INVESTIGATING THE IMPACT OF ANTIOXIDANT SUPPLEMENTATION ON MUSCLE ANTIOXIDANT STATUS AND THE SKELETAL MUSCLE PROTEOME IN THOROUGHBRED RACEHORSES

By

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ABSTRACT

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Glutathione (GSH) and Coenzyme Q10 (CoQ10) are potent cellular antioxidants that work to mitigate oxidative stress arising from reactive oxygen species (ROS) production. GSH is not well absorbed because it is broken down into individual amino acids in the small intestines. Cysteine is the rate limiting amino acid in GSH synthesis but is not well absorbed and is instead largely produced from methionine intracellularly. N-acetyl cysteine (NAC) supplementation has been studied as an alternative to cysteine or GSH supplements and has been shown to increase the amount of circulating cysteine and increase transport activity for this reduced form in humans. GSH concentrations in skeletal muscle have been measured in only a few studies in other species. Neither GSH nor NAC have been previously studied in the skeletal muscle of horses.

CoQ10 is an electron transporter in the mitochondrial electron transport chain (ETC) where it transfers electrons either from complex I or complex II to complex III. Within the ETC, complex I has been shown to be the primary source of ROS during exercise in comparison to the other complexes. Due to its location and function, CoQ10 can also function as a potent membrane bound antioxidant and mitigate ROS produced through the ETC. CoQ10's function as an antioxidant and electron transporter has not been studied in great depth in horses. Branched chain amino acid (BCAA) supplements are three essential amino acids that have been shown to have positive effects on protein synthesis through the mammalian target of rapamycin pathway

(mTOR). BCAA can serve as an energy source in skeletal muscle where they are directly metabolized in the tricarboxylic acid cycle. There are a limited number of studies of the impact of BCAA supplementation on equine skeletal muscle and none have looked at BCAA combined with antioxidant supplements.

The overarching objective of this dissertation was to evaluate the impact of antioxidant supplementation on fit, healthy Thoroughbred horses. The first study supplemented NAC and CoQ10 to maximally exercising Thoroughbreds to determine its effect on the redox equilibrium and skeletal muscle proteome. We determine that NACQ increases muscle GSH concentrations post exercise while increasing TCA cycle enzymes and enhancing capacity for cellular NADPH production. The second study measured the effect of a single dose of differing amounts and formulations of CoQ10 on plasma CoQ10 concentrations. We determined that individual horses have different absorption responses, with 50% showing no response. The third study analyzed chronic CoQ10 supplementation's effect on plasma CoQ10 concentrations, concentrations or activities of skeletal muscle antioxidants, mitochondrial respiration, and the skeletal muscle proteome. Results supported CoQ10's function as an antioxidant and ability to alter the contribution of complex I and complex II to electron transfer without increasing mitochondrial volume density. The final study of this dissertation analyzed the impact of NAC and BCAA supplementation on sub-maximally exercising Thoroughbreds by measuring antioxidant status and alterations to the skeletal muscle proteome before and after exercise. This study identified no changes in skeletal muscle GSH concentrations and ROS before or after exercise but did find differentially expressed proteins within the ETC, redox reactions, and glycolysis after submaximal exercise. All supplements warrant further investigation in horses with myopathies.

To Chacos: If love could have saved you, you would have lived forever

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KEY TO ABBREVIATIONS

ACh	Acetylcholine
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
ANOVA	Analysis of Variance
BCA	Bicinchoninic Acid
BCAA	Branched Chain Amino Acids
Ca^{2+}	Calcium
Cat	Catalase
CoQ10	Coenzyme Q10
CHAC1	Glutathione specific gamma-glutamylcyclotransferase 1
CK	Creatine Kinase
CRYAB	αβ-crystallin
CS	Citrate Synthase
Cys	Cysteine
DNA	Deoxyribonucleic Acid
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
ETC	Electron Transport Chain
FDR	False Discovery Rate
FH	Fumarate Dehydrogenase
GGT	Gamma-glutamyl transferase
GPx	Glutathione Peroxidase

GSH	Glutathione, reduced
GSSG	Glutathione, oxidized
GR	Glutathione Reductase
h	Hour
HPLC	High Performance Liquid Chromatography
IDH2	Isocitrate Dehydrogenase, NADP dependent
KER	Kentucky Equine Research
kg	Kilogram
MDA	Malondialdehyde
MFM	Myofibrillar Myopathy
mg	Milligram
min	Minute
m/s	Meters per second
NAC	N-Acetyl Cysteine
NACQ	N-Acetyl Cysteine and Coenzyme Q10
NAD	Nicotinamide adenine dinucleotide, oxidized
NADH	Nicotinamide adenine dinucleotide, reduced
NADP	Nicotinamide adenine dinucleotide phosphate, oxidized
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced
nmol	nanomoles
NNT	NAD(P) transhydrogenase
OGDH	2-oxoglutarate dehydrogenase
RNS	Reactive Nitrogen Species

ROS	Reactive Oxygen Species
SOD	Superoxide Dismutase
TMT	Tandem mass tag
μg	Microgram
μL	Microliter

CHAPTER 1

SKELETAL MUSCLE STRUCTURE AND OXIDO-REDUCTASE PATHWAYS

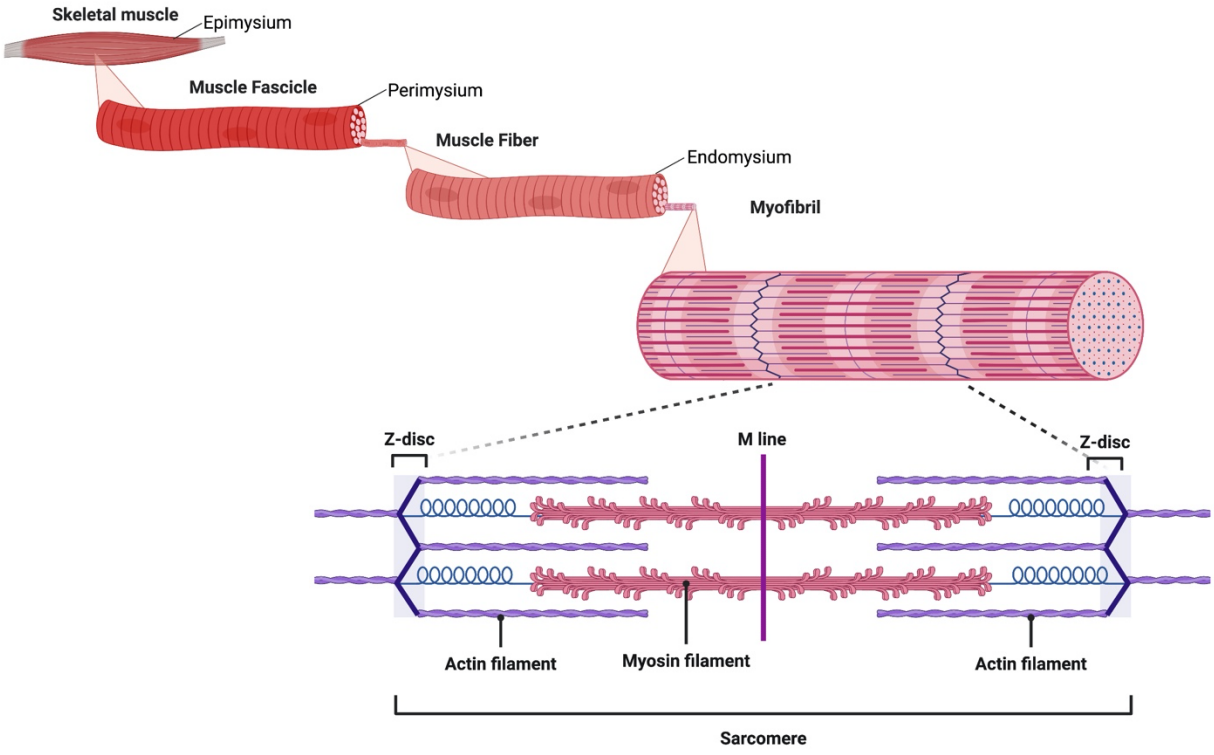
SKELETAL MUSCLE

Skeletal muscle is essential for posture, respiration, and locomotion (Martin, Timmons et al. 2015). Unlike other tissues, skeletal muscle fibers are multinucleated and post-mitotic, which means that activation of satellite cells is necessary for myofibers to regenerate (Martin, Timmons et al. 2015).

Skeletal muscle structure

Muscle fibers contain a multitude of myofibrils that are composed of a series of sarcomeres. The sarcomere is in essence the functional unit of skeletal muscle contraction and is bordered by Z-discs that act to anchor the actin filaments and give skeletal muscle its striated appearance (Martin, Timmons et al. 2015). The M-line lies in the middle of each sarcomere and stabilizes the myosin filaments. When skeletal muscle contracts, the Z-discs of each sarcomere move closer together due to the rachet-like movement of myosin's globular heads along the actin filaments (Klein 2013, Martin, Timmons et al. 2015). Depending upon the individual muscle, approximately 10,000 sarcomeres form a linear series within a single myofibril (Martin, Timmons et al. 2015). Myofibrils are approximately 1-2μm in diameter and run from tendon to tendon within the muscle fiber. Muscle fibers are enwrapped by a thin layer of connective tissue called the endomysium (Martin, Timmons et al. 2015). Multiple muscle fibers together form a fascicle which is encased by a connective tissue layer called the perimysium and all fascicles within a muscle are insheathed by the outer connective tissue layer called the epimysium (Figure 1.1). An extensive array of organelles within each fiber are vital for muscle function, especially for energy generation, muscle contraction and relaxation.

Figure 1.1. Skeletal muscle structure. The skeletal muscle contractile unit is the sarcomere which is bordered by Z-discs. Multiple sarcomeres in a linear series form a myofibril within a muscle fiber which is encased by the endomysium. Multiple muscle fibers are grouped together in fascicles insheathed by the perimysium. Finally, multiple fascicles form a skeletal muscle that is encased by the epimysium. Figure created with Biorender.com.



Skeletal muscle fiber types

Each muscle fiber contains specific types of myosin heavy chains that define the muscle fiber types; slow-twitch type I, fast-twitch type 2A and fast-twitch type 2X. Intermediated fiber types do also exist that contain a combination of IIA and IIX myosin. The type I fibers are considered slow-twitch fibers because their myosin heavy chains hydrolyze ATP at a slow rate and considered fatigue resistant because they largely produce ATP through oxidative phosphorylation within mitochondria (Klein 2013, Frontera and Ochala 2015, Martin, Timmons et al. 2015). Type IIX fibers have the fastest rate of ATP hydrolysis, are recruited at high intensity exercise, and contain fewer mitochondria resulting in a greater reliance on energy through anaerobic glycolysis which enhances their sensitivity to fatigue (Klein 2013, Frontera and Ochala 2015, Martin, Timmons et al. 2015). Type IIA fibers are intermediate in terms of speed of contraction and mitochondrial content making them more fatigue resistant than type 2X fibers.

Skeletal muscle contraction and relaxation

Skeletal muscle has the unique ability to contract and relax voluntarily and independently of other muscle groups (Martin, Timmons et al. 2015). Contraction begins with a nerve impulse at the neuromuscular junction that results in the release of acetylcholine (ACh) which in turn depolarizes the motor end plate. An impulse then travels down the sarcolemma to the transverse tubule where activation of the voltage-gated calcium channel signals the ryanodine receptor to release calcium (Ca^{2+}) from the sarcoplasmic reticulum. As cytosolic Ca^{2+} is increased, the thin filaments reveal the binding site on actin facilitating myosin-actin crossbridge formation. With crossbridge cycling, myosin globular heads move along the actin filament shortening the

sarcomere and producing muscle contraction. For muscle relaxation to occur, adenosine triphosphate (ATP) must be hydrolyzed to release the myosin globular head from actin. To prevent further interaction between myosin and actin, Ca^{2+} is actively transported back into the sarcoplasmic reticulum through the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) which is an ATP-dependent transporter. Contraction and relaxation of skeletal requires that ATP is rapidly generated during exercise to facilitate cycles of rapid contraction and relaxation cycle.

During a bout of exercise, initially creatine phosphate and glycolytic pathways utilizing glucose, or glycogen sustain the supply of ATP. In the presence of oxygen, fat, glycogen, or glucose can be aerobically metabolized to generate ATP. Glycogen is metabolized to form glucose-1 phosphate which is further metabolized to form pyruvate by glycolysis. Pyruvate is transported into mitochondria where it is processed by the Krebs cycle to generate NADH and $FADH_2$, both of which are utilized by the mitochondrial electron transport chain (ETC) to generate ATP carbon dioxide and water. In the absence of sufficient oxygen or when rapid generation of ATP is required, anaerobic glycolysis generates ATP from the conversion of pyruvate to lactate which produces NAD^+ to facilitate further metabolism of glucose to pyruvate.

Sedentary individuals fatigue quickly during high-speed exercise because their low mitochondrial content results in early reliance on anaerobic glycolysis, reaching the lactate threshold of 4 mmol/L at a slower speed. As sedentary individuals become more active, the number of mitochondria increase within actively recruited muscle fibers and energy is generated through aerobic metabolism during exercise. This increases the speed at which they reach the

lactate threshold. Training adaptation increase the time to fatigue of an athlete because more ATP are generated from the Krebs cycle and ETC than from anaerobic glycolysis.

Oxidative Phosphorylation

In the ETC, the electrons from NADH and $FADH_2$ go through a chain of protein complexes that increase the proton gradient across the inner mitochondrial membrane that is linked to generation of ATP. The proton gradient increases the acidity in the intermembrane space and creates an electrical difference with a positive charge outside and a negative charge inside. The mitochondrial ETC has five distinct complexes that include NADH Ubiquinone Oxidoreductase e (complex I), Succinate Dehydrogenase (complex II), Cytochrome C Reductase (complex III), Cytochrome C Oxidase (complex IV), and ATP synthetase (complex V). Complexes I through IV create the proton gradient. Once the proton gradient is formed, complex V uses chemiosmosis to create an ATP molecule by combining a phosphate molecule with adenosine diphosphate (ADP). Electrons do not move between the complexes easily; there are two important carrier proteins that are required to do the work. Coenzyme Q10 (CoQ10) is the carrier protein that transports the electron from both complexes I and II to complex III (Spindler, Beal et al. 2009). Reduced CoQ10 also serves as an antioxidative preventing lipid peroxidation of the mitochondrial membrane by free radicals (Spindler, Beal et al. 2009). Cytochrome C is the carrier protein that transports electrons from complex III to complex IV and is thought to be one of the rate-limiting steps of the ETC. While both CoQ10 and Cytochrome C are vital to the production of ATP, they are also important antioxidants because of their ability to both decrease the creation of reactive oxygen species (ROS) as well as act as antioxidants to reduce ROS (Korshunov, Krasnikov et al. 1999, Pereverzev, Vygodina et al. 2003, Littarru and Tiano 2007).

ROS are generated due to electron leak for complexes I and III in the mitochondrial electron transport. Because CoQ10 and Cytochrome C lie closest to this source of ROS they impact ROS production.

SKELETAL MUSCLE OXIDO-REDUCTASE PATHWAYS

Reactive oxygen species

Reactive oxygen and reactive nitrogen species (ROS, RNS) are chemically reactive molecules containing oxygen or nitrogen, respectively. Examples of ROS molecules include superoxide ions, hydrogen peroxide, hydroxyl ions, alkoxyl radicals, and peroxyl radicals. RNS molecules include nitric oxide, nitrogen dioxide, and peroxynitrite. These molecules provide both positive and negative feedback to the cell. Their positive contributions arise from the fact that a degree of ROS/RNS generation stimulates an increase in the cellular antioxidant pool and generates signals indicating that the cell is unhealthy triggering apoptosis. However, when the amount of ROS and RNS produced vastly outweighs the antioxidant capacity of the cell, this has the negative consequence of oxidative stress with oxidation of proteins, lipids, and nucleic acids (Ferreira and Reid 2008). RNS molecules in excess can disrupt vital cellular processes like ETC, calcium regulation, and myosin cross-bridge cycling. ROS molecules are found at highest concentrations in the areas where they are produced, primarily complexes I and III of the ETC, because ROS are unstable and short lived (Ferreira and Reid 2008). This is one reason why CoQ10 and Cytochrome C are important antioxidants within the ETC and why SOD, which neutralizes ROS, is highly abundant within the mitochondria. Since the mitochondria are the primary sources of ROS/RNS production, antioxidants such as CoQ10 that are found in the mitochondria have been a focus of investigation in humans and lab animal species.

Mitochondrial antioxidants

Mitochondrial antioxidants are the first line of defense against radicals and reactive molecules. They are essential to generate and protect critical cell functions. There are four primary

antioxidants within mitochondria, three are enzymatic, superoxide dismutase (SOD), catalase (Cat) and glutathione peroxidase (GPx), and one non-enzymatic, glutathione (GSH) (Figure 1.2). SOD is one of the only antioxidants that can reduce superoxide molecules into hydrogen peroxide molecules. This is an important conversion because superoxide molecules are created through the mitochondrial ETC. Both Cat and GPx are antioxidants that reduce hydrogen peroxide to water (Ighodaro and Akinloye 2019). GPx can work individually using selenium as a cofactor or simultaneously with GSH (Tapiero, Townsend et al. 2003, Ighodaro and Akinloye 2019).

$$O_2^{\cdot-} + e^- + 2H^+ \rightarrow H_2O_2 \text{ (SOD reaction)}$$

$$2H_2O_2 \rightarrow 2\ H_2O + O_2 \text{ (Catalase reaction)}$$

$$H_2O_2 \rightarrow H_2O \text{ (GPx reaction)}$$

GSH is a ubiquitous antioxidant composed of three amino acids glutamate, cysteine and glycine (Mari, Morales et al. 2009). GSH donates a hydrogen to reduce hydrogen peroxide to water by creating a disulfide bond with another glutathione molecule.

$$2GSH + H_20_2 \rightarrow GSSG + 2\ H_2O$$

To retain cellular antioxidant capacity, it important that these antioxidants are rapidly reduced from their oxidized state. Glutathione reductase (GR) reduces oxidized glutathione (GSSG) molecules to maintain a high concentration of GSH and a low concentration of GSSG. Glutathione reductase is an FAD containing enzyme that uses NADPH to reduce the disulfide bond in GSSG (Pai and Schulz 1983).

$$GSSG + NADPH \rightarrow 2\ GSH + NADP^+ \text{ (GR reaction)}$$

These antioxidants work together in an overlapping system to reduce ROS and prevent oxidative stress. One way the system works together is by using multiple antioxidants to convert superoxide molecules to water in the mitochondria. Initially, superoxide dismutase converts the superoxide into hydrogen peroxide. Then, catalase, glutathione, or glutathione peroxidase convert hydrogen peroxide to two water molecules, or one water molecule and two hydroxyl radicals (Figure 1.2).

Other thiol-based antioxidants include the peroxiredoxin and thioredoxin families. Most of these molecules are found within the cytosol however, peroxiredoxin III and V and thioredoxin 1 and 2, as well as thioredoxin reductase have been found within mammalian mitochondria (Miranda-Vizuete, Damdimopoulos et al. 2000, Cao, Lindsay et al. 2007). The peroxiredoxin family of antioxidants not only reduces free radicals or hydrogen peroxide molecules, but also signal the cell when the antioxidant capacity has become overwhelmed which would lead to oxidative damage or even cellular death (Brown, Cox et al. 2010). Thioredoxins work a little differently than other antioxidants in that, instead of reducing radicals, they reduce other antioxidants rejuvenating their oxidative capacity. One of the primary functions of thioredoxin 1 and thioredoxin reductase is to reduce the peroxiredoxin antioxidants as well and other thiols like glutathione (Lu and Holmgren 2014). However, thioredoxin 2 is one of the only know proteins that can reduce protein disulfide bonds (Miranda-Vizuete, Damdimopoulos et al. 2000). The thioredoxin family demonstrate how thiols work in concert in order to maintain optimal antioxidant capacity. The three families of cysteine-based antioxidants, glutathione, peroxiredoxins and thioredoxins make up the first and second line of defense against free radicals and reactive molecules making them crucial components of the antioxidant system.

Vitamin C, Vitamin E and Selenium

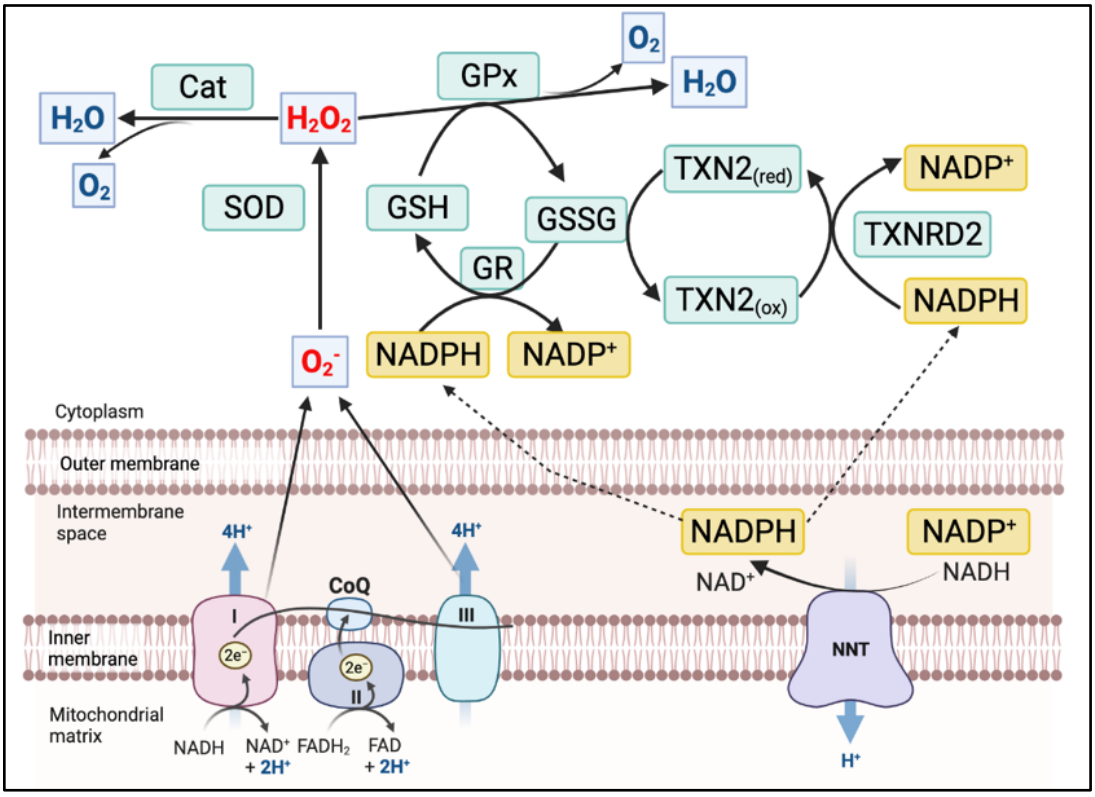
Certain vitamins that are exogenously absorbed in most species serve as important free radical scavengers reducing hydroxyl radicals. These vitamins include water-soluble ascorbic acid (vitamin C) (Bendich, Machlin et al. 1986), the fat-soluble tocopherol family (vitamin E) (Niki and Noguchi 2004) and β-carotene (vitamin A) (Young and Lowe 2018). Although ascorbate (vitamin C) was identified as an essential nutrient in 1922, it took over 40 years to discover that, similar to GSH, vitamin C regenerates oxidized vitamin E (Chan 1993). Ascorbate also scavenges free radicals such as peroxides resulting in the oxidized form of ascorbate, semidehydroascorbate (SDHA). SDHA is then reduced by SDHA reductase using NADH as a cofactor (Green and O'Brien 1973). As a lipid-soluble molecule, vitamin E has a vital role in protecting against lipid peroxidation in membranes (Niki and Noguchi 2004, Young and Lowe 2018). Selenium is a mineral that has an important role as a component of the antioxidant GSH-Px (Tapiero, Townsend et al. 2003). As an essential component of GSH-Px, Se acts synergistically with α-tocopherol to counteract lipid peroxidation (Tapiero, Townsend et al. 2003). Vitamins C and E are a secondary line of defense against reactive oxygen species.

Metal binding proteins

Iron within the mitochondria is utilized for synthesis of cofactors essential to the function of enzymes involved in oxidation-reduction reactions. Iron concentrations are heavily regulated by ferritin and it's signaling cascade which has the capacity to decipher between the need to sequester or release iron in the blood stream. Other metal binding proteins include metallothionein that contain sulfhydryl groups allowing them to bind with heavy metal ions such as zinc in the cytosol (Formigari, Irato et al. 2007). The sulfhydryl groups also allow these

molecules to act as antioxidants and scavenge free radicals such as hydroxyl radicals (Viarengo, Burlando et al. 2000, Formigari, Irato et al. 2007). With regard to free radical scavenging, the metal binding antioxidants are essentially the last line of defense.

Figure 1.2. Mitochondrial electron transport chain, reactive oxygen species, and antioxidants including enzymatic and non-enzymatic. Figure adapted from *A Panoramic Overview of Mitochondria and Mitochondrial Redox Biology* (Kim 2014). O_2^-, superoxide anion; SOD, superoxide dismutase; H_2O_2, hydrogen peroxide; Cat, catalase; GPx, glutathione peroxidase; GSH, reduced glutathione; GSSG, oxidized glutathione; GR, glutathione reductase; TXN2, thioredoxin 2; TXNRD2, thioredoxin reductase 2; NNT, NADP transhydrogenase; NADPH, nicotinamide adenine dinucleotide phosphate. Figure created with Biorender.com.



ANTIOXIDANTS PREVIOUSLY EVALUATED IN EQUINE STUDIES

The effect of vitamin E and selenium (Se) deficiencies have been studied in horses for over four decades. Vitamin E is needed to prevent lipid peroxidation (Maylin, Rubin et al. 1980) and Se is a cofactor for GPx (Maylin, Rubin et al. 1980, Ighodaro and Akinloye 2019).

A deficiency of Se has been shown to cause severe muscle fiber degeneration in young domestic animals such as foals, lambs and calves which is believed to arise from oxidative stress in the muscle of young animals (Ammerman and Miller 1975, Lofstedt 1997). In adult horses, deficiencies in vitamin E and Se were postulated to play a role in a form of exercise-induced muscle degeneration called azoturia or tying-up syndrome (Maylin, Rubin et al. 1980). A study of Standardbred horses with tying up syndrome, however, found that the concentrations of serum and skeletal muscle vitamin E and blood GPx activity were actually higher in horses that experienced tying up than control horses, likely as a result of zealous supplementation (Roneus and Hakkarainen 1985). Plasma GPx activity is used to assess blood Se concentrations because Se concentrations and GPX activity are significantly positively correlated (Maylin, Rubin et al. 1980).

One of the first vitamin E studies in horses that investigated the effect of supplemental vitamin E (DL-α-tocopheryl acetate) on exercised horses utilized 3 different daily doses: placebo, 80 international units (IU), and 300 IU. The study consisted of 19 Thoroughbreds and Quarter Horses between the ages of 4-15 yr. Horses were exercised for 20-30 min/d at speeds of 4-8 m/s for 5 d/wk during the duration of this study. As horses became more conditioned, the duration at the high-speed exercise increased while remaining in the 20-30 min/d timeframe. This study also

utilized a submaximal exercise test to identify effects of different doses on oxidative stress after exercise. This study found that, to maintain proper muscle and blood concentrations of vitamin E, it is important to at least supplement horses with a minimum of 80 IU/day of DL-α-tocopheryl acetate, a synthetic form of vitamin E. This study, however, did not find any statistically significant difference between the 3 doses on muscle oxidative damage measured as thiobarbituric acid reactive substances (TBARS) and therefore concluded that "vitamin E status of the horses did not seem to affect the integrity of skeletal muscle following repeated submaximal exercise" (Siciliano, Parker et al. 1997). This is in agreement with a study that supplemented horses with 10,000 IU and 5,000 IU of DL-α-tocopheryl acetate which also failed to see any antioxidant effect of supplementation in intensely exercising horses measuring oxidative stress as lipid hydroperoxides (Williams and Carlucci 2006).

Vitamin E is comprised of a chromonal ring with a side chain that can either be saturated (tocopherol) or unsaturated (tocotrienol) and each of these have 4 isoforms (α, β, χ, and δ) which all have different methylation status and different locations of the side chain (Fagan, Harris et al. 2020). Additionally, there are up to 8 different stereoisomers for each isoform of vitamin E based on the orientation of chiral carbons on the side chain. The isoform α-tocopherol is known to be the most biologically active and is a potent radical scavenging antioxidant (Amazan, Rey et al. 2012, Finno and Valberg 2012, Fagan, Harris et al. 2020). In a supplementation trial performed by Brown et al., 19 horses of multiple different breeds were utilized to determine the effects of two different formulations of natural vitamin E, RRR-α-tocopherol (Brown, Valberg et al. 2017). This study consisted of three different supplementation groups including a control group (n=5), 5000 IU/d RRR-α-tocopheryl acetate powder (Elevate® Maintenance Powder, Kentucky

Performance Products, Versailles, KY), and one group that was gradually transitioned from 5000 IU/d RRR-α-tocopherol water dispersed liquid (Elevate® water soluble, Kentucky Performance Products) to 5000 IU/d of the RRR-α-tocopheryl acetate powder (Brown, Valberg et al. 2017). This study found that, with the RRR-α-tocopheryl acetate powder it took the horses 47 days to significantly increase their serum α-tocopherol concentrations whereas with the RRR-α-tocopherol liquid the α-tocopherol concentrations were significantly increased within 6 days and remained increased at that level throughout the study even while tapering to the acetate powder. They also found that, with the liquid α-tocopherol supplementation, the plasma concentrations were significantly higher than both the acetate powder and the control groups. This led the conclusion that it is extremely important to select the appropriate formulation of α-tocopherol to achieve adequate concentrations (Brown, Valberg et al. 2017). Specifically, the micellized RRR-α-tocopherol stereoisoform is the form that is best absorbed and biologically active (Finno and Valberg 2012, Fagan, Harris et al. 2020).

Although vitamin E and selenium have been extensively investigated as antioxidant supplements in horses, it is clear from a review of the literature that there are other important antioxidants that have yet to be thoroughly investigated in horses. This includes CoQ10 as well as thiol-based antioxidants.

Coenzyme Q10

CoQ10 is known as ubiquinol in the reduced state and ubiquinone in the oxidized state. One of the problems with CoQ10 as an oral supplement is the poor bioavailability of commercially-available supplements (Mantle and Dybring 2020). Human studies have reported no significant

difference in plasma CoQ10 concentrations following administration of ubiquinone or ubiquinol formulations and significant inter-subject variation in baseline plasma levels and bioavailability of CoQ10, irrespective of the CoQ10 form (ubiquinone/ubiquinol) administered (Singh, Niaz et al. 2005). Intestinal absorption of CoQ10 appears to be highly variable among individuals and independent of the form of CoQ10 (ubiquinone/ubiquinol) administered (Singh, Niaz et al. 2005).

One formulation of CoQ10 that has been developed to enhance absorption in horses is a microencapsulated crystalline ubiquinone which is water-soluble (Hydro Q-sorb) (Sinatra, Chopra et al. 2013, Sinatra, Jankowitz et al. 2014). In a 30 day supplementation trial using 800 mg/day of Hydro-Q-Sorb given to six unexercised 2-year old Thoroughbreds, more than a 2-fold increase in serum CoQ10 from baseline occurred (Sinatra, Chopra et al. 2013). In the second study, two groups of five Thoroughbreds in race training received 1900 mg/day and 3400 mg/day of CoQ10 for several months (Sinatra, Jankowitz et al. 2014). Horses were lightly exercised for 8 weeks followed by 9 weeks of high intensity exercise. Blood samples were obtained in the morning on the low intensity exercise day and immediately after exercise on the high intensity exercise test day. With 1900mg of hydro-soluble ubiquinone, a 3-fold increase from baseline in plasma CoQ10 occurred after light exercise and 2-fold increase from baseline after high intensity. With 3400mg of hydro-soluble ubiquinone, an 8 fold increase in plasma CoQ10 from baseline occurred with light exercise and a 3 fold increase was maintained after high intensity exercise (Sinatra, Jankowitz et al. 2014).

Another formulation of CoQ10 used in horses is powdered cyclodextrin-encapsulated ubiquinone (CAVAQ10 and Vital Paste 7.5%). In a 14-day study of sedentary Warmblood horses beginning an exercise regime, supplementation of 800mg cyclodextrin-encapsulated ubiquinone (Vital Paste 7.5%) with 990 IU of RRR-α-tocopheryl acetate did not show an increase in plasma CoQ10 from baseline for that treatment group (Svete, Vovk et al. 2021). The Svete et al. study did, however, report an effect of co-supplementation with α-tocopheryl acetate on plasma CoQ10 concentrations when compared to the placebo group. Of note, however, the placebo was a separate group of horses housed by owners under separate conditions (Svete, Vovk et al. 2021). Thus, individual variation and different environments, rather than an effect of a relatively low dose of poorly bioavailable vitamin E, could explain the differences between placebo and supplemented groups in the Svete et al. study.

Both of these previous equine CoQ10 supplements utilize an oxidized form of CoQ10 which then must be reduced within the body prior to having an antioxidant effect. A recent formulation of CoQ10 has been developed that is a micellized form of the reduced form of CoQ10 ubiquinol. This formulation encapsulates ubiquinol into fat particles that are <10 nm to enhance absorption as a lipid soluble molecule (Ruiz, Tibary et al. 2021). Further research is needed to assess the absorption and ability of this formulation of CoQ10 and its capacity to act as an antioxidant in horses. Studies are needed to compare the effect of formulation and dose of CoQ10 on using the same group of horses.

ANTIOXIDANT AND AMINO ACID SUPPLEMENTS THAT MAY BENEFIT SPECIFIC MYOPATHIES

Myofibrillar Myopathy in horses

Myofibrillar Myopathy (MFM) is a late onset myopathy in horses with exercise intolerance that is defined by features of myofibrillar disarray, streaming of Z discs, and ectopic accumulation of proteins such as desmin in muscle fibers (Valberg, Nicholson et al. 2017). Desmin is a readily oxidized cytoskeletal protein that acts to keep Z-discs in alignment. A diagnosis of MFM is established in light microscopy by observing desmin aggregation in either gluteal or semimembranosus muscle biopsy samples (Williams 2019). MFM has been identified in two breeds of horses: Arabians and Warmbloods.

In Arabian horses, MFM manifests by 11 to 15 years of age as muscle pain, stiffness and muscle fiber degeneration after prolonged exercise such as endurance rides (Valberg, McKenzie et al. 2016, Valberg, Perumbakkam et al. 2018). Transcriptomic and proteomic profiling of Arabians with MFM and control Arabians found decreased expression of peroxiredoxin 6, is a cysteine-based antioxidant, as well as altered expression of genes required to produce cysteine from methionine after exercise (Valberg, Perumbakkam et al. 2018). Based on these findings, MFM in Arabian horses was suggested to be associated with a deficiency in cysteine-based antioxidants, predisposing them to oxidative stress and desmin aggregation (Valberg, Perumbakkam et al. 2018).

In Warmblood horses, the clinical onset of MFM appears to be evident by 8 years of age with desmin aggregation apparent on average by 11 years of age (Valberg, Nicholson et al. 2017).

Warmbloods with MFM have signs of exercise intolerance, stiffness reluctance to go forward and engage the hindquarters, and mild lameness that has no underlying orthopedic cause (Lewis, Nicholson et al. 2017, Valberg, Nicholson et al. 2017, Williams, Bertels et al. 2018). The specific etiology for MFM in horses is likely complex. Mutations in genes known to cause MFM in humans have not been identified in Warmblood horses with MFM (Williams, Velez-Irizarry et al. 2021). Transcriptomic and proteomic profiling of muscle from MFM in Warmblood horses did identify altered expression of several Z-disc proteins and proteins in complex I of the ETC in comparison to control horses (Williams, Velez-Irizarry et al. 2021). Additionally, decreased protein expression of catalase, as well as increased gene expression of *CHAC1* (gamma-glutamylcyclotransferase) was identified in MFM compared to control horses (Williams, Velez-Irizarry et al. 2021). Th enzyme encoded by *CHAC1* specifically degrades GSH. Deficiencies of antioxidants in this breed could also impact the muscles' ability to reduce ROS and prevent oxidative stress (Williams, Velez-Irizarry et al. 2021).

Owners of MFM horses report that the majority of horses managed with a traditional low starch, high fat diet and exercise daily do not achieve a satisfactory level of improvement (Williams, Bertels et al. 2018). Based on the reported alterations in peroxiredoxins and *CHAC1*, the question arises as to whether provision of cysteine-based antioxidants or CoQ10 could improve the performance of MFM horses. One antioxidant that has been shown *in vitro* to prevent desmin aggregation is N-acetyl cysteine (NAC) (Whitehead, Pham et al. 2008, Segard, Delort et al. 2013, Delort, Segard et al. 2019). A mouse model of MFM also showed a significant decrease in desmin aggregation when supplemented with NAC (Whitehead, Pham et al. 2008, Delort, Segard et al. 2019). There are currently no studies that have looked at the effects of NAC on muscle in

horses. One equine study supplemented oral NAC at 20 mg/kg BW and showed that it had an anti-inflammatory effect in the endometrium (Witte, Melkus et al. 2012). It would be of great interest to determine the impact of NAC on healthy horse muscles and then subsequently on muscle from MFM horses.

Glutathione

The availability of cysteine is the rate limiting step in the synthesis of GSH because mammalian cells do not store cysteine and it must be synthesized from the essential amino acid methionine (Kwon and Stipanuk 2001). Thus, concentrations of tissue GSH are dependent on cysteine synthesis or circulation of its oxidized form, cystine, to skeletal muscle where it is transported into the myoplasm and reduced to cysteine (Yu and Long 2016). Cysteine itself is easily oxidized and has a relatively unstable shelf life while cystine (oxidized cysteine) is poorly soluble and therefore poorly absorbed (Droge 2005, Atkuri, Mantovani et al. 2007). For this reason, studies have utilized NAC as an oral supplement to increase the pool of unbound cysteine and thereby impact GSH concentrations (Droge 2005, Atkuri, Mantovani et al. 2007, Sato, Tamba et al. 1999). There is only one supplementation study of NAC in horses and there are no studies that have measured skeletal muscle GSH concentrations in horses.

Skeletal muscle GSH concentrations have been measured previously in several other species showing a wide range of concentrations depending on the method of analysis and the specific muscle (Table 1.1) (Martensson and Meister 1989, Ji, Fu et al. 1992, Marin, Kretzschmar et al. 1993, Luo, Hammarqvist et al. 1996, Hammarqvist, Luo et al. 1997, Hammarqvist, Andersson et al. 2005, Dam, Mitchell et al. 2012, Michailidis, Karagounis et al. 2013, Morin, Guiraut et al.

2019). The primary muscle that has been studied in humans is the quadriceps (quadriceps femoris) which is comprised of four separate muscles one of which is the vastus lateralis, a predominantly fast twitch muscle (~60% fast twitch muscle fibers) (Staron, Hagerman et al. 2000). Concentrations of GSH in this muscle range from 1.2 -1.5 nmol/mg WW (Table 1.1). The primary muscle that is biopsied in horses is the gluteal muscle which is made up of approximately 80% fast twitch muscle fibers (Aldrich, Velez-Irizarry et al. 2021). Thus, it would be interesting to compared GSH concentrations in horse muscle to other species provided a similar analytical method is used.

Table 1.1. Glutathione concentrations in skeletal muscle across different species. Each study is presented with the species it was measured in, type of analysis performed, exercise type, muscle group, and concentrations in their respective units.

Study	Species	Analysis	Exercise	Muscle	[GSH]	
Martensson and Meister, 1989 (Martensson and Meister 1989)	Mice	glutathione disulfide reductase-5,5'-dithiobis(2-nitrobenzoate) recycling method	No	Quadriceps	0.77 ± 0.53 nmol/mg WW	mean ± SD
				Skeletal muscle mitochondria	5.70 ± 0.30 nmol/mg protein	
				Heart	1.15 ± 0.071 nmol/mg WW	
				Heart mitochondria	11.50 ± 0.40 nmol/mg protein	
Ji *et al.,* 1992 (Ji, Fu et al. 1992)	Rat	HPLC method	Pre	Superficial Vastus Lateralis	0.55 ± 0.05 nmol/mg WW	mean ± SEM
				Deep Vastus Lateralis	1.5 ± 0.02 nmol/mg WW	
				Soleus	3.2 ± 0.2 nmol/mg WW	
			25m/min 5% grade	Superficial Vastus Lateralis	0.45 ± 0.05 nmol/mg WW	
				Deep Vastus Lateralis	1.7 ± 0.3 nmol/mg WW	
				Soleus	3.5 ± 0.2 nmol/mg WW	
			25m/min 10% grade	Superficial Vastus Lateralis	0.6 ± 0.05 nmol/mg WW	
				Deep Vastus Lateralis	1.8 ± 0.2 nmol/mg WW	
				Soleus	3.2 ± 0.2 nmol/mg WW	

Table 1.1 (cont'd)

Dam *et al.,* 2012 (Dam, Mitchell et al. 2012)	Rats	HPLC and detected at 350nm	No	Quadriceps	1.40 ± 0.03 nmol/mg WW	mean ± SEM
Morin *et al.,* 2019 (Morin, Guiraut et al. 2019)	Guinea Pig	Capillary electrophoresis/UV	No	Gastrocnemius	10 ± 2 nmol/mg protein	mean ± SD
Marin *et al.,* 1993 (Marin, Kretzschmar et al. 1993)	Dog	Spectrophotometrically at 412nm	Untrained	Gastrocnemius	1.56 ± 0.17 nmol/mg WW	mean ± SD
			Trained		2.22 ± 0.24 nmol/mg WW	
Luo *et al.,* 1996 (Luo, Hammarqvist et al. 1996)	Human	HPLC separation with fluorescent detection at excitation 394nm and emission 480 nm	No	Quadriceps femoris	1.43 ± 0.17 nmol/mg WW	mean ± SEM
Hammarqvist *et al.,* 1997 (Hammarqvist, Luo et al. 1997)	Human	HPLC separation with fluorescent detection at excitation 394nm and emission 480 nm	No	Vastus Lateralis	1.42 ± 0.041 nmol/mg WW	mean ± SD
Hammarqvist *et al.,* 2005 (Hammarqvist, Andersson et al. 2005)	Human	HPLC separation with fluorescent detection at excitation 394nm and emission 480 nm	No	Quadriceps femoris	1.21 ± 0.15 nmol/mg WW	mean ± SD
Michailidis *et al.,* 2013 (Michailidis, Karagounis et al. 2013)	Human	Spectrophotometrically at 375nm	Pre	Vastus Lateralis	55.0 ± 3.00 nmol/mg protein	mean ± SD
			2 h post		43.0 ± 3.00 nmol/mg protein	
			48 h post		34.0 ± 2.00 nmol/mg protein	
			8 d post		47.0 ± 3.00 nmol/mg protein	

Branched Chain Amino Acids

Branched Chain Amino Acids (BCAA) are a group of 3 essential amino acids including leucine, isoleucine, and valine. These amino acids cannot be produced by the body but instead must be consumed in the diet. BCAA are unique because they can be metabolized directly by skeletal muscle as an energy source (Klein 2013). A human study evaluated the effect of the consumption of a sports drink containing 45% leucine, 30% valine, and 25% isoleucine multiple times before, during, and following exercise bouts (Karlsson, Nilsson et al. 2004). A significant increase in phosphorylation of the 70-kDa S6 protein kinase ($p70^{S6k}$) protein occurred 1hr and 2hr following the exercise (Karlsson, Nilsson et al. 2004). The enzyme $p70^{S6k}$ is part of the mammalian target of rapamycin (mTOR) signaling pathway (Karlsson, Nilsson et al. 2004). The mTOR signaling pathway serves as a regulator of cellular metabolism, growth, proliferation, and survival and has a strong impact on muscle mass by increasing gene transcription and muscle protein synthesis (Wang and Proud 2006, Laplante and Sabatini 2009). There are many ways to increase the activity of the mTOR pathway including dietary leucine supplementation (Wang and Proud 2006, Laplante and Sabatini 2009). Because myopathies such as MFM are characterized by gradual muscle atrophy, BCAA supplementation of MFM horses could potentially decrease muscle loss as an adjunct to NAC's effect on glutathione concentrations. Before conducting such a study in MFM horses, however, it is important to study the effect of BCAA in combination with other antioxidants in healthy equine muscle.

RESEARCH SIGNIFICANCE AND OBJECTIVES

Significance

Elite equine athletes require a large amount of ATP to fuel maximal exercise leading to the potential production of excessive ROS and oxidative stress. To prevent oxidative stress, muscle must reduce the generated ROS through an adequate source of reduced antioxidants. If oxidative stress does occur, detrimental effects such as lipid, protein, and DNA oxidation may occur. Fully investigating the effects of antioxidant supplementation in healthy horses needs to proceed investigation of the effect of supplementation on horses with myopathies in order to isolate the effects of the supplement alone from an interaction between disease and supplementation.

Overarching objective

The overarching objective of this dissertations is to evaluate the impact of antioxidant supplementation on healthy Thoroughbred horses and determine their intracellular effects. Defining the effects of antioxidant supplements in healthy horses could lead to future studies investigating their impact on disease processes involving oxidative stress. A combination of hypothesis-driven research as well as discovery-based techniques like proteomics and transcriptomics were utilized to determine redox equilibriums, protein expression, gene expression, and pathways impacted by antioxidant and BCAA supplementation in healthy Thoroughbred horses.

Aim 1 The impact of chronic supplementation of NAC and CoQ10

Hypothesis: Provision of NAC and CoQ10 (NACQ) as an oral supplement will alter the redox equilibrium of healthy equine muscle by enhancing thiol-based antioxidants, increasing CoQ10 content, and altering mitochondrial protein expression.

Objectives:

1. To determine the effects of NACQ before and after maximal exercise on skeletal muscle GSH, CoQ10, and ROS concentrations as well as mitochondrial antioxidant activity.
2. To determine the effects of NACQ on the skeletal muscle proteome.

Aim 2 The impact of acute supplementation of CoQ10

Hypothesis: Micellized ubiquinol will have better plasma absorption relative to a standard dose of microencapsulated crystalline ubiquinone and provision of with RRR-α-tocopherol will further increase plasma absorption of micellized ubiquinol.

Objectives:

1. To determine the plasma absorption of a single dose of 1600 mg hydro-soluble ubiquinol compared to single administration of 1600 – 3200 mg of micellized ubiquinol.
2. To determine if co-supplementation of micellized ubiquinol and RRR-α-tocopherol increases plasma CoQ10 concentrations.

Aim 3 The impact of chronic supplementation of CoQ10

Hypothesis: Provision of micellized ubiquinol as a chronic oral supplement will enhance CoQ10 concentrations in both the skeletal muscle and plasma, alter the skeletal muscle redox equilibrium, and enhance both mitochondrial protein expression and ETC function.

Objectives:

1. To determine the effect of >30d CoQ10 supplementation on the skeletal muscle CoQ10 and ROS concentrations.
2. To determine the effect of >30d CoQ10 supplementation on the skeletal muscle proteome.
3. To determine the effect of >30d CoQ10 supplementation on the mitochondrial electron transport chain.

Aim 4 The impact chronic supplementation of NAC and BCAA

Hypothesis: Provision of chronic NAC and BCAA supplementation will enhance thiol-based antioxidants, alter the redox equilibrium of muscle, and alter proteomic profiles after submaximal exercise in comparison to a placebo.

Objectives:

1. To determine the effects of NAC and BCAA before and after submaximal exercise on the skeletal antioxidant and ROS concentrations.
2. To determine the effects of NAC and BCAA before and after submaximal exercise on the skeletal muscle proteome.

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CHAPTER 2

THE IMPACT OF N-ACETYL CYSTEINE AND COENZYME Q10 SUPPLEMENTATION ON CONCENTRATIONS OF GLUTATHIONE, COENZYME Q10 AND THE SKELETAL MUSCLE PROTEOME IN FIT THOROUGHBRED HORSES

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ABSTRACT

Horses have one of the highest skeletal muscle oxidative capacities amongst mammals which combined with a high glycolytic capacity could perturb redox status during maximal exercise. We determined the effect of 30 days of oral Coenzyme Q10 and N-acetyl-cysteine supplementation (NACQ) on muscle glutathione (GSH), cysteine, ROS and Coenzyme Q10 concentrations and the muscle proteome in 7 maximally exercising Thoroughbred horses using a placebo and randomized cross-over design. Gluteal muscle biopsies were obtained the day before and 1 hr after maximal exercise. Concentrations of GSH, cysteine, Coenzyme Q10 and ROS were measured, and citrate synthase, glutathione peroxidase, and superoxide dismutase activities analyzed. GSH increased significantly 1 hr post exercise in the NACQ group ($p=0.022$) whereas other antioxidant concentrations/activities were unchanged. TMT proteomic analysis revealed 40 differentially expressed proteins with NACQ out of 387 identified, including upregulation of 13 mitochondrial proteins (TCA cycle and NADPH production), 4 Z disc proteins, and down regulation of 9 glycolytic proteins. NACQ supplementation significantly impacted muscle redox capacity after intense exercise by enhancing muscle glutathione concentrations and increasing expression of proteins involved in the uptake of glutathione into mitochondria and the NAPDH-associated reduction of oxidized glutathione without any evident detrimental effects on performance.

INTRODUCTION

Horses have one of the highest skeletal muscle oxidative capacities amongst mammals with evolutionary adaptations in heart size and lung capacity maximizing aerobic metabolism (Essen-Gustavsson 1986). During aerobic muscle contraction, both ATP and reactive oxygen species (ROS) are produced in the mitochondria. Physiological amounts of ROS generated in skeletal muscle serve important adaptive signaling functions to prevent muscle fatigue, however, excessive ROS can overwhelm antioxidant capacity causing oxidative damage to proteins, lipids, and DNA with detrimental effects on muscle function (Shadel and Horvath 2015). Both nonenzymatic and enzymatic cellular antioxidants reduce ROS. Glutathione (γ-l-glutamyl-l-cysteinylglycine) is the most abundant nonenzymatic antioxidant in mammalian cells (Ribas, Garcia-Ruiz et al. 2014). It maintains the thiol status of critical proteins via cysteine's sulfhydryl group and defends against ROS via its reducing capacity (Ferreira and Reid 2008). Glutathione is synthesized endogenously from glutamate, cysteine and glycine with cysteine availability being the rate limiting step in synthesis (Kwon and Stipanuk 2001). Mammalian cells do not have a large storage pool of free cysteine, rather, cysteine is either synthesized from methionine (Kwon and Stipanuk 2001) or circulated to skeletal muscle in its oxidized form, cystine, where it is transported into the myoplasm and reduced to cysteine (Yu and Long 2016).

Enhancing plasma or tissue glutathione concentrations can be accomplished in other species, including humans, by increasing the availability of cysteine (Droge 2005). Human studies have utilized N-acetylcysteine (NAC) because cysteine is easily oxidized, has a relatively unstable shelf life, and its disulfide cystine is poorly soluble in water affecting absorption (Droge 2005, Atkuri, Mantovani et al. 2007). NAC increases the unbound pool of circulating cysteine and cells

exhibit high transport activity for this reduced form whereas transport activity for the larger disulfide cystine is relatively low (Sato, Tamba et al. 1999). One study has supplemented horses with oral NAC at 20 mg/kg and showed that NAC has an anti-inflammatory effect in the endometrium (Witte, Melkus et al. 2012). Although muscle concentrations or activities of antioxidants such as vitamin E (Roneus and Hakkarainen 1985, Roneus, Hakkarainen et al. 1986, Brown, Valberg et al. 2017) have been studied in supplementation trials, concentrations of skeletal muscle glutathione and many other antioxidants have not been reported in horses.

Ubiquinone, also known as coenzyme Q10, (CoQ10; 2,3 dimethoxy-5 methyl-6-decaprenyl benzoquinone) is another potent cellular antioxidant. CoQ10 serves an additional essential role in the mitochondrial electron transport system where it accepts electrons from complex I (NADH ubiquinone oxidoreductase) and complex II (succinate ubiquinone reductase) and transfers them to complex III (ubiquinol cytochrome c reductase) (Garrido-Maraver, Cordero et al. 2014). CoQ10 supplementation has been recommended for human athletes and for treatment of myopathies, CoQ10 deficiencies, neurodegenerative and cardiovascular disease in humans (Garrido-Maraver, Cordero et al. 2014). In horses, plasma CoQ10 concentrations have been shown to increase with oral supplementation of 1-3.4 g/day (Sinatra, Jankowitz et al. 2014, Ruiz, Tibary et al. 2021). Only one study has evaluated muscle CoQ10 concentrations in horses (Thueson, Leadon et al. 2019). In that study, fit Thoroughbred horses supplemented for 21 days with 1 g/day of oral CoQ10 had significantly increased gluteal muscle CoQ10 concentrations (Thueson, Leadon et al. 2019).

As elite athletes, Thoroughbred racehorses have maximal oxygen consumption that is two to three times greater than elite human athletes and a higher mitochondrial mass than many other species (Hoppeler and Weibel 1998, Poole 2003). Thus, it seems likely that Thoroughbred horses produce large amounts of ROS during maximal exercise which could have detrimental physiological impacts if ROS overwhelm antioxidant capacity. High levels of ROS have been measured in equine cultured myoblasts during hypoxia but not in skeletal muscle biopsies to the authors' knowledge (Ceusters, Mouithys-Mickalad et al. 2012). Enhancing the most ubiquitous antioxidant, glutathione, and the most potent antioxidant CoQ10 in skeletal muscle could have beneficial effects on skeletal muscle in equine athletes and horses with myopathies impacted by oxidative stress (Valberg, McKenzie et al. 2016, Bookbinder, Finno et al. 2019).

The purpose of the present study was to determine the effect of oral supplementation of NAC and CoQ10 (NACQ) on skeletal muscle ROS and enzymatic and nonenzymatic antioxidant concentrations/activities in exercising Thoroughbred horses. A second objective was to determine if NACQ supplementation had an impact on the skeletal muscle proteome.

METHODS

Horses

Seven Thoroughbred horses (5 geldings and 2 mares, 4.7 ± 2.1 y) were galloped over 1½ miles on the track 3 d/wk and walked (10 min) and trotted (20 min) on an automated exercise machine 3 d/wk. Horses were turned out for 3-6 h/d in a grass paddock for 3d/wk on exercise days and 8 h/d on their rest day. Throughout the trial, horses were fed timothy hay ad libitum and 2.1 kg/500 kg body weight of concentrate 3 times/d on exercise days and 2-3 times/d on rest days (OBS Racing Blend, Ocala Breeders Feed & Supply Ocala, FL) (Table 2.1). The amount fed was adjusted based on weekly body weights.

Study design

A randomized cross-over design was used where horses were supplemented with a placebo (40 g of an electrolyte supplement containing 9.6 g sodium, 2 g potassium, 16 g chloride, 3.6 g calcium and 1.2 g magnesium (Race Recovery, KER) once/d) or NACQ for 30 days. Daily NACQ consisted of 1.6 g CoQ10 (Nano Q10, KER Versailles, KY), 10 g NAC and the same electrolyte supplement as the placebo. The dose of CoQ10 and NAC was divided into the morning and evening concentrate and horses were watched to ensure complete consumption.

Test protocol

On Day 30 and Day 60, horses performed an exercise test consisting of a ½ mile (0.8 km) breeze on the track at the horse's top speed while wearing a heart rate monitor with GPS (KER Clockit Polar monitor). During the second exercise test on Day 60, the same experienced rider was instructed to replicate the GPS-determined speed from the Day 30 exercise test.

Table 2.1. Guaranteed analysis of the OBS Racing Blend concentrate provided to the horses throughout the study at 2.1 kg/500 kg body weight.

Nutrient	Amount
Crude Protein (Min)	12.0%
Lysine (Min)	0.7%
Crude Fat (Min)	8.0%
Crude Fiber (Max)	9.0%
Calcium (Min)	0.6%
Calcium (Max)	0.7%
Phosphorus (Min)	0.5%
Selenium (Min)	0.5ppm
Zinc (Min)	145ppm
Vitamin A (Min)	11,000 IU/kg
Vitamin D (Min)	1,100 IU/kg
Vitamin E (Min)	211 IU/kg

Muscle samples

The day prior to the exercise test (Day 29, 59), horses were sedated with 200 mg xylazine IV and a percutaneous needle biopsy of the gluteus medius muscle was obtained from a standardized site 16cm along a line from the highest point of the tuber coxae to the tail head using local anesthesia (Lindholm and Piehl 1974). The muscle biopsy procedure was repeated using the alternate side one hour after the exercise test on Day 30 and 60. Muscle samples were immediately flash-frozen in liquid nitrogen.

Blood samples

Jugular venous blood samples were obtained prior to the exercise test and 10 min, 1 h and 4 h after the exercise test. A buffy coat from EDTA tubes was obtained from each horse and frozen at -80ºC. Plasma samples were kept chilled on ice or in the refrigerator, centrifuged within two hours of collection, frozen on dry ice and shipped with muscle samples to the laboratory on dry ice where they were stored at -80ºC until analyses.

Blood sample analysis

Plasma lactate concentrations were analyzed using a YSI lactate analyzer (YSI incorporated, Yellow Springs, OH). Plasma creatine kinase (CK) and gamma-glutamyl transferase (GGT) activity was assayed in pre-exercise and 4 hr post-exercise samples at the Veterinary Diagnostics Laboratory at Michigan State University using standard methods.

DNA was isolated from 250uL buffy coat using the Qiagen DNA extraction kit (Qiagen, Hilden, Germany). Myostatin genotypes were determined using primers designed to amplify a 150bp

region containing a previously identified single nucleotide polymorphism (g.66493737C>T) as previously described (Hill, McGivney et al. 2010, Petersen, Valberg et al. 2014). Sanger sequencing and Sequencher software (Gene Codes Corporation, Ann Arbor, MI) were used to determine myostatin genotypes.

Muscle homogenate

For the ROS, cysteine, and glutathione assays, approximately 30 mg of muscle was homogenized in radioimmunoprecipitation assay buffer (RIPA). Protein content of the homogenate was determined using a pierce BCA assay kit (Thermo Scientific, Waltham, MA).

Reactive oxygen species

ROS were analyzed in resting and post-exercise muscle samples using 10 µl of the homogenate diluted 20x in RIPA buffer. An oxidant sensitive fluorescent probe kit (OxiSelect STA-347, Cell BioLabs) was used to measure total ROS which included reactive nitrogen species, including hydrogen peroxide, nitric oxide, peroxyl radical, and peroxynitirite anion in resting and 1 hr post-exercise muscle samples. Sample concentrations were measured using Synergy h1 plate reader (Biotek, Winooski, VT) with 480nm excitation and 530nm emission in a 96-well black plate with white wells. All samples and standards were measured in triplicate and concentrations were measured as hydrogen peroxide (H_2O_2) equivalence.

Thiols

Cysteine and glutathione concentrations were analyzed in resting and post-exercise muscle homogenates using liquid chromatography tandem mass spectrometry (LC/MS). Protein in the

homogenate was reduced using Tris(2-carboxyethyl)phosphine hydrochloride in water (pH 7) containing N-ethylmaleimide which binds to the active sulfhydryl group stopping any subsequent reactions. An internal standard of 20uM GSH ammonium salts D-5 (Toronto Research Chemicals, North York, Ontario, Canada) was added to all samples and standards. Chromatographic separation of cysteine and glutathione was achieved using a Phenomenex Kinetex 1.7um EVO C18 100A (50 x 2.1mm) (Phenomenex, Torrance, CA) column with a gradient elution consisting of acetonitrile and deionized water with 0.1% formic acid. Multiple reaction monitoring optimized using Waters Empower 4 software was used for detection of ions generated by cysteine and glutathione in the LC/MS.

CoQ10

CoQ10 analysis of resting muscle samples was performed at the Michigan State University Mass Spectrometry and Metabolomics Core using a high-resolution/accurate-mass (HR/AM) UHPLC-MS/MS system consisting of a Thermo Vanquish UHPLC interfaced with Thermo Q-Exactive according to Pandey 2018 (Pandey, Riley et al. 2018). Approximately 10 mg of tissue was homogenized in a 95:5 ethanol:2-propanol solution containing 500 ng/mL CoQ4 internal standard with 125 μg of butylated hydroxytoluene pre-dried in the homogenization tube. CoQ10 was extracted from this homogenate by adding 400 μL of hexane then 200 μL milli-Q water. The organic phase was collected, evaporated and then reconstituted in 2 mL of ethanol containing 0.3 M hydrochloric acid. 10 uL of sample was injected onto a Waters Acquity BEH-C18 UPLC column (2.1x100 mm) and eluted using a 5 minute isocratic flow of 5 mM ammonium formate in 2-propanol/methanol (60:40 v/v) at 0.3 ml/min. Compounds were ionized by electrospray operating in positive ion mode with a spray voltage of 3.5 kV, capillary temperature of

256.25°C, probe heater temperature of 412.50°C, and S-Lens RF level of 50. Spectra were acquired using a full MS/all ion fragmentation method at 70,000 resolution, AGC target of 1e6, and mass range of m/z 150-1000. The normalized collision energy for the AIF scans was set to 22V. Data were processed using Xcalibur software version 4.1.31.9.

Glutathione Peroxidase and Superoxide Dismutase

Glutathione peroxidase and superoxide dismutase activities were measured in resting and post-exercise muscle samples using colorimetric assay kits (Cayman Chemical, Ann Arbor, MI) according to manufacturer's instructions. For the superoxide dismutase assay, 10mg of tissue was homogenized in pH 7.2 buffer containing 20mM 2-[4-(2-hydroxyethyl)piperazine-1-yl]ethanesulfonic acid, 1mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, 210mM mannitol, and 70mM sucrose. For the glutathione peroxidase assay, 10mg of tissue from was homogenized in pH 7.5 buffer containing 50mM Tris-HCl, 5mM ethylenediaminetetraacetic acid, and 1mM dithiothreitol. The reducing agent BCA assay kit (Thermo Scientific) was used to determine protein content for glutathione peroxidase and superoxide dismutase homogenates.

Citrate Synthase

Citrate synthase (CS) activity, a marker for mitochondrial volume density, was assayed in resting muscle samples. Approximately 10 mg of gluteal muscle was homogenized in 0.1 M phosphate buffer (pH 7.3) and the activity of citrate synthase was determined fluorometrically at 25°C according to Essen and Henrickson et al., 1984 (Essen-Gustavsson and Henriksson 1984).

Statistical analysis

Data was analyzed for normality using a Shapiro-Wilks normality test. Maximal heart rates during the exercise test were compared between placebo and NACQ using a paired t-test. Plasma lactate concentrations, GGT, and CK activities were analyzed using a repeated measure analysis of variance (rmANOVA). Data for ROS, GSH, and cysteine did not pass normality and were log transformed. An rmANOVA with Bonferroni post hoc testing was used to analyze GSH, cysteine, ROS, SOD, GPx, and CoQ10. A paired t-tests were used to analyze CS activities. Analyses were performed using GraphPad Prism software version 8.2.0 (GraphPad Software San Diego, CA). Significance was set at $P<0.05$.

Muscle proteomics

Proteomic analysis was performed on the 29- and 59-Day resting muscle samples obtained from the 5 geldings in the study using a tandem mass tag 11-plex MS/MS quantification analysis at the Michigan State University Proteomics Core. Protein was extracted from muscle samples using a radioimmunoprecipitation lysis buffer and protease inhibitor and pelleted prior to submission. From each sample, 500 μg of protein was digested in trypsin with a Filter-Aided Sample preparation protocol and spin ultrafiltration unit cutoff of 30,000Da (Wisniewski, Zougman et al. 2009). Reverse phase C18 SepPaks were used to de-salt the resulting peptides (Waters Corporation) which were then dried by vacuum centrifugation. Peptide quantification was verified by colorimetric peptide concentration with 5 μL from each sample digest using a pierce BCA assay kit (Thermo Scientific). Isobaric labeling, gel fractionation and LC/MS/MS analysis were performed as previously described (Valberg, McKenzie et al. 2016).

Quantitative data analysis

Scaffold Q+ (version Scaffold_4.9.0, Proteome Software Inc., Portland, OR) was used to quantitate TMT Label Based Quantitation peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at greater than 99.9% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii, Keller et al. 2003). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins sharing significant peptide evidence were grouped into clusters. Channels were corrected by the matrix in all samples according to the algorithm described in i-Tracker (Shadforth, Dunkley et al. 2005). Normalization was performed iteratively (across samples and spectra) on intensities, as described in Statistical Analysis of Relative Labeled Mass Spectrometry Data from Complex Samples Using ANOVA (Oberg, Mahoney et al. 2008). Medians were used for averaging. Spectra data were log-transformed, pruned of those matched to multiple proteins, and weighted by an adaptive intensity weighting algorithm. Of 108665 spectra in the experiment at the given thresholds, 79921 (74%) were included in quantitation. Differentially expressed proteins were determined by applying Permutation Test with unadjusted significance level $P < 0.003$ corrected by Benjamini-Hochberg. Significant proteins were grouped according to their cellular functions.

RESULTS

Exercise test, heart rates, and lactate concentrations

The maximum speed attained (NACQ 16.1 ± 0.7 m/s; placebo 16.1± 0.9 m/s) and maximum heart rate (NACQ 206 ± 8.34 bpm; placebo 207 ± 9.38 bpm) did not differ between NACQ and placebo (P=0.69) or between Day 30 and Day 60 (P=0.58). Plasma lactate concentrations were significantly higher (P<0.0001) at 10 min post exercise than all other timepoints and did not differ between NACQ and placebo (P=0.68) (Figure 2.1).

Plasma CK and GGT activities increased significantly from rest to 4 h post exercise (CK P<0.001, GGT P= 0.008) and did not differ significantly between placebo and NACQ (CK P=0.64, GGT P<0.99) (Table 2.2).

ROS, glutathione, and cysteine concentrations

The average coefficient of variance between replicates across all samples was 3.9%. There was no significant difference in muscle ROS between NACQ and placebo either before or after exercise (Table 2.3). ROS concentrations did not change significantly with exercise on either the NACQ or the placebo and showed wide individual variation. Muscle glutathione concentrations increased significantly by 36% after exercise (P=0.022) on NACQ compared to placebo whereas cysteine concentrations did not differ significantly (P=0.40) (Figure 2.2).

Glutathione peroxidase and superoxide dismutase activity

There was no significant difference in muscle glutathione peroxidase or superoxide dismutase activities in resting or post exercise muscle between NACQ and placebo (Table 2.3).

Figure 2.1. The concentration of plasma lactate in horses supplemented with NACQ compared to placebo, before, during and after an exercise test at a maximal gallop. Lactate concentrations were significantly higher 10 min post exercise than at all other timepoints (P<0.0001) and were not significantly different between placebo and NACQ.

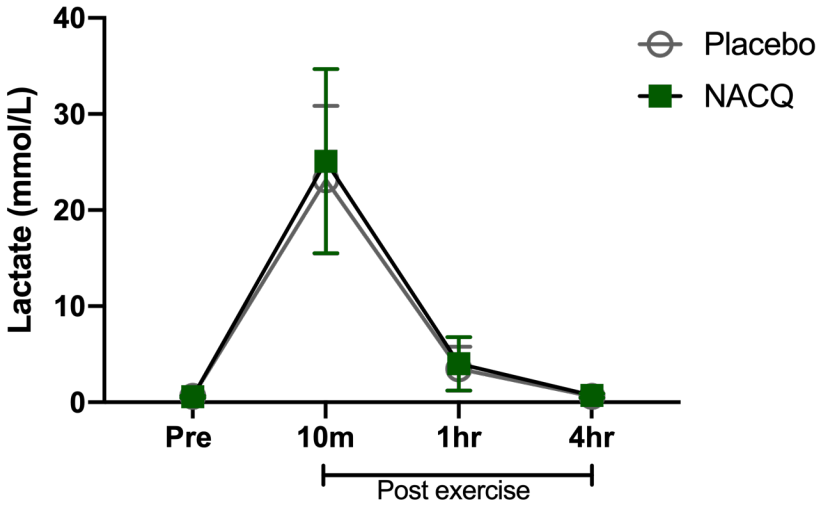


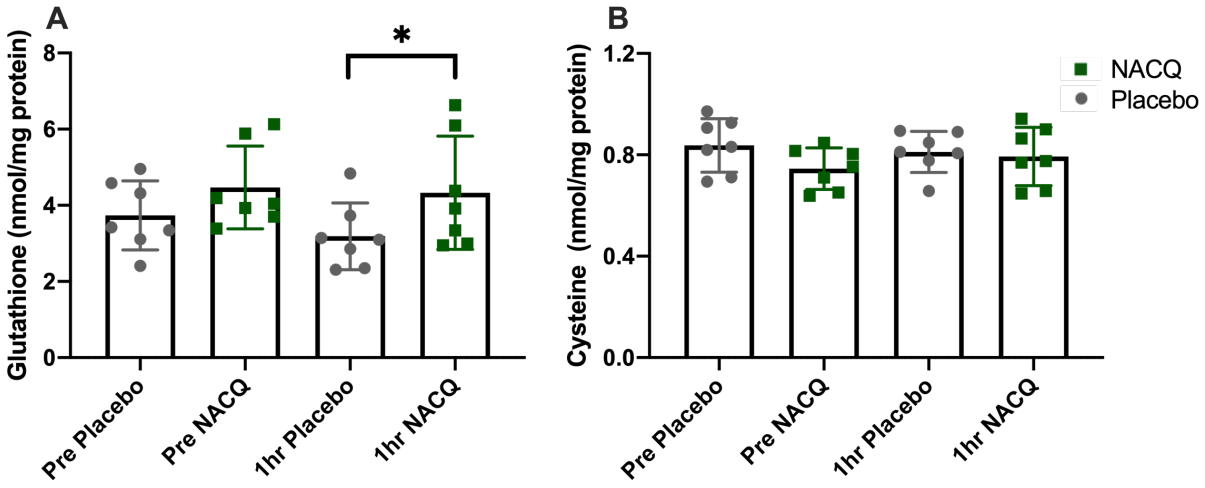
Table 2.2. Plasma gamma-glutamyl transferase (GGT) and creatine kinase (CK) activity in N-acetyl cysteine and coenzyme Q10 (NACQ) and placebo supplemented horses. Differences in superscripts indicate significant differences with supplementation or exercise. There were no significant effect of NACQ/placebo supplementation, however there was a significant effect of exercise on gamma glutamyl transferase (GGT) (P=0.008) and creatine kinase (CK) activities (P<0.001).

	GGT (U/L)	CK (U/L)
	Mean ± Sd	**Mean ± Sd**
Placebo Pre	18.7 ± 6.0^{a}	190.0 ± 44.3^{a}
Placebo 4 h Post	25.0 ± 8.0^{b}	446.1 ± 171.2^{b}
NACQ Pre	20.0 ± 7.2^{a}	278.4 ± 151.4^{a}
NACQ 4 h Post	22.6 ± 5.7^{b}	387.4 ± 146.2^{b}

Table 2.3. Skeletal muscle reactive oxygen species (ROS), Coenzyme Q10 (CoQ10) concentrations and activities of glutathione peroxidase (GPx) and superoxide dismutase (SOD) in NACQ and placebo supplemented horses. Differences in superscripts indicate significant differences with supplementation or exercise. There were no significant effects of exercise or NACQ/placebo supplementation.

	Supplement	Rest	1 h post-exercise	P value
ROS	NACQ	16.51 ± 6.14 [a]	14.68 ± 5.60 [a]	
(uM H_2O_2 equivalence)	Placebo	13.81 ± 4.83 [a]	17.55 ± 5.00 [a]	0.4779
CoQ10	NACQ	7090 ± 2898 [a]	7653 ± 3632 [a]	
(ng/mg protein)	Placebo	7679 ± 2442 [a]	8560 ± 416 [a]	0.8352
GPx	NACQ	13.81 ± 4.83 [a]	17.55 ± 5.00 [a]	
(mmol/min/mg protein)	Placebo	25.53 ± 5.08 [a]	21.52 ± 5.53 [a]	0.2065
SOD	NACQ	7.178 ± 1.238 [a]	6.604 ± 0.792 [a]	
(U/mg protein)	Placebo	7.377 ± 1.551 [a]	7.390 ± 1.187 [a]	0.6191

Figure 2.2. A. Gluteal muscle glutathione concentrations in horses at rest and one h after exercise with NACQ (green) or placebo (grey) supplementation. One-hour post-exercise glutathione concentrations were significantly higher in NACQ horses than placebo (P=0.022) **B.** Muscle cysteine concentrations at rest and 1 hr after exercise in horses supplemented with NACQ or placebo. Concentrations were not significantly different (P=0.40).



CoQ10 and citrate synthase

Resting muscle CoQ10 concentrations did not differ significantly between NACQ and placebo or pre and post exercise samples. Considerable interindividual variation was found in muscle CoQ10 concentrations. CS activity did not differ between NACQ and placebo (P=0.36) or between muscle samples obtained on Day 29 and Day 59 (P=0.88). CoQ10 concentrations were significantly moderately correlated with CS activity (r=0.56, P=0.003) (Figure 2.3A). Muscle CoQ10 expressed as CoQ10 concentration/unit CS activity did not differ significantly between NACQ and placebo (P=0.99) (Table 2.3, Figure 2.3B).

Myostatin genotypes

One horse was homozygous for the g.66493737C>T variant previously associated with a sprinter phenotype, two horses were homozygous wild type previously associated with a stayer phenotype and four horses were heterozygous (McGivney, Browne et al. 2012). There did not appear to be a particular pattern of CoQ10 concentrations or response to CoQ10 supplementation amongst the 3 genotypes although there were too few horses for a statistical analysis (Figure 2.4).

Figure 2.3. A. Correlation between muscle Coenzyme Q10 concentrations and CS activity in resting gluteal muscle samples (R=0.56, P=0.003). **B.** The ratio of CoQ10/CS in horses supplemented with NACQ or placebo. No significant differences were apparent (P=0.99).

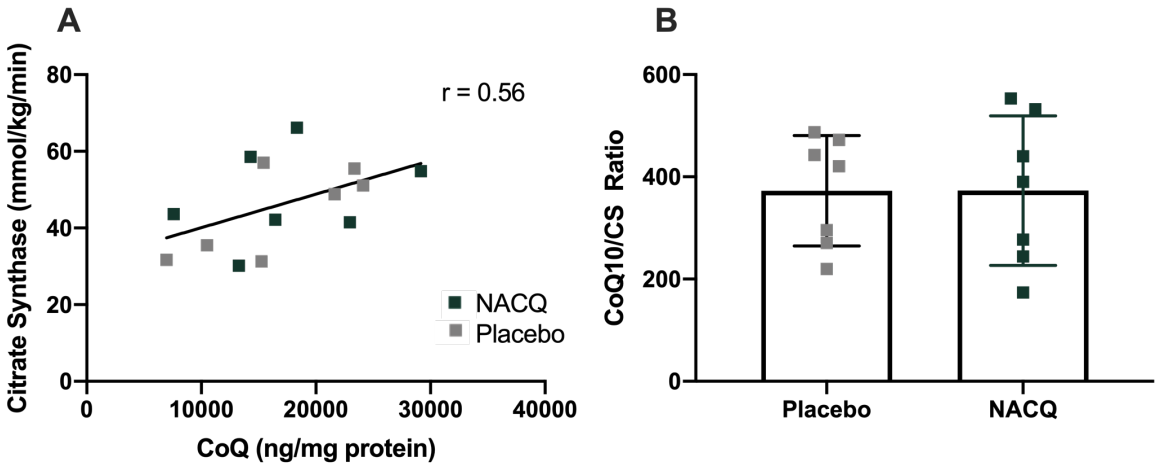
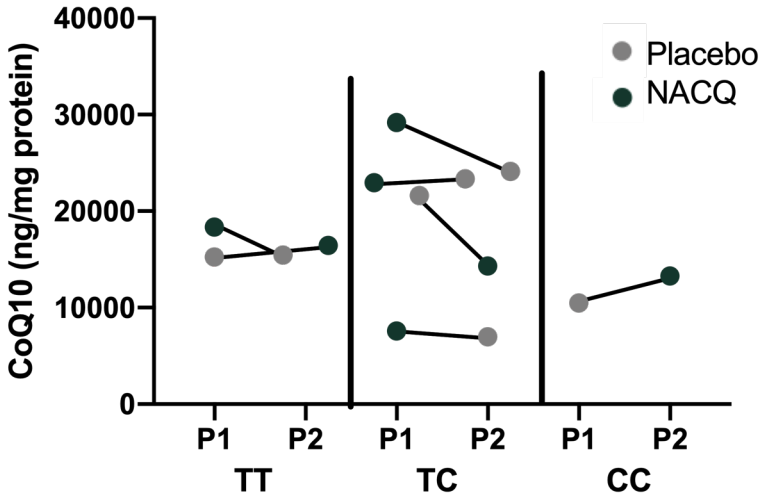


Figure 2.4. Myostatin genotypes and CoQ10 concentrations in horses on the placebo (grey) and on NACQ (green). The order of the points represents the order in which horses were fed the placebo or NACQ in the randomized block design.



Proteomics

There were 387 unique total proteins identified in the proteomic dataset with 40 proteins differentially expressed between NACQ and placebo: 22 with increased expression and 18 decreased expression (Table 2.4). Proteins with increased expression largely localized to the mitochondrion and the sarcomeric Z disc with 3 appearing to be blood-borne (albumen, apolipoprotein A1, serum macroglobulin) (Table 2.4). Mitochondrial proteins included 5 proteins involved in the generation of NADH in the tricarboxylic acid cycle, 3 protein subunits in the electron transfer system, and 3 proteins involved in long chain fatty acid metabolism or transport (Table 2.4, Figure 2.5). The upregulated citric acid cycle proteins included two enzymes that produce succinyl CoA, isocitrate dehydrogenase (IDH2) and 2-oxogluterate dehydrogenase (OGDH). OGDH as well as an additional upregulated protein in the mitochondrial inner membrane, NADP transhydrogenase (NNT), generate nicotinamide adenine dinucleotide phosphate (NADPH) which is required to reduce oxidized glutathione. Upregulated proteins in the sarcomere were either components of the Z disc or a Z disc associated chaperone protein (CRAYB) that prevents protein misfolding. The sarcomeric Z-disc defines the lateral borders of the sarcomere and is important for mechanical stability of contractile filaments.

Proteins with decreased expression were localized to the sarcoplasmic reticulum, the sarcomere, and the myoplasm (Table 4). This included -glutamate O-methyltransferase (ARMT1) which is involved in the synthesis of cysteine from methionine, fast twitch 2X myosin (MYH1), the fast-twitch sarcoplasmic reticulum ATPase (ATP2A1), and nine glycolytic/gluconeogenic enzymes (Table 2.4).

Several antioxidant proteins were expressed in the proteomic dataset but were not differentially expressed including mitochondrial superoxide dismutase [Mn], superoxide dismutase [Cu-Zn], catalase isoform X1, glutathione S-transferase P, cluster of glutathione S-transferase Mu 1 and mitochondrial thioredoxin-dependent peroxide reductase.

Table 2.4. The gene identification for a protein, protein name, log_2 fold change and P value adjusted for multiple test corrections in 5 geldings supplemented with NACQ compared to placebo.

Gene ID	Protein Name	Adjusted P value	Log_2 Fold Change
Increased expression			
MB	Myoglobin	1.90E-03	0.22
	Mitochondria		
	TCA cycle		
IDH2	Isocitrate Dehydrogenase [NADP] Mitochondrial	2.10E-04	0.12
ACO2	Aconitate Hydratase Mitochondrial	5.00E-04	0.11
MDH2	Malate Dehydrogenase Mitochondrial	4.00E-03	0.11
FH	Fumarate Hydratase Mitochondrial	2.00E-03	0.11
OGDH	2-Oxogluterate Dehydrogenase Mitochondrial Isoform X3	8.70E-04	0.09
	Electron Transfer System		
COX5A	Cytochrome C Oxidase Subunit 5A Mitochondrial	8.90E-04	0.18
COX4I1	Cytochrome C Oxidase Subunit 4 Isoform 1 Mitochondrial	5.00E-04	0.13
ATP5F1B	ATP Synthase Subunit beta Mitochondrial	1.00E-04	0.10
	Fat metabolism		
ACADVL	Very Long-Chain Specific Acyl-CoA Dehydrogenase Mitochondrial	1.00E-03	0.11
ASCL1	Long-Chain-Fatty-Acid-CoA Ligase 1 Isoform X2	2.70E-03	0.10
FABP3	Fatty Acid-Binding protein Heart	1.00E-03	0.27
	Other		
NNT	NAD(P) Transhydrogenase Mitochondrial Isoform X1	3.00E-03	0.07
VDAC2	Voltage-Dependent Anion-Selective Channel Protein 2	2.00E-03	0.14
	Sarcomere Proteins		
	Z disc		
CRYAB	Alpha-Crystallin B Chain	1.00E-04	0.28
ACTN2	Alpha-Actinin-2 Isoform X1	1.00E-04	0.16
FLNC	Filamin-C Isoform X1	1.30E-04	0.12
PDLIM5	PDZ and LIM Domain Protein 5 Isoform X9	7.20E-04	0.12
	Myosin		
MYBPC1	Myosin-Binding Protein C Slow-Type Isoform X2	1.00E-04	0.11
	Blood Proteins		
ALB	Serum Albumin Precursor	1.00E-04	0.38
APOA1	Apolipoprotein A-I	1.00E-03	0.34
LOC100061692	Alpha-2 Macroglobulin	3.50E-03	0.22

Table 2.4 (cont'd)

Gene ID	Protein Name	Adjusted P value	Log_2 Fold Change
	Decreased expression		
	Cysteine Synthesis		
ARMT1	Protein-Glutamate O-Methyltransferase	7.30E-04	-0.40
	Glycolysis/Gluconeogenesis		
PFKM	ATP-Dependent 6-Phosphofructokinase Muscle Type Isoform X3	2.00E-03	-0.07
PKM	Pyruvate Kinase PKM Isoform M1	1.00E-04	-0.10
LDHA	L-lactate Dehydrogenase A Chain	1.80E-04	-0.10
FBP2	Fructose -1,6-bisphosphatase isozyme 2	4.00E-03	-0.11
GPI	Glucose-6-Phosphate Isomerase	1.00E-04	-0.14
PHKA1	Phosphorylase B Kinase Regulatory Subunit alpha Skeletal Muscle Isoform X1	1.00E-03	-0.14
PHKB	Phosphorylase B Kinase Regulatory Subunit beta	2.00E-03	-0.14
ENO3	Beta-Enolase	1.00E-04	-0.15
PGM1	Phosphoglucomutase-1 Isoform X2	1.00E-04	-0.15
	Sarcomere		
MYOM1	Myomesin-1 Isoform X4	1.00E-03	-0.08
MYOM2	Myomesin-2 Isoform X3	1.00E-04	-0.12
MYH1	Myosin-1 Isoform X1	2.70E-04	-0.11
MYBPC2	Myosin-Binding Protein C Fast-Type	1.00E-04	-0.17
MYLK2	Myosin Light Chain Kinase 2 Skeletal/Cardiac Muscle	2.50E-03	-0.20
	Calcium Regulation		
SRL	Sarcalumenin Isoform X1	1.00E-03	-0.09
ATP2A1	Sarcoplasmic/Endoplasmic Reticulum Calcium ATPase 1 Isoform X1	1.00E-04	-0.17
ANXA6	Annexin A6 Isoform X1	1.00E-04	-0.13

DISCUSSION

The present study examined the impact of 30 d of supplementation of NACQ on non-enzymatic (glutathione, CoQ10) and enzymatic (glutathione-peroxidase and superoxide dismutase) antioxidants in skeletal muscle as well as the effect on the muscle proteome in fit Thoroughbred horses. Results showed that NACQ supplementation significantly enhanced post-exercise glutathione concentrations, enhanced proteins involved in reduction of oxidized glutathione and enhanced proteins involved in mitochondrial oxidative energy metabolism and the Z disc while decreasing glycolytic enzyme and fast-twitch type 2X myosin protein expression.

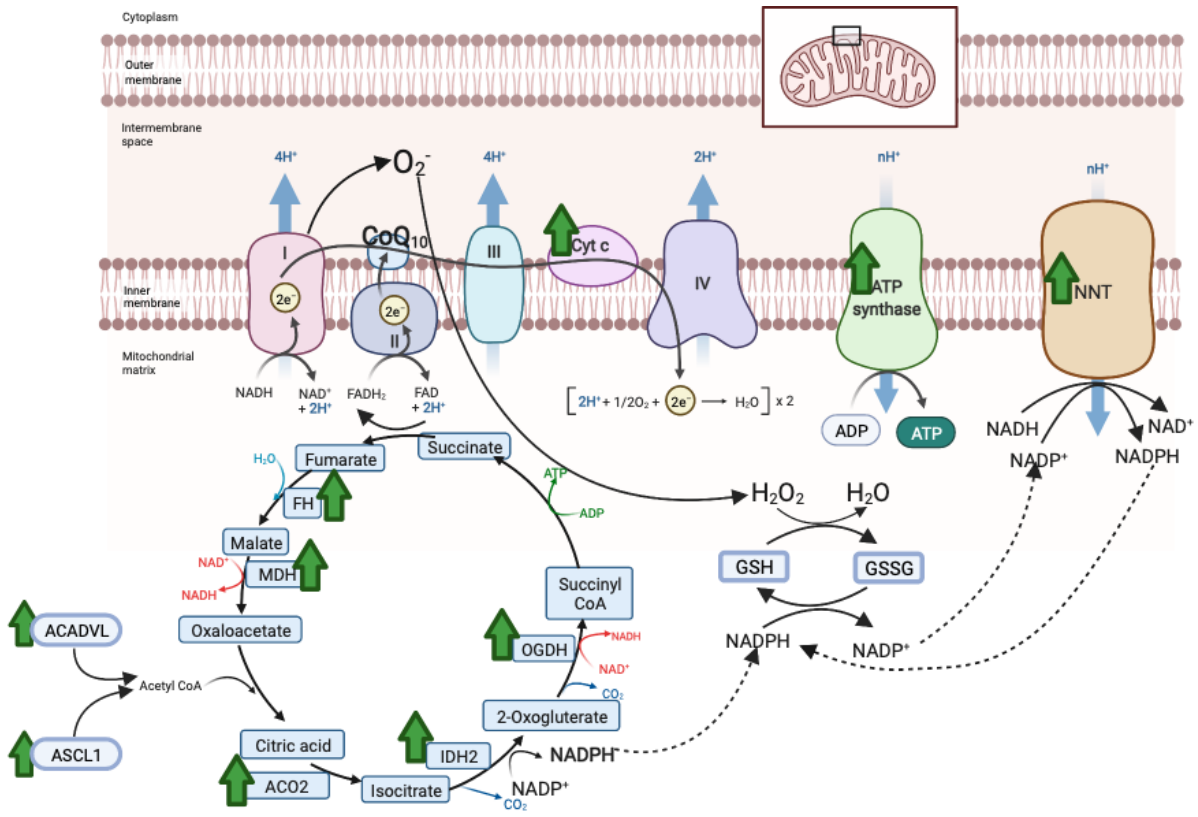
Glutathione concentrations have been previously measured in skeletal muscles samples from several different species resulting in wide ranges of concentrations depending on the method of analysis and the muscle group sampled (Martensson and Meister 1989, Ji, Fu et al. 1992, Marin, Kretzschmar et al. 1993, Luo, Hammarqvist et al. 1996, Hammarqvist, Luo et al. 1997, Hammarqvist, Andersson et al. 2005, Dam, Mitchell et al. 2012, Michailidis, Karagounis et al. 2013, Morin, Guiraut et al. 2019). When predominantly fast twitch muscle such as the human vastus lateralis or "quadriceps" (~60% fast twitch muscle fiber; glutathione 1.2 -1.4 nmol/mg ww) (Staron, Hagerman et al. 2000) were compared to Thoroughbred gluteal muscle (~80% fast twitch muscle fibers 0.85 nmol/kg ww) (Aldrich, Velez-Irizarry et al. 2021) measured by HPLC, horses appear to have approximately 40% lower glutathione concentrations than humans. This could be in part be due to the higher fast-twitch fiber type composition of horse gluteal muscle compared to human vastus lateralis or intriguingly, equine muscle could have a lesser capacity to synthesize glutathione or an enhanced turnover of glutathione with exercise.

Glutathione concentrations were significantly higher (↑ 35%) 1 h post-exercise in horses on NACQ compared to placebo but did not differ significantly between treatments at rest. This agrees with a study of human athletes taking 60 mg/kg oral NAC that showed a significant increase in glutathione concentrations (~14%) 2 h after, but not before, exercise in vastus lateralis muscle on supplementation (Michailidis, Karagounis et al. 2013). The reason for the post-exercise increase in glutathione could be stimulation of enzymes involved in glutathione synthesis by exercise combined with increased availability of muscle cysteine (Marin, Kretzschmar et al. 1993). Oral NAC provides a readily absorbable form of cysteine that liberates endogenous, protein-bound cysteine in plasma (Radtke, Coles et al. 2012) and enhances transport of cysteine into muscle cells by the gamma-glutamyl cycle (Atalay, Seene et al. 1996, Medved, Brown et al. 2004). A measurable increase in skeletal muscle cysteine concentrations was not detected on NACQ, which could have been due to rapid incorporation of soluble cysteine into thiol-based antioxidants and other proteins. After exercise, gamma-glutamyl transferase had significantly increased serum activity in our horses which could have facilitated rapid cysteine transport into muscle and subsequent synthesis of glutathione in the 1h post-exercise samples. A potential reduction in synthesis of cysteine from methionine in horses on NACQ was supported by the finding that glutamate O-methyltransferase (ARMT1) had decreased protein expression in NACQ versus placebo supplemented muscle. ARMT1 is one of the enzymes required to synthesize cysteine from methionine (Schubert, Blumenthal et al. 2003).

An enhanced ability to shuttle glutathione into mitochondria and to reduce oxidized glutathione in horses on the NACQ supplement was supported by proteomic data. Fumerate (FH) and 2-oxoglutarate dehydrogenase (OGDH) which produce malate/2-oxoglutarate for the SLC25A

mitochondrial glutathione shuttle had increased expression on NACQ vs placebo (Figure 2.5). Additionally, isocitrate dehydrogenase (IDH2) and NADP+ transporter (NNT) which generate NADPH to reduce glutathione also had increased expression (Schiaffino, Reggiani et al. 2015) (Figure 2.5). Taken together, the results of our study support the ability of NACQ supplementation to enhance post-exercise muscle glutathione concentrations, likely through increased muscle cysteine availability and also the potential ability of NACQ to enhance the capacity of muscle to reduce oxidized glutathione.

Figure 2.5. Proteins with significantly increased expression (green arrow) in the mitochondria of horses on NACQ compared to placebo. Horses with NACQ supplementation had upregulation of 2 cytochrome C subunits, 1 subunit of ATP synthase, NAD(P) transhydrogenase (NNT), fumarate hydratase (FH), malate dehydrogenase (MDH), 2-oxoglutarate dehydrogenase (OGDH), NADP dependent isocitrate dehydrogenase (IDH2), aconitase (ACO2), Very Long-Chain Specific Acyl-CoA Dehydrogenase Mitochondrial (ACADVL), and Long-Chain-Fatty-Acid-CoA Ligase 1 Isoform X2 (ASCL2). Created with Biorender.com.



Enzymatic antioxidants identified either in the proteome or by measuring enzymatic activities in muscle samples were not altered by the NACQ supplementation. The ability of a number of these antioxidants to continue functioning as reducing agents, however, could be indirectly enhanced by supplementation with NACQ by providing a ready supply of glutathione which is reciprocally oxidized in the process of reducing oxidized glutathione peroxidase or thioredoxins and peroxiredoxins (Ezraty, Gennaris et al. 2017).

Coenzyme Q10 was the additional antioxidant in the NACQ supplement. Similar to studies in healthy humans, we did not find a significant increase in muscle CoQ10 concentrations after CoQ10 supplementation. Neither a 14 d trial with an oral fast-melt CoQ10 formulation (manufacture's recommended dose 100 mg/d) (Cooke, Iosia et al. 2008) nor a 28 d trial using 150mg oral capsule supplementation produced an increase in muscle CoQ10 in healthy subjects (Zhou, Zhang et al. 2005). Muscle CoQ10 concentrations were shown, however, to increase with supplementation in humans with deficiencies in enzymes required to synthesize CoQ10 (Garrido-Maraver, Cordero et al. 2014). Concentrations of muscle CoQ10 in our healthy horses were found to be similar to those measured in healthy humans using a similar MS/MS analysis (Montero, Sanchez-Alcazar et al. 2008). The absence of an increase in CoQ10 muscle concentrations in supplemented horses during the present study could be due to could have arisen from inhibition of extraneous muscle CoQ10 absorption due to optimal endogenous muscle concentrations.

There have been previous equine studies measuring the effect of CoQ10 supplementation on plasma CoQ10 concentrations (Sinatra, Chopra et al. 2013, Sinatra, Jankowitz et al. 2014, Svete,

Vovk et al. 2021). Two of the studies utilized oral CoQ10 supplementation (HydroQ-Sorb; GelTec/Tishcon Corp., Westbury, NY) with one study feeding 800 mg/d for 60d (Sinatra, Chopra et al. 2013) and the other utilizing two different doses of 1.9g and 3.4g as acute doses to measure their effect on exercise (Sinatra, Jankowitz et al. 2014). The third study utilized supplementation with both 800 mg CoQ10 (Vital Paste 7.5%, Valens Int. d. o. o., Šenčur, Slovenia) and 1.8 IU/kg BW/day Vitamin E (d-α-tocopherol acetate; natural vitamin E-oil, Natural Wealth, Bohemia, NY, USA) for 14 days (Svete, Vovk et al. 2021). The first two studies found an increase in plasma CoQ10 concentrations (Sinatra, Chopra et al. 2013, Sinatra, Jankowitz et al. 2014); however, this was attenuated with exercise in the second study (Sinatra, Jankowitz et al. 2014) and the third study found an increase in plasma CoQ10 when supplemented with both CoQ10 and Vitamin E (Svete, Vovk et al. 2021). It has not been established whether an increase in plasma CoQ10 concentration correlates with an increase in muscle CoQ10 concentrations.

Our results are in contrast to a previous equine study in which 6 fit Thoroughbred horses were supplemented with 1g of ubiquinol (Recovery Sport, Anlon Nutrition, Kilcullen, Ireland) for 3 weeks using a cross-over design (Thueson, Leadon et al. 2019). The previous study found a significant increase in muscle CoQ10 concentrations which was largely driven by 2/6 individual horses. Muscle CoQ10 concentrations in the previous study were reported to be similar to concentrations in human muscle measured using an HPLC method. Notably, CoQ10 concentrations measured by HPLC in the previous equine study were approximately 100x lower than those measured in our study using an MS/MS method (Thueson, Leadon et al. 2019). The differences in results between the two equine studies could be due to methodological differences,

differences in gastrointestinal absorption of the different CoQ10 supplements or the small number of horses and the large individual variability in both CoQ10 concentrations and responses to supplementation. Unfortunately, serial blood samples coordinated with oral supplementation were not obtained in the present or the previous study of equine muscle CoQ10 concentrations (Thueson, Leadon et al. 2019).

It has been suggested that wide individual variation of CoQ10 concentrations in horses could be linked to myostatin genotypes based on a study that inferred CoQ10 concentrations from mitochondrial complex I + III and complex II + III activities (Rooney, Porter et al. 2017). Although the number of horses with diverse genotypes in our study was small, visual inspection of the data did not identify a relationship between myostatin genotype and either muscle CoQ10 concentrations measured by MS/MS or responses to CoQ10 supplementation (Figure 2.4).

The near-maximal exercise tests used in our study was performed on a standard racetrack and appeared highly repeatable as there were no differences in maximal speed, maximal heart rate or plasma lactate concentrations between the first and second exercise tests. Our cross-over design, however, did not allow us to evaluate any potential long-term benefit of NACQ supplementation on athletic performance; nor did the use of combined NAC and CoQ10 supplementation allow us to differentiate alterations caused by NAC from those caused by CoQ10. The cross-over design did mitigate any potential effects of training in this study, as confirmed by muscle CS activity which was not significantly different between Day 29 and 59 in our study. In a field setting, volatile Thoroughbreds require muscle biopsies to be obtained following a cool down period and not immediately after exercise. At one hour after exercise, an increase in ROS was not identified

and no differences were found in ROS between NACQ and placebo before or after exercise. Unfortunately, our study could not ascertain whether or not high amounts of ROS were generated during exercise without further serial biopsies. A previous study found an increase in plasma malondialdehyde (MDA) activity after exercise in untrained horses in both the placebo and the CoQ10 groups suggesting that ROS is generated during exercise (Svete, Vovk et al. 2021). The increase in MDA was attenuated by additional supplementation with Vitamin E (Svete, Vovk et al. 2021).

The TMT proteomic analysis used in the present study evaluated the relative abundance of muscle proteins with NACQ supplementation relative to the placebo. The study assessed comparisons within the same individual minimizing intra-individual variability common with repeated measures. The log_2FC differences identified were relatively small, however, the biological relevance of the changes was supported by the fact that proteins with related functions had similar patterns of differences. The general tendency in the proteomic analysis was for an increase in proteins involved in oxidative metabolism and a decrease in proteins involved in glycolytic metabolism indicating a relative shift toward a more oxidative phenotype. This shift was not sufficient enough to produce an increase in CS activity, which is often used as a marker for enhanced mitochondrial volume density in horses (Hoopeler, Jones et al. 1987). The functional impact of the small yet significant alterations in the muscle proteome were not assessed in the present study.

The ability of the supplement to enhance glutathione as well as, redox sensitive chaperone proteins such as CRYAB has applications for diseases like myofibrillar myopathy (MFM)

(Forrest, Al-Sarraj et al. 2011, Fittipaldi, Mercatelli et al. 2015). Arabian horses with MFM have Z-disc disruption and alterations in pathways of cysteine synthesis with decreased expression of another thiol-based antioxidant peroxiredoxin that can be reduced by glutathione (Valberg, McKenzie et al. 2016). Warmblood horses with MFM have alterations in Z disc proteins, enrichment of pathways of oxidative stress and markedly increased expression of the gene *CHAC1* which encodes an enzyme that degrades glutathione (Williams, Velez-Irizarry et al. 2021). The results of the present study suggest that evaluating the impact of NACQ supplementation on horses with MFM is warranted. Concerns have been raised in human medicine about the antioxidant supplementation impairing training adaptations mediated by ROS signaling (Droge 2005). None of the horses in the present study, however, had any impairment of exercise responses while on the NACQ supplement and there was no impact on CS activity, an indicator of training response.

CONCLUSION

In conclusion, supplementation of fit Thoroughbred horses with N-acetyl cysteine and CoQ10 for 30 days appears to impact muscle redox status without any evident detrimental effects on performance. Muscle glutathione concentrations after intense exercise were significantly increased and expression of proteins involved in the uptake of glutathione into mitochondria and the reduction of oxidized glutathione were enhanced.

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CHAPTER 3

PLASMA ABSORPTION OF ORAL COENZYME Q10: EFFECT OF DOSE AND FORMULATION

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ABSTRACT

Coenzyme Q10 (CoQ10) is a potent antioxidant, as well as an integral component of the mitochondrial electron transport chain. CoQ10 exists in the oxidized form (ubiquinone) and the reduced form (ubiquinol). Both ubiquinone and ubiquinol are commercially available CoQ10 supplements, however, there are few equine studies of the oral absorption of CoQ10. The purpose of this study was to determine plasma CoQ10 (ubiquinol + ubiquinone) concentrations following four different doses (0mg – 3200mg) of micellized ubiquinol given once orally compared to 1600mg of hydro-soluble ubiquinone. In addition, the effect of administering 800 mg of micellized ubiquinol combined with 2000 IU of micellized α-tocopherol on plasma CoQ10 concentrations was evaluated. Six unfit Thoroughbred horses (11.7 ± 2.5 yrs; 3 geldings and 3 mares) were used in a Latin square design. Plasma samples were taken before supplementation, then every 2 hr for up to 8 hr following supplementation and a final sample was taken 24 hr after supplementation. Concentrations of CoQ10 were measured by mass spectrometry and compared by repeated measures ANOVA. Large interindividual variation in response to both forms of CoQ10 was apparent. There was no statistically significant change in plasma CoQ10 over time for any dose or formulation with no time by treatment effect. We conclude that wide individual variation exists in the response of fit Thoroughbred horses to 800 – 3200 mg of oral micellized ubiquinol and hydro-soluble ubiquinone with or without additional supplementation with RRR -a tocopherol.

INTRODUCTION

Coenzyme Q10 (CoQ10), 2,3-dimethoxy-5-methyl-6-decaprenyl-1,4-benzoquinone, is a potent fat-soluble antioxidant present in intracellular membranes. CoQ10 exists within the body in the oxidized form (ubiquinone) and the reduced form (ubiquinol); these two redox forms are continually inter-converted within cells as part of the normal function of CoQ10 (Bhagavan and Chopra 2006, Mantle and Dybring 2020). In the blood, CoQ10 is transported as ubiquinol (bound to low-density lipoprotein- and very low-density lipoprotein- cholesterol), irrespective of the initial dietary form (ubiquinone or ubiquinol) (Mantle and Dybring 2020). Adequate amounts of tissue CoQ10 (ubiquinone + ubiquinol) ensure that redox homeostasis is maintained, allows vitamins C and E to be reduced to their active form and thereby prevents oxidative stress (Cooke, Iosia et al. 2008).

CoQ10 is found in the highest concentrations in the inner mitochondrial membrane (Bentinger, Brismar et al. 2007). Here, in addition to being an antioxidant, it also acts as a redox carrier, activator of uncoupling proteins, and modulator of the permeability transition pore (Bentinger, Brismar et al. 2007). As a redox carrier, CoQ10 transfer protons from complexes I or II to complex III in the mitochondrial electron transport chain generating adenosine triphosphate (ATP) via oxidative phosphorylation (Hargreaves 2003, Bhagavan and Chopra 2006). Strenuous exercise increases reactive oxygen species and can induce a relative CoQ10 deficiency by increasing energy demands on the mitochondria and resultant reactive oxygen species (Cooke, Iosia et al. 2008, Deichmann, Lavie et al. 2010).

The ability of CoQ10 to act both as a potent antioxidant and an integral component of oxidative phosphorylation, makes it an attractive nutraceutical. Oral CoQ10 has been recommended for humans with cardiovascular disease, neurodegenerative disease, myopathies, and CoQ10 deficiencies (Bhagavan and Chopra 2006, Garrido-Maraver, Cordero et al. 2014). Recently, it has also been recommended for horses with myopathies (Pagan 2020). CoQ10 has also been investigated for its potential to enhance athletic performance in human athletes (Cooke, Iosia et al. 2008). Because physiologic concentrations of CoQ10 do not fully saturate the mitochondrial receptors, even a small increase in the CoQ10 concentration of mitochondrial membranes can lead to an increase in mitochondrial respiration (Littarru and Tiano 2007).

One issue with CoQ10 supplementation is poor bioavailability, which is impacted by the chemical formulation of CoQ10 (Mantle and Dybring 2020). Some CoQ10 supplements that have been used in horses contain microencapsulated crystalline ubiquinone that is formulated to be water-soluble (CAVAQ10 and Hydro Q-sorb). Oral supplementation of 0.8-3.4g/day of this formulation to horses has been reported to increase plasma and serum CoQ10 concentrations (Sinatra, Chopra et al. 2013, Sinatra, Jankowitz et al. 2014, Ruiz, Tibary et al. 2021). Recent technical improvements in encapsulation methods have made more bioactive ubiquinol supplements available (Mantle and Dybring 2020). A micellized form of ubiquinol has recently been developed that encapsulates ubiquinol in fat particles that are < 10 nm to enhance absorption of this lipid-soluble molecule (Tibary et al. 2021). Another means that has been explored to enhance CoQ10 absorption in horses is co-administration of CoQ10 and vitamin E (Svete, Vovk et al. 2021). Although there are separate equine studies looking at these CoQ10

formulations, there are no studies that have compared the effect of formulation and dose on absorption of CoQ10 in the same group of horses.

The overall goal of the present study of Thoroughbred horses was to determine the absorption of several different doses of micellized ubiquinol relative to a standard dose of microencapsulated crystalline ubiquinone. Our first objective was to compare plasma absorption of a single administration of 1600 mg of hydro-soluble ubiquinone to a single administration of 1600 – 3200 mg of micellized ubiquinol. The second objective was to determine if co-supplementation of micellized ubiquinol and vitamin E (RRR-α-tocopherol) enhanced plasma CoQ10 concentrations.

METHODS

Horses

Six Thoroughbred geldings aged 11.7 ± 2.5 yrs were included in the study. Throughout the 42-week study, horses had ad libitum access to compressed timothy hay and were fed 1kg of concentrate twice a day (HKJC Eclipse; Kentucky Equine Research (KER), Versailles, KY, USA). One kg of HKJC Eclipse is guaranteed to include 3.2 Mcal digestible energy, 13.6% crude protein, 8.3% crude fat, 32.1% non-structural carbohydrate, and 18.7% neutral detergent fiber. Horses were also supplemented once daily with 60g MicroMax (KER, Versailles, KY) (Table 3.1). All horses were monitored to ensure consumption of the CoQ10 supplements and RRR-α tocopherol top-dressed on their feed. Horses were exercised on a treadmill or mechanical walker 5 days/wk throughout the trial. Treadmill exercise 2 days/wk consisted of 11 min of walk, trot, and canter on a 3-degree slope. Mechanical walker exercise 3 days/wk consisted of 35 min of walk and trot. Horses were turned out for 4-6 hr/day in a grass paddock 6 days/wk and stalled for 36-40 hr prior to and during collection day.

Study design

A 6 x 6 Latin square design was used where horses were supplemented with six different treatments top-dressed on the morning grain meal as a single dose. Horses were observed to determine that all grain was consumed. Each period consisted of a 24-hr collection day followed by a 6-day washout. Treatments were placebo (water), 1600mg hydro-soluble ubiquinone (CAVAQ10, Wacker Chemical Corp., Munich, Germany), 800mg micellized ubiquinol (M-Uol) (Nano-CoQ10, KER, Versailles, KY), 1600mg M-Uol, 3200mg M-Uol, 800mg M-Uol + 2000IU micellized RRR-α-tocopherol (Nano-E, KER, Versailles, KY).

Table 3.1. Guaranteed analysis of 60 g MicroMax supplement provided to horses once daily.

Nutrient	Amount
Calcium	1500 mg
Phosphorus	1000 mg
Magnesium	600 mg
Iodine	0.95 mg
Selenium	0.95 mg
Copper	72.5 mg
Zinc	220 mg
Manganese	120 mg
Iron	92.5 mg
Vitamin A	21,145 IU
Vitamin D	2,114.5 IU
Vitamin E	190 IU
Biotin	0.25 mg

Blood samples

An indwelling catheter was placed in the jugular vein 30-60 min prior to the first collection timepoint and removed after the 8hr collection after which a 24 hr sample was collected via venipuncture. The highest plasma concentration (Cmax) of the ingested CoQ10 is reached after approximately 6 hours in humans (range 5-8hr) (Bhagavan and Chopra 2006, Mantle and Dybring 2020). Blood samples were placed into lithium heparin tubes immediately pre-feeding (0hr), 2, 4, 8, and 24 hr post feeding. Samples were immediately centrifuged at 4°C for 10 min to isolate the plasma which was then frozen at -20°C for 7- 42 days until transfer to -80°C where it was stored prior to analysis.

CoQ10 Analyses

Plasma CoQ10 analysis (ubiquinone + ubiquinol) was performed at the Michigan State University Mass Spectrometry and Metabolomics Core using a high-resolution/accurate-mass (HR/AM) UHPLC-MS/MS system consisting of a Thermo Vanquish UHPLC interfaced with Thermo Q-Exactive according to Pandey 2018 (Pandey, Riley et al. 2018). Approximately 45 μL of plasma was diluted in 255 μL of a 95:5 ethanol:2-propanol solution containing 500 ng/mL CoQ4 internal standard with 125 μg of butylated hydroxytoluene pre-dried in the tube. CoQ10 was extracted from this precipitate by adding 400 μL of hexanes then 200 μL milli-Q water. The organic phase was collected, evaporated, and then reconstituted in 200 μL of ethanol containing 0.3 M hydrochloric acid. 10 μL of sample was injected onto a Waters Acquity BEH-C18 UPLC column (2.1x100 mm) and eluted using a 5-minute isocratic flow of 5 mM ammonium formate in 2-propanol/methanol (60:40 v/v) at 0.3 ml/min. Compounds were ionized by electrospray operating in positive ion mode with a spray voltage of 3.5 kV, capillary temperature of

256.25°C, probe heater temperature of 412.5°C, and S-Lens RF level of 50. Spectra were acquired using a full MS/all ion fragmentation method at 70,000 resolution, AGC target of 1e6, and mass range of m/z 150-1000. The normalized collision energy for the AIF scans was set to 22V. Data were processed using Xcalibur software version 4.1.31.9. Protein content of the plasma was determined using a pierce Bicinchoninic Acid assay kit (Thermo Scientific, Waltham, MA).

Statistical analysis

A one-way analysis of variance (ANOVA) was used to examine the effect of time on plasma CoQ10 concentrations in the placebo. A mixed-model ANOVA was utilized to compare placebo and all treatments and timepoints using a Tukey multiple comparison test. Principal Component Analysis (PCA) was performed to investigate any clustering of individual horses. Statistical analysis was performed using GraphPad Prism software (GraphPad, San Diego, CA, USA) version 9.2.0. Significance was set at $P < 0.05$.

RESULTS

CoQ10 concentrations

When horses were fed the placebo, plasma CoQ10 concentrations did not differ significantly over the 24 h test collection (p=0.99) (Figure 3.1A and B). Plasma CoQ10 concentrations also did not differ significantly when comparing the 1600 mg of hydro-soluble ubiquinone response to the response to any of the M-Uol doses [800 mg (p=0.98), 1600 mg (p=0.96), 3200 mg (p=0.66)] (Figure 3.1C and D). Nor were there significant differences in plasma CoQ10 concentrations among the three doses of M-Uol (800 mg p=0.71, 1600 p=0.74, and 3200 p=0.99 respectively). There also was no significant difference in plasma CoQ10 concentrations comparing 800 mg M-Uol with 800 mg M-Uol + 2000 IU d-α tocopherol (p=0.62) (Figure 3.1E and F).

There was a significant individual horse effect on plasma CoQ10 concentrations (P<0.001). Graphical depiction of the change from baseline in plasma CoQ10 concentrations in each horse revealed three horses that had at least one time point where plasma CoQ10 concentrations were 2-fold higher than baseline for at least one dose of M-Uol where others showed a 2-fold decrease from baseline for at least one dose of M-Uol (Figure 3.2). In contrast, three horses had very little change in plasma CoQ10 with M-Uol (Figure 3.2). Principle component analysis utilizing all CoQ10 concentrations for each dose/form of CoQ10 showed clustering of horses into three groups (Figure 3.3). PC1 accounted for 27.33%, PC2 accounted for 24.68%, PC3 accounted for 16.90%, PC4 accounted for 15.77, PC5 accounted for 9.49, and PC6 accounted for 5.83% of the total variance.

Figure 3.1. Plasma CoQ10 concentrations in horses prior to (0) and for up to 24 hr post supplementation represented as either mg/mL or ng/mg protein. **A and B.** Placebo (blue), **C and D**. 1600mg M-Uol (green), 3200mg M-Uol (magenta), 1600mg hydro-solubilized ubiquinone (HS-Uone; red), **E and F**. 800mg micellized ubiquinol (M-Uol) (aqua), and 800mg M-Uol + 2000 IU RRR-α tocopherol (M-Uol+E; orange). There were no significant differences in plasma CoQ10 concentrations among the treatments (p=0.13).

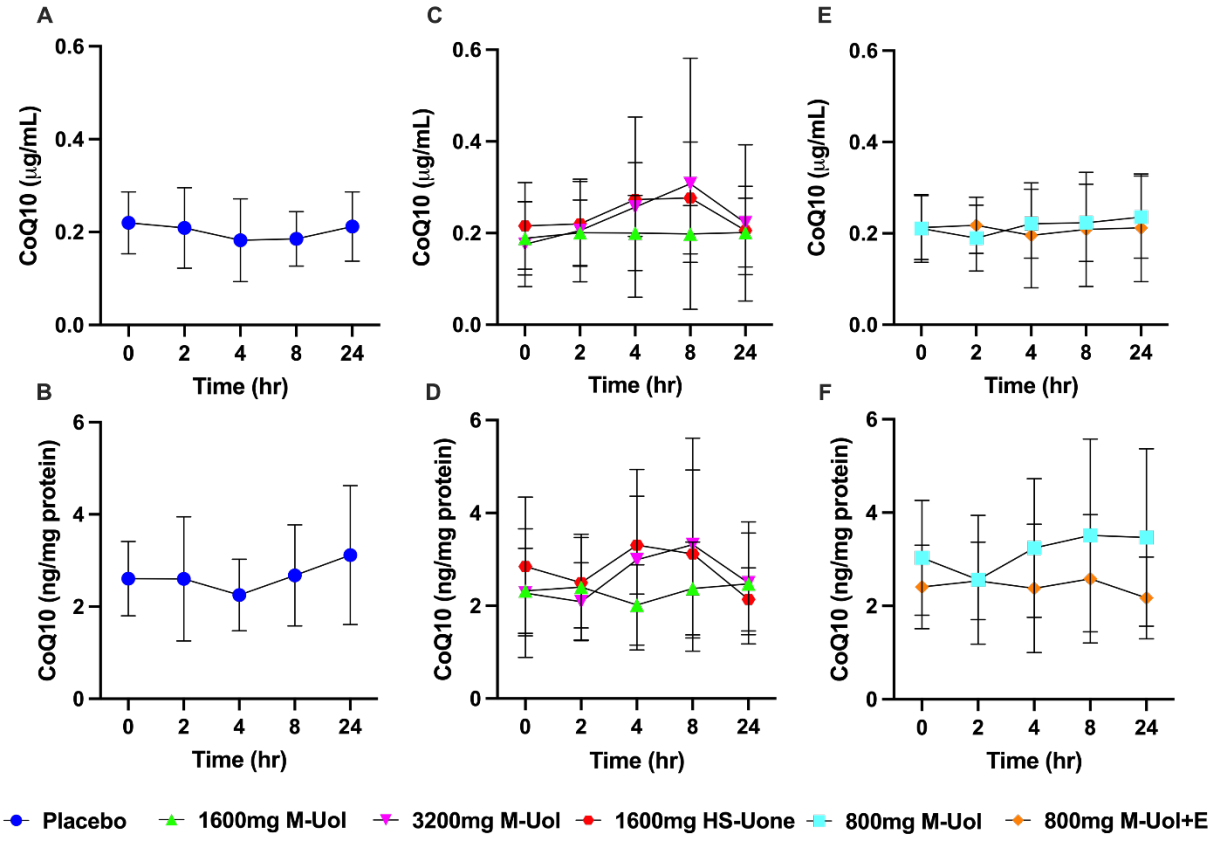


Figure 3.2. The change from baseline (0 hr) in plasma CoQ10 concentrations of individual horses from 2 to 24 hr after supplementation. Placebo (royal blue), 800mg micellized ubiquinol (M-Uol) (ice blue), 1600mg M-Uol (green), 3200mg M-Uol (magenta), 800mg M-Uol + 2000 IU d-α tocopherol (M-Uol+E; orange), and 1600mg hydro-solubilized ubiquinone (HS-Uone; red).

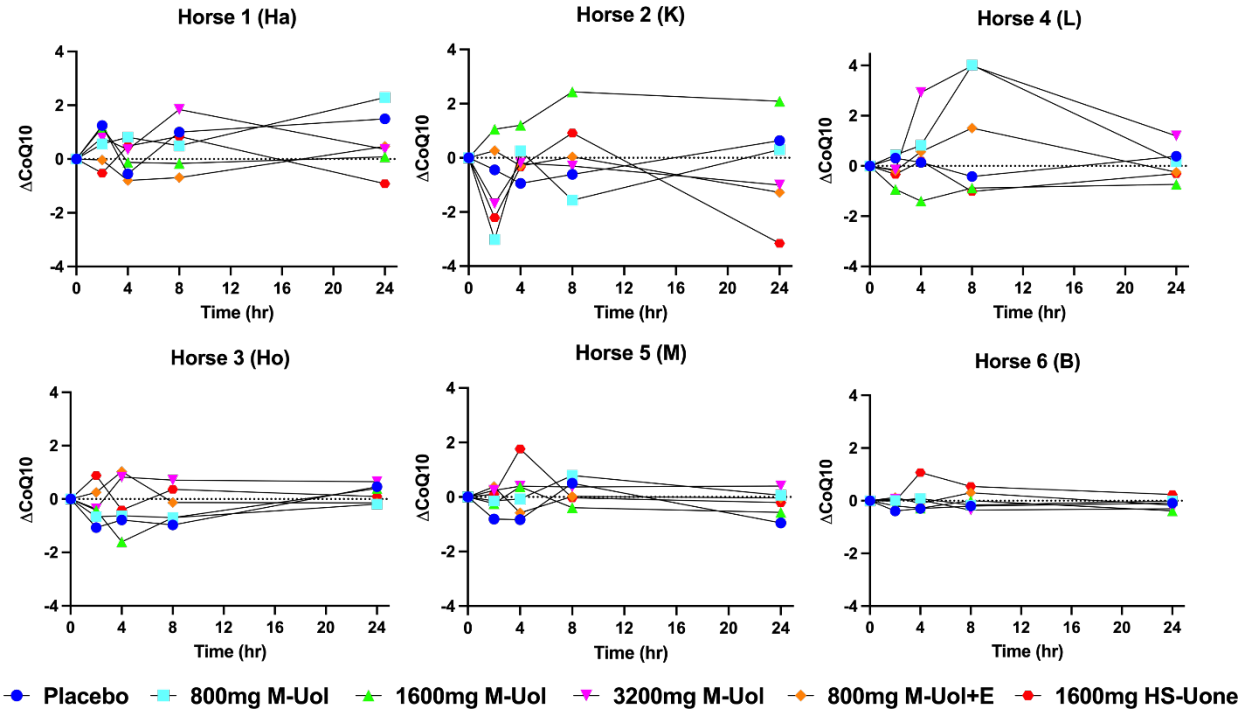
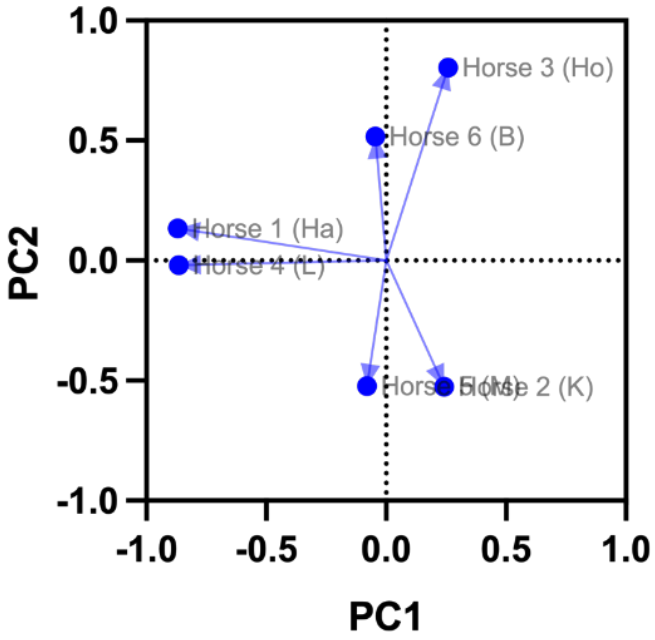


Figure 3.3. Principle Component Analysis (PCA) of the plasma CoQ10 concentration for all 6 supplementation doses organized by individual horse. Not the large differences between individuals in their response to supplementation. PC1 explained 27.3% of the variation and PC2 explained 24.7% of the variation.



DISCUSSION

The present study assessed plasma absorption after a single administration of three different doses of micellized ubiquinol, a combination of micellized ubiquinol and RRR-α-tocopherol and hydro-solubilized ubiquinone. Baseline plasma CoQ10 concentrations in our study ranged from 0.18 to 0.22μg/mL, which is similar to plasma CoQ10 concentrations in previous equine studies (0.226 ± 0.043 μg/mL) and lower than concentrations reported in humans, 0.34 to 1.64 μg/mL (Bhagavan and Chopra 2006, Sinatra, Chopra et al. 2013). CoQ10 concentrations did not significantly differ from baseline on the placebo, the three doses of micellized ubiquinol, hydro-solubilized ubiquinone or with co-supplementation of RRR-α tocopherol. There was, however, a significant effect of individual horse on the absorption of both formulations of CoQ10.

One of the formulations of CoQ10 that has been developed to enhance absorption is a powdered cyclodextrin-encapsulated ubiquinone (CAVAQ10). In our study, a single 800mg/horse dose of this formulation of ubiquinone did not result in an increase in plasma CoQ10 concentrations (ubiquinone + ubiquinol) in Thoroughbreds performing light exercise. This agrees with another study that fed 800 mg of an inclusion complex of ubiquinone with β-cyclodextrin (Vital Paste 7.5%) to horses of a variety of breeds over 14-days (Svete, Vovk et al. 2021). Although that study found no increase CoQ10 concentrations compared to baseline with supplementation (2.47 ± 1.20 versus 2.48 μg/mL) or placebo (2.94 ± 0.82 μg/mL) in agreement with our study, the reported plasma CoQ10 concentrations were 10 times higher than those in the present study and other studies. In humans, plasma CoQ10 concentrations range from 0.34 -1.64 μg/mL with a mean across studies of 0.58 μg/mL (Bhagavan and Chopra 2006). Variability exists within humans such that males have higher CoQ10 concentrations than females and concentrations vary

between races (Bhagavan and Chopra 2006). The higher plasma CoQ10 concentrations measured by Svete et. al. 2021 compared to our study could be due to methodological differences, breed differences, and the differences that appear to exist among individual animals (Svete, Vovk et al. 2021).

Two previous equine studies have also looked at formulations of microencapsulated solubilized ubiquinone (HydroQ-Sorb) (Sinatra, Chopra et al. 2013, Sinatra, Jankowitz et al. 2014). In one 30-day supplementation trial using 800 mg/day of Hydro-Q-Sorb given to six unexercised 2-year old Thoroughbreds, more than a 2-fold increase in serum CoQ10 from baseline occurred (Sinatra, Chopra et al. 2013). In the second study, two groups of five Thoroughbreds in race training received 1900 mg/day and 3400 mg/day of CoQ10 for several months (Sinatra, Jankowitz et al. 2014). Horses were lightly exercised for 8 weeks followed by 9 weeks of high intensity exercise with two horses failing to complete the exercise program. Blood samples were obtained in the morning on the low intensity exercise day and immediately after exercise on the high intensity exercise test day. With 1900mg of hydro-soluble ubiquinone, a 3-fold increase from baseline in plasma CoQ10 occurred after light exercise and 2-fold increase from baseline after high intensity. With 3400 mg of hydro-soluble ubiquinone, an 8-fold increase in plasma CoQ10 from baseline occurred with light exercise and a 3 fold increase was maintained after high intensity exercise (Sinatra, Jankowitz et al. 2014). In our study, similar baseline concentrations of CoQ10 were measured compared to the Sinatra studies, however, neither a single dose of 1600 mg of hydro-soluble ubiquinone, nor 800 – 3200 mg of micellized ubiquinol increased plasma CoQ10 concentrations. It is possible this is related to chemical formulations of CoQ10 even though micellized ubiquinol has better absorption than hydro-solubilized

ubiquinone (Ruiz, Tibary et al. 2021). Alternatively, a more likely explanation for the differences is that our study used a single dose of CoQ10 versus the Sinatra et al. studies which supplemented hydro-soluble ubiquinone for > 30 days.

Wide interindividual variation in response to CoQ10 supplementation was clearly present in our equine study. CoQ10 concentrations at 8 hr on 3200 mg ubiquinol in our study ranged 7-fold between individual horses. Our findings mirror that of a human study that reported no significant difference in plasma CoQ10 levels following administration of ubiquinone or ubiquinol formulations and significant inter-subject variation in baseline plasma levels and bioavailability of CoQ10, irrespective of the CoQ10 form (ubiquinone/ubiquinol) administered (Singh, Niaz et al. 2005). Intestinal absorption of CoQ10 appears to be highly variable among individuals and independent of the form of CoQ10 (ubiquinone/ubiquinol) administered (Singh, Niaz et al. 2005).

Another means suggested to try and enhance CoQ10 absorption in horses is co-supplementation with vitamin E (Svete, Vovk et al. 2021). In our study, co-supplementation of 800 mg of micellized ubiquinol combined with 2000 IU of highly absorbable micellized RRR-α-tocopherol (Brown, Valberg et al. 2017) did not result in an increase in plasma CoQ10. In a 14-day study of sedentary Warmblood horses beginning an exercise regime, supplementation of 800mg cyclodextrin-encapsulated ubiquinone with 990 IU of RRR-α-tocopheryl acetate also did not show an increase in plasma CoQ10 from baseline for that treatment group (Svete, Vovk et al. 2021). The Svete et al. study did, however, report an effect of co-supplementation with α-tocopheryl acetate on plasma CoQ10 concentrations when compared to the placebo group. Of

note, however, the placebo was a separate group of horses housed by owners under separate conditions (Svete, Vovk et al. 2021). Thus, individual variation and different environments, rather than an effect of a relatively low dose of poorly bioavailable vitamin E, could explain the differences between placebo and supplemented groups in the Svete et al. study.

CONCLUSION

In conclusion, the results of our study show that Thoroughbred horses have variable responses to a single dose of CoQ10 regardless of dose, formulation, or addition of RRR-α tocopherol. When an increase in plasma CoQ10 occurred after supplementation, the peak occurred approximately 8hr post supplementation. Further studies are needed to elucidate the effects of chronic supplementation of micellized ubiquinol on plasma and muscle CoQ10 concentrations.

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CHAPTER 4

IMPACT OF COENZYME Q10 SUPPLEMENTATION ON SKELETAL MUSCLE RESPIRATION, ANTIOXIDANTS AND THE MUSCLE PROTEOME IN THOROUGHBRED HORSES

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ABSTRACT

Coenzyme Q10 (CoQ10) is an essential component of the mitochondrial electron transfer system and potent antioxidant commonly used as a nutritional supplement. The impact of CoQ10 supplementation on mitochondrial electron transport or the muscle proteome is largely unknown. The purpose of this study was to determine the effect of CoQ10 supplementation on plasma/muscle CoQ10 concentrations, antioxidant balance, the proteome, and mitochondrial respiration. In a randomized cross-over design, six Thoroughbred horses received 1,600 mg/d CoQ10 or no supplement (control) for 40-d periods separated by a 60-d washout period. Plasma/muscle samples were taken at the end of each period. Plasma/muscle CoQ10 and glutathione (GSH) concentrations were measured using mass spectrometry, antioxidant activities by fluorometry, mitochondrial enzyme and oxidative stress by colorimetry, and mitochondrial respiratory capacities by high-resolution respirometry. A mixed linear model with period, supplementation, and period × supplementation as fixed effects and horse and time as repeated effects was used. Proteomics was performed using a tandem mass tag 11-plex analysis and permutation testing with FDR < 0.05. Plasma CoQ10 concentrations increased 4 hr post CoQ10 supplementation (P=0.04). Concentrations of muscle CoQ10 (P=0.07), GSH (P=0.75), and malondialdehyde (P=0.47), as well as activities of superoxide dismutase (P=0.16) and catalase (P=0.66) did not differ whereas glutathione peroxidase activity (P=0.003) decreased on CoQ10. Intrinsic complex II (E_{CII}) capacity was higher and contribution of complex I was lower (FCR_{PCI} and FCR_{PCIG}) when horses were supplemented CoQ10 with no impact of CoQ10 on mitochondrial volume density. Decreased expression of complexes I, III, and IV as well as tricarboxylic acid (TCA) cycle enzymes when on CoQ10 were noted in proteomics. We conclude that the decreased expression of TCA enzymes that produce NADH and subunits of complex I

which utilizes NADH together with enhanced electron transfer capacity via complex II versus complex I with CoQ10 supplementation support an impact of CoQ10 supplementation on muscle energetics and mitochondrial respiration.

INTRODUCTION

Coenzyme Q10 (CoQ10), 2,3-dimethoxy-5-methyl-6-decaprenyl-1,4-benzoquinone, is a potent fat-soluble antioxidant present in highest concentrations in the inner mitochondrial membrane (Bentinger, Brismar et al. 2007). There CoQ10 acts as a redox carrier, transferring protons from complexes I or II to complex III in the mitochondrial electron transfer system generating adenosine triphosphate (ATP) via oxidative phosphorylation (Hargreaves 2003). Amongst its other functions, CoQ10 acts as a redox carrier, activator of uncoupling proteins, and modulator of the permeability transition pore (Bentinger, Brismar et al. 2007).

In chapter 3 of this dissertation, we showed that there was wide interindividual variation in plasma CoQ10 concentrations following a single dose of two forms of CoQ10, a micellized ubiquinol or a hydro-soluble ubiquinone. In chapter 2 of this dissertation, we found that muscle CoQ10 concentrations did not increase with 30 days of supplementation of 1,600 mg of micellized ubiquinol combined with 10 g N-acetyl cysteine. There were, however, significant changes in the muscle proteome that primarily involved mitochondrial tricarboxylic acid cycle (TCA) proteins. Whether this altered mitochondrial protein expression was the result of CoQ10 or N-acetyl cysteine supplementation could not be discerned from this study. A supplementation trial using CoQ10 alone would be necessary to determine the sole effect of CoQ10 on the muscle proteome.

Although muscle CoQ10 concentrations did not increase with micellized ubiquinone supplementation in Chapter 2, it is possible that CoQ10 enhances skeletal muscle mitochondrial respiratory function. A previous study of human patients with mitochondrial myopathies found

that CoQ10 supplementation increased skeletal muscle mitochondrial function measured by phosphorous magnetic resonance spectroscopy, suggesting that the efficiency of respiration was increased independent of mitochondrial enzyme deficits (Barbiroli, Frassineti et al. 1997). To the authors' knowledge, the effect of CoQ10 supplementation on mitochondrial oxidative phosphorylation has not been assessed using newer techniques such as high resolution respirometry (HRR). Respirometry assesses the respiratory capacities of the mitochondrial electron transfer system. Specifically, it can separately quantify capacities of complex I and II as well as measure capacities of electron transfer both coupled and uncoupled from ATP production (Li, White et al. 2016, White, Warren et al. 2017). HRR has recently been used to evaluate effects of aging or selenium supplementation in horses using saponin-permeabilized muscle fibers (Li, White et al. 2016, White, Warren et al. 2017). Application of HRR to muscle samples obtained from horses after chronic CoQ10 supplementation would be an ideal way to determine if CoQ10 supplementation impacts mitochondrial respiration.

We hypothesized that supplementation with 1,600 mg CoQ10 (micellized ubiquinol) for 30 days would result in a CoQ10 plasma dose response 4 hr following supplementation. Further, we hypothesized that chronic CoQ10 supplementation would increase mitochondrial oxidative phosphorylation capacities, alter antioxidant balance, and alter mitochondrial protein expression.

The first objective of our study was to determine the effect of at least 30 days of 1,600 mg CoQ10 supplementation on plasma CoQ10 concentrations before and after a single dose. The second objective was to determine the effect of 30 days of 1,600 mg CoQ10 supplementation on skeletal muscle CoQ10 concentrations and muscle antioxidant balance. The third objective was

to determine the effect of CoQ10 supplementation on mitochondrial electron transport. The fourth objective was to determine the effect of CoQ10 supplementation on the muscle proteome.

METHODS

Horses

Six Thoroughbred horses (3 geldings and 3 mares, 8.7 ± 2.2 y, 542.3 ± 20.8 kg BW) were used for the present study. Horses were primarily housed in grass paddocks in groups of 3 based on sex for 19 to 20 hr/d and were otherwise stalled in 3.7 × 3.7 m bedded stalls prior to and following daily exercise. Horses had *ad libitum* access to grass and water while in paddocks and had *ad libitum* access to timothy hay and water while stalled (Table 4.1). The basal diet was formulated to meet or slightly exceed dietary requirements for horses in light work (NRC 2007). Horses were fed OBS Sport grain (Ocala Breeders Supply, Ocala, FL) BID which has 3.3 Mcal/kg digestible energy, 13.3% crude protein, 11.2% crude fat, 16.2% non-structural carbohydrates, and 34.3% neutral detergent fiber. Each animal was fed between 4 and 5 kg/d of OBS in order to maintain a body condition score (BCS) of 5 to 6 (Henneke, Potter et al. 1983).

Horses exercised 6 d/wk with an alternating schedule of treadmill and under saddle exercise. Three d/wk, horses were exercised on a high-speed treadmill for 20 min/d at a 3 to 5° incline at a walk, trot, canter (6 to 7 m/s), and gallop (8 to 10 m/s). The remaining 3 d/wk, horses walked for 30 min on a 6-horse panel mechanical walker measuring approximately 20.1 m in diameter (EquiGym, LLC, Lexington, KY) followed by 30 min under saddle exercise at the walk, trot, and canter. Horses were accustomed to this exercise program 7 to 8 wk prior to initiation of the study.

Table 4.1. Nutrient composition of timothy hay and grass offered to horses. All nutrients are presented on a 100% dry matter (DM) basis.

Nutrient	Compressed Timothy Hay	Grass Pasture
DE, Mcal/kg	2.08	1.97
CP, %	12.75	18.05
ADF, %	34.90	34.80
NDF, %	59.75	59.90
Starch, %	0.9	2.15
Crude Fat, %	2.87	3.88
Ca, %	0.38	0.59
P, %	0.22	0.42
Mg, %	0.23	0.28
K, %	1.83	2.62
Na, %	0.02	0.03
Fe, ppm	148.00	276.50
Zn, ppm	28.00	47.50
Cu, ppm	7.00	66.00

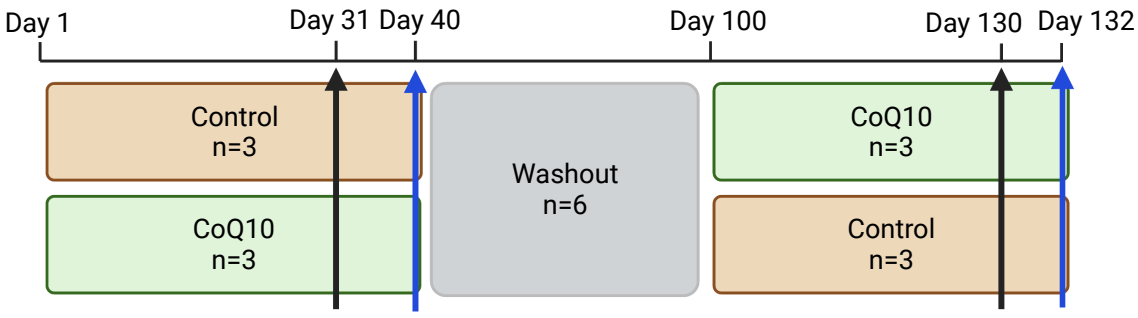
Study design

A randomized cross-over study design was utilized for the muscle study with two 30-d periods separated by a 60-d washout (Figure 4.1). During the first period, horses were randomly assigned to receive either 1) supplementation with 1,600 mg CoQ10/d through 15 mL of Nano-Q10 (Kentucky Equine Research, Versailles KY) divided into two doses and administered orally with a dosing syringe or 2) a control treatment in which horses received no additional supplementation. Following the washout period, horses switched treatment groups for the second supplementation period. During the washout period, all horses received only the control diet and continued the same weekly exercise protocol. The plasma response to chronic CoQ10 supplementation was assessed following collection of 30 d muscle samples by extending the CoQ10 supplementation for an additional 10 days after period 1 and after two days at the end of period 2 (Figure 4.1). This difference in timing of plasma dose responses between period 1 and 2 was due to unforeseen circumstances.

Blood samples

Jugular venous blood samples were obtained through venipuncture at the end of the period 1 and period on d 40 and 132, respectively, approximately 16 hr after the last dose of CoQ10 (pre) as well as 4 hr after a single dose of 1,600 mg CoQ10 or no supplementation (control) (Figure 4.1). Samples were collected in lithium heparin tubes, centrifuged within 30 min of collection, frozen on dry ice, and shipped with muscle samples to the laboratory on dry ice where they were stored at -80°C until analysis.

Figure 4.1. The randomized block design used for control and CoQ10 supplementation with a 60-day washout period between treatment periods. Muscle biopsies were taken 30 days following the beginning of supplementation (black arrows) and effect of CoQ10 administration on plasma concentrations was evaluated 4 h after supplementation subsequent to muscle biopsies (blue arrows). Figure created with BioRender.com.



Muscle samples

Gluteus medius muscle samples were collected on day 31 and 130 prior to CoQ10 supplementation and daily exercise (White et al., 2016). Horses were sedated with intravenous administration of detomidine hydrochloride (Dormosedan; Zoetis Services LLC, Parsippany, NJ) at doses recommended by the manufacturer. The collection site was clipped free of hair and surgically cleaned with povidone scrub and 70% ethyl alcohol. 0.1 to 0.5 mL of 2% Lidocaine (VetOne, MWI Veterinary Supply Company, Boise, ID) was administered intradermally to desensitize the area. A 14-gauge needle was used to puncture the skin followed by insertion of a 14-gauge tissue collection needle (SuperCore TM Semi-Automatic Biopsy Instrument, Argon Medical Devices, Frisco, TX) to a fixed depth of 5 cm to collect muscle tissue. Tissue was collected from the left side of the horse on d 31 and the right side of the horse on d 130. Samples were aliquoted and either flash frozen in liquid nitrogen and stored at -80°C until future analyses or placed in mitochondrial preservation solution (BIOPS; 10 mM Ca-EGTA buffer, 0.1 μM free calcium, 20 mM imidazole, 20 mM taurine, 50 mM K-MES, 0.5 mM dithiothreitol, 6.56 mM $MgCl_2$, 5.77 mM ATP, and 15 mM phosphocreatine; pH 7.1) and stored on ice or at 4°C until high-resolution respirometry was performed. Upon finishing collection, the site was cleaned with 70% ethyl alcohol and sealed using aluminum bandage (AluShield Spray, MWI Veterinary Supply Company, Boise, ID).

CoQ10 Analysis

CoQ10 analysis of both plasma and muscle samples was performed at the Michigan State University Mass Spectrometry and Metabolomics Core using a high-resolution/accurate-mass (HR/AM) UHPLC-MS/MS system consisting of a Thermo Vanquish UHPLC interfaced with

Thermo Q-Exactive according to Pandey 2018 (Pandey, Riley et al. 2018). Data was analyzed as described (Chapters 2 and 3) briefly, approximately 10 mg of tissue was homogenized in 500 μL of 95:5 ethanol:2-propanol solution containing 500 ng/mL CoQ4 internal standard with 125 μg of butylated hydroxytoluene pre-dried in the homogenization tube with a bead homogenizer (bullet blender, Next Advance, Troy, NY, USA). CoQ10 was extracted with a 2:1 solution of hexanes to water, the organic layer was removed and evaporated. Within 24 hr of quantification, the samples were reconstituted in 2 mL of ethanol containing 0.3 M hydrochloric acid. For the plasma samples, 45 μL of undiluted plasma was added to 255 μL of the same 95:5 ethanol:2-propanol solution as used with the tissue. Samples were further processed as described above other than they were reconstituted in 200 μL of the ethanol:0.3M hydrochloric acid solution. Data were processed using Xcalibur software version 4.1.31.9 and accounted for different final dilution volumes.

Muscle Mitochondrial Enzyme Activities

Citrate synthase (CS) and cytochrome *c* oxidase (CCO) activities were determined as measures of mitochondrial function and volume density, respectively, using kinetic colorimetry (Spinazzi, Casarin et al. 2012, Li, White et al. 2016). Previously cryopulverized (Spectrum™ Bessman Tissue Pulverizer; Spectrum Laboratories, Inc., Rancho Dominguez, CA) muscle powder was sonicated in sucrose homogenization buffer (20 mM Tris, 40 mM KCl, 2 mM EGTA, 250 mM sucrose) with 1 part 5% detergent (n-Dodecyl β-D-maltoside; Sigma D4641) 3 times for 3 seconds each while on ice. Samples were centrifuged at 11,000 × *g* for 3 min at 0°C, and the supernatant homogenate was aliquoted and stored at -80°C until analysis. Enzyme activities were measured using a microplate reader (Synergy H1, BioTek Instruments, 237 Winooski, VT,

USA). Briefly, CS activity was determined by measuring the initial rate of reaction of free CoA-SH with DTNB at 412 nm at 37ºC, and CCO activity was determined by measuring the linear rate of oxidation of fully reduced cytochrome *c* at 550 nm at 37°C. Both assays utilized 80-fold diluted muscle homogenate and were analyzed in duplicate. Intra-assay and inter-assay CV for CS activity was 2.3% and 2.8%, respectively. Intra-assay CV for CCO activity was 2.4%. Enzyme activities were normalized to total protein content which was determined using the Coomassie Bradford Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA). CCO activity was also normalized to CS activity (intrinsic) as a measure of function per mitochondria within the sample (Larsen, Nielsen et al. 2012).

High-Resolution Respirometry

Muscle samples collected into ice-cold BIOPS and stored on ice or at 4°C were analyzed for mitochondrial oxidative phosphorylation (P) and electron transfer system (E) capacities using high-resolution respirometry within 24 h of collection. Immediately prior to analysis, samples were permeabilized as described previously (Li, White et al. 2016). Permeabilized fibers were then rinsed in mitochondrial respiration solution (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) and placed on a rocker for 10 min at 4ºC. Approximately 1.5 to 2.5 mg (wet weight) of rinsed fibers were then immediately added to each chamber of an Oroboros Oxygraph-2k (O2k; Oroboros, Innsbruck, Austria) containing MiR06 (MiR05 + 280 U/mL catalase) and 20 mM creatine. Chambers were maintained at 37ºC and in hyperoxic conditions (200 to 650 µM O_2) through addition of 200 mM H_2O_2. The previously described (Latham, Fenger et al. 2019) substrate uncoupler inhibitor titration protocol for this study was as follows:

1) complex I substrates, pyruvate (5 mM) and malate (1 mM), to determine mitochondrial proton leak (LEAK); 2) adenosine diphosphate (ADP; 2.5 mM), to quantify complex I-supported P (P_{CI}); 3) glutamate (10 mM), an additional complex I substrate (P_{CIG}); 4) cytochrome *c* (cyt *c,*10 μM), to measure integrity of the outer mitochondrial membrane; 5) the complex II substrate, succinate (10 mM), to measure maximal P (P_{CI+II}); 6) uncoupler carbonyl cyanide 3-chlorophenylhydrazone (CCCP, 0.5μM steps), to attain maximal noncoupled E (E_{CI+II}); 7) a complex I inhibitor, rotenone (0.5μM), to measure complex II-supported E (E_{CII}); and 8) a complex III inhibitor, antimycin A (2.5 μM), to quantify non-mitochondrial residual O_2 consumption. All data were normalized to residual O_2 consumption. Respiration data are presented either relative to tissue weight (integrative), CS activity (mitochondrial volume density; intrinsic) or as a ratio of contribution to maximal electron transfer capacity (flux control ratio, FCR).

Muscle proteomics

Proteomic analysis was performed on muscle samples from 5 horses in both treatment groups using an 11-plex plates for tandem mass tag MS/MS quantification analysis at the Michigan State University Proteomics Core. Horses were selected based on the respirometry data. Protein was extracted from muscle samples using a radioimmunoprecipitation lysis buffer and protease inhibitor and pelleted prior to submission. Protein concentrations were determined by standard BCA assay. From each sample, 500 mg of protein was digested in trypsin with a Filter-Aided Sample preparation protocol and spin ultrafiltration unit cutoff of 30,000Da (Wisniewski, Zougman et al. 2009). Reverse phase C18 SepPaks were used to de-salt the resulting peptides (Waters Corporation, Milford, MA, USA) which were then dried by vacuum centrifugation.

Peptide quantification was verified by colorimetric peptide concentration using 5 mL from each sample digest. Isobaric labeling, gel fractionation and LC/MS/MS analysis were performed.

Proteomic analysis was performed using Scaffold Q+ version 5.0.1 (Proteome Software Inc., Portland, OR). Peptide identifications were accepted if they could be established at greater than 95.0% probability by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at greater than 99.9% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii, Keller et al. 2003). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins sharing significant peptide evidence were grouped into clusters. Channels were corrected by the matrix in all samples according to the algorithm described in i-Tracker (Shadforth, Dunkley et al. 2005). Normalization was performed iteratively (across samples and spectra) on intensities, as described in Statistical Analysis of Relative Labeled Mass Spectrometry Data from Complex Samples Using ANOVA (Oberg, Mahoney et al. 2008). Medians were used for averaging. Spectra data were log-transformed, pruned of those matched to multiple proteins, and weighted by an adaptive intensity weighting algorithm. Differentially expressed proteins were determined by applying Permutation Test with an adjusted p value of $P < 0.0027$ corrected by Benjamini-Hochberg. Significant proteins were grouped according to their cellular functions.

Antioxidants analysis

Glutathione (GSH) was measured using a high-performance liquid chromatography mass spectrometry analysis as previously described (Chapter 2). Briefly, approximately 30mg of tissue

was homogenized in a bead homogenizer with 500uL 1x radioimmunoprecipitation assay (RIPA) buffer, reduced with Tris(2-carboxyethyl)phosphine hydrochloride and N-ethylmaleimide. An internal standard of 20uM GSH ammonium salt D-5 (Toronto Research Chemicals, Toronto, Ontario, Canada) was utilized to standardize all samples and standards. Chromatographic separation was performed using a Phenomenex Kinetex 1.7um EVO C18 100A (50 x 2.1mm) (Phenomenex, Torrance, CA) column as previously described (Chapter 2).

Muscle glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase (Cat) activities were measured using colorimetric assay kits (Cayman Chemical, Ann Arbor, MI) according to manufacturer's instructions. For the SOD assay, 10mg of tissue was homogenized in a bead homogenizer using 500 μL buffer at pH 7.2 buffer containing 20mM 2-[4-(2-hydroxyethyl)piperazine-1-yl]ethanesulfonic acid, 1mM ethylene glucol-bis(b-aminoethyl ether)-N,N,N',N'-tetraacetic acid, 210mM mannitol, and 70mM sucrose and centrifuged at 1,500 × *g* for 5 min at 4°C. For the GPx assay, 10mg of tissue from was homogenized in 500 μL pH 7.5 buffer containing 50mM Tris-HCl, 5mM ethylenediaminetetraacetic acid, and 1mM dithiothreitol and centrifuged at 10,000 × *g* for 15 min at 4°C. For the Cat assay, 10mg of tissue was homogenized in 500 μL pH 7.0 buffer containing 50mM potassium phosphate and 1mM ethylenediaminetetraacetic acid and centrifuged at 10,000 × *g* for 15 min at 4°C. All supernatants were collected immediately and analyzed in triplicate.

The standard BCA assay kit (Thermo Scientific) was used to determine protein concentrations for CoQ10 and GSH. The reducing agent BCA assay kit (Thermo Scientific) was used to determine protein content for Cat, GPx, and SOD homogenates.

Malondialdehyde (MDA) analysis

Using a commercially available kit (Northwest Life Science Specialties, Vancouver, WA), muscle malondialdehyde (MDA) concentration, a marker of lipid peroxidation, was measured as previously described (White and Warren 2017). Briefly, cryopulverized muscle powder was diluted 1 mg tissue (wet weight) to 10 µL of assay buffer then sonicated while on ice and centrifuged at 11,000 × *g* for 10 min at 0°C. The supernatants were collected and stored at -80°C until analysis. Samples were analyzed in triplicate with intra-assay and inter-assay CV of 2.2% and 1.6%, respectively. Malondialdehyde concentration was normalized to total protein quantified by the Coomassie Bradford Protein Assay kit (Thermo Fisher Scientific).

Statistical analysis

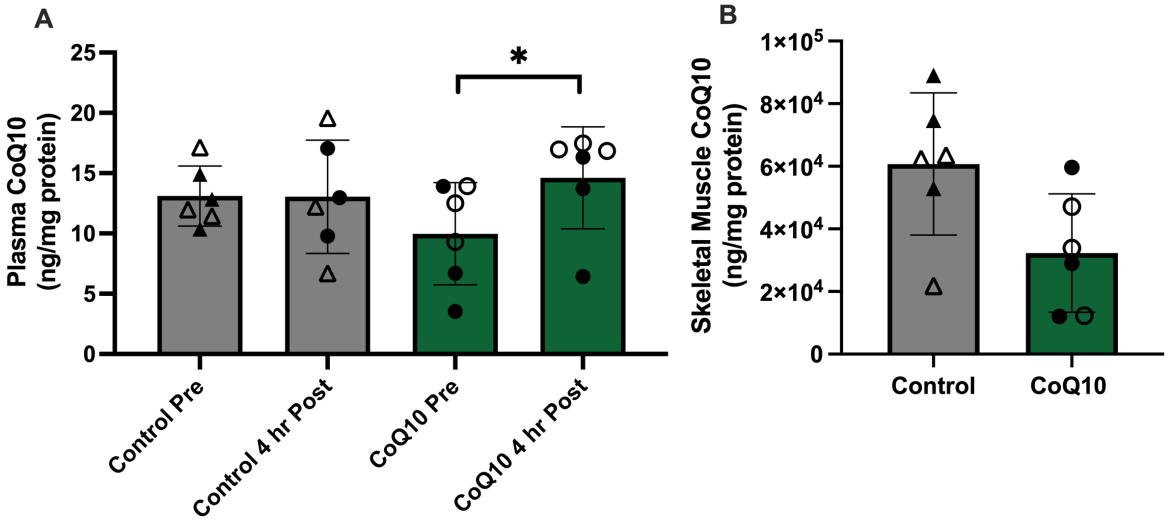
Plasma CoQ10 concentrations were analyzed as change from baseline concentrations (4 hr post – pre supplementation). All data was analyzed using PROC MIXED in SAS (version 9.4, SAS Institute, Inc., Cary, NC) a mixed model equation with supplementation (CoQ10 or control), period (period 1 or 2), and supplementation × period as fixed effects and horse as a repeated effect. Significance was set at $p<0.05$ and data are presented as means ± SD. Graphs were created using GraphPad Prism software version 9.3.1 (GraphPad Software San Diego, CA).

RESULTS

Plasma and skeletal Muscle CoQ10 concentrations

The plasma CoQ10 dose response 4 hr post supplementation of CoQ10 was greater when horses were supplemented with CoQ10 compared to the control diet (P=0.04) (Figure 4.2A). Muscle CoQ10 concentrations were not significantly different between CoQ10 treatment and control diet (P=0.07) (Figure 4.2B).

Figure 4.2. Plasma and muscle CoQ10 concentrations. **A.** Plasma CoQ10 concentration in plasma before (Pre) and 4hr post supplementation in the control and CoQ10 supplementation groups. No statistically significant difference was observed in control but in CoQ10 supplemented horses there was a statistically significant increase in plasma CoQ10 4hr post supplementation. **B.** Skeletal muscle CoQ10 concentrations did not significantly differ between control and CoQ10 treatments. Open symbols represent CoQ10 supplementation during the first supplementation period and closed symbols are CoQ10 supplementation during the second supplementation period



Mitochondrial enzyme activities and capacities

CS and COX activities were unaffected by CoQ10 supplementation, period, or supplementation × period interaction.

Integrative (per mg tissue) LEAK (Figure 4.3A), P_{CI} (Figure 4.3B), P_{CIG} (Figure 4.3C), P_{CI+II} (Figure 4.3D), E_{CI+II} (Figure 4.3E), or ECII (Figure 4.3F) were unaffected by CoQ10 supplementation, period, or supplementation × period interaction.

Intrinsic LEAK (Figure 4.4A), P_{CI} (Figure 4.4B), and P_{CIG} (Figure 4.4C) did not differ with CoQ10 supplementation, period, or treatment × period interaction. Intrinsic P_{CI+II} (Figure 4.4D) and E_{CI+II} (Figure 4.4E) capacities tended to be higher when horses received CoQ10 compared to control ($P \leq 0.08$) with no period or supplementation × period interaction. There was a significant supplementation × period interaction on intrinsic E_{CII} ($P = 0.05$); intrinsic E_{CII} was greater in CoQ10 versus control group following period 1 ($P = 0.005$) but not after period 2 (Figure 4.4F).

The flux control ratio (FCR) for LEAK (FCR_{LEAK}) was not impacted by CoQ10 supplementation, period, or supplementation × period interaction (Figure 4.5A). FCR_{PCI} ($P \leq 0.03$) and FCR_{PCIG} ($P = 0.01$) were significantly lower with CoQ10 supplementation compared to control with no period or treatment × period interaction (Figure 4.5B, C). FCR_{PCI+II} was not significantly different with CoQ10 supplementation, however, FCR_{PCI+II} was greater following period 2 compared to period 1($P = 0.05$) (Figure 4.5D). FCR_{ECII} did not differ with CoQ10 supplementation, period, or supplementation × period interaction (Figure 4.5E).

Figure 4.3. Integrative (per mg tissue) mitochondrial capacities in the gluteus medius of fit Thoroughbred horses before and after 30d supplementation of CoQ10 or control diet. Symbol differences represent the period of supplementation, open symbols represent CoQ10 supplementation during the first supplementation period and closed symbols are CoQ10 supplementation during the second supplementation period.

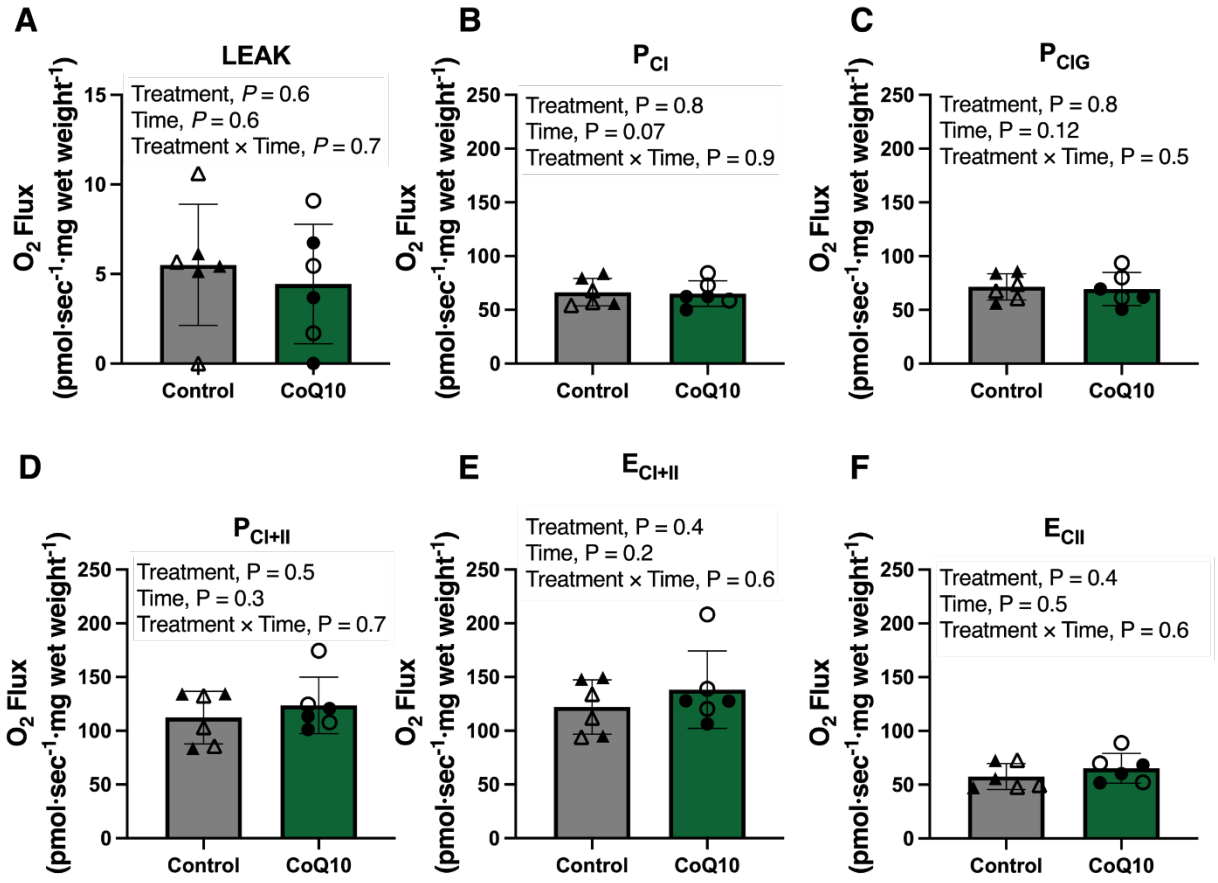


Figure 4.4. Intrinsic (relative to CS activity) mitochondrial capacities in the gluteus medius muscle of fit Thoroughbred horses before and after 30d supplementation of CoQ10 or control diet. Symbol differences represent the period of supplementation, open symbols represent CoQ10 supplementation during the first supplementation period and closed symbols are CoQ10 supplementation during the second supplementation period. Within periods, different letters indicate significant treatment differences.

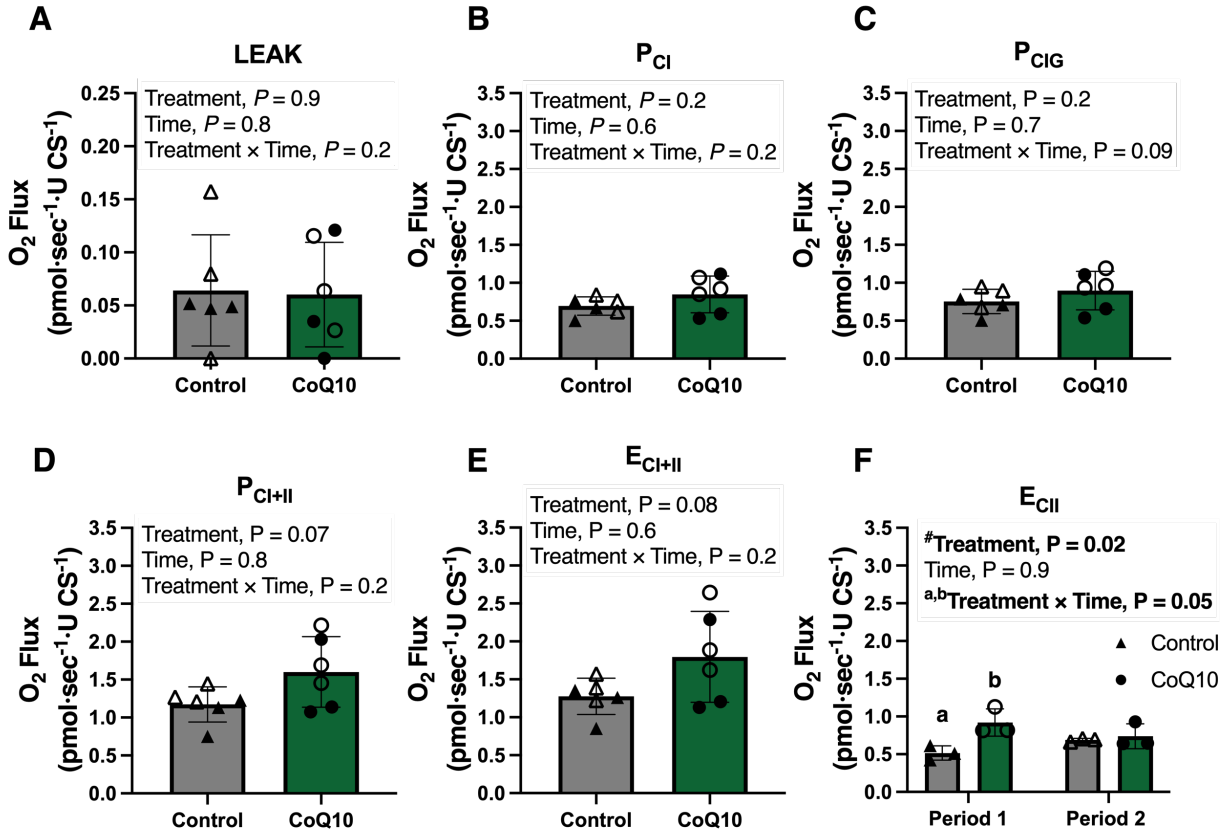
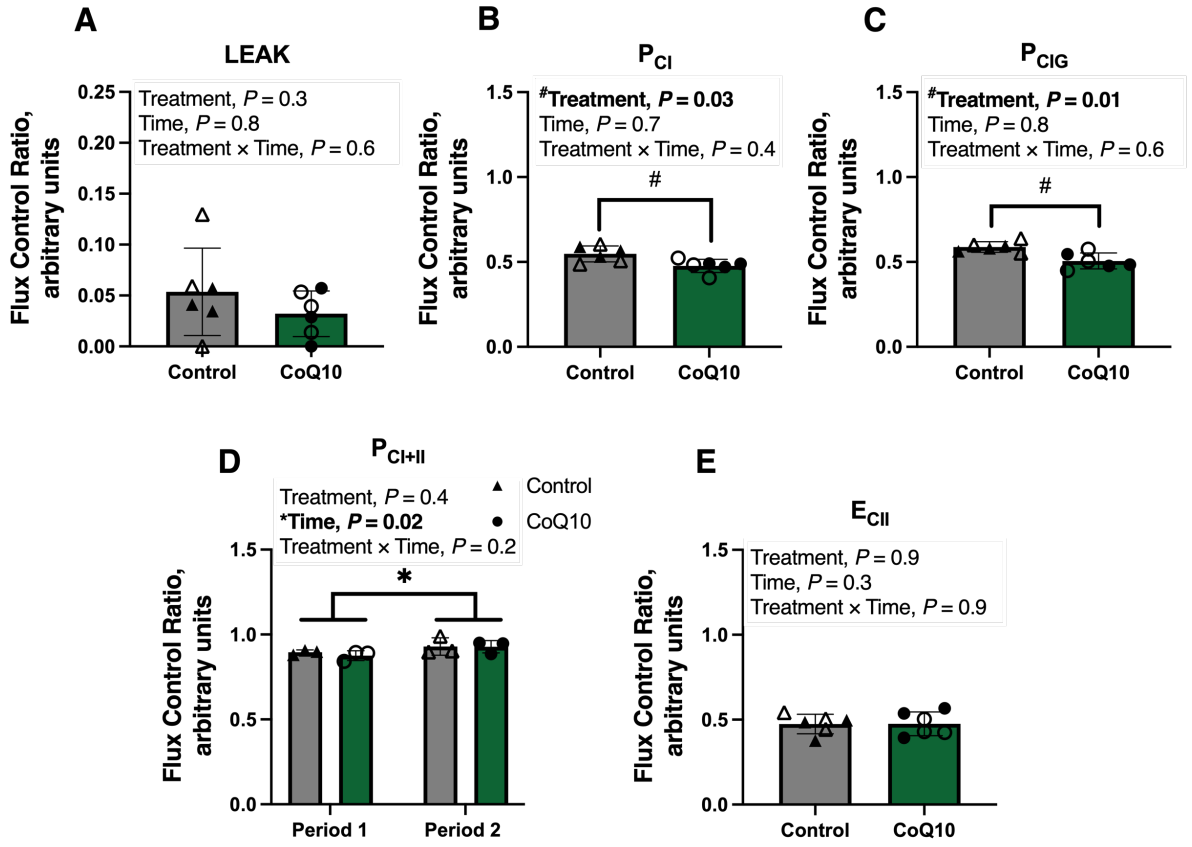


Figure 4.5. Flux control ratios (FCR) of gluteus medius muscle samples from fit Thoroughbred horses before and after 30d supplementation of CoQ10 or control diet. #Regardless of time, Control significantly different from CoQ10 ($P \leq 0.05$). *Across treatments, day 30 significantly differs from day 126 ($P \leq 0.05$).



Proteomics

There were 834 total unique proteins identified. Of those, 38/834 were differentially expressed, 8 with increased expression and 30 with decreased expression comparing CoQ10 supplementation with the control (Table 4.2). All the proteins found within mitochondria were down regulated with CoQ10 supplementation including two subunits of complex I, two subunits of complex III, and four subunits of ATP synthase. Other differentially expressed mitochondrial proteins included four enzymes in TCA cycle, five enzymes in fatty acid metabolism, two voltage-dependent ion channels, one chaperone protein, and a membrane channel for GSH import into the mitochondria (Table 4.2, Figure 4.6). The down regulated TCA cycle proteins include malate dehydrogenase (MDH2), aconitate hydrase (ACO2), isocitrate dehydrogenase 2 (IDH2), and 2-oxogluterate dehydrogenase (OGDH). Within the inner mitochondrial membrane there was an additional electron transfer protein that was downregulated, NAD(P) transhydrogenase (NNT), which generates nicotinamide adenine dinucleotide phosphate (NADPH). In addition, seven proteins in the sarcomere and sarcoplasmic reticulum, four in glycolysis and gluconeogenesis, and two miscellaneous proteins were differentially expressed (Table 4.2).

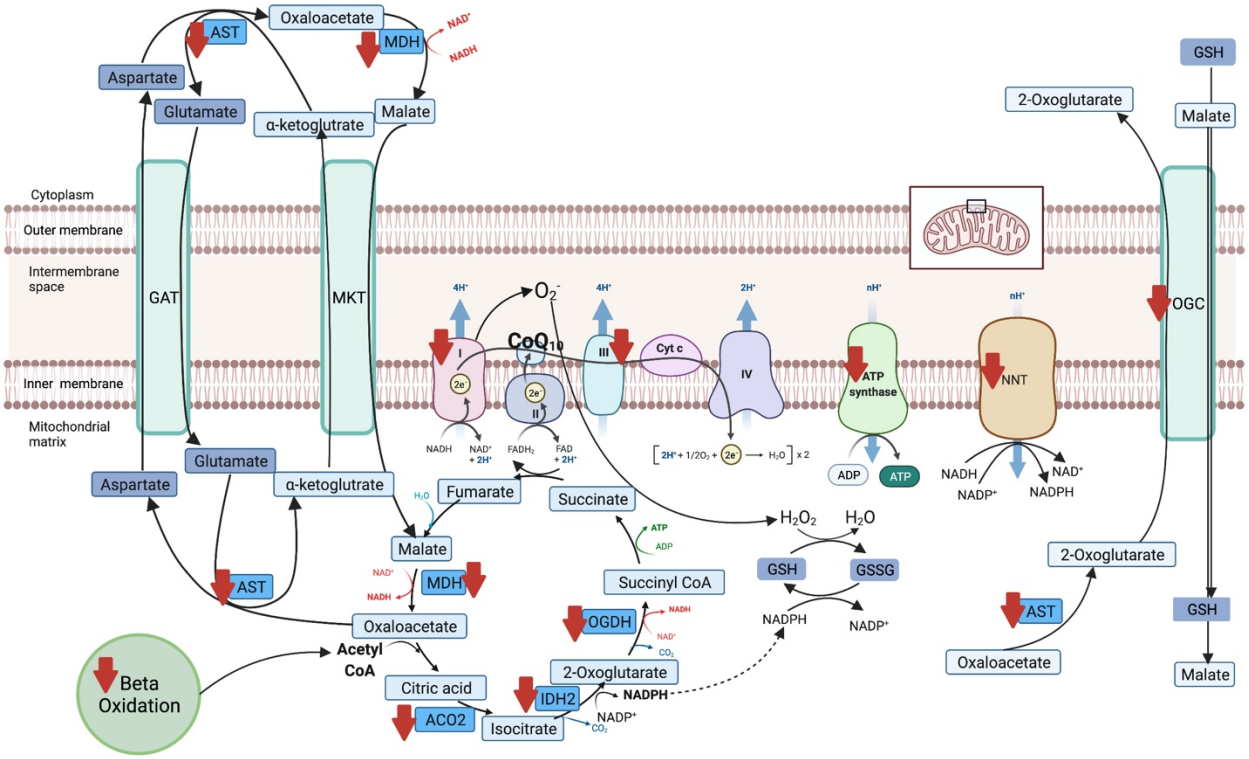
Table 4.2. The gene identification for protein, protein name, p value adjusted for multiple test corrections, and log_2 fold change differential expression for the proteomics analysis with CoQ10 compared to control.

Gene ID	Protein Name	Adjusted P Value	Log_2 Fold Change
	Mitochondria		
NDUFS8	NADH dehydrogenase [ubiquinone] iron-sulfur protein 8, mitochondrial	0.002	-0.12
NDUFB5	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 5, mitochondrial	0.001	-0.11
UQCRFS1	cytochrome b-c1 complex subunit Rieske, mitochondrial	0.002	-0.09
UQCRH	cytochrome b-c1 complex subunit 6, mitochondrial isoform X2	0.001	-0.14
ATP5F1c	ATP synthase subunit gamma, mitochondrial isoform X3	0.00091	-0.11
ATP5ME	ATP synthase subunit e, mitochondrial	0.0007	-0.14
ATP5PD	ATP synthase subunit d, mitochondrial isoform X2	0.00029	-0.11
MT-ATP6	ATP synthase subunit alpha, mitochondrial	< 0.0001	-0.09
PHB2	prohibitin-2	0.001	-0.07
OGDH	Cluster of 2-oxoglutarate dehydrogenase, mitochondrial isoform X3	0.00097	-0.08
IDH2	Cluster of isocitrate dehydrogenase [NADP], mitochondrial	0.001	-0.07
ACO2	aconitate hydratase, mitochondrial	< 0.0001	-0.09
MDH2	malate dehydrogenase, mitochondrial	< 0.0001	-0.09
NNT	NAD(P) transhydrogenase, mitochondrial isoform X1	< 0.0001	-0.06
HADHA	trifunctional enzyme subunit alpha, mitochondrial	0.00046	-0.06
ACAA2	3-ketoacyl-CoA thiolase, mitochondrial	0.002	-0.03
ETFB	electron transfer flavoprotein subunit alpha, mitochondrial	0.001	-0.12
ACADVL	very long-chain specific acyl-CoA dehydrogenase, mitochondrial isoform X6	< 0.0001	-0.05
CRAT	carnitine O-acetyltransferase isoform X1	< 0.0001	-0.06
AST	aspartate aminotransferase, mitochondrial	0.00044	-0.08
Aifm1	apoptosis-inducing factor 1, mitochondrial isoform X1	0.00098	-0.1

Table 4.2 (cont'd)

Gene ID	Protein Name	Adjusted P Value	Log_2 Fold Change
DLD	dihydrolipoyl dehydrogenase, mitochondrial	0.00038	-0.07
SLC25A	phosphate carrier protein, mitochondrial isoform X1	0.001	-0.09
VDAC1	voltage-dependent anion-selective channel protein 1	0.00082	-0.07
VDAC2	voltage-dependent anion-selective channel protein 2	0.001	-0.09
	Sarcomere and SR		
MYBPC2	myosin-binding protein C, fast-type	< 0.0001	0.09
MYL1	myosin light chain 3	0.00012	-0.17
MYL2	myosin regulatory light chain 2, ventricular/cardiac muscle isoform	0.00037	-0.12
MYLK2	myosin light chain kinase 2, skeletal/cardiac muscle	0.00058	0.12
MYOM1	myomesin-1 isoform X4	0.00065	0.04
TNNT1	troponin I, slow skeletal muscle	0.001	-0.15
RYR1	Cluster of ryanodine receptor 1 isoform X1	0.00078	0.04
	Glycolysis/gluconeogenesis		
PYGM	Cluster of glycogen phosphorylase, muscle form	0.00056	0.08
AGL	glycogen debranching enzyme isoform X1	0.0003	0.06
LDHB	L-lactate dehydrogenase B chain isoform X1	0.001	-0.07
PPP1R3A	protein phosphatase 1 regulatory subunit 3A	0.0014	0.09
	Miscellaneous		
PLIN4	perilipin-4 isoform X5	0.001	-0.12
CA3	carbonic anhydrase 3	< 0.0001	0.09

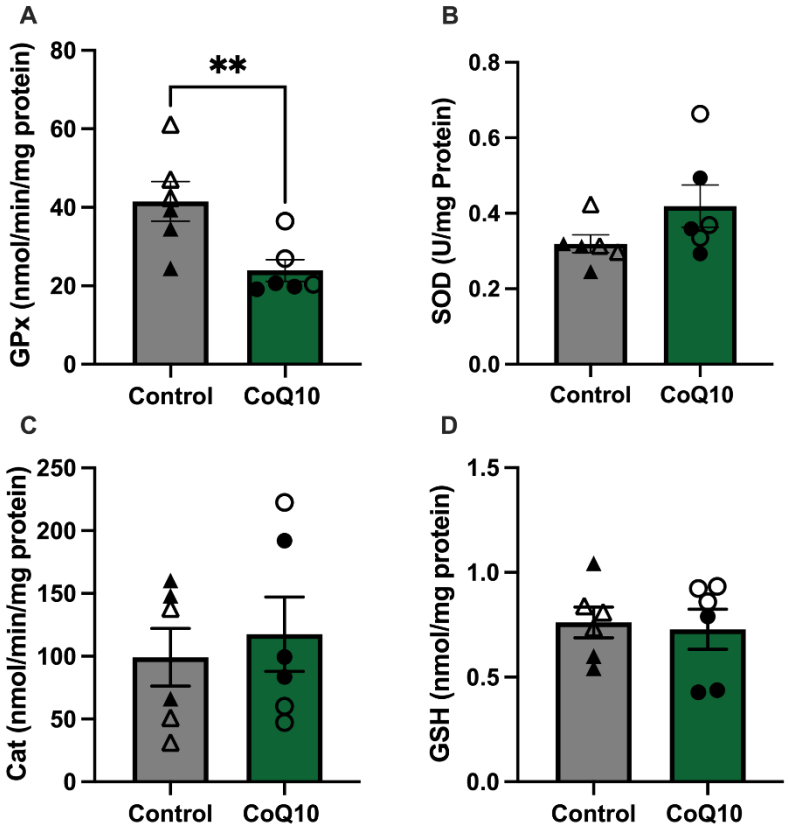
Figure 4.6. Proteins with significantly decreased expression (red arrow) in the mitochondria of horses on CoQ10 compared to control. Horses with CoQ10 supplementation had down regulation of 2 subunits of complex I, 2 subunits of complex III, and 4 subunits of ATP synthase, NAD(P) transhydrogenase (NNT), malate dehydrogenase (MDH), 2-oxogluterate dehydrogenase (OGDH), NADP dependent isocitrate dehydrogenase (IDH2), aconitase (ACO2), aspartate aminotransferase (AST), and the 2-oxoglutarage transport channel (OGC). Created with BioRender.com.



Antioxidants

GPx activity was significantly lower with CoQ10 supplementation compared to control (P=0.003) with a significant supplement × time interaction (P=0.02) (Figure 4.7A). No significant differences were found in SOD or Cat activity or GSH concentrations (SOD, P=0.16; Cat, P=0.66; GSH, P=0.75) (Figure 4.7B, C, D). No differences were found in MDA concentrations between control (48.2 ± 2.7 pmol/mg protein) and CoQ10 treatment (52.0 ± 4.4 pmol/mg protein) groups *(*P=0.47).

Figure 4.7. Skeletal muscle antioxidant activities (SOD, GPx, Cat) and GSH concentrations relative to protein concentrations. **A.** Activity of GPx in control and CoQ10 treatment groups. with significantly lower activity in the CoQ10 supplemented horses (*P*=0.003). **B**. Activities of SOD in control and CoQ10 treatment groups were not significantly different with treatment (*P*=0.17). **C**. Activities of Cat in control and CoQ10 treatment groups were not significantly different with treatment (*P*=0.66). **D**. GSH concentrations in control and CoQ10 treatment groups were not significantly different (*P*=0.75). Open symbols represent CoQ10 supplementation during the first supplementation period and closed symbols CoQ10 supplementation during the second supplementation period. ** signifies *P*<0.01.



DISCUSSION

The present study assessed the impact of 30 d or more of supplementation with 1600 mg CoQ10 on plasma and muscle CoQ10 concentrations, mitochondrial oxidative phosphorylation, the muscle proteome and muscle antioxidant status. We found that plasma, but not muscle, CoQ10 concentrations increased with CoQ10 supplementation, and that the contribution of complex I relative to II to intrinsic oxidative phosphorylation decreased with CoQ10 supplementation. Proteomic data revealed small but significantly decreased expression of mitochondrial proteins including complexes I, III and IV as well as proteins involved in shuttling GSH into mitochondria. Antioxidant status was altered with decreased GPx activity on CoQ10 supplementation with no indication of oxidative stress assessed by MDA. Thus, chronic CoQ10 supplementation appears to impact mitochondrial respiration and antioxidant status, favoring substrates supplying $FADH_2$ for complex II rather than NADH for complex I.

A significant increase in plasma CoQ10 concentrations 4 hr after supplementation following 30 – 40 days of supplementation with micellized ubiquinol contrasts our previous findings of no increase following a single dose of 1600 mg micellized ubiquinol or hydro-soluble ubiquinone CoQ10 (Chapter 3). In agreement with this finding, a study of 41 fit or unfit males and females found no significant differences in plasma CoQ10 concentrations between supplemented or placebo groups after a single dose of CoQ10 (fast-melt CoQ10 formulation) (Cooke, Iosia et al. 2008). Following 14 days of CoQ10 supplementation (100 mg fast-melt CoQ10 BID), however, plasma CoQ10 concentrations in the subjects in this study were significantly higher in the CoQ10 supplemented groups compared to the control group (Cooke, Iosia et al. 2008). CoQ10 is rapidly taken up by the liver after ingestion and then a limited amount is normally released into

the blood bound to circulating lipoproteins (Bhagavan and Chopra 2006). We postulate that the increase in plasma CoQ10 with chronic, but not acute CoQ10 supplementation is the result of saturation of liver CoQ10 content resulting in greater release of CoQ10 into the plasma levels with chronic supplementation.

Muscle CoQ10 concentrations in the present study did not increase with supplementation, in agreement with our previous study of CoQ10 and N-acetylcysteine supplementation of Thoroughbred racehorses for 30 days (Chapter 2). This contrasts a study by Thueson et al. that found higher CoQ10 concentrations in muscle samples following 10 and 21 d of supplementation of 1 g of ubiquinol (Recovery Support, Anlon Nutrition, Kilcullen, Ireland) in fit Thoroughbreds (Thueson, Leadon et al. 2019). The Thueson et al. study, however, utilized different methodology for measuring CoQ10 and had concentrations that were 100x lower than those measured in our study. In agreement with our study, the human study by Cooke et al. found no differences in muscle CoQ10 concentrations after 14 days of CoQ10 supplementation and wide inter-individual variation in CoQ10 concentrations (Cooke, Iosia et al. 2008). It is possible that muscle CoQ10 concentrations are saturated when levels are adequate and therefore no additional CoQ10 is taken up into muscle fibers. CoQ10 is highly lipophilic, has a high molecular weight and poor aqueous solubility which could limit its absorption and muscle uptake (Greenberg and Frishman 1990).

An impact of CoQ10 supplementation on skeletal muscle was suggested by the significant differences in protein expression identified within the muscle proteome and by differences in muscle GPx activity. The expression of 2 subunits of complex I, 2 subunits of complex III, and 4

subunits of complex V were significantly decreased as well as expression of TCA cycle proteins, and enzymes involved in fat metabolism (Table 4.2 and Figure 4.6). Each complex in the ETC contains multiple subunits, however, interestingly, the down regulated subunits were of utmost importance to electron transport. In complex I, the subunits with decreased expression are those that directly transfer electrons from complex I to CoQ10 (Lin, Zheng et al. 2021) and, in complex III, the down regulated Rieske subunit directly transfers electrons from complex III to cytochrome C (Erecinska, Wilson et al. 1976) (Figure 4.6). The fold change of expression of the subunits is relatively small but their statistical significance and roles indicate that CoQ10 supplementation impacted the muscle proteome and potentially affects electron transfer.

One of the primary goals of this study was to determine if CoQ10 supplementation impacted mitochondrial respiration. We found no differences in CS activity between CoQ10 supplemented and control horses, indicating that mitochondrial volume density was not impacted by CoQ10 supplementation. Integrative (relative to mg of protein) maximal oxidative phosphorylation and electron transfer capacities were unaffected by CoQ10 supplementation. However, intrinsic (relative to citrate synthase activity) maximal oxidative phosphorylation and electron transfer capacities differed. Horses supplemented with CoQ10 had a greater intrinsic complex II-supported electron transfer capacity during the first period and a decreased contribution of complex I (FCR_{PCI} and FCR_{PCIG}) to maximal electron transfer capacity compared to controls. These data suggest that CoQ10 supplementation alters mitochondrial reliance on electrons supplied to complex I versus II. In addition to linking electron transfer from complexes I and II to complex III, CoQ10 can also form a functional structure called the CoQ10-junction (Hidalgo-Gutierrez, Gonzalez-Garcia et al. 2021). This junction primarily facilitates the reduction of

sulfur-based molecules that donate electrons directly to CoQ10 where they can be transferred to complex III provided there is ongoing $FADH_2$-mediated electron transport through complex II (Hidalgo-Gutierrez, Gonzalez-Garcia et al. 2021). One reason CoQ10 supplementation could decrease the contribution of complex I to electron transport could be that the additional provision of CoQ10 enhances electron transfer via the CoQ10 junction. In addition, the proteome identified decreased expression of MDH2, OGDH, and ACO2 which, within the TCA cycle, generate NADH, the substrate for complex I. Reduced TCA cycle activity could reduce the flow of electrons to complex I. Thus, our results suggest that CoQ10 supplementation in horses has the potential to impact the contributions of complex I and complex II to electron transport.

Complex I is thought to be the primary generator of reactive oxygen species (ROS) compared to the other complexes of the electron transfer system (Duong, Levitsky et al. 2021). A decreased use of complex I might lead to decreased production of ROS. In humans, CoQ10 supplementation suppressed hydrogen peroxide (H_2O_2) levels during leak respiration, which is the state where mitochondrial ROS production is greatest (Pham, Macrae et al. 2020). In the present study we did not identify evidence of oxidative stress based on measurement of MDA activity. It is important to recognize, however, that muscle samples were taken at rest and that daily exercise was submaximal. Thus, our study design did not permit assessment of ROS generation when it would be at its highest, during maximal exercise. Future research is warranted to determine if CoQ10 supplementation impacts markers of oxidative stress in maximally exercising horses. This would be especially interesting considering the possibility that CoQ10 supplementation seems to favor electron transfer via complex II over complex I which could lower the production of ROS.

Both CoQ10 and GSH are potent nonenzymatic antioxidants. CoQ10 supplementation of horses in the present study resulted in a significant decrease in activity of an enzymatic antioxidant GPx which serves to reduce oxidized proteins either on its own or using GSH as a co-factor. Notably, the proteomic data identified decreased expression of proteins involved in shuttling GSH into mitochondria. Expression of SLC25A protein channel, which exports 2-oxoglutarate (2-OG) from the mitochondria and imports malate and GSH into the mitochondria, was decreased (Figure 4.6). Additionally, IDH2, which produces 2-OG, and AST, which can convert oxaloacetate to 2-OG, had decreased expression all suggesting a decreased need for shuttling GSH into mitochondria. A decreased need to import GSH into mitochondria could arise from the potent antioxidant capacity of supplemented CoQ10.

CONCLUSION

In conclusion, chronic CoQ10 supplementation in fit Thoroughbred horses increased plasma CoQ10 4 hr after supplementation, without impacting resting muscle CoQ10 concentrations. CoQ10 altered mitochondrial oxidative phosphorylation capacities by increasing the intrinsic capacity of complex II-supported electron transfer after the first period while lowering the contribution of complex I relative to maximal electron transfer. In support, we also found decreased expression of subunits of complex I, III, and V, and TCA proteins that produce the NADH that supplies complex I in CoQ10 supplemented horses. A potential compensatory decrease in GPx activity was found in horses receiving the potent CoQ10 antioxidant with concomitant downregulation of proteins involved in shuttling GSH into the mitochondria. Taken together, in addition to functioning s an antioxidant, CoQ10 in equine muscle appears to alter electron transfer through complex II over complex I without increasing mitochondrial volume density.

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CHAPTER 5

IMPACT OF AMINO ACID AND N-ACETYL CYSTEINE SUPPLEMENTATION ON SKELETAL MUSCLE ANTIOXIDANT STATUS AND PROTEOME IN THOROUGHBRED RACEHORSES

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ABSTRACT

Equine muscle disorders such as myofibrillar myopathy (MFM) are characterized by muscle atrophy and alterations in genes/proteins that produce or degrade cysteine-based antioxidants. Currently there is no published treatment for MFM. One potential means to counter muscle atrophy is provision of dietary branched chain amino acids (BCAA) and a means to enhance cysteine-based antioxidants is supplementation with N-acetyl cysteine (NAC). Prior to prescribing such supplements for MFM horses, it is important to assess their impact in healthy horses. The purpose of this study was to determine the effect of NAC+BCAA supplementation on the skeletal muscle redox equilibrium and proteome of healthy Thoroughbred horses. In a randomized cross over study design, six fit Thoroughbred horses received 10 g NAC and 20 g BCAA (10 g leucine, 5 g isoleucine, and 5 g valine) or no supplement (placebo) for 32-d separated by a 21-d washout period. Pre-exercise samples were taken one day prior to a submaximal standardized exercise test and post-exercise samples were taken 1 hr following exercise. Glutathione (GSH) and cysteine concentrations were measured using mass spectrometry and reactive oxygen species (ROS) were measured using fluorometry with statistical analysis performed with a repeated measure ANOVA. Proteomics was performed using 3 tandem mass tag 11-plex analyses and the statistical analysis was performed using a mixed linear mode. Apart from lameness in one horse, horses remained in good health and normal body weight throughout the study. Prior to exercise, only one protein was differentially expressed between supplemented and placebo groups, cytochrome b-c1. An increase in proteins in the sarcomere or proteasomal pathways was not detected before or after submaximal exercise in NAC+BCAA compared to placebo. There was increased expression of mitochondrial proteins in both complexes I (N=3) and III (N=2), differential expression of glycolytic proteins (N=6) as

well as altered expression of proteins involved in redox (N=6) and protein metabolic pathways (N=4) 1 hr after exercise on NAC+BCAA. Concentrations of GSH, cysteine, and ROS did not differ between treatment or with exercise. We conclude that NAC+BCAA supplementation to submaximally exercising healthy horses did not alter muscle glutathione or cysteine concentrations or myofibrillar protein expression but did produce small increases expression of select mitochondrial and antioxidant proteins following submaximal exercise. The potential benefit of this supplement for horses with muscle atrophy and redox imbalances requires further investigation.

INTRODUCTION

Myofibrillar myopathy (MFM) has been identified in two horse breeds (Arabian and Warmbloods) and is associated with late onset of clinical signs of exercise intolerance, stiffness, reluctance to go forward or engage the hindquarters, and mild lameness that has no underlying cause (Valberg, McKenzie et al. 2016, Lewis, Nicholson et al. 2017, Valberg, Nicholson et al. 2017, Williams, Bertels et al. 2018). The clinical presentation of MFM is also associated with gradual muscle atrophy particularly when horses are not being exercised (Valberg, Nicholson et al. 2017). The gold standard diagnostic technique for identifying MFM is through a muscle biopsy where desmin aggregation is typically apparent on average at 11 years of age in affected horses (Valberg, McKenzie et al. 2016, Valberg, Nicholson et al. 2017). Research indicates that MFM is a complex myopathy involving alterations pathways centralized to the sarcomere, extracellular matrix and redox balance (Valberg, McKenzie et al. 2016, Lewis, Nicholson et al. 2017, Valberg, Nicholson et al. 2017, Williams, Bertels et al. 2018). Transcriptomic and proteomic profiling of MFM in Warmblood horses identified several Z-disc proteins with altered expression when compared to control horses (Williams, Velez-Irizarry et al. 2021) Decreased protein expression of complex I, the mitochondrial antioxidant, catalase, as well as increased gene expression of *CHAC1* (gamma-glutamylcyclotransferase), encoding an enzyme that specifically degrades GSH, was identified in MFM Warmblood horses (Williams, Velez-Irizarry et al. 2021). Arabian horses with MFM had decreased expression of the cysteine-based antioxidant peroxiredoxin 6 as well as decreased post-exercise expression of genes encoding proteins involved in cysteine synthesis (Valberg, Perumbakkam et al. 2018).

Since MFM is characterized by both muscle atrophy and disturbances in cysteine-based antioxidants, we hypothesized that clinical signs could be alleviated by providing a supplement that increased cysteine-based antioxidants and enhanced muscle protein synthesis. However, before evaluating the effect of a supplement in an uncontrolled field study of MFM horses, it is important to study the effects of a potential dietary supplement in a controlled trial with healthy research horses.

Branched Chain Amino Acids (BCAA) are a group of 3 essential amino acids including leucine, isoleucine, and valine. Research studies in humans have shown that provision of BCAA shortly after intense exercise can enhance muscle mass (Tipton and Wolfe 2004). Leucine is believed to be a key driver of muscle mass enhancement because it can activate the mammalian target of rapamycin (mTOR) pathway which is a cellular signaling cascade that regulates cellular metabolism, growth, proliferation, and survival by increasing transcription for protein synthesis (Wang and Proud 2006, Laplante and Sabatini 2009). In humans, leucine has been shown to increase phosphorylation of $p70^{S6k}$, an activator of the mTOR pathway, 1 hr and 2 hr following exercise (Karlsson, Nilsson et al. 2004). In horses, increasing the protein content of the equine diet by 0.25 and 0.5 g of crude protein/kg BW has also been shown to activate the mTOR pathway (Loos, McLeod et al. 2020). Thus, BCAA supplementation could be a means to enhance protein synthesis and muscle mass in horses.

Glutathione (γ-l-glutamyl-l-cysteinylglycine; GSH) is the most ubiquitous cysteine-based antioxidant in mammalian cells (Ribas, Garcia-Ruiz et al. 2014) with cysteine availability being the rate-limiting step in GSH synthesis (Kwon and Stipanuk 2001). In chapter 2 of this

dissertation, we showed that 30 d of supplementation with 10 g of N-acetyl cysteine (NAC) and 1,600 mg Coenzyme Q10 increased muscle concentrations of glutathione (GSH) 1 hr post maximal exercise by approximately 35%. We also found that there was a significant impact of NAC and CoQ10 supplementation on the muscle proteome primarily involving tricarboxylic acid cycle proteins and the capacity to generate NADPH for the reduction of oxidized GSH. Thus, NAC could be an ideal supplement to enhance GSH concentrations in muscle of MFM horses.

We hypothesized that supplementation of NAC and BCAA to fit sub-maximally exercising Thoroughbred horses would alter the redox equilibrium of muscle by enhancing thiol-based antioxidants and alter proteomic profiles consistent with enhanced protein synthesis.

The first objective of the present study was to compare the effect of 32 d of 10 g NAC and 20 g BCAA supplementation (NAC+BCAA) or placebo on skeletal muscle ROS, GSH and cysteine concentrations in Thoroughbred horses before and after a submaximal exercise tests. The second objective was to compare the effect NAC+BCAA supplementation or placebo on the skeletal muscle proteome before and after submaximal exercise.

METHODS

Study design

In a randomized cross-over design with a 21d washout period, six Thoroughbred horses (4 geldings and 2 mares, 5.7 ± 2.8 y) were supplemented with a placebo (15 mL applesauce once daily) or NAC+BCAA for 31d. Daily NAC+BCAA consisted of 10 g NAC and 20 g BCAA (10 g leucine, 5 g isoleucine, and 5 g valine) suspended in 15 mL applesauce once daily (Figure 5.1). At the end of each supplementation period, on days 31-32 and days 84-85, horses performed an exercise test. Muscle biopsies were obtained one day prior to the exercise tests on days 30 and 83 and one hour after the exercise test on days 31-32 and 84-85 (Figure 5.1). Jugular venous blood samples were obtained prior to the exercise test and 10 min after the exercise test. One horse failed to complete the second post-exercise sampling for the study due to an injury on d 82, however, pre-exercise samples were utilized in the analysis.

Diets

Throughout the trial, horses had ad libitum access to timothy hay and were fed 6 kg concentrate 3 times/d scheduled as 3 kg AM, 1 kg pre-exercise, and 2 kg PM meals (Table 5.1) (OBS Racing Blend, Ocala Breeders Feed & Supply Ocala, FL). Horses received 60 g loose salt once daily top dressed on feed.

Figure 5.1. The randomized block design used for placebo and NAC + BCAA supplementation with a 21-day washout period between treatment periods. Pre exercise muscle biopsies were taken 30 days following the beginning of supplementation (black arrows) and exercise test and post exercise muscle biopsies were taken 31-32 days following the beginning of supplementation (blue arrows). Figure created with BioRender.com.

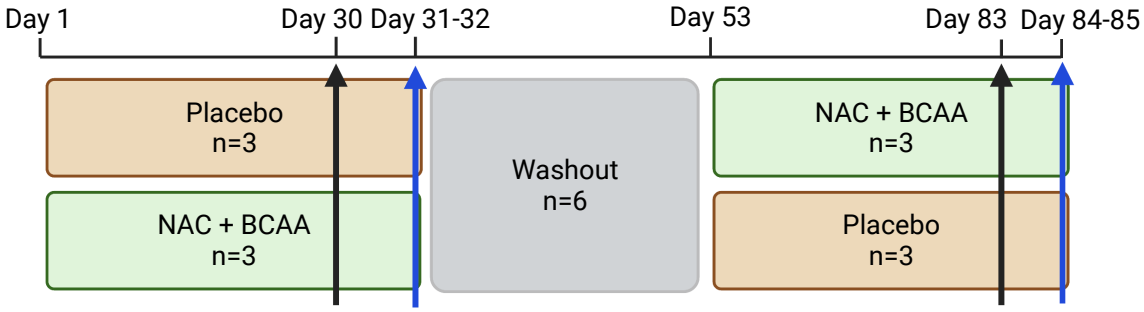


Table 5.1. Guaranteed analysis of the OBS Racing Blend concentrate provided to the horses throughout the study at 2.1 kg/500 kg body weight.

Nutrient	Amount
Crude Protein (Min)	12.0%
Lysine (Min)	0.7%
Crude Fat (Min)	8.0%
Crude Fiber (Max)	9.0%
Calcium (Min)	0.6%
Calcium (Max)	0.7%
Phosphorus (Min)	0.5%
Selenium (Min)	0.5 ppm
Zinc (Min)	145 ppm
Vitamin A (Min)	11,000 IU/kg
Vitamin D (Min)	1,100 IU/kg
Vitamin E (Min)	211 IU/kg

Exercise

Horses were conditioned on the track 3 d/wk and walked (30 min) on an automated exercise machine 3 d/wk throughout the trial. Track conditioning consisted of a gradual increase in distance from 6 furlongs to 10 furlongs while maintaining the same submaximal intensity of approximately10m/s speed. During the washout period track exercise was reduced to speeds of 8-10 m/s. Horses were turned out for approximately 16 hr/d overnight in grassy pastures.

Test protocol

On days 31 or 32 and days 84 or 85, horses performed an exercise test consisting of a 10-furlong gallop at 12-13 m/s speed while wearing a heart rate monitor with GPS (KER Clockit Polar monitor). Three horses exercised on day 31 and three on day 32 based on availability of the jockey. The horses were exercised in the same order for the first and second exercise tests. During the second exercise test on days 84-85, the same experienced rider was instructed to replicate the GPS-determined speed from the previous exercise test. Heart rates, speeds and plasma lactates were compared between the two tests to ensure they were of the same intensity. Horses were fed the NAC+BCAA 1 h prior to the exercise test.

Muscle samples

One day prior to the exercise tests on days 30 and 83, horses were sedated with 200 mg xylazine IV and a percutaneous needle biopsy of the gluteus medius muscle was obtained from a standardized site 16cm along a line from the highest point of the tuber coxae to the tail head using local anesthesia (Lindholm and Piehl 1974). The muscle biopsy procedure was repeated

using the alternate side one hour after the exercise test on days 31-32 and 84-85. Muscle samples were immediately flash-frozen in liquid nitrogen and stored at -80ºC until analysis.

Blood samples

Jugular venous blood samples were obtained prior to the exercise test and 10 min after the exercise test. Plasma samples were kept chilled on ice or in the refrigerator, centrifuged within 30 minutes of collection and frozen and stored at -4ºC until analysis.

Plasma lactates

Plasma lactate concentrations were analyzed using a YSI lactate analyzer according to manufacturer's guidelines (YSI incorporated, Yellow Springs, OH).

Muscle homogenates

For the ROS, cysteine, and glutathione assays, approximately 30mg of muscle was homogenized in bead homogenizer (bullet blender, Next Advance, Troy, NY, USA) in 500 µL of radioimmunoprecipitation assay buffer (RIPA) with protease inhibitors (cOmplete protease inhibitor, Sigma-Aldrich, St. Louis, MO, USA). Protein content of the homogenate was determined using a pierce BCA assay kit (Thermo Fisher Scientific, Waltham, MA, USA).

Reactive oxygen species

ROS were analyzed in resting and post-exercise muscle samples using 10 µl of the homogenate diluted 20x in 1x RIPA buffer. An oxidant sensitive fluorescent probe kit (OxiSelect STA-347, Cell BioLabs, San Diego, CA, USA) was used to measure total ROS which included reactive

nitrogen species, including hydrogen peroxide, nitric oxide, peroxyl radical, and peroxynitrite anion in resting and 1 h post-exercise muscle samples as previously described (Chapter 2). All samples and standards were measured in triplicate and concentrations were measured as hydrogen peroxide (H_2O_2) equivalence per mg protein.

Thiols

Cysteine and glutathione concentrations were analyzed in resting and post-exercise muscle homogenates using liquid chromatography mass spectrometry (LC/MS) as previously described (Chapter 2 and 4). Briefly, protein in the homogenate was reduced using Tris(2-carboxyethyl)phosphine hydrochloride in water (pH 7) containing N-ethylmaleimide which binds to the active sulfhydryl group stopping any subsequent reactions. An internal standard of 20μM GSH ammonium salts D-5 (Toronto Research Chemicals, Toronto, Ontario, Canada) was added to all samples and standards. Chromatographic separation of cysteine and glutathione was achieved using a Phenomenex Kinetex 1.7um EVO C18 100A (50 x 2.1mm) (Phenomenex, Torrance, CA, USA) column with a gradient elution consisting of acetonitrile and deionized water with 0.1% formic acid. Multiple reaction monitoring optimized using Waters Empower 4 software was used for detection of ions generated by cysteine and glutathione in the LC/MS.

Statistical analysis

Data was analyzed for normality using a Shapiro-Wilks normality test. Plasma lactate concentrations were analyzed using a repeated measure ANOVA. Data for ROS, GSH, cysteine did not pass normality and were log transformed. A repeated measure ANOVA with Bonferroni post hoc testing was used to analyze GSH, cysteine, and ROS. Analyses were performed using

GraphPad Prism software version 9.2.0 (GraphPad Software, San Diego, CA, USA). Significance was set at $P<0.05$.

Muscle proteomics

Proteomic analysis was performed for both treatment groups for pre-muscle samples from six horses and for both treatment groups for post-exercise muscle samples from three horses using three 11-plex plates for tandem mass tag MS/MS quantification analysis at the Michigan State University Proteomics Core. Post-exercise samples included the three horses of the most similar ages. Protein was extracted from muscle samples using a radioimmunoprecipitation lysis buffer and protease inhibitor and pelleted prior to submission. From each sample, 500mg of protein was digested in trypsin with a Filter-Aided Sample preparation protocol and spin ultrafiltration unit cutoff of 30,000Da (Wisniewski, Zougman et al. 2009). Reverse phase C18 SepPaks were used to de-salt the resulting peptides (Waters Corporation, Milford, MA, USA) which were then dried by vacuum centrifugation. Peptide quantification was verified by colorimetric peptide concentration using 5mL from each sample digest. Isobaric labeling, gel fractionation and LC/MS/MS analysis were performed.

Quantitative data analysis

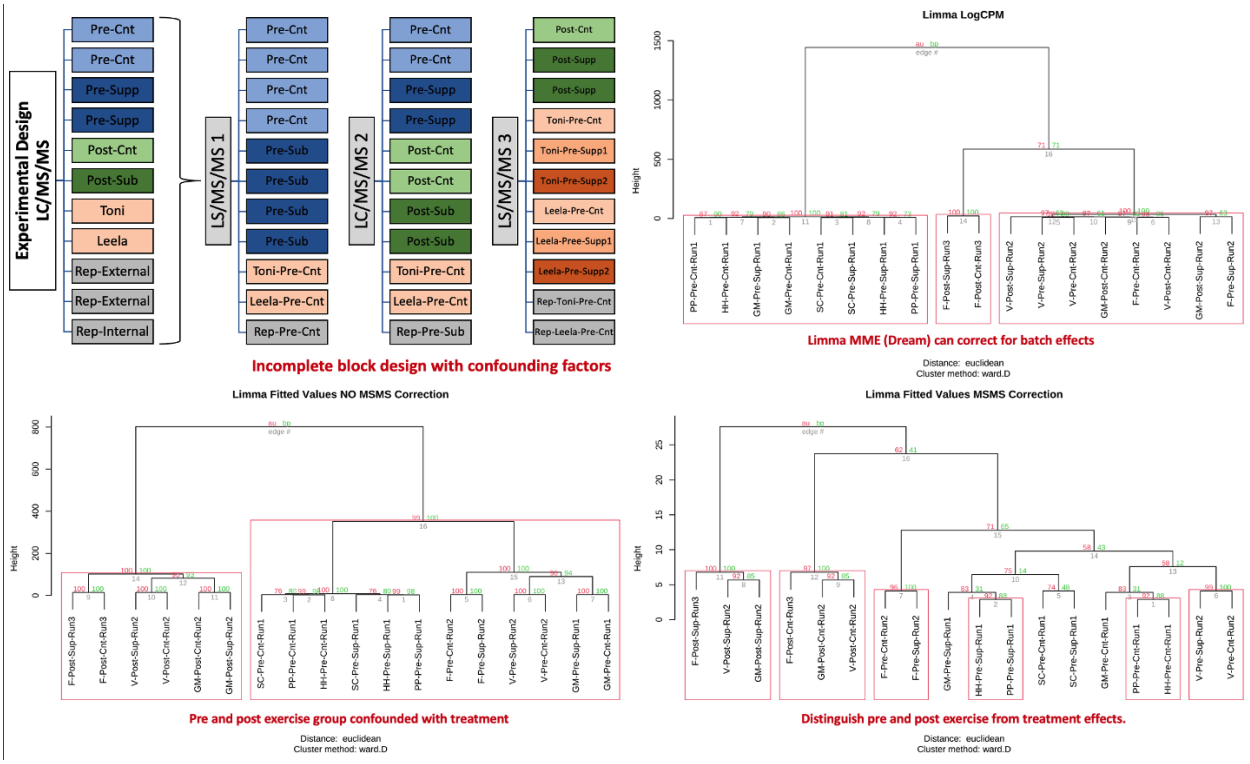
The mapped peptides were probabilistically validated using Scaffold Q+ (v5.0.1, Proteome Software Inc., Portland, OR) using an FDR ≤ 0.01 confidence filter. Peptide identifications were accepted if they could be established at greater than 95.0% probability by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at an FDR ≤ 0.01 and contained at least two identified peptides. Proteins that contained similar peptides and

could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins sharing significant peptide evidence were grouped into clusters. Of 70,695 spectra in the experiment at the given thresholds, 45,800 (65%) were included in quantitation. Protein spectra data were pruned of those matched to multiple proteins, and the median peptide spectra per protein used as the protein spectral estimates. Between-sample normalization was applied to the estimated protein expressions using the trimmed mean of M-values (TMM) (Robinson, McCarthy et al. 2010). The protein spectral counts were then transformed to approximate a Gaussian distribution by calculating the $\log_2$ counts per million (CPM), which is the $\log_2$ of protein spectral counts and scale-normalized library size ratio. The mean-variance trend of protein expression was estimated and incorporated in the variance modeling of the DE analysis as precision weights to account for observational level and sample-specific parameters shared across genes (Ritchie, Phipson et al. 2015). The experimental design was organized as a complete block design across LC/MS/MS run. However, unforeseen circumstances caused the design to shift to an incomplete block design with confounding factors (Figure 5.2).

A mixed model equation (MME) was used to estimate differential protein expression between treatment accounting for repeated measures as random animal effects (variance Partition Bioconductor) (Hoffman and Roussos 2021). A t-test statistics was used to determine covariates to include in the MME, effects of batch, age, sex, block, and treatment were evaluated with random effect variance estimated with restricted maximum likelihood (REML) and heteroskedastic error variance modeled with precision weights. The precision weights incorporate the uncertainty of protein quantitation in the MME as an estimate of the relationship

between the magnitude of protein expression and the sampling variance. The variance of the error is heteroskedastic which was the estimated precision weights across all samples for that protein. The variance for the fixed effects was estimated using the post hoc calculation $\hat{\sigma}_{\beta j}^2 = var(X_j \beta_j)$. Moderate t-tests were used to identify differentially expressed proteins (DEP) between treatments by testing the null hypothesis, $H_0: L\beta = 0$ versus the alternative $H_a: L\beta \neq 0$ where L is the contrast matrix for treatment comparisons. Estimating the random effects of the MME changes the degrees of freedom of the null distribution of hypothesis tests. Therefore, to estimate the degrees of freedom for the hypothesis tests for each protein, the Satterthwaite approximation was used. Multiple test correction was performed using a false discovery rate (FDR) of 0.10 to identify differentially expressed proteins (DEP) between treatments.

Figure 5.2. LC/MS/MS Array Design. The three LC/MS/MS arrays contained incomplete blocks of treatment that confounded treatments effects with batch effects. A mixed model equation was implemented to account for repeated measures and batch effects. The dendrograms highlight the effects of batch (top right), confounding batch with treatment and exercise group effects (bottom left), and the fitted values from the MME after accounting for batch effects (used in hypothesis tests). The statistical model implemented in this study was able to differentiate treatment effects.



RESULTS

Lactate concentrations

Plasma lactate concentrations were significantly higher ($P<0.0033$) at 10 min post-exercise compared to pre-exercise but did not differ between NAC+BCAA and placebo ($P=0.92$) (Pre: NAC+BCAA 0.58 ± 0.16 mmol/L, placebo 0.58 ± 0.14 mmol/L; 10 min post: NAC+BCAA 10.0 ± 4.6 mmol/L, placebo 10.6 ± 10.0).

ROS, glutathione, and cysteine concentrations

There was no significant difference in muscle ROS between NAC+BCAA and placebo either before or after exercise (Figure 5.3). ROS concentrations did not change significantly with exercise on either the NAC+BCAA or the placebo and showed wide individual variation. Muscle glutathione and cysteine concentrations were not significantly different between treatments or prior to and following exercise (Figure 5.4).

Figure 5.3. Skeletal muscle reactive oxygen species (ROS) in NAC+BCAA and placebo supplemented horses. There were no significant effects of exercise or supplementation (P=0.17).

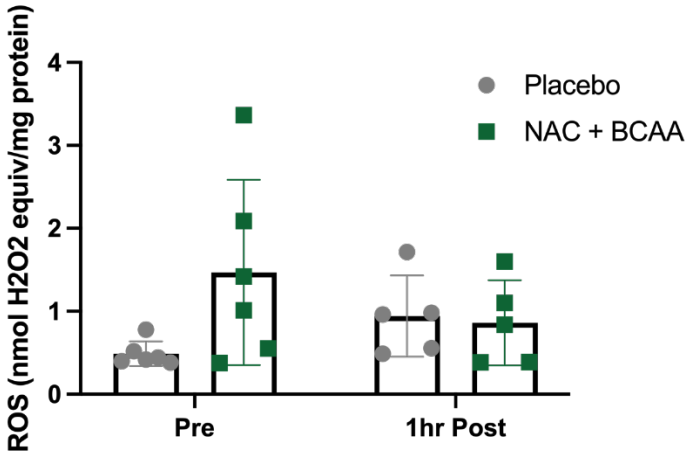
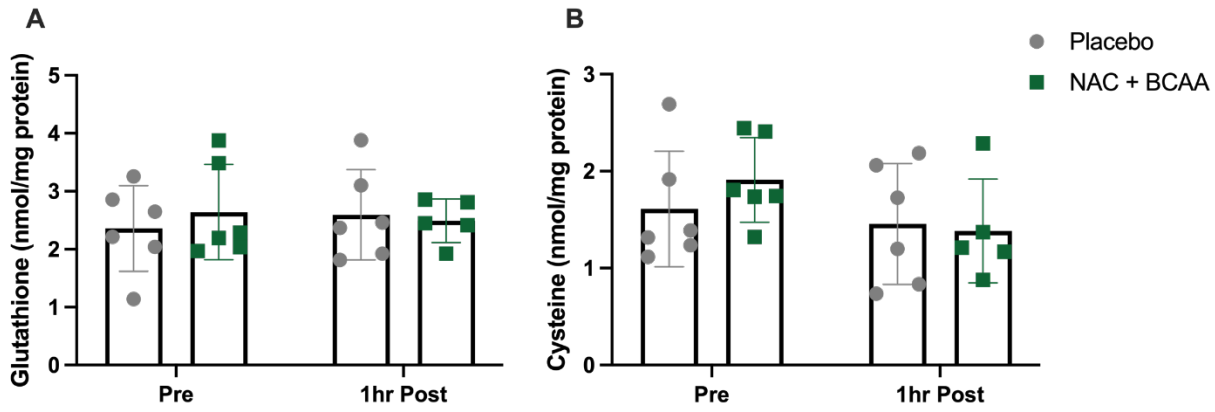


Figure 5.4. A. Gluteal muscle glutathione concentrations in horses at rest and one h after exercise with NAC+BCAA (green) or placebo (grey) supplementation. There were no significant differences between treatments or time periods (P=0.67) **B.** Muscle cysteine concentrations at rest and 1 h after exercise in horses supplemented with NACQ or placebo were not significantly different (P=0.23).



Proteomics

A total of 740 proteins were identified in the proteomic dataset.

Pre-exercise: One protein was differentially expressed between NAC+BCAA and placebo treatments before exercise. Cytochrome b-c1 complex (UQCRC1), which catalyzes electron transfer from ubiquinol to cytochrome C, had a $\log_2$ fold change of 0.19 on the supplement.

Post-exercise: One hr post submaximal exercise, 50 differentially expressed proteins were identified (Table 5.2). A subset of these involved cysteine and antioxidants. The selenocysteine protein (Selenow) had one of the highest $\log_2$ fold increases in expression and it is involved in muscle growth and antioxidant function (Noh, Park et al. 2010, Ko, Lee et al. 2019). Expression of a cysteine-based thioredoxin, superoxide dismutase and two markers for oxidative stress also had significantly decreased expression (Table 5.2). S-methyl-5'-thioadenosine phosphorylase, a methionine salvage protein, had the largest decrease in expression.

Several mitochondrial proteins were differentially expressed. Within the electron transport chain, 3 subunits of complex I (↑NDUFA9, ↑NDUFA10, ↓NDUFAB1), 2 subunits of complex III (↑UQCRC1, ↑UQCRC2), and 2 subunits of ATP synthase (↑ATP5MG, ↓ATP5PF) were differentially expressed. Several tricarboxylic acid cycle proteins were also differentially expressed in NAC + BCAA versus placebo including aconitase (↑ACO) and two subunits of NAD dependent isocitrate dehydrogenase (↑IDH3A, ↓IDH3B) (Table 5.2).

Several myoplasmic proteins were differentially expressed including synaptopodin 2-like protein isoform X1 (↑SYNPO2L). Proteins involved in protein synthesis were down regulated with NAC+BCAA supplementation included plasminogen activator inhibitor 1 RNA-binding protein (SERBP1), elongation factor 1-alpha-2 (EEF1A2), and translationally controlled tumor protein (TPT1). Proteins involved in protein metabolism that were differentially expressed with NAC+BCAA supplementation included alpha-1-antiproteinase 2 (↑SPI2) aspartyl aminopeptidase (↓DNPEP), cathepsin D (↓CTSD), and prolyl endopeptidase (↓PREP). Differentially expressed glycolysis/gluconeogenesis proteins included 2 glyceraldehyde-3-phosphate dehydrogenases (↓GAPDHS, ↓GAPDH), phosphoglucomutase-1 isoform X2 (↓PGM1), glycogenin-1 (↓GYG1), and UTP-glucose-1phosphate uridylyltransferase (↑UGP2) (Table 5.2).

Table 5.2. The gene identification for a protein, protein name, log_2 fold change and P value adjusted for multiple test corrections in 5 horses supplemented with NAC+BCAA compared to placebo 1hr following submaximal exercise test.

Gene ID	Protein name	Adjusted P Value	Log_2 Fold Change
	Methionine/Cysteine Metabolism		
LOC100065392	S-methyl-5'-thioadenosine phosphorylase isoform X2 [MTAP]	3.92E-02	-1.08
	MITOCHONDRIA		
	Electron transport		
NDUFA9	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 9 mitochondrial	6.83E-02	0.50
NDUFA10	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 10 mitochondrial isoform X1	9.33E-02	0.42
NDUFAB1	acyl carrier protein mitochondrial isoform X1	4.33E-02	-0.66
UQCRC1	Cluster of cytochrome b-c1 complex subunit 1 mitochondrial	2.15E-02	0.18
UQCRC2	cytochrome b-c1 complex subunit 2 mitochondrial	6.02E-02	0.23
ATP5MG	ATP synthase subunit g mitochondrial	9.33E-02	0.43
ATP5PF	Cluster of ATP synthase-coupling factor 6 mitochondrial	5.96E-02	-0.45
	TCA cycle		
IDH3B	isocitrate dehydrogenase [NAD] subunit beta mitochondrial isoform X2	6.83E-02	-0.79
IDH3A	Cluster of isocitrate dehydrogenase [NAD] subunit alpha mitochondrial	9.33E-02	0.30
ACO2	aconitate hydratase mitochondrial	8.53E-02	0.16
	Other		
NIT2	omega-amidase NIT2 isoform X1	6.07E-02	-0.55
ECI1	enoyl-CoA delta isomerase 1 mitochondrial isoform X1	5.85E-02	0.38
MRPS36	28S ribosomal protein S36 mitochondrial	6.07E-02	-0.36
PHB2	prohibitin-2	7.70E-02	0.41
	Redox		
GLO1	lactoylglutathione lyase	2.15E-02	-0.49
SOD2	superoxide dismutase [Mn] mitochondrial precursor	4.81E-02	-0.29
TXNDC17	thioredoxin domain-containing protein 17	8.94E-02	-0.44
AKR1B1	aldose reductase	5.25E-02	-0.23

Table 5.2 (cont'd)

Gene ID	Protein name	Adjusted P Value	Log_2 Fold Change
HSPB6	heat shock protein beta-6	8.94E-02	-0.34
SELENOW	selenoprotein W	6.02E-02	0.64
	MYOPLASM		
	Protein Synthesis		
SERBP1	Cluster of plasminogen activator inhibitor 1 RNA-binding protein	3.99E-02	-0.91
EEF1A2	Cluster of elongation factor 1-alpha 2	6.02E-02	-0.39
TPT1	Cluster of translationally-controlled tumor protein	8.94E-02	-0.31
	Sarcomeric Proteins		
SYNPO2L	synaptopodin 2-like protein isoform X1	6.79E-02	0.29
	CK/Glycolysis/Gluconeogenosis		
CKM	creatine kinase M-type	3.92E-02	-0.48
GAPDHS	Cluster of glyceraldehyde-3-phosphate dehydrogenase testis-specific	2.15E-02	-0.77
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	3.92E-02	-0.30
PGM1	phosphoglucomutase-1 isoform X2	9.67E-02	-0.26
GYG1	glycogenin-1	4.81E-02	-0.34
UGP2	Cluster of UTP--glucose-1-phosphate uridylyltransferase	6.83E-02	0.49
	Protein Metabolism		
SPI2	alpha-1-antiproteinase 2 isoform X1	6.83E-02	0.97
DNPEP	Cluster of aspartyl aminopeptidase	5.96E-02	-0.59
CTSD	cathepsin D	6.83E-02	-0.36
PREP	prolyl endopeptidase	9.54E-02	-0.27
	Calcium Regulation		
ANK1	ankyrin-1 isoform X14	6.07E-02	0.18
CASQ1	calsequestrin-1	6.02E-02	-0.49
CA3	carbonic anhydrase 3	5.85E-02	-0.29
S100A11	protein S100-A11	9.33E-02	-0.28
	Cytoskeleton		
TMSB4X	thymosin beta-4	2.15E-02	-1.28
ACTB	Cluster of actin cytoplasmic 1	6.83E-02	-0.70
CAP2	Cluster of adenylyl cyclase-associated protein 2	2.15E-02	0.66
	Lipid Transport		
APOH	beta-2-glycoprotein 1	8.37E-02	0.73

Table 5.2 (cont'd)

Gene ID	Protein name	Adjusted P Value	Log_2 Fold Change
LOC100057425	fatty acid-binding protein adipocyte	6.83E-02	-0.37
	Miscellaneous		
RTN2	reticulon-2 isoform X2	4.81E-02	0.43
MB	myoglobin	5.96E-02	-0.32
RAB1B	ras-related protein Rab-1B	2.15E-02	-0.65
NUGGC	Cluster of nuclear GTPase SLIP-GC	8.37E-02	-0.67
LOC100146489	histone H2A.Z	9.33E-02	-0.75
ACTN4	Cluster of alpha-actinin-4	6.02E-02	0.79

DISCUSSION

The present study examined the impact of 32 d of supplementation with 10 g NAC and 20 g BCAA on the skeletal muscle antioxidant status measured as GSH and ROS as well as the effect of supplementation on the muscle proteome in Thoroughbred horses performing submaximal exercise. Concentrations of GSH, cysteine, or ROS did not differ before or after exercise comparing NAC+BCAA supplementation to placebo. An enhancement of sarcomeric proteins indicative of increased protein synthesis was not detected on NAC+BCAA by proteomic analysis. Only one differentially expressed protein was identified at rest between supplemented and un-supplemented animals, however, 1 hr following submaximal exercise, 50 differentially expressed proteins were identified. Most of these proteins were found within the mitochondria, glycolysis/glycogen synthesis, and oxidation/reduction pathways potentially indicating a mild shift in energy metabolism. It appears that NAC+BCAA produces little detectable enhancement of sarcomeric proteins in horses with normal muscle mass, consuming a balanced diet and exercising at submaximal speeds.

Total energy intake and daily protein intake are important for athletes to ensure that enough protein is available to support both muscle turnover and metabolic adaptations to exercise (Torre-Villalvazo, Aleman-Escondrillas et al. 2019). BCAA are of particular importance because leucine increases muscle protein synthesis, preserves or restores muscle mass and BCAA serve as substrates for the TCA cycle (Pasiakos, McClung et al. 2011, Breen and Churchward-Venne 2012, Churchward-Venne, Burd et al. 2012, McNurlan 2012, Torre-Villalvazo, Aleman-Escondrillas et al. 2019). In horses, 14 weeks of supplementation with 20 g lysine and 15 g threonine to young (≤10 yr) and aged (≥20 yr) horses significantly increased scores of muscle

mass compared to horses receiving the basal diet alone (Graham-Thiers and Kronfeld 2005). In the present study, we used a global proteomic approach to determine if supplementation with 20g/d of BCAA 1 h prior to exercise would impact the relative expression of muscle proteins. If the BCAA enhanced muscle mass in the present study, we would have expected to see an increase in expression of sarcomeric proteins on NAC+BCAA versus placebo because sarcomeric proteins such as myosin makes up 25-30% of total muscle protein (Wilborn and Willoughby 2004). We found no significant difference in the expression of myosin or other contractile proteins, however, before or after exercise in horses supplemented with NAC+BCAA compared to those that were not supplemented. It is plausible that the horses in our study had optimal amounts of protein for their activity level from their basal diet and therefore additional amino acids had little impact.

The mammalian target of rapamycin (mTOR) is a signaling pathway that can be regulated by the branched-chain amino acid leucine (Pasiakos, McClung et al. 2011). A preliminary study of healthy Thoroughbreds exercising at moderate intensity assessed the effect of similar concentrations of NAC and BCAA used in the present study on activators of mTOR using Western blot techniques[1]. Moderate exercise resulted in activation of protein kinase B (Akt) and P70-S6 kinase 1 ($p70^{S6k}$), both activators of mTOR, however, there was no difference in their activation comparing supplementation with NAC or BCAA[1]. In the present study, on NAC+BCAA, 2/50 differentially expressed proteins were in the mTOR pathway, SERBP1 and EEF1A2. Cleavage of SERBP1 can be mediated by $p70^{S6k}$ and impacts lipid synthesis (Cuyas,

[1] A. Hauss, C. Loos, A. Gerritsen, K. Urschel, J. Pagan. (2021). Effect of branched-chain amino acid and N-acetylcysteine supplementation post-exercise on muscle mTOR signaling in exercising horses. Journal of Equine Veterinary Science **100** 103524

Corominas-Faja et al. 2014). Decreased expression of SERBP1 could indicate increased cleavage which would increase mTOR activation. Decreased expression of EEF1A2, an elongation factor that inhibits p53 was also identified in the proteomic data post-exercise. When p53 is inhibited, mTOR is activated (Pellegrino, Calvisi et al. 2014). Since EEF1A2 has decreased expression, it could be allowing more activation of p53 which would then decrease the activation of mTOR. Thus, there is little evidence to support mTOR activation and enhanced protein synthesis in horses on the NAC+BCAA diet based on our study. Further investigation of the role of BCAA in activation of mTOR and enhancing muscle development in our study would require western blot analyses and potentially a lower protein diet combined with a maximal exercise session.

It is important to note, that a lack of impact of NAC+BCAA on sarcomeric proteins in healthy horses in the present study does not imply it would not have an impact on horses with myopathies characterized by suboptimal protein synthesis or enhanced protein degradation. BCAA supplementation increased muscle protein synthesis in patients with sarcopenia from surgery or cancer (Choudry, Pan et al. 2006, Kitajima, Takahashi et al. 2018, Ko, Wu et al. 2020). In mice with induced skeletal muscle atrophy, BCAA supplementation attenuated the effects of the atrophy particularly in type 2 muscle fibers (Yamanashi, Kinugawa et al. 2020). Notably, in MFM horses, desmin aggregation occurs in type 2 fibers. MFM is also with differential expression of Z disc proteins including SYNPOL2 (Williams, Velez-Irizarry et al. 2021). SYNPOL2 is involved in the Z-disc's role of sensing, integrating, and transducing biomechanical stress signals into signaling responses (Williams, Velez-Irizarry et al. 2021). The present study found increased expression of SYNPOL2 in the NAC+BCAA supplemented horses supporting further study of NAC+BCAA in horses with MFM horses.

Although sarcomeric proteins were not impacted by NAC+BCAA supplementation, there was a clear interaction of NAC+BCAA and exercise on the gluteal muscle proteome such that 50 differentially expressed proteins were identified 1 hr post-exercise comparing NAC+BCAA to the placebo. In comparison, only one differentially expressed protein was identified between treatments in our proteomic analysis of resting samples. The NAC+BCAA supplement resulted in alterations in mitochondrial protein expression following exercise. Most of the differential expression of mitochondrial proteins post-exercise had increased expression comparing supplement to placebo, including 2 subunits each of both complex I and III. One subunit of complex I had decreased expression in the NAC+BCAA supplemented group, however, this subunit is also involved in protection from insulin resistance outside of its role in complex I (Zhang, Hou et al. 2019). There was disagreement in the direction of differential expression in subunits of ATP synthase with both subunits being involved in the stalk portion of ATP production. Concomitantly, we found a decrease in glycolytic proteins including GAPDH, PGM1, and GYG1. Thus, in healthy horses, the NAC+BCAA supplement had a significant but relatively minor impact on metabolic proteins in terms of the $\log_2$ fold change with a general pattern of increased oxidative and decreased glycolytic proteins.

Muscle ROS concentrations in the present study did not differ with supplementation or exercise which agrees with our previous study (Chapter 2). However, both studies assessed muscle concentrations 1 hr post exercise due to the excitable nature of Thoroughbred horses which requires a cool down period prior to muscle sampling. This cool down period may have allowed time for the muscle to clear ROS produced during exercise and therefore neither study could ascertain whether high amounts of ROS were generated during exercise. In addition, the

submaximal exercise intensity used in the present study was less likely to generate ROS than a maximal exercise test (Sureda, Ferrer et al. 2009). To the authors' knowledge, no study has assessed a serial analysis of muscle ROS concentrations beginning immediately following different types of exercise and repeated until 1 hr following exercise which would assist in interpreting the 1 hr post exercise concentrations measured in our study.

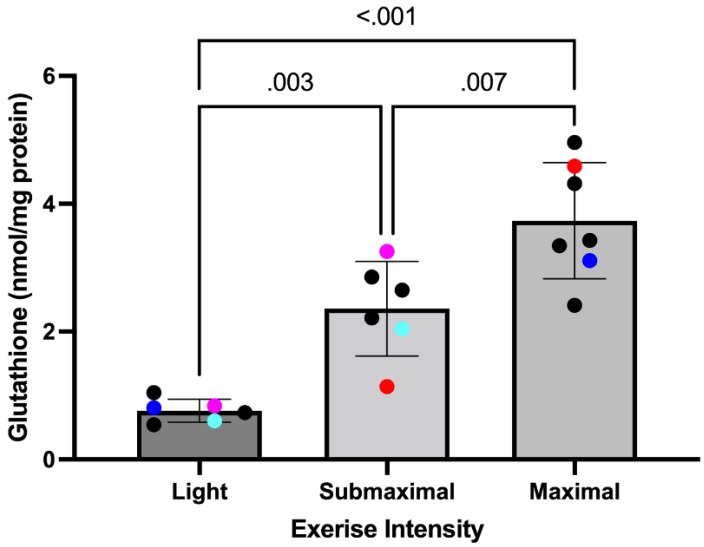
There was also a shift in redox protein expression in the proteomic data. A cysteine-based protein selenocysteine (Selenow) with antioxidant function had one of the highest log_2 fold (Noh, Park et al. 2010, Ko, Lee et al. 2019). Additionally, expression of a cysteine-based thioredoxin, superoxide dismutase and two markers for oxidative stress also had significantly decreased expression (Table 5.2). A decreased need for methionine, a cysteine precursor, was suggested by the largest decrease in expression of S-methyl-5'-thioadenosine phosphorylase, a methionine salvage protein. We did not, however, identify a change in concentrations of the cysteine-based antioxidant GSH in gluteal muscle of NAC+BCC supplemented horses before or after exercise.

The lack of change in muscle GSH concentrations in resting muscle in horses on the NAC+BCAA in the present study agrees with our previous study of 30 d NACQ supplementation in Thoroughbred horses (Chapter 2). Furthermore, a lack of change in resting muscle GSH concentrations agrees with a human study (60 mg/kg oral NAC) (Michailidis, Karagounis et al. 2013). The lack of change in GSH concentrations likely reflects adequate resting GSH concentrations and minimal production of ROS at rest. Following a maximal exercise test, when ROS is often generated, a significant increase in GSH concentrations has been documented, both in horses on NACQ (35%) and humans on NAC (14%) (Chapter 2)

(Michailidis, Karagounis et al. 2013). It is possible that the lack of increase in GSH post-exercise in the present study was the result of the training and exercise test only incorporating submaximal compared to the maximal exercise. The choice of submaximal exercise was made by the owner of the horses who feared they were not fit enough to perform a maximal exercise test without injury.

Muscle concentrations of GSH in horses have only been measured during the studies presented in this dissertation. Because all studies in this dissertation used the same high performance liquid chromatography analysis to measure gluteus medius muscle GSH concentrations, we were able to compare concentrations in the control and placebo groups pre-exercise across studies. Horses were fed similar basal diets with the biggest difference being supplement or exercise intensities among the studies. In chapter 2 of this dissertation, in the placebo group pre-exercise, mean muscle concentrations of 3.7 ± 0.9 nmol/mg protein were found in horses performing maximal exercise intensities with peak fitness. In the present study with sub-maximally exercising horses, the mean concentration of GSH was 2.4 ± 0.7 nmol/mg protein in the placebo group pre-exercise. In chapter 4 of this dissertation, horses performing treadmill and under-saddle exercise (light intensity) had the lowest mean GSH concentrations of 0.8 ± 0.1 nmol/mg protein in the control group. Horses used in multiple studies each consistently showed an increase in GSH with increasing exercise intensity. Thus, muscle GSH concentrations appear to increase with the intensity of training in healthy horses (Figure 5.5). The lack of impact of NAC on muscle GSH in the present study compared to the study in Chapter 2 could be due to the lower exercise intensity used for the horses in the current study resulting in less ROS and less stimulus to enhance muscle GSH concentrations.

Figure 5.5. Skeletal muscle GSH concentrations across different exercise intensities represented as nmol/mg protein. Concentrations shown were pre exercise on the control supplement for each individual study. Exercise intensities include light (chapter 4), submaximal (present study), and maximal (chapter 2). Different colored points represent the same horse used in different studies. Data analyzed using a one way ANOVA and GraphPad Prism 9.3.1.



There were limitations in the present study that are important to consider. The timing of BCAA supplementation relative to exercise has been shown to affect its impact on muscle mass in humans. Both adding 0.75 g or 3 g of leucine to a drink following resistance exercise increased muscle protein synthesis for 1-3 hr following the exercise bout (Churchward-Venne, Burd et al. 2012). Supplementing 5.6 g of BCAA following resistance exercise was also shown to increase muscle protein synthesis 22% within 4 hr of an exercise bout in another human study (Jackman, Witard et al. 2017). Equine studies have shown that amino acid supplementation can increase plasma amino acid concentrations after exercise (Urschel, Geor et al. 2010, Nostell, Essen-Gustavsson et al. 2012), however no studies have analyzed the impact of post-exercise oral BCAA supplementation on mTOR activity or muscle protein synthesis. It could be possible that by supplementing BCAA before exercise in the present study, we missed the optimal window for increasing muscle protein synthesis, however, studies have shown that feeding 189 mg lysine/kg BW/d significantly increases plasma BCAA concentrations and activation of gluteal muscle mTOR pathways measured 90 min after administrations (Loos, McLeod et al. 2020). Thus, elevations in BCAA would be expected to exist during the exercise test in the present study and be maintained 1 hr post exercise period. It is also possible that we did not see any increase in sarcomeric protein content because exercise performed was not high intensity exercise such as resistance exercise which is what the human studies analyzed.

CONCLUSION

In conclusion, NAC+BCAA supplementation did not alter the relative amount of sarcomeric proteins in skeletal muscle but did increase expression of some cysteine-based antioxidants in fit horses 1 hr after submaximal exercise. After exercise, NAC+BCAA also resulted in small increases in two subunits of complexes I and III in the mitochondrial ETC and decreased expression of glycolytic enzymes. Further studies of the impact of the NAC+BCAA supplement on horses with myopathies such as MFM should be performed to determine potential beneficial effects in animals with muscle atrophy and altered redox balance.

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CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

Background

Elite equine athletes will require a large amount of ATP to be produced to fuel an exercise bout either through aerobic pathways such as the mitochondrial electron transport chain (ETC) or through anaerobic pathways such as glycolysis. During a bout of exercise there is the potential to produce reactive oxygen species (ROS) which in excessive amounts can lead to oxidative stress. Complex I in the ETC has been implicated as the primary generator of ROS during exercise compared to the other complexes (Duong, Levitsky et al. 2021). Coenzyme Q10 (CoQ10) is an electron transporter within the ETC that is responsible for transferring electrons from either complex I or complex II to complex III. CoQ10 is also a potent membrane-bound antioxidant and due to its location in relation to complex I it could potentially mitigate ROS production. CoQ10 is a lipophilic molecule that has been studied as a nutritional supplement in humans but has not been studied in great depth on its effect as an antioxidant or on skeletal muscle especially in horses.

Glutathione (GSH) is a ubiquitous, peptide, non-enzymatic antioxidant that can be produced from 3 amino acids with cysteine containing the sulfhydryl group responsible for its antioxidant status. GSH concentrations have only been studied a handful of times in skeletal muscle. In human studies, it has been shown that N-acetyl cysteine (NAC) is a readily available source of cysteine that can be used to produce GSH (Droge 2005). Other amino acid supplements such as branched chain amino acids (BCAA) have not been studied in conjunction with antioxidant supplementation. It is imperative to fully investigate the effects of antioxidant supplementation

in healthy horses prior to investigating the effect of supplementation on horses with myopathies to understand the effects and ensure the safety and efficacy of the supplementation in diseases.

Impact of NAC and CoQ10 in maximally exercising Thoroughbreds

The first study of this dissertation hypothesized that the provision of NAC and CoQ10 (NACQ) as an oral supplement will alter the redox equilibrium of healthy equine muscle by enhancing thiol-based antioxidants, increasing CoQ10 content, and altering mitochondrial protein expression. Our objectives were to measure changes in the redox equilibrium through skeletal muscle concentrations of GSH, CoQ10, and ROS, as well as the activity of GPx and SOD, and to measure the relative abundance of muscle proteins through a proteomic analysis.

The first objective was accomplished through biochemical analyses including fluorometric, colorimetric, and mass spectrometry analyses to identify the concentrations and activities of the different antioxidants as well as the concentrations of ROS. In horses supplemented with NACQ, there was a significant increase (35%) in GSH concentrations 1 hr post-maximal exercise compared to the control group. There were no significant differences in other antioxidant or ROS measurements. Our finding that CoQ10 concentrations in the skeletal muscle did not increase is in direct contrast to a study that found that 1 g of ubiquinol supplementation did increase muscle concentrations of CoQ10 (Thueson, Leadon et al. 2019). The concentrations measured in the previous study were approximately 100x lower than those measured in this study, casting doubt on this analysis. Thus, the NACQ supplement appears capable of augmenting post-exercise GSH GSH without impacting muscle COQ10. The absence of an increase in CoQ10 muscle concentrations in supplemented horses could be due to optimal endogenous muscle concentrations or lack of absorption through the gastrointestinal tract.

The second objective was to identify the differences in muscle proteins through a proteomic analysis of NACQ supplemented horses compared to the controls. Forty proteins were identified to be differentially expressed in the NACQ group with 22 having increased expression and 18 with decreased expression. There were two primary groups of proteins with differential expression including mitochondrial proteins (13) which all had increased expression and glycolytic/gluconeogenic (9) which all had decreased expression. During the maximal exercise testing there were no differences in the production of plasma lactate between NACQ and control supplementation groups indicating that the decreased expression of the glycolytic proteins did not have a functional effect.

Supplementation with NACQ increased mitochondrial proteins involved with the reduction of oxidized glutathione as well as the ability to shuttle GSH into mitochondria. Increased expression of TCA cycle enzymes included NADP-dependent isocitrate dehydrogenase (IDH2) produce 2-oxoglutarate, which can be utilized by the SLC25A mitochondrial glutathione shuttle to bring GSH into the mitochondria. Additionally, IDH2 and NADP+ transhydrogenase (NNT) are able to generate NADPH which can reduce oxidized glutathione (Schiaffino, Reggiani et al. 2015). NACQ appears to not only enhance post-exercise GSH concentrations but also increase the capacity to reduce oxidized GSH within the mitochondria.

Overall, NACQ was able to alter the mitochondrial antioxidant equilibrium by increasing concentrations of GSH 1 hr post exercise while also increasing the capacity to import GSH into the mitochondria and to produce NADPH which is required to reduce oxidized GSH.

Impact of a single dose of CoQ10

The second study of this dissertation hypothesized that micellized formulation of ubiquinol will have better plasma absorption relative to a standard dose of microencapsulated crystalline ubiquinone and that provision of RRR-α-tocopherol will further increase plasma absorption of the micellized ubiquinol. Our objectives for this study were to determine the optimal dose of micellized ubiquinol (1,600-3,200 mg) compared to the standard 1,600 mg hydro-soluble ubiquinone. The second objective was to determine if co-supplementation with 2,000 IU of RRR-α-tocopherol would increase the absorption of micellized ubiquinol.

The first objective was accomplished by measuring the response to a single dose of micellized ubiquinol or hydro-soluble in 6 Thoroughbred horses across a 24 hr period following supplementation. Plasma samples were collected at time 0 (pre-supplementation), 2 hr, 4 hr, 8 hr, and 24 hr post supplementation for all doses. Plasma CoQ10 concentrations were expected to peak between 4 and 8 hrs post-supplementation, however there were no significant differences in CoQ10 concentrations from baseline at any time point and supplementation amount. Overall, this study did not find evidence of absorption of CoQ10 regardless of dose or formulation following a single dose.

The second objective was accomplished by measuring the response to a single dose of micellized ubiquinol (800 mg) compared to the same dose with the addition of 2,000 IU of RRR-α-tocopherol across a 24 hr period following supplementation. There were no differences in plasma absorption of micellized ubiquinol with or without the additional supplementation of RRR-α-tocopherol. This was in direct contrast to another recently published study that analyzed the

effects of 800 mg cyclodextrin-encapsulated ubiquinone with 990 IU of RRR-α-tocopheryl acetate which found that co-supplementation with α-tocopheryl acetate increased plasma CoQ10 concentrations when compared to the placebo group (Svete, Vovk et al. 2021). However, the placebo group for this study was a separate group of horses housed under separate conditions as the supplemented group in contrast to our study where each horse received each dose in a Latin-square design. Therefore, individual variation and environmental effects could explain the differences between the placebo and supplemented groups in the Svete et al. study and individual differences may have impacted our study.

Our findings were similar to a human study that reported no significant difference in plasma CoQ10 levels following supplementation with either ubiquinone or ubiquinol formulations. Similarly to what we found, they also found significant inter-individual variation in baseline plasma levels and bioavailability of CoQ10, irrespective of the CoQ10 form (ubiquinone/ubiquinol) administered (Singh, Niaz et al. 2005)

Overall, it appears that intestinal absorption of CoQ10 is highly variable among individuals and independent of the form of CoQ10 (ubiquinone/ubiquinol) administered.

Impact of CoQ10 on fit Thoroughbreds

The third study of this dissertation hypothesized that supplementation with 1,600 mg CoQ10 (micellized ubiquinol) for 30 days would result in a CoQ10 plasma dose response 4 hr following supplementation. Further, we hypothesized that chronic CoQ10 supplementation would increase mitochondrial oxidative phosphorylation capacities, alter antioxidant balance, and alter mitochondrial protein expression.

The first objective of our study was to determine the effect of at least 30 days of 1,600 mg CoQ10 supplementation on plasma CoQ10 concentrations before and after a single dose. The second objective was to determine the effect of 30 days of 1,600 mg CoQ10 supplementation on skeletal muscle CoQ10 concentrations and muscle antioxidant balance. The third objective was to determine the effect of CoQ10 supplementation on mitochondrial electron transport. The fourth objective was to determine the effect of CoQ10 supplementation on the muscle proteome.

The first objective was accomplished by measuring the effects of a single 1,600 mg dose of micellized ubiquinol on plasma CoQ10 concentrations 4 hr after supplementation following >30 d of supplementation. A significant increase in plasma CoQ10 within 4 h of CoQ10 dosing following 30-40 d of supplementation was found which contrasts the previous study that found no increase in plasma CoQ10 concentrations after a single dose of CoQ10 and no previous supplementation.

The second objective was accomplished by measuring the impact of CoQ10 supplementation on antioxidant concentrations and activities, and oxidative stress. Skeletal muscle CoQ10 concentrations did not differ between the control and CoQ10 supplementation group. There were also no differences seen in GSH or MDA concentrations, or SOD or Cat activities between supplementation groups. However, there was a significant decrease in GPx activity with CoQ10 treatment which indicates a shift in the antioxidant balance. Since biopsies were taken at rest prior to daily exercise, we did not expect there to be an increase in MDA concentrations as a measure for oxidative stress due to the long period of time between the biopsy and the previous exercise bout. However, it would have shown oxidative stress if the horses were producing enough ROS in their daily exercise to overwhelm the antioxidant cycle and cause oxidative damage. This was not the case in these animals and therefore we can say that even with a decrease in GPx activity, the antioxidant capacity was sufficient to mitigate ROS produced during daily training.

The third objective was accomplished by utilizing high-resolution respirometry to identify the functionality of each complex in the electron transfer system. The capacities of each complex were analyzed in three different ways; integrative which measures per mg tissue, intrinsic which measures relative to citrate synthase activity, and flux control ratios which is a ratio of individual complexes to total oxidative phosphorylation. There were no differences identified in any of the integrative measures of any complex or total oxidative phosphorylation. Intrinsic measures found a significant increase in the capacity of complex II with CoQ10 treatment (E_{CII}) and identified a treatment by period interaction indicating that period 1 influenced this finding more than period 2. Flux control ratios identified a significant decrease in the contribution of complex I to total

oxidative phosphorylation ($FCRP_{CI}$ and $FCRP_{CIG}$) on the CoQ10 supplement compared to the control. Total oxidative phosphorylation (P_{CI+II}) also had a time effect where all horses regardless of treatment had significantly higher oxidative phosphorylation in the second period as opposed to the first. Overall, this suggests that CoQ10 supplementation in horses has the potential to impact the contributions of complex I and complex II to electron transport and appears to have an impact on skeletal muscle even though total concentrations do not increase.

The final objective was accomplished using a tandem mass tag 11-plex proteomic analysis to identify differentially expressed proteins in the CoQ10 supplemented group compared to the controls. This analysis identified 38 differentially expressed proteins in the CoQ10 group out of a total of 834 identified proteins with 30 of them being down regulated and 8 having up regulation. All the differentially expressed proteins located withing the mitochondria had decreased expression including two subunits of complex I, two subunits of complex III, and four subunits of ATP synthase. There was also a decrease in the SLC25A shuttle, 5 enzymes in beta oxidation as well as 4 TCA cycle enzymes including malate dehydrogenase (MDH2), aconitate hydrase (ACO2), isocitrate dehydrogenase 2 (IDH2), and 2-oxogluterate dehydrogenase (OGDH). These findings are interesting because they seem to align with the differences seen in the high-resolution respirometry data.

From this study it appears that CoQ10 is able to alter the antioxidant equilibrium by decreasing the activity of GPx and decreasing the expression of the SLC25A shuttle which is used to import GSH and malate into the mitochondria. There is an increase in complex II capacity seen in the intrinsic mitochondrial respiration data while complex I contribution to total oxidative

phosphorylation appears to be lower. This is further supported by the decrease in TCA cycle enzymes such as MDH2 that aid in the production of NADH as a substrate for complex I. It is possible that with the decreased contribution from complex I, these horses are producing less ROS since that is the primary generator in the ETC (Duong, Levitsky et al. 2021).

Overall, it appears that CoQ10 supplementation in moderately fit horses can function both as an antioxidant as well as alter electron transfer by increasing the capacity of complex II while decreasing the contribution of complex I to total electron transfer without increasing mitochondrial volume density.

Impact of NAC and BCAA in sub-maximally exercising Thoroughbreds

The fourth study of this dissertation hypothesized that the provision of NAC and branched chain amino acids (BCAA) would alter the redox equilibrium by enhancing thiol-based antioxidants and alter proteomic profiles reflective of increased protein synthesis after submaximal exercise in comparison to a placebo.

The first objective of this study was to determine the effect of NAC+BCAA supplementation on muscle concentrations of GSH, cysteine, and ROS before and 1 hr after submaximal exercise. The second objective of this study was to determine the supplement and exercise effect on the skeletal muscle proteome.

The first objective was accomplished by measuring the concentrations of GSH and cysteine using high performance liquid chromatography mass spectrometry and measuring the concentrations of ROS through a fluorescent analysis. No significant differences were seen in concentrations of GSH, cysteine, or ROS with or without NAC+BCAA supplementation or before or after exercise.

The second objective was accomplished by performing a tandem mass tag proteomic analysis and analyzing identified peptides with a mixed model equation to identify differentially expressed proteins in the NAC+BCAA supplemented groups compared to placebo. This analysis identified 1 protein in the pre exercise samples that was differentially expressed between NAC+BCAA and placebo but identified 50 differentially expressed proteins 1 hr after exercise between NAC+BCAA out of a total of 740 identified proteins. No differences were seen in

sarcomeric proteins indicating that either the optimal window for increased muscle protein synthesis was missed with supplementation or that these horses had ample amount of sarcomeric proteins for their activity level. There was an increase in expression of 2 subunits of both complex I and complex III of the mitochondrial ETC as well as 6 decreased glycolytic enzymes. There was also increased expression of cysteine based antioxidants and altered expression of other redox proteins.

From this study it appears that NAC+BCAA supplementation did not increase the expression of sarcomeric proteins, nor did it alter concentrations of GSH, cysteine, or ROS before or after submaximal exercise. However, it can alter the mitochondrial protein expression including subunits of the ETC and glycolytic enzymes while increasing proteins involved with redox reactions.

Overall, NAC+BCAA could benefit horses with myopathies with muscle atrophy or altered redox states such as MFM and this should be studied further to determine other benefits within a diseased population.

Conclusion

This dissertation identifies the beneficial effects of supplementation with antioxidants and amino acids in healthy and fit Thoroughbred horses. It also highlighted the importance of considering exercise intensity and obtaining samples post-exercise when studying the impact of antioxidants in horses. Supplementation of NAC and CoQ10 in maximally exercising horses can increase concentrations of GSH 1 hr following exercise while also increase the TCA enzymes and

increase the capacity to produce NADPH which can reduce oxidized GSH. A single dose of CoQ10 is not effective in increasing plasma concentrations, however, interindividual variation is extensive. Chronic CoQ10 supplementation can alter the contribution of Complex 1 and II to electron transfer in the ETC, alter the redox equilibrium by decreasing GPx activity, and decrease the expression of proteins used to import GSH into the mitochondria. NAC and BCAA can increase the expression of cystine based redox proteins while increasing expression of select subunits within ETC complexes. Overall, all supplementations used in this dissertation should be further studied in horses with myopathies such as MFM.

Suggestions for continued work

As with most scientific evaluations of nutritional supplements, there is no definitive answer, and further investigation should be done.

The first study was unable to evaluate which antioxidant was responsible for the different impacts seen. It is reasonable to believe that supplementation with NAC was responsible for increasing concentrations of GSH 1 hr post-exercise, however supplementation with the same amount of NAC did not increase GSH concentrations before or after exercise in the fourth study with supplementation of NAC and BCAA. When further analyzing this phenomenon, we realized that GSH concentrations are strongly dependent on the general fitness levels and exercise intensities of the horses. When we evaluated GSH concentrations across our studies, we found that the same horse had significantly different GSH concentrations depending on the intensity of training in that study.

The first study and the third study both utilized chronic supplementation of CoQ10, however, with different exercise intensities we saw different responses. In the third study we identified a decreased contribution of complex I in the ETC to total oxidative phosphorylation with an increased intrinsic capacity of complex II. However, due to the time of the biopsies and the relative fitness level of these horses we are unsure if this decreased contribution of complex I also decreased the production of ROS. It would be interesting to evaluate the effect of exercise intensities on mitochondrial respiration. It would also be interesting to determine if there are differences in concentrations of muscle GSH to further support the differences seen in this dissertation. One way this could be studied in Thoroughbred horses specifically would be to

measure GSH concentrations and mitochondrial respiration in Thoroughbred racehorses, Thoroughbred horses used for 3-day eventing, pleasure-ridden Thoroughbreds, and Thoroughbred broodmares that have been out of exercise for a designated period.

Supplementation with amino acids in conjunction with antioxidants in healthy horses was performed to ensure their efficacy prior to supplementing horses with myopathies. Specific myopathies such as myofibrillar myopathy (MFM) have been associated with possible increases in ROS and increases in gene expression (*CHAC1*) that encodes an enzyme that degrades GSH. It would be interesting to identify the effects of these supplements on Warmbloods with MFM, but that might be an unascertainable study. Limitations to performing a well-controlled study would include the cost of housing, training, and general upkeep of a large enough group of horses to ensure the study had enough power to identify differences. It would also be difficult to collect a group of MFM horses to utilize for research and even more difficult to find a group of age- and sex-matched healthy control Warmbloods. If these limitations could be surpassed it would be interesting to identify baseline concentrations for cellular antioxidants such as GSH or CoQ10 healthy Warmblood horses and in MFM Warmblood horses prior to identifying any effect of supplementation. Since this study found an effect of fitness level it would be important to measure these concentrations in horses performing the same level of exercise. Once normal values for control and MFM horses were established it would be interesting to analyze the effects of NAC, BCAA, or CoQ10 on MFM horses individually evaluate the response.

While primarily focused on developing a supplement for horses with MFM for this dissertation, it would also be interesting to identify the effect of either NAC or CoQ10 on other equine

myopathies. One myopathy that might also benefit from supplementation with antioxidants is recurrent exertional rhabdomyolysis (RER). RER is another complex myopathy in horses and is associated with increased release of sarcoplasmic reticulum calcium in the muscle that can cause episodes of rhabdomyolysis. Transcriptomic and proteomic analysis identified decreased expression of mitochondrial proteins and increased mitochondrial gene expression. NAC or CoQ10 as potent antioxidants could mitigate some of the effects seen in the mitochondria of RER horses but should be studied separately to evaluate the individual responses to each antioxidant.

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