8-6-2021

Oxidative stress biomarkers in blood plasma of moderately exercised horses

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A Thesis
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Master of Science
in Agricultural Science
in the Department of Animal and Dairy Sciences

Mississippi State, Mississippi

August 2021

Name: Elizabeth Catesby Ott

Date of Degree: August 6, 2021

Institution: Mississippi State University

Major Field: Agricultural Science

Major Professor: Clay Cavinder

Title of Study: Oxidative stress biomarkers in blood plasma of moderately exercised horses

Pages in Study: 51

Candidate for Degree of Master of Science

Equine athletes are subjected to environmental and physical stressors resulting in oxidative stress that can negatively impact performance. Oxidative stress can result in lipid peroxidation, cell damage, and DNA degradation leading to physiological dysfunction and increased instance of disease. It has been established that humans are able to adapt to oxidative stress when exposed to extended periods of high-intensity exercise, however, this has yet to be established in the equine model. In the present study, we sought to establish patterns of oxidative stress expression immediately following exercise and adaption to prolonged exposure to exercise training in the equine model. Results indicate horses express changes in oxidative stress biomarkers at the onset of exercise training but adapt with prolonged exercise regimes. Future research should focus on mitigation techniques and therapeutics for oxidative stress in equine athletes.

DEDICATION

To my wonderful mother, who has always encouraged me to chase my dreams no matter how they shift

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

The United States equine industry generates approximately $122 billion dollars for the US economy annually, both through direct and indirect spending. Throughout the country, over 7 million horses participate in racing, showing, or recreational activities every year (Jones, 2017). Mississippi's equine industry, with around 120,000 horses, generates $1.15 million in economic impacts annually with 90% of that horse population participating in showing and pleasure (Myles, 2008).

Horses are transported all over the country to participate in competitions and are subject to a variety of environmental and physical stressors on a regular basis. Many horse owners rely on unproven supplements and complicated feed strategies to combat temperament and exercise related performance issues. Trailering to and from shows, along with increased exercise loads during competition, is often thought to have a negative effect on the horse's performance, however there is little research investigating the specific mechanisms involved and effected by these stressors. The induction of Oxidative Stress (OS) brought on by physical stressors could help explain the decline in performance of many equine athletes when presented at competition.

Oxidative stress is often negatively associated with athletic performance due to its potentially damaging effects to unprotected tissues and cells, however, recent research in human athletes has indicated that regular exercise can increase antioxidant response, decreasing overall OS over time (Parker et al., 2014; Zuo et al., 2015). Despite this positive effect, unaccustomed or

exhaustive exercise can result in an imbalance of in the oxidant/ antioxidant ratio, causing muscle, tissue, and cell damage (Aguiló et al., 2005; Davies et al., 1982; He et al., 2016; Thirumalai et al., 2011). Horses presented at competition are exposed to continuous days of exercise without rest, deviating from their regular exercise routine, as well as an increase in exercise load for the duration of the competition. This increased exercise load and resulting increase in OS may be a contributor to decreased performance seen at competition, due to damage to muscle tissue and decreased force production within skeletal muscle (Powers & Jackson, 2008; M. Reid et al., 1993; Steinbacher & Eckl, 2015a; Wan et al., 2017).

The majority of equine research regarding OS has been performed on subjects participating in endurance and racing competitions (Brkljača Bottegaro et al., 2018; de Moffarts et al., 2004; Gondim et al., 2009; Smarsh & Williams, 2017), with data taken only immediately before and after exercise. While research pertaining to OS in human athletes is abundant, there is little information on OS in horses induced by moderate exercise or exercise repeated over extended periods of time. Understanding the immediate effects of exercise on OS production, as well as the effects of repeated exercise on OS levels over time, will give horse owners and trainers better insight on the effects of exercise on equine athletes, and how to develop training and showing programs to combat induction of OS.

Oxidative stress can be defined as an imbalance between free radicals (FR) and antioxidants in the body. Free radicals are known as atoms containing one or more unpaired electrons in an atomic orbital. These unpaired electrons make atoms unstable and highly reactive. The most common cause of FR formation is the synthesis of ATP, which produces reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Pham-Huy et al., 2008). Immune functions can benefit from low levels of ROS and RNS in the body, however, when the body is

unable to neutralize the ROS/RNS present, they can have detrimental effects and result in OS. This process can result in the altered structure of proteins, lipids, and DNA, and can damage cell membranes (Valko et al., 2007).

Investigation of research into the effects of OS on equine athletes led to the development of the currently discussed research. Analysis of available equine related literature, and issues present within the equine field led researchers to investigate the capacity that stressors associated with competition have to elicit significant OS response in equine athletes. The objectives of this study were to determine baseline parameters for OS biomarkers during rest and exercise, compare baseline measurements of OS biomarkers to those following exercise training, and investigate the persistence of OS biomarkers following exercise.

CHAPTER II

LITERATURE REVIEW

Free Radicals and Oxidative Stress

Oxygen free radicals – more commonly referred to as reactive oxygen species (ROS) and reactive nitrogen species (RNS) – and their effect on OS across a multitude of species has been a topic in human and veterinary medicine now for over fifty years. Upon the discovery of oxygen toxicity in the early 1950's (Gerschman et al., 1954) there have been numerous articles regarding the production of ROS and the effects they have on biological systems (Beckman & Ames, 1998; Lykkesfeldt & Svendsen, 2007; Pham-Huy et al., 2008; Powers & Jackson, 2008). It is now understood that ROS and RNS play a number of roles within living systems, and have the potential to be both beneficial and harmful (Valko et al., 2007).

Moderate and low concentrations of ROS and RNS are necessary for maintenance of normal cell function, acting as regulatory mediators in signaling processes (Li, 2013) along with regulation of vascular tissue and ensuring the maintenance of redox homeostasis (Dröge, 2002). It is well known that the production of ROS can be identified in most cell types, acting as secondary messengers in a variety of signal transduction pathways (Valko et al., 2007). Reactive Oxygen Species can also be seen working as part of the body's defense mechanism, used to attack microbes eliciting an inflammatory response in the body (Li, 2013).

Although the benefits of low concentrations of ROS and RNS are now widely recognized, accumulation of these compounds can be dangerous to living systems. The buildup

of ROS and RNS causes an imbalance in the oxidant/antioxidant ratio maintained by the body, causing OS (Pham-Huy et al., 2008). Oxidants can be classified into radicals, a compound with an unpaired electron in the outer orbital, and non-radicals. A ROS/RNS radical status is relatively unrelated to the reactive potential of the molecule, rendering this classification system of little use. Buettner et al. (1993) created a more applicable classification system, according to an oxidants one-electron reduction potential (Lykkesfeldt & Svendsen, 2007). This reduction potential is analogous to the oxidant's reactive potential, effectively organizing these oxidants by the potential to cause harm. Free radicals include hydroxyl ($OH^{\bullet}$), superoxide ($O_2^{\bullet-}$), nitric oxide ($NO^{\bullet}$), nitrogen dioxide ($NO_2^{\bullet}$), peroxyl ($ROO^{\bullet}$), and lipid peroxyl ($LOO^{\bullet}$), with $OH^{\bullet}$ acknowledged as the most reactive species (Buettner, 1993).

Oxidative stress occurs when the body is unable to neutralize or destroy excess ROS/RNS, causing oxidative damage that can alter and destroy cell membranes and other structures such as proteins, lipids, lipoproteins and deoxyribonucleic acid (DNA). The hydroxyl radical is capable of damaging or destroying all structural aspects of a DNA molecule (Valko et al., 2007), and is the causative agent of lipid peroxidation when combined with peroxynitrite (Pham-Huy et al., 2008).

Antioxidant Defense System

Prolonged exposure to damaging oxidants has led organisms to develop defense mechanisms to help prevent oxidative damage to cells and tissues throughout the body. These defense mechanisms have developed into a variety of systems including preventative mechanisms, repair mechanisms, antioxidant and physical defenses (Cheeseman & Slater, 1993). Antioxidants are primarily responsible for protecting cells and tissues throughout the body from

oxidative damage by ROS, which is achieved by inactivating or transforming oxidants. Transformation of oxidants by antioxidant enzymes results in less reactive forms of the oxidative molecules, or oxidative molecules that can further react with antioxidant enzymes (Kirschvink et al., 2008).

The hydroxyl radical along with the O_2 anion and NO are responsible for a wide variety of issues that are associated with oxidative damage, which has required the body to adapt an effective method to combat this damage. Antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione-peroxidase (GPx) play a large role in catalyzing damaging oxidants in the body. These enzymes are responsible for transforming the hydroxyl radical into the O_2 anion, which is then reduced further into hydrogen peroxide (H_2O_2) and water through catalytic activity (Fridovich, 1995; Valko et al., 2007).

It is well established that exercise results in an increase in OS within the body (Davies et al., 1982; Li, 2013; Powers et al., 2011; Sachdev & Davies, 2008; Smarsh & Williams, 2017). With this ROS increase there is also an increase in the antioxidant levels during exercise (Aguiló et al., 2005; Fisher-Wellman & Bloomer, 2009; Parker et al., 2014). The presence of both antioxidants and oxidants is known as the oxidant/antioxidant ratio and can be used to measure the antioxidant response to the presence of ROS. In cases of extreme or exhaustive exercise, there is often a spike in the oxidant levels which shows an imbalance in the oxidant/antioxidant ratio, which when the bodies antioxidant defense system is no longer capable of keeping up with the ROS specie generation caused by intense exercise (Kirschvink et al., 2008).

When exposed to moderate-high intensity exercise continuously over time, an adaptive response within the antioxidant defense system is stimulated, increasing the ability to combat OS and maintain redox homeostasis in more extreme conditions (He et al., 2016; Parker et al., 2014;

Zuo et al., 2015). This elevated antioxidant response increases an individual's threshold for preventing OS related injuries when faced with exhaustive exercise, however, cannot prevent OS completely (Aguiló et al., 2005). There is extensive research investigating the mechanisms of antioxidant adaptation and its effects on athletes, with numerous papers and review articles dedicated to breaking down the components responsible for this adaption (Davies et al., 1982; Ji, 2008; M. B. Reid, 2001; Sachdev & Davies, 2008; Sen, 1995). Common themes established across the literature suggest that specific antioxidant response trends can be seen across species when experiencing exercise induced oxidative stress. Previous research consistently indicates that SOD levels increase in magnitude relative to exercise intensity (de Moffarts et al., 2004; Higuchi et al., 1985; Leeuwenburgh et al., 1994). Increases in GPx activity in response to increased exercise has also been recorded by numerous authors, however there is some discrepancy amongst experiments, resulting in less consistent findings for this parameter (Aguiló et al., 2005; Ji, 2008; Margis et al., 2008). Other physical parameters have also been indicated to effect the onset and severity of OS response such as age and sex (Kamper et al., 2009; Leeuwenburgh et al., 1994; Smarsh & Williams, 2017). These themes are not only represented within human research, but can be identified within equine research as well, with equine research investigating age effects and exercise response on the on-set and severity of OS as well as the antioxidant response demonstrated.

Age effect on OS markers has also been investigated by Smarsh et al. (2017) in a study measuring MDA, NO, GPx, and GSH-T in blood, muscle, and erythrocyte samples. An exercise training regime was used to measure OS biomarkers in unfit yearlings and mares. These data demonstrated that exercise training improved antioxidant status in both mares and yearlings, however there were more significant effects on antioxidant status of the mares, who showed

lower preliminary antioxidant levels. Age appeared to have an effect on antioxidant capacity, with the yearlings showing a higher antioxidant capacity in comparison to the older mares (Smarsh & Williams, 2017).

In a study by Bottegaro et al. (2018) examining the oxidative status of endurance horses, OS biomarker levels were analyzed before and after participating in endurance races of varying lengths. Through blood, plasma, and erythrocyte analysis the levels of antioxidant and oxidant markers in the blood were studied. The determination of reactive oxygen metabolites (d-ROMs), along with the biological antioxidant potential (BAP) and MDA levels were the oxidant biomarkers studied in this experiment. Although there were increases in MDA, d-ROMS and BAP, this study suggests that BAP levels were the most reliable OS marker, with little variation within and between horses (Brkljača Bottegaro et al., 2018). However, this study was limited in its information pertaining to fitness level of the horses analyzed in each race and failed to determine adequate baseline blood analysis for proper comparison to post-race biomarker levels. The horse population studied in this experiment was transported varying distances to participate in the competition and allowed a minimum of two hours of adjustment time before the initial pre-exercise blood sample was taken. Still, blood samples of horses two-hours post transportation may show elevated OS markers, as OS biomarkers have been detected post transportation (Onmaz et al., 2011), and have been seen in the blood up to 48 hours after induction of stress (Moffarts et al., 2004). These variables may be responsible for some of the discordant changes seen in the results of this study.

Detecting Oxidative Stress

Identifying OS by quantifying free radicals can be extremely difficult or impossible depending on the free radical being studied. The ability to study these free radicals directly is

complicated due to their exceedingly short half-life, coupled with their highly reactive nature. Due to these factors, free radicals are most often studied by identifying by-products or end products of free radical reactions. By measuring the number of byproducts found in a sample, the approximate level of ROS that were present within the body can be determined (Sachdev & Davies, 2008).

Each free radical has an individual reaction pathway that it undergoes within the body, and subsequently produces a unique byproduct that can be studied. For example, lipid peroxidation can be measured indirectly by analyzing the levels of malondialdehyde (MDA) found within the body, while glutathione (GH) and glutathione-peroxidase (GPx) ratios can be used to determine the prevalence of H_2O_2.

Glutathione Peroxidase

Glutathione Peroxidases make up a family of multiple isozymes that are most commonly known for their role in catalyzing the reduction of organic hydroperoxides along with H_2O_2. The reduction of these molecules results in water or corresponding alcohols and is conducted using electrons donated from GSH (Margis et al., 2008). The 4 major Se dependent isozymes found within mammalian tissues are GPx1-4, which fall under the category of selenoproteins as they contain a selenocysteine center. The selenocysteine center acts as a catalyst, ensuring a fast reaction with H_2O_2 molecules along with a quick reduction by GSH (Brigelius-Flohé & Maiorino, 2013).

GPxs play a major role in managing H_2O_2 homeostasis. Following the breakdown of the O_2 anion by SOD into H_2O_2 and $O_2^{\bullet-}$, GPx is responsible for continuing to break down the byproduct's left behind by the O_2 anion. Glutathione peroxidase utilizes reduced glutathione (GSH) to reduce H_2O_2, resulting in oxidized glutathione and water (Fukai & Ushio-Fukai, 2011).

Glutathione reductase (GR) is responsible for reducing oxidized glutathione to GSH, ensuring adequate levels of GSH are available for GPx reactions moving forward (Austin et al., 1988). Disruption to homeostasis of H_2O_2 within various signaling cascades can be detrimental, resulting in possible damages to numerous body systems, including the insulin signaling pathway while also playing a role in carcinogenesis and apoptosis regulation (Brigelius-Flohé & Maiorino, 2013).

Superoxide Dismutase

The $O_2^{\bullet-}$molecule is one of the most prevalent and damaging free radicals found within the body, which has resulted in the development of the SOD enzyme, a key player in the antioxidant defense system. Superoxide dismutase is responsible for breaking down the $O_2^{\bullet-}$ radical, separating it into H_2O_2 and oxygen (Halliwell & Gutteridge, 2015; Powers et al., 1999). Along with its primary role, SOD is also responsible for inhibiting inactivation of NO (Fukai & Ushio-Fukai, 2011). Location and molecular composition of the SOD enzyme differs between the three isozymes found within skeletal muscle. The three isozymes are ion bound to different metals, with 2 binding to Cu-Zn and the third to Mn. The Mn-SOD enzyme is located within the mitochondrial matrix, while one Cu-Zn SOD is located within the cytosol, and the other Cu-ZN SOD is located within the extracellular matrix (Fukai & Ushio-Fukai, 2011; Halliwell & Gutteridge, 2015; Powers et al., 1999).

Superoxide dismutase is responsible for maintaining the correct proportions of $O_2^{\bullet-}$. Cellular metabolism naturally produces vascular $O_2^{\bullet-}$ which aids in the creation of numerous other ROS within the body. Fluctuations in the $O_2^{\bullet-}$ levels within the body have been documented to alter vascular tone and gene expression and result in inflammation and cellular

growth (Fukai & Ushio-Fukai, 2011). The role SOD plays as a defense against the deactivation of NO is crucial in order to maintain NO role as an anti-inflammatory and anticoagulant within the body. The reaction of $O_2^{\bullet-}$ with NO results in the production of a strong oxidant peroxynitrite ($ONOO^-$). The generation of this molecule is involved in conditions leading to endothelial and mitochondrial dysfunction (Fukai & Ushio-Fukai, 2011; Guzik & Harrison, 2006). Due to the involvement of nitric oxide, oxidative stress plays a large role in the cardiovascular system, and is a known player in the development of cardiovascular disease (Lakshmi et al., 2009).

Oxidant generation is largely prevented in the electron transport chain of the mitochondrial matrix, where electron leakage can result in radicalizing neutral molecules into damaging oxidants (Barja, 1999). Binding of free transition metals such as Cu and Fe to ROS contributes to the damaging effects these molecules have on cells and tissues throughout the body. Generation of oxidants is prevented by the presence of enzymatic complexes located in the mitochondrial respiratory chain, as well as the presence of proteins responsible for binding to free Cu and Fe (Kirschvink et al., 2008). The presence of proteins responsible for binding to free transition metals limits the damaging effects of these metals, and decreases the capacity for ROS generation (Beckman & Ames, 1998; Valko et al., 2007).

Pathologies of Oxidative Stress

Transition metal bound ROS are one of the many causes of OS that are involved in and associated with diseases spanning both humans and animals, with transition metals playing a role in neurological disorders such as Alzheimer's disease (Smith et al., 2000). Recent research has indicated that ROS-bound Cu and Fe disrupt oxidative homeostasis, altering senile plaques present in this condition, with modifications characteristic of oxidative damage (Bagheri et al., 2018). Copper and Fe accumulation has been reported to be significantly elevated throughout

multiple regions of the brain most commonly effected by changes seen in Alzheimer's patients (Bagheri et al., 2018; Chen & Zhong, 2014). Neurological diseases are largely effected by plaque buildup within the brain which can be attributed to an oxidant/ antioxidant imbalance within these tissues (Chen & Zhong, 2014; Smith et al., 2000). Equine neurological diseases such as equine grass sickness have also been associated with OS, however, further research in this area is necessary to determine the role of OS on the development of this condition (McGorum et al., 2003).

Lower airway diseases such as asthma (Sahiner et al., 2011) and recurrent airway obstruction (RAO) (Art et al., 1999), in humans and horses respectively, have identified OS as a contributor to the onset and severity of these diseases. The endogenous and exogenous ROS produced during OS and inhaled from the environment are strong contributors to the inflammatory response seen within the respiratory system in those with these conditions (Art et al., 1999; Sahiner et al., 2011). Investigation into the effects of OS on asthma patients has shown that those suffering from this condition have significantly lower levels of antioxidants such as SOD, GSH, CAT, and increased levels of ROS such as MDA, NO, and $O_2^{\bullet -}$ in the epithelial lining fluid and alveoli of the lungs, indicating higher baseline levels of OS than that of healthy patients (Church & Pryor, 1985; Comhair & Erzurum, 2010; Sahiner et al., 2011). Production of ROS as well as SOD levels within the airways of asthmatic patients correlate to the severity of reactivity, demonstrating the direct role OS plays in the onset and severity of this condition (Calhoun et al., 1992; Sanders et al., 1995).

Due to the apparent symptomatic and pathophysiologic similarities between asthma and RAO, it has been speculated that RAO onset and severity is caused by similar mechanisms found in human asthma, however, there is little research investigating asthmas equine counterpart

(Deaton, 2006). It is clear that exposure to exogenous allergens, predominantly organic dust produced by hay and other forages, elicits a neutrophilic inflammation, resulting in antioxidant consumption and OS (Art et al., 2006; Brazil, 2000). Further research is needed to develop a better understanding of how OS effects the pathophysiology of RAO.

Malondialdehyde

One of the most common indicators of lipid peroxidation is the presence of aldehydes such as malondialdehyde (MDA), which are derived from the peroxidation of polyunsaturated fatty acids. Aldehydes are known to compound the effects of oxidative damage to cells and surrounding tissues (Del Rio et al., 2005; Singh et al., 2014). These molecules are highly reactive and have a long half-life in comparison to other by products of OS, increasing the ability of these molecules to interact with and damage biomolecules such as nucleic acids and proteins. Interaction of aldehydes with other biomolecules can result in extensive, and often irreversible damage to cell structures and functionality (Del Rio et al., 2005). Arachidonic acid metabolism is a major contributor the production of MDA, and can be found in a variety of locations throughout the body including cell membranes, as well as brain, muscle and liver tissue (Singh et al., 2014).

Analysis of MDA levels is a well-established method for monitoring lipid peroxidation in different biological systems and is often used to monitor various health disorders (Singh et al., 2014). Samples are most commonly analyzed for MDA by testing for thiobarbituric acid (TBA) activity, or thiobarbituric acid-reactive species (TBARS). It is well established that an increase in MDA levels after exercise bouts is common (Sachdev & Davies, 2008).

The use of TBARS analysis to determine MDA levels has been used extensively in equine research to show increased incidence of lipid peroxidation following exercise. When

investigating the effects of exercise on plasma antioxidant capacity, White et al. (2001) utilized a plasma TBARS analysis to demonstrate an increase in lipid peroxidation in thoroughbred racehorses. Results from this study showed a significant increase from baseline in TBARS following high intensity exercise in horses untreated with ascorbate, indicating that high intensity exercise elicits a significant increase in lipid peroxidation due to increased MDA concentrations brought on by OS (White et al., 2001).

In a similar experiment by Gondim et al. (2009), analysis of plasma TBARS was used to detect differences in lipid peroxidation levels in horses before and after endurance races. Measurements were taken over the span of 3 days, demonstrating the pattern of lipid peroxidation when horses were exposed to continued endurance exercise. When compared to baseline (taken before d 1 or racing), TBARS levels increased significantly following d 1 of racing and then plateaued, showing no significant increase between d 1-3 of racing. The lack of significant difference in TBARS levels from d 1-3 of racing indicates that continued exposure to oxidative stress does not compound lipid peroxidation as exposure continues (Gondim et al., 2009). Understanding of TBARS level trends was limited by sample times within this experiment, as samples for d 1-3 of racing were only taken after the race was complete, and therefore could not be compared to levels prior to racing each day. This did not allow for comparison between increases in TBARS levels for each racing day, which could have shown whether significant differences were present between the beginning and end of the race for each racing day. Measurement of TBARS before racing on d 1-3 could have also been used to indicate the length of time lipid peroxidation persists within the body after racing.

Oxidative Stress in Skeletal Muscle

While measuring the ROS directly produced during exercise can be beneficial, OS can also be measured by examining macrophysiological changes within the subject. These changes can be seen in the contractility of muscles, fatigue endurance capacity, vasodilation, blood flow, and tetanic force output. These macrophysiological changes are most often seen in skeletal muscle, due to the high incidence of OS during exercise (Sahlin et al., 2010; Steinbacher & Eckl, 2015b; White et al., 2001). It is well established that ROS produced during exercise can have a positive effect on antioxidant expression when muscles are routinely exercised, however, overloading muscles in extreme exercise surpasses the body's ability to protect against drastic increases in free radical formation within the muscle (Davies et al., 1982). This accumulation of ROS can be detrimental to skeletal muscle, resulting in muscle fatigue and atrophy (Steinbacher & Eckl, 2015b).

Skeletal muscle is a highly reactive muscle group that is responsible for voluntary movement. Moderate to heavy exercise causes increased strain on skeletal muscle, and is known to result in OS, through increased production of free radicals within the muscle fibers. Following the discovery of OS, investigators have documented the negative and positive effects of OS on skeletal muscle and have established that repeated exposure to moderate to high intensity exercise can increase antioxidant capacity over time (Powers & Jackson, 2008; Steinbacher & Eckl, 2015b). This increase in antioxidants reduces the damage caused by free radicals during exercise and can result in a number of physiological changes to the muscle group such as fiber type transformation and increased force generation. These changes allow the muscle to develop an increased capacity for aerobic metabolism and increase the muscles resistance to fatigue and atrophy.

Muscle fatigue is defined as a decrease in maximal force or power production in response to contractile activity (Degens & Veerkamp, 1994; Wan et al., 2017). Extreme muscle fatigue can cause decreased physical performance in both humans and horses and can be caused by a number of factors. The most common cause of muscle fatigue in exercising horses is overloading muscle with moderate to high intensity exercise, which can cause oxidative damage (Powers & Jackson, 2008). Recent research has indicated certain ROS are routinely released by contracting skeletal muscle both at rest and during exercise. Hydrogen peroxide, SO, and NO are all ROS associated with the contraction of skeletal muscle and are believed to be released both into the muscle fibers and into the muscles extracellular space (Patwell et al., 2004).

Force production of skeletal muscle is dependent on the presence of ROS in unfatigued muscle, with basal levels of ROS necessary for normal force production (M. B. Reid, 2001; Supinski & Callahan, 2007). If ROS basal levels decrease or become depleted, force production decreases, however, small increases in ROS levels coincide with an increase in force production. This increase is seen only with mild increases in ROS levels, as there is a threshold for beneficial effects of ROS (M. Reid et al., 1993; M. B. Reid et al., 1998). High levels of ROS reverse the positive effects on force production, causing a decrease in the isometric force generated by the skeletal muscle fibers (M. Reid et al., 1993). The relationship of ROS with force production in unfatigued muscles is variable, and the effects of ROS on muscle is determined in a dose-dependent manner.

The decrease in force production seen in the presence of high levels of ROS can be associated with the contractile use of the skeletal muscles (Supinski & Callahan, 2007). This information leads to the conclusion that the continuous contraction of muscle fibers during exercise may result in the buildup of high levels of ROS in the muscle, resulting in a decrease in

force production. Using this information in relation to athletes, authors speculated that the buildup of ROS is the cause of muscle fatigue during or after high intensity or prolonged exercise (Degens & Veerkamp, 1994). These ROS products of muscle contractions are produced on the cellular level, and are attributed to multiple cellular functions within the body, including electron transport chain leakage within mitochondria and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Davies et al., 1982; Halliwell & Gutteridge, 2015).

Oxidative Stress: Mitochondria

Initial research into the source of free radicals hypothesized that mitochondria located within the myofibrils of the muscle were responsible for leaking lone electrons from the electron transport chain (Davies et al., 1982). Research indicated that between 1-4% of O_2 consumed by the mitochondria was converted into SO and released into the cell and extracellular membranes (Sachdev & Davies, 2008). Complex(s) I and III of the electron transport chain haven been consistently identified as the processes predominantly responsible for production and leakage of $O_2^{\bullet-}$ into the cell (Barja, 1999; Powers & Jackson, 2008).

Research prior to this publication shows contrasting data which indicates the estimated production of ROS by mitochondria reported by Sachdev & Davies (2008) was overestimated by several orders of magnitude. St-Pierre et al. (2002) estimated that the portion of oxygen converter to ROS throughout the electron transport chain is approximately 0.15% opposed to the 1-4% estimated by Sachdev & Davies (2008; St-Pierre et al., 2002). This would indicate that electron transport chain leakage is not the leading contributor to the production of OS in skeletal muscle as was originally thought.

Increased body temperature has also been associated with an increase in generation of ROS (specifically O_2^- and H_2O_2) due to mitochondrial uncoupling, suggesting mitochondria

undergo a temperature-dependent loss of respiratory control as temperature increases (Salo et al., 1991). It is known that uncoupled mitochondria are responsible for the generation of O_2^- and H_2O_2 at significantly higher rates than properly coupled mitochondria. This temperature dependent reaction paired with the increased body temperature exhibited during exercise is indicated in the increased 'leakage' of ROS during moderate to high intensity exercise (Sachdev & Davies, 2008).

Quindry et al. (2013) reported on the effects of external temperature on OS expression in athletes during moderate intensity exercise. Throughout this experiment, core body temperature was monitored while participants used a stationary cycle to achieve moderate intensity exercise in three controlled environments consisting of neutral (20°C), cold (7°C), and warm (33°C). Significantly elevated levels of OS biomarkers were recorded in participants following exercise in the warm environment, but not when exercising in the neutral or cold environments (Quindry et al., 2013). These findings indicate that increased core body temperature due to hyperthermic environmental conditions increases OS during moderate intensity exercise in human athletes.

When investigating the effects of external environments on OS and athletic performance in equine athletes, Mills et al. (1996) subjected equine athletes to prolonged variable-intensity and short-term high-intensity exercise within 2 environments consisting of cool (20°C; 40%RH) and hot/humid (30°C; 80%RH) conditions. These exercise protocols were designed to mimic equine competition scenarios seen in racing or the speed and endurance phase of 3 d eventing. Strong correlations were seen between exercise in hot/humid conditions and increased OS biomarkers (GSSH, lipid hydroperoxides) in comparison to OS biomarkers seen in prolonged or short-term exercise in cool conditions (Mills et al., 1996). These findings are consistent with those seen in human athletes (Quindry et al., 2013) and other animal models (Kaldur et al., 2014)

indicating that external temperature during exercise has the potential to increase OS through the uncoupling of mitochondria due to increasing core temperature in exercising subjects. This information should be considered when determining parameters for further research in oxidative stress trials in equine athletes.

Oxidative Stress: Nicotinamide Adenine Dinucleotide Phosphate

Outside of mitochondrial related OS generation, there are a variety of proposed causes, including the generation of O_2 by NADPH oxidase. Nicotinamide adenine dinucleotide phosphate oxidase is responsible for the generation of O_2 from molecular oxygen by transferring electrons from an NADPH molecule in order to radicalize molecular oxygen (Halliwell & Gutteridge, 2015). Reactive oxygen species production within skeletal muscle has been linked to NADPH oxidase during exercise. The presence of NADPH oxidase throughout multiple cellular locations within muscle fibers – including sarcoplasmic reticulum, transverse tubules, and the sarcolemma – has resulted in the postulation that increased contractile movements of muscle fibers during exercise leads to excess ROS production (Powers et al., 2011). While there are no proven uses of NADPH oxidase within the muscle fibers, there is evidence that the generation of O_2 influences the release of Ca by the sarcoplasmic reticulum (Jackson, 2008). Studies have shown the NADPH oxidase enzymes can be found in both cardiac (Cherednichenko Gennady et al., 2004) and skeletal (Xia et al., 2003) muscle fibers. Affecting this Ca release mechanism could result in changes in muscle contraction and its resulting force production.

Multiple studies have indicated the O_2 is released into the extracellular space following the contraction of skeletal muscle fibers (Patwell et al., 2004; M. Reid et al., 1993; M. B. Reid et al., 1998). Electron transfer across the plasma membrane can then be achieved by the plasma membrane redox systems present in muscle fibers and other cells. The release of O_2 into cytosol

and the extracellular space could result in serious repercussions throughout the body, possibly causing a cascade of ROS generation throughout the surrounding membranes.

CHAPTER III

OXIDATIVE STRESS BIONARKERS IN BLOOD PLASMA OF MODERATELY EXERCISED HORSES

Introduction

Oxidative stress is a detrimental aspect of various physiological processes and has been well documented as a component in a variety of diseases and disorders and is strongly linked to the aging process (Barja, 1999; Chen & Zhong, 2014; Fukai & Ushio-Fukai, 2011). Exercise has been proven to increase incidence of OS is both humans and rats and is known to cause damage to muscle components which can result in muscle soreness and decreased force production in effected areas (Powers & Jackson, 2008; M. Reid et al., 1993; Steinbacher & Eckl, 2015b).

Oxidative stress occurs when the body is unable to neutralize or destroy excess ROS/RNS, causing oxidative damage that can alter and destroy cell membranes and other structures such as proteins, lipids, lipoproteins and DNA (Valko et al., 2007). This process is known to have a negative effect on athletes, causing decreases in performance and overall well-being (Davies et al., 1982; Ji, 2008; M. B. Reid, 2001; Sachdev & Davies, 2008; Sen, 1995). When investigated in the equine model, OS is known to occur after exposure to increased physical stress such as trailering and increased exercise load, which are commonly seen in horses participating in the equine show/ competition circuit (Andriichuk et al., 2016; Niedźwiedź et al., 2013; Smarsh & Williams, 2017).

Research in human medicine has shown that continued exposure to OS can cause adaptation of the antioxidant response to increase production of antioxidants within the body, resulting in decreased free radicals and decrease damage to body structures (de Moffarts et al., 2004; Traustadóttir et al., 2012).

The current study was designed to determine the effect of stress induced by moderate exercise on oxidative biomarkers in blood plasma. This study used exercise as a model to induce OS to understand potential OS in various situations including trailering, new environment, and acutely increased performance demands associated with the equine show circuit.

Materials & Methods

Experimental Design

Stock-type horses (n = 6; 6-16 ± 3 y) were housed at the Mississippi State University Horse Unit in 8 × 8 m auxiliary stalls. The first 2 wk were the acclimation period for horses to adjust to housing, diet, and exercise, followed by an 8-wk experimental period. Horses were stalled continuously during the project, with no free turn out, but exercised routinely via the project exercise procedure. All horses were fed an average of 20 lbs/d of bermudagrass hay. Nutrena 12% Stock and Stable pelleted grain was fed twice daily to the horses to maintain their desired body condition score of 5.5. The body condition score was evaluated at the beginning of the study and every 2 wk thereafter (Henneke et al., 1983).

Exercise Protocol

Horses were subject to a standardized moderate-intensity exercise procedure following the NRC guidelines (Council, 2007), consisting of 30% walk, 55% trot, and 15% canter, with a target heart rate (HR) of 90 BPM. Prior to the start of the current study, all horses were subject to

a 2-wk acclimation period, with 25% of the experimental exercise intensity in wk 1 and 50% intensity in wk 2 (Table 2.1). Immediately following the acclimation period, the experimental exercise procedure was conducted. This exercise regimen consisted of 1h exercise bouts, 3 d/wk over an 8 wk period.

Heart rates were monitored using Hylofit™ heart rate monitors attached to the saddle girth (Hylonome LLC, Wilton, Conneticut). Monitors were placed on the left side of the horse along the heart girth with the electrodes at the base of the heart girth and midway below the withers, as directed by the manufacturer. The electrodes were lubricated with ultrasound gel to ensure accurate reading. Three HR monitors were used, and horses HRs were monitored individually, and exercise adjustments were made when the minimum HR requirement was not met for each gait (walk: 70 BPM; trot: 95 BPM; canter: 115 BPM).

Table 3.1 Protocol to meet moderate intensity exercise requirements.

Duration	Gait
4 min	Walk
8 min	Trot
3 min	Walk
5 min	Canter
8 min	Trot
4 min	Walk
8 min	Trot
4 min	Walk
5 min	Canter
8 min	Trot
3 min	Walk

Blood Sample Collection

Blood samples were collected before exercise (-60 min), immediately upon completion of exercise (0 min), and at 30, 60, and 90 min after exercise. Samples were collected during wk 1, 2, 7 and 8, while the exercise procedure was conducted for the entire 8 wks. At each time point, blood samples were collected via jugular venipuncture into 10 mL vacutainer tubes containing potassium heparin or silicone coating for later separation of blood plasma and serum (Becton, Dixon and Company, Franklin Lakes, NJ). Blood plasma was centrifuged immediately after collection for 10 mins at 2,000 × g, aliquoted into 1.5 mL microcentrifuge tubes, and frozen on dry ice, and stored at -80°C until analysis.

Sample Analysis

Analysis of blood plasma TAC concentrations were completed using a Trolox Equivalent Antioxidant Capacity (TEAC) assay. ABTS stock solution was created using mM 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt and 2.45 mM potassium persulfate, which was then diluted to an absorbance of 0.8 to 0.9 using DI water. Plasma samples were

diluted 1:10 before 10uL of diluted sample were combined in 200 μL of ABTS to measure absorbance.

Plasma homogenates were analyzed for MDA concentrations using a modified version of the TBARS extraction procedure developed by Draper et al. (Draper et al., 1993). Malondialdehyde was extracted in 10% trichloroacetic acid with 50 ppm of BHT. The extracted sample was reacted with 0.02 mM TBA solution and measured at 532 nM. Absorbance was compared with MDA authentic solution and results were expressed as μM of MDA.

Blood plasma concentrations of GPx were determined using a Glutathione Peroxidase Assay Kit (Cayman Chemical, Ann Arbor, MI). Plasma was diluted by a factor of 1:2 with provided sample buffer. Diluted sample was then reacted with NADPH and GPx Co-substrate. Activity was compared with GSH control solution and results were expressed as nmol/ min.

Plasma SOD concentrations were determined using a Superoxide Dismutase Assay Kit (Cayman Chemical, Ann Arbor, MI). Plasma was diluted by a factor of 1:5 with provided sample buffer. Diluted sample was reacted with radical detector and xanthine oxidase. Activity was measured at absorbance 440 nm and compared with SOD standard solution and results were expressed in U/ mL.

All assays were performed according to the manufacturer's instructions within 6 mo of sample collection.

Statistical Analysis

Data were analyzed as a split-plot design in time (repeated measurement) using a generalized linear mixed model with wk, day, time, and their interactions as fixed effects and animal within a combination of wk × day as random effect. The analysis of variance was

performed by the GLIMMIX procedure of SAS 9.4 (SAS Institute, Cary, NC). The selection of the appropriate covariance structure for the repeated measurement was based on three default Information Criteria calculated by SAS in the smaller-is-better format (AIC, Akaike's Information Criteria; AICC, AIC Corrected; and BIC, Bayesian Information Criteria; (Kincaid, 2005)), resulting in a first-order autoregressive structure being used. Means, if differing, were separated by a protected t-test using the LSMEANS statement. If three-way wk × day × time interaction was significant, all time points were only compared with the baseline values at -60 min on d 1 of wk 1 to reduce the total number of pair-wise comparisons and overall experiment-wise error rate. Actual probability values were reported.

Results

Thiobarbituric Acid Reactive Substances

A wk x d x time interaction ($P < 0.001$) for TBARS was observed in exercised horses. Blood plasma concentrations of TBARS activity ranged from 2.750 ± 0.177 to 4.920 ± 0.177 μM malondialdehyde within these data. Horses displayed a baseline measurement of 2.700 ± 0.177 μM malondialdehyde on d 1 of wk 1 immediately before exercise. This value will be used for comparison against all other measurements. While there are some variations between wk, most TBARS plasma concentrations remained at baseline, except for select measurements from wk 1 and 7, and most measurements from d 1 and 2 of wk 2. Concentrations of TBARS decreased by 0.525 ± 0.177 to 0.625 ± 0.177 μM malondialdehyde throughout wk 1 (Figure 3.1). Decreases were seen 30 and 60min after exercise on d 1 of wk 1 ($P \leq 0.037$), 60min after exercise on d 2 of wk 1 ($P \leq 0.037$), and 90min after exercise on d 3 of wk 1 ($P \leq 0.016$), while all other wk 1 plasma concentrations remained at baseline ($P \geq 0.097$). Significant spikes from baseline in TBARS concentrations were seen at all collection times following exercise on d 2 of wk 2, with a continuation of this spike visible both before and immediately after exercise on d 3 of wk 2 ($P < 0.001$). On d 2 of wk 2 TBARS concentrations increased by 1.210 ± 0.177, 2.150 ± 0.177, 2.220±0.177, and 2.060 ± 0.177 μM malondialdehyde at 0, 30, 60, and 90-min post-exercise respectively, and increased by 2.030, and 1.300 μM malondialdehyde at -60 and 0-min after exercise on d 3 of wk 2 ($P \leq 0.001$; Figure 3.1). On d 1 of wk 7 TBARS concentrations decreased by 0.500 ± 0.177 to 0.625 ± 0.177 μM malondialdehyde immediately before exercise until 60-min post exercise ($P \leq 0.047$) before returning to baseline, while all other wk 7 measurements remained at baseline concentrations ($P \geq 0.063$; Figure 3.2). There were no significant changes from baseline observed throughout wk 8 ($P \geq 0.273$; Figure 3.2).

Glutathione Peroxidase

A wk x d x time interaction ($P < 0.001$) for GPx blood plasma activity were observed in exercised horses. Activity of GPx ranged from 107.5 ± 14.8 to 255.9 ± 14.8 μM/min within these data. Horses displayed a baseline measurement of 172.6 ± 14.8 μM/min. Most time points has similar blood plasma GPx activity to the baseline value, however; plasma GPx activity peaked on d 1 of wk 2 following exercise and remained elevated at min -60 and 0, before returning to baseline on d 2 of wk 2 (Figure 3.5). The GPx activity was increased by 74.7 ± 14.8 and 63.4 ± 14.8 μM/min at 60 and 90-min after exercise on d 1 of wk 2 ($P \leq 0.003$), and 77.0 ± 14.8, 83.4 ± 14.8, and 48.2 ± 14.8 μM/min at -60, 0, and 30-min on d 2 of wk 2, respectively ($P \leq 0.023$). Horses displayed reduced GPx activity in blood plasma in comparison to baseline after exercise on d 2 of wk 7 and activity remained at these levels into d 3 of wk 7 immediately before and after exercise before returning to baseline 60-min after exercise on d 3 of wk 7 (Figure 3.6). There were no significant changes from baseline seen throughout wk 8, with activity remaining within the range of 174.8 ± 14.8 to 205.4 ± 14.8 μM/min for all measurements ($P \geq 0.102$).

Superoxide Dismutase

A wk x d x time interaction ($P < 0.001$) for SOD activity was observed in exercised horses. The blood plasma SOD activity ranged from 138.3 ± 3.9 to 182.8 ± 3.9 U/ml within these data. Horses displayed a baseline SOD activity of 167.2 ± 3.9 U/ml on d 1 of wk 1 immediately before exercise. This measurement will be used for comparison against all others. Throughout wk 1 and 2, significant changes in SOD activity were observed. Blood plasma SOD activity increased from baseline at 30-min post exercise on d 1 of wk 1 ($P = 0.003$), while all other measurements on d 1 of wk 1 remained at baseline ($P \geq 0.253$). Decreases in activity from

baseline of 19.4 ± 3.9 and 19.6 ± 3.9 U/ml were observed on d 2 of wk 1 at 60 and 90-min following exercise and continued d 3 of wk 1 at all collection times with decreases ranging between 11.9 ± 3.9 and 19.4 ± 3.9 U/ml ($P \leq 0.009$; Figure 3.3). This decrease in activity continued into d 1 of wk 2 at -60, 0, and 60-min after exercise ($P \leq 0.022$), before returning to baseline 90-min following exercise. A decrease in plasma SOD activity of 12.6 ± 3.9 U/ml is also seen at the final collection on d 3 of wk 2 ($P \leq 0.018$). At all other collection times SOD plasma activity remained at baseline throughout wk 1 and 2 ($P \geq 0.064$). Significant changes from baseline were observed in most measurements taken throughout wk 7 and 8, with significant decreases seen within each d of wk 7 as well as d 1 and 2 of wk 8 ($P \leq 0.043$; Figure 3.4), and significant increases observed on d 3 of wk 8 ($P \leq 0.029$). Decreases in activity ranged from 10.5 ± 3.9 to 29.0 ± 3.9 throughout wk 7 and 8 ($P \leq 0.043$). Enzyme activity was lower at min -60 and 90 on d 1 of wk 7 and remained lower immediately before and after exercise on d 2, before returning to baseline at min 30 for the remainder of d 2 ($P \leq 0.023$). All observations on d 3 of wk 7 showed decreases from baseline which continued d 1 and 2 of wk 8 ($P \leq 0.043$). Increased plasma SOD concentration ($P \leq 0.029$) was observed on d 3 of wk 8 at all collection times, except at 30-min post-exercise.

Total Antioxidant Capacity

The main effect of day was significant ($P = 0.037$) for blood plasma concentrations of TAC in exercised horses. The TACvalue ranged from 0.370 ± 0.012 to 0.399 ± 0.012 mM of trolox equivalence from d 1 to 3 (Figure 3.7). The blood plasma concentrations of TAC on d 2 was 0.399, 7.5% greater than on d 3 ($P = 0.013$), with an increase of 0.029 ± 0.012. The TAC value did not differ between d 1 and 2 or d 1 and 3 ($P = 0.450$; Figure 3.7).

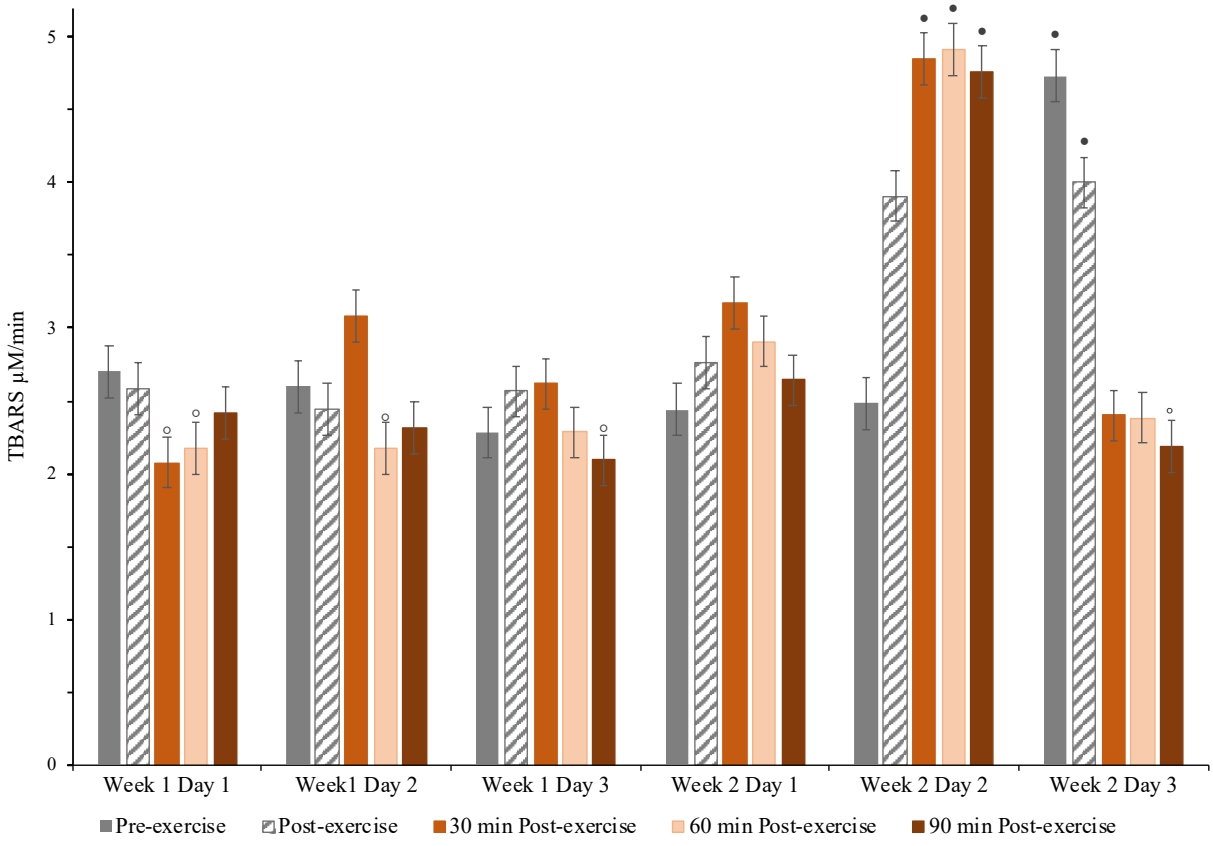

Figure 3.1 Blood plasms concentrations of thiobarbituric acid reactive substances (TBARS) on d 1, 2, and 3 of wk 1 and 2 for moderately exercised horses. All data are represented as the mean ± standard error. °denotes a decrease from baseline, •denotes an increase from baseline ($P < 0.050$).

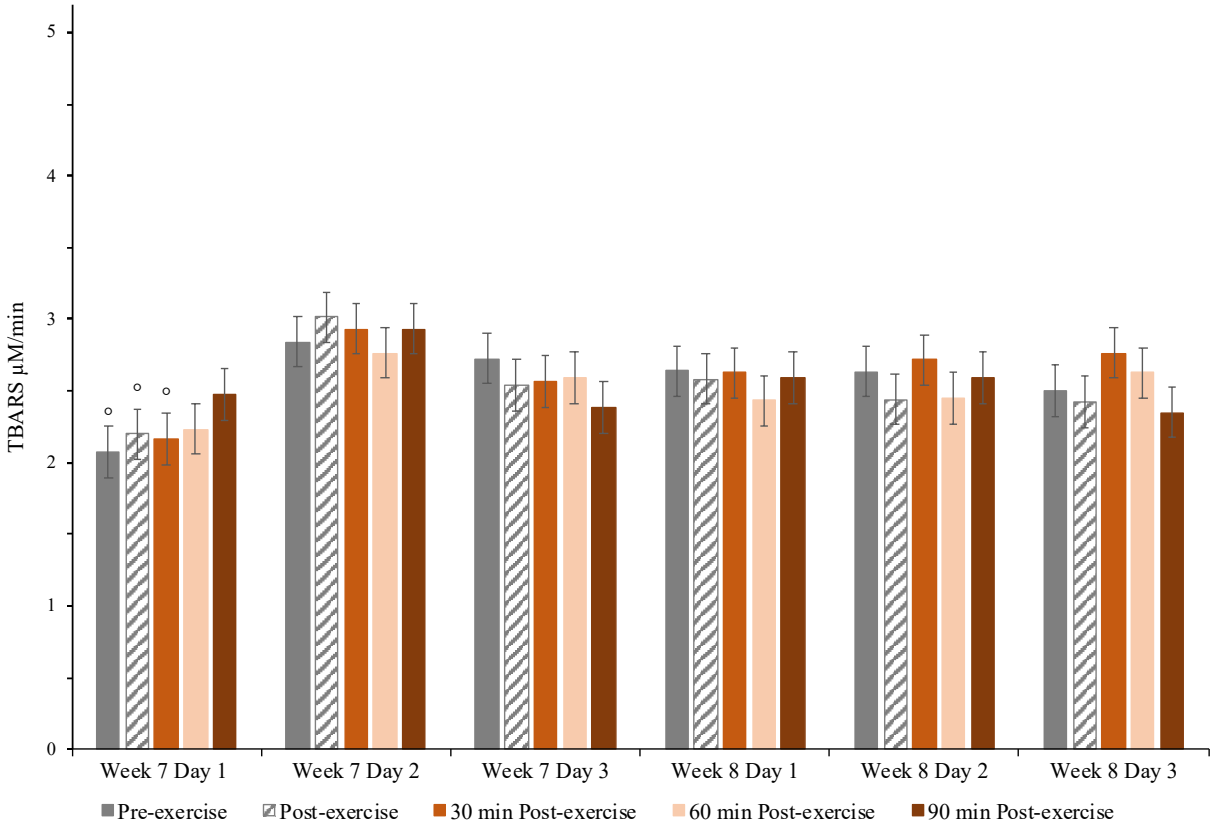

Figure 3.2 Blood plasma concentrations of thiobarbituric acid reactive substances (TBARS) on d 1, 2, and 3 of wk 7 and 8 for moderately exercised horses. All data are represented as the mean ± standard error. °denotes a decrease from baseline, •denotes an increase from baseline ($P < 0.050$).

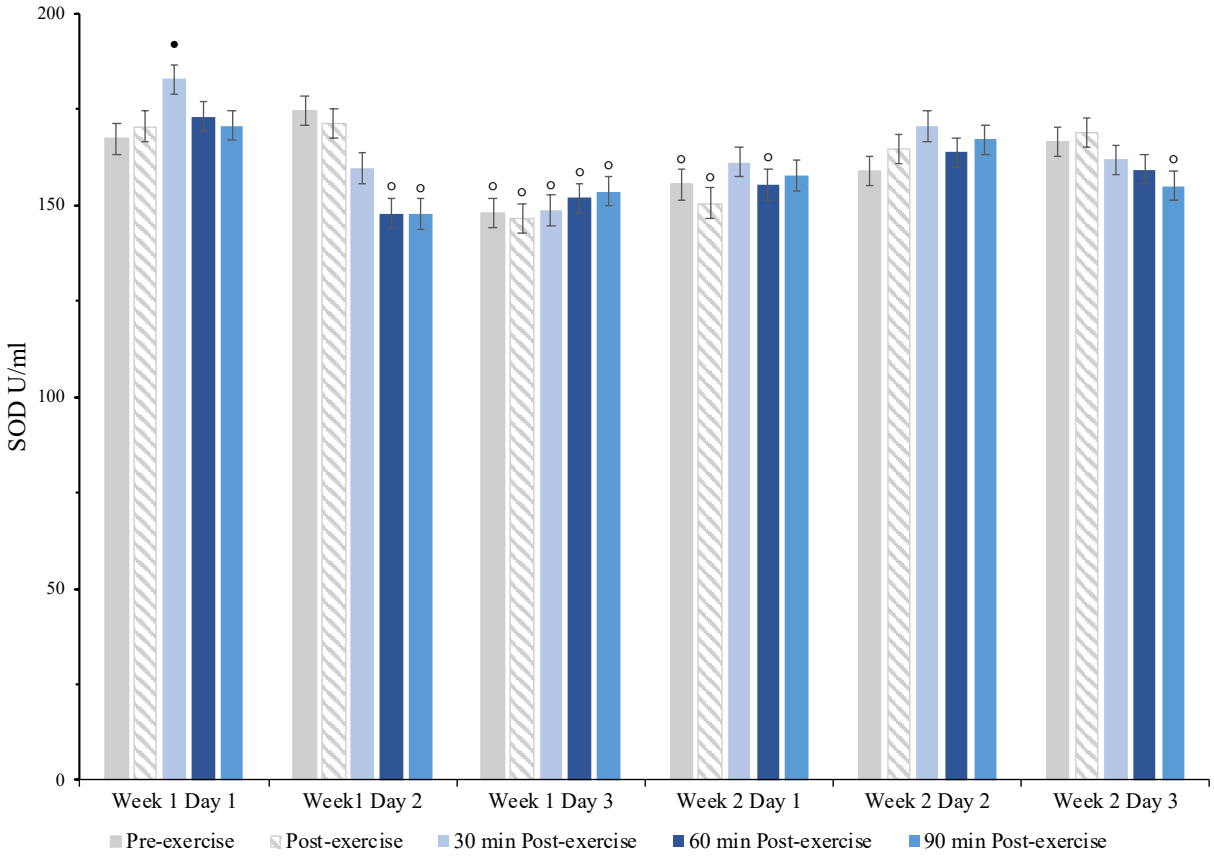

Figure 3.3 Blood plasma concentrations of superoxide dismutase (SOD) activity on d 1, 2, and 3 of wk 1 and 2 for moderately exercised horses. All data are represented as the mean ± standard error. °denotes a decrease from baseline, •denotes an increase from baseline ($P < 0.050$).

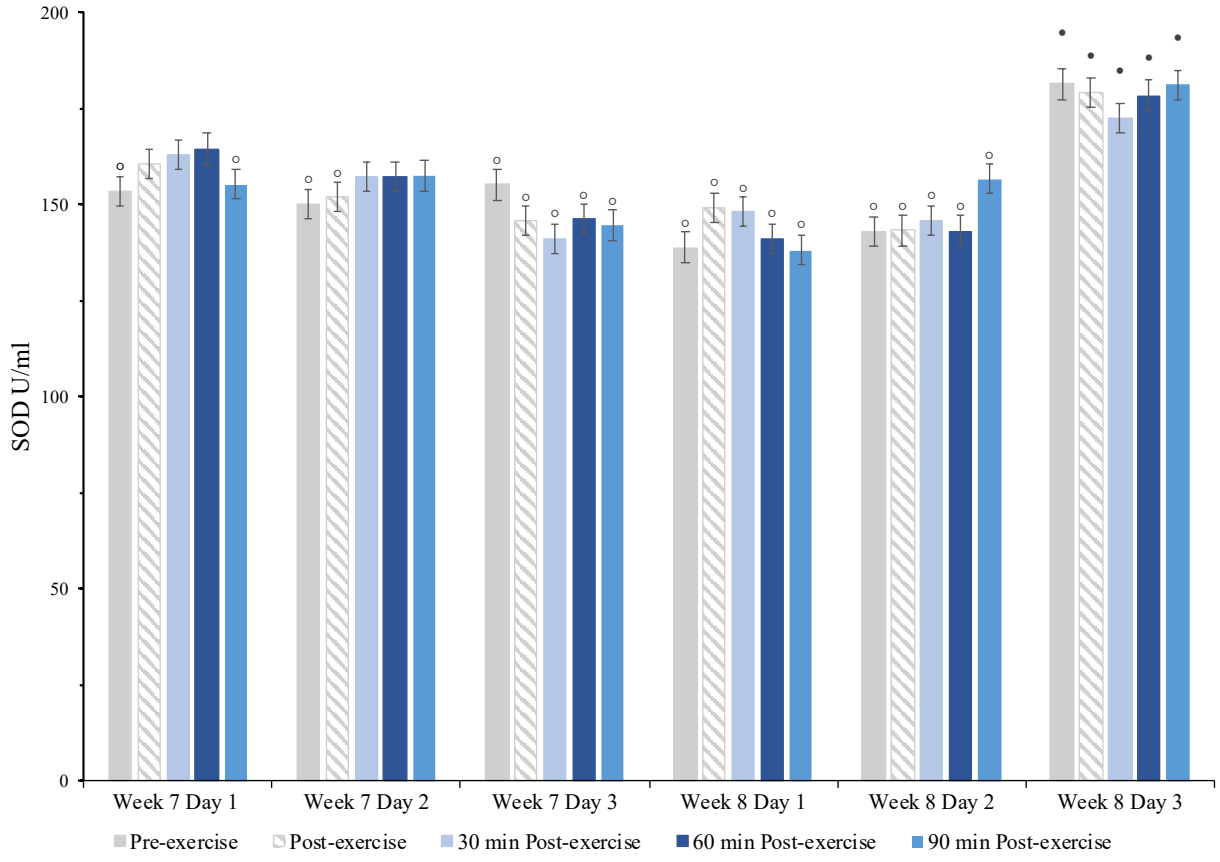

Figure 3.4 Blood plasma concentrations of superoxide dismutase (SOD) activity on d 1, 2, and 3 of wk 7 and 8 for moderately exercised horses. All data are represented as the mean ± standard error. °denotes a decrease from baseline, •denotes an increase from baseline ($P < 0.050$).

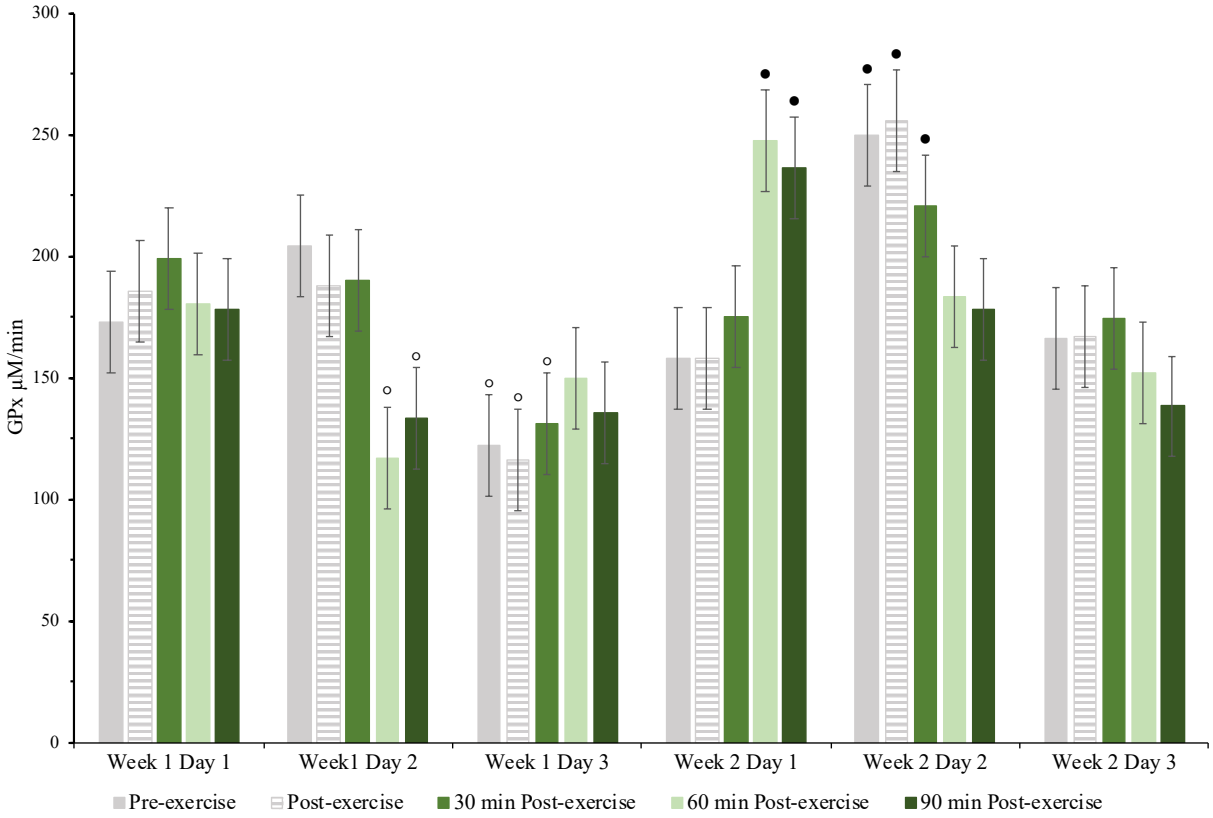

Figure 3.5 Blood plasma concentrations of glutathione peroxidase (GPx) activity on d 1, 2, and 3 of wk 1 and 2 for moderately exercised horses. All data are represented as the mean ± standard error. °denotes a decrease from baseline, •denotes an increase from baseline ($P < 0.050$).

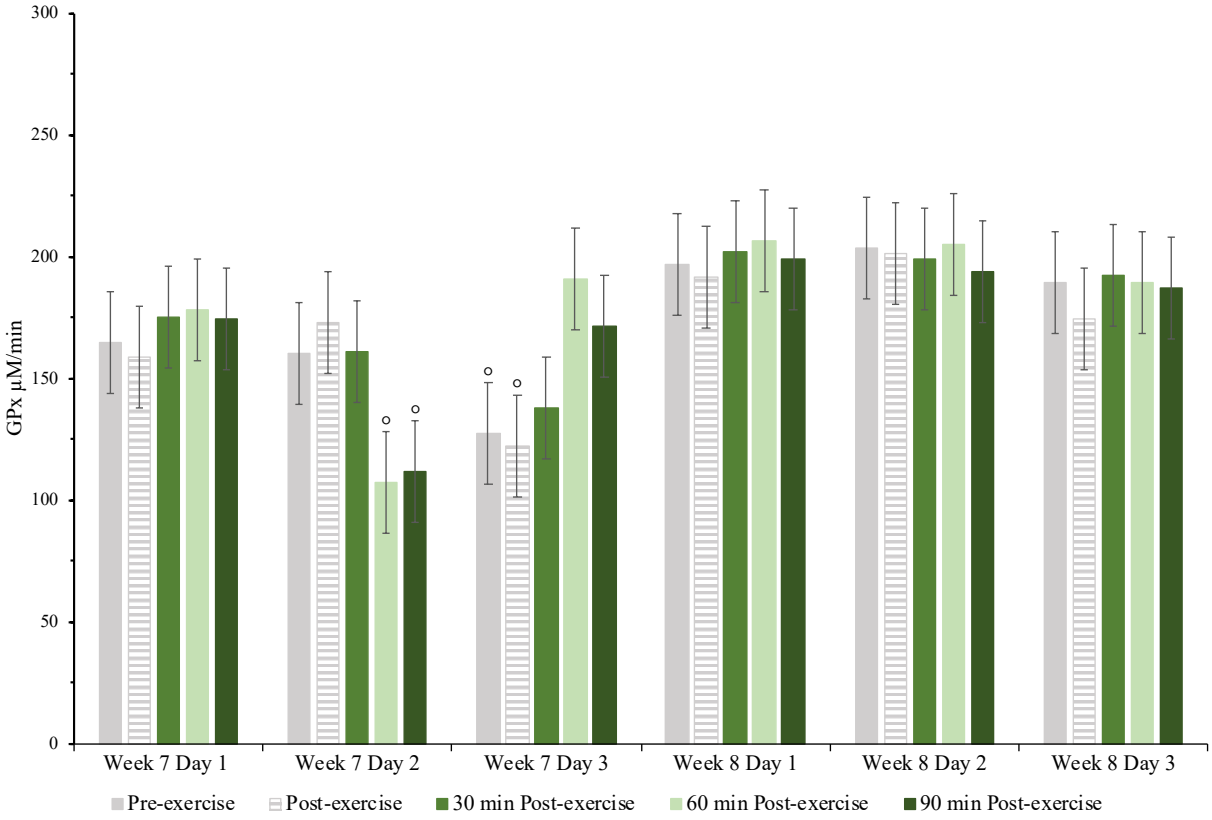

Figure 3.6 Blood plasma concentrations of glutathione peroxidase (GPx) activity on d 1, 2, and 3 of wk 7 and 8 for moderately exercised horses. All data are represented as the mean ± standard error. °denotes a decrease from baseline, •denotes an increase from baseline ($P < 0.050$).

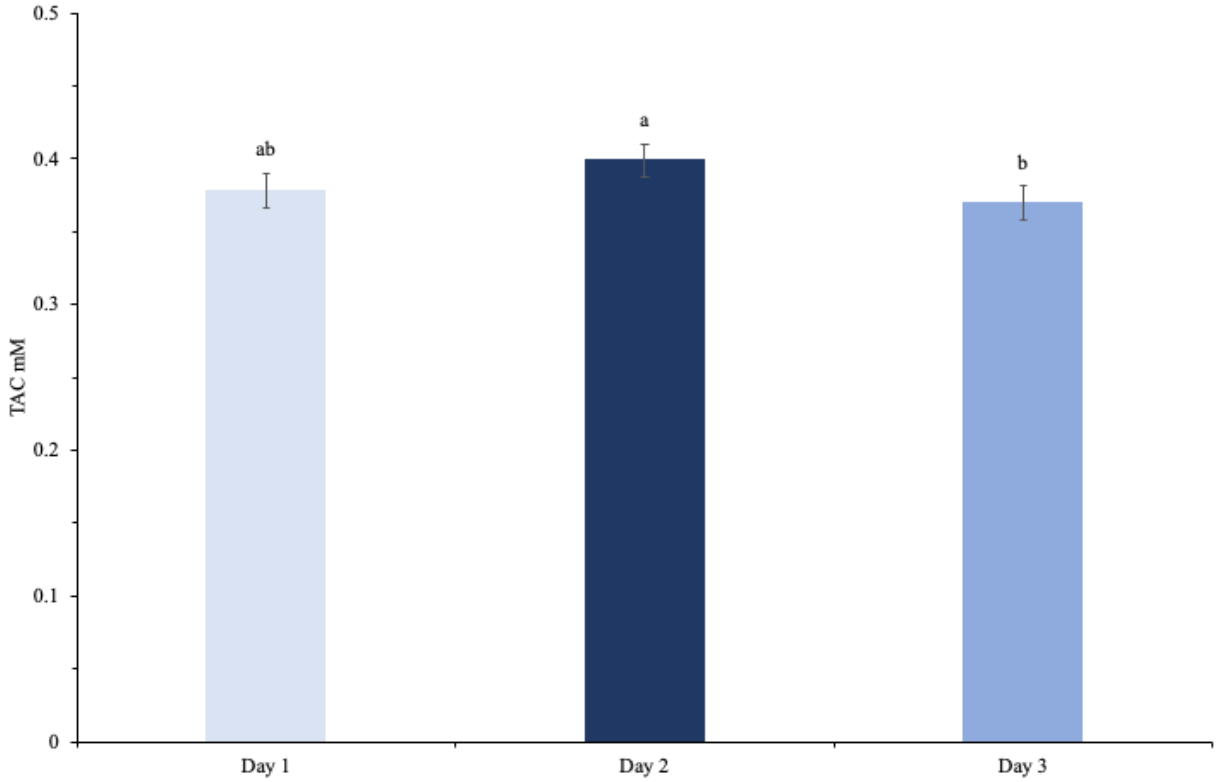

Figure 3.7 Blood plasma concentrations of total antioxidant capacity (TAC) for d 1, 2, and 3 between all wk for moderately exercised horses. All data are represented as the mean ± standard error. •denotes an increase from baseline ($P < 0.050$)

Discussion

This study aimed to identify patterns of expression of acute and chronic OS in athletes when exposed to extended periods of exercise training. This experimental design allowed us to gain more insight on how the body adapts to OS exposure immediately after each exercise bout, as well as how the response to stress adapts over time. This information could help equine trainers and riders better understand the conditioning process for incoming or recovering athletes, and aid in future research in the development of techniques for reducing OS created by numerous stressors experienced by equine athletes.

The oxidant responses to exercise in horses varies among numerous forms of exercise (such as treadmill, race, endurance, and jumping), as well as other parameters such as breed and fitness level (Brkljača Bottegaro et al., 2018; de Moffarts et al., 2004; Fazio et al., 2016; Piccione et al., 2014; Williams et al., 2004). There is little information about the effects of prolonged moderate intensity exercise training on unfit horses, although there is evidence that training has a positive effect on the oxidant/antioxidant equilibrium in the body of both humans and horses (de Moffarts et al., 2004; Traustadóttir et al., 2012). The current research indicated that although TBARS values were changed early in wk 2, the antioxidant system (TAC), GPx, and SOD activity were the responses of the animal's body in an oxidative stress event, showcasing the horse's ability to adapt to OS when exposed to exercise training.

This study shows that some blood antioxidant markers in healthy Stock type horses change in response to exercise, including the ability to adapt to prolonged exposure to moderate intensity exercise. Blood plasma concentrations of TBARS stabilized at baseline following an initial response to exercise seen within the first 2 wk of sampling. The initial decrease in TBARS concentrations following exercise during the first wk is consistent with previous findings when

analyzing an immediate response to a single bout of exercise (Andriichuk et al., 2016). Andriichuk et al. (2016) produced results showing lipid peroxidation after exercise; however, samples were only collected after 1 exercise bout, so comparisons can only be made to the first d of exercise training in our study. A study similar to the one presented here was conducted on trained and untrained mares and yearlings and showed similar MDA results in the untrained yearling group as demonstrated by the TBARS concentrations in our study (Smarsh & Williams, 2017). The untrained yearling group had the largest spike of plasma MDA concentrations during the second wk of exercise which is consistent with our study's findings. This spike in wk 2 could be attributed to a buildup of TBARS within the body following numerous exercise bouts, before adaptation or recovery from the effects of repeated exercise. In comparison with Smarsh and Williams (2017), our results differed in the 7th wk of exercise, as they showed an increase in MDA concentrations while our study exhibited a decrease on d 1 of wk 7 with no significant changes for the remainder of wk 7 or 8. These differences may be due to dissimilarities is exercise protocol, as Smarsh and Williams (2017) protocol was dependent on a graded exercise test and adjusted throughout the program. Exercise protocols varied in time and intensity and were adjusted multiple times over the duration of their project. These adjustments may not have allowed the horses to adapt to the administered exercise since it was not consistent.

The reduction pattern seen in SOD activity was somewhat consistent with the reduction in GPx activity seen in the first 2 wk, both showing reduced enzymatic activity on d 2 and 3 of wk 1. Glutathione Peroxidase and SOD activity patterns were different in wk 2 of collections, as GPx concentrations increased while SOD activity returned to baseline. Superoxide Dismutase concentrations decreased for all measurements starting d 3 of wk 7 and remained at these concentrations until d 3 of wk 8 where they showed an increase from baseline for all

measurements. Blood plasma GPx concentrations remained at baseline for the majority of wk 7 and 8, only showing a decrease at the end of d 2 and beginning of d 3 of wk 7. There are conflicting reports for behavior of SOD and GPx in exercising horses which make it difficult to determine if this study produced an accurate representation of typical antioxidant behavior when exposed to persistent exercise training. Multiple studies have indicated that elevated antioxidant activity is common following exercise, with Williams and Carlucci (2006) as well as Lamprecht and Williams (2012) reporting increases in antioxidant activity. These reports differed from the present study, as they investigated the OS response to a single exercise bout in horses unaccustomed to the exercise protocol. This experimental design doesn't provide insight on the effects of training on the adaptation of the OS response. de Moffarts et al. (2004) differed from the former studies, as they implemented an 8 wk interval training program following an initial 4 wk period of adaptation to exercise. Interestingly, de Moffarts et al. (2004) recorded a 100% increase in SOD plasma concentrations over a 12 wk period, but no response within 60 min of a single exercise bout as reported by the previously mentioned authors (Lamprecht & Williams, 2012; Williams & Carlucci, 2006).

Contrasting results regarding antioxidant activity have been recorded by a number of authors showing no difference (Marlin et al., 2002) or a decrease in activity following exercise (Hargreaves et al., 2002; Ono et al., 1990). It is interesting to note that 2 of these studies were conducted on horses fit for endurance racing at the time of sample collection. This could contribute to the contrasting results, as these horses would most likely be adapted to the high intensity exercise expected in competition. With this in mind, one could infer that these horses are already adapted to the increase in OS associated with each exercise routine. This would indicate that the recorded response in these reports is showing a decrease from the elevated blood

plasma concentrations recorded in untrained horses exposed to a single exercise bout. This is supported in the present study by the changes seen between the first 2 wk of sampling and the last 2 wk.

The decrease in blood plasma concentration of SOD found in the present study is indicative of increased superoxide radical levels within the body, indicating an increased level of OS. The majority of decreased SOD activity was recorded after exercise on d 2, as well as before and after exercise on d 3, specifically in wk 1 and 7. This decrease may be due to an inability of the horse's antioxidant response to maintain normal levels of SOD when battling continuous OS caused by prolonged exercise training. The increased SOD activity seen on d 1 of wk 1 is more consistent with the literature (Andriichuk et al., 2016; de Moffarts et al., 2004; Lamprecht & Williams, 2012; Williams & Carlucci, 2006), although a large portion of literature on SOD concentrations in horses is reporting data collected following 1 exercise bout, without extended periods of exercise training (Andriichuk et al., 2016; de Moffarts et al., 2004; Lamprecht & Williams, 2012; Williams & Carlucci, 2006). Without more data to compare SOD concentration response following repeated exercise bouts, it is difficult to determine if SOD concentrations throughout this trial are an accurate representation of the equine population. The increased SOD concentrations seen in the final wk of the present study gives the implication that horses are able to maintain higher levels of SOD after adapting to the exercise protocol and its accompanying OS.

Although blood plasma concentrations of GPx fluctuated in the first 2 wk of our study, they ultimately stabilize at baseline by the final sampling period, showing no changes in GPx concentrations from baseline at the conclusion of exercise training. These data are consistent with research investigating equine exercise (Balogh et al., 2001; de Moffarts et al., 2004), but is

inconsistent with a number of reports in human medicine (Deaton & Marlin, 2003; Leeuwenburgh et al., 1994; Somani et al., 1995), which show increased blood plasma concentrations of GPx activity after prolonged exercise training. Results of the current study show an increase in GPx concentrations during the second wk of exercise training, which is consistent with human trials; however, the increased concentrations return to baseline for most of the final 2 wks of exercise training.

Dietary levels of Se could effect blood GPx concentrations within the body, as Se is known to play a role in maintenance of the glutathione antioxidant response in horses (Brummer et al., 2013; Caple et al., 1978). Because all horses were fed the same diet throughout this study, Se levels were consistent between horses, although the diet could have limited the ability for this biomarker to respond adequitely if Se levels were low. Unfortunately Se concentrations within blood were not calculated throughout this project so we are unable to determine if this mineral remained at adequate levels. We hypthosize that the return of plasma concentraitons of GPx to baseline indicates the horses have adapted to the exercise protocol which allows GPx concentrations to remain at baseline levels due to decreased incidence of free radicals throughout the body.

Significant increase of blood plasma concentrations of TAC on d 2 of each wk were obtained. When analyzed, TAC blood concentrations increased on d 2 before returning to baseline on d 3, with no differences from baseline seen following exercise. Ukrainian warmblood and Holsteiner horses saw no change in blood plasma concentrations of TAC before or after undergoing an exercise test (Andriichuk et al., 2016), which is consistent the current trial. We speculate that the increase in TAC seen on d 2 corresponds to the increase in other OS biomarkers (TBARS and GPx) seen on this same day. Increased blood plasma concentrations of

TAC was most likely a response to increased oxidants in the body incited by multiple days of exercise. The reduction back to baseline on d 3 indicates recovery from the oxidant overload experienced on d 2 and may be evidence of the horse adjusting to the effects of exercise at the end of each week.

A variety of evidence has been accumulated that suggests regular exercise improves the bodies response to oxidative damage. This idea is supported by the findings within the current study, as lipid peroxidation levels remain mostly at baseline throughout the last 2 wk of sampling, following an extended period of physical training. The stabilization of TBARS levels and GPx activity in the final 2 wk of exercise training indicates that the horses were adapted to the exercise protocol. The increase in SOD activity seen on the final day of exercise training indicates a decrease in the superoxide anion concentration within the body, therefore decreasing incidence of oxidative stress, which aligns with the theory that horses adapted to the exercise protocol.

Other avenues of stress that are common in the equine industry include trailering, acutely increased performance demands, and introduction to new environments and animals. There is currently very little research investigating the OS response to these stressors, aside from a study conducted by Niedźwiedź et al. (2013) that indicated horses exhibit higher levels of OS biomarkers after a 12 h period of trailering. Other physical stressors associated with the equine show industry need to be further investigated in order to properly understand the causes of OS in the equine model, as well as the degree of oxidant imbalance experienced by these athletes.

Conclusion

The current study showed that horses exposed to increased levels of physical stress experience changes in OS biomarkers following its onset. It was also demonstrated that

prolonged exposure to stress can result in adaptation of the stress response. This study used exercise as a model to create physical stress; however, horses are exposed to a variety of physical stressors such as trailering, showing, and environmental changes, on a regular basis that may produce similar effects in the equine OS response. Understanding the initial and extended response seen in the equine athlete when exposed to new stressors is crucial in determining how to prevent oxidative damage in future athletes. Further research identifying methods to reduce OS is necessary and has the potential to improve performance as well as the overall well-being of the equine athlete.

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