THE IMPACT OF AGE AND EXERCISE ON ANTIOXIDANTS AND OXIDATIVE STRESS IN HORSES

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ABSTRACT OF THE DISSERTATION

The Impact of Age and Exercise on Antioxidants and Oxidative Stress in Horses

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The present study examined the effect of acute exercise and intensive exercise training on the oxidative stress, antioxidant status, and muscle metabolism of yearlings and mature mares. Objective one was to look at the effect of repeated biopsies on muscle inflammation and oxidative stress. The pattern of biopsies does impact oxidative stress occurrence, with an alternating corner pattern resulting in less oxidative stress than biopsies taken in a row. Objective two was to look at levels of oxidative stress and antioxidants in the blood and skeletal muscle of horses after an acute bout of exercise. It was found that some markers were affected by acute exercise, but overall the test was not sufficient to induce severe oxidative stress, thus the intensity of acute exercise does impacts the level of oxidative stress endured by the horse. Objective three was to compare the effects of exercise training on oxidative stress and antioxidant status between yearlings and mares. It was found that training did significantly improve antioxidant status and reduce oxidative stress in the trained mares, while the trained

yearlings did not have as significant changes in either regard. Objective four was to compare the effects of acute exercise before and after exercise training on oxidative stress, antioxidants, cortisol, and creatine kinase in two yearlings and mares. When challenged to acute exercise before training the mares had significantly higher levels of oxidative stress and cortisol and lower antioxidant status as compared to the yearlings. After exercise training, trained mature mares had lower levels of oxidative stress compared to prior training, and when challenged to acute exercise had lower oxidative stress and cortisol. The yearlings had few significant changes in oxidative stress after training and in response to acute exercise. In conclusion, young, maturing horses had lower levels of oxidative stress and cortisol, and higher levels of antioxidants, as compared to mature mares. Training can help reduce levels of oxidative stress in mature mares, while in young horses training is not as influential in reducing oxidative stress, suggesting that their young age is the most important defense against exercise-induced oxidative stress.

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INTRODUCTION

The musculoskeletal system is a common area for injury in horses undergoing intense exercise; this is a particular concern for young growing horses, who could suffer permanent damage to their muscle and potentially end a promising athletic career. Skeletal muscle is not only extremely adaptable to exercise; it is also an important site of oxidative stress. While oxidative radicals are important physiologically as signaling molecules for many different pathways, chronic and excessive oxidative stress and inflammation may cause damage to proteins and DNA. In horses work has been done looking at oxidative stress markers in the blood, but very little work has been done analyzing skeletal muscle oxidative stress. In addition, while it is also known that oxidative stress plays a role in the aging process, it is not well understood if/how oxidative stress impacts individuals as they mature and develop into adulthood. Therefore, the age of a horse undergoing exercise may affect their ability to recover and adapt from intense exercise. Research is limited in the yearling horse that is novel to exercise and just starting an exercise training program. With an exercise training protocol, horses may be able to enhance their ability to cope with excessive oxidative stress and inflammation from exercise. This doctoral project aims to look at the combined effects of age and exercise on oxidative stress and antioxidant status in the horse in both the circulatory system and skeletal muscular system.

CHAPTER ONE: **Literature Review**

Young Racehorses and Injury Prevalence

Before a horse can compete and perform competitively there are many hurdles it has to clear. One such challenge is being able to remain healthy and sound through training. Young racehorses, in particular, are very susceptible to injury; a study done by Rossdale et al. (1985) found that the greatest number of days lost to training was caused by lameness (67.6%). Of these, lameness due to muscle comprised 18%, the second most common source of injury behind foot-related injuries (19%). In addition, the younger the racehorse, the greater the likelihood for injury; one study found that 2-year-old Thoroughbreds were almost three times more likely than 3-year-old Thoroughbreds to have a musculoskeletal injury (Cogger et al., 2008). Many of these injuries are due to a strenuous training schedule, with little time for rest and recovery in between bouts of exercise.

It has been suggested that early but gradual introduction of young racehorses to small amounts of high speed exercise may be beneficial to their musculoskeletal system (Verheyen et al., 2005). This gradual introduction to high speed exercise is important in order to prevent possible lameness. The growth and development of the musculature is affected by intensive exercise, since adaptations to exercise occur primarily in the musculoskeletal and cardiorespiratory systems. A training protocol that is too intense will lead to lameness or overtraining, which is defined as the imbalance between training and recovery. Overtraining can produce a long-term decreased performance and impaired ability to train successfully (de Graaf-Roelfsema et al., 2007; McArdle et al., 2007; Hamlin et al., 2002; Tyler et al., 1996). Rivero (2007) points out that there is an

upper limit in training where no further muscular adaptations can be obtained, and if a horse is trained past this limit it could lead to overtraining and subsequent injury. Overtraining is a concern more so for older horses who are trained rigorously, but young horses in training could be at a higher risk for musculoskeletal injuries. The shock of constant, strenuous exercise can overwhelm young racehorses, leading to (but not limited to) decreases in body weight, plasma cortisol concentrations and packed cell volume (Hamlin et al., 2002). Finally, many trainers start young racehorses on a standardized training regime, since they cannot yet know the training capacity of each horse (Leleu and Jens, 2010). This is not ideal, since every horse will respond to exercise differently, and a level of exercise that may be optimal for one horse could result in lameness for another horse.

Skeletal Muscle

There are three different types of muscle found in the body – cardiac, smooth, and skeletal. Skeletal muscle is the muscle that acts on bones to propel, twist, and otherwise move the body. Skeletal muscle is extremely adaptive, and can undergo both morphological and biochemical changes due to the influences of both aging and exercise. Bundles of muscle fiber cells make up the functional units of skeletal muscle; within the muscle fibers are myofibrils, and within the myofibrils are sarcomeres (Berchtold et al., 2000; Stehle et al., 2009). The middle of the sarcomere is the M-line, while the outer edges are the Z-discs; the Z-discs contain thick and thin filaments. Thin filaments consist of actin, the troponin complex, and tropomyosin. Thick filaments consist of myosin,

myosin light chains, and myosin binding protein C. From the myosin, heads or cross-bridges protrude at regular intervals.

In order to produce movement, skeletal muscles contract and relax to propel the body. The contraction and relaxation of the muscle is controlled by the release of calcium. An action potential at the transverse tubules causes the release of calcium from the sarcoplasmic reticulum. Calcium will then bind to troponin C, resulting in a cascading event where the cross-bridges of myosin bind with actin, splitting ATP and exerting a force on the thin filament which will pull the filament to the sarcomere center and cause a contraction. The removal of calcium from troponin C breaks the bind of the cross-bridge and actin, thus returning the sarcomere to its original length and relaxing the muscle (Stehle et al., 2009).

Muscle Fiber Types. Two different fiber types make up skeletal muscle; Type I (slow-twitch) and Types IIA and IIB (fast-twitch). The classification of fiber types is based mainly on the pH lability of mATPase activity; the amount of mitochondria present and the width of the Z-bands can also used to distinguish fiber types (Berchtold et al., 2000). The cross-bridges of type I fibers bind slowly due to the slow hydrolysis of ATP. The ATP of type I fibers is mainly derived from oxidative phosphorylation following the oxidation of fatty acids and glucose. In addition, their high number of capillaries, low levels of glycogen, and high activity of enzymes such as succinate dehydrogenase (SDH) make type I fibers highly oxidative. This allows type I fibers to be resistant to fatigue with slow speed of action, making them ideally suited for prolong bouts of aerobic exercise, such as endurance exercise (Rivero and Piercy, 2008; Snow and Valberg, 1994; Dubowitz et al., 1973).

In type II fibers, the cross-bridges bind faster, creating force more rapidly. These fibers rely on ATP generated from glycogenolysis amd glycolysis; additionally, type II fibers have higher amounts of glycogen and glycolytic enzymes such as phosphofructokinase (PFK) and lactate dehydrogenase (LDH) (Lindholm and Piehl, 1974; Snow and Guy, 1980; Snow and Valberg, 1994). Type IIA fibers are both glycolytic and oxidative, with an intermediate resistance to fatigue and a fast speed of action. Type IIB fibers are glycolytic and fatigue quickly, but with a fast speed of action and high force capacity; type IIB fibers have a maximal velocity of shortening that is three times higher than type IIA fibers (Rivero and Piercy, 2008). Type IIA and B fibers are used for faster, shorter bouts of anaerobic exercise, such as a sprint. Type II fibers have been found to have higher rates of oxidative stress. One such example is the leakage of reactive oxygen species from mitochondria, which is two- to threefold greater in type II muscle fibers than type I muscle fibers (Powers and Jackson, 2008).

The ratios and histological makeup of these fiber types can vary considerably in mammals; factors such as age, sex, and breed all have an impact on fiber type. Size does matter in mammals; smaller mammals typically have higher concentrations of mitochondria in type IIA fibers, while larger mammals have more mitochondria in type I fibers. Age also plays an important role in fiber type ratios. It has been documented in humans and rodents that aging leads to a gradual loss of muscle fibers, as well as a selective atrophy of type II fibers (Berchtold et al., 2000). Exercise also plays a role in muscle fiber transformation; in humans it has been shown that endurance training leads to an increase in type I fibers, while sprint training (repeated bouts of 30 second high intensity exercise) leads to an increase in type IIA and IIB fibers (Berchtold et al., 2000).

Equine Skeletal Muscle. More than half of a mature horse's body weight is comprised of skeletal muscle. Most mammals have 30-40% of their body weight in skeletal muscle, while non-athletic horses have 42%, and athletic horses (such as Thoroughbreds and Standardbreds) have 55% of their body weight comprised of skeletal muscle. Equine skeletal muscle has considerable potential to adapt during exercise training, which can influence strength, resistance to fatigue (or stamina), and maximum velocity of shortening of muscle fibers (Snow and Valberg, 1994). The main forms of muscle adaptation to training include hypertrophy, where myofibers increase in size, and remodeling of the myofibers, where enzymatic and structural characteristics such as fiber types are altered. The breed of horse, age, sex, and level of fitness all impact the degree to which these muscle adaptations can occur; to date, in Standardbreds the common muscular responses to training do not show hypertrophy but do show changes in enzymatic and structural characteristics (Rivero 2007).

The equine muscle most commonly used to study aspects of exercise is the middle gluteal muscle, since it is known to play a major role in locomotion, has an important function in the propulsion of the hind leg, and is relatively easy to procure samples via biopsies (Roneus and Lindholm, 1991). There is great variation in equine skeletal muscle, and as mentioned before, factors such as breed, age, sex, and physical fitness all have an impact. Within the middle gluteal muscle the following breed variations have been recorded: Thoroughbreds and Quarter Horses have the highest percentage of type II fibers (80-90%), followed by Standardbred and Andalusians with a moderate amount of type II fibers (75%) (Snow and Valberg, 1994; Snow and Guy, 1981; Lindholm and Piehl, 1974). Age also plays an important role in muscle characteristics, particularly in

growing horses. Horses do not complete their growth until about 5 years of age, and during those five years their muscle characteristics change considerably. Muscle growth can also be impacted if the young horse is in an exercise program. Several studies have looked at foals from birth to 1 year of age, and have found that overall, there is a slight increase in type I fibers, a consistent increase in type IIA fibers, and a decrease in type IIB fibers (Eto et al., 2003; Dingboom et al., 2002; Dingboom et al., 1999; Rivero et al., 1993; Essen-Gustavsson et al., 1983). Several studies looking at Thoroughbred racehorses that have undergone an exercise training program have found an increase in type IIA:IIB fibers and many times hypertrophy of type I fibers (Eto et al 2003; Yamano et al., 2002; Eaton et al., 1999; Miyata et al., 1999).

Oxidative Stress

Oxidative stress is a disturbance in the oxidant/antioxidant balance, in favor of oxidants. These oxidants (also known as reactive oxygen species or ROS) may damage lipids, protein, and DNA, which can decrease athletic performance (Deaten et al., 2003). The main oxidants produced are superoxide and nitric oxide, but others include hydrogen peroxide, hydroxyl radicals, peroxynitrite, and hyperchlorite. There are also multiple secondary radical species that are produced as a result of chain reactions with ROS (Powers and Jackson, 2008). The key site of oxidative stress is the skeletal muscle, specifically within the mitochondria (Figure 1). In the mitochondria, complexes I and II of the electron transport chain have been identified as the key sites of electron leakage. The leaked electrons are scavenged by oxygen to produce superoxide; in humans it is

estimated that 2-5% of oxygen consumed is reduced to superoxide by mitochondria (Powers and Jackson, 2008; Sachdev and Davies, 2008). Most free radicals are hard to detect in the body via assays due to their high reactivity and/or short half-lives, therefore end products or by-products of radical-induced reactions are used as measurements of oxidative stress. Two of the most common markers used to detect free radical production after exercise are products of lipid peroxidation and glutathione oxidation (Sachdev and Davies 2008).

It is important to note that ROS do play an important role in normal physiological activities. One such example is the production of superoxide by phagocytosing cells to kill invaded bacteria. Another is the up-regulation of endogenous defense systems to eliminate xenobiotics or pro-carcinogens. Hydrogen peroxide can be considered a regulator of cell death pathways and exposure can augment the expression of key antioxidant enzymes in myotubes (Franco et al., 1999). In addition, many ROS contain products of lipoxygenases which have been partially characterized as mediators of inflammatory responses or metabolic regulators (Brigelius-Flohe 2009). Generations of low to moderate concentrations of oxidants beneficially affect endurance development, aging, and the metabolic syndrome. In addition, during exercise ROS production may not necessarily be detrimental; it can be required for normal force production in skeletal muscle, the development of training-induced adaptation in endurance performance, and the induction of endogenous defense systems (Powers et al., 2011; Powers et al., 2010; Brigelius-Flohe 2009). The harmful effects of ROS are seen only when they are in excessive and chronic amounts in the body, such as during times of disease or after

intense exercise. Long, intense bouts of exercise daily lead to chronically high amounts of oxidative stress, which increases the risk for muscle damage to occur.

Lipid Peroxidation. The basic structure of biological membranes was first established by Singer and Nicolson (1971; 1972), in which they proposed the fluid mosaic model of mammalian cell membranes. In this model, the membrane consists of a phospholipid bilayer, with the hydrophilic phosphate heads facing the aqueous solutions inside and outside the cell, and the hydrophobic tails contained within the center of the membrane. Throughout the membrane are globular proteins, glycoproteins, and glycolipids. A large number of phospolipids consist of polyunsaturated fatty acids (PUFAs). The double bonds found in PUFAs make the lipid bilayer extremely susceptible to oxidation.

Lipid peroxidation occurs when a hydroxyl radical abstracts a hydrogen atom from a lipid, thus neutralizing the lipid but forming a lipid radical at the same time (Sachdev and Davies, 2008; Powers and Jackson, 2008). This lipid radical has an unpaired electron; this electron typically is donated to oxygen, which forms a peroxy lipid radical and is thus able to react with other fatty acids, causing a lipid peroxidation chain (Figure 2). This chain causes damage to the membrane by altering the fluidity and increasing the rigidity, which allows proteins within the membrane to be more easily attacked and impairs essential membrane functions (Kirschvink et al., 2008; Powers and Jackson, 2008). The main product of lipid peroxidation is malondialdehyde (MDA), and is a commonly assayed marker of oxidative stress.

Nitric Oxide. Many free radicals are produced with oxidative stress; one such radical is nitric oxide (NO). This important radical and signal molecule is generated by nitric oxide synthases (NOS) in a process where NOS catalyzes the oxidation of L-arginine, resulting

in NO and citrulline as a by-product. There are three different isoforms of NOS; the first, inducible NOS (iNOS), is transcriptionally upregulated by cytokines and plays an important role in the inflammation process. The other two forms, endothelial (eNOS) and neuronal (nNOS), are dependent on calcium and calmodulin (Mills et al., 1996) and are normally expressed in skeletal muscle. Fast-twitch muscle fibers strongly express nNOS and is localized to the muscle sarcolemma, while eNOS is localized to the muscle mitochondria (Powers and Jackson 2008). During muscle contractions, calcium plays an important role by binding to troponin-tropomyosin in the actin filaments, which ultimately leads to muscle contractions. The extracellular signals that increase cytosolic calcium also activates muscle nNOS, so there is also an increase in NO production seen with muscle contractions (Stamler and Meissner 2001). The main source of NO released from the skeletal muscle is typically nNOS (Powers and Jackson 2008). The major actions of NO in cells relate to its ability to bind to the ferrous iron in guanyl cyclase and the subsequent formation of the second messenger cGMP.

While NO is known predominately for its role in regulating vascular tone in systemic and pulmonary circulation as a major vasodilator (Mills et al., 1996), NO also plays an important role in oxidative stress. Not only is NO a weak reducing agent, it can react with oxygen to form nitric dioxide, and, most importantly, reacts extremely rapidly with superoxide to form peroxynitrite (Powers and Jackson 2008). Peroxynitrite is a strong oxidizing agent and can lead to the depletion of thiol groups, damage to DNA, and nitration of proteins. This reaction is the primary reaction when both superoxide and NO are present; as a result, the bioavailability of both molecules decreases. The scavenging of superoxide by NO also results in increased force production during submaximal

contractions; since endogenous production of nitric oxide modulates skeletal muscle force contraction, a decrease in the bioavailability of nitric oxide can increase force production (Stamler and Meissner 2001; Powers and Jackson 2008). Finally, it is also important to note that due to the extremely short half-life of NO, it is very hard to measure *in vitro*. Thus, measurement of the total nitrates (NO_3) and nitrites (NO_2) are used as an indirect measurement of NO.

Antioxidants

Antioxidants are part of the defense system against oxidants. They prevent generation of ROS, inactivate oxidants, and limit the deleterious effects of oxidants by enabling repair of oxidative damage. An array of antioxidants, both enzymatic and nonenzymatic, can be found in the skeletal muscle. These antioxidants exist in a variety of places, including the fiber of skeletal muscle (in the cytoplasm and mitochondria) and both the extracellular and vascular space (Powers and Jackson, 2008).

The principal antioxidant enzymes include superoxide dismutase, glutathione peroxidase, and catalase. Superoxide dismutase (SOD) catalyzes the dismutation of superoxide anions to hydrogen peroxide and oxygen (Reid 2001). In mammals, three isoforms of SOD exist (SOD1, SOD2, SOD3), and all require a redox active transition metal in the active site to accomplish the breakdown of the superoxide anion. Among the three isoforms, SOD1 requires copper-zinc as a cofactor and is found in the cytosol and mitochondrial space, SOD2 uses manganese and is located in the mitochondrial matrix, and SOD3 requires copper-zinc and is located in the extracellular space. Hydrogen

peroxide is enzymatically dehydrated to water and molecular oxygen by catalase. Catalase is another important antioxidant enzyme, and is widely distributed throughout the cell. Iron is a required cofactor that is attached to the active site of the enzyme (Powers et al 2008). Glutathione peroxidase (GPx) catalyzes the reduction of hydrogen peroxide to water and reduces organic hydroperoxides to alcohol (Reid 2001).

Vitamins C and E and glutathione are also antioxidants found in the muscle. Vitamin E is part of a family of lipid-soluble antioxidants that function primarily to protect muscle membranes against oxidation. Vitamin C (ascorbic acid) directly scavenges ROS and facilitates redox cycling of vitamin E (Reid 2001). There are also many nonenzymatic antioxidants which exist in varying concentrations in cells throughout the body. These include GSH, uric acid, bilirubin, and many others (Powers et al., 2008).

Glutathione and Glutathione Peroxidase. Unlike MDA and NO, which are products of lipid peroxidants and oxidants, respectively, glutathione oxidation involves enzymatic and nonenzymatic antioxidants. In glutathione oxidation, glutathione peroxidase (GPx) uses glutathione (GSH) to donate a pair of hydrogen ions to reduce hydrogen peroxide to two water molecules (Figure 3). This reaction oxidizes GSH to glutathione disulfide (GSSG). Another enzyme, glutathione reductase, then converts GSSG back to GSH, thus allowing the cycle to start again. Among the nonenzymatic antioxidants, GSH is probably the most important since as mentioned previously, it serves as a substrate for GPx to eliminate hydrogen peroxide. There are five different forms of GPx in mammals; each one differs in substrate specificity and cellular localization. These varying isoenzymes can reduce a large variety of hydroperoxides, making GPx an important

antioxidant in the process of protecting against oxidative stress (Powers and Jackson, 2008).

Exercise, Aging and Oxidative Stress

Every animal is different in physiological aspects that affect the onset of oxidative stress, including age, training status and dietary intake of subjects. Much research has been done looking at the effects of exercise on oxidative stress (Fisher-Wellman and Bloomer, 2009), and it has been seen that single bouts of exercise (anaerobic and aerobic) can induce oxidative stress (Dillard et al., 1978; Powers and Jackson, 2008). In humans, typically a decrease in reduced GSH, an increase in oxidized GSH, and no change in total GSH has been seen after acute aerobic exercise in the blood (Fisher-Wellman and Bloomer, 2009; Michailidis et al., 2007; Steinberg et al., 2006; Laaksonen et al., 1999; Szczesniak et al., 1998; Sen et al., 1994). Activity of GPx in the blood have been reported to increase, decrease, or not change at all in humans challenged to acute aerobic exercise (Laaksonen et al., 1999; Buczynski et al., 1991; Akova et al., 1991; Vider et al., 2001). Reports on MDA in humans after acute aerobic exercise have varied; though many have seen an increase, others report no change in MDA levels after exercise (Fisher-Wellman and Bloomer, 2009). It is evident from these results that levels of exercise-induced oxidative stress can vary considerably due to factors such as exercise intensity and duration, as well as specific characteristics of the individual undergoing exercise.

The effects of exercise induced ROS have been observed not only in the blood, but in skeletal muscle as well. The different fiber types typically exhibit different levels

of ROS, with type II fibers exhibiting higher levels of ROS (Powers et al., 2011). Furthermore, the magnitude of exercise-induced increases in SOD activity in muscle fibers is greatest in type I and type IIA (Powers and Jackson, 2008). Similar to SOD, the amount of glutathione peroxidase (GPx) present in skeletal muscle differs between fibers, with type I containing the highest level of GPx. Also similar to SOD, exercise increases the amount of GPx activity (Powers et al. 2008). Research is beginning to show that acute bouts of exercise are beneficial and necessary for muscle growth and adaptation (Powers et al., 2011). The concern for muscle damage and potential injury is when there are chronic, elevated levels of oxidative stress.

The aging process is defined as a progress decline in cellular function and a decrease in a cell's ability to maintain homeostasis (Figueiredo et al., 2009). It has been widely acknowledged that oxidative stress plays a role in the aging process. Harman in 1956 identified the "Free Radical Theory of Aging," which theorized that continuous attacks by free radicals in the body contribute to the degradation of cells. Since then, multiple other sources have linked oxidative stress with aging (Anandan et al., 2013; Barja 2004; Yu, 1996) and confirmed that rates of lipid peroxidation have been shown to increase with age (Dillard et al., 1978; Spiteller 2007).

Little is known about the rate of change in oxidative stress as an individual goes through maturation. It has been theorized that since children endure greater oxygen cost during exercise, as well as rely more on aerobic metabolism, that they may have higher levels of oxidative stress (Cooper et al., 2004). Several studies with children and adolescents have found that children that undergo an exercise bout do experience oxidative stress (Bentitez-Sillero et al., 2011; Gougoura et al., 2007; Santos-Silva et al.,

2001). Still, there are very few studies looking at maturation and oxidative stress; while one study found reduced glutathione and increased lipid peroxidation in pubescent boys versus prepubescent boys (Perez-Navero et al., 2009), another found no difference in GSH between pubescent boys versus prepubescent boys after exercise. Several studies in rats have found that older rats have increased lipid peroxidation and increased levels of oxidative stress as compared to young rats. Lawler et al. (1993) compared 4 month old rats to 24 month old rats, and found evidence for increased oxidation in skeletal muscle. Anandan et al., (2013) compared 2-3 month old rats to 20-24 month old rats and found lower antioxidant levels in cardiac muscle of the aged rats. Based on the little research there is, the role of oxidative stress in maturation has yet to be clearly defined, and more research is needed.

Inflammation and Muscle Damage

Damage to skeletal muscle causes a variety of problems. The first problem is the increased permeability of muscle membranes and even tears in muscle fibers, which can cause the release of creatine kinase (CK), myoglobin, and troponin – all markers of muscle membrane damage. Additional effects of muscle damage are the tearing of portions of the muscle's connective tissue, damage to the vasculature (which could lead to the formation of a haematoma), and alteration in calcium regulation in the muscle cells, thus affecting muscle contractions (McArdle et al., 2007; Smith et al., 2008). Muscle damage is associated with elevated oxidative stress, inflammation, and levels of cortisol. Immediately after exercise-induced muscle damage, neutrophils enter the

muscle and begin the removal of degraded proteins and cell debris, followed by monocytes and macrophages (Tidball 2005; Li et al., 2001; Figure 4). In turn, these cells are capable of producing oxidants and secreting pro-inflammatory cytokines, such as tumor necrosis factor –alpha (TNF-α), interleukin 1 (IL-1), and interleukin 6 (IL-6), ultimately causing oxidative stress and inflammation (Konig et al., 2001). This pathway has been better examined in humans than in horses. While prior studies in horses have looked at pro-inflammatory cytokines, to date neutrophils have not been shown in muscle biopsies. Finally, elevated levels of cortisol occur after high-intensity exercise, which can initiate excessive protein breakdown and tissue wasting.

The muscle-derived enzyme CK makes ATP available for contraction by phosphorylation of ADP. Increased concentrations of CK in serum are associated with muscle damage or injury; however, there have been physiological increases in CK without any visible evidence of muscle damage. It has been shown in the horse that CK levels increase after strenuous exercise; light exercise typically does not show any increase in CK. Prolonged, moderate-intensity exercise has also been shown to increase CK; thus, both intensity and duration of exercise impact the release of CK from skeletal muscle (Kingston 2008). Circulating TNF-α has been seen to increase (662.9 ± 1046 pg/ml vs. 733.3 ± 1079 pg/ml) after aerobic work in endurance horses (Holbrook et al., 2010), but changes due to training in horses has not been well studied. It is known that age does play a role in the immune system, with aged horses having a reduced immune function (Horohov et al., 1999), but looking at young horses with exercise training has yet to be studied with this objective in mind.

Cortisol. Cortisol is a glucocorticoid that is released by the adrenal cortex in response to the binding of adrenocorticotropic hormone (ACTH) to the adrenal cortex. This hormone has effects on glucose, protein, and free fatty acid metabolism, and mobilizes these energy reserves in times of stress, such as with exercise. Multiple studies have documented acute increases in the plasma concentration of cortisol in response to submaximal, maximal, and supramaximal exercise (Mastorakos et al., 2005; Consitt et. al., 2002; Luger et al., 1987).

This effect has been seen in horses as well, with peak cortisol levels in horses are generally observed 30-60 minutes after exercise (Gordon et. al., 2007; McKeever 2002; Nagata et al., 1999). Training does have an influence on cortisol, though it depends on the intensity and duration; it has been shown that peak post-exercise cortisol concentrations are reached earlier in trained horses and trained horses also have a faster cortisol recovery time (McKeever 2002).

Similar to oxidative stress, age plays a role in the endocrine response in horses, but of the few studies that have been done, cortisol was looked at in the aged horse. Horohov et al. (1999) found that after an acute bout of exercise, while both young (7.5±0.7 years) and aged (25.3±1.1 years) mares had an increase in cortisol after exercise, the younger mares had higher levels of cortisol at heart rates of 180 and 200 beats/minute. Malinowski et al. (2006) looked at three age groups of mares: young (6.8±0.4 years), middle-aged (15.2±0.74 years), and old (27.0±0.72 years). After acute exercise, the young and middle-aged mares had increased cortisol that was elevated for 40 minutes post exercise; old mares had no difference in cortisol levels after exercise. After an exercising training protocol, similar results were obtained in an acute exercise

test, with the exception that the middle-aged mares had elevated cortisol for 60 minutes post exercise. Comparing cortisol concentrations of young (1-2 years old) horses to mature (8-12 years old) with their response to both acute exercise and exercise training has not been studied.

Oxidative Stress, Antioxidants, and Horses

In horses, research looking at MDA, NO, GSH, and GPx in relation to exercise has varied greatly, both in results and exercise used to induce oxidative stress. As mentioned, MDA is the main product of lipid peroxidation, and prior exercise studies have looked at levels of both MDA and thiobarbituric acid reactive substances (TBARS) as interchangeable measurements of lipid peroxidation. Prior equine studies have found after intense exercise elevated levels erythrocyte MDA (Matsuki et al 2001), and whole blood MDA (Ceylan et al 2009). Elevated levels of plasma TBARS have also been seen after longer aerobic exercise (Marlin et al., 2002). Levels of total GSH in erythrocytes have been seen to decrease after acute intense exercise (de Moffarts et al., 2006) and longer aerobic exercise (Marlin et al., 2002). Activity of GPx has shown a decrease after intense exercise (Ono et al., 1990) or no change after exercise (Brady et al., 1976). Finally, levels of nitrites (NO_2) and nitrates (NO_3) have been seen to increase in the whole blood of horses after 4 hr of exercise (Ceylan et al 2009). Conversely, Lamprecht et al (2009) found that the intensity of exercise undergone by horses impacted changes in concentrations of total nitrites in plasma after exercise. Less rigorous exercise tests resulted in no change in total nitrites, moderate exercise resulted in increased total nitrite

concentration afterwards, and the most rigorous exercise test resulted in decreased nitrite concentrations after exercise. A decrease in total nitrates and nitrites was found again in a subsequent exercise study using a rigorous exercise test (Lamprecht and Williams 2012).

In human and laboratory animal studies have shown evidence of ROS in skeletal muscle after intense exercise. Increased lipid peroxidation, decreased NO levels and increased GSH have all been observed in rat skeletal muscle after acute intense exercise (Balci and Pepe, 2012; Bejma and Li, 1999; Venditti and Di Meo, 1996; Powers and Jackson, 2008). Few studies have looked at these markers of oxidative stress in the gluteal muscle of the horse after intense exercise. A study done by Kinnunen et al (2005a) did not find any change in GPx activity in the gluteus medius muscle in horses after exercise. Total MDA has been seen to increase in the middle gluteal muscle of horses at 10 or 24 hours after exercise (Matsuki et al. 1991). With the gluteal muscle being a site of ROS production, it is important to determine total concentrations of oxidants and how they contribute to muscle damage and soreness after exercise, as circulatory levels of markers of oxidative stress may not fully reflect damage and merely show leakage out of cells. It has been seen with histology that minor muscle damage can occur as a result of oxidative stress after exercise (Kinnunen et al., 2005b). Looking at a combination of four different markers (MDA, NO, GSH, and GPx) in both blood and skeletal muscle in combination with intense exercise has yet to be examined in horses.

Few studies have looked specifically at the effects of age on oxidative stress in horses. Williams et al. (2008) found no difference in erythrocyte total GSH, GPx, or lipid hydroperoxides (LPO) after a bout of acute exercise between mature (12 ± 2 years)

and aged (22 ± 2 years) mares. Beyond this study, none are focused on comparing different ages of horses with regards to oxidative stress. However, it is interesting to note that many of the studies that have found differences in concentrations of markers of oxidative stress after acute exercise (Ono et al., 1990; Avellini et al., 1999; Richardson et al., 2006; Ceylan et al., 2009) used horses that were 3 years old or younger. Fewer studies have looked at mature (8-15 years old) horses and acute exercise (Lamprecht et al., 2009, 2012). The comparison of the oxidative response to exercise by young (18 – 36 months) and mature horses has yet to be looked at; this is especially important to look at, since it is known that young horses undergo a huge amount of growth and muscle adaptation during the first two years of life (Roneus and Lundholm, 1991), and exercise can play an important role in how their muscle grows and adapts.

Equine Exercise Training

Exercise training may have an effect on the concentrations of antioxidants and markers of oxidative stress in horses, but again, few studies have looked at this. Williams et al (2008) found that horses challenged to an acute bout of exercise after 8 weeks of training had higher concentrations of erythrocyte GSH-T compared to concentrations seen after an acute bout of exercise prior to training. The authors of the study theorized that the increase in erythrocyte GSH-T seen after the exercise training enabled horses to then better cope with oxidative stress after an intense bout of exercise. A study done by Siciliano et al (1997) did not see any change in concentrations of TBARS in skeletal muscle after 90 days of exercise training. Another study (Avellini et al., 1995) noted a

decrease in GPx concentrations in erythrocytes after 60 days of training. Due to the limited knowledge and conflicting results on this topic, more research is needed to determine if there is an effect of exercise training on markers of oxidative stress and to what benefit or detriment it has in the horse.

There has been more work done in rodents in regards to exercise training and its impact on antioxidants. A study done by Venditti and Di Meo (1996) in rats found increased concentrations of GPx in skeletal muscle after training. Another study by Powers et al. found in rats training-induced changes in GPx and SOD, but these changes were muscle specific (1994). A third study in rats found increased levels of total GSH and decreased levels of NO after training in the gastrocnemius muscle (Balci and Pepe, 2012). Multiple studies have shown that endurance exercise training can promote 20-112% increases in the activities of SOD in exercised human muscle; again, the differences in SOD activity increase were due to variations in exercise used and specific muscles/fiber types studied (Powers and Jackson, 2008). Extensive work with skeletal muscle fibers has shown increases in ROS after exercise (Powers and Jackson, 2008; Ferreira and Reid 2007; Murrant and Reid 2001), but alterations due to exercise training are unclear. Based on these results, there is strong evidence to support changes in antioxidant status as a result of exercise training, and can be seen in horses as well; the impact of training on oxidative stress is less clear. The increase in antioxidants after training is thought to be an adaptive response to increased levels of oxidative stress production.

Summary

This doctoral project aims to show how intensive exercise affects the antioxidant status, oxidative stress, inflammation, and cortisol concentrations in the skeletal muscle and blood of different age groups of horses. While oxidative stress and inflammation are necessary for muscle growth and development, an excessive amount could overwhelm the musculoskeletal system and lead to muscle damage. A definitive level where oxidative stress is no longer beneficial and becomes detrimental is unfortunately unknown for any species due to the typically short half-lives of ROS and their small *in vivo* concentrations. Furthermore, limited knowledge exists on how an exercise training protocol can affect the concentrations of markers of oxidative stress and inflammation. Altogether, these factors lead to the difficulty in defining what an "excessive amount" of oxidative stress truly is in the athletic horse. This knowledge will aid in ultimately determining if a moderate level of exercise training and acute bouts of intense exercise are enough to cross the threshold needed into increased oxidative stress, which could potentially lead to poor performance and increased risk of muscular injury.

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Abbreviations

NO = nitric oxide

GSH-T = total glutathione

GPx = glutathione peroxidase

TBARS = thiobarbituric acid reactive substances

MDA = malondialdehyde

BHT = butylated hydrotoulene

GXT = graded exercise test

IET = interval exercise test

RSET = repeated sprints exercise test

TP = total protein

Hct = hematocrits

HR = heart rate

CK = creatine kinase

TNF-α = tumor necrosis factor alpha

ROS = reactive oxygen species

PRE = sample taken immediately before an exercise test

POST = sample taken immediately after an exercise test

Figures

Figure 1. Sites of production for superoxide, nitric oxide, and other reactive oxygen species in the skeletal muscle. Courtesy of: Powers et al., 2011. DOI: 10.1002/cphy c100054 Copyright © 2011 American Physiological Society.

Figure 2. Schematic pathway of lipid peroxidation. Courtesy of: Bocci et. al., 2011. DOI 10.1186/1479-5876-9-66 Copyright ©2011 Bocci et al; licensee BioMed Central Ltd.

Figure 3. The cycle of glutathione oxidation. Glutathione peroxidase (GPx) uses glutathione (GSH) to donate a pair of hydrogen ions to reduce hydrogen peroxide to two water molecules. This oxidizes GSH to glutathione disulfide (GSSG). Glutathione reductase, then converts GSSG back to GSH.

Figure 4. Process of inflammation and oxidant production after injury to a muscle cell. Courtesy of: Tidball 2005. DOI: 10.1152/ajpregu.00454.2004. Copyright © 2005 American Physiological Society.

Figure 1.

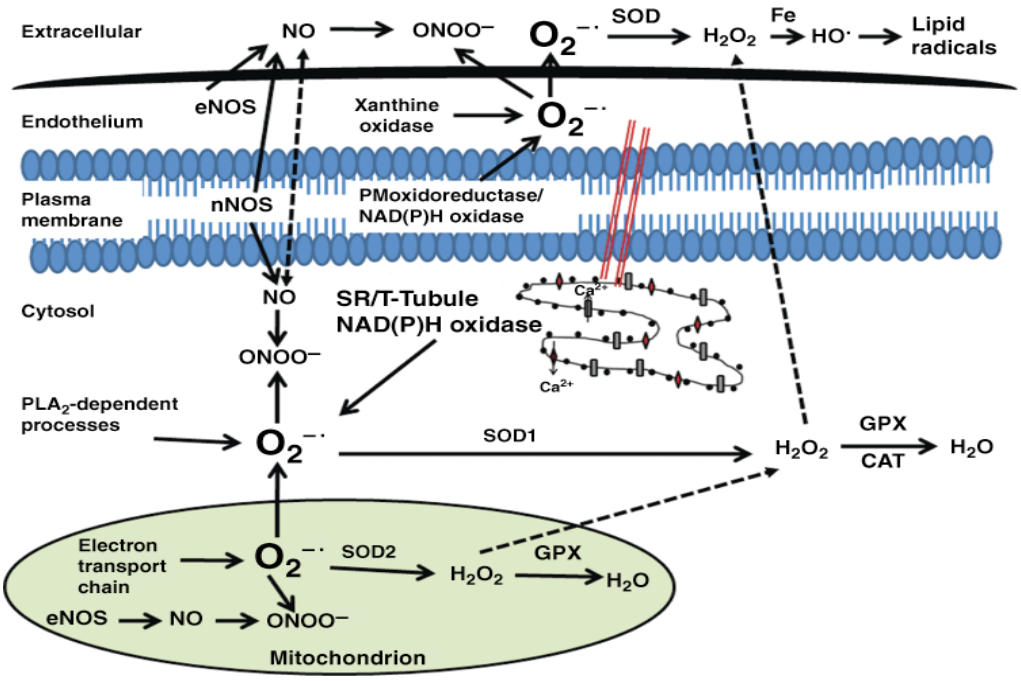


Figure 2.

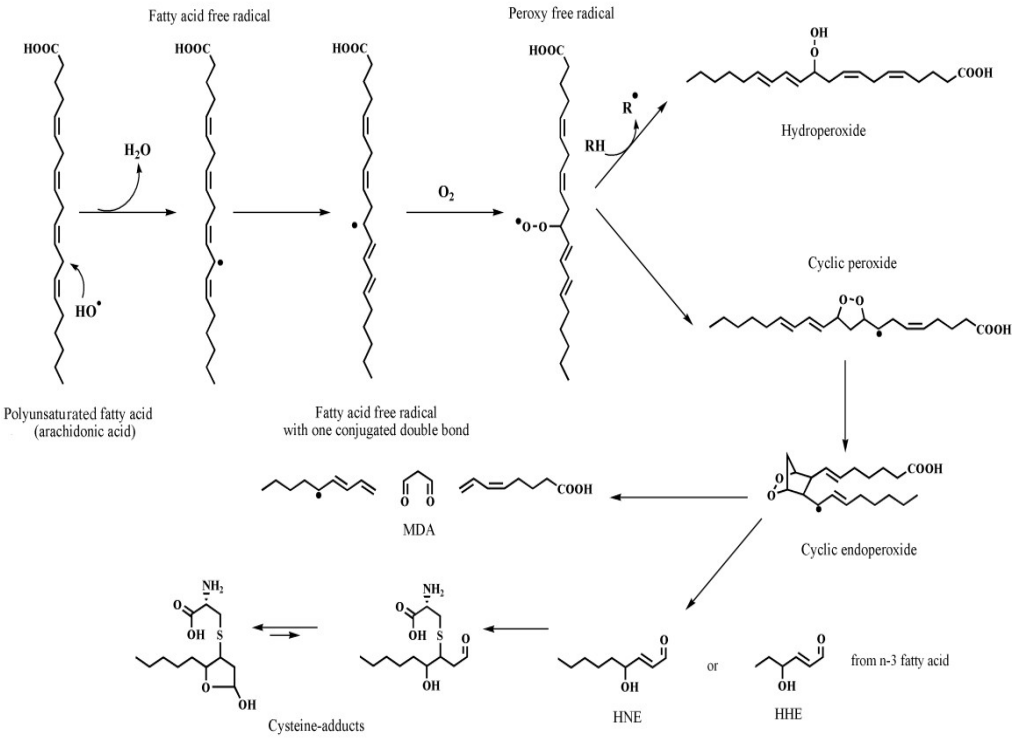


Figure 3.

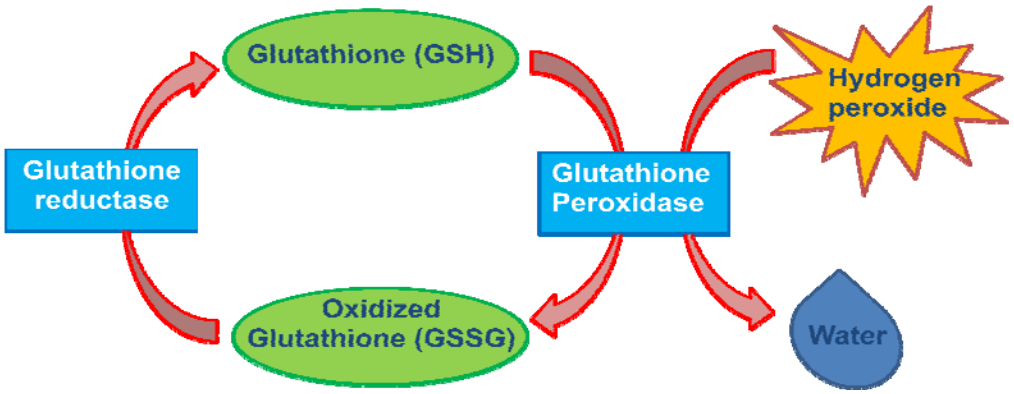
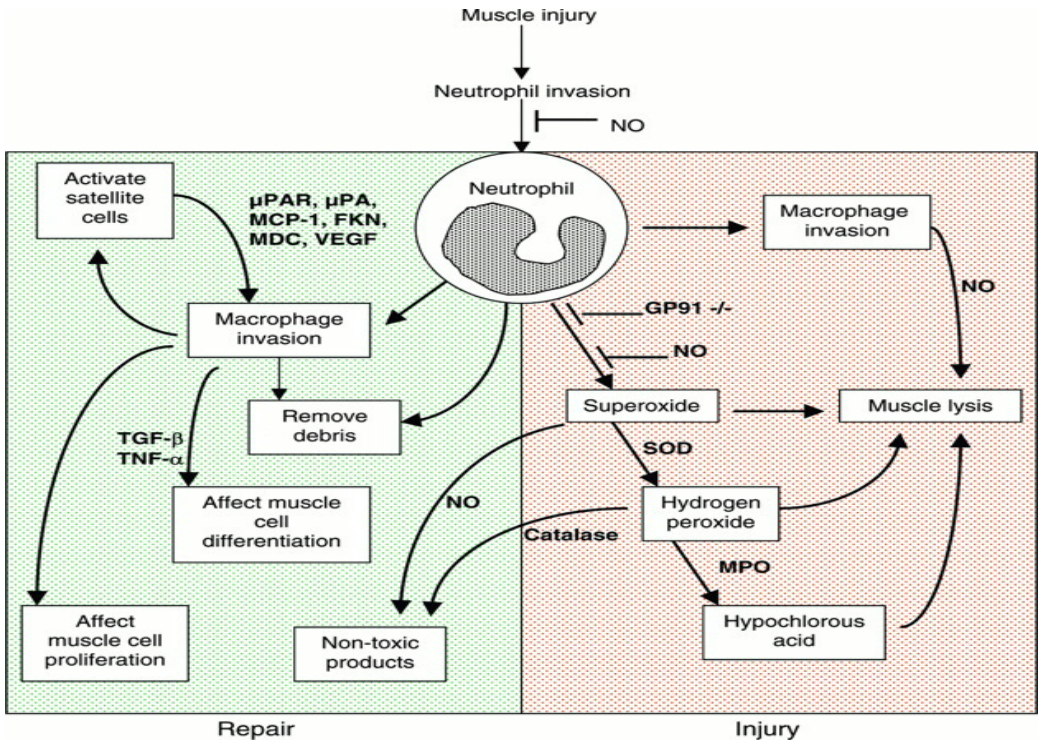


Figure 4.



Research Objectives and Hypotheses

1) To analyze and validate a repeatable method of performing muscle biopsies on the middle gluteal muscle of horses in a 24-hour period.

   Hypothesis for Aim 1: Concentrations of malondialdehyde (MDA) will be altered if repeated muscle biopsies are performed in the middle gluteal muscle in a row or in a quadrant pattern.

2) To compare markers of oxidative stress in skeletal muscle and plasma in exercising horses and non-exercising horses.

   Hypothesis for Aim 2: Blood and skeletal muscle concentrations of malondialdehyde (MDA), total glutathione (GSH-T), glutathione peroxidase (GPx), nitric oxide (NO), will be altered in horses after an intense bout of exercise, with increased levels of MDA and lowered levels of GSH-T, GPx, and NO.

3) To compare markers of oxidative stress and antioxidant status in skeletal muscle and circulation in two different age groups of horses (mature and young) after an acute bout of exercise.

Hypothesis for Aim 3: Young exercising horses will have higher oxidative stress and lower antioxidant status as indicated by MDA, GSH-T, GPx, and NO, than the mature exercising horses after a single bout of intense exercise

4) To compare these same markers in skeletal muscle and blood two different age groups of horses (mature and young) before and after 8-weeks of exercise training.

Hypothesis for Aim 4: Young exercising horses will have higher oxidative stress and lower antioxidant status as indicated by MDA, GSH-T, GPx, and NO than the mature exercising horses after a single bout of intense exercise, before exercise training begins. After all horses undergo an exercise training protocol for eight weeks, it is hypothesized that the young and mature horses will be better able to handle the stress of exercise, as evidenced by a quicker recovery to normal cardiovascular and respiratory rates and increased antioxidant status.

CHAPTER TWO: **A validation of equine skeletal muscle biopsy and analysis of oxidative stress.**

ABSTRACT

Different muscle biopsy techniques exist for horses and a specific comparison of the muscles each technique samples has not been done. Furthermore, there is concern that the actual process of the biopsy can induce inflammation and oxidative stress leading to compounded results in exercise studies. The hypothesis of this study was that concentrations of MDA will be altered depending on if repeated muscle biopsies are performed in the middle gluteal muscle in a row or in a quadrant pattern, thus creating an inflammatory response. Four Standardbred mares (12 ± 4 years old; 537± 17 kg) were used to compare 4 biopsies done either in a row pattern or alternating corner patter in the middle gluteal muscle (row was done on the left gluteal, and quadrant was done on the right gluteal). In addition, two commonly used biopsy sites were analyzed for the specific muscle that they sample from. There was an effect of method for the biopsies (P=0.03), with significantly higher concentrations of MDA found at 24H in the row pattern than the quadrant pattern (P=0.03). It was also found that though both biopsy sites do in fact sample the middle gluteal muscle, previously published techniques directly samples the middle gluteal, while the technique in question had to go through the superficial gluteal before reaching the middle gluteal muscle. Therefore, it was determined that the alternating corner pattern for the biopsy sites induce less lipid peroxidation than the row pattern, and the technique previously developed is the most accurate method for sampling from the middle gluteal muscle.

Introduction

The muscle biopsy is an important tool in understanding muscle physiology. One such aspect is oxidative stress, which is a disturbance in the oxidant/antioxidant balance, in favor of oxidants. These oxidants (also known as reactive oxygen species or ROS) may damage lipids, protein, and DNA (Deaton et al., 2003). A common marker of oxidative stress is malondialdehyde (MDA), the main product of lipid peroxidation. Lipid peroxidation occurs when a hydroxyl radical abstracts a hydrogen atom from a lipid, thus neutralizing the lipid but forming a lipid radical at the same time (Sachdev and Davies, 2008; Powers and Jackson, 2008). This reaction continues in a cascading chain of lipid peroxidation, potentially damaging and disrupting the cell membrane. A key site of oxidative stress is in the skeletal muscle, so middle gluteal muscle biopsies are critical in determining the concentrations of markers of oxidative stress, such as MDA. Much work has been done looking at MDA in equine blood (Ceylan et al., 2009; Marlin et al., 2002; Matsuki et al., 2001), but little has been done in the skeletal muscle (Matsuki et al., 1991).

Oxidative stress and inflammation are closely linked. After muscle damage occurs, neutrophils enter the muscle and begin the removal of degraded proteins and cell debris, followed by monocytes and macrophages (Konig et al., 2001). In turn, these cells are capable of producing oxidants and secreting pro-inflammatory cytokines. With muscle biopsies, and in particular multiple biopsies within a 24-hour period, there is the risk for inflammation from the actual biopsy process. Previous reports in both humans and horses are few and conflicting; while some studies find that repeated biopsies do have an effect on inflammation (Wagner 2011; Guerra et al., 2011; Malm et a., 2000),

others have not (Liburt et al., 2010). Repeated muscle biopsies are necessary to monitor changes in concentrations of oxidative stress and inflammation markers after exercise. However, since the muscle biopsy is invasive, there is a risk for oxidative stress and inflammation occurring from the mere act of taking a muscle biopsy confounding any results from exercise. While there are studies looking at inflammation occurrence from biopsies, none have looked at oxidative stress. By comparing concentrations of MDA from muscle biopsies, it is possible to estimate the level of oxidative stress undergone by the mere act of a biopsy.

Charriére and Duchenne first described the muscle biopsy technique in 1865, when they used it to look at muscular dystrophy in humans. In equine exercise physiology, the muscle biopsy technique has been used for over 40 years; it was first used as a tool to analyze fiber composition and enzyme activity of skeletal muscle. Lindholm and Piehl (1974) were the first to describe a method for equine muscle biopsies. They describe a technique specific to the gluteus medius muscle. Briefly, the biopsy site was chosen 10 cm dorso-caudal to the trochanter major of the tuber coxae. After shaving and sterilizing, and making a 5 mm skin incision, muscle samples weighing 20-40 mg were obtained using a Bergström needle. Another commonly cited technique is by Snow and Guy (1976). They looked at several muscles including the triceps brachii, deltoideus, vastus lateralis, biceps femoris, semitendinosus, and gluteus medius. Though they do not describe how they decided on an area of the muscle, it is noted that the chosen site is shaved and sterilized, a 1 cm incision is made, and 50-100 mg of muscle is collected using a Bergström needle.

Another technique to determine biopsy sites has been used to collect muscle samples. The biopsy site is measured by drawing a line from the tuber coxae to the fullest point of the belly of the semitendinosus muscle, then finding the midway point and adding a perpendicular line and setting the biopsy site 6 cm from the spine (Manso Filho et. al., 2009; Byrd et. al., 1989). Though all of these methods have been used in various studies, a comparison of the biopsy sites has yet to be looked at; it is important to compare these techniques and analyze which skeletal muscles are actually being sampled.

By determining if concentrations of MDA are altered in a standing horse model by taking multiple muscle biopsies in the same area in a 24 hour period. The hypothesis of this study was that concentrations of MDA will be altered depending on if repeated muscle biopsies are performed in the middle gluteal muscle in a row or in a quadrant pattern, thus creating an oxidative stress and inflammatory response.

Materials and Methods

Comparison of biopsy sites.

Two common areas for biopsy sites were measured out and then dissected on a mare euthanized for medical reasons. The first site was measured as described by Lindholm and Piel (1974); the biopsy site was chosen 10 cm dorso-caudal to the trochanter major of the tuber coxae. The second site was measured by drawing a line from the tuber coxae to the fullest point of the belly of the semitendinosus muscle, then finding the midway point and adding a perpendicular line to the spine (Manso Filho et. al., 2009; Byrd et. al., 1989). The cranial medial corner of the shaved 5 x 5 cm square

was set 6 cm laterally from the spine (Figure 1). The skin was surgically removed from the sites, and the muscles were dissected out. Biopsies were taken using a 6 mm Bergström needle (Lindholm and Piehl, 1974) in order to see where exactly the needle was sampling (Figure 2).

Comparison of repeated biopsies patterns.

Four Standardbred mares (12 ± 4 years old and weighing 537± 17 kg) were used. Biopsies were performed on the left and right middle gluteal muscle. They were measured by drawing a line from the tuber coxae to the fullest point of the belly of the semitendinosus muscle, then finding the midway point and adding a perpendicular line (Figure 1). The biopsy site was set at 6 cm from the spine, and a mirror site was measured on the opposite hindquarter. Biopsies for both sides were performed in a 5 x 5 cm square, and were collected at 4 timepoints: 0, 30 min, 2 H and 24 H. Muscle biopsies were taken from muscle at a depth of 5 cm using a 6 mm Bergström needle (Lindholm and Piehl, 1974). On the left side the biopsies were performed in a row, perpendicular to the spine, with 2 cm between each biopsy site (Figure 2). On the right side, biopsies were performed in alternating corners of the square (i.e. the 0 sample was taken in the cranial medial corner of the square, the 30 min sample was taken in the caudal lateral corner, the 2 H was taken in the caudal medial corner, and the 24 H sample was taken in the cranial lateral corner). Muscle samples were rinsed in saline solution and then snap frozen in liquid nitrogen and stored at -80°C until homogenization.

Sample Analysis

Muscle samples were weighed and approximately 100 mg of wet tissue was then added to 1 mL of 1x RIPA buffer (Cayman Chemicals, Ann Arbor, MI, USA). Samples were homogenized (Potter S Homogenizer; Sartorius-Stedim, Bohemia, NY, USA) for two minutes at 300 rpm and then aliquoted into microcentrifuge tubes. Each aliquot was vortexed, then centrifuged for 10 min at 8160 x *g* at 4°C. After spinning, final aliquots were pipetted into new microcentrifuge tubes and stored at -80°C until assay analysis.

Muscle homogenates were analyzed for MDA concentrations using a thiobarbituric acid reactive substances (TBARS) assay (Cayman Chemicals, Ann Arbor, MI, USA). Total protein concentrations of the muscle homogenates were determined using the BioRad Protein Assay (BioRad Laboratories, Hercules, CA, USA). A 1:10 dilution was used for all samples.

Statistical Analysis

Data are presented as mean ± SE unless otherwise noted. Data was statistically analyzed using a general linear model ANOVA in SAS (Version 9.1, Cary, NC), and *post hoc* analysis was performed using the Tukey method.

Results

Comparison of biopsy sites.

It was found that though both biopsy sites do in fact sample the middle gluteal muscle, the technique by Lindholm and Piel (1974) directly samples the middle gluteal, while the technique modified from Manso Filho et. al. (2009) and Byrd et. al. (1989) had to go through the superficial gluteal before reaching the middle gluteal muscle (Figure 3A, B).

Comparison of repeated biopsies patterns.

There was a significant effect for pattern (P=0.03). Specifically, the 24H sample taken via the row pattern had significantly higher concentrations of MDA than the 24H sample taken via the quadrant pattern (1.69 ± 0.83 versus 0.786 ± 0.04 nmol/mg protein, respectively; P=0.02). The 24H sample taken via the row pattern had significantly higher concentration of MDA as compared with the 0 sample (1.69 ± 0.83 versus 0.746 ± 0.19 nmol/mg protein, respectively; P=0.02). There was also a trend for the 24H sample on the left to be higher than the 30 min (P=0.10) or 2H (P=0.08) samples also taken via the row pattern. There was no significant difference between either pattern at timepoint 0, nor were there any significant differences in MDA between 0, 30 min, 2H , and 24H taken via the quadrant pattern (Figure 4).

Discussion

The comparison of biopsy sites found that both techniques do result in samples collected from the middle gluteal muscle. Due to the anatomy of the horse however, the biopsy site as described by Lindholm and Piehl (1974) is an easier site to sample, since the Bergström needle is directly inserted into the middle gluteal. Conversely, with the technique modified from Manso Filho et. al. (2009) and Byrd et. al. (1989), the needle must be inserted through the superficial gluteal muscle before reaching the middle gluteal. In addition, if the sample site is set slightly more caudal, samples obtained may be solely from the superficial gluteal, as the muscle increases in thickness. Thus, to ensure conformity in samples, both within a study and for comparisons to others, it is clear that the site used by Lindholm and Piehl (1974) is ideal for measuring the middle gluteal muscle in horses, which for the sake of this dissertation will be the site used in Chapters 4 and 5.

It is apparent from this study that the pattern of muscle biopsies does play a role in subsequent oxidative stress. The row pattern generated more oxidative stress by 24H than the quadrant, with a continual rise in MDA. The quadrant pattern had no increases in MDA through the 24H. While no studies have looked at the effects of repeated biopsies on oxidative stress in horses, two equine studies have analyzed markers of inflammation in the gluteal muscle. Wagner (2011) found that repeated biopsies in a row resulted in increased expression of IFN-gamma mRNA and decreased expression of IL-beta mRNA from the initial biopsy to the final biopsy 5 days later. The pattern used by Wagner (2011) set the biopsies at 1 cm apart, versus the current study set the biopsies at 2 cm apart. Liburt et al., (2010) found no change in the mRNA expression of IFN-gamma

or IL-beta in 24 hours, but it is not known what pattern was used to collect the biopsies. The difference in biopsy site locations could result in differences in inflammation. This study showed that the row pattern results in increased oxidative stress within 24 hours; coupled with prior research showing increased inflammation in a row pattern over 5 days, it appears that this is not an ideal way to sample repeated biopsies. The inflammation and oxidative stress accrued though the biopsies could compound any effects of exercise.

Conclusion

The biopsy sampling technique developed by Lindholm and Piehl is the most accurate method for consistent, repeatable sampling from the middle gluteal muscle in the horse. In addition, when performing the muscle biopsies, a quadrant pattern is recommended over a row pattern to ensure lower amounts of lipid peroxidation and inflammation. The pattern of muscle biopsies sampled within 24 hours can affect levels of oxidative stress, most significantly at 24 hours post exercise. Further exploration into alternative biopsy patterns (such as a row, but with nonconsecutive biopsies, or variations on the quadrant pattern) would be worthwhile to explore, in the goal of finding a sampling pattern that induces the lowest possible oxidative stress and inflammation.

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Figure Captions.

Figure 1. Determination of biopsy site by drawing a line from the point of the tip to the point of the hindquarters, then finding the midway point and adding a perpendicular line. The biopsy site was set at 6 cm from the spine, and a mirror site was measured on the opposite hindquarter.

Figure 2. Depiction of the two methods of muscle biopsy site used. The quadrant pattern is on the left, and the row pattern on the right.

Figure 3. An approximate comparison of the biopsy sites used by Lindholm and Piehl (1974) versus the site location adapted from Manso Filho et. al. (2009) and Byrd et. al.(1989). (**A)** The red circle denotes the biopsy site location as described by Lindholm and Piehl. The red box denotes the biopsy site location described by the current study (adapted from Manso Filho et. al. (2009) and Byrd et. al.(1989). **(B)** The biopsy needle is shown inserted into the red square, through the superficial gluteal (identified by the green arrow) and into the middle gluteal muscle (identified by the red arrow).

Figure 4. Malondialdehyde (MDA) concentrations in skeletal muscle of horses at 0, 30 minutes, 2 hours and 24 hours, with the blue bars representing horses with biopsies performed in a quadrant pattern, and the red bars representing horses with biopsies

performed in a row. Data are presented as the mean ± SE. *Significance was set at $P < 0.05$.

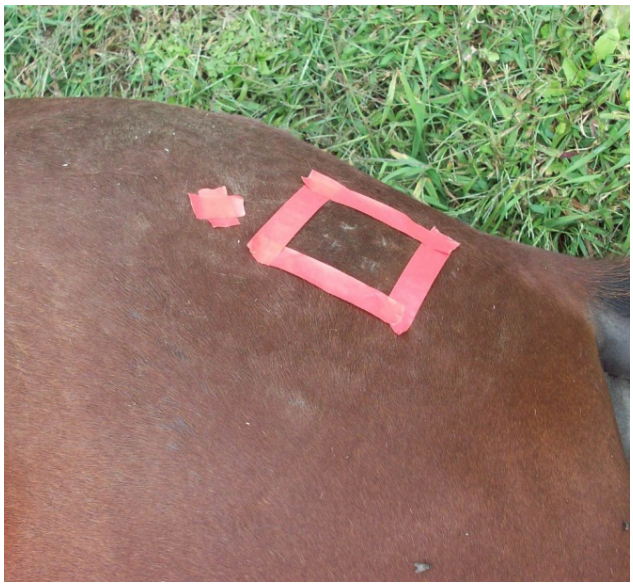
Figures

Figure 1.

Figure 2.

Figure 3.

A.



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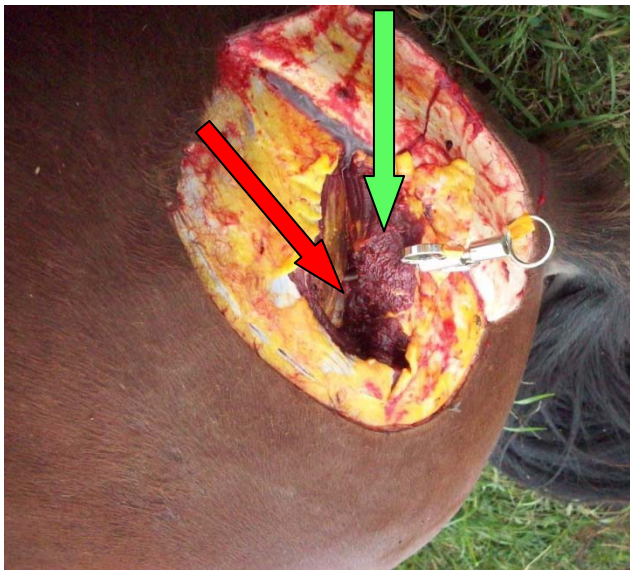

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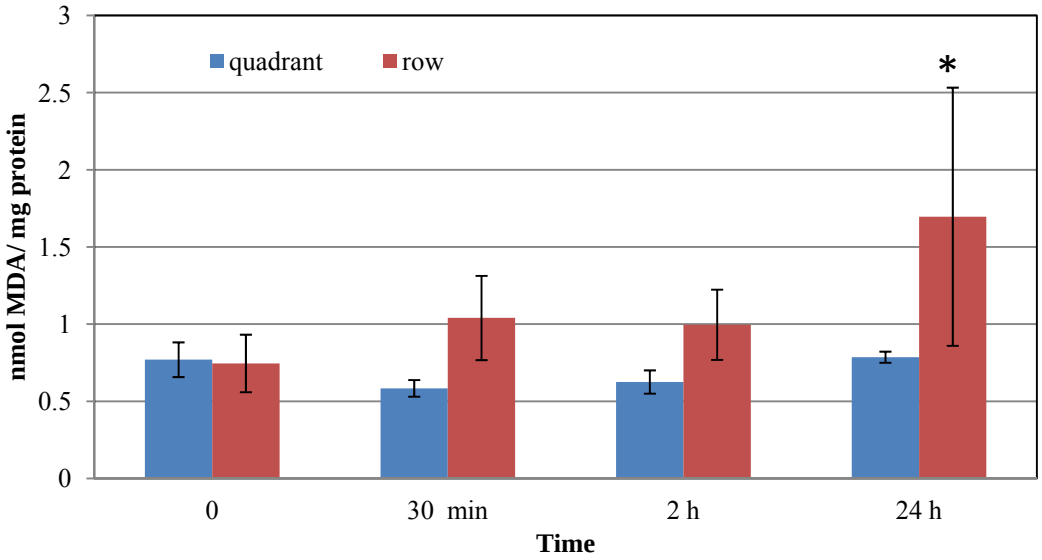
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CHAPTER THREE: **Oxidative stress and antioxidant status in equine skeletal muscle and blood after intense exercise in Standardbred mares: A pilot study**

ABSTRACT

The objective of this pilot study was to compare markers of oxidative stress in skeletal muscle and blood of intensely exercising horses and non-exercising horses. Using a randomized cross-over design, six mature, healthy unfit Standardbred mares were divided into two groups of three; one stood in a set of restraining stocks, the other completed an Interval Exercise Test (IET). Blood and middle gluteal muscle samples were collected before exercise and at intervals up to 24 h of recovery. Samples were analyzed for hematocrit (Hct), plasma total protein (TP), total muscle and erythrocyte glutathione (GSH-T) and glutathione peroxidase (GPx), and muscle and plasma malondialdehyde (MDA) and nitric oxide (NO). Data was statistically analyzed using a general linear model ANOVA. Hematocrit and TP were significant for treatment, sample and a treatment by sample interaction ($P < 0.0001$). The main effect of sample time was significant for erythrocyte GSH-T and erythrocyte GPx ($P=0.0005$; $P=0.0012$, respectively). The main effect of treatment was significant for erythrocyte GPx ($P=0.004$) and plasma NO ($P=0.007$). In addition, erythrocyte GSH-T was higher at 1.5H in the exercised horses than in the standing controls ($P=0.05$). There were no differences with treatment or sample for muscle MDA, NO, GPx or GSH-T. The results suggest that the intensity of acute exercise is directly correlated with the amount of oxidative stress induced post exercise.

Introduction

Oxidative stress is a disturbance in the oxidant/antioxidant balance, in favor of oxidants. Reactive oxygen species (ROS) may damage lipids, protein, and DNA, all of which can decrease athletic performance (Deaton et al., 2003). A key site of oxidative stress is the skeletal muscle (Powers and Jackson, 2008); with intense exercise, the amount of ROS produced increases in the muscle, and is subsequently released into the bloodstream. Excessive and chronic amounts of ROS after intense exercise cause damage to the muscles via lipid peroxidation.

The horse is an exceptional athlete for a variety of reasons (including, but not limited to high maximal oxygen uptake and large reserve of splenic red blood cells), but also of importance is the musculoskeletal system. More than half of a mature horse's body weight is comprised of skeletal muscle, in contrast to many other mammals (Rivero, 2007). The middle gluteal muscle plays a major role in locomotion and has an important function in the propulsion of the hind leg (Roneus and Lindholm, 1991). Intense bouts of exercise put the muscle under considerable stress, and can lead to increased oxidative stress (Kirschvink et al., 2008; Marlin et al., 2002). High levels of oxidative stress and inflammation could be a factor in subsequent muscle injuries.

Malondialdehyde (MDA), nitric oxide (NO), total glutathione (GSH-T), and glutathione peroxidase (GPx) are four markers commonly used to measure oxidative stress. Malondialdehyde (MDA) is the main product of lipid peroxidation, and is associated with the reduction of cellular function, and aggregation and polymerization of cellular compounds (Matsuki et al., 1991). Prior exercise studies have looked at levels of both MDA and thiobarbituric acid reactive substances (TBARS) as interchangeable measurements of lipid peroxidation. Nitric oxide (NO), a free radical generated by nitric

oxide synthase, is increased with muscle contractions. Superoxide reacts rapidly with NO to form peroxynitrite which is a strong oxidizing agent (Powers and Jackson, 2008). In glutathione oxidation, glutathione peroxidase (GPx) uses glutathione (GSH) to donate a pair of hydrogen ions to reduce hydrogen peroxide to two water molecules. This reaction oxidizes GSH to glutathione disulfide (GSSG). Another enzyme, glutathione reductase, then converts GSSG back to GSH, thus allowing the cycle to start again (Powers and Jackson, 2008).

Prior equine studies have found after exercise elevated levels of plasma TBARS (Marlin et al., 2002), erythrocyte MDA (Matsuki et al., 2001), and whole blood MDA (Ceylan et al., 2009). Levels of GSH-T in erythrocytes have been seen to decrease after long exhaustive exercise (Marlin et al., 2002). Similarly, levels of GPx have also shown a decrease after acute intense exercise (Ono et al., 1990). Finally, levels of nitrites (NO_2) and nitrates (NO_3) in both whole blood and plasma have been seen to increase (Ceylan et al., 2009; Lamprecht et al., 2009), but plasma nitrites and nitrates have also shown a decrease in concentration after intense exercise (Lamprecht and Williams, 2012; Lamprecht et al., 2009).

The studies mentioned above examined the blood and plasma of horses (Lamprecht and Williams, 2012; Ceylan et al., 2009; Lamprecht et al., 2009; Marlin et al., 2002; Matsuki et al., 2001; Ono et al., 1990), but few studies have looked at these markers of oxidative stress in the gluteal muscle after intense exercise (Matsuki et al., 1991; Siciliano et al., 1997; Kinnunen et al., 2005a). With the gluteal muscle being an important site of ROS production, it is important to determine total concentrations of oxidants and how they contribute to muscle damage and soreness after exercise, as

circulatory levels of markers of oxidative stress may not fully reflect damage. In humans and laboratory animals studies have shown that muscle contractions during intense exercise produces high levels of ROS (Powers and Jackson, 2008; Venditti and Di Meo, 1996).

Looking at a combination of four different markers in both blood and skeletal muscle in combination with intense exercise has yet to be examined in horses. Therefore, the objective of this pilot study was to compare markers of oxidative stress in skeletal muscle and plasma in exercising horses and non-exercising horses. The hypothesis of this study was that levels of MDA, GSH-T, GPx, and NO would be altered after an intense bout of exercise in the blood and skeletal muscle of horses. Specifically, levels of all markers would increase after exercise, and with the exception of NO (which will remain elevated through 24 hours), all markers would return to levels seen prior to exercise by the 24 hr sample. In contrast, the levels of these markers should not change in the skeletal muscle or blood of non-exercising horses.

Materials and Methods

Animals and Experimental Design

The Rutgers University Institutional Animal Care and Use Review Board approved all methods and procedures used in this experiment. Six mature Standardbred mares (12±4 years old and weighing 537±17 kg) participated in the study. All horses were healthy, but unfit (not undergoing consistent forced exercise in a two to three month period previous to Trial 1), were housed at the Rutgers University Equine Facilities on 3-acre exercise dry-lots, and were familiar with running on a high speed equine treadmill. The mares were divided randomly into two groups of three (A and B). The study was

designed as a crossover; in the first part of the study (TRIAL 1), group A exercised, while group B was the standing control. After a period of 1 month, group A was the standing control, while group B exercised (TRIAL 2).

One week prior to this study the six mares performed a graded exercise test to determine the speed at maximum heart rate. This speed was used to standardize the following acute exercise test. The exercise protocol used to exercise the mares was an Interval Exercise Test (IET; Williams and Carlucci, 2006). The test was run on a high speed treadmill (Sato I, Equine Dynamics, Inc., Lexington, KY) asset at a fixed 6% grade. The mares began the exercise with a 2 minute walk at 1.5 m/s; they then increased to 4 m/s for 8 minutes. The warm up was followed by two intervals each consisting of 2 minutes at 100% maximum heart rate (9-10 m/s) with 4 minutes at 4 m/s in between (Williams and Carlucci, 2006). During the exercise test all mares wore a heat rate monitor (Polar Equine Heart Rate Monitor; FitMed Inc., Mill Valley, CA, USA) to determine heart rate at various time points.

Sample Collection

Intravenous catheters (Angiocath, 14 gauge, Becton Dickson, Inc., Parsippany, NJ) were inserted percutaneously into the left jugular using aseptic techniques and local anesthesia at least 30 min prior to the first blood sample to allow for adaptation to the catheter. The catheters were kept patent with sterile heparinized physiological saline solution at a working concentration of 10 U/ml. Blood was collected at 30 min prior (-30 min) and immediately prior to the exercised horses IET start time (PRE), immediately

after (POST), 30 min, 1, 1.5, 2, and 24 H after exercise. At each time point, blood samples (20 mL) were taken and immediately aliquoted into two pre-chilled vacutainer tubes containing sodium heparin (Becton Dickson, Inc., Franklin Lakes, NJ), inverted, and placed in ice. Of the two collection tubes, one was immediately analyzed for hematocrits (Hct) using microhematocrit technique (CritSpin® S-120, Iris Sample Processing, Westwood, MA, USA), and then red blood cells were extracted. Briefly, 500 μL of whole blood was transferred from the collection tube to a microcentrifuge tube and was centrifuged at 2500 x *g* for 5 min at 4°C. The plasma supernatant was discarded and the remaining erythrocytes were washed with 500 μL of ice-cold 0.9% sodium chloride solution, thoroughly vortexed and centrifuged a second time as described above. The saline supernatant was removed and discarded, and the remaining erythrocytes were lysed with 1 mL of ice-cold distilled deionized water. Samples were then immediately stored at -80°C until further analysis.

The other sodium heparin collection tube was immediately centrifuged for 10 min at 1500 x *g* at 4°C. Plasma was then analyzed for total protein (TP) using digital refractometry (Palm Abbe Veterinary Refractometer, MISCO Inc., Cleveland, OH, USA), and then aliquoted into microcentrifuge tubes. For plasma samples to be used in the thiobarbituric acid reactive substances (TBARS) assay, 100 μL of butylated hydrotoluene (BHT) was added; the sample was mixed well and then stored at -80°C, along with the untreated plasma samples until further analysis.

Muscle biopsy time points for all horses were 30 min prior to the exercised horses IET start, 10 min, 2, and 24 h after exercise. Muscle biopsies were taken from the middle gluteal muscle at a depth of 5 cm using a 6 mm Bergström needle (Lindholm and Piehl,

1974). Muscle biopsies were performed in a 5 x 5 cm square, in alternating corners of the square (i.e. the PRE sample was taken in the cranial medial corner of the square, the POST sample was taken in the caudal lateral corner, the 2 H was taken in the caudal medial corner, and the 24 H sample was taken in the cranial lateral corner). This method is adapted from a previous protocol showing no differences in the selected assays after repeated biopsies (Chapter Two). The right and left middle gluteal muscles were used for biopsies; one side was selected randomly for the biopsies in TRIAL 1, while the other side was used for the biopsies in TRIAL 2. Each muscle biopsy site was given a subcutaneous injection of 5 cc of lidocaine prior to the scalpel incision and biopsy. After rinsing with saline, muscle samples were immediately frozen (snap frozen) in liquid nitrogen and stored at -80°C until homogenization.

Sample Analysis

Muscle samples were weighed and approximately 100 mg of wet tissue was then added to 1 mL of 1x phosphate buffered saline (PBS) solution. Samples were homogenized (Potter S Homogenizer; Sartorius-Stedim, Bohemia, NY, USA) for two minutes at 300 rpm and then aliquoted into four microcentrifuge tubes at 1:1 dilutions containing the specific buffer needed for each assay: TBARS (RIPA buffer, Cayman Chemicals, Ann Arbor, MI, USA), NO (PBS buffer), GSH-T (metaphosphoric acid (MPA) buffer), and GPx (Tris-HCl, pH 7.5, containing 5 m*M* EDTA and 1 m*M* 2-mecaptoethanol). Each aliquot was vortexed, then centrifuged for 10 min at 8160 x *g* at 4°C. After spinning, final aliquots were pipetted into new microcentrifuge tubes, then stored at -80°C until assay analysis.

Plasma and muscle homogenates were analyzed for total nitrate/nitrite concentrations using a NO Assay (Bioassays Systems, Hayward, CA, USA; interassay CV = 7.9%; intraassay CV = 4.5%) and MDA concentrations using a TBARS assay (Cayman Chemicals, Ann Arbor, MI, USA; interassay CV = 6.2%; intraassay CV = 1.9%). Erythrocyte lysate and muscle were analyzed for GPx activity and GSH-T concentrations using a Bioxytech GPx-340 (OXIS Research, Portland, OR; interassay CV = 4.1%; intraassay CV =1.44%) and GSH-400 Assay, respectively (OXIS Research, Portland, OR; interassay CV = 4.9%; intraassay = 1.4%).

Total protein concentrations of the erythrocyte lysate and muscle homogenates were determined using the BioRad Protein Assay (BioRad Laboratories, Hercules, CA, USA). A 1:10 dilution was used for all samples; for the muscle homogenates, the aliquot was from the homogenate in 1x PBS solution.

All assays were performed according to manufacturer's instructions approximately 1 month after collection. All samples were performed in duplicate, and all assays were read using a micro-plate reader (Spectramax 340, Molecular Devices, Sunnyvale, CA).

Statistical Analysis

Data are presented as mean ± SE unless otherwise noted. Data was statistically analyzed using a linear mixed effects model, with sample time and exercise as main effects, using the R program (statistical environment for analysis; R Development Core Team, 2011). Post hoc comparisons of means were performed using the Tukey test with significance set at $P = 0.05$.

Results

Heart Rates and Time to Exercise Completion

The average peak heart rate for the first interval of the IET was 213 ± 8.5 beats per min, and the average peak heart rate for the second interval of the IET was 219 ± 5.2 beats per min. The average length of time spent at the first interval was 109.2 ± 7.6 sec, and the average length of time spent at the second interval was 53 ± 8.5 sec. The average length of time for the entire IET was 16.56 ± 0.4 min.

Blood and Muscle Results

Hematocrits and TP were significant for main effects treatment and sample and a treatment by sample interaction (Table 1; $P < 0.0001$). The exercised horses' hematocrits and TP values peaked at POST (Hct, 57.31 ± 2.35 %; TP, 7.38 ± 0.36 g dl^{-1}) and returned to baseline concentrations by 24 H (Hct, 40.53 ± 2.90%; TP, 6.18 ± 0.20 g dl^{-1}). Standing horses had no effect of sample time on hematocrits or TP (average Hct, 38.25 ± 0.65 %; average TP, 6.11 ± 0.04 g dl^{-1}; $P > 0.1$).

The main effect of treatment (exercise vs. standing) was significant for erythrocyte GPx activity (P=0.004) and plasma NO concentration (P=0.007; Figure 1). The main effect of sample time was significant for erythrocyte GSH-T concentration and erythrocyte GPx activity (P=0.0005; P=0.0012, respectively). Erythrocyte GPx and GSH-T steadily increased from POST to a peak at 1.5H (94 ± 0.32 mU/mg and 0.871 ± 0.11 nmol /mg protein, respectively; Figure 2), and then returned to PRE values at 24H. In addition, erythrocyte GSH-T concentration was higher 1.5H in the exercised mares than in the standing controls (P=0.05; Figure 3). Numerically, erythrocyte GPx was also

higher at 1.5H in the exercising mares than in the standing controls, but it was not significant.

There were no significant differences with treatment or sample time for muscle MDA, NO, GPx or GSH-T (Table 2). It should be noted that there were similar numerical changes across all markers in the muscle in the exercised mares, suggesting a possible impact of exercise (no consistent, significant changes were observed in the standing controls). Exercised mares had numerically lower concentrations of NO, GSH-T, and MDA, and lower activity of GPx at 2H, but higher at 24H as compared to pre exercise values.

Discussion

In this study, exercised mares had few significant differences in antioxidant status and oxidative stress than the standing controls. Other results of this study indicate that the exercise test used (IET) did challenge the mares physically as shown by the time to completion of the test, average peak heart rates, and hematocrits and total protein value. In addition, though none of the markers showed a significant difference in the skeletal muscle, consistent numerical changes in all markers were noticed over time in the exercised mares, who had lower concentrations of NO, GSH-T, and MDA, and lower activity of GPx at 2H but higher at 24H, as compared to pre exercise samples.

In this study there were no significant effects of sample time or treatment on plasma and muscle MDA. In several other equine studies, MDA and/or TBARS have been found to increase after exercise. Marlin et al. (2002) found elevated levels of plasma TBARS in horses after competing in a 140-km endurance race, while Matsuki et

al. (2001) found elevated erythrocyte MDA after an acute treadmill test. Ceylan et al. (2009) found elevated whole blood MDA after 4 hours of exercise. Decreases in erythrocyte TBARS has also been seen in horses after exercise (Balogh et al., 2001), as well as no change in lipid peroxides after a 1000 meter sprint (Ono et al., 1990).

Few studies in horses exist that analyze muscle MDA; in the equine middle gluteal muscle, elevated total and protein-bound MDA have been shown after acute exercise, but no difference was found in free MDA (Matsuki et. al., 2001). In the present study, total MDA was analyzed in the plasma and muscle, while protein-bound was not. In rat studies there are conflicting reports on muscle MDA and the effect of exercise; one study found no difference in MDA in the gastrocnemius muscle in male and females rats after acute exercise (Balci and Pepe, 2012), while another study found increases in MDA in the deep vastus lateralis muscle of young and old rats (Bejma and Ji, 1999). A further possible explanation for the lack of change in lipid peroxidation is that the mares were physiologically equipped to handle any oxidative stress that occurred as a result of exercise. Lipid peroxidation occurs when cell membranes are broken down; if the exercise did not generate sufficient ROS to achieve lipid peroxidation, then the cell membranes may have remained intact and unharmed, and thus no changes in MDA would occur. This may account for the variations in MDA concentrations found after exercise both in humans (Powers and Jackson, 2008; Sachdev and Davies, 2008) and horses (Marlin et al., 2002: Matsuki et al., 2001; Ceylan et. al., 2009). A final reason for discrepancies in MDA concentrations could be due to methodical variations; the TBARS assay and the HPLC methods vary. A criticism for measuring TBARS is the lack of

specificity, though the present study did use BHT to remedy that problem, as BHT has been shown in the past to increase specificity (Halliwal and Chirico, 1993).

In the present study erythrocyte GSH-T was increased at 1.5H post exercise, and GPx had a trend to also increase at 1.5H post exercise. Levels of GSH-T in erythrocytes have been seen to decrease after endurance exercise in horses (Marlin et al., 2002) and after acute exercise over jumps (Balogh et al., 2001). However, studies have also seen no change in erythrocyte GSH-T after an acute exercise test (Kinnunen et al., 2005a), or increase after acute intense exercise (Williams and Carlucci, 2006; Ceylan et al., 2009; Lamprecht and Williams, 2012). Activity of erythrocyte GPx have shown a decrease after intense treadmill exercise (Ono et al 1990), or an immediate increase after acute exercise (Williams and Carlucci, 2006; Lamprecht and Williams, 2012). There were no differences in muscle GSH-T or GPx in this study. Prior studies have found change in muscle GPx activity was found after acute exercise in horses (Kinnunen et al., 2005a), and no change found in rats in GSH-T in the deep vastus lateralis or gastrocnemius after acute exercise (Bejma and Ji, 1999; Balci and Pepe, 2012). The lack of change in muscle GSH-T concentration and GPx activity in the present study is consistent with these reported studies. Similar to MDA muscular concentrations, if the cell membranes were not harmed by lipid peroxidation, then antioxidants and other molecules would not have leaked out into the system; the circulatory changes in GPx activity and GSH-T concentration may not have been from skeletal muscle, but perhaps other sources in the body.

In the present study plasma NO had an overall exercise effect, with mares increasing plasma NO concentration at 24H. Prior studies have shown concentrations of

nitrites (NO_2) and nitrates (NO_3) to increase or decrease after intense exercise in both whole blood and plasma of horses (Ceylan et al., 2009, Lamprecht et al., 2009; Lamprecht and Williams, 2012). Lamprecht et al. (2009) showed that the intensity of the exercise bout has a significant impact on the subsequent oxidative stress in horses. A treadmill test of short duration (<9 minutes) had no effect on plasma total nitrites. A test similar to the one in this study (IET) resulted in an increase in plasma total nitrites 2 hours and 24 hours post exercise. In the present study, no significant change was seen in NO in the skeletal muscle due to exercise. In the rat, decreased levels of NO have been seen in the gastrocnemius after exhaustive exercise (Balci and Pepe, 2012). To date, no studies have looked at the effects of exercise on skeletal muscle NO in the horse, and even in other species there is extremely limited data. The increase in plasma NO concentration but lack of change in muscle NO concentration indicates the source of plasma NO may not have been skeletal muscle and was instead a result of vasodilation during exercise.

Conclusion

This study showed that the intensity and duration of an acute treadmill test are both important factors in determining if an oxidative stress response will occur after exercise. Given the limited changes in oxidative stress and antioxidant status, the mares in this study appear to have been able to cope physiologically with any exercise induced oxidative stress. It is plausible that a more strenuous and longer exercise test would be more likely to elicit an oxidative stress and inflammation response. Furthermore, while there are many studies looking at oxidative stress and antioxidant status in the blood of

horses, there is a lack of data available on oxidative stress in the skeletal muscle. While prior research has indicated that the skeletal muscle a key site of oxidative stress, given the changes in antioxidant status further research is needed to determine the effects of exercise of varying duration and intensity on equine skeletal muscle.

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Figure Captions.

Figure 1. Plasma nitric oxide (NO) concentrations (mg/dL) in exercising and standing horses for sample times 30 minutes before exercise (-30), immediately before exercise (PRE), immediately after exercise (POST), +30, 1H, 1.5H, 2H, and 24H after exercise.

Figure 2. Erythrocyte glutathione peroxidase (GPx) activity (mU/mg protein) in exercising and standing horses for sample times 30 minutes before exercise (-30), immediately before exercise (PRE), immediately after exercise (POST), +30, 1H, 1.5H, 2H, and 24H after exercise.

Figure 3. Erythrocyte glutathione (GSH-T) concentrations (nmol/mg protein) in exercising and standing horses for sample times 30 minutes before exercise (-30), immediately before exercise (PRE), immediately after exercise (POST), +30, 1H, 1.5H, 2H, and 24H after exercise. *****Denotes difference between exercising and standing horses within sampling time ($P < 0.05$).

Figures

Figure 1.

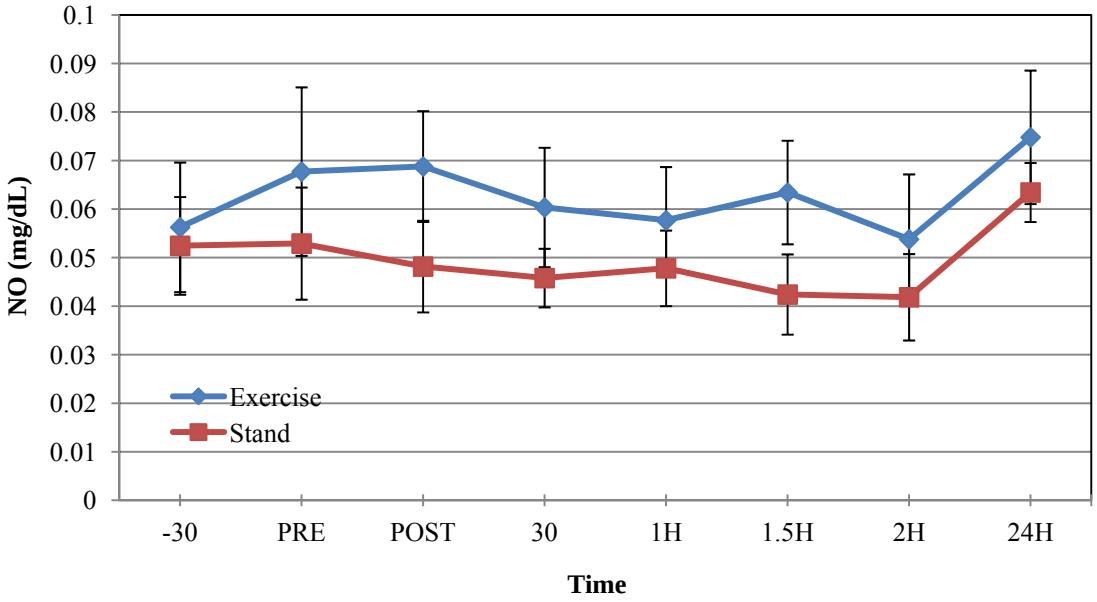


Figure 2.

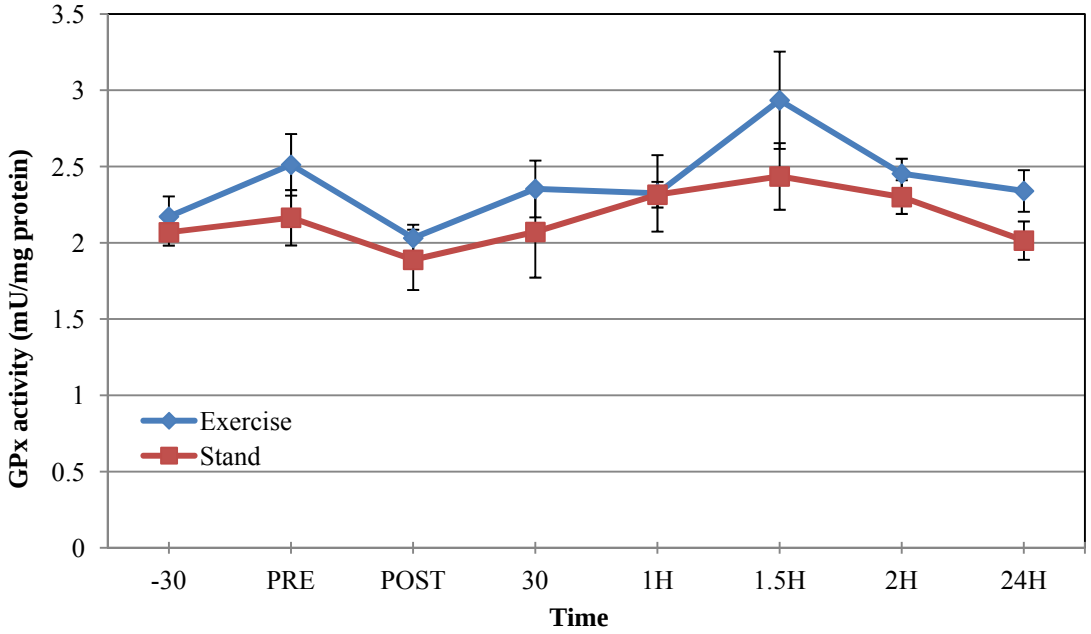
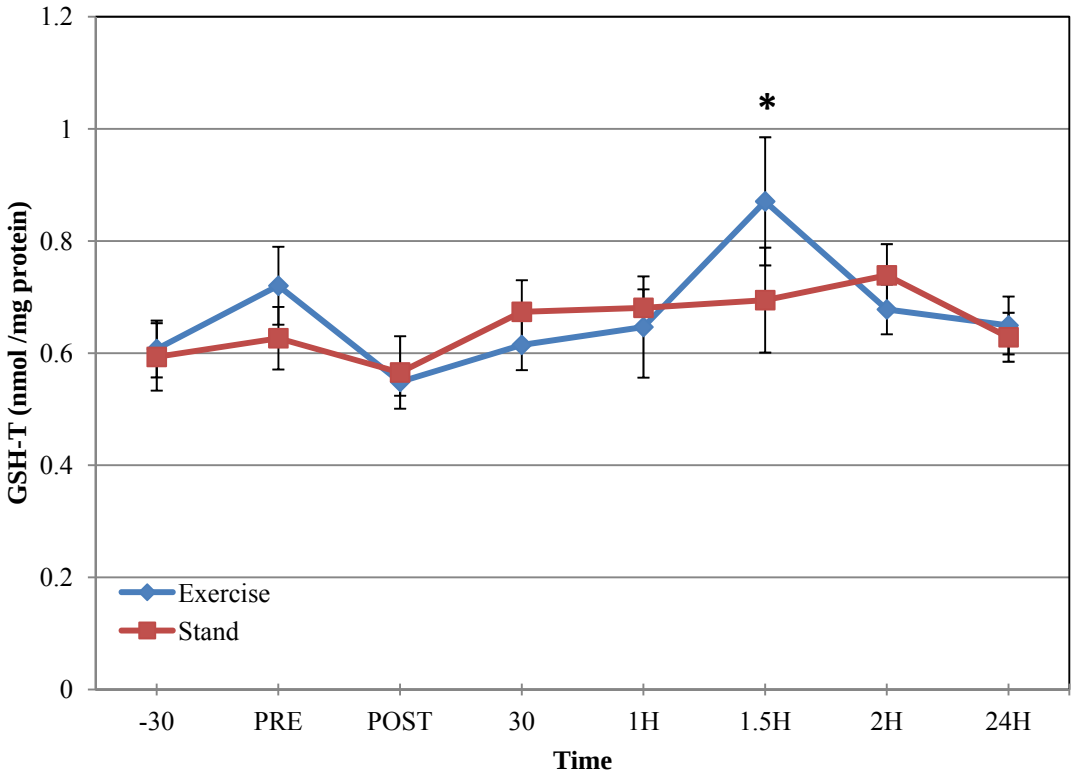


Figure 3.


1.2
1
0.8
0.6
0.4
0.2
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GSH-T (nmol /mg protein)
*
Exercise
Stand
-30
PRE
POST
30
1H
1.5H
2H
24H
Time

Tables

Table 1. Hematocrit (Hct), plasma total protein (TP), erythrocyte total glutathione (RBC GSH-T), and plasma malondialdehyde (plasma MDA) in exercising and standing horses for sample times -30, PRE, POST, +30, 1H, 1.5H, 2H, and 24H. Data is presented as the mean ± SE. Letters a, b, c denote differences across sampling time for exercise horses ($P < 0.05$).

	-30	PRE	POST	+30	1H	1.5H	2H	24H
Hct (%)								
Stand	40.23±1.5	41.88±1.9	39.79±1.6	35.81±1.2	36.85±1.3	34.20±1.2	37.63±2.0	40.31±2.5
Exercise	41.18±1.6[a]	40.52±1.7[a]	57.31±2.4[b]	43.04±1.6[a]	41.21±1.6[a]	37.90±0.8[a]	43.33±1.9[a]	40.53±2.9[a]
TP (mg dl^{-1})								
Stand	6.18±0.1	6.18±0.1	6.23±0.1	6.05±0.1	5.97±0.1	5.95±0.1	6.03±0.2	6.31±0.1
Exercise	6.25±0.2[a]	6.26±0.1[a]	7.38±0.4[b]	6.2±0.1[a]	6.28±0.1[a]	6.27±0.1[a]	6.40±0.1[a]	6.18±0.2[a]
MDA(nmol/mL)								
Stand	10.11±1.3	8.03±1.3	8.90±1.4	9.29±2.2	10.77±2.1	10.13±1.6	10.01±1.9	12.22±0.8
Exercise	10.91±1.1	10.22±1.3	9.29±1.3	9.94±2.0	12.43±1.3	15.34±6.4	9.76±1.7	10.38±1.6

Table 2. Comparison of muscle total glutathione (GSH-T), malondialdehyde (MDA), glutathione peroxidase (GPx) and nitric oxide (NO) in exercising and standing horses for sample times PRE, POST, 2H, and 24H. Data is presented as the mean ± SE.

	PRE	POST	2H	24H
GSH-T (nmol/mg)				
Stand	17.21±2.49	15.15±2.24	13.28±1.83	15.74±2.61
Exercise	16.17±2.61	13.72±1.54	9.63±1.50	21.39±9.37
MDA (nmol/mg)				
Stand	1.83±0.52	1.46±0.36	1.75±0.32	1.68±0.39
Exercise	1.72±0.35	1.80±0.38	1.14±0.18	2.08±1.06
GPx (mU/mg)				
Stand	25.99±7.28	23.95±4.50	22.64±6.35	28.57±8.36
Exercise	30.04±6.69	22.73±5.37	16.53±3.75	43.31±24.06
NO (μmol/mg)				
Stand	1.81±0.54	2.61±0.79	3.70±1.84	2.68±0.76
Exercise	2.25±0.64	1.97±0.47	1.73±0.72	4.61±1.77

CHAPTER FOUR: **The effect of age and exercise training on oxidative stress in equine skeletal muscle and blood.**

ABSTRACT

The hypothesis of this study was that yearlings would have higher levels of oxidative stress as measured by malondialdehyde (MDA), total glutathione (GSH-T), glutathione peroxidase (GPx), and nitric oxide (NO) than mature mares before training, but after training, mares and yearlings would have similar levels of oxidative stress. Ten yearling Standardbred fillies (18 ± 2.4 months) and 10 mature Standardbred mares (13 ± 2.1 years) were split into trained and non-trained groups. Trained horses were exercised 5 day/wk for seven weeks; all twenty horses performed GXTs prior to week 0 and on weeks 2, 5, and 7; blood and muscle samples were collected on those weeks and analyzed for GSH-T, GPx, MDA, and NO. Data was statistically analyzed using a linear mixed effects model, with Tukey as a post hoc analysis and is presented as mean ± SE ($P = 0.05$). At week 0, the yearlings had lower plasma MDA concentrations ($P=0.056$) and higher muscle GSH-T and NO concentrations ($P=0.03$) than the trained mares. At week 9 plasma NO concentrations were lower in trained mares than in the trained yearlings ($P=0.007$). Trained mares increased muscle MDA and decreased plasma MDA concentrations from week 0 to 9 ($P<0.01$). All groups increased plasma NO concentrations at week 2 ($P<0.05$) but returned to baseline levels by week 9; all mares increased muscle NO concentrations by week 9 ($P<0.03$). Trained mares and yearlings had increased erythrocyte GPx activity at weeks 7 and 9 and GSH-T concentration at

week 7 ($P<0.05$). All mares increased muscle GSH-T concentration by week 9 ($P<0.05$). Yearlings did not initially have higher levels of oxidative stress as hypothesized. The mares had higher lipid peroxidation and lower antioxidant status in the muscle prior to training which supports the theory of increased oxidative stress with aging. The trained mares did improve their antioxidant status and oxidative stress levels, resulting in levels similar to the yearlings.

Introduction

Many important physiological processes require oxidative stress; examples include production of superoxide by phagocytosing cells to kill invaded bacteria; hydrogen peroxide regulation of cell death pathways; and the role of reactive oxygen species (ROS) as mediators of inflammatory responses or metabolic regulators (Brigelius-Flohe 2009; Franco et al 1999; Powers et al., 2011; Powers et al., 2010). Oxidative stress is not always considered beneficial though, for it is well documented in humans and rats that exercise can induce oxidative stress (Dillard et al., 1978; Powers and Jackson, 2008), and high, chronic levels of ROS have been linked to muscle damage and soreness following exercise in humans (Arent et al., 2010; Urso and Clarkson, 2003; Clarkson and Thompson, 2000). The prevalence of oxidative stress as a result of exercise (both acute aerobic bouts and endurance) have been well documented in the horse (Lamprecht and Williams 2012; Ceylan et al 2009; Lamprecht et al., 2009; de Moffarts et al., 2006; Marlin et al., 2002; Matsuki et al 2001; Ono et al., 1990).

Lipid peroxidation, a common route of ROS production, can damage muscle cells, as hydroxyl radicals cause a cascading chain damaging the lipid bilayer and breaking the membrane. Malondialdehyde (MDA) is a common marker of lipid peroxidation (Kirschvink et al., 2008; Powers and Jackson, 2008). Nitric oxide (NO), though well known for its role in vasodilation, also contributes to oxidative stress, as it reacts extremely quickly with superoxide to form peroxynitrite, a ROS which can damage DNA and proteins (Stamler and Meissner 2001; Powers and Jackson 2008). Antioxidants are often thought of as the defense system again excessive levels of oxidants. Two such examples are the antioxidants glutathione (GSH) and glutathione peroxidase (GPx), which work in a cycle to reduce hydrogen peroxide to two water molecules.

In addition, oxidative stress is linked with aging (Anandan et al., 2013; Spiteller 2007; Barja 2004; Yu, 1996; Dillard et al., 1978). Though the association with aging has been made, the effect of oxidative stress on individuals as they go through puberty and into adulthood is not clear. Higher oxidative stress could be found in children as compared to adults, since they endure a greater oxygen cost during exercise and rely more on aerobic metabolism (Cooper et al., 2004). Differences in oxidative stress in young and aged rats have been found as well (Anandan et al., 2013; Lawler et al., 1993).

It is plausible that oxidative stress levels differ between young and mature horses as well, given what has been found so far in humans and rats. One equine study looked at two different ages of horses (mature and old) and no difference was found in erythrocyte total GSH or GPx after a bout of acute exercise (Williams et al., 2008). It is known that the age of a horse undergoing exercise does affect other physiological aspects, such as cortisol, maximal oxygen uptake, and maximal heart rate (Betros et al., 2002; Horohov et

al., 1999; McKeever and Malonowski, 1997) so it is likely that this also applies to oxidative stress. Young horses (18-24 months) have not been studied for levels of oxidative stress, both at rest and after exercise, but given that they are undergoing a large amount of growth and development during this time, oxidative stress could play an important role.

An exercise training protocol could help to blunt the effect of oxidative stress that occurs after intense exercise. Research done with humans has found that there is an increase in activity of antioxidant enzymes after training (Powers and Jackson, 2008), and research in rats has also found this (Venditti and Di Meo, 1996; Powers et al., 1994). Prior equine research has not shown consistent evidence that exercise training can alter levels of antioxidants and oxidative stress markers as a horse becomes more conditioned (Williams et al., 2008; Siciliano et al., 1997; Avellini et al., 1995). This could be due to differences in training protocols (duration, intensity, length) as well as differences in the horses (age, sex, breed). In the young growing horse that is novel to an exercise training program, there is no research to date looking at oxidative stress.

The objective of this study was to compare markers of oxidative stress in skeletal muscle and blood of horses of different ages and fitness levels. The hypothesis was that young horses would have higher levels of oxidative stress as measured by MDA, GSH-T, GPx, and NO than mature horses before an exercise training protocol; after training has concluded, mares and yearlings will have similar levels of oxidative stress.

Materials and Methods

Animals and Experimental Design

The Rutgers University Institutional Animal Care and Use Review Board approved all methods and procedures used in this experiment. Ten yearling Standardbred fillies (Y; 18 ± 2.4 months) and 10 mature Standardbred mares (M; 13 ± 2.1 years) were split into two groups; trained (T; 7 mares, 7 yearlings) and untrained (NT; 3 mares, 3 fillies). Therefore the four groups in this study were yearlings trained and non-trained (YT and YNT, respectively) and mares trained and non-trained (MT and MNT, respectively). All the mares were healthy, but unfit (not undergoing consistent forced exercise in a two to three month period prior to the study). All the yearlings were healthy, unfit, and novel to exercise; prior to the start of the study they were acclimated to the equine exerciser and high speed equine treadmill. All twenty horses were housed at the Rutgers University Equine Facilities on 3-acre exercise dry-lots; the mares remained outside continuously, while the yearlings were outside 0800-1600 with the remainder of the night spent inside 4 m x 4 m box stalls. All horses were fed grain twice daily to meet additional nutrient requirements; the mares were fed UltraActive (1.1 kg/d) and the yearlings were fed Ultra Broodmare/Yearling (1.1 kg/d) (Pennfield Feed, Lancaster, PA) (Table 1), in addition to forage (Table 2). Nutrient intake for mares and yearlings is shown in Table 3.

Training Protocol

Trained horses were exercised five days a week for seven weeks using an equine exerciser. Based on maximum heart rates determined by graded exercise tests (GXTs), the intensity of the workout averaged 50-60% of their maximum heart rate for a duration of 20-50 minutes (started with 20 min and worked up in intensity and duration with 50 min at 60% max by week 7). Heart rates were monitored by use of the Polar Heart Rate System (Polar Equine Heart Rate Monitor; FitMed Inc., Mill Valley, CA, USA) (Figure 1). All twenty horses performed GXTs prior to week 0 and on weeks 2, 5, and 7 (for trained horses, this replaced one day on the exerciser for these weeks).

Sample Collection

Muscle biopsies were taken from the middle gluteal muscle at a depth of 5 cm using a 6 mm Bergström needle (Lindholm and Piehl, 1974). Muscle biopsies were performed in a 5.1 x 5.1 cm square, in alternating corners of the square (as explained in Chapter 2). The left middle gluteal muscle was used for biopsies. Each muscle biopsy site was given a subcutaneous injection of 5 mL of lidocaine prior to the scalpel incision and muscle biopsy. After rinsing with saline, muscle samples were immediately frozen (snap frozen) in liquid nitrogen and stored at -80°C until homogenization.

Blood and muscle biopsies from the middle gluteal muscle were collected between 0800 and 1100 prior to the daily exercise, as well as prior to the start of exercise training (week 0 and 9). At each time point, blood samples were taken via jugular venipuncture and immediately put into pre-chilled 10 mL vacutainer tubes containing

either sodium heparin or EDTA (Becton Dickson, Inc., Franklin Lakes, NJ); tubes were inverted and placed back on ice. Blood was also collected into 10 mL non-chilled serum separator tubes (Becton Dickson, Inc., Franklin Lakes, NJ); tubes were not inverted and were placed in racks at room temperature.

Of the two sodium heparin collection tubes, one was used for red blood cells extraction. Briefly, 500 μL of whole blood was transferred from the collection tube to a microcentrifuge tube and was centrifuged at 2500 x *g* for 5 min at 4°C. The plasma supernatant was discarded and the remaining erythrocytes were washed with 500 μL of ice-cold 0.9% sodium chloride solution, thoroughly vortexed and centrifuged a second time as described above. The saline supernatant was removed and discarded, and the remaining erythrocytes were lysed with 1 mL of ice-cold distilled deionized water. Samples were then immediately stored at -80°C until further analysis.

The other sodium heparin collection tube was immediately centrifuged for 10 min at 1500 x *g* at 4°C and plasma was collected. For plasma samples to be used in the thiobarbituric acid reactive substances (TBARS) assay for measurement of MDA, 100 μL of butylated hydrotoluene (BHT) was added; the sample was mixed well and then stored at -80°C, along with the untreated plasma samples until further analysis.

The EDTA tubes were centrifuged for 10 min at 1500 x *g* at 4°C. Plasma was collected and stored at -80°C. Serum tubes remained at room temperature for approximately 45 minutes to allow for blood clotting and were centrifuged for 15 minutes at 1200 x *g* at 10°C. Serum aliquots were then collected and stored at -80°C.

Sample Analysis

Muscle samples were weighed and approximately 100 mg of wet tissue was then added to 1 mL of 1x phosphate buffered saline (PBS) solution. Samples were homogenized (Potter S Homogenizer; Sartorius-Stedim, Bohemia, NY, USA) for two minutes at 300 rpm and then aliquoted into four microcentrifuge tubes at 1:1 dilutions containing the specific buffer needed for each assay: TBARS (RIPA buffer, Cayman Chemicals, Ann Arbor, MI, USA), NO (PBS buffer), GSH-T (metaphosphoric acid (MPA) buffer), and GPx (Tris-HCl, pH 7.5, containing 5 m*M* EDTA and 1 m*M* 2-mecaptoethanol). Each aliquot was vortexed, then centrifuged for 10 min at 8160 x *g* at 4°C. After spinning, final aliquots were pipetted into new microcentrifuge tubes and stored at -80°C until assay analysis.

Plasma and muscle homogenates were analyzed for total nitrate/nitrite concentrations using a NO Assay (Bioassays Systems, Hayward, CA, USA; interassay CV = 7.9%; intraassay CV = 4.5%) and MDA concentrations using a TBARS assay (Cayman Chemicals, Ann Arbor, MI, USA; interassay CV = 6.2%; intraassay CV = 1.9%). Erythrocyte lysate and muscle were analyzed for GPx activity and GSH-T concentrations using a Bioxytech GPx-340 (OXIS Research, Portland, OR; interassay CV = 4.1%; intraassay CV =1.44%) and GSH-400 Assay, respectively (OXIS Research, Portland, OR; interassay CV = 4.9%; intraassay = 1.4%).

Total protein concentrations of the erythrocyte lysate and muscle homogenates were determined using the BioRad Protein Assay (BioRad Laboratories, Hercules, CA, USA). A 1:10 dilution was used for muscle samples and a 1:100 dilution was used for

erythrocyte lysate samples; for the muscle homogenates, the aliquot was from the homogenate in 1x PBS solution.

All assays were performed according to manufacturer's instructions approximately 1 month after collection. All samples were performed in duplicate, and all assays were read using a micro-plate reader (Spectramax 340, Molecular Devices, Sunnyvale, CA).

Statistical Analysis

Data will be presented as mean ± SE unless otherwise noted. All analyses were done in the context of a linear mixed model with random intercepts for horse. The use of random intercepts for horse helps account for autocorrelation of the observations on an individual horse over time. Significant initial analyses were followed by Tukey's Honestly Significant Difference (HSD) test for pairwise comparisons with significance set at $P = 0.05$. Tukey's HSD controls the Type I error rate in an individual analysis, but there was no other adjustment for multiple testing. Analyses were done using the R program (statistical environment for analysis; R Development Core Team, 2011).

Results

General fitness

Weights and body condition scores of all mares and yearlings over the course of the eight weeks of training are shown in Table 4. There were no significant differences between training and non-training within ages, so results have been combined. As

shown, overall the mares lost weight from week 0 to week 9 (559±12.7 kg versus 534±12.3 kg), while the yearlings overall gained weight from week 0 to week 9 (407±10.5 kg versus 425±8.4 kg). There were no significant changes in BCS for any of the horses. Table 5 shows the average maximum speeds obtained (m/s) and time to completion of exercise test during GXTs prior to exercise training and at weeks 2, 5, and 7. Both trained mares and trained yearlings increased maximum speeds obtained (Mares: 9.2±0.2 m/s at PRE GXT versus 10.2±0.4 m/s for GXT at week 7; Yearlings: 9.6±0.1 m/s at PRE GXT versus 11.0±0.3 m/s for GXT at week 7) and time to completion of exercise (Mares: 6.6±0.2 min at PRE GXT versus 7.6±0.4 min for GXT at week 7; Yearlings: 7.0±0.1 min at PRE GXT versus 8.3±0.3 min for GXT at week 7). MNT and YNT groups did not improve in speed, but the MNT group did improve slightly in time (6.4±0.2 min at PRE GXT versus 7.0±0.4 min for GXT at week 7).

Group Effects

There was a significant effect of week by group ($P<0.0001$) for plasma MDA (Figure 2A). At week 0, there was a trend for a group effect ($P=0.056$), with the YNT having lower concentrations of plasma MDA than the MT ($P=0.09$) and MNT ($P=0.07$). At week 2, there was a significant group effect ($P=0.015$, with the MT having lower concentrations of plasma MDA than YT ($P=0.003$). At week 5, there was a group effect ($P<0.0001$), with both the YT and YNT having lower plasma MDA concentrations than the MT ($P<0.001$) and MNT ($P=0.015$ and $P=0.005$, respectively). There were no group

effects for weeks 7 and 9 for plasma MDA concentrations. There was no effect of group for muscle MDA concentrations (Figure 2B).

For plasma NO there was no overall group effect however, there was a group effect at week 9 (P=0.025), with the MT having lower plasma NO concentrations than the YT (P=0.007; Figure 3A). In muscle NO there was also no overall group effect however, at week 0 there was a group effect (P=0.03), with the MT having lower muscle NO concentrations as compared to the YT (P=0.01; Figure 3B).

There was no main effect of group on erythrocyte or muscle GPx activity (Figure 4A & B). For erythrocyte GSH-T, there was not a main effect of group (Figure 5A). There was however, a group effect at week 0 for muscle GSH-T (P=0.03), with the MT having lower muscle GSH-T concentrations than the YT and YNT (P=0.04; P=0.05, respectively; Figure 5B).

Week Effects

Overall for the groups there was a week effect (P =0.0003) for plasma MDA with lower plasma MDA concentrations at Week 9 as compared to week 0 (P = 0.008) and week 2 (P = 0.0004; Figure 2A). Within the groups, there was an effect of week on plasma MDA for all (P<0.0.002) except the MNT. For MT, plasma MDA concentrations were higher at week 0 as compared with weeks 2, 5, and 9 (P=0.006; P=0.01; P<0.001, respectively). At week 5, plasma MDA concentrations were higher than at week 9 (P=0.002). For YT, plasma MDA concentrations were higher at week 2 than at weeks 0, 5, and 9 (P=0.002; P<0.001; P<0.001, respectively). For YNT, plasma MDA

concentrations were lower at week 0 than at weeks 2 or 7 ($P<0.001$; $P=0.007$, respectively), higher at week 2 than at weeks 5 or 9 ($P<0.001$), and at week 7, plasma MDA concentrations were higher than at weeks 5 ($P=0.001$). For muscle MDA, there was an effect of week ($P<0.0001$; Figure 2B). Specifically, in the MT group, at week 0 there was lower concentrations of MDA as compared to weeks 2, 5, 7, and 9 ($P<0.007$).

There was an effect of week for all groups with plasma NO ($P<0.05$; Figure 3A). Week 2 had higher plasma NO concentrations than weeks 0, 5, 7, and 9 ($P<0.001$), and week 5 had lower plasma NO concentrations than weeks 0 and 9 ($P<0.001$; $P=0.01$, respectively). In the MNT group, plasma NO concentrations were higher at week 2 than at week 5, 7, and 9 ($P=0.007$; $P=0.02$; $P=0.04$, respectively). In the YNT group, plasma NO concentrations were higher at week 2 than at weeks 0, 5, 7, and 9 ($P<0.001$). In the MT group, plasma NO concentrations were higher at week 2 than at weeks 0, 5, 7, and 9 ($P<0.0001$). In the YT group, plasma NO concentrations were higher at week 2 than at weeks 0, 5, 7, and 9 ($P=0.002$; $P<0.001$; $P<0.001$; $P=0.02$, respectively). In addition in the YT group, plasma NO concentrations were lower at week 5 than at week 9 ($P=0.01$). There was an effect of week for muscle NO ($P=0.03$; Figure 3B), with higher muscle NO concentrations at week 9 than week 0 ($P=0.01$). Within the groups, the MNT had a week effect for muscle NO ($P=0.001$), with muscle NO concentrations lower at week 0 than weeks 2 or 9 ($P<0.0001$), higher at week 2 than weeks 5 or 7 ($P<0.0001$), and higher at week 9 than weeks 5 and 7 ($P<0.0001$). In addition, in the MT muscle NO concentrations were lower at week 0 than at week 9 ($P=0.03$).

There was a main effect of week on erythrocyte GPx activity ($P<0.001$), with weeks 7 and 9 having higher erythrocyte GPx activity than weeks 0, 2, and 5 ($P<0.04$;

P<0.03; P<0.001, respectively; Figure 4A). Differences were seen in both the YT and MT groups across the weeks, but not YNT or MNT groups. For MT, erythrocyte GPx activity was lower at week 0 than at weeks 7 or 9 (P<0.001; P=0.04), higher at week 7 than at weeks 2 or 5 (P=0.005; P<0.001, respectively), and higher at week 9 than 5 (P=0.02). In the YT, erythrocyte GPx activity was lower at week 2 than at weeks 7 and 9 (P=0.03; P=0.04, respectively) and lower at week 5 than at weeks 7 and 9 (P=0.002; P=0.004, respectively). There were no main effects of week on muscle GPx activity (Figure 4B).

In erythrocyte GSH-T there was a week effect (P<0.0001; Figure 5A). Week 7 had higher erythrocyte GSH-T concentrations than weeks 0, 2, and 9 (P<0.001), and week 5 had higher erythrocyte GSH-T concentrations than week 0 (P=0.04). In the MNT group, erythrocyte GSH-T concentrations were lower at week 0 than at weeks 7 or 9 (P<0.001; P=0.006). In the MT group, erythrocyte GSH-T concentrations were higher at week 7 than at weeks 0 and 5 (P=0.004; P=0.03). In the YT group, erythrocyte GSH-T concentrations were higher at week 7 than at weeks 0, 2, and 9 (P=0.04; P=0.006; P=0.002, respectively). There was a main effect of week for muscle GSH-T (P<0.0002; Figure 5B), with lower muscle GSH-T concentrations at week 0 than weeks 2, 5, 7, and 9 (P<0.05). In the MT group, muscle GSH-T concentrations were lower at week 0 than at weeks 2, 5, 7, and 9 (P<0.001). In the MNT group, muscle GSH-T concentrations were lower at week 0 than at weeks 2, 7, and 9 (P<0.001; P=0.002: P=0.05, respectively). There were no weekly differences in muscle GSH-T concentrations for any of the yearlings.

Discussion

Yearlings did not begin their exercise training with higher levels of oxidative stress in muscle or blood as compared to the mares as originally hypothesized. There were significant changes to many of the markers in the blood and muscle throughout training, but these fluctuations did not always result in an overall significantly different change when comparing weeks 0 and 9. In general, the trained mares had the most adaptive response to exercise training, while the non-trained yearlings had the fewest changes over the eight weeks.

A limitation to this training program was the loss of a week of training at week 4 due to Hurricane Sandy. Power was lost at the training facility for 6 days, and unfortunately due to time restraints the lost week of training was unable to be made up at the end of the study. Detraining effects do occur with inactivity, but typically in horses these detraining effects are not seen until 5-6 weeks of inactivity (Snow and Valberg, 1994; Rivero 2007). In this study, the horses had only been trained for 3 weeks prior to the hurricane, so it might be possible given their limited exercise adaptations at the end of week 3 that the inactivity could have begun to reverse training effects. However, as heart rates were measured consistently through training, and it was found that the horses' heart rates were the same in week 5 as they were in week 3, so the horses may not have been affected by the loss of training in week 4.

Group effects

The main differences between groups were as follows: at week 0, both trained and nontrained yearlings had lower plasma MDA concentrations (P=0.056) and higher muscle GSH-T and NO concentrations (P=0.03) than the MT group. At week 9 plasma NO concentrations were lower in MT than in the YT group (P=0.007). No group effects were seen with erythrocyte GPx activity and GSH-T concentration and muscle MDA concentration and GPx activity in the current study.

Lipid peroxidation and oxidative stress are both believed to increase with age (Dillard et al., 1978; Spiteller 2007), but few equine studies at this time have looked at age and oxidative stress. A prior study by Williams et al. (2008) found no difference in plasma lipid hydroperoxides (LPO; formed in an earlier step of lipid peroxidation than MDA) between mature (12 years) and aged mares (22 years). The current study shows several age effects suggesting the yearlings had lower oxidative stress (as evident by lower plasma MDA and higher muscle NO) and higher antioxidant status (as evident by higher muscle GSH-T) prior to training.

Concentrations of markers in muscle and blood did not typically correlate in this study, for example with MDA, there were no differences between groups in muscular MDA concentrations, but there were in plasma concentrations. Prior oxidative stress work has typically assumed the main site of ROS production to be the mitochondria of skeletal muscle cells (Powers and Jackson, 2008). However, more recent research suggests that this may not be true. Several reports have shown that ROS production in mitochondria is higher in state 4 (basal) respiration than in active state 3 respiration,

when maximal ADP is stimulated (Di Meo and Venditti, 2001; Adhihetty et. al., 2005; Anderson and Neufer 2006). Other sites in the cell where ROS are produced include the sarcoplasmic reticulum, transverse tubules, and the sarcolemma (Powers and Jackson, 2008). The differences in MDA concentrations in plasma versus skeletal muscle may be that the source for plasma MDA is solely the sarcolemma, the cell membrane of the skeletal muscle, and not in fact the mitochondria.

The yearlings had lower plasma NO than the mares prior to training. Nyberg et al. (2012) found that aging reduce NO bioavailability in older humans (~64 years old) as compared to young humans (~23 years old), as evidenced by measuring NO metabolites in arterial and venous circulation and the muscle interstitium. Nitric oxide is highly reactive with superoxide, so it stands to reason that if oxidative stress increases with age, then the amount of nitric oxide could decrease with age as higher levels of superoxide react with the nitric oxide. This theory could explain the higher NO concentration found in the skeletal muscle of the yearlings prior to training when compared to the mares. After training, the lower plasma NO concentrations in the trained mares appear to be an effect of training and improving oxidative stress status.

Given the lack of equine studies comparing different age groups in skeletal muscle, a comparison with oxidative stress in rat skeletal muscle is mentioned here. No age differences (8 months versus 24 months) were found in MDA and GSH-T in the deep vastus lateralis in one study (Bejma and Ji, 1999), while a second study found evidence of increased lipid peroxidation in aged versus young rates (24 months vs. 4 months) in the deep vastus lateralis and soleus muscles, and increased GSH-T also in the soleus muscle of aged rats (Leeuwenburgh et al., 1994).

A comparative species approach does have its limitations, particularly when looking at equine skeletal muscle versus human and rodent skeletal muscle. Several studies have shown slight species variation in skeletal fiber types and muscle. Differences in myosin heavy chain (MHC) immunohistochemistry between equine skeletal muscle and rodent skeletal muscle have found variations in type IIB muscle fibers. Equine type 2B fibers are comparable to type 2x fibers by IHC methods, while histochemical methods put together type 2a/x fibers as type 2B fibers. Equine type 2B fibers do not correspond with rodent type 2B fibers (Valberg 2014; Linnane et al., 1999). Matoba et al. (1985) compared fiber types of 10 mammalian species, and were able to divide the species into two groups based on the stability of IIA1 fibers to copper inclusion after pretreatment in solutions of varying acidity. While rats, mice, and humans fell into the group with a reciprocal relationship between lability to acid and stability to copper, while horses (along with cats and asses) were in a group where a parallel relationship was found between lability and stability. However, cats and rats were observed in a separate study to have similar concentrations of ATP, phosphocreatine, and inorganic phosphate in the soleus muscle (Kushmerick et al., 1992). Finally, a study by Kelso et al. (1985) compared several biochemical components of the gastrocnemius, soleus, plantaris, and middle gluteal muscle of the horse and rat. Comparisons were based on wet and dry weight, protein, and total creatine concentrations, and researchers found that components such as ATP, phosophocreatine and glycogen are best compared when related to total creatine concentrations and not by weight. Additionally, the equine middle gluteal muscle had the highest glycogen concentration of all the muscles studied

between the horse and rat. Overall evidence points to species differences in the identification of specific fiber types as well as varying levels of biochemical components.

Week effects

The main differences within the groups were as follows: MT group increased muscle MDA and decreased plasma MDA from week 0 to 9 ($P<0.01$). All groups increased plasma NO at week 2 ($P<0.05$) but returned to baseline levels by week 9; all mares increased muscle NO concentration by week 9 ($P<0.03$). The MT and YT groups had increased erythrocyte GPx at weeks 7 and 9 and GSH-T at week 7 ($P<0.05$), while the MNT group had increased erythrocyte GSH-T at weeks 7 and 9 ($P<0.01$). There were no differences in muscle GPx activity. All mares increased muscle GSH-T concentrations by week 9 ($P<0.05$). Overall, the MT group had the most differences by week 9, followed by YT.

Fluctuations were most evident in the concentrations of plasma MDA and NO, particularly in the first 5 weeks of training. The fluctuations in plasma MDA for both trained groups may have been due to a loss of training for 1 week due to the hurricane at week 4; the horses in training may have been already experiencing training adaptations by week 4, and the loss of a week of exercise may have had a detraining effect, although the fact that heart rates in week 3 and 5 were the same suggest that no detraining occurred. The YNT group had increased plasma MDA at week 2 similar to the YT group. As for the increased plasma NO at week 2 for all horses, possibly the difference is due to the assay itself and not due to a difference with the horses. No dietary changes were

made at week 2, and the missed week of training did not occur until week 4. Given the large volume of samples processed, multiple kits were needed for analysis. Though standards were run with each batch of samples, week 2 plasma samples may have had technical issues (incubation times, extraction precision) that impacted concentrations of NO.

While plasma NO concentrations did increase at week 2, there were no significant differences between weeks 0 and 9. The trained yearlings did have a numerical increase in plasma NO concentrations at week 9 as compared to week 0, and trained mares had a numerical decrease in plasma NO at week 9 as compared to week 0, it was not significant. A prior study using horses on a jumping circuit for 5 weeks found plasma NO concentrations steadily increased over 5 weeks (Marañón et al., 2008). The mares and yearlings in the present study may have physiologically been able to keep up with oxidative stress production so that it was not detrimental to them. Research has shown that oxidative stress is most evident in animals in a disease state or compromised physiological functions. The current study did not have any sick or unhealthy horses (to the researcher's knowledge and observations) so the lack of change for plasma NO may simply be because all horses were healthy.

The MT group appears to have benefited the most from training in terms of lowered oxidative stress and increased antioxidant status. In the circulatory system, plasma MDA concentrations decreased and erythrocyte GSH-T concentration and GPx activity increased. Prior human research has found increases in activity of antioxidant enzymes after training (Powers and Jackson, 2008). Research in rats has also found exercise training to increase antioxidant enzyme activity (Venditti and Di Meo, 1996;

Powers et al., 1994). The results in the current study agree with the results in a similar equine study examining training and oxidative stress; Sciliano et al. (1999) found 3 year old stallions had decreased plasma MDA after 70 days of training.

Vitamin E (along with selenium) is an important part of the antioxidant defense system, especially in its role of maintaining the integrity of cellular membranes. Selenium is a component of GPx and can help to protect against vitamin-E deficiencies. The National Research Council (NRC) recommends that an adult, sedentary horse receive 1 IU/kg body weight daily of vitamin E. Adults with light exercise should receive 1.6 IU/kg body weight vitamin E daily, and growing horses should receive 2 IU/kg body weight vitamin E daily (NRC 2007). In the current study, the YNT group received 666 IU/day (or 1.6 IU/ kg body weight vitamin E daily), YT received 987 IU/day (or 2.4 IU/ kg body weight vitamin E daily), and all mares received 483 IU/day of vitamin E(or 0.88 IU/ kg body weight vitamin E daily). Unfortunately, while the trained yearlings did receive sufficient amounts of vitamin E, the mares, and particularly the trained mares, did not. However, the trained mares did increase erythrocyte GSH-T concentrations and GPx activity by week 7 and with prior work showing that training improves antioxidant status, it appears that training despite the lower levels of vitamin E did not lead to a decrease in their antioxidant status. The trained mares did decrease in erythrocyte GSH-T concentration from week 7 to 9, which may have been due to both the increased intensity and duration of the exercise training they experienced in the final two weeks, and the low vitamin E. With sufficient vitamin E levels the mares may have sustained their higher GSH-T concentrations through week 9. An unfortunate limitation of the study was that blood levels of vitamins E could not be measured due to financial constraints of the

project. Additionally, dietary selenium could not be measured in the hay analysis and was therefore not calculated, which could have contributed to the mares decrease in GSH-T concentrations.

Though improvements were found in the circulatory system of the trained mares, in the skeletal muscle concentrations of MDA, NO, and GSH increased, while GPx activity did not change. These results again indicate that the source of circulatory ROS and antioxidants is not just the skeletal muscle, contrary to long held beliefs (Powers and Jackson, 2008). Other potential sources during exercise could be the heart, lungs, white blood cells, and blood vessels. Comparison with rat skeletal muscle finds a variety of results. One study found in rats that after exercise training for eight weeks (5 days/week) female rats had increased MDA in the gastrocnemius muscle while male rats had no difference, but all rats had decreased NO in the gastocnemius (Balci and Pepe, 2012). Another study with male rats that were trained for 10 weeks found no difference lipid peroxidation of the deep vastus lateralis and soleus muscles, increased GPx activity in the deep vastus lateralis, and decreased GSH-T in the soleus but not the deep vastus lateralis muscle (Leeuwenburgh et al., 1994). Nyberg et al., (2012) found that active older adults had higher skeletal muscle protein levels of NO synthase as compared to sedentary older adults. A study in rats found increased levels of total GSH after training in the gastrocnemius muscle (Balci and Pepe, 2012).

Trained yearlings had increased antioxidant status in the circulatory system only, as shown by increased erythrocyte GPx activity and GSH-T concentration. Williams et al. (2008) found no effect of age or training on erythrocyte GPx activity, but did find increased erythrocyte GSH-T concentration after acute exercise after 8 weeks of training,

Avellini et al. (1999) found decreased erythrocyte GPx after 60 days of training, and Marañón et al. (2008) found decreased erythrocyte GPx in jumpers after 5 weeks of competing. Increased GPx in lymphocytes were found after 70 days of training in horses, but it is important to note that the horses were supplemented with vitamin E as well (Siciliano et al., 1999). In the current study, while the yearlings were not supplemented with Vitamin E, their dietary levels were sufficient for the exercise they were involved in. But, similar to the mares that were trained, the trained yearlings had a decrease in erythrocyte GSH-T from week 7 to 9; this may have been due to the increased intensity and duration of the exercise training they experienced in the final two weeks. In those final two weeks of training, supplementing with vitamin E may have maintained GSH-T concentrations and potentially increased GPx activity.

The MNT and YNT groups both had fluctuations in marker concentrations over the training period. While by week 9 the YNT group had no differences in markers as compared to week 0, the MNT group actually had increased muscle GSH-T and NO concentration by week 9 and increased erythrocyte GSH-T through week 7 and 9. The YNT group did perform bi-weekly GXTs, and this sudden change from pasture life to exercise may have resulted in more membrane lipid peroxidation and leakage of MDA into circulation. In other words, their novelty to exercise prior to training may have had an impact. As for the MNT group, they too had the biweekly GXTs, which might have allowed for minor adaptations to exercise. A further explanation may be the sensitivity of the assays in detecting changes of muscular oxidative stress and antioxidants. Several researchers have proposed that while oxidative stress does contribute to aging, the relative amounts of ROS produced in mammals is relatively low (Barja, 2004). If ROS

production was much higher, then aging would occur faster and result in shorter life spans. However, it is thought that rate of mitochondrial ROS generation is inversely correlated with maximum longevity. While there are no defined amounts of ROS based on mammalian size, given the relative size of the mares (559±12.7 kg at week 0) and the long life of horses in general, the relative ROS production in a healthy horse may be rather low. Combined with assay sensitivity in readings, differences in muscle ROS concentrations and antioxidant activity could be due to the low concentrations in the horse's bodies and difficulty reading those concentrations, thus allowing for fluctuations and changes in concentrations.

Conclusion

The yearlings did not have higher levels of oxidative stress than the mares prior to exercise training as hypothesized, but appeared to have lower levels of oxidative stress and higher antioxidant status. The mares had higher plasma lipid peroxidation and lower levels of muscle glutathione and nitric oxide, evidence of higher oxidative stress levels prior to training. Exercise training improved antioxidant status in the trained mares and yearlings, with more significant effects seen in the trained mares. However, markers in the circulatory system and the skeletal muscle did not always have similar changes, indicating that other areas in the body may be responsible for ROS and antioxidant production, particularly during exercise training. The non-trained horses did improve their antioxidant status and reduced levels of oxidative stress, suggesting that biweekly workouts can have an impact on oxidative stress. While the trained yearlings did

improve their antioxidant status, their age appears to have been more important in their lowered oxidative stress than exercise training. Further research is needed to explore other possible areas in the body for ROS and antioxidant production, as well as continuing research looking at the relationship between age and oxidative stress.

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Figure Captions

Figure 1. Depiction of data from the Polar Heart rate Program as typically seen in a training session. Time for training session is shown on the x-axis, and % of heart rate max is shown on the y-axis. Each colored line represents a horse engaging in the training session.

Figure 2. (A) Concentrations of plasma malondialdehyde (MDA) at weeks 0, 2, 5, 7, and 9 for mature non-trained (M NT), mature trained (M T), young non-trained (Y NT), and young trained (Y T). Letters a,b denote overall differences between weeks at $P<0.007$. Letters m,n denote differences between groups within individual weeks at $P<0.05$; (**B)** Concentrations of skeletal muscle malondialdehyde (MDA) at weeks 0, 2, 5, 7, and 9. Letters x,y denote differences between weeks within O T at $P<0.007$. All data is presented as the means ± SE.

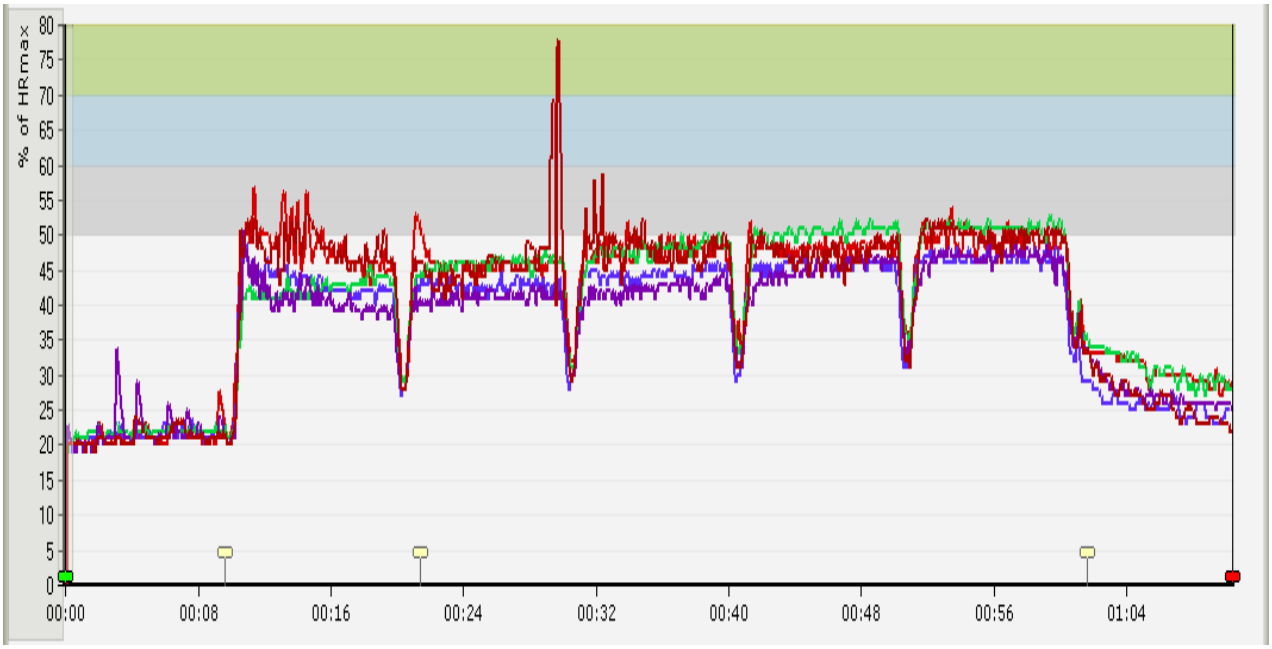
Figure 3. (A) Concentrations of plasma nitric oxide (NO) at weeks 0, 2, 5, 7, and 9 for mature non-trained (M NT), mature trained (M T), young non-trained (Y NT), and young trained (Y T). Letters a,b denote overall differences between weeks at $P<0.01$. Letters x,y denote differences between weeks within individual groups at $P<0.02$; **(B)** Concentrations of skeletal muscle nitric oxide (NO) at weeks 0, 2, 5, 7, and 9. Letters a,b denote overall differences between weeks at $P<0.01$. Letters x,y, and z denote differences between weeks within individual groups at $P<0.003$. All data is presented as the means ± SE.

Figure 4. (A) Activity of erythrocyte glutathione peroxidase (GPx) at weeks 0, 2, 5, 7, and 9 for mature non-trained (M NT), mature trained (M T), young non-trained (Y NT), and young trained (Y T). Letters a,b denote overall differences between weeks at $P<0.04$. Letters x,y denote differences between weeks within individual groups at $P<0.02$; **(B)** Activity of skeletal muscle glutathione peroxidase (GPx) at weeks 0, 2, 5, 7, and 9. All data is presented as the means ± SE.

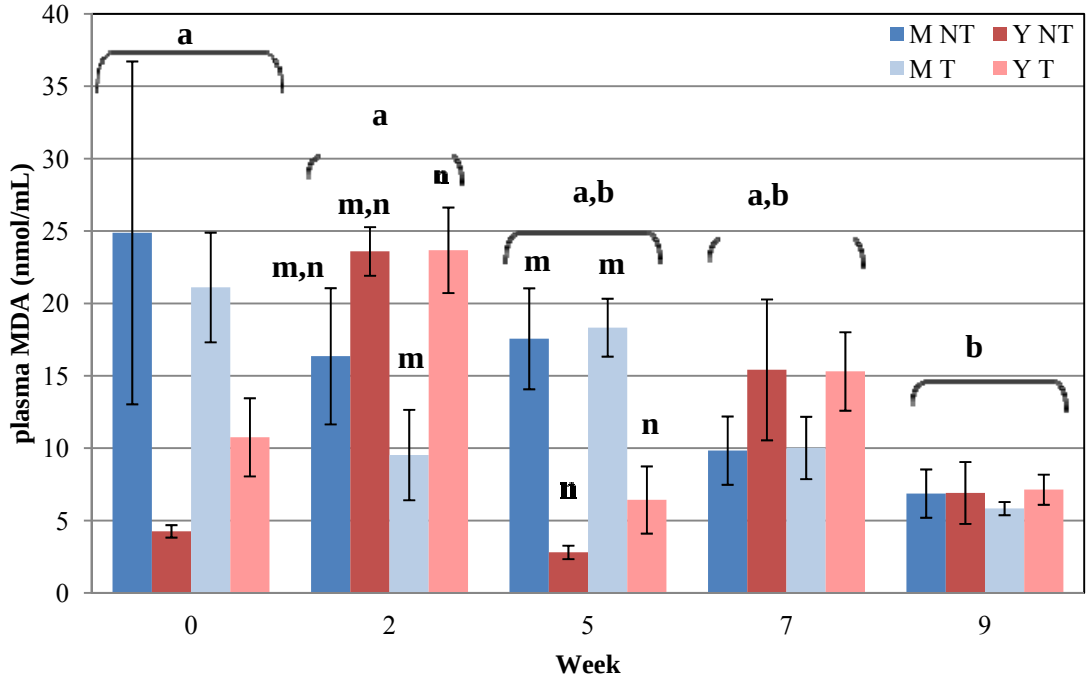
Figure 5. (A) Concentrations of erythrocyte total glutathione (GSH-T) at weeks 0, 2, 5, 7, and 9 for mature non-trained (M NT), mature trained (M T), young non-trained (Y NT), and young trained (Y T). Letters a,b denote overall differences between weeks at $P<0.04$. Letters x,y denote differences between weeks within individual groups at $P<0.04$; **(B)** Concentrations of skeletal muscle total glutathione (GSH-T) at weeks 0, 2, 5, 7, and 9. Letters a,b denote overall differences between weeks at $P<0.05$. Letters x,y denote differences between weeks within individual groups at $P<0.04$. Letters m,n denote differences between groups within individual weeks at $P<0.05$. All data is presented as the means ± SE.

Figures

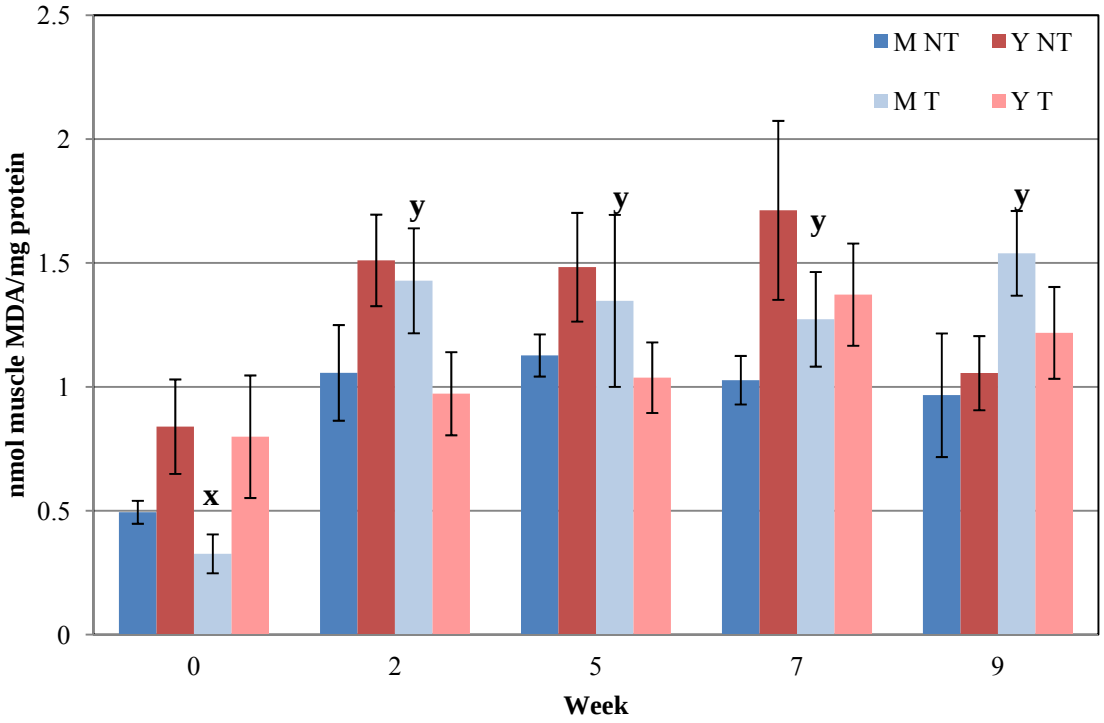
Figure 1.



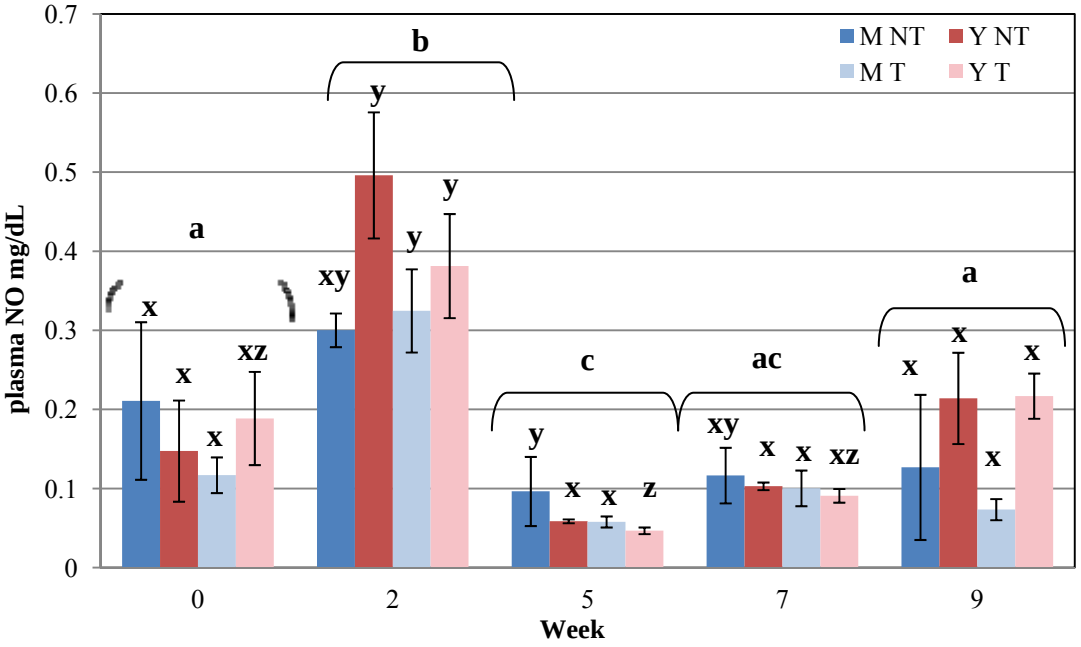
2A.


plasma MDA (nmol/mL)
Week
M NT
Y NT
M T
Y T
a
a
a,b
a,b
b
m,n
m,n
m
n
m
m
n
n
0
2
5
7
9
0
5
10
15
20
25
30
35
40

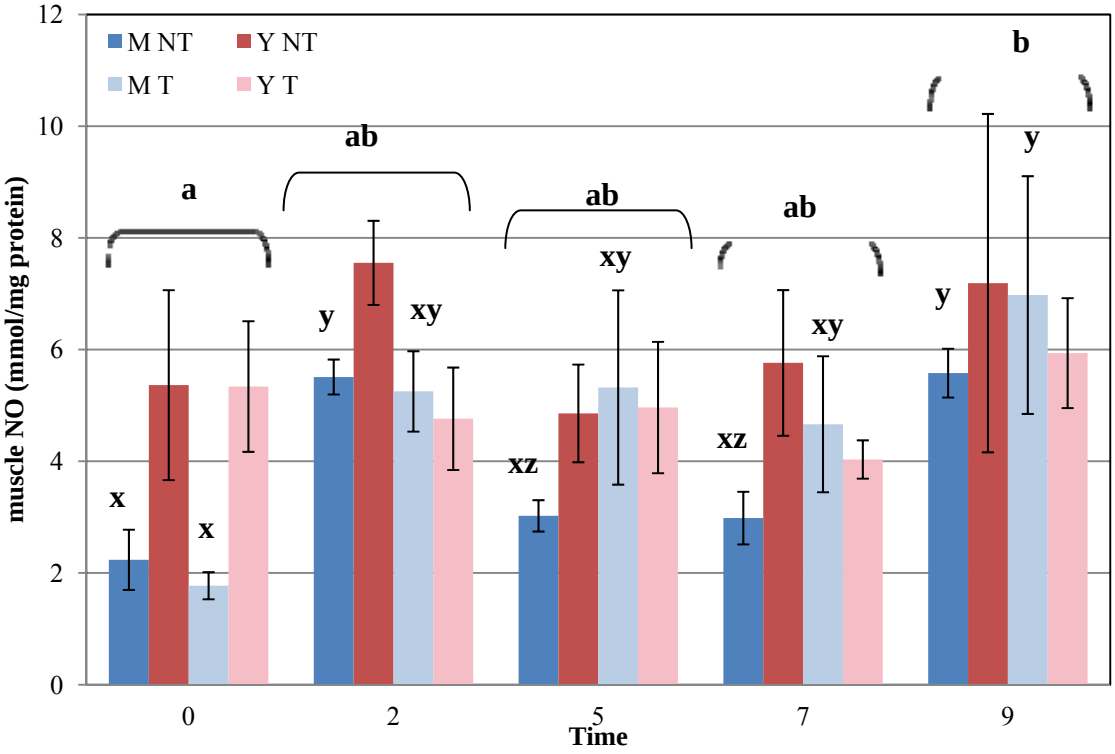
2B.


nmol muscle MDA/mg protein
Week
M NT
Y NT
M T
Y T
x
y
y
y
y
0
2
5
7
9
0
0.5
1
1.5
2
2.5

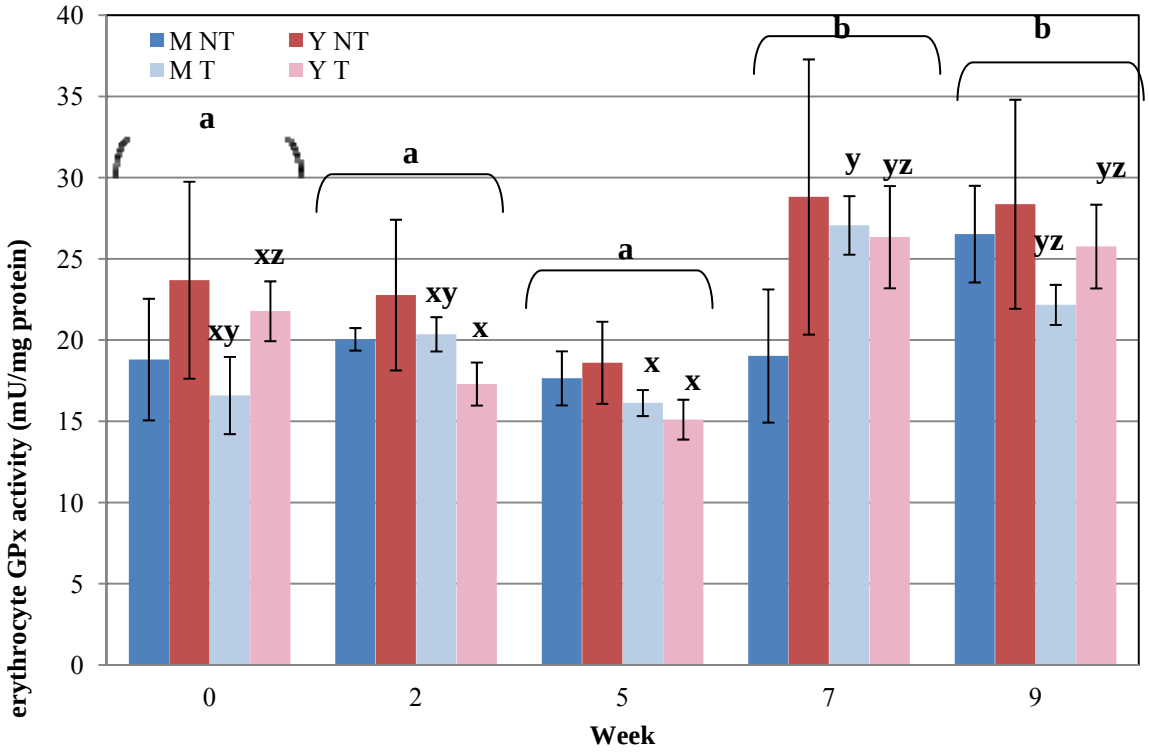
3A.


M NT
Y NT
M T
Y T
plasma NO mg/dL
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
2
5
7
9
Week
a
b
c
ac
a
x
x
x
xz
xy
y
y
y
y
x
x
x
z
xy
x
x
xz
x
x
x
x

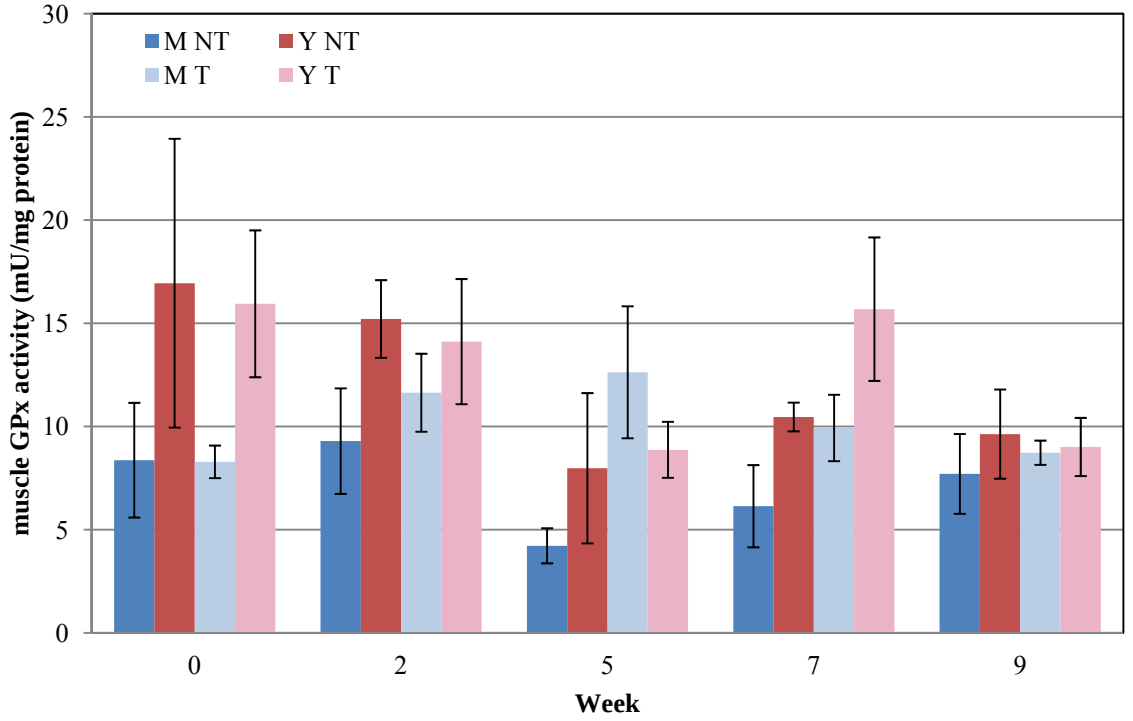
3B.


M NT
Y NT
M T
Y T
muscle NO (mmol/mg protein)
12
10
8
6
4
2
0
0
2
5
7
9
Time
a
ab
ab
ab
b
x
x
y
xy
xz
xy
xz
xy
y
y
y

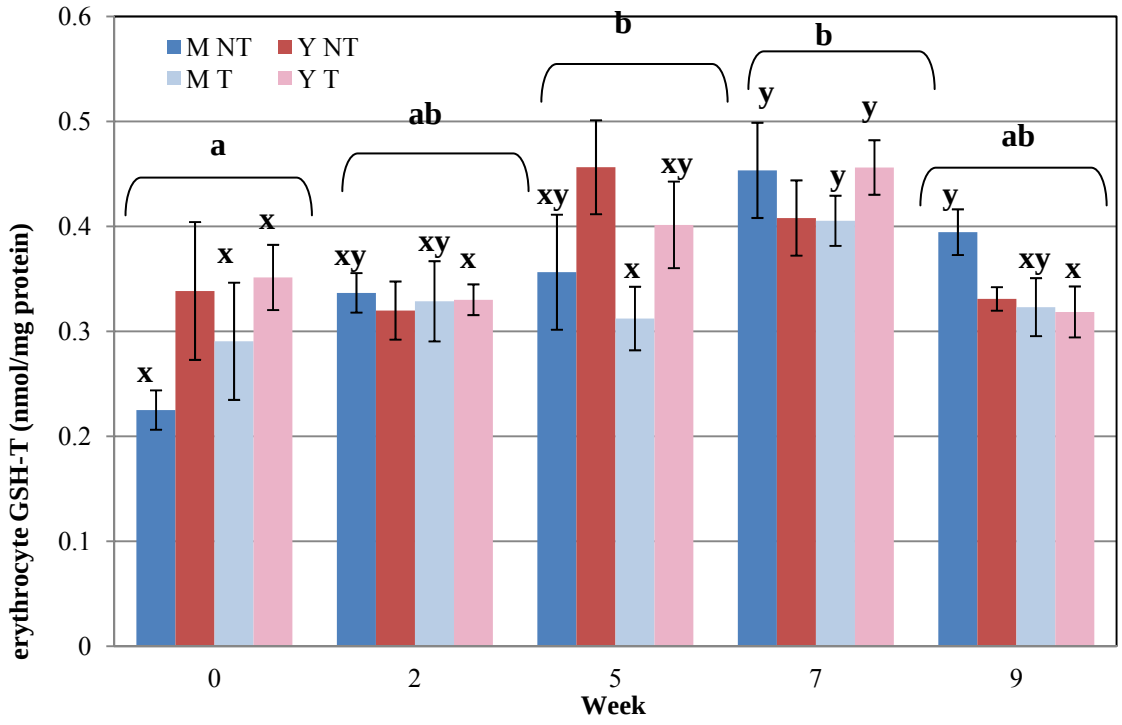
4A.


M NT
Y NT
M T
Y T
erythrocyte GPx activity (mU/mg protein)
Week
0
2
5
7
9
a
a
a
b
b
xy
xz
xy
x
x
x
y
yz
yz
yz

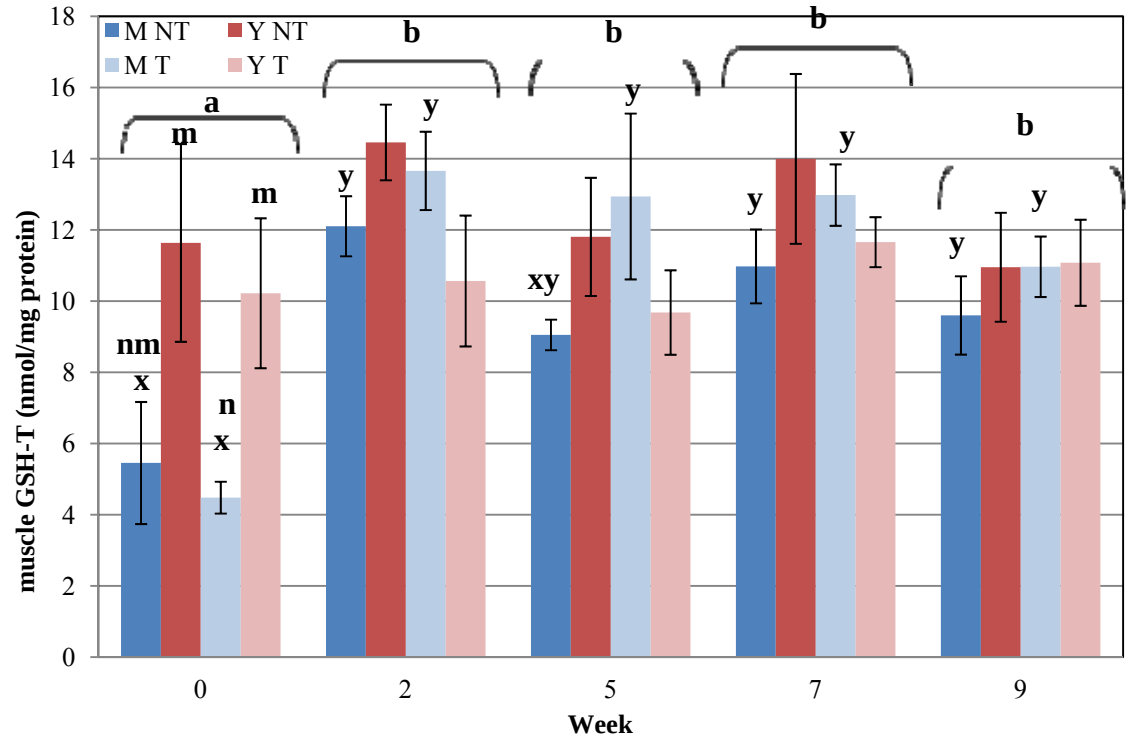
4B.


M NT
Y NT
M T
Y T
muscle GPx activity (mU/mg protein)
Week
0
2
5
7
9

5A.


M NT
Y NT
M T
Y T
erythrocyte GSH-T (nmol/mg protein)
Week
0
2
5
7
9
0.1
0.2
0.3
0.4
0.5
0.6
a
ab
b
b
ab
x
xy
y

5B.


M NT
Y NT
M T
Y T
muscle GSH-T (nmol/mg protein)
Week
0
2
5
7
9
2
4
6
8
10
12
14
16
18
a
b
b
b
b
nm
n
m
x
xy
y

Tables

Table 1. Nutrient composition of grain for mature mares and yearlings. Nutrients are expressed on a 100% dry matter basis[a].

Nutrient[b]	Ultra - Broodmare Yearling	Ultra - Active Textured
DE, Mcal/kg	2.94	3.17
CP, %	14.8	12.2
Fat , %	6	5.5
Fiber – max, %	10.1	5.1
Lysine, %	0.7	0.57
Ca – min, %	0.5	0.6
Ca – max, %	0.9	0.8
P – min, %	0.5	0.5
P – max, %	0.7	0.7
Mg, %	0.21	0.19
K, %	0.8	0.61
Na, %	0.36	0.38
Fe, ppm	163	128
Zn, ppm	206	185
Cu, ppm	64	40
Mn, ppm	134	115
Cl, %	0.53	0.6
S, &	0.27	0.21
I, ppm	0.77	0.5
Co, ppm	0.27	0.2
Se, ppm	1.17	0.86
Mo, ppm	0.8	0.6
Starch – max, %	25	40
Vitamin E, IU/kg	440	236
Vitamin A, IU/kg	23502	17097

[a]Analyses were provided by Pennfield Feed, Lancaster, PA.

[b]DE, digestable energy; CP, crude protein.

Table 2. Nutrient composition of hay for mature mares and yearlings. Nutrients are expressed on a 100% dry matter basis[a].

Nutrient[b]	Timothy Grass	Alfalfa Orchard Grass Mix
CP, %	8.7	16.4
ADF, %	40.5	38.1
NDF, %	62.1	52.6
NFC, %	23.2	22.8
Starch, %	0.2	1.2
WSC, %	12.4	8.9
ESC, %	7.2	6.2
Fat, %	2.1	2.3
Ash, %	5.88	9.3
TDN, %	59	56.5
Ca, %	0.71	0.60
P, %	0.23	0.34
Mg, %	0.14	0.26
K, %	1.42	2.76
Na %	0.30	0.02
Fe, ppm	66	133
Zn, ppm	17	18.5
Cu, ppm	6	9.5
Mn, ppm	24	29.5
Mo, ppm	1.8	1.55
Se, ppm[c]	0.03	0.30
S, %	0.13	0.19
Chloride Ion, %	0.12	0.35
Lysine, %	0.34	0.71
DE, Mcal/lb	0.97	0.98
Vitamin E, IU/kg[c]	19.5	16.5
Vitamin A, IU/kg[c]	21340	26000

[a]Analyses were performed by Dairy One DHIA Forage Testing Laboratory, Ithaca, NY.

[b]CP, crude protein; ADF, acid detergent fiber; NDF, neutral detergent fiber; NFC, nonfiber carbohydrates; WSC, water soluble carbohydrates; ESC, simple sugars; TDN, total digestible nutrients; DE, digestible energy.

[c]Nutrients values were obtained from NRC (1989) estimations.

Table 3. Nutrient intake of mares throughout the trial and yearlings both untrained or pre-training and yearlings after they were in the third week of training. These nutrient intakes were balanced to meet NRC recommended intakes for horses at this status.

Nutrient[b]	Yearlings, untrained & pre-training	Yearlings, training	Mature Mares
Total feed, lb	18.5	20.0	21.0
DE, Mcal/lb	18.9	20.9	21.8
CP, %	1027	1127	877
Ca, g	55	61	68
P, g	26	30	27
Cu, mg	101.3	129.3	103.6
Zn, mg	354	495	392
Se, mg	2.51	3.31	1.45
Mn, mg	340	430	354
I, mg	0.5	0.8	0.7
Lysine, g	41.3	46.0	35.5
Mg, g	17	19	14
K, g	154	159	124
Vitamin E, IU/day	666	987	483
Vitamin A, IU/day	191,246	202,602	198.274

[b]DE, digestible energy; CP, crude protein.

Table 4. Comparisons of body weights (kg) and body condition scores (BCS) for the mature mares and yearlings at weeks 0, 2, 5, 7, and 9 of training. Data are presented as the mean ± SE.

	Mares		Yearlings	
Week	**Kg**	**BCS**	**Kg**	**BCS**
0	559±12.7	6.1±0.2	407±10.5	5.5±0.1
2	542±14.2	6.2±0.2	408±9.9	5.5±0.1
5	543±12.2	6.0±0.2	413±8.4	5.4±0.2
7	537±12.6	6.1±0.2	419±7.6	5.5±0.1
9	534±12.3	6.0±0.3	425±8.4	5.5±0.1

Table 5. Average maximum speed obtained (m/s) and time to completion (min) of graded exercise tests (GXTs) for trained and non-trained mares and yearlings before exercise training (PRE) and at weeks 2, 5, and 7. Data are presented as the mean ± SE.

	Trained Mares		**Non-trained Mares**		**Trained Yearlings**		**Non-trained Yearlings**	
Week	**m/s**	**min**	**m/s**	**min**	**m/s**	**min**	**m/s**	**min**
PRE	9.2±0.2	6.6±0.2	9.2±0.2	6.4±0.2	9.6±0.1	7.0±0.1	9.7±0.2	7.2±0.2
2	9.6±0.2	7.0±0.2	9.2±0.2	6.7±0.2	10.1±0.1	7.4±0.2	10.0±0.3	7.3±0.3
5	9.6±0.3	6.9±0.3	9.5±0.3	6.9±0.3	10.6±0.2	7.9±0.2	9.8±0.2	7.3±0.1
7	10.2±0.4	7.6±0.4	9.7±0.4	7.0±0.5	11.0±0.3	8.3±0.3	9.7±0.3	7.1±0.4

CHAPTER FIVE: **The effect of oxidative stress on equine skeletal muscle and blood after acute exercise in mature horses and yearlings before and after exercise training**

ABSTRACT

The hypothesis of this study was that yearling would have higher levels of oxidative stress in the blood and muscle as measured by malondialdehyde (MDA), total glutathione (GSH-T), glutathione peroxidase (GPx), and nitric oxide (NO), cortisol and creatine kinase (CK) than mares after a single bout of intense exercise; after training the mares and yearlings would have similar levels of oxidative stress after a second bout of intense exercise. Ten yearling Standardbred fillies (18 ± 2.4 months) and 10 mature Standardbred mares (13 ± 2.1 years) ran a repeated sprints exercise test (RSET1) on a treadmill. After 8 weeks of training, all 20 horses ran another treadmill test (RSET2). Blood and muscle samples were taken before and periodically after each RSET. Blood samples were analyzed for hematocrit (Hct), total protein (TP), MDA, NO, GPx, GSH-T, cortisol, and CK; muscle samples were analyzed for MDA, NO, GPx, and GSH-T. Analyses were done in the context of a linear mixed model with random intercepts for horse, and data is presented as mean ± SE ($P<0.05$). In RSET1 yearlings had lower concentrations of TP, Hct, plasma MDA and higher concentrations of muscle MDA and GSH-T, erythrocyte GSH-T and higher erythrocyte GPx activity than the mares ($P<0.05$). In RSET2 few differences existed between age and treatment groups. In RSET2, the trained mares had lower plasma TP, plasma MDA, muscle GSH-T and higher muscle

MDA, and NO as compared to RSET2. Trained yearlings had higher muscle MDA (only at 2H) and NO and erythrocyte GPx (at POST and 30) in RSET2 as compared to RSET1. Yearlings started with lower oxidative stress and higher antioxidant status, but after training mares had improved so that few differences were seen between groups in RSET2. While age was the important defense for the yearlings, training was the best way for the mares to improve oxidative stress and antioxidant status.

Introduction

Many important physiological processes require oxidative stress, such as the production of superoxide by phagocytosing cells to kill invaded bacteria, regulation of cell death pathways by hydrogen peroxide, and the role of reactive oxygen species (ROS) as mediators of inflammatory responses or metabolic regulators (Brigelius-Flohe 2009; Franco et al 1999; Powers et al., 2011; Powers et al., 2010). Oxidative stress is not always considered beneficial. It is well documented that exercise can induce oxidative stress (Dillard et al., 1978; Powers and Jackson, 2008), and high, chronic levels of ROS have been linked to muscle damage and soreness following exercise (Arent et al., 2010; Urso and Clarkson, 2003; Clarkson and Thompson, 2000). Malondialdehyde (MDA) is a common marker of lipid peroxidation (Kirschvink et al., 2008; Powers and Jackson, 2008). Lipid peroxidation begins with hydroxyl radicals scavenging a hydrogen atom from a lipid and ultimately cause a cascading chain lipid peroxidation that can lead to damage to lipid bilayer and breaking of the membrane. Nitric oxide (NO), though well known for its role in vasodilation, also contributes to oxidative stress, as it reacts

extremely quickly with superoxide forming peroxynitrite, which can damage DNA and proteins (Stamler and Meissner 2001; Powers and Jackson 2008). Antioxidants are often thought of as the defense system again excessive levels of oxidants. Two such examples are the antioxidants glutathione (GSH) and glutathione peroxidase (GPx), which work in a cycle to reduce hydrogen peroxide to two water molecules.

In addition, oxidative stress is linked with aging (Anandan et al., 2013; Spiteller 2007; Barja 2004; Yu, 1996; Dillard et al., 1978), with increased rates of lipid peroxidation and increased generation of reactive oxygen species (ROS). Though the association with aging has been made, the effect of oxidative stress on maturation is not clear. It has been theorized that since children endure a greater oxygen cost during exercise, as well as rely more on aerobic metabolism, that they may have higher levels of oxidative stress (Cooper et al., 2004). Additionally, studies in children have seen an increase in oxidative after exercise (Bentitez-Sillero et al., 2011; Gougoura et al., 2007; Santos-Silva et al., 2001), but comparisons with adults and exercise have not been studied.

The prevalence of oxidative stress as a result of exercise (both acute aerobic bouts and endurance) have been well documented in the horse (Lamprecht and Williams 2012; Ceylan et al 2009; Lamprecht et al., 2009; de Moffarts et al., 2006; Marlin et al., 2002; Matsuki et al 2001; Ono et al., 1990). The effect of age on levels of oxidative stress in the horse is not as well known; only one study has looked at two different ages of horses (mature and old) and no difference was found in erythrocyte total GSH or GPx after a bout of acute exercise (Williams et al., 2008). However, the age of a horse undergoing exercise does affect other physiological aspects, such as cortisol, maximal oxygen uptake,

and maximal heart rate (Betros et al., 2002; Horohov et al., 1999; McKeever and Malonowski, 1997) so it is likely that this also applies to oxidative stress. Young horses (18-24 months) have not been studied for levels of oxidative stress, both at rest and after exercise, but given that they are undergoing a large amount of growth and development during this time, oxidative stress could play an important role.

Damage to muscle fiber cells causes the release of creatine kinase (CK) from these muscles. In the horse, CK activity has typically been shown to increase after exercise (Hargreaves et al., 2002; Lejeune et al., 2010; Serteyn et al., 2010), though others have found little to no change in CK activity after exercise (Kedzierski and Bergero, 2006; Dalh et al., 2006: Seeherman and Morris 1990). Again, the effect of maturation has not been looked at with CK. Coristol released by the adrenal cortex in times of stress, such as with exercise. Multiple equine studies have documented peak cortisol levels 30-60 minutes after exercise (Gordon et. al., 2007; McKeever 2002; Nagata et al., 1999). The effects of age on cortisol have been studied in the aged horse (>20 years; Horohov et al., 1999; Malinowski et al., 2006) population, but studying mature horses with the maturing horse again has not been examined.

The hypothesis of this study was that young exercising horses will have higher levels of oxidative stress and lower antioxidant status as measured by malondialdehyde (MDA), total glutathione (GSH-T), glutathione peroxidase (GPx), and nitric oxide (NO), as well as higher cortisol and creatine kinase (CK) than the mature exercising mares when challenged to a single bout of intense exercise. After 8 weeks of exercise training, when challenged to a second bout of intense exercise, the trained yearlings and mares will have similar levels of MDA, GSH-T, GPx, NO, cortisol, and CK.

Materials and Methods

Animals and Experimental Design

The Rutgers University Institutional Animal Care and Use Review Board approved all methods and procedures used in this experiment. Ten yearling Standardbred fillies (Y; 18 ± 2.4 months) and 10 mature Standardbred mares (M; 13 ± 2.1 years) were split into two groups; trained (T; 7 mares, 7 yearlings) and untrained (NT; 3 mares, 3 fillies). Therefore the four groups in this study were yearlings trained and non-trained (YT and YNT, respectively) and mares trained and non-trained (MT and MNT, respectively). All the mares were healthy, but unfit (not undergoing consistent forced exercise in a two to three month period prior to the study). All the yearlings were healthy, unfit, but also novel to exercise; prior to the start of the study they were acclimated to the equine exerciser and high speed equine treadmill. All twenty horses were housed at the Rutgers University Equine Facilities on 3-acre exercise dry-lots; the mares remained outside continuously, while the yearlings were outside 0800-1600, and brought in 4 m x 4 m stalls for the remainder. All horses were fed a commercial grain concentrate twice daily to meet additional nutrient requirements (NRC 2007); the mares were fed UltraActive (1.1 kg/d; Pennfield Feed Inc, Lancaster, PA) and the yearlings were fed Ultra Broodmare/Yearling (1.1 kg/d; ; Pennfield Feed Inc, Lancaster, PA) (Table 1), in addition to forage (Table 2). Nutrient intake for mares and yearlings is shown in Table 3.

Prior to the start of exercise training, a graded exercise test (GXT) was performed in order to determine the speed at maximum heart rate of all exercising horses. During

the GXT, the animals ran on a high speed horse treadmill (Sato I^3) up a fixed 6% grade. The test started at an initial speed of 4 $m \cdot s^{-1}$ for 1 min. Speed was then increased to 6 $m \cdot s^{-1}$ followed by incremental 1 $m \cdot s^{-1}$ increases every 60 s (omitting 5 $m \cdot s^{-1}$), until the horses reached fatigue. Fatigue is defined as the point where the horse could not keep up with the treadmill despite humane encouragement.

At the beginning and the end of exercise training a repeated sprints exercise test (RSET1) was performed by all 20 horses. The RSET began with a 2 minute walk at 1.5 m/s followed by 4 minutes of trotting at 4 m/s. The warm up was followed with 2 minute sprints at 7, 8, 9, and 10 m/s, with a 2 minute walk at 1.5 m/s in between each sprint (Lamprecht et al., 2009).

Trained horses were exercised five days a week for seven weeks using an equine exerciser. Based on maximum heart rates determined by graded exercise tests (GXTs), the intensity of the workout averaged 50-60% of their maximum heart rate for a duration of 20-50 minutes (started with 20 min and worked up in intensity and duration with 50 min at 60% max by week 7). All twenty horses performed GXTs prior to week 0 and on weeks 2, 5, and 7 (for trained horses, this replaced one day on the exerciser for these weeks). At the end of training, all twenty horses performed a second RSET (RSET2).

Sample Collection

Muscle biopsies were taken from the middle gluteal muscle at a depth of 5 cm using a 6 mm Bergström needle (Lindholm and Piehl, 1974). Muscle biopsies were performed in a 5.1 x 5.1 cm square, in alternating corners of the square (as explained in

Chapter 2). The right middle gluteal muscle was used for biopsies. Each muscle biopsy site was given a subcutaneous injection of 5 mL of lidocaine prior to the scalpel incision and muscle biopsy. Samples were taken immediately prior to exercise (PRE), immediately after exercise (POST), and 2H, 6H, and 24H after exercise. After rinsing with saline, muscle samples were immediately frozen (snap frozen) in liquid nitrogen and stored at -80°C until homogenization.

Intravenous catheters (Angiocath, 14 gauge, Becton Dickson, Inc., Parsippany, NJ) were inserted percutaneously into the left jugular using aseptic techniques and local anesthesia at least 30 min prior to the first blood sample to allow for adaptation to the catheter. The catheters were kept patent with sterile heparinized physiological saline solution at a working concentration of 10 U/ml. Blood sample time points for all horses was immediately prior to exercise (PRE), immediately after exercise (POST), 30 minutes, 1 hour, 1.5 hours, 2 hours, 6 hours, and 24 hours after exercise. At each time point, blood samples were taken and immediately aliquoted into pre-chilled 10 mL vacutainer tubes containing either sodium heparin or EDTA (Becton Dickson, Inc., Franklin Lakes, NJ); tubes were inverted and placed back on ice. Blood was also collected into 10 mL non-chilled serum separator tubes (Becton Dickson, Inc., Franklin Lakes, NJ); tubes were not inverted and were placed in racks at room temperature.

Of the two sodium heparin collection tubes, one was immediately analyzed for hematocrits (Hct) using a microhematocrit technique (CritSpin® S-120, Iris Sample Processing, Westwood, MA, USA), and then red blood cells were extracted. Briefly, 500 µL of whole blood was transferred from the collection tube to a microcentrifuge tube and was centrifuged at 2500 x *g* for 5 min at 4°C. The plasma supernatant was discarded and

the remaining erythrocytes were washed with 500 μL of ice-cold 0.9% sodium chloride solution, thoroughly vortexed and centrifuged a second time as described above. The saline supernatant was removed and discarded, and the remaining erythrocytes were lysed with 1 mL of ice-cold distilled deionized water. Samples were then immediately stored at -80°C until further analysis.

The other sodium heparin collection tube was immediately centrifuged for 10 min at 1500 x *g* at 4°C. Plasma was then analyzed for total protein (TP) using digital refractometry (Palm Abbe Veterinary Refractometer, MISCO Inc., Cleveland, OH, USA), and then aliquoted into microcentrifuge tubes. For plasma samples to be used in the thiobarbituric acid reactive substances (TBARS) assay, 100 μL of butylated hydrotoluene (BHT) was added; the sample was mixed well and then stored at -80°C, along with the untreated plasma samples until further analysis.

The EDTA tubes were centrifuged for 10 min at 1500 x *g* at 4°C. Plasma was collected and stored at -80°C. Serum tubes remained at room temperature for approximately 45 minutes to allow for blood clotting and were centrifuged for 15 minutes at 1200 x *g* at 10°C. Serum aliquots were then collected and stored at -80°C.

Sample Analysis

Muscle samples were weighed and approximately 100 mg of wet tissue was then added to 1 mL of 1x phosphate buffered saline (PBS) solution. Samples were homogenized (Potter S Homogenizer; Sartorius-Stedim, Bohemia, NY, USA) for two minutes at 300 rpm and then aliquoted into four microcentrifuge tubes at 1:1 dilutions containing the specific buffer needed for each assay: TBARS (RIPA buffer, Cayman

Chemicals, Ann Arbor, MI, USA), NO (PBS buffer), GSH-T (metaphosphoric acid (MPA) buffer), and GPx (Tris-HCl, pH 7.5, containing 5 m*M* EDTA and 1 m*M* 2-mecaptoethanol). Each aliquot was vortexed, then centrifuged for 10 min at 8160 x *g* at 4°C. After spinning, final aliquots were pipetted into new microcentrifuge tubes and stored at -80°C until assay analysis.

Plasma and muscle homogenates were analyzed for total nitrate/nitrite concentrations using a NO Assay (Bioassays Systems, Hayward, CA, USA; interassay CV = 7.9%; intraassay CV = 4.5%) and MDA concentrations using a TBARS assay (Cayman Chemicals, Ann Arbor, MI, USA; interassay CV = 6.2%; intraassay CV = 1.9%). Erythrocyte lysate and muscle were analyzed for GPx activity and GSH-T concentrations using a Bioxytech GPx-340 (OXIS Research, Portland, OR; interassay CV = 4.1%; intraassay CV =1.44%) and GSH-400 Assay, respectively (OXIS Research, Portland, OR; interassay CV = 4.9%; intraassay = 1.4%). Plasma samples were analyzed for cortisol using a Cortisol ^{125}I RIA Kit (MP Biomedicals, Orangeburg, NY; interassay CV = 3.6%; intraassay = 6.1%). Serum samples were analyzed for CK by Marshfield Labs (Marshfield, WI).

Total protein (TP) concentrations of the erythrocyte lysate and muscle homogenates were determined using the BioRad Protein Assay (BioRad Laboratories, Hercules, CA, USA). A 1:10 dilution was used for muscle samples and a 1:100 dilution was used for erythrocyte lysate samples; for the muscle homogenates, the aliquot was from the homogenate in 1x PBS solution.

All assays were performed according to manufacturer's instructions approximately 2-5 months after collection. All samples were performed in duplicate, and

all assays were read using a micro-plate reader (Spectramax 340, Molecular Devices, Sunnyvale, CA).

Statistical Analysis

Data will be presented as mean ± SE unless otherwise noted. All analyses were done in the context of a linear mixed model with random intercepts for horse. The use of random intercepts for horse helps account for autocorrelation of the observations on an individual horse over time. Significant initial analyses were followed by Tukey's Honestly Significant Difference (HSD) test for pairwise comparisons with significance set at P = 0.05. Tukey's HSD controls the Type I error rate in an individual analysis, but there was no other adjustment for multiple testing. Analyses were done using the R program (statistical environment for analysis; R Development Core Team, 2011).

Results

RSETs

A comparison of the time to completion in RSET1 and RSET2 for all groups, as well as time spent at or above 90% heart rate maximum, is shown in Table 4. All groups did increase their total time running the RSET after training. In addition, prior to exercise training, only 1 mare was able to complete the exercise test (RSET1). After exercise training, 2 trained mares and 4 trained yearlings were able to complete all steps of the RSET2.

Age Effects: Before Training

In RSET1 there was an effect of age on hematocrits (P=0.002). There were no group effects within ages, so results shown are combined for groups within age (Figure 1A). The yearlings had a lower Hct than the mares at PRE, 30, 1.5H, 2H, and 24H (P< 0.03). There was a trend in the yearlings for lower levels of Hct than the mares at 1H and 6H (P=0.06; P=0.08, respectively). There was an age effect for TP (P<0.0001). There were no group effects within ages, so results shown are combined for groups within age (Figure 2A). The yearlings had lower levels of TP than the mares at PRE, POST, 30 , 1H, 1.5H, 2H, 6H, and 24H (P<0.0001).

There was an effect of age on plasma MDA concentration in RSET1 (P=0.003). There were no group effects within ages, so results shown are combined for groups within age (Figure 3A). The mares had higher plasma MDA concentration than the yearlings at PRE, 30, 1H, 1.5H, 2H, 6H, and 24H (P < 0.01). In muscle MDA in RSET1, there was an effect of age (P=0.002; Figure 3B). The yearlings had higher muscle MDA concentrations than the mares at PRE, 2H, 6H, and 24H (P=0.03; P=0.01; P=0.03; P=0.03, respectively), and there was a trend for the yearlings to have higher muscle MDA concentrations than the mares at POST (P=0.07).

In plasma NO there was an effect of sample time by age (P<0.0001). There were no group effects within ages, so results shown are combined for groups within age (Figure 4A). The yearlings had higher concentrations of plasma NO at 24H as compared to the mares (P=0.02). There was an effect of age on muscle NO (P<0.0001); yearlings

had higher muscle NO concentration at PRE, POST, 2H, 6H, and 24H as compared to the mares ($P<0.02$; Figure 4B).

For erythrocyte GSH-T, there no group effects within ages, so results shown are combined for groups within age (Figure 5A). The yearlings had higher concentrations of erythrocyte GSH-T at PRE, POST, and 2H ($P<0.05$) than the mature mares. There was also age effect for muscle GSH-T ($P=0.001$). Again, there were no group effects within ages, so results shown are combined for groups within age (Figure 5B). The mares had lower concentrations of muscle GSH-T at PRE, 2H, 6H, and 24 H than the yearlings ($P<0.03$). There were no effects of sample time on muscle GSH-T.

There was an effect of age for erythrocyte GPx in RSET1 ($P=0.0002$). There were no group effects within ages, so results shown are combined for groups within age (Figure 6A). The yearlings had higher erythrocyte GPx activity at POST, 1H, 1.5H and 2H ($P<0.02$) than the mares. There was also an effect of age for muscle GPx ($P=0.05$; Figure 6B). The yearlings had higher muscle GPx activity at PRE than the mares ($P=0.02$).

There was an age effect on plasma cortisol concentration ($P=0.01$). There were no differences in sample times between the YT and YNT in RSET1, so their results have been combined (Figure 7A). There was a group effect ($P<0.02$), with the MNT group having higher plasma cortisol concentration than the MT, YT, and YNT at both POST ($P<0.02$) and at 2H ($P<0.03$). At 24H, there was a group effect ($P=0.03$), with the MNT having higher plasma cortisol concentration than the YT or YNT groups ($P<0.01$; $P=0.02$, respectively). There were no age effects in RSET1 for serum CK (Figure 8A).

Age Effects: After Training

In RSET2 there was an effect of group for Hct (P=0.003) and a trend for an effect of sample time by group for Hct (P=0.06; Figure 1B). The MNT group had higher Hct than the YNT, MT, and YT groups at POST, 30, 1H, 1.5H, and 2H (P=0.004; P=0.01; P=0.004; P=0.002; P=0.01, respectively). There was also a group effect at 24H with MNT having higher Hct than YNT and MT (P<0.03). There was an effect of group and sample time by group on plasma TP (P=0.03). At POST, MT had a higher TP concentration than YT (P=0.01) and MNT had a higher TP concentration than YNT and YT (P=0.04; P=0.001, respectively; Figure 2B). At 30 (P=0.03), YT had lower TP than the MNT and MT groups (P=0.03).

There were no differences between the groups in plasma or muscle MDA concentration in RSET2 (Figure 3C). In plasma NO, there was an effect of group and sample time by group on (P=0.04; P=0.003, respectively). There was a group effect at the PRE sample (P=0.02), with the MT group having a lower plasma NO concentration than the YT group (P=0.01; Figure 4C). There was a group effect at the POST sample (P=0.01), with MT having a lower plasma NO concentration than YT and YNT (P<0.001; P=0.04, respectively). There was a group effect at 24H (P<0.0001), with MT and MNT having lower plasma NO concentrations than YT (P<0.0001) and YNT (P<0.0001). There were no differences between the groups for muscle NO concentration.

There were no differences between the groups for muscle or erythrocyte GSH-T in RSET2. In RSET2, there was an effect of group on erythrocyte GPx (P=0.05; Figure

6C). At POST the YNT group had lower erythrocyte GPx activity than the MNT group (P=0.03). There were no differences between the groups for muscle GPx activity.

For plasma cortisol, there was a trend for a group effect at POST (P=0.06), with the MT group having lower cortisol than the MNT and YT groups (P=0.09; P=0.05, respectively; Figure 7B). There were no other differences between groups at the other sample times. There were no group effects in RSET2 for serum CK (Figure 8B).

Exercise Effects: Before Training

Table 4 shows the average time to completion for RSET1; all horses took 19-20 minutes to complete the test. In RSET1 there was an effect of sample time on Hct (P<0.0001) in the mature mares and the young horses , with POST being higher as compared to PRE, 30, 1H, 1.5H, 2H, 6H, and 24H (P<0.001). Total protein had an effect of sample time and sample time by age (P<0.0001). Within the mares, there was an effect of sample time (P<0.001), with plasma TP concentration was higher at POST as compared to PRE, 30, 1H, 1.5H, 2H, 6H, and 24H (P<0.001); plasma TP concentration was also was higher at 24H as compared to PRE, 1H,1.5H, and 2H (P<0.001; P=0.001; P<0.001; P=0.004, respectively). In the yearlings, plasma TP concentration was higher at POST as compared to PRE, 30, 1H, 1.5H, 2H, 6H, and 24H (P<0.001). The yearlings also had a higher TP concentration at 24H as compared with PRE, 30, 1H, and 1.5H (P=0.01; P=0.04; P=0.005; P=0.007, respectively).

In plasma MDA there was an effect of sample time and sample time vs. age (P=0.04; P=0.004, respectively). There was an effect of sample time in the mares for

plasma MDA (P=0.02), with higher plasma MDA at POST as compared to 30 and 2H (P=0.02; P=0.01, respectively). In the yearlings there was an effect of sample time (P=0.03), with higher plasma MDA at 24H as compared to POST and 30 (P=0.02; P=0.03, respectively). There was no effect of sample time on muscle MDA.

In plasma NO there was an effect of sample time (P=0.0003). The mares had a sample time effect (P<0.0001), with lower plasma NO concentrations at 24H as compared to PRE, POST, 30, 1H, 1.5H, and 2H (P<0.001), and lower at 6H as compared to POST and 30 (P=0.05; P=0.01, respectively). The yearlings also had a sample time effect (P<0.0001), with lower plasma NO concentration at 6H as compared to PRE, POST, and 24H (P=0.05; P=0.02; P<0.001, respectively) and higher at 24H as compared to 1H, 1.5H, and 2H (P=0.05; P=0.01; P=0.01, respectively). There was no effect of sample time on muscle NO concentration in RSET1.

There was no effect of sample time on erythrocyte and muscle GSH-T concentrations for RSET1. There was an effect of sample time for erythrocyte GPx activity in RSET1 (P=0.0002). In the mares erythrocyte GPx activity was higher at 24H as compared to PRE, POST, or 1H (P=0.04; P=0.001; P=0.01, respectively). There was no effect on sample time in the yearlings for erythrocyte GPx activity, and no effect for muscle GPx activity for both yearlings and mares.

There was an effect of sample time (P<0.0001) on plasma cortisol concentration. At POST the plasma cortisol concentration was higher as compared to PRE, 2H, 6H, or 24H in all horses (P<0.001); the plasma cortisol concentration also was higher at 2H as compared to 6H (P=0.03) in all horses. For serum CK activity there was an effect of

sample time (P<0.0001). There were no group or age effects, so results are combined (Figure 8A). Serum CK activity was higher at 2H, 6H, and 24H as compared to PRE (P<0.001) and POST (P<0.04), and was higher at 6H as compared to 2H (P<0.001).

Exercise Effects: After Training

Table 4 shows the average time to completion for RSET2; all horses took 20-22 minutes to complete the test. In RSET2, there was an effect of sample time for Hct (P<0.0001). For all groups, Hct was higher at POST as compared to PRE, 30, 1H, 1.5H, 2H, 6H, and 24H (P<0.01). In plasma TP concentrations there was an effect of sample time and sample time by group (P<0.001). With TP, all four groups had an effect of sample time (P<0.0001), with higher plasma TP concentration at POST as compared to PRE, 30, 1H, 1.5H, 2H, 6H, and 24H (P<0.001). In the YT group, there were additional sample effects, with higher plasma TP concentration at 6H as compared to PRE, 30, 1H, and 1.5H (P<0.03) and higher at 24H as compared to PRE and 1.5 (P=0.007; P=0.04, respectively).

In plasma MDA there was an effect of sample time (P=0.001). In the MT, there was an effect of time (P=0.02), with a higher plasma MDA concentration at 30 as compared to 1.5H (P=0.01). There were no differences in muscle MDA concentrations (Figure 3C).

In plasma NO, there was an effect of sample time on the YNT (P=0.03), with lower plasma NO concentrations at 6H as compared to PRE, POST, 30, and 1H (P=0.02; P<0.001; P=0.01; P=0.03, respectively) (Figure 4C).). In the YT group, there was an

effect of sample time (P<0.0001). The YT had higher plasma NO concentrations at POST as compared to 1H, 1.5H, 2H, 6H, and 24H (P < 0.05). The YT had a lower plasma NO concentration at 6H as compared to PRE, 30, 1H, 1.5H, 2H, and 24H (P<0.001). There was an effect of sample time for the MT (P=0.01), with higher plasma NO concentrations at 24H as compared to POST (P=0.02). There was no effect of sample time on plasma NO concentration in the MNT group. There were no differences in muscle NO concentrations.

With erythrocyte GSH-T concentrations there was an effect of sample time by group (P=0.01) but no sample time effects on muscle GSH-T concentrations. For erythrocyte GPx activity there was an effect of sample time (P=0.002). The MT had lower GPx activity at POST as compared to 24H (P=0.03) and the YT had lower GPx at POST and 1.5H as compared to PRE (P=0.03; P=0.08, respectively). There were no effects of sample time in the non-trained groups for erythrocyte GPx activity. In muscle GPx there was an effect of sample time (P=0.04). The MNT had higher muscle GPx activity at 24H as compared to PRE, POST, and 2H (P=0.04; P=0.02; P=0.04, respectively). There were no sample time effects in the yearlings and MT group for muscle GPx activity.

For plasma cortisol there was an effect of sample time (P<0.0001). The MNT had a higher plasma cortisol concentration at POST as compared to PRE, 6H, and 24H (P<0.001), and a higher plasma cortisol concentration at 2H as compared to 6H (P<0.001; Figure 7B). The MT had a higher plasma cortisol concentration at POST as compared to PRE, 6H, and 24H (P<0.001; P<0.001; P=0.03), and a higher plasma cortisol concentration at 2H as compared to 6H (P=0.01). The YT had a higher plasma cortisol

concentration at POST as compared to PRE, 2H, 6H, and 24H (P<0.001), and a higher plasma cortisol concentration at 24H as compared to 6H (P=0.02). The YNT had no difference in sample time for plasma cortisol concentrations. In RSET2 there was an effect of sample time on serum CK activity (P=0.0001; Figure 8B). Serum CK activity was higher at 2H, 6H, and 24H as compared with PRE (P<0.02), and was higher at 6H as compared to POST (P=0.03).

Training Effects

The YT, YNT, and MT had differences in Hct between the RSETs. The YT had higher Hct at 1.5H in RSET1 as compared to 1.5H in RSET2 (P=0.05), the YNT had higher Hct at 2H in RSET1 as compared to 2H in RSET2 (P=0.01), and the MT had higher Hct in RSET1 at 24H as compared to the same timepoint in RSET2 (P=0.01). The MNT group had higher plasma TP concentrations in RSET1 at PRE, POST, 30, 1H, 1.5H, 2H, 6H, and 24H as compared to the same timepoints in RSET2 (P < 0.01; Figure 2C). The MT had higher plasma TP concentrations in RSET1 at PRE, POST, 30, 1H, 1.5H, 2H, 6H, and 24H as compared to the same timepoints in RSET2 (P<0.0001).

The MT and MNT had differences in plasma MDA concentrations between the RSETs (Figure 3D). The MT had lower plasma MDA concentrations in RSET2 as compared to RSET1 at PRE, POST, 30, 1H, 1.5H, 2H, 6H, and 24H (P < 0.0004). The MNT had lower plasma MDA concentrations in RSET2 as compared to RSET1 at PRE, 30, 1H, 1.5H, 2H, 6H, and 24H (P < 0.04). For muscle MDA, the MT had higher muscle MDA concentrations in RSET2 as compared to RSET1 at PRE, POST, and 2H

(P<0.0001). In addition, the YNT had higher muscle MDA concentrations in RSET2 as compared to RSET1 at POST (P=0.05), and the YT had higher muscle MDA concentrations in RSET2 as compared to RSET1 at 2H (P=0.0003).

The MT and YT had differences in muscle NO concentrations between RSET1 and RSET2. The MT had higher muscle NO concentrations in RSET2 at PRE, 6H, and 24H as compared to the same timepoints in RSET1 (P=0.01; P=0.03; P=0.01, respectively) (Figure 4D). The YT had higher muscle NO concentrations in RSET2 at POST and 2H (P=0.01; P=0.03, respectively) as compared to 6H in RSET1.

The MNT and MT had differences in erythrocyte GSH-T concentrations between RSET1 and RSET2. The MNT had higher erythrocyte GSH-T concentrations at PRE and POST (P=0.01; P=0.05, respectively) in RSET1 as compared to the same timepoints in RSET2. In the MT, there was lower muscle GSH-T concentrations at PRE, POST, 2H, and 6H (P<0.03), in RSET1 as compared to the same timepoints in RSET2. The YT had higher erythrocyte GPx activity in RSET1 at POST and 30 as compared to the same timepoints in RSET2 (P=0.002; P=0.05, respectively; Figure 6D).

The MT had a higher plasma cortisol concentration at 2H (P=0.02) in RSET1 compared to RSET2 (Figure 7C) and the MNT had higher plasma cortisol concentrations at POST, 2H and 24H (P=0.02; P=0.03; P=0.05, respectively) in RSET1 as compared to RSET2. There was a trend for the YT to have higher serum CK activity at POST and 2H (P=0.06) in RSET2 as compared with the same timepoints in RSET1.

Discussion

In this study, the age of the horses was a significant factor in their response to acute exercise. The yearlings had lower levels of oxidative stress and higher levels of antioxidants prior to exercise, and after RSET1 had lower levels of exercise-induced oxidative stress than the mares. When challenged to RSET2 after training, the mares post exercise had a reduction in oxidative stress. Training did not have as large an impact on the yearlings, suggesting that their age is the most important defense mechanism against oxidative stress.

Age Effects: Before Exercise

In RSET1 the main age effects were as follows: yearlings had lower concentrations of TP, Hct, plasma MDA and higher concentrations of muscle MDA, muscle GSH-T, erythrocyte GSH-T and higher erythrocyte GPx activity than the mares through the majority of RSET1. Yearlings had higher plasma NO concentrations at 24H as compared to the mares, and had higher muscle GPx activity at PRE as compared to the mares. The MNT had overall higher plasma cortisol concentrations than the MT or either groupsof yearlings. No age effect was seen with serum CK activity.

It is known that aging has an impact on the cardiovascular system; this includes decreases in maximal heart rates, cardiac output, and resting plasma volumes (McKeever and Malinowski, 1997). This in turn can lead to a lack of thermoregulation and increased sweat rates during and after intense exercise. One possible reason that the yearlings had lower concentrations of TP and HCt is that their thermoregulatory response to exercise

was lower than the mares, and had smaller volumes of fluid shifted out of the cardiovascular system towards sweat production. However, a study by McKeever and Malinowski found no differences in total protein between mature horses (5.3±0.8 years) and aged horses (22±0.4 years) at rest and at exercise (1999). Another possible reason for the difference in TP and Hct is that maturity could impact the cardiovascular system. Spensley et al. (1987) found in foals (from age 2 days – 24 weeks) that plasma, red blood cells, total blood, and extracellular fluid are all impacted by maturation. It was shown that while absolute plasma volume, RBC, and total blood volume progressively increased from 2 weeks through 24 weeks, plasma, RBC and total blood volumes decreased relative to body weight increase (Spensley et al., 1987). Horses do not fully mature until ~4 years of age, changes in plasma volume and other aspects of circulation could still occur in yearlings.

The higher levels of GSH-T and GPx in the yearlings could be due to several factors. The first is that the diet of the mares and yearlings differed in concentration of Vitamin E. As mentioned in Chapter 4, while the yearlings had sufficient levels of vitamin E (2.4 IU/daily; NRC recommends 2 IU/kg daily for growing horses), all mares received 0.88 IU/ daily (NRC recommends horses in light exercise receive 1.6 IU/kg daily and adult horses receive 1 IU/kg daily of vitamin E), which, especially for the mares in training, was not a sufficient amount. Lowered vitamin E in the diet could lead to lower GPx activity and GSH-T concentration in the mares. Prior work in horses has found no differences in erythrocyte GSH-T concentrations between mature mares (12 ± 2 years old) and aged mares (22 ± 2 years), also fed slightly different diets (Williams et al., 2008).

A further reason for increased GSH-T concentration and GPX activity in the skeletal muscle is due to age differences in fiber type distribution. Prior work in horses has found adult Standardbreds to have an increase in the percentage of type I and decrease in type IIB fibers in the middle gluteal (Ronéus and Lindholm, 1991), while work in Standardbred foals (6 months – 1 year old) has found an increase in the percentage of type IIA fibers, no change in type I fibers, and a slight decrease in type IIB (Essen-Gustavsson et. al., 1983); these changes continue through 1-2 years of age. The yearlings and mares in the current study therefore probably had different percentages of type I, IIA, and IIB muscle fibers in the middle gluteal, which could have an impact on concentrations of ROS and antioxidants. In studies looking at humans and rodents, levels of antioxidants such as GPx have been found to be higher in type I and type IIA muscles, while ROS production has been found to be higher in type II muscles (Powers 2011; Powers and Jackson, 2008). In rats, one study has found no differences in muscular GSH-T between young and old groups (Bejma and Ji, 1999), while another found increased GSH-T in the soleus muscle of aged rats as compared to younger rats (Leeuwenburgh et al., 1994).

Perez-Navero et al. (2009) found higher rates of lipid peroxidation in pubescent boys as compared to prepubescent boys, suggesting that lipid peroxidation levels can vary with maturation. In the current study, the increased plasma lipid peroxidation in the mares correlates with findings in humans. As for the increased MDA in the muscle of the yearlings, to date no equine studies have examined this, but as mentioned, fiber types have varying levels of ROS production, and it has been reported that type II fibers have

more production of ROS. The yearlings with potentially higher percentages of type II fibers would be a possible reason for higher muscle MDA concentrations.

The MNT group had higher cortisol levels than the MT group, suggesting a large effect of individual variation on plasma cortisol levels. By chance, it seems that the MNT group had individuals with a higher stress response to exercise, resulting in higher plasma cortisol levels post exercise. The yearlings had lower levels of plasma cortisol after exercise than the mares. Prior work in horses looking at the effects of age has analyzed mature horses (either young or middle-aged) with aged horses (25-27 years old). Horohov et al., found that mature horses (~7 years) had higher cortisol during exercise at heart rates of 180 and 200 beats/minute and no differences post exercise (1999). Malinowski et al. looked at three age groups of mares: young (6.8±0.4 years), middle-aged (15.2±0.74 years), and old (27.0±0.72 years); after acute exercise, the young and middle-aged mares had increased cortisol that was elevated for 40 minutes post exercise; old mares had no difference in cortisol levels after exercise (2006). Liburt (2011) found that after acute treadmill exercise, young mares (~7 years) had higher levels of cortisol as compared to old mares (~22 years). While it appears that aged mares have a blunted cortisol response to exercise, in young, growing horses such as in the present study, there also appears to be reduced plasma cortisol after exercise as compared to mature mares. The yearlings may have been better able to cope with the challenge of acute exercise, thus the lowered plasma cortisol response.

Age Effects: After Training

In RSET2 the main group effects were as follows: the MNT had higher Hct than the MT groups and all yearlings through the majority of RSET2. At POST all mares had higher TP than the YT (and MNT was higher than the YNT at POST as well). The MT had lower plasma NO at PRE, POST, and 2H than YT. At POST the YNT had lower erythrocyte GPx activity than the MNT, and there was a trend at POST for MT to have lower plasma cortisol than MNT and YT. There were no group differences for muscle MDA, NO, GSH-T concentrations and GPx activity, plasma MDA concentrations, erythrocyte GSH-T concentrations, and serum CK activity.

The lower plasma NO in the trained mares could be an indicator of two things; one is that the intense final two weeks of training had reduced the bioavailability of NO due to increased scavenging by higher concentrations of superoxide. An additional reason may be an increased level of vasodilation during training in the mares as compared to the yearlings. In addition, all mares still had higher plasma TP than the yearlings at POST exercise, indicating that the mares were more challenged in thermoregulating and shifting fluid to sweat production.

Overall there were much fewer differences between the groups in RSET2. Training could have been one reason for the lack of differences, particularly for the mares. However, there was not improvement in all markers in RSET2. With the training, one would expect the horses to have lowered oxidative stress and higher antioxidant status, as prior studies have shown exercise training to do. A possible limitation is that the RSETs did not match the training in terms of exercise intensity.

While the horses did perform GXTs every two weeks, in order for maximal improvement in RSET2, acute exercise should have been performed more than every two weeks.

Exercise Effects: Before Training

The main exercise effects in RSET1 are as follows: For all horses, Hct, plasma TP, and cortisol was lower at POST and serum CK increased from 2H to 24H. Mares had lower plasma MDA at POST as compared to 30 and 2H, lower plasma NO by 24H, and had higher erythrocyte GPx activity by 24H. The yearlings had lower plasma MDA at 24H as compared to POST and 30 and plasma NO was lower at 6H but higher at 24H. There were no exercise effects for any horses for erythrocyte GSH-T and muscle MDA, NO, GSH-T, or GPx, and yearlings had no exercise effects for erythrocyte GPx.

The predominant exercise-induced changes in the circulatory system of the mares and yearlings were found with plasma MDA and NO. Unlike many prior equine studies (Matsuki et al 2001; Ceylan et al 2009; Marlin et al., 2002), there was not an increase in plasma MDA with exercise. With the plasma NO, the yearlings had a significant increase by 24H while the mares had a significant decrease. Studies have shown a decrease in plasma NO following exercise in mature mares (Lamprecht et al., 2009; Lamprecht and Williams, 2012). It has been theorized that the lack of NO post-exercise is due to its increased uptake by superoxide to form peroxynitrite, and thus increasing levels of oxidative stress (Powers and Jackson, 2008; Nyberg et al., 2012). Additionally, the reduced NO would also be a result of its use in vasodilation and vasobrochiation. The lower plasma NO in the mares could be an indicator that greater levels of superoxide

were present and uptaking nitric oxide, as well as the increased cardiovascular response in the mares, thus reducing NO bioavailability. The yearlings with, the increase in plasma NO at 24 hours, could be an indicator that the yearlings recovered more quickly from the exercise-induced oxidative stress and had a lesser cardiovascular exercise response, and so there was a shorter reduction in bioavailability.

No exercise effects were seen in muscle for any of the oxidative stress marker or antioxidants. Prior research in the equine middle gluteal muscle has reported after exercise elevated total and protein-bound MDA (Matsuki etal., 2001) as well as no change in GPx activity (Kinnunen et al; 2005). This may be due to difference in the duration of the exercise test (25 vs. 20 minutes) or could be due to differences in measurement of MDA. As theorized in Chapter 4, not all ROS and antioxidants in the circulatory system originate in the skeletal muscle. The lack of change in the skeletal muscle could be due to other sources in the body for ROS and antioxidants. The cell membranes of the skeletal muscle cells may not have been damaged sufficiently by lipid peroxidation to cause leaking of various molecules into the circulatory system. Nitric oxide is known to be a major vasodilator and is found in abundance in the circulatory system. Studies in rats have also shown an increase in muscle MDA after exercise (Venditti and Di Meo, 1996) and a decrease in NO in the gastrocnemius (Balci and Pepe, 2012).

In addition, exercise did not have an effect of erythrocyte GSH-T in the yearlings. There was a trend for erythrocyte GSH-T to be higher at 24H post exercise as compared to immediately post exercise. Prior equine studies have shown an increase in erythrocyte GSH-T immediately after acute treadmill exercise (Williams et al., 2008; Lamprecht and

Williams, 2012) and after acute gallop bouts (Chiaradia et al., 1998). Other equine studies have shown no changed in erythrocyte GSH-T after acute exercise (Kinnunen et al., 2005; Kirshvink et al., 2002; Mills et al., 1996), while Marlin et al., (2002) found lower erythrocyte GSH-T in horses after an endurance race. While there was no effect of exercise on erythrocyte GPx activity in the yearlings, the mares had higher levels of GPx at 24H post exercise at compared prior to exercise. In prior equine studies, GPx has increased immediately after acute exercise (Williams et al., 2008; Lamprecht and Williams, 2012), decreased immediately after acute exercise (Ono et al., 1990), or remained unchanged (Brady et al., 1977). The increase at 24H post exercise may be compensating for increased oxidative stress induced by the exercise as GPx works to clear the body of ROS.

Consistent with prior equine studies, all groups had higher plasma cortisol concentrations and CK activity after exercise (Gordon et. al., 2007; McKeever 2002; Nagata et al., 1999; Hargreaves et al., 2002; Lejeune et al., 2010; Serteyn et al., 2010). The MT group did have lower cortisol immediately post exercise as compared to the MNT and the YT groups. While the difference between the groups of mares is attributed to training, the difference between yearlings and MT may be due to the fact that the training protocol may have been more of a challenge for the mares.

Exercise Effects: After Training

The main exercise effects in RSET2 are as follows: All horses had higher Hct and TP at POST and higher serum CK at 2H, 6H, and 24H. The MT group had higher plasma

MDA at 30 as compared to 1.5H, higher plasma NO at 24H as compared to POST, and lower erythrocyte GPx at POST and 1.5H as compared to PRE. The YT had higher TP at 6H and 24H as compared to PRE, lower plasma NO at 6H, lower erythrocyte GPx at POST and 1.5H as compared to PRE. The MNT group had higher muscle GPx at 24H. All groups except the YNT group had higher plasma cortisol at POST. There were no exercise effects on erythrocyte GSH-T and muscle MDA, NO, or GSH-T.

In RSET2, the MT group had consistently lower plasma NO than the other groups, and by 24 hours, similar to RSET1, all mares had lower plasma NO concentrations. The yearlings had lower plasma NO at 6H post exercise, but significantly higher plasma NO at 24H post exercise. Again, the reduction in NO in the mares could indicate increased scavenging by high levels of superoxide, resulting in increased oxidative stress. The increase of plasma NO by 24 hours in the young horses also indicated a faster recovery from the exercise.

Williams et al. (2008) found that horses challenged to a GXT after exercise training still had higher levels of erythrocyte GSH-T post exercise. However, in that study an effect of exercise was also seen prior to training on GSH-T; this study had no exercise effect on GSH-T before or after training. Studies with rats have found no difference in levels of GSH-T in the gastrocnemius before and after exercise training, which differs from the present study, which found increased muscle GSH-T after training.

In RSET2, the MT and YT groups had a decrease in erythrocyte GPx immediately after exercise. No effects of exercise were found in the non-trained groups on erythrocyte

GPx. The immediate decrease in erythrocyte GPx in the trained groups is similar to results found by Ono et al. (1990), where erythrocyte GPx activity decreased in thoroughbred racehorses immediately post exercise; their reasoning for the decreased GPx activity was that since the horses were in the midst of training, their bodies were already at lower antioxidant levels, so the exercise bout proved to be more challenging than if the horses were well rested. In the current study, this could also be an explanation as to why erythrocyte GPx decreased in all trained horses - they were exercise training and already challenging their bodies daily to exercise-induced oxidative stress. When these trained horses were challenged acutely in RSET2, their GPx activity was depleted more significantly than horses not exercising daily.

Once again in the skeletal muscle there were no exercise effects for MDA, GSH-T or NO concentrations. However, there was a difference in GPx activity, with the MNT group having higher muscle GPx activity at 6H and 24H post exercise. It has been mentioned already that the mares were being fed lower levels of vitamin E than recommended; however, the MNT were closer in achieving the recommended daily dose of vitamin E. So in RSET2, it is possible in the MNT group there were sufficient amounts of GPx activity in the muscle to eliminate any increases in oxidative stress. The MT group not only consuming even lower vitamin E than recommended for their level of training but also undergoing strenuous exercise training in the last two weeks, may not have showed any increase in GPx activity in the muscle because they simply did not have enough systemic GPx to combat the oxidative stress.

Training Effects

The main effects of training are as follows: In RSET2, the MT group had lower plasma TP, plasma MDA, muscle GSH-T and higher muscle MDA, and NO. The YT had higher muscle MDA (only 2H) and NO and erythrocyte GPx (at POST and 30) in RSET2, as well as a trend for higher serum CK (only POST and 2H). The MNT group had lower plasma TP and MDA and higher erythrocyte GSH-T (only PRE and POST) and plasma cortisol. The YNT had higher muscle MDA (POST).

All mares had lower TP in RSET2, which may have been due to the time of the year when RSET2 occurred. The horses ran RSET1 in early October while RSET2 occurred in mid-December, which may have impacted levels of sweat production and fluid shifts in the cardiovascular system. The warmer ambient temperature in October would have resulted in higher sweat production than in December, and thus the lower plasma TP in December regardless of the temperature controlled interior of the treadmill facility.

Both groups of mares had lower plasma MDA in RSET2; while this may be a result of exercise training for the MT group, it is possible that the biweekly GXTs performed by all horses did have a slight training impact on the MNT group. In a study by Avellini et al. (1999), horses had increased peroxidation of erythrocytes after 60 days of training. Chiaradia et al. (1998) found that after 3 months of exercise training, an acute bout of exercise still increased plasma MDA. In contrast to their lower plasma MDA concentrations, the MT group had higher muscle MDA in RSET2, as did all yearlings. Studied in rats have shown both increases in muscle MDA after training (Balci

and Pepe, 2012) or no difference in muscle MDA after training (Balci and Pepe, 2012; Leeuwenburgh et al., 1994). The yearlings had no differences in muscle or plasma MDA after training; this could be due to the training not being intense enough for them to achieve exercise adaptations, or that their young age allows their bodies to provide sufficient buffers against oxidative stress.

Both trained groups had increased muscle NO in RSET2 as compared to RSET1. To our knowledge this is the first study to look at the effects of training on muscle NO. A study looking at jumpers found decreased plasma NO after 5 weeks of competition (Marañón et al., 2008), and a prior study with rats found reduced NO in the gastocnemius muscle after 8 weeks of training (Balci and Pepe, 2012).

The only differences seen in GSH-T and GPx were in the mares, which had higher muscle GSH-T through RSET2 as compared to RSET1, and the YT group, which had lower erythrocyte GPx activity in RSET2 as compared to RSET1 post exercise. In addition, the lack of differences between groups for erythrocyte suggest training was able to improve antioxidant status in the mares; though why the MNT group also was no different from the others is unclear; the intermittent GXTs may have helped to improve their antioxidant status as well. The mares who were in training may have had reduced levels of GPx in response to exercise because their systems were already challenged by the daily bouts of training.

When comparing the RSETs, all mares had higher cortisol levels in RSET1 as compared to RSET2. Thus, once again while training may have improved plasma cortisol levels in the MT group, the biweekly GXTs may have also helped the MNT

group to improve their stress response. The yearlings had no difference in cortisol levels between RSETs; training may not have been strenuous enough for the yearling's training to improve their stress response to exercise. Liburt found that the effect of age disappeared after young (7.3±0.6 years) and old (22.0±0.7 years) horses were trained and subjected to another acute exercise test (2011). Malinowski et al. (2006) found that after training, while the young (6.8±0.4 years) and old (27.0±0.2 years) groups had similar response to acute exercise, the middle aged group (15.2±0.74 years), had cortisol elevated for 60 minutes as compared to 40 minutes prior to training.

No effect of training was seen with serum CK in any horses. Lindner et al. (2006) looked at horses trained for 6 weeks, at either 1, 2, or 3 times per week; he found that the horses exercise 1 or 2 times per week, when challenged to exercise had increased CK 12 hours post exercise, but returned to pre-exercise levels 24 hours post exercise. The horses exercise 3 times/week had no difference in CK levels after exercise. While the current study found no difference due to training, all horses still had increased serum CK activity due to the acute exercise test.

A limitation to this study was that inflammation was not examined. Oxidative stress and inflammation are closely linked, with neutrophils and machrophages capable of producing ROS in efforts to eliminate pathogens and damaged tissue in the body (Konig et al., 2001). But it is unclear if the ROS produced during inflammation is a source of overall increased systemic oxidative stress. Additionally, while pro-inflammatory cytokines, such as tumor necrosis factor –alpha (TNF-α), interleukin 1 (IL-1), and interleukin 6 (IL-6) have been examined in the circulatory system of horses and found to increase concentrations with acute and endurance exercise (Holbrook et al., 2010;

Horohov et. al., 1999; Steltosova et al., 2006) there has yet to be evidence of these markers in equine skeletal muscle. Evidence does exist for ROS to stimulate cytokine release in murine myocytes *in vitro* (Kosmidou et al., 2002), but again, *in vivo* studies have conflicting reports (Smith et al., 2008).

Furthermore, inflammation and cortisol are also linked. Cortisol is known to suppress the immune system, which may allow for slight muscle damage and subsequent remodeling of the skeletal muscle (McKeever et al, 2014). Again, measuring markers of inflammation would have allowed better insight as to whether muscle damage was occurring.

A further limitation to this study was that of the various hormones involved in the hypothalamic-pituitary- adrenal axis (HPAA), only plasma cortisol was measured. Corticotropic releasing hormone (CRH) from the hypothalamus stimulates the release of adrenocorticotropic hormone (ACTH) from the pituitary gland, which finally stimulates the release of cortisol from the adrenal gland. In horses, increase in ACTH have been seen post exercise (Nagata et al., 1999), leading to a subsequent increase in cortisol. By measuring ACTH and CRF, a more complete picture of the impact of exercise and exercise training would have been possible.

Conclusion

The importance of young age on oxidative stress and antioxidant status was quite evident in the current study. While most prior studies have focused on the impact of oxidative stress on aged animals, this study found that young growing horses have less

oxidative stress and higher antioxidant status than mares. This was contrary to the expectation that the yearlings would have higher levels of oxidative stress due to the novel exercise they were performing. In addition, training effects were most evident in the mares than in the yearlings, suggesting that their initial higher levels of oxidative stress were improved with training. Interestingly, the non-trained mares often had similar results to the trained mares, leading to speculation that though the non-trained mares only ran a sprint test once every two weeks, it was still sufficient exercise to improve antioxidant status and lessen oxidative stress. The yearlings, on the other hand, were physiologically able to handle the challenge of exercise-induced oxidative stress prior to training, and thus there was little additional improvement that training could offer.

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Figure Captions

Figure 1. (A) Hematocrit (Hct) during RSET1 at sample times PRE, POST, 30 min, 1, 1.5, 2, 6, and 24 H post exercise for mares and yearlings. *denotes differences in ages at each sample time at $P < 0.03$. ** denotes differences in ages at each sample time at $P < 0.08$. **(B)** Hematocrit (Hct) during RSET2 at sample times PRE, POST, 30 min, 1, 1.5, 2, 6, and 24 H post for mature non-trained (M NT), mature trained (M T), young non-trained (Y NT), and young trained (Y T). Letters x,y denote differences between groups within sample times at $P<0.05$.

Figure 2. (A) Total Protein (TP) in RSET1 at sample times PRE, POST, 30 min, 1, 1.5, 2, 6, and 24 H post exercise for mares and yearlings. *denotes differences in ages at each sample time at $P < 0.0001$. **(B)** Total Protein (TP) in RSET2 at sample times PRE, POST, 30 min, 1, 1.5, 2, 6, and 24 H post for mature non-trained (M NT), mature trained (M T), young non-trained (Y NT), and young trained (Y T). Letters x,y denote differences between groups within sample times at $P<0.05$. **(C)** Total Protein (TP) at sample times PRE, POST, 30 min, 1, 1.5, 2, 6, and 24 H post exercise for mature trained in RSET1 (R1 M T), mature trained in RSET2 (R2 M T), mature non-trained in RSET1 (R1 M NT), mature non-trained in RSET2 (R2 M NT). For both mature trained and non-trained TP was higher for RSET 1 than RSET 2 at each sample time ($P < 0.05$).

Figure 3. (A) Concentrations of plasma malondialdehyde (MDA) for RSET 1 at sample times PRE, POST, 30 min, 1, 1.5, 2, 6, and 24 H post exercise for mares and yearlings.

*denotes differences in ages at each sample time at P < 0.01. (**B)** Concentrations of skeletal muscle malondialdehyde (MDA) for RSET 1 at sample times PRE, POST, 2, 6 and 24H for old and young horses. *denotes differences in ages at each sample time at P < 0.03. ** denotes differences in ages at each sample time at P < 0.07. **(C)** Concentrations of plasma malondialdehyde (MDA) at sample times PRE, POST, 30 min, 1, 1.5, 2, 6, and 24 H post exercise for mature non-trained (M NT), mature trained (M T), young non-trained (Y NT), and young trained (Y T). **(D)** Concentrations of skeletal muscle malondialdehyde (MDA) at sample times PRE, POST, 2, 6 and 24H for mature non-trained (M NT), mature trained (M T), young non-trained (Y NT), and young trained (Y T). **(E)** Concentrations of plasma malondialdehyde (MDA) at sample times PRE, POST, 30 min, 1, 1.5, 2, 6, and 24 H post exercise for mature trained in RSET1 (R1 M T),mature trained in RSET2 (R2 M T), mature non-trained in RSET1 (R1 M NT), mature non-trained in RSET2 (R2 M NT).

Figure 4. (A) Concentrations of plasma nitric oxide (NO) for RSET 1 at sample times PRE, POST, 30 min, 1, 1.5, 2, 6, and 24 H post exercise for mares and yearlings. *denotes differences in ages at each sample time at P < 0.001. (**B)** Concentrations of skeletal muscle nitric oxide (NO) for RSET 1 at sample times PRE, POST, 2, 6 and 24H for mares and yearlings. *denotes differences in ages at each sample time at P < 0.02. **(C)** Concentrations of plasma nitric oxide (NO)at sample times PRE, POST, 30 min, 1, 1.5, 2, 6, and 24 H post exercise in RSET2 for mature non-trained (M NT), mature trained (M T), young non-trained (Y NT), and young trained (Y T). Letters x,y denote differences between groups within sample times at P < 0.04. **(D)** Concentrations of

skeletal muscle nitric oxide (NO) at sample times PRE, POST, 2, 6 and 24H post exercise for old trained horses in RSET1 (R1 MT) and mature trained in RSET2 (R2 MT). Letters x,y denote differences between RSETS within ages at each sample time at P<0.03.

Figure 5. (A) Concentrations of erythrocyte total glutathione (GSH-T) for RSET 1 at sample times PRE, POST, 30 min, 1, 1.5, 2, 6, and 24 H post exercise for mares and yearlings. *denotes differences in ages at each sample time at P < 0.05. (**B)** Concentrations of skeletal muscle total gluthathione (GSH-T) for RSET 1 at sample times PRE, POST, 2, 6 and 24H for mares and yearlings. *denotes differences in ages at each sample time at P < 0.03.

Figure 6. (A) Activity of erythrocyte glutathione peroxidase (GPx) for RSET 1 at sample times PRE, POST, 30 min, 1, 1.5, 2, 6, and 24 H post exercise for mares and yearlings. *denotes differences in ages at each sample time at P < 0.02. (**B)** Concentrations of skeletal muscle glutathione peroxidase (GPx) for RSET 1 at sample times PRE, POST, 2, 6 and 24H for mares and yearlings. *denotes differences in ages at each sample time at P < 0.02. ** denotes differences in ages at each sample time at P < 0.05. **(C)** Activity of erythrocyte glutathione peroxidase (GPx) for RSET 2 at sample times PRE, POST, 30 min, 1, 1.5, 2, 6, and 24 H post exercise for mature non-trained (M NT), mature trained (M T), young non-trained (Y NT), and young trained (Y T). Letters x,y denote differences between groups within sample times at P < 0.03. **(D)** Activity of erythrocyte glutathione peroxidase (GPx) at sample times PRE, POST, 30 min, 1, 1.5, 2, 6, and 24 H

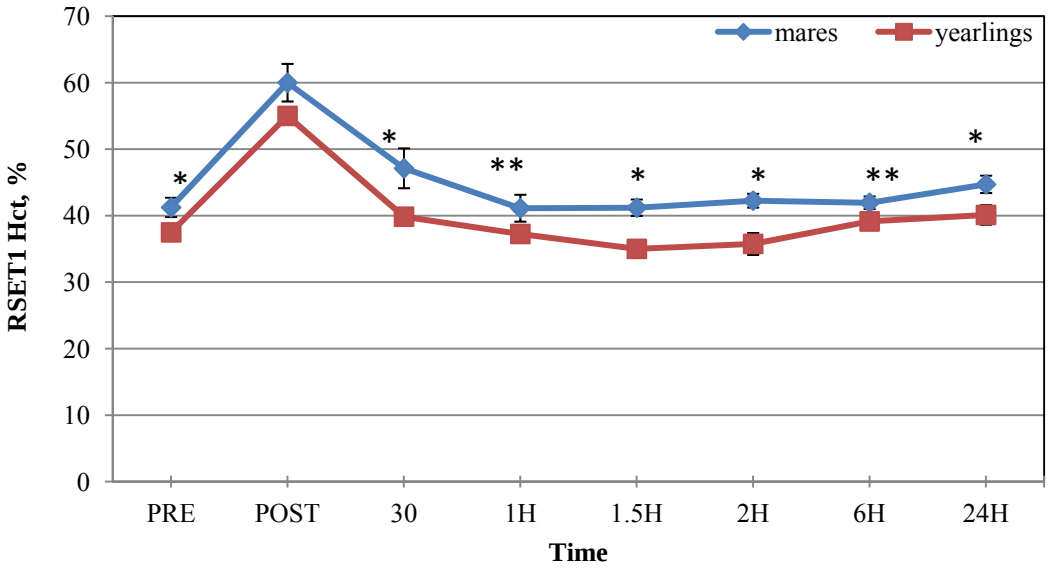
post exercise for young trained horses in RSET1 (R1 YT) and young trained horses in RSET2 (R2 YT). *denotes differences in ages at each sample time at $P < 0.05$. ** denotes differences in ages at each sample time at $P < 0.07$.

Figure 7. (A) Concentrations of plasma cortisol for RSET1 at sample times PRE, POST, 2, 6, and 24 H post exercise for yearlings, mature non-trained (M NT) and mature trained (M T). Letters x,y denote differences between groups within sample times at $P < 0.03$. (**B)** Concentrations of plasma cortisol for RSET 2 at sample times PRE, POST, 2, 6 and 24H for mature non-trained (M NT), mature trained (M T), young non-trained (Y NT), and young trained (Y T). Letters a,b denote differences between overall sample times at $P < 0.05$. Letters x,y denote differences between groups within sample times at $P < 0.05$. **(C)** Concentrations of plasma cortisol at sample times PRE, POST, 2, 6, and 24 H post exercise for mature trained in RSET1 (R1 M T), mature trained in RSET2 (R2 M T), mature non-trained in RSET1 (R1 M NT), mature non-trained in RSET2 (R2 M NT). Letters a,b denote differences between the M T group at each sample times at $P < 0.02$. Letters x,y denote differences between the M NT group within sample times at $P < 0.05$.

Figure 8. (A) Activity of serum creatine kinase (CK) for RSET1 at sample times PRE, POST, 2, 6, and 24 H post exercise for mares and yearlings. Letters x,y denote differences overall groups between sample times at $P < 0.001$. (**B)** Activity of serum creatine kinase (CK) for RSET 2 at sample times PRE, POST, 2, 6 and 24H for mature non-trained (M NT), mature trained (M T), young non-trained (Y NT), and young trained (Y T). Letters a,b denote differences between overall sample times at $P < 0.02$.

Figures

1A.



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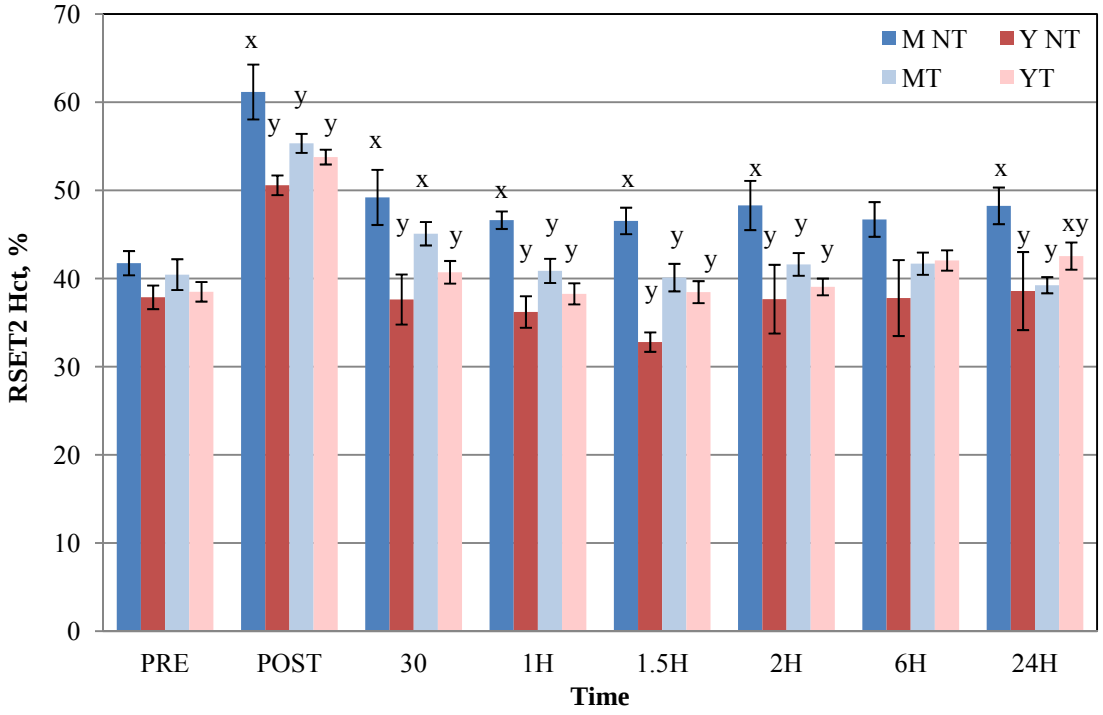

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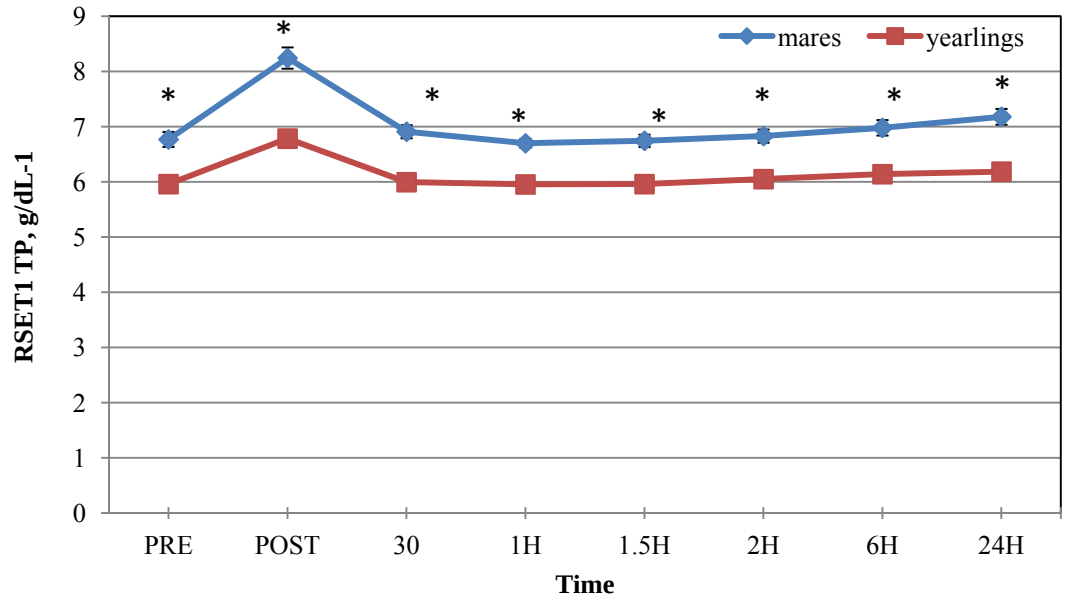
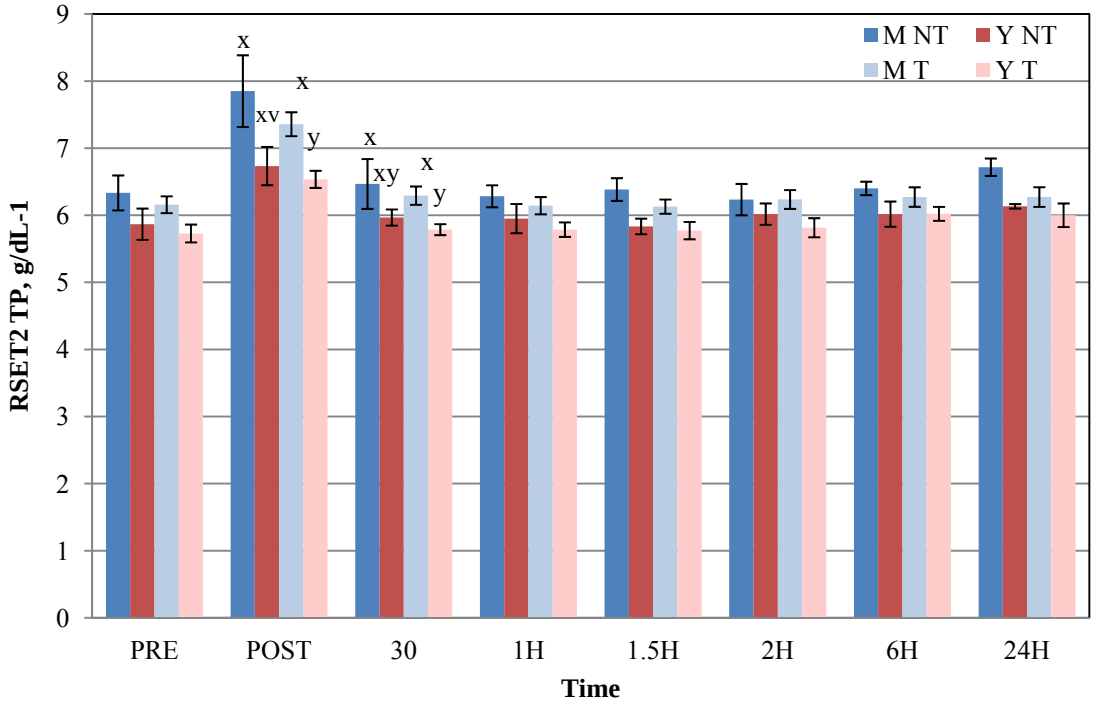
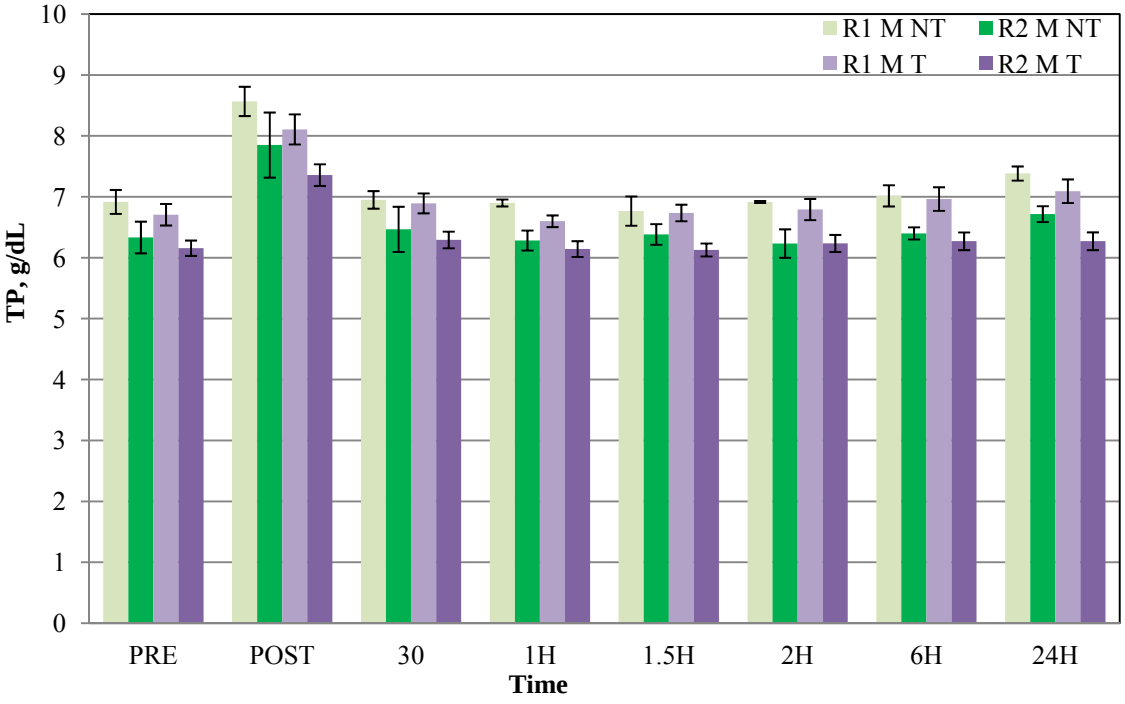

RSET1 TP, g/dL-1
mares
yearlings
PRE
POST
30
1H
1.5H
2H
6H
24H
Time

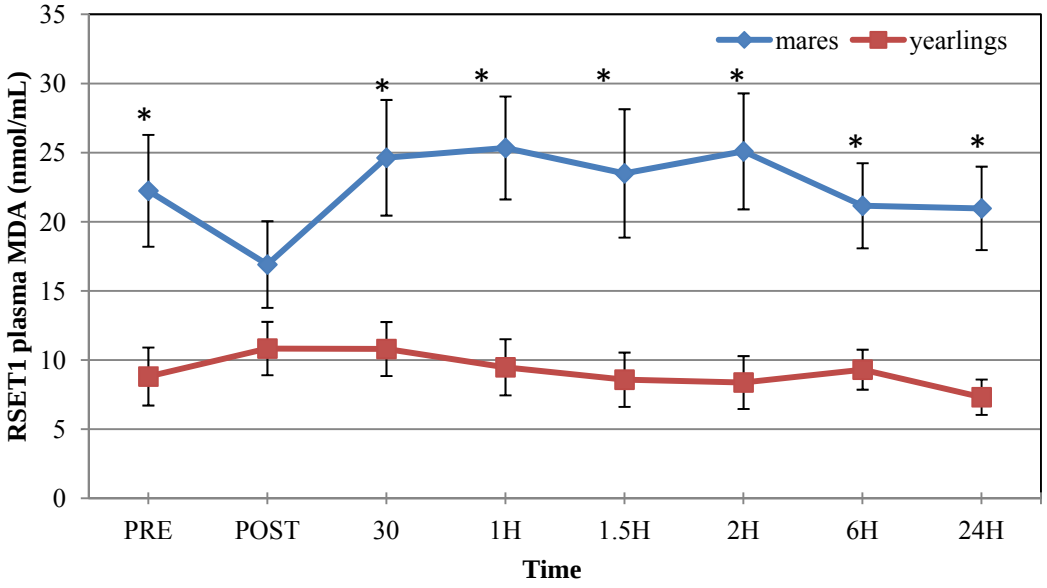
2B.


RSET2 TP, g/dL-1
M NT
Y NT
M T
Y T
PRE
POST
30
1H
1.5H
2H
6H
24H
Time

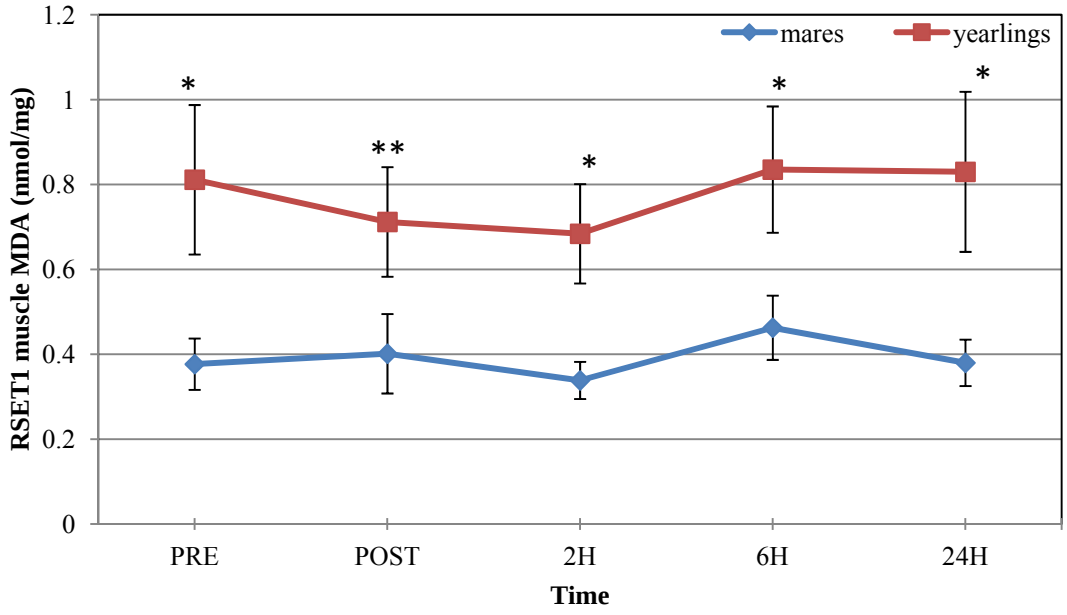
2C.


R1 M NT
R2 M NT
R1 M T
R2 M T
TP, g/dL
0
1
2
3
4
5
6
7
8
9
10
PRE
POST
30
1H
1.5H
2H
6H
24H
Time

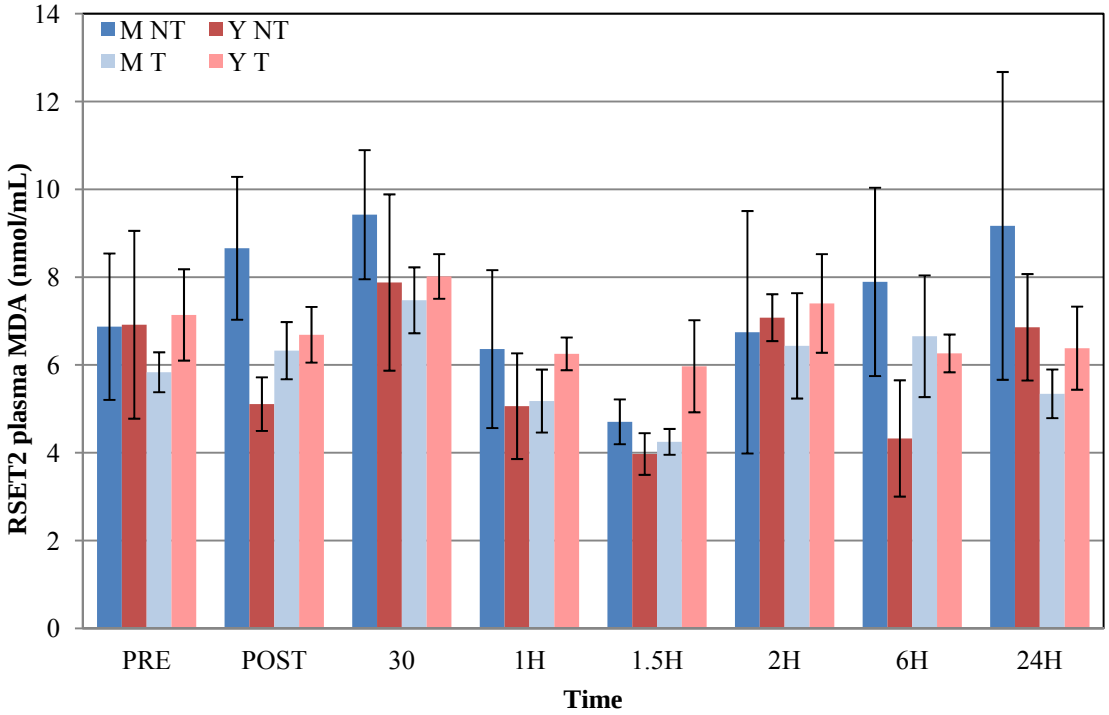
3A.


mares
yearlings
RSET1 plasma MDA (nmol/mL)
0
5
10
15
20
25
30
35
*
PRE
POST
30
1H
1.5H
2H
6H
24H
Time

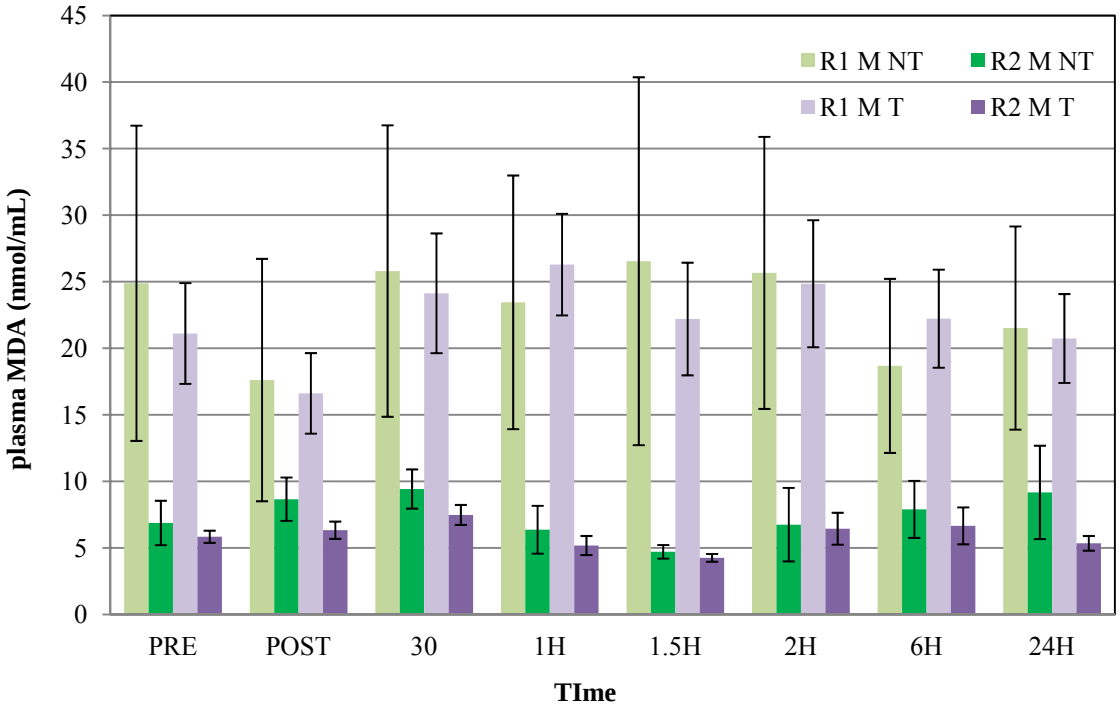
3B.


1.2
1
0.8
0.6
0.4
0.2
0
RSET1 muscle MDA (nmol/mg)
mares
yearlings
*
**
*
*
*
PRE
POST
2H
6H
24H
Time

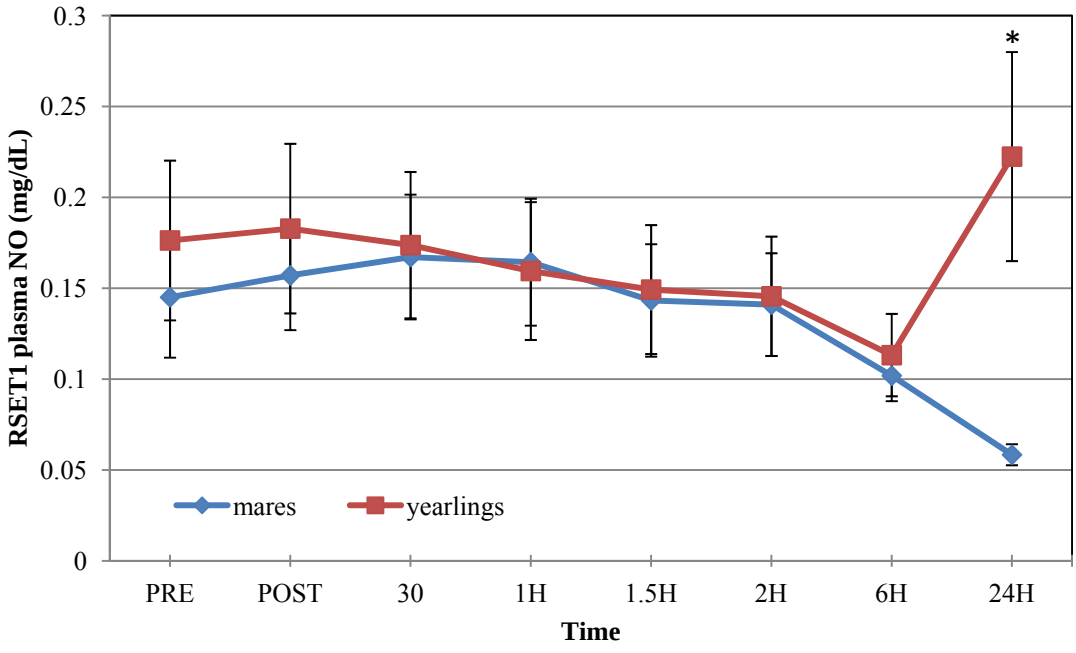
3C.


14
12
10
8
6
4
2
0
RSET2 plasma MDA (nmol/mL)
M NT
Y NT
M T
Y T
PRE
POST
30
1H
1.5H
2H
6H
24H
Time

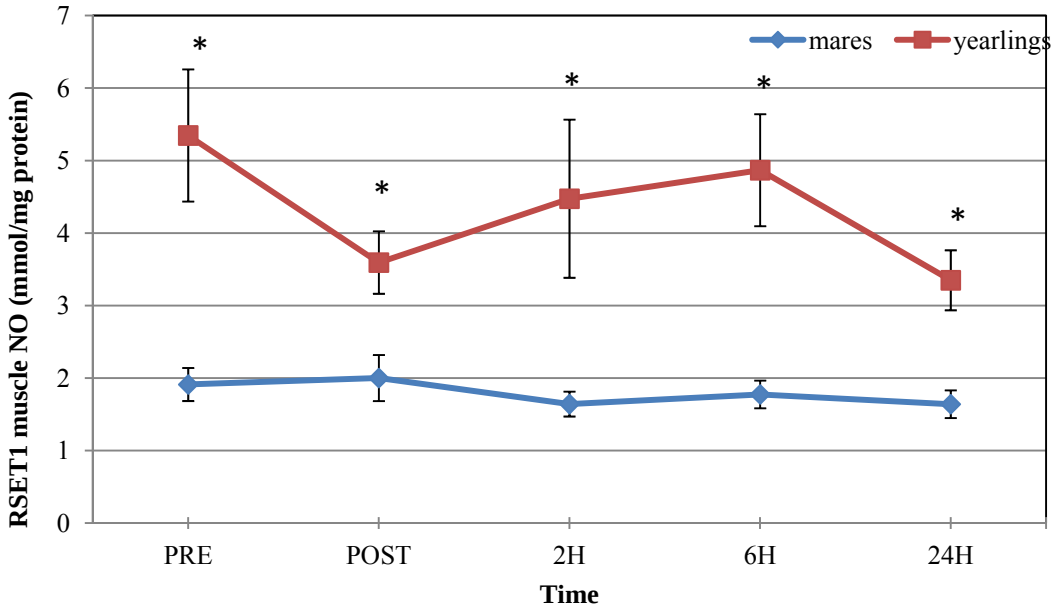
3D.


45
40
35
30
25
20
15
10
5
0
plasma MDA (nmol/mL)
R1 M NT
R2 M NT
R1 M T
R2 M T
PRE
POST
30
1H
1.5H
2H
6H
24H
TIme

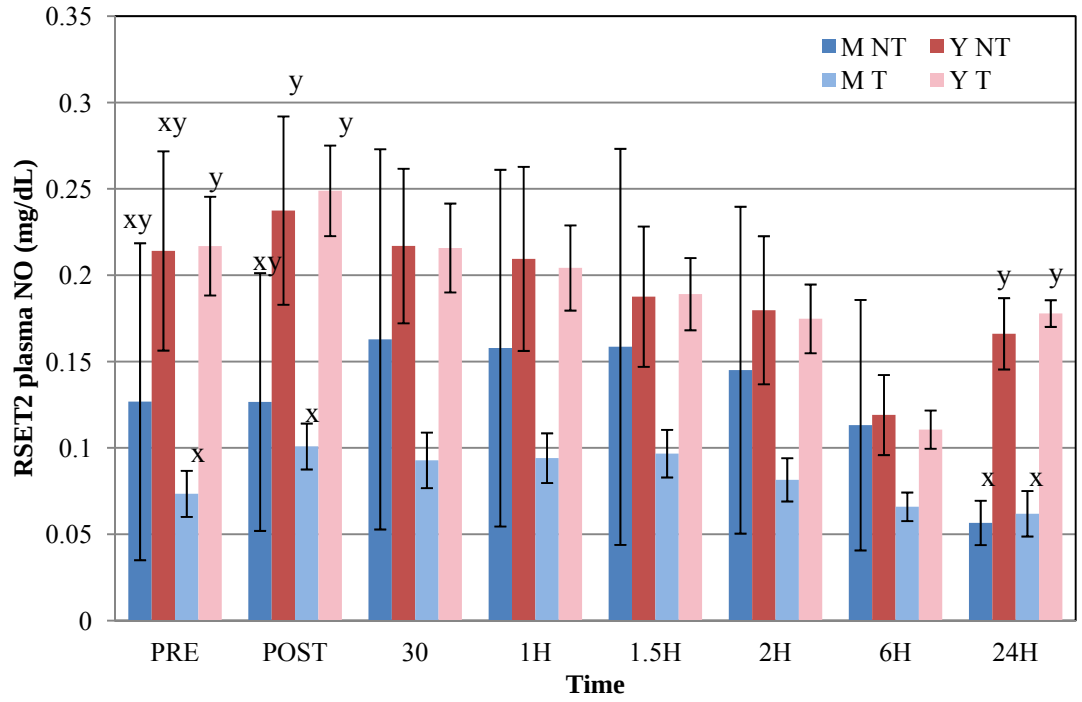
4A.


0.3
0.25
0.2
0.15
0.1
0.05
0
RSET1 plasma NO (mg/dL)
*
mares
yearlings
PRE
POST
30
1H
1.5H
2H
6H
24H
Time

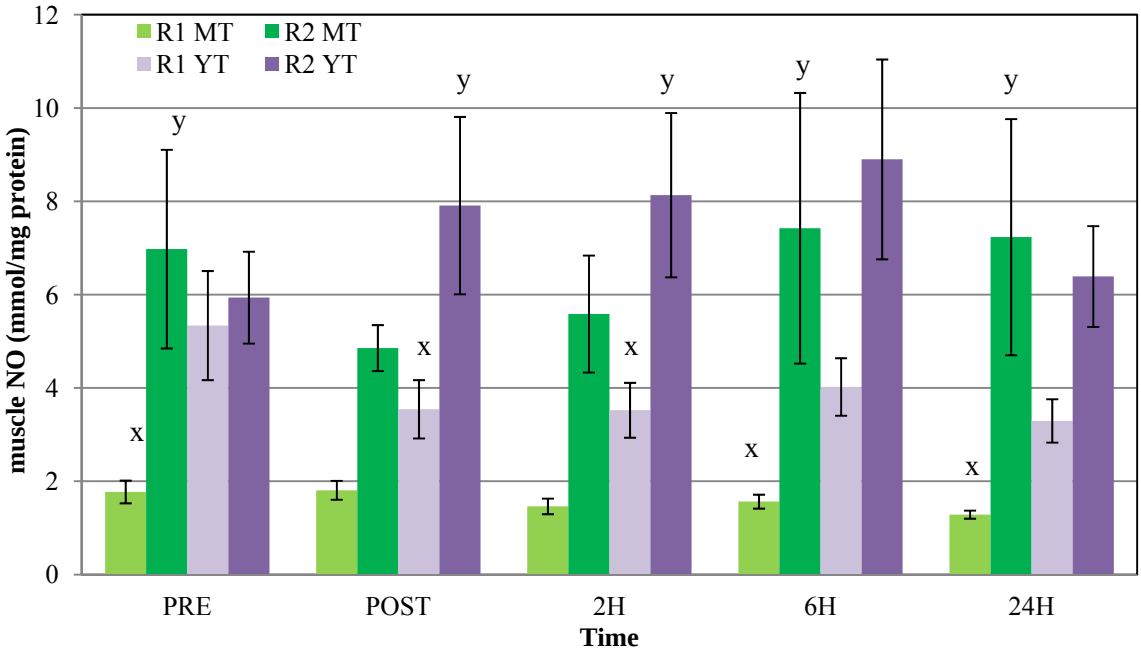
4B.


RSET1 muscle NO (mmol/mg protein)
7
6
5
4
3
2
1
0
mares
yearlings
*
*
*
*
*
PRE
POST
2H
6H
24H
Time

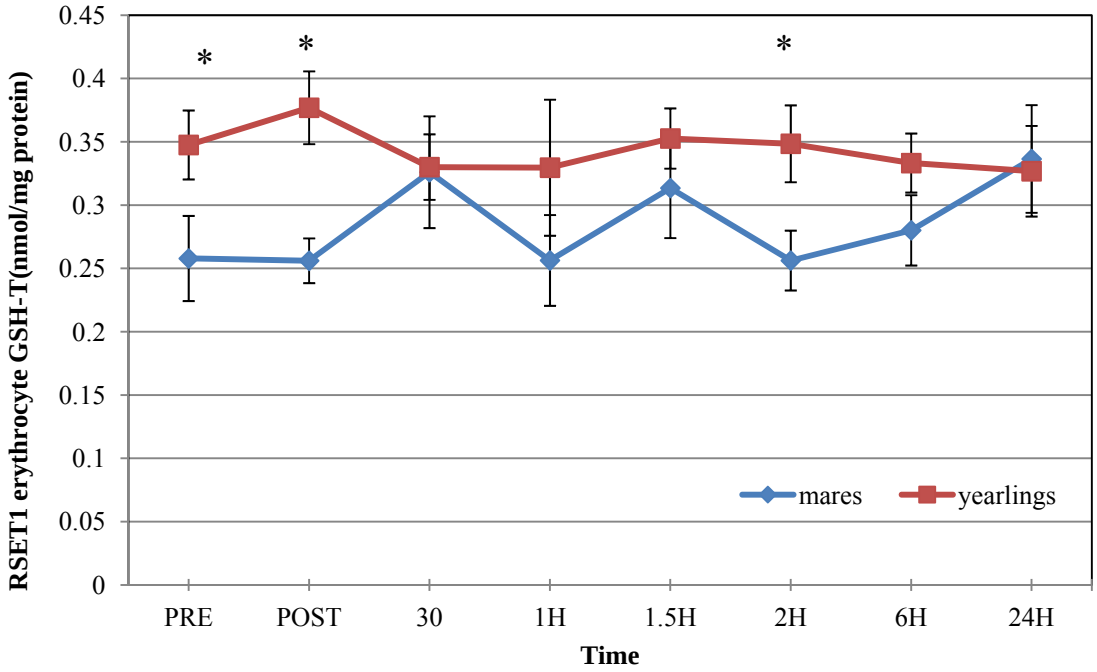
4C.


RSET2 plasma NO (mg/dL)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
M NT
Y NT
M T
Y T
xy
y
xy
y
xy
y
x
x
x
y
y
PRE
POST
30
1H
1.5H
2H
6H
24H
Time

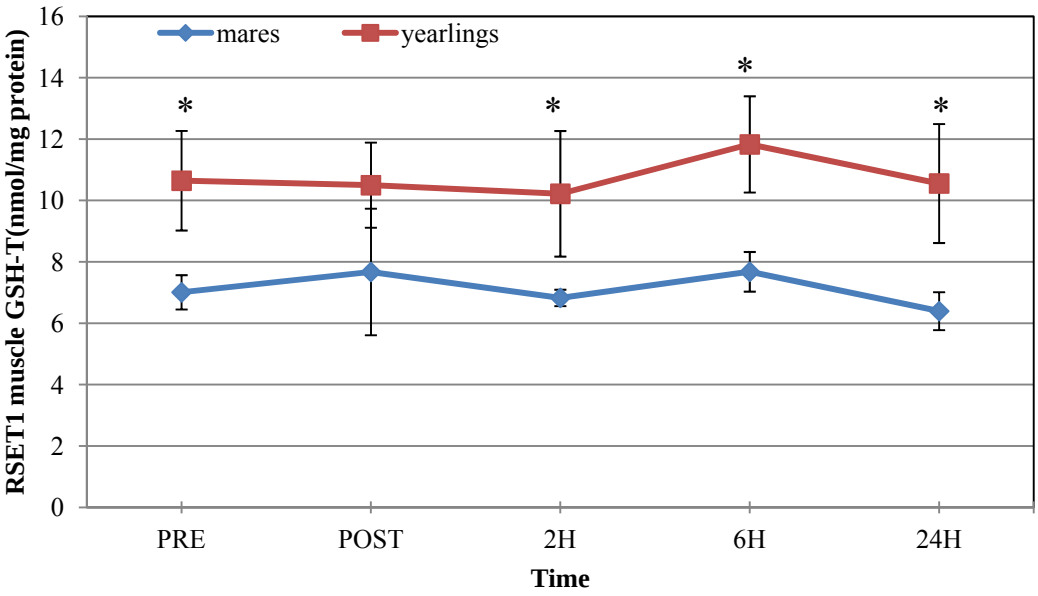
4D.


R1 MT
R2 MT
R1 YT
R2 YT
muscle NO (mmol/mg protein)
0
2
4
6
8
10
12
x
y
PRE
POST
2H
6H
24H
Time

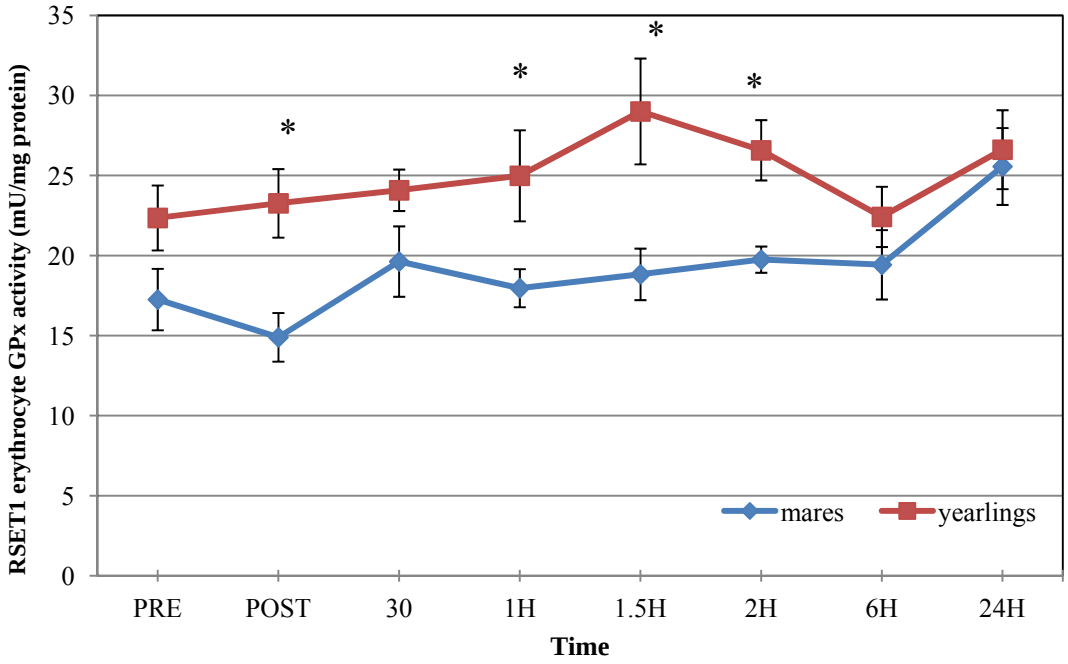
5A.


RSET1 erythrocyte GSH-T(nmol/mg protein)
0
0.05
0.1
0.15
0.2
0.25
0.3
0.35
0.4
0.45
*
mares
yearlings
PRE
POST
30
1H
1.5H
2H
6H
24H
Time

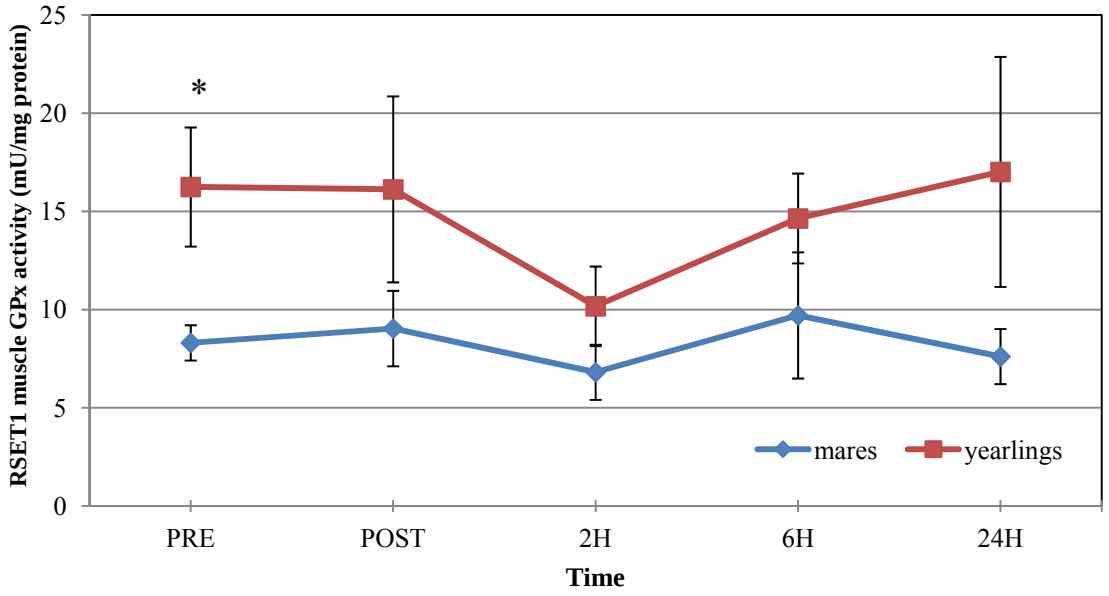
5B.


mares
yearlings
RSET1 muscle GSH-T(nmol/mg protein)
16
14
12
10
8
6
4
2
0
*
*
*
*
PRE
POST
2H
6H
24H
Time

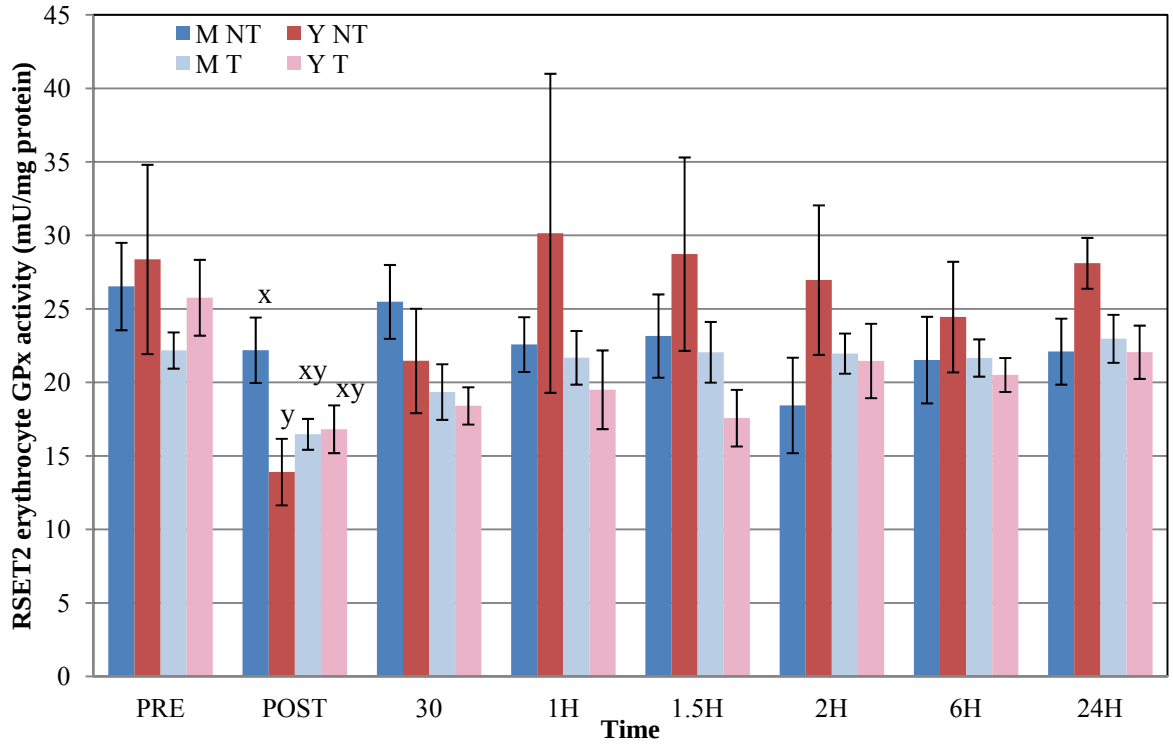
6A.


RSET1 erythrocyte GPx activity (mU/mg protein)
35
30
25
20
15
10
5
0
*
*
*
*
mares
yearlings
PRE
POST
30
1H
1.5H
2H
6H
24H
Time

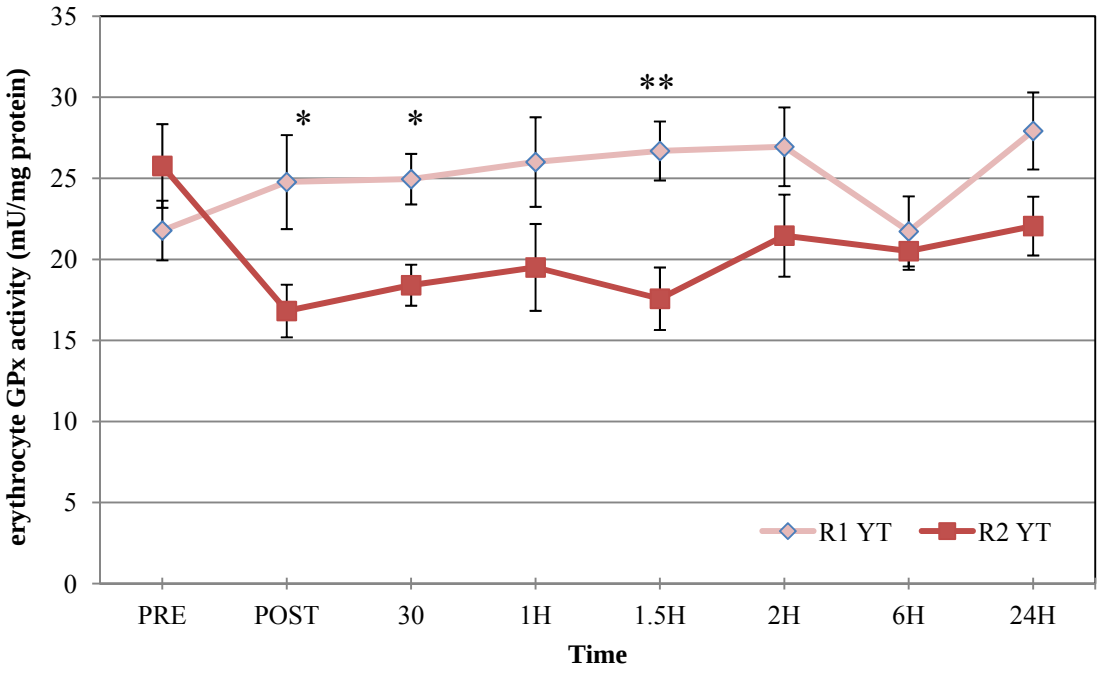
6B.


RSET1 muscle GPx activity (mU/mg protein)
25
20
15
10
5
0
*
mares
yearlings
PRE
POST
2H
6H
24H
Time

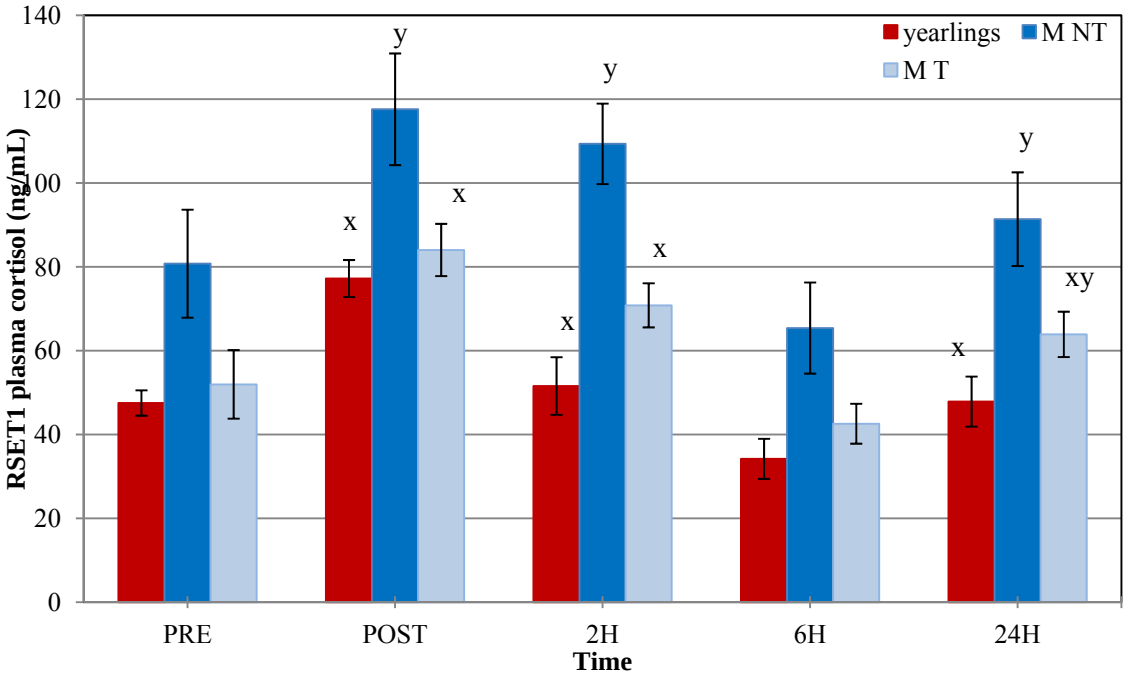
6C.


RSET2 erythrocyte GPx activity (mU/mg protein)
45
40
35
30
25
20
15
10
5
0
M NT
Y NT
M T
Y T
x
y
xy
xy
PRE
POST
30
1H
1.5H
2H
6H
24H
Time

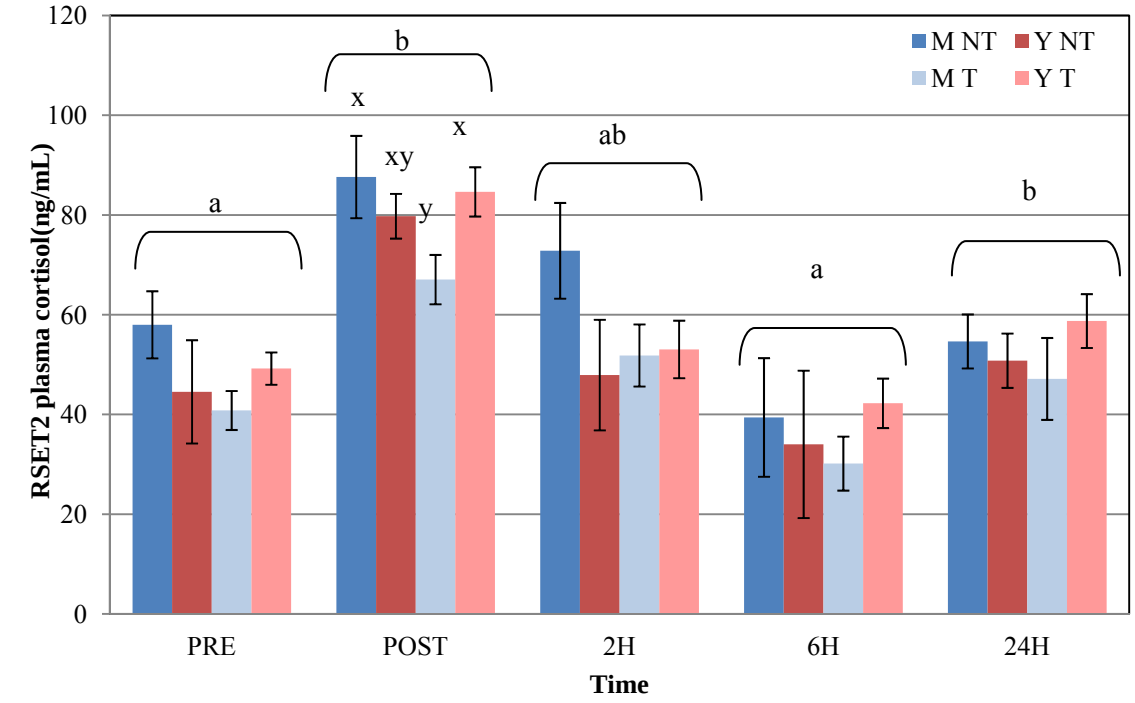
6D.


35
30
25
20
15
10
5
0
erythrocyte GPx activity (mU/mg protein)
*
*
**
R1 YT
R2 YT
PRE
POST
30
1H
1.5H
2H
6H
24H
Time

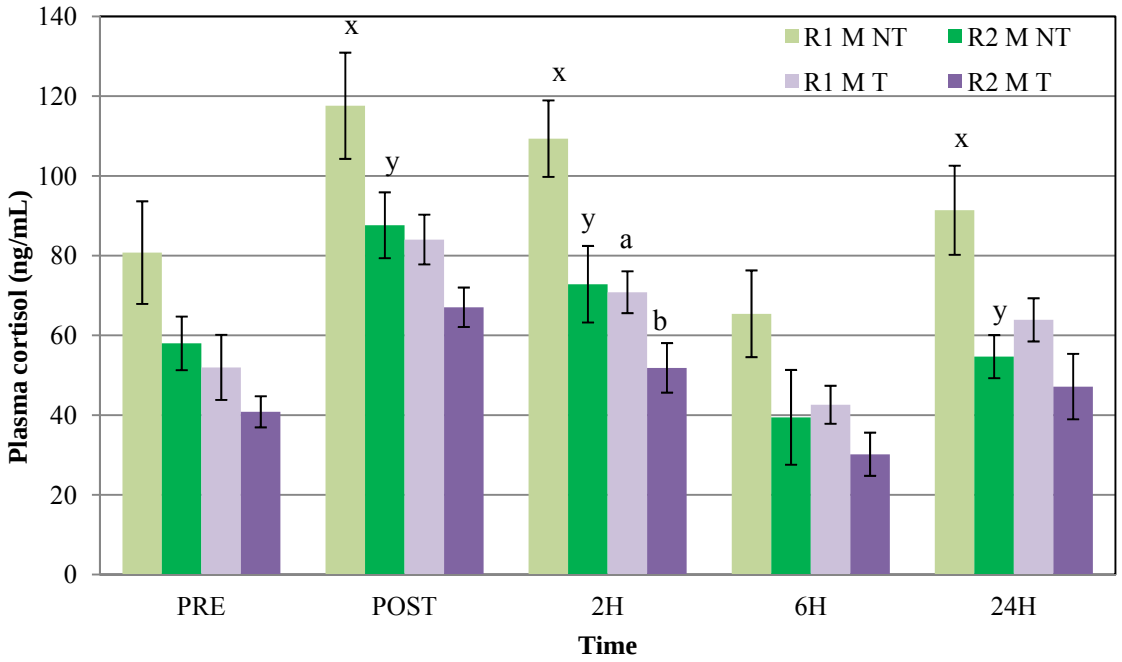
7A.


140
120
100
80
60
40
20
0
RSET1 plasma cortisol (ng/mL)
yearlings
M NT
M T
x
y
x
y
x
x
y
xy
PRE
POST
2H
6H
24H
Time

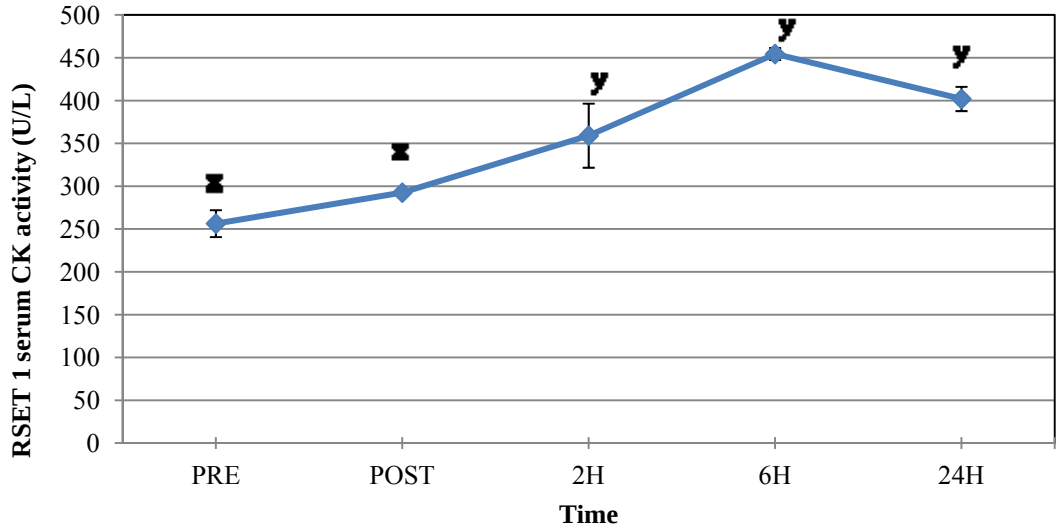
7B.


RSET2 plasma cortisol(ng/mL)
120
100
80
60
40
20
0
M NT
Y NT
M T
Y T
a
b
x
xy
y
x
ab
a
b
PRE
POST
2H
6H
24H
Time

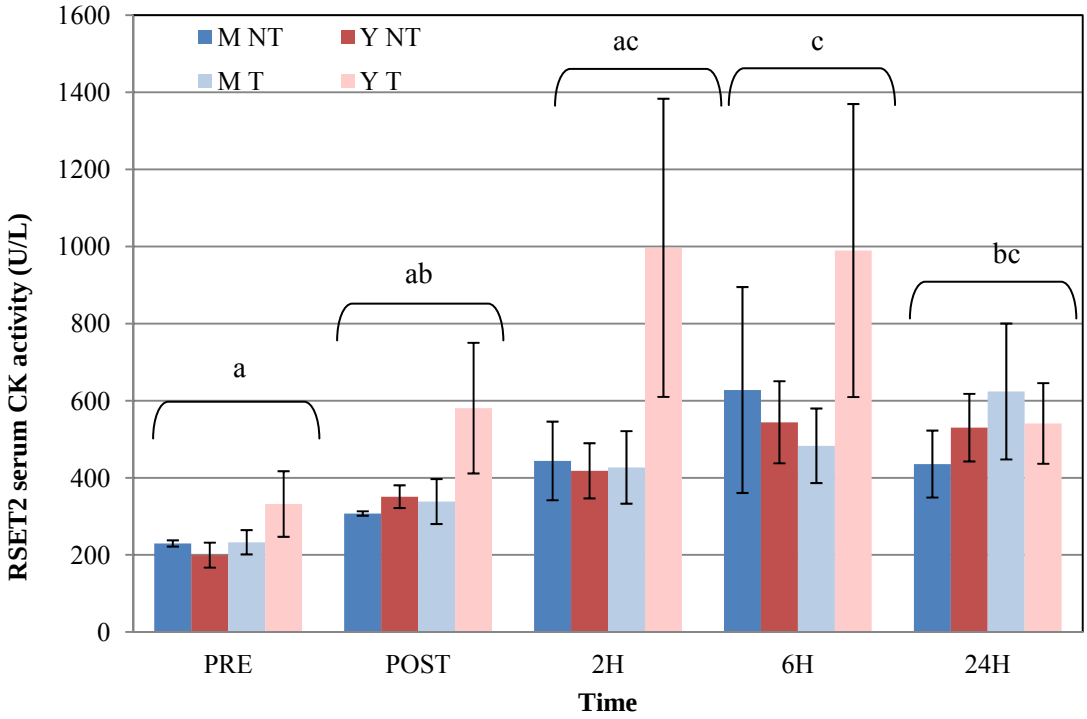
7C.


Plasma cortisol (ng/mL)
140
120
100
80
60
40
20
0
R1 M NT
R2 M NT
R1 M T
R2 M T
x
y
x
y
a
b
x
y
PRE
POST
2H
6H
24H
Time

8A.


RSET 1 serum CK activity (U/L)
500
450
400
350
300
250
200
150
100
50
0
x
x
y
y
y
PRE
POST
2H
6H
24H
Time

8B.


RSET2 serum CK activity (U/L)
1600
1400
1200
1000
800
600
400
200
0
M NT
Y NT
M T
Y T
a
ab
ac
c
bc
PRE
POST
2H
6H
24H
Time

Tables

Table 1. Nutrient composition of grain for mature mares and yearlings. Nutrients are expressed on a 100% dry matter basis[a].

Nutrient[b]	Ultra - Broodmare Yearling	Ultra - Active Textured
DE, Mcal/kg	2.94	3.17
CP, %	14.8	12.2
Fat , %	6	5.5
Fiber – max, %	10.1	5.1
Lysine, %	0.7	0.57
Ca – min, %	0.5	0.6
Ca – max, %	0.9	0.8
P – min, %	0.5	0.5
P – max, %	0.7	0.7
Mg, %	0.21	0.19
K, %	0.8	0.61
Na, %	0.36	0.38
Fe, ppm	163	128
Zn, ppm	206	185
Cu, ppm	64	40
Mn, ppm	134	115
Cl, %	0.53	0.6
S, &	0.27	0.21
I, ppm	0.77	0.5
Co, ppm	0.27	0.2
Se, ppm	1.17	0.86
Mo, ppm	0.8	0.6
Starch – max, %	25	40
Vitamin E, IU/kg	440	236
Vitamin A, IU/kg	23502	17097

[a]Analyses were provided by Pennfield Feed, Lancaster, PA.

[b]DE, digestable energy; CP, crude protein.

Table 2. Nutrient composition of hay for mature mares and yearlings. Nutrients are expressed on a 100% dry matter basis[a].

Nutrient[b]	Timothy Grass	Alfalfa Orchard Grass Mix
CP, %	8.7	16.4
ADF, %	40.5	38.1
NDF, %	62.1	52.6
NFC, %	23.2	22.8
Starch, %	0.2	1.2
WSC, %	12.4	8.9
ESC, %	7.2	6.2
Fat, %	2.1	2.3
Ash, %	5.88	9.3
TDN, %	59	56.5
Ca, %	0.71	0.60
P, %	0.23	0.34
Mg, %	0.14	0.26
K, %	1.42	2.76
Na %	0.30	0.02
Fe, ppm	66	133
Zn, ppm	17	18.5
Cu, ppm	6	9.5
Mn, ppm	24	29.5
Mo, ppm	1.8	1.55
Se, ppm[c]	0.03	0.30
S, %	0.13	0.19
Chloride Ion, %	0.12	0.35
Lysine, %	0.34	0.71
DE, Mcal/lb	0.97	0.98
Vitamin E, IU/kg[c]	19.5	16.5
Vitamin A, IU/kg[c]	21340	26000

[a]Analyses were performed by Dairy One DHIA Forage Testing Laboratory, Ithaca, NY.

[b]CP, crude protein; ADF, acid detergent fiber; NDF, neutral detergent fiber; NFC, nonfiber carbohydrates; WSC, water soluble carbohydrates; ESC, simple sugars; TDN, total digestible nutrients; DE, digestible energy.

[c]Nutrients values were obtained from NRC (1989) estimations.

Table 3. Nutrient intake of mares throughout the trial and yearlings both untrained or pre-training and yearlings after they were in the third week of training. These nutrient intakes were balanced to meet NRC recommended intakes for horses at this status.

Nutrient[b]	Yearlings, untrained & pre-training	Yearlings, training	Mature Mares
Total feed, lb	18.5	20.0	21.0
DE, Mcal/lb	18.9	20.9	21.8
CP, %	1027	1127	877
Ca, g	55	61	68
P, g	26	30	27
Cu, mg	101.3	129.3	103.6
Zn, mg	354	495	392
Se, mg	2.51	3.31	1.45
Mn, mg	340	430	354
I, mg	0.5	0.8	0.7
Lysine, g	41.3	46.0	35.5
Mg, g	17	19	14
K, g	154	159	124
Vitamin E, IU/day	666	987	483
Vitamin A, IU/day	191,246	202,602	198.274

[b]DE, digestible energy; CP, crude protein.

Table 4. Comparison of RSET1 (R1) and RSET2 (R2) time to completion for mares trained (T) and non-trained (NT) and yearlings trained (T) and not trained (NT),as well as time spent at 90% heart rate maximum. Data are presented as the mean ± SE.

	Mares		Yearlings	
	T	NT	T	NT
R1 (min)	20.2±0.7	18.8±0.8	20.5±0.3	19.3±0.4
R2 (min)	21.1±0.3	20.3±0.3	21.9±0.1	20.9±0.6
R1 90% HR max (min)	6.0±0.7	5.6±0.2	5.9±0.4	5.5±1.3
R2 90% HRmax (min)	6.4±0.4	6.3±0.3	6.1±0.5	6.1±1.1

OVERALL DISCUSSION AND SUMMARY

The role of oxidative stress in muscular injury and adaptation is still hotly debated. While it is now generally accepted that oxidative stress is necessary, the limit at which oxidative stress becomes harmful to the body is difficult to say, because of the many factors that can influence oxidative stress, as well as every individual's ability to cope with it. In the horse, the wide variability seen in exercise-induced oxidative stress produced, or not produced, proves just how difficult it is to determine "normal" levels of oxidative stress. This doctoral project strived to add more information to the puzzle of the impact of oxidative stress on the equine athlete.

Given that muscle biopsies are vital to studying oxidative stress within skeletal muscle, ensuring that the act of taking a muscle biopsy did not produce oxidative stress which would confound any results was quite important. In addition, it is well known that there is variation among the distribution of muscle fiber types within muscles; research with rats has also shown how samples from different muscles result in very different levels of oxidative stress. Thus validating the chosen site for future biopsies was also critical. This project validated the biopsy site in the middle gluteal muscle of the horse, and showed how the pattern of biopsies taken can impact levels of oxidative stress. This knowledge is important for further research in oxidative stress in the muscle, for it ensures that biopsies taken in conjuncture with exercise studies will not have confounded results from there mere act of taking a muscle sample.

Few equine studies have compared markers of oxidative stress in the skeletal muscle and blood, particularly of horses after exercise and at rest, so this project looked

next at this situation. The exercise test chosen resulted in only mild increases in oxidative stress in the blood, and no changes in the muscle. While many variations have been seen in prior equine studies as whether or not exercise will affect oxidative stress, it has always been generally assumed that the main site of production of oxidative stress was the mitochondria of the skeletal muscle. However, given the lack of similarity with changes in the blood and skeletal muscle with oxidative stress markers, consideration must be given to the fact that other sites in the body (heart, lungs, etc.) are playing an important role during exercise to produce ROS and antioxidants. So while many times the variations in intensity and duration of exercise are cited as the main reasons for differences in oxidative stress, perhaps the issue is that there are multiple sources in the body for ROS production, and that different exercises impact these sites differently. Furthermore, while the current studies strove to use a variety of markers of oxidative stress and antioxidants, it may be that given the large variety in ROS and antioxidants, it is difficult to pick the "ideal" markers to represent changes in oxidative stress. As more research is done in this field, perhaps we will gain a better understanding of these ROS and antioxidant to specific types of exercise.

Not only does the type of exercise endured affect oxidative stress, but age and training were considered important factors as well. The findings that young growing horses have considerably less oxidative stress and higher levels of antioxidants as compared to mature mares is novel; no prior knowledge of oxidative stress in the growing yearling was known. Differences between ages were found not only in the blood, but the skeletal muscle as well, and their responses to exercise varied as a result of this; often the acute exercise did not even elicit a response for a specific marker of oxidative stress or

antioxidants. With training, the mares had many more significant improvements than the yearlings. It was interesting to see over training that many markers of oxidative stress had week to week fluctuations. The reasons for this are not clear, but one might be that the progressive increase in speed and intensity of the training bouts could have impacted the resting levels of oxidative stress and antioxidants. The other interesting observance was the improvement of the non-trained mares. Though not training daily, a biweekly sprint seemed to help them improve oxidative stress and antioxidant status as well. The implications of this, particularly for mature horses, could be that even minimal, regular exercise can improve the response to oxidative stress. Changes were not seen in the non-trained yearlings over the training period, so it appears that biweekly sprints did not impact them as much as the mares. Few differences were seen between trained and non-trained yearlings; part of this may be that the yearlings were naturally inclined to be more active in the pastures; it was typical to see them voluntarily playing and running around. So while the non-trained group was not formally trained, their general inclination to undergo voluntary exercise in the field may have helped them cope with oxidative stress. This information is useful for the implementation of future training programs for yearlings and young horses, for it shows that training in young horses in order to maintain or keep low levels of oxidative stress may not be necessary, provided the young horses are given plenty of time with access to open areas to run and play. Future work looking at yearlings with less access to pasture exercise (i.e. stall kept) would be interesting in seeing how much of an impact this activity has on oxidative stress.

A further consideration based on this study is that the trained mares had more significant improvements in oxidative stress and antioxidant status than the yearlings.

While age is one factor in this difference, the other may be due to dietary differences. The mares were accidentally fed diets low in vitamin E, while the yearlings were not. In clinical settings, high oxidative stress is seen in disease states or nutritionally deficient states. While in healthy animals no additional antioxidants are needed, this is not the case in sick animals. While the mares in the current study were not sick, they were not receiving the full nutrients they needed for this level of exercise training. So perhaps while exercise can lower oxidative stress and improve antioxidant status in compromised animals, such as the mares in this study, exercise may not be as beneficial (in terms of oxidative stress) for healthy, younger individuals.

Prior research with age and oxidative stress typically focuses on the older, aging animal; in the horse this would be an animal at least 20 years old. While now much can be said on the role of oxidative stress with aging, extremely limited data looks at oxidative stress and maturation. This study clearly demonstrates the differences in antioxidant and oxidative stress levels between the young growing horse and the mature adult horse. Given that horses are not fully mature until 4 years of age, future work looking at oxidative stress and antioxidants between 2, 3, and 4 year olds would be important to continue the work of analyzing oxidative stress and maturation.

Finally, an important aspect of this study was that no antioxidant supplementation was used. Antioxidants have become wildly popular as supplements, for both human and equine athletes. Yet the efficacy of these supplements is rarely known; many times people will take, or give their horses, supplements with no way to prove that they work. This study showed two important points; the first is that exercise training can improve the oxidative stress levels and antioxidant status of mature mares without any

supplementation. In just eight weeks of training, mares reduced lipid peroxidation levels and improved levels of antioxidants. The second point is that the young horses prior to training already had high levels of antioxidants and low levels of oxidative stress as compared to the mares. Supplementing antioxidants for young horses starting training would, in this case, seem unnecessary. Also importantly, training only slightly improved oxidative stress and antioxidants in the young horses; while the training protocol may not have been strenuous enough to elicit a significant training response, their age may have also played a large role. Further analysis of the changes in oxidative stress as a horse continues to mature would help to show how levels of oxidative stress and antioxidants also change.