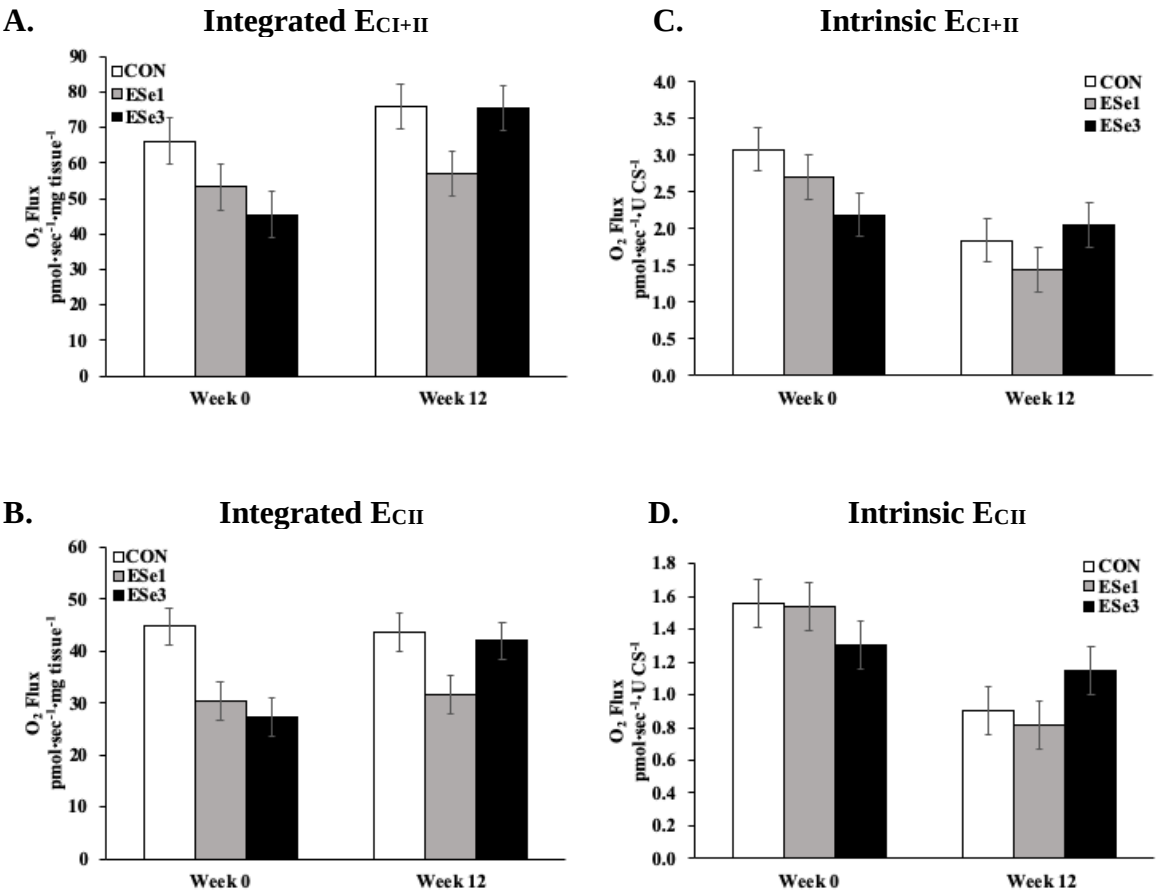


Figure 4. (A) Integrated (relative to tissue wet weight) electron transport capacity (E) with complex I and II substrates (E_{CI+II}), (B) integrated E with complex II substrates (E_{CII}), (C) intrinsic (relative to citrate synthase activity; CS) E_{CI+II}, and (D) intrinsic E_{CII} in the gluteus medius of two-year-old Quarter Horses before (week 0) and after (week 12) 12 wk of undergoing submaximal exercise training and receiving 1 of 3 custom-formulated concentrates: 1) 0.1 mg Se/kg DM plus 100 IU vitamin E/kg DM (CON), 2) 0.1 mg Se/kg DM plus no added vitamin E (ESe1), or 3) 0.3 mg Se/kg DM plus no added vitamin E (ESe3). Overall effects of dietary treatment (P = 0.072, P = 0.015, P = 0.391, P = 0.510), time (P = 0.014, P = 0.053, P = 0.003, P = 0.002), and treatment × time (P = 0.121, P = 0.146, P = 0.156, P = 0.153) for panels A, B, C, and D, respectively.

The fractional control of P_{CI} and P_{CII} increased from wk 0 to 12 in all horses ($P \leq 0.02$; Fig. 5A and Fig. 5B). Additionally, a trend for a time × treatment interaction was noted for fractional control of P_{CI} (P = 0.09; Fig. 5A); the CON group tended to increase from wk 0 to 12 (P = 0.09) while ESe1 and ESe3 remained unchanged from wk 0 to 12. Fractional control of E_{CII} was greater in CON than ESe1 (P = 0.03) and ESe3 (P = 0.03; Fig. 5C). However, fractional control of P_{CI} was greater in ESe1 (P = 0.03) and ESe3 (P = 0.01; Fig. 5A), indicating that the

CON group relied more heavily on complex II and the reduced vit E groups relied more heavily on complex I.

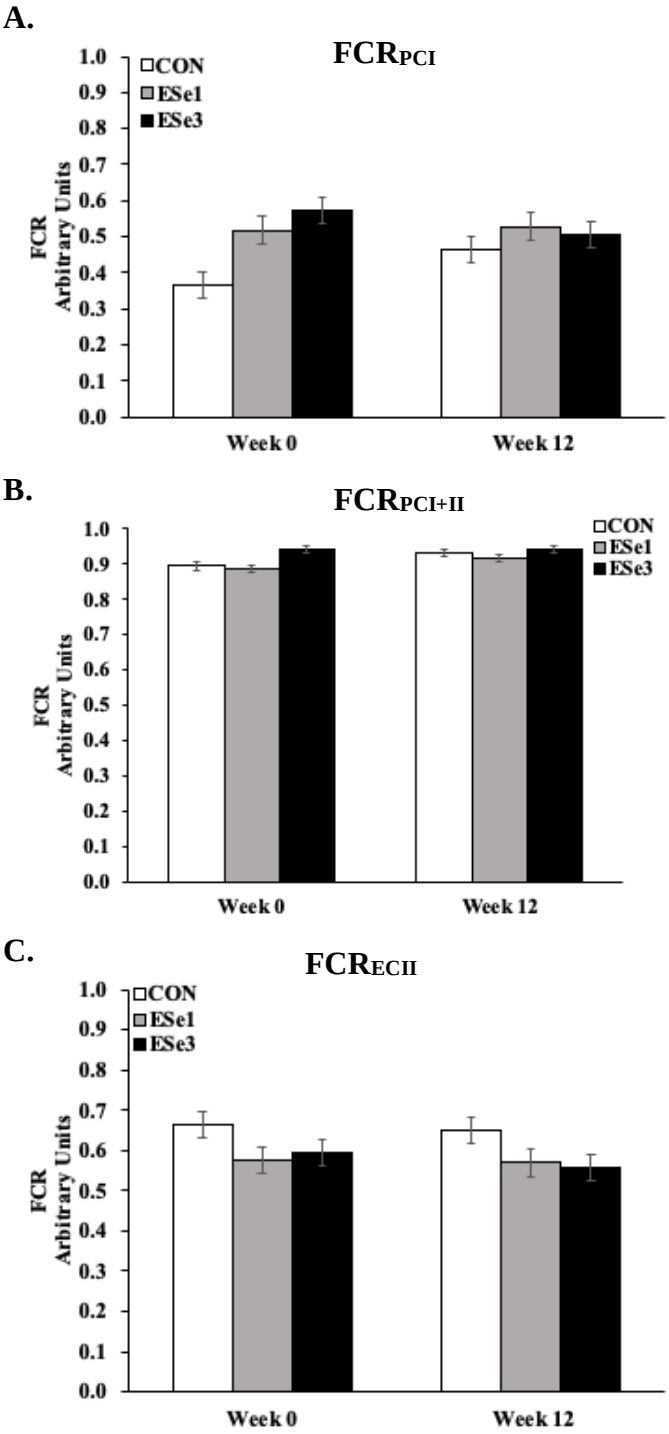


Figure 5. (A) Fractional control ratio (FCR) of oxidative phosphorylation with complex I substrates (FCR_{PCI}), (B) FCR with complex I and II substrates (FCR_{PCI+II}) and (C) FCR with complex II substrates (FCR_{ECII}) in the gluteus medius of two-year-old Quarter Horses before (week 0) and after (week 12) 12 wk of undergoing submaximal exercise training and receiving 1 of 3 custom-formulated concentrates: 1) 0.1 mg Se/kg DM plus 100 IU vitamin E/kg DM (CON), 2) 0.1 mg Se/kg DM plus no added vitamin E (ESe1), or 3) 0.3 mg Se/kg DM plus no added vitamin E (ESe3). Overall effects of dietary treatment ($P = 0.028$, $P = 0.296$, $P = 0.048$), time ($P = 0.619$, $P = 0.003$, $P = 0.442$), and treatment × time ($P = 0.086$, $P = 0.938$, $P = 0.897$) for panels A, B, and C, respectively.

CHAPTER V

DISCUSSION

Several antioxidants work together in a system to maintain mitochondrial health (Niki et al., 1995). Enzymatic defenses, such as selenium dependent-GPx, are responsible for the detoxification of peroxides and protection of the cell (Borek et al., 1986; Ghazi et al., 2012). Nonenzymatic defenses, such as vitE, function by directly removing ROS (Borek et al., 1986; Ghazi et al., 2012). Excess ROS can cause oxidative damage therefore skeletal muscle antioxidant systems upregulate in response to stressors (Ji, 2008). Additionally, Se has been shown to enhance the antioxidant effects of vitE (Ghazi et al., 2012). This relationship between Se and vitE is why the current study investigated elevated levels of Se coupled with reduced vitE.

Statistically, serum Se levels increased over the course of the study in all horses, but remained between 190 and 210 μg/L, which is similar to levels previously reported in mature Thoroughbred horses (White et al., 2016). Most studies in horses show an increase in serum Se following as few as 4 wk of supplementation with various levels of Se (Brummer, et al., 2013; Calamari, et al., 2009; Richardson et al., 2006; White et al., 2016; White et al., 2017); however, these studies did not remove vitE from the diet. This may be the reason the current study did not note differences in serum Se between treatment groups.

Citrate synthase activity is used as a marker of mitochondrial number in skeletal muscle (Larsen et al., 2012). The increase in CS activity from wk 0 to 12 in all horses in the current study suggests that mitochondrial density increased over the 12-wk experimental period but was not effected by Se or vitE levels. This is in direct contrast with a previous study that reported increased CS activity in the GM of Quarter Horse yearlings receiving 0.3 mg Se/kg DM

compared to horses receiving 0.1 mg Se/kg DM (White et al., 2017). The reason for the different results in the current study could be due to the difference in vitE intakes between the two studies. The current study only compared 0.1 to 0.3 mg Se/kg DM with no added vitE while the study by White et al. (2017) compared 0.1 to 0.3 mg Se/kg DM with added vitE. The current results are also in contrast to work in humans that reported increased mitochondrial area, measured by electron microscopy, in the vastus lateralis after 10 wk of endurance training and organic Se supplementation (Zamora et al., 1995). However, the same study found that training combined with Se had a dampening effect on mitochondrial proliferation compared to training alone, due to larger increases in mitochondrial number in the training group in the absence of supplemental Se (Zamora et al., 1995). The reason for the attenuation of mitochondrial biogenesis in response to supplemental Se may be explained by a reduction in signaling due to less degradation as a result of the antioxidant properties of Se (Tessier et al., 1995). However, this dampening effect was not observed in the present study. A more reasonable explanation for difference in results in the current study is the difference in diets. Our study decreased vitE levels with a simultaneous increase in Se levels; the above-mentioned studies did not change dietary vitE.

While the current study showed an increase in CS activity, the effects of exercise cannot be isolated from the effects of growth due to study design. Previous work in yearling Quarter Horses showed that 9 wk of submaximal exercise training increased integrated CCO activity in the GM muscle but did not affect CS and intrinsic CCO activities; however, similar changes in these measures were reported in non-exercised control horses, suggesting growth may have masked adaptations due to exercise training (White et al., 2017). Regardless, this study highlighted the limited effects of submaximal exercise training on mitochondria as measured by CS and CCO activities in growing horses. In 3- to 4-yr-old Thoroughbred horses, 18 wk of high-

intensity exercise training (90 to 110% VO_2 max) on a treadmill at a 6° incline resulted in an increase in CS activity in the GM (Kitaoka et al., 2012). Similarly, Arabian horses subjected to endurance exercise training at 80% VO_2 max for 50-80 min/d, 4 d/wk for 12 wk exhibited an increase in type I and IIa fibers with a concomitant decrease in type IIx fibers in mature Arabian horses (D'Angelis et al., 2008). It is generally accepted that greater mitochondrial measures are positively correlated with the percentage of oxidative type I and IIa fibers. This differential response to exercise training based on intensity has been well documented in humans (Leek et al., 2001; Vigelso et al., 2014). Therefore, the adaptive process noted following more intense exercise regimes in previous studies in horses may not have been evident in the current study due to the relatively low level of exercise in which the horses were engaged.

Cytochrome *c* oxidase activity is closely associated with oxidative function (Larsen et al., 2012). In the current study, integrated CCO activity and oxidative (P) and electron transport (E) capacities increased from wk 0 to 12 while intrinsic CCO activity and P and E capacities decreased. Combined with the aforementioned increase in CS activity, these results suggest an increase in mitochondrial number but a decrease in function and capacity at the level of the individual mitochondria. Contrasting results were reported in a similar study utilizing Quarter Horse yearlings, which showed 9 wk of submaximal exercise training increased integrated $P_{CI+II,}$ and intrinsic $P_{CI,}$ $P_{CI+II,}$ and E_{CI+II} in the GM (White et al., 2017). However, in mature Arabian horses, 10 wk of endurance training resulted in an increase in integrative $P_{CI,}$ but no change in P_{CI+II} in the GM and triceps brachii muscles, suggesting improvements in CI-linked oxidative phosphorylation only following training (Votion et al., 2010). The difference in results is likely due to the younger age of the horses in the current study and difference in training protocols between studies. Addionally, breed differences likely play a role in adaptations to exercise, as

Arabians have more oxidative muscle fibers overall than Quarter Horses (Rivero, 2007) and breed affects mitochondrial capacity and utilization of complexes within the electron transport system at as early as 6 mo of age (Latham, et al., 2019).

Comparisons of these measures following exercise training tend to vary widely between species. In general, it is well known that endurance exercise training improves the oxidative capacity of muscles and causes a switch toward more oxidative fiber types (Booth et al., 2015; Drake et al., 2015). An increase in P_{CI} and P_{CI+II} in the gastrocnemius muscle of rats was demonstrated after only 10 d of treadmill exercise (Daussin et al., 2012). However, increases in P_{CI+II} and E_{CI+II} in the vastus lateralis muscle were noted after 10 wk of endurance training in men (Pesta et al., 2011). Species differences in muscle adaptations make comparisons of results across studies difficult. However, the horse may be a superior research model for human sports medicine to rodents due to similarities in adaptation timing.

Horses in CON had higher P_{CI+II}, E_{CI+II}, and E_{CII} than ESe1 throughout the study. However, CON was not significantly different than ESe3 for $P_{CI,}$ P_{CI+II}, or $E_{CI+11.}$ The CON group was likely similar to ESe3 due to the elevated levels of Se compensating for the reduced vitE. In support of this theory, rats supplemented with Se in the form of selenite at 0.2 mg/kg per d for 7 d showed less suppression of mitochondrial respiratory complex activities following hypoxia compared to non-supplemented animals (Mehta et al., 2012). Additonally, Se has been shown to enhance the activity of mitochondrial complexes I and IV, while the effect of vitE on these complexes has not been conclusively determined (Wesselink et al., 2019). More research is needed to determine the exact negative implications of removing vitE from the diet of horses, and the proper inclusion rate of Se to overcome these decrements.

Limitations of this study include the lack of intensity in the submaximal exercise program. While the current protocol was intended to mimic common industry practices, intensity of exercise for the horses was often limited by the progression of the student in the course in which the horses were enrolled. Consequently, the low intensity of the exercise was likely insufficient to elicit changes in oxidative capacity of the muscle. Additionally, inclusion of a non-exercised control group would have allowed for differentiation of responses to exercise training. Effects of Se supplementation without compounding effects of removing vitE would also have been useful. As tested, responses to elevated Se alone were unable to be discerned. These additional groups would allow for a more accurate comparison of the role Se and vitE play in skeletal muscle mitochondrial oxidative capacity and biogenesis.

Overall, increased levels of Se in the form of EconomasE at 0.3 mg Se/kg DM may be beneficial to horses with low vitE intake. Due to the high costs of vitE, EconomasE may provide a solution to providing the optimum level of essential antioxidants in feedstuffs for young exercising horses. This study also provides a novel approach for measuring mitochondrial capacity in horse skeletal muscle, which may be advantageous for horse owners, trainers, and veterinarians looking to optimize the horse's athletic potential or diagnoses and treat skeletal muscle myopathies. Future studies are needed to better understand the role of antioxidants in equine skeletal muscle mitochondrial capacity.

CHAPTER VI

SUMMARY

The importance of antioxidants and their role in reducing reactive oxygen species is well understood. The role of antioxidants, particularly Se and vitE, in mitochondrial oxidative capacity and biogenesis is not as well characterized. Several studies have shown that increased levels of Se above the current NRC requirement resulted in increased mitochondrial number and oxidative capacity. However, adaptations to elevated dietary Se coupled with decreased vitE has not been determined. In this study, CS and integrated CCO activity increased over time but were not affected by dietary treatment. Intrinsic CCO, oxidative phosphorylation, and electron transport capacities decreased from wk 0 to 12. This suggested a decrease in individual mitochondrial function. However, while ESe1 horses had lower mitochondrial capacities than CON, ESe3 was generally similar to CON. This finding suggests feeding EconomasE™ to provide 0.3 mg Se/kg DM may prevent adverse effects of removing 100 IU dietary vitE/kg DM on mitochondria in young horses. Future studies should investigate the separate and combined effects of exercise, Se, and vitE before a recommendation can be made regarding the optimum levels of these antioxidants for growing and exercising horses.

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