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INFLAMMATORY AND ANTIOXIDANT STATUS OF  
HORSES UNDERGOING INTENSE EXERCISE AND  
NUTRITIONAL SUPPLEMENTATION

by

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Dr. Carey A. Williams and Dr. Carol A. Bagnell

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## ABSTRACT OF THE DISSERTATION

### Inflammatory and Antioxidant Status of Horses Undergoing Intense Exercise and Nutritional Supplementation

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Markers of inflammation and antioxidant status were used to partially characterize physiological responses to a single bout of intense, exhaustive exercise in healthy Standardbreds, and to evaluate anti-inflammatory and antioxidant properties of an antioxidant enzyme supplement, with a particular focus on joint health. Main objectives were to 1) identify a model of exercise-induced inflammation by comparing inflammatory responses to different modes of exercise, 2) evaluate effects of supplemental superoxide dismutase (SOD) on exercise-induced inflammatory response and antioxidant status, and 3) evaluate effects of repeated arthrocentesis, exercise, and SOD supplementation on markers of inflammation and cartilage metabolism, in horses. For the first study, a repeated sprint exercise test was identified as the most strenuous compared to interval- and graded exercise tests. This was based upon post-exercise systemic increases in pro-inflammatory cytokine transcripts, decreases in circulating

nitric oxide (NO), and peak heart rate response. The second study showed increases in interferon-gamma, interleukin-1 beta, and interleukin-10 cytokine transcripts, upregulated antioxidant defenses (SOD, total glutathione, glutathione peroxidase), and a decline in plasma NO, following intense exercise. Supplemental SOD (3000 IU/d for 6 wks) had no detectable influence on markers of performance, inflammatory response, or antioxidant status relative to intense exercise, and compared to the placebo control. The third study showed that repeated arthrocentesis in the same joint did not alter synovial fluid concentrations of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), although anabolic cartilage metabolism, measured by chondroitin sulfate-846 (CS), was elevated at the last sample, indicating sensitivity to repeated synovial fluid aspiration. Following exercise, increases in PGE<sub>2</sub> relative to pre-exercise concentrations, and transient increases in CS, indicated healthy adaptive responses to exercise. Similar to the second study, supplemental SOD had no influence on PGE<sub>2</sub> or CS, suggesting it was not an effective solution for reducing exercise-induced inflammation or improving antioxidant status in horses. These studies contribute to a limited working knowledge of inflammatory and antioxidant responses to intense exercise, and may be useful when differentiating between adaptive responses and early pathological changes resulting from exercise-induced stress in horses.

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## **Introduction**

Exercise-induced inflammation and oxidative stress are problematic in the equine athlete resulting in impaired health, decline in quality of life, lost training time and millions of dollars in veterinary expenses. Equine athletes, like their human counterparts, suffer from challenges to the immune system related to exercise, transportation stress, injury, and disease just to name a few. Post-exercise problems associated with increased expression of inflammatory markers and oxidative stress can range from the mild symptoms of delayed-onset muscle soreness to debilitating problems related to soft tissue, joint and bone damage. Joint pain associated with tissue micro-damage and inflammatory disease is of significant concern to the equine industry. With the high prevalence, exorbitant cost, and debilitating nature of joint disease, it would be prudent to focus on preventative strategies rather than treatments. Early detection and treatment of joint inflammation could mean the difference between development of a career-ending debilitating condition or the integration of preventative measures that reduce prevalence of chronic conditions and severity of degenerative processes before they become problematic. The proposed research is intended to investigate the effectiveness of an oral antioxidant supplement to reduce inflammation, boost antioxidant status, and prevent excessive oxidative damage in exercising horses, both systemically and in carpal and tarsal joints. Identification of an oral supplement with antioxidant and anti-inflammatory properties would provide an alternative to (invasive) pharmaceutical therapy and a more practical and desirable way to prevent and/or treat chronic inflammatory disorders in horses.

## **Literature Review**

### **Significance of Inflammation and Oxidative Stress**

Inflammation and oxidative stress have been implicated in the pathogenesis of many debilitating chronic diseases in humans including, but not limited to Alzheimer's disease (Simpson et al., 2009), Parkinson's disease (Rojo et al., 2009), Multiple Sclerosis and stroke (Kaur and Ling, 2008), cancer (Butt and Sultan, 2009), atherosclerosis and chronic kidney disease (Fassett et al., 2009). The oxidative stress and inflammatory pathways are not mutually exclusive as there is documentation of complex relationships among various inflammatory, pro- and antioxidant mediators (Cuzzocrea and Reiter, 2001). Cytokines are hormone-like proteins which help mediate inflammatory responses to various stimuli. As reviewed by Peake et al. (2007) several factors can influence cytokine production during exercise. These include muscle glycogen breakdown, interactions between immune cells and stress hormones, lipopolysaccharide production, endogenous antioxidant enzyme activity, calcineurin-nuclear factor activated T cells, production of heat shock proteins and exercise-induced oxidative stress. Oxidative stress-mediated cytokine production occurs through changes in the redox-equilibrium due to alterations in redox-sensitive signal transduction pathways via activation of nuclear transcription factor  $\kappa$ B (NF- $\kappa$ B; Li et al., 1998). Reactive oxygen species (ROS) can also activate neutrophils to produce pro-inflammatory cytokines, as has been demonstrated in rabbit lungs under hyperoxic conditions (Mikawa et al., 1995). Additionally, certain cytokines can in turn mediate the production of ROS and their signaling (Rovin et al., 1997; Haddad and Harb, 2005) in addition to activating NF-  $\kappa$ B (Li et al., 1998). Pro-

inflammatory cytokines can prime neutrophils to migrate to sites of inflammation where they initiate the release of ROS (Zhang et al., 2002) and are considered important mediators of oxidative stress (Haddad and Harb, 2005). Cytokines specifically influence the redox equilibrium via upregulation of antioxidant enzymes and changes in reduced to oxidized glutathione shuttling and recycling (Chen et al., 1998). Exercise-induced upregulation of pro-inflammatory cytokines was shown to increase manganese superoxide dismutase activity in treadmill exercised rats which was considered cardio-protective within the context of ischemic injury (Yamashita et al., 1999). The superoxide anion is also a key mediator of inflammatory responses possibly through neutrophil infiltration and adhesion (Salvemini et al., 1999). Furthermore, this free oxygen radical has cell signaling properties through induction of the inflammatory pathway via the NF- $\kappa$ B and activator protein-1 (AP-1) transcriptional factors (Udipi et al., 1999). Specifically induction of NF- $\kappa$ B results in the expression of adhesion molecules, cytokines, acute-phase response proteins and pro-inflammatory enzymes. There are important roles for pro- and antioxidants in altering mediators integral to pathologic and homeostatic inflammatory pathways and vice versa, however care must be taken to consider the multi-faceted consequences when enhancing or blunting any component within these pathways (Marikovsky et al., 2003).

Equine athletes suffer from challenges to the immune system and inflammation related to exercise (Auer, 1989; Bertone et al., 2001; Petersen et al., 2004; Firth, 2006). Inflammation is a fundamental response to infection or trauma and is manifested in a number of common equine diseases. Joint inflammation and ROS formation are common physiological responses initiated by stressors including exercise, transportation,

competition, injury and illness (Firth, 2006). Inflammatory processes result in the release of cytokines, which mediate the wide range of symptoms associated with trauma and infection. Specifically, oxygen-derived free radicals and inflammatory processes in the joint result in compromised viscoelastic properties of synovial fluid and tissue degradation, causing pain and mechanical instability (Bertone et al., 2001). Post-exercise problems associated with increased expression of inflammatory markers can range from the mild symptoms of delayed-onset muscle soreness to debilitating problems related to soft tissue, joint and bone damage (Auer et al., 1989; Brenner et al., 1999). Chronic inflammation associated with the aforementioned, can lead to tissue destruction, subsequent poor performance and a likely end to the athletic career of a horse (Petersen et al., 2004).

### **Epidemiology of Joint Disease in Horses**

Joint discomfort in horses is the most common cause of lameness, diminished athletic performance and quality of life, economic burden, and animal loss (Kidd et al., 2001). In the early 1980's, it was reported that 67.6% of the lost training days for racehorses were attributable to lameness (Rossdale et al., 1985). Similar data indicated that over 80 % of total days lost from training in 2 and 3 yr old Thoroughbred race horses were attributable to lameness, and 14 % of those days lost were due to joint injuries (Dyson et al., 2008). A study conducted by the National Animal Health Monitoring System reported that 50 % of the equine operations surveyed, reported having at least one horse with lameness during the previous year (1999). Leg or joint problems were the most commonly perceived cause of lameness in the spring and winter seasons (USDA,

2000). Other epidemiological studies conducted by the USDA (USDA, 2001; USDA, 2007) have shown that in 1998 costs of lameness related to lost use of horses, veterinary services, drugs, and care came to an estimated total between \$678 million and \$1 billion. Totals were estimated based on 8.5 to 13.7 lameness cases per 100 horses costing an average \$432 per case in equids aged six months or older (USDA, 2001). Furthermore, 7.7% of the deaths in 2005 were linked to lameness issues (USDA, 2007).

In Standardbred racehorses, there is a near equal distribution between hindlimb and forelimb lameness resulting from use trauma (Hinchcliff et al., 2004a). The carpus, or knee of the horse, is located on the forelimb where the radius/ulna and cannon bone come together to form the diarthroidial joint. Diseases of the carpus most commonly affect racehorses, and in general, the carpus is the most common articular injury site and likely the most common source of lameness. Degenerative changes of and within the synovium, joint capsule, articular cartilage, subchondral bone, and ligaments result from the repeated stress of training and performance (Firth, 2006). The mid-carpal joint is a predisposed site of lameness in race horses, especially young Standardbreds and Thoroughbreds. It is typical for most young racehorses to develop some form of synovitis early in their training due to the failure of tissues to adapt properly to the repeated physical stress (Pool, 1996).

The tarsus, or hock of the horse, is located on the hindlimb where the tibia and cannon bone come together to form this complex joint. Within the hock, distal tarsitis remains the most common lameness. Osteoarthritis (OA) is commonly found in the distal intertarsal joint and tarsometatarsal joints which is largely composed of periarticular soft-tissue (Hinchcliff et al., 2004a). Inflammation and OA in the tarsal joints can be caused

by several factors including poor conformation, athletic use, and angular limb deformities centered on the hock. Degenerative joint disease of the tarsometatarsal joint may contribute to tarsal lameness more often and more severely than the other small tarsal joints. Most likely, this is caused by eccentric joint loading, sliding and shear forces or axial loading placed on the distal rows of tarsal bones during competition (Hinchcliff et al., 2004a). Sheer forces have been demonstrated to modulate nitric oxide production and chondrocyte matrix macromolecule metabolism in adult bovine articular chondrocytes under *in vitro* conditions (Das et al., 1997). A study by Grondahl and Dolvik (1993) reported that 14.3% of 753 young Standardbred trotters sampled were diagnosed with osteochondrosis in the tibiotarsal joint and 11.8% were diagnosed with bony fragments in the palmar/plantar portion of the metacarpo- and metatarsophalangeal joints. Overall, sixty percent of all lameness problems are related to OA (McIlwraith, 2005), and horses most commonly affected are three-day-event horses, jumpers, barrel racers, Standardbred race horses, reining and dressage horses (Hinchcliff et al., 2004a).

***Biomarkers of Joint Metabolism and Disease.*** Biomarkers, as termed by Billingham (2002), are products of normal metabolism within tissues, that may increase or decrease when the homeostatic environment is altered due to disease and/or injury. Biomarkers can be used to identify changes in anabolic and catabolic processes in the synovial joint. They have been useful in identifying exercise-induced changes in both synovial fluid and serum, although they are not particularly useful in determining the stage or severity of joint pathologies (McIlwraith 2005). Biomarkers can further be categorized as biochemical or immunological. They differ in the methodologies by which they are detected and may be direct or indirect constituents of joint metabolism

(McIlwraith, 2005).

Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) is a prostanoid, or biologically active lipid product of the cyclooxygenase-2 influenced metabolism of arachidonic acid, which serves predominantly as a mediator of inflammation and a relaxant of smooth muscle. As an indirect biomarker of joint disease, it is an indicator of local inflammation in synovial fluid. Increased concentrations of PGE<sub>2</sub> have been shown to occur in inflamed (Tung et al., 2002; Au et al., 2007; Heinecke et al., 2009) and osteoarthritic joint tissues of horses (May et al., 1994; Chan et al., 2005; van den Boom et al., 2005; Frisbie et al., 2008), where they compromise the cartilage matrix by decreasing its content of proteoglycan (Tietz and Chrisman, 1975; Lipiello et al., 1978) as well as initiating a localized pain response via sensitization of peripheral nociceptor terminals (Tchetina et al., 2007). Prostaglandin E<sub>2</sub> most likely originates from articular tissues and is important for the maintenance of local cartilage homeostasis (Amin et al., 1997; Murakami et al., 1998; von Rechenberg et al., 2000). Therefore, PGE<sub>2</sub> production is not dependent upon leukocyte infiltration into the intraarticular space in the case of joint inflammation (Sturge et al., 1978). Increased PGE<sub>2</sub> concentrations of 22.5 pg ml<sup>-1</sup> or greater are considered an excellent indicator of synovitis (McIlwraith, 2005; Frisbie et al., 2008) and predictor of equine joint disease (Bertone et al., 2000; Kirker-Head, et al., 2000). Studies have shown induced increases in the concentration of PGE<sub>2</sub> blood plasma after intense exercise in humans (Peake et al., 2005), although an analogous increase has not been detected in equine plasma (Mitten et al., 1995).

Common biomarkers of catabolic processes in joints include, but are not limited to, total sulfated glycosaminoglycans (GAGs; Farndale et al., 1986; Alwan et al., 1991;

Frisbie et al., 2008), keratan sulfate, a proteoglycan component of aggrecan which indicates cartilage turnover (Thonar et al., 1985; Alwan et al., 1990; Todhunter et al., 1997; Okumura and Fujinaga, 2000; Caron et al., 2002; Okumura et al., 2002; Celeste et al., 2005), type II collagen fragments (Billinghurst et al., 2001; McIlwraith, 2005), and cartilage oligomeric matrix protein, also a component of tendon, menisci, and synovium, which can be evaluated in equine serum and synovial fluid (Skioldebrand et al., 2001; Misumi et al., 2002; Arai et al., 2005; McIlwraith, 2005). Other biomarkers of anabolic joint metabolism include carboxypeptide of type II collagen (CPII), a measure of type II collagen synthesis (Celeste et al., 2005; McIlwraith, 2005; Frisbie et al., 2008), chondroitin sulfate (Caterson et al., 1983; McIlwraith, 2005), and the epitope of chondroitin sulfate (CS-846 epitope; Celeste et al., 2005; McIlwraith, 2005; Frisbie et al., 2008), as a marker of cartilage turnover and aggrecan synthesis. Exercise-induced increases in CPII and CS-846 in serum and synovial fluid have been documented in horses, and positive correlations have been noted between these two biomarkers (Frisbie et al., 2008).

### **Inflammation and Cytokine Theory of Disease**

Inflammation is a fundamental response to infection or trauma and is manifested in a number of common equine diseases such as osteoarthritis and tendonitis. Control of the inflammatory response is largely due to self-controlling innate immune mechanisms and brain-derived immunoregulatory output via the autonomic nervous system (Pavlov and Tracey, 2004). Inflammatory processes are mediated by activated macrophages, monocytes and other immune cells (Pavlov and Tracey, 2004). The inflammatory

cascade results in the release of small hormone-like proteins, termed cytokines. Although the inflammatory response is usually countered rapidly by endogenous anti-inflammatory agents, excessive production of pro-inflammatory cytokines or their production in the wrong biological context may lead to situations of chronic inflammation. The cytokine theory of disease attributes the clinical manifestation of disease to the overproduction of cytokines resulting in a chronic uncontrolled inflammatory state (Czura and Tracey, 2005). Cytokines such as tumor necrosis factor alpha (TNF $\alpha$ ), interleukin-1 beta (IL-1 $\beta$ ), interleukin-6 (IL-6), interferon gamma (IFN $\gamma$ ), and interleukin-10 (IL-10) have pleiotropic biological activities which mediate the wide range of symptoms associated with trauma and infection, as well as effects on other elements of the immune system. In order for homeostasis and health to be maintained, cytokine production is essential and must be controlled and balanced (Tracey, 2007).

***Tumor necrosis factor alpha.*** Tumor-necrosis factor alpha is a cytokine, produced by macrophages in response to injurious stimuli and infection, responsible for the cardinal clinical designations of inflammation including heat, redness, swelling, and pain (Tracey, 2002). Tumor necrosis factor alpha has also been implicated in chronic, low grade inflammation, which is prevalent in disease states such as type-II diabetes and cardiovascular disease (Peake et al., 2005). Systemically, TNF $\alpha$  is responsible for activating other immune cells to release cytokines such as interleukin-1 (IL-1) and other inflammatory mediators including eicosanoids, nitric oxide and ROS. This pro-inflammatory response mediates vasodilation, the recruitment of leukocytes, and an increase in vascular permeability (Pavlov and Tracey, 2004). However, a systemic inflammatory response with sustained elevated levels of TNF $\alpha$  and ROS can be

destructive to tissues via reduced cardiac output, induced microvascular thrombosis, systemic capillary leakage syndrome, and growth inhibition of hemopoietic cells (Sakashita et al., 1994; Tracey, 2002). Systemic increases in TNF $\alpha$  activity and gene expression following intense exercise has been demonstrated in horses (Barton et al., 2003; Streltsova et al., 2006; Donovan et al., 2007).

***Interleukin-1 beta.*** Interleukin-1 $\beta$  and TNF $\alpha$  either independently or synergistically, are the primary pro-inflammatory cytokines involved in joint degradation. Interleukin-1 induces cartilage catabolism, decreases proteoglycan synthesis, upregulates PGE<sub>2</sub> (Morris et al., 1990; Dvorak et al., 2002; McIlwraith, 2005), collagenase production, and matrix metalloproteinase activity (Smith et al., 1989). These pro-inflammatory cytokines also upregulate osteoclast activity, increase superoxide anion formation (Iwasaki et al., 1990), and regulate pain (Billinghurst et al., 1995; Frisbie et al., 2002; Punzi et al., 2002). They are also strong activators of IL-10 synthesis, an anti-inflammatory cytokine, providing a negative feedback mechanism through which pro-inflammatory cytokines are down-regulated (Moldoveanu et al., 2001). Specifically, IL-1 $\beta$  upregulates a variety of genes including those that up regulate its own expression as well as that of IL-6 (Moldoveanu et al., 2001). Interleukin-1 $\beta$  also induces the enzymes necessary for the synthesis of leukotrienes, prostaglandins and nitric oxide (Moldoveanu et al., 2001). Upregulation of the IL-1 receptor level and a deficiency in interleukin-1 receptor antagonist (IL-1Ra) level, contributes to excessive production of nitric oxide in osteoarthritic tissue (Fernandes et al., 2002).

***Interleukin-6.*** Interleukin-6 is known to have a diverse repertoire of physiological activities both pro- and anti-inflammatory in nature as reviewed by

Pedersen and Fischer (2007). This inflammatory response cytokine is stimulated by TNF $\alpha$  and IL-1 $\beta$  and is also thought to counter the systemic cytokine response by inhibiting the release of TNF $\alpha$  and IL-1 $\beta$  as well as promoting immunosuppression through promotion of IL-1Ra, and IL-10 secretion (Starkie et al., 2003; Peake et al., 2005). Studies where elevated levels of IL-6 have been detected after intense exercise may render it a legitimate biomarker for fatigue and skeletal muscle inflammation (Suzuki et al., 2002). Chondrocytes as well as synoviocytes and articular fibroblasts are thought to produce IL-6 (Guerne et al., 1990; Bunning et al., 1990). It has also been implicated in OA pathogenesis via a negative feedback mechanism that attenuates proteolytic damage in the joint. Interleukin-6 is thought to contribute to the pathogenesis of OA by increasing inflammatory cell content in synovium, stimulating chondrocyte proliferation causing thickening of the articular cartilage, and enhancing IL-1-stimulated synthesis of matrix metalloproteinases (MMPs) and decreasing proteoglycan synthesis (Fernandes et al., 2002). Studies in humans have shown exercise-induced increases in systemic IL-6 production (Weinstock et al., 1999; Goebel et al., 2000). In two equine studies, elevated IL-6 concentration in synovial fluid was determined to be a good predictor of joint disease due to a high correlation with synovial fluid white blood cell count (Bertone et al., 2001) and high levels in joints with chip fractures (Ley et al., 2007).

***Interferon gamma.*** Interferon gamma is considered a pro-inflammatory cytokine that serves to activate macrophages, augment the synthesis of other pro-inflammatory cytokines, and induce nitric oxide synthase and reactive oxygen up-regulation and production (Moldoveanu et al., 2001; Ainsworth et al., 2003). It is a glycoprotein produced by T-helper 1 and natural killer cells in response to viral infection, chemical

and immune stimulation (Elenkov, 2004; Ijzermans and Marquet, 1989). Increases in systemic IFN $\gamma$  in humans follows light (Kimura et al., 2001) and moderate exercise (Baum et al., 1997). One equine study demonstrated increases in circulating IFN $\gamma$  transcript following intense exhaustive exercise in horses (Streltsova et al., 2006) as well. Increases in IFN $\gamma$  following intense exhaustive exercise in humans, has not been reported in the literature which may indicate a post-exercise immunosuppressed state (Haahr et al., 1991; Northoff et al., 1998; Weinstock et al., 1999; Kimura et al., 2001; Suzuki et al., 2002).

***Interleukin-10.*** Lastly, IL-10 is produced by T-helper 2 cells and is involved in the deactivation of monocytes and an anti-inflammatory cytokine pattern via inhibition of T-helper 1 and natural killer cell activation. Anti-inflammatory activities of this cytokine include decreased IL-1 $\beta$ , TNF $\alpha$ , and MMP production, as well as inhibition of PGE $_2$  release and reactive oxygen radical suppression. It also up-regulates IL-1Ra and tissue inhibitor of metalloproteinases (Fernandes et al., 2002). One study demonstrated that mononuclear cells derived from synovial fluid induced the production of IL-10 after stimulation with bacterial antigens. In turn, IL-10 reduced TNF $\alpha$  and IFN $\gamma$  levels (Punzi et al., 2002).

***Role of Cytokines in Joint Disease.*** Cytokines play a central role in the pathophysiology of inflammatory joint disease (Punzi et al., 2002). The inflammatory cytokines thought to be involved in the pathogenesis of equine joint disease include TNF $\alpha$ , IL-1 $\beta$ , IL-6, and IFN $\gamma$ . Cytokines induce the production of degradative enzymes, specifically MMPs, which break down the extra articular matrix of articular cartilage and stimulate prostaglandins creating additional inflammation and pain. Cytokines also

prevent the compensatory effort of chondrocytes to restore integrity to degraded extracellular matrix (Fernandes et al., 2002). Matrix metalloproteinases, specifically MMP-1, MMP-2, and MMP-9, play a pivotal role in joint degeneration by degrading cartilage and subsequently releasing sGAG, a major component of articular cartilage. Prostaglandins, specifically PGE<sub>2</sub>, are released from IL-1 and TNF $\alpha$  stimulated chondrocytes and cause vasodilation, proteoglycan depletion from cartilage, increased pain perception, bone demineralization and promotion of plasminogen activator secretion (McIlwraith, 2005).

Specifically, TNF $\alpha$ , IL-1 $\beta$ , and IL-6 are pro-inflammatory cytokines integrally involved in synovial metabolism and have been reported to stimulate articular cartilage matrix catabolism (Bertone et al., 2001; McIlwraith, 2005). In human patients with varying arthropathies, IL-1 $\beta$ , IL-6, and TNF $\alpha$  were found in the synovial fluid. Interleukin-1 $\beta$ , TNF $\alpha$ , and to a lesser extent IL-6, are produced by the synovial membrane, diffuse into chondrocytes which in turn produce inflammatory cytokines (Punzi et al., 2002). These cytokines in particular, have been implicated as mediators and markers indicative of exercise-induced joint pathology. Normal, short-term or adaptive responses to exercise have not been clearly differentiated from the onset of disease in horses, making early detection of pathological changes systemically and in joints, difficult to detect. Characterization of cytokine response to intense, exhaustive exercise in healthy horses would help elucidate their role in the etiology of inflammatory disorders, providing valuable insight regarding a healthy adaptive response, versus early onset of disease. This information could be useful in efforts to prevent chronic and debilitating conditions in athletic horses before they become career ending.

## **Oxidative Stress and Exercise**

Oxidative stress can be defined as an imbalance of ROS and antioxidant defenses in favor of oxidants (Urso and Clarkson, 2003). Physical activities, varying in intensity, have been shown to increase the generation of ROS in horses (reviewed in Kirschvink et al., 2008). This has been documented in endurance horses (Frankiewicz-Jozko and Szarska, 2000; Hargreaves et al., 2002; Marlin et al., 2002; Williams et al., 2004), pentathlon horses (Balogh et al., 2001), Marremmana racehorses (Chiaradia et al., 1998), Thoroughbred racehorses (Ishida et al., 1999; White et al., 2001), Standardbred trotters (Kinnunen et al., 2005a,b), and horses undergoing different physical treadmill exercise trials, both moderate and intense (Mills et al., 1997; Avellini et al., 1999; Deaton et al., 2002). Reactive oxygen species can be defined as oxygen-containing molecules including oxygen-derived free radicals (molecules with an unpaired electron) and several non-radical derivatives, which are produced by oxidative reactions and are more reactive than atmospheric oxygen (Sjodin et al., 1990; Sen, 1995; Noguchi and Niki, 1999). The formation of ROS is not necessarily damaging or harmful, and in fact is necessary for several metabolic and immune functions. However, the overproduction of these ROS results in damage to lipids, proteins, and genetic material, which manifests itself as decreased physical performance (Clarkson, 1995), muscular fatigue (Friden and Lieber, 1992), muscle damage (Dekkers et al., 1996; Goldfarb, 1999), and overtraining syndrome (Tiidus, 1998).

Reactive oxygen species are formed from either the re-oxygenation of a hypoxic environment or from inflammatory cytokine activity resulting from mitogen activated protein kinase pathways (MAPK) up-regulated during cellular damage (Fehrenbach and

Schneider, 2006). Several mechanisms are involved in the formation of ROS *in vivo*. With exercise comes a need for increased oxygen uptake to meet energy demands. Maximal oxygen uptake ( $\text{VO}_{2\text{max}}$ ) in horses can reach  $200 \text{ ml O}_2 \text{ kg}^{-1} \text{ min}^{-1}$  during exercise (Jones and Lindstedt, 1993; Young et al., 2002), an increase of about 30 times compared to that of oxygen intake at rest (Butler et al., 1993). During mitochondrial respiration most of the oxygen consumed is converted to carbon dioxide and water, although an incomplete reduction of 1 to 5% of the oxygen results in the formation of ROS, specifically superoxide radicals ( $\text{O}_2^\bullet$ ; Clarkson and Thompson, 2000). Increased production of  $\text{O}_2^\bullet$  can occur during ischemic muscular work as a result of xanthine oxidase-catalyzed degradation of hypoxanthine (Konig et al., 2001). Furthermore, exercise-induced muscle damage may result in the activation of polymorphonuclear neutrophils (PMN) and subsequent oxidative burst which has been shown to increase ROS formation (Konig et al., 2001). Other mechanisms among others, thought to result in the formation of ROS include the loss of calcium homeostasis in stressed myocytes, catecholamine auto-oxidation, and enhanced cytokine production with activation of NF- $\kappa$ B (Konig et al., 2001; Urso and Clarkson, 2003).

***Role of ROS in Arthropathies.*** In the absence of sufficient antioxidants, ROS are major contributors to joint pathogenesis (Auer et al., 1992; Dimock et al., 2000). Research has shown elevated levels of free radicals in the synovial fluid of diseased equine joints (Dimock et al., 2000; McIlwraith, 2005). Reactive oxygen species, such as superoxide anion, hydroxyl radicals and hydrogen peroxide can be released from damaged or inflamed joint tissues. This results in the depolymerization of hyaluronan, the major component of synovial fluid that arises from proteoglycans, and the cleavage of

proteoglycans and collagen fibrils (Greenwald and Moy, 1979; Greenwald and Moy, 1980; Wong et al., 1981; Betts and Cleland, 1982). As hyaluronan is depolymerized it loses its viscoelasticity and lubrication properties. With the loss of proteoglycans and collagen, there comes a loss in mechanical stability of the cartilage matrix further increasing joint tissue vulnerability to ROS and mechanical stress. Upon attack by ROS, proteins are also modified by the addition of carbonyl groups into amino acid residues (Dimock et al., 2000).

***Biomarkers of Oxidative Stress and Antioxidant Status.*** Several biomarkers of oxidative stress and antioxidant status can be measured in the blood and synovial fluid. Nitric oxide (NO) is a by-product of the oxidation of L-arginine to citrulline, catalysed by the nitric oxide synthase (NOS) isoenzymes, and is considered a highly reactive, cytotoxic free radical. Nitric oxide has a short half life and its oxidation results in the formation of nitrite which can then be oxidized by oxyhemoglobin to produce nitrate and methemoglobin (Sureda et al., 2006). Nitrite and nitrate are strongly correlated with NO and are often quantified as indirect markers of NO production in biological fluids (Sastry et al., 2002). Nitric oxide has many important physiological roles including maintenance of cardiovascular homeostasis, basal vasodilator tone, immune regulation, smooth muscle proliferation, and respiratory modulation (Sureda et al., 2006). It is also thought that NO is a physiologic mediator of inflammation and oxidative stress, and it may have a specific role in the pathogenesis of arthropathies, however its role in the synovial joint needs further investigation (Karan et al., 2003). Nitric oxide catalyses the IL-1-induced inhibition of proteoglycan synthesis (Bird et al., 1997), plays a role in chondrocyte apoptosis (Kim et al., 2003) and was demonstrated to activate matrix metalloproteinases

(Murrell et al., 1995). It has also been shown that NO is quantifiable in synovial fluid during an inflammatory response (Dimock et al., 2000; van den Boom et al., 2005). In clinical cases of osteoarthritis, NO concentrations were elevated in synovial fluid (Farrell et al., 1992; Johnston and Fox, 1997), as well as in cartilage and subchondral bone from equine osteoarthritic metacarpophalangeal joints (van der Harst et al., 2006).

Furthermore, concurrent induction of cyclooxygenase-2 (COX-2) and inducible NOS by cytokines may occur in inflamed tissue where NO may stimulate COX-2 to produce more prostaglandins (Johnston and Fox, 1997). Although the specific role of NO in the pathogenesis of degenerative joint disease has not been elucidated, production by synovial fibroblasts and chondrocytes has been documented (Johnston and Fox, 1997) as well as the expression of inducible NOS in equine chondrocytes (Tung et al., 2002), thereby supporting a role for NO in the pathogenesis of equine arthropathies.

Creatine kinase (CK) is a muscle enzyme that phosphorylates ADP from creatine phosphate to form ATP for muscular contraction (Hinchcliff et al., 2004b). It is also a biomarker of muscle membrane leakage and indirectly muscle damage resulting from exercise (Hoffman et al., 2004). Lipid peroxidation and muscle membrane leakage, as indicated by increased plasma levels of CK during endurance exercise in horses, have been correlated with and can be considered an indirect indication of antioxidant status and oxidative stress (Frank et al., 2000; Hargreaves et al., 2002; Williams et al., 2004).

Superoxide dismutases (SOD) are enzymatic antioxidant defenses along with glutathione peroxidase, and catalase. Superoxide dismutases can be categorized as metalloenzymes which exert their action on the same substrate by catalyzing the same chemical reaction despite different amino acid sequences and several distinct components

(Staninger, 2006). There are several isoforms of SOD, each with a functional metal group, including copper/zinc SOD ( $M_r = 16,000 - 33,000$ ; Lerch and Ammer, 1981; Marklund, 1984), manganese SOD ( $M_r = 80,000$ ; Marklund, 1984), iron SOD, and there is recent evidence indicating the existence of additional isozymes including selenium, nickel, and cadmium SOD (Fridovich, 1975; Staninger, 2006). In mammalian tissue, the first copper/zinc SOD (Cu/Zn-SOD), previously known as haemocuprein or erythrocuprein, was discovered by McCord and Fridovich (1969). Shortly thereafter, the manganese-SOD was discovered in chicken liver (Weisiger and Fridovich, 1973a), and mammalian tissue (McCord et al., 1977; Marklund, 1978). One other mammalian SOD, extracellular-SOD (EC-SOD), was isolated from human lung tissue by Marklund (1982). Cu/Zn-SOD has been found to be highly conserved among species including that of horse liver, bovine and human erythrocytes, and yeast (Lerch and Ammer, 1981). Superoxide dismutases are found in most eukaryotic cells both in the cytosol (Cu/Zn-SOD) and mitochondria (Mn-SOD) as well as in the extracellular space (EC-SOD) and it has been shown that these enzymes are specific to their location and have distinct properties (Weisiger and Freidovich, 1973b).

Specifically, SOD catalyze the dismutation of superoxide anions into oxygen and hydrogen peroxide, thereby preventing the formation of the hydroxyl radical (Fridovich, 1975). It has been widely recognized that such enzymes provide a defense system, essential for the survival of aerobic organisms (Beyer, 1991). One study demonstrated that a 50-60 % decrease in Cu/Zn SOD activity resulted in 50 % cell death via an apoptotic mechanism, most likely the NO-peroxynitrite pathway, in PC12 rat pheochromocytoma (neuronal) cells (Troy et al., 1996). Another study by Lynch et al.

(1997) demonstrated that 68 % decrease in Cu/Zn-SOD activity resulted in a 58 % increase in vascular superoxide formation and subsequent increase in lipid peroxidation, as well as decreased endothelial NO-mediated arterial relaxation. Extra-cellular-SOD is thought to be directly responsible for NO bioavailability, which is important for many important physiological processes (Rush et al., 2002; Jung et al., 2003). A study conducted by Fukai et al. (2000) suggests that NO upregulates EC-SOD expression via a feed-forward mechanism, thereby preventing the formation of peroxynitrite and maximizing NO bioavailability, as was demonstrated in human and murine vascular tissues. By scavenging superoxide anions, thus preventing NO from combining with superoxide anions thereby preventing peroxynitrite formation, SOD increases the bioavailability of NO and prevents the formation of a potent oxidant with detrimental biological effects. Extracellular SOD has been shown to increase with exercise training thereby improving the redox state (Fukai et al., 2000; Rush et al., 2002) although both in chronic and acute inflammatory states, the increased production of the superoxide anion can surpass the endogenous SOD scavenging capacity. Relatively low SOD activity in equine plasma ( $18.7 \pm 2.4$  units  $\text{ml}^{-1}$ ), compared to that of other species including rabbits ( $636 \pm 207$ ), mice ( $400 \pm 60$ ), rats ( $332 \pm 23$ ), cows ( $126 \pm 14$ ), sheep ( $97.9 \pm 5.8$ ), pigs ( $56.0 \pm 14$ ) and humans ( $26.3 \pm 3.6$  units  $\text{ml}^{-1}$ ) has been reported (Marklund et al., 1982; McCord, 1974). Similarly, low SOD activity in equine synovial fluid ( $7 \pm 2$  units  $\text{ml}^{-1}$ ) has also been documented (Auer et al., 1989). It has also been shown that in trained Thoroughbred racehorses the oxidant/antioxidant equilibrium undergoes significant changes in response to training and racing and that supplementation with an antioxidant

mixture helped improve the hydrophilic, lipophilic and enzymatic antioxidant blood capacity (deMoffarts et al., 2005b).

Glutathione peroxidase (GPx) is another endogenous antioxidant enzyme found in mitochondria, cytosol and cellular membranes, that metabolizes hydrogen peroxide, among other organic peroxides, and is specific to its substrate glutathione, which serves as an electron donor (Konig et al., 2001; Powers and Jackson, 2008). Glutathione peroxidase is also considered a selenoenzyme or tetrameric protein with four identical subunits each containing a selenocysteine residue that serves as a redox catalyst (Arthur, 2000). The response of GPx to exercise in horses is conflicting in the literature. Studies have reported decreases in erythrocyte GPx activity following exercise (Brady et al., 1978; Ono et al., 1990; Williams et al., 2005) and exercise training (deMoffarts et al., 2005b), while other studies demonstrated increases in GPx activity following exercise when compared to pre-exercise values in horses (Frankiewicz-Jozko and Szarska, 2000; Hargreaves et al., 2002). Varying exercise intensities and modalities, fitness status of horses, sample collection timing, as well as analytical methodologies may account for the differences between studies, making it difficult to compare results across studies.

The main non-enzymatic antioxidants include ascorbic acid (Vitamin C),  $\alpha$ -tocopherol (Vitamin E), glutathione, carotenoids, and flavonoids. Glutathione is a ubiquitous thiol antioxidant and forms a redox buffer system when coupled with its disulfide oxidized form, and also plays a role in modulating signal transduction during times of oxidant stress (Haddad and Harb, 2005). It is abundant in the cytosol, mitochondria and nuclei of cells where it maintains the redox state of critical protein sulphhydryls necessary for DNA repair and expression. The ratio of oxidized glutathione

(glutathione disulphide; GSSH) to reduced glutathione (GSH) is also a good indicator of oxidative stress (Valko et al., 2007). Glutathione has many protective roles including serving as a cofactor for several detoxifying enzymes against oxidative stress, assisting in amino acid transport through plasma membranes, scavenging hydroxyl radicals and singlet oxygen, detoxifying hydrogen peroxide and lipid peroxides, and regeneration of Vitamins C and E to their active forms (Valko et al., 2007). Several studies have reported exercise-induced changes in erythrocyte total glutathione and in each case the glutathione response was inversely related to the corresponding GPx response in horses. Studies have reported increases in total glutathione following acute and endurance exercise (Brady et al., 1978; Williams et al., 2005), while no change in total glutathione was found in Standardbreds (deMoffarts et al., 2005a), decreases in total glutathione were found in trained eventing horses (deMoffarts et al., 2005a), as well as during and following endurance exercise in horses when compared to pre-exercise values (Frankiewicz-Jozko and Szarska 2000; Hargreaves et al., 2002).

### **Exogenous Superoxide Dismutase**

There are a substantial number of studies, as reviewed by Yasui and Baba, (2006), that support the efficacy of exogenously administered SOD as an anti-inflammatory and antioxidant agent. Intravenous administration of a SOD-mimic in a rat model of inflammation and ischemia attenuated pro-inflammatory cytokine release, reduced lactate dehydrogenase, neutrophil infiltration, malondialdehyde, and myeloperoxidase in blood and tissue components (Salvemini et al., 1999). Another study found similar effects after administering an intramuscular injection of recombinant human SOD to rat and mouse

models of inflammation, where the SOD inhibited formation of pro-inflammatory cytokines as well as lipid peroxidation of tissues (Zhang et al., 2002). In a model of hepatic fibrosis in rats (bile duct ligation), treatment with an intravenous bolus of SOD polyanionic conjugate (DIVEMA) resulted in a reduction in free oxygen radicals and intrahepatic ROS production (Swart et al., 1999). Liposome-encapsulated SOD topical (sub-gingival) application for 6 wks reduced inflammation in a canine model of periodontal inflammation (Petelin et al., 2000).

Evidence in the literature also suggests that different oral formulations of SOD such as bovine erythrocyte liposome encapsulated-SOD (Regnault et al., 1996) and Glisodin (P.L THOMAS & Co, Inc, Morristown, NJ, USA) are bioavailable as well as effective in reducing markers of inflammation and oxidative stress in several species. Glisodin is a water dispersible form of SOD derived from melon (*Cucumis melo* LC) in combination with a patented wheat-derived gliadin biopolymer enteric coating which is a 40% hydro-alcoholic soft gel spray that is spray-dried using maltodextrin for support. The gliadin biopolymer has been shown to delay the release of SOD in an environment mimicking that of the gastrointestinal system allowing it to avoid protein (SOD) degradation prior to enterocyte uptake (Vouldoukis et al., 2004a). Gliadin biopolymers have also been shown to further improve delivery of the active ingredient. This is achieved via delayed enzyme release and bioadhesive properties which preserve the enzyme activity throughout the gastrointestinal tract, promote binding to the intestinal epithelial barrier, and increase intestinal permeability by increasing zonulin release and the opening of enterocyte tight junctions (Dugas, 2002; Drago et al., 2006). Mice orally supplemented with Glisodin for 28 days showed a rise in circulating antioxidant enzyme

activity which was correlated with an increased erythrocyte resistance to oxidative stress-induced hemolysis (Vouldoukis et al., 2004b). Furthermore, supplemented mice also exhibited increases in hepatic antioxidant defenses which were correlated with a significant decrease in hepatocyte apoptosis in the presence of Sin-1 (peroxynitrite chemical donor) when compared to unsupplemented controls ( $p < 0.001$ ; Vouldoukis et al., 2004b). In a pre-clinical rodent model of type-II diabetes, supplementation of an orally available gliadin/SOD supplement (Oxykine) reduced diabetes-induced oxidative stress and renal mesangial cell injury thereby slowing the progression and acceleration of this disease (Naito et al., 2005). In another case, peritoneal macrophages activated by an intra-peritoneal injection of IFN $\gamma$  and stimulated with IgG1/anti-IgG1 immune complex (IgG<sub>1</sub>IC), collected from mice after a 28 day oral supplementation of Glisoden<sup>®</sup> exhibited a reduction in TNF $\alpha$  production and elevated IL-10 production as well as decreased superoxide, nitric oxide, and peroxynitrite concentrations *ex vivo* (Vouldoukis et al., 2004a).

Treatment of IgG<sub>1</sub>IC-stimulated, IFN $\gamma$ -activated macrophages, with crude cantaloupe melon extract, inhibited superoxide anion and peroxynitrite production *in vitro* (Vouldoukis et al., 2004a). Furthermore, IgG<sub>1</sub>IC stimulated macrophages exhibited a reduced TNF $\alpha$  production and an increase in IL-10 production in the presence of CME *in vitro* (Vouldoukis et al., 2004a). Supplementation of gliadin/SOD in human soccer athletes has been shown to improve recovery from exercise and reduce oxidative stress. Specifically, improvements in the velocity at which lactate threshold is reached and a decrease in the magnitude of 8-iso PGF<sub>2</sub> $\alpha$  response were observed (Arent et al., 2009). Similarly, favorable results from a study in which college football athletes were

supplemented with a proprietary nutraceutical drink blend containing SOD, coenzyme Q10 and beta glucans during a 7 week training period were found (Arent et al., 2007). Data showed anti-inflammatory (reduced IL-6), antioxidant (reduced 8-iso PGF<sub>2</sub> $\alpha$ ), and reduced muscle membrane leakage (reduced CK) effects both after acute and chronic bouts of exercise when control and supplement groups were compared at the culmination of the training period. In another human study, healthy volunteers were supplemented with an orally effective gliadin/SOD and exposed to 100% oxygen breathing at 2.5 atmosphere absolute (ATA) for 60 minutes (Muth et al., 2004). Results indicated reduced damage to DNA strands as well as reduced blood isoprostane levels in the experimental group when compared to the placebo group. Similarly, a study by Kick et al. (2007) demonstrated that after 14 days of oral supplementation of cantaloupe melon extract and wheat gliadin biopolymer in a porcine aortic cross-clamping ischemia/reperfusion injury model, that DNA damage, spinal cord apoptosis, and NO release were all reduced. Another study, demonstrated that addition of SOD to bovine synovial fluid was protective against hyaluronic acid depolymerization by superoxide derived hydroxyl radicals *in vitro*, thereby preserving the viscoelastic properties characteristic of healthy synovial fluid (McCord, 1974). Palosein, an FDA-approved, injectable form of a Cu-Zn protein with high SOD activity derived from bovine liver, generically named Orgotein (Huber, 1981), has also been shown to ameliorate free radical-induced (superoxide) reduction of equine synovial fluid viscosity (Auer et al., 1990) and hypoxanthine induced hyaluronic acid degradation in human synovial fluid *in vitro* (Betts and Cleland, 1982).

Superoxide dismutase supplementation in other species has been shown to be

beneficial in reducing oxidative stress and inflammation, however clinical data from equine trials are needed. Once we understand the disease process and relationships associated with systemic and localized joint inflammation plus the role oxidative stress plays in these pathways, the effectiveness of various nutritional supplements can be evaluated and appropriate combinations of treatment and new preventative management strategies for chronic diseases can be used effectively and economically in equine athletes (Gutierrez et al., 1997; Goodrich and Nixon, 2004).

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### Abbreviations

AP-1 = activator protein-1	NOS = nitric oxide synthase
$\beta$ -GUS = beta glucuronidase	$O_2^{\cdot -}$ = Superoxide anion radical
CK = creatine kinase	OA = osteoarthritis
CON = control	PEAK = sample taken at fatigue
COX-2 = cyclooxygenase-2	PGE <sub>2</sub> = prostaglandin E <sub>2</sub>
CPII = carboxypeptide II	PMN = polymorphoneutrophils
CS-846 = epitope of chondroitin sulfate	qRT-PCR = quantitative real-time polymerase chain reaction
GAG = glycosaminoglycan	REC = recovery sample following exercise
GPx = glutathione peroxidase	ROS = reactive oxygen specie
GSH = glutathione reduced	RT = rectal temperature
GSH-T = total glutathione	SF = synovial fluid
GXT = graded exercise test	SOD = superoxide dismutase
Hct = hematocrit	TAS = total antioxidant status
HR = heart rate	TBARS = thiobarbituric acid
IET = interval exercise test	TNF $\alpha$ = tumor necrosis factor alpha
IFN $\gamma$ = interferon gamma	TP = total protein
IL-1 $\beta$ = interleukin-1 beta	TRT = experimental treatment group
IL-6 = interleukin-6	
IL-10 = interleukin-10	
LA = lactate	
MMP = matrix metalloproteinase	
NO = nitric oxide	

## Research Objectives

The high incidence of exercise-induced injury and chronic diseases involving inflammation and oxidative stress has a negative impact on the health, performance and welfare status of equine athletes. Additionally, there is a general lack of reliable data regarding the usefulness and efficacy of nutritional supplements intended to remedy or prevent exercise-induced inflammation and oxidative stress in horses. Therefore, science-based inquiry regarding the identification and efficacy of practical nutritional interventions intended to protect against exercise-induced inflammatory and oxidant stress is warranted. The objectives and hypotheses for the following research studies are as follows:

### Journal Article 1

***Rationale:*** Inflammation is most often studied within the context of acute injury or a disease state. Furthermore, studies evaluating effects of exercise in horses are often done in the field, providing limited experimental control. A controlled, repeatable framework or model that could be used to evaluate effects of exercise and nutritional interventions for related injury or disease would be useful. Additionally, changes in cytokine transcripts and nitric oxide in response to different modes of exercise have not been previously documented in healthy horses. Therefore, this study was conducted in a controlled environment using a high- speed equine treadmill in order to identify the best model of exercise-induced inflammation in healthy horses for use in subsequent studies. Due to the

integral role cytokines and nitric oxide play in both the maintenance of health and development of disease, this type of data is an important addition to the literature that can be used as a benchmark for typical inflammatory responses to exercise in horses that may be useful in distinguishing between adaptive responses and early pathological processes.

***Objective:*** To compare exercise-induced inflammatory responses in horses undergoing three different treadmill exercise tests of varying intensities.

Responses to exercise were based on cytokine gene expression in peripheral blood, plasma and synovial fluid total nitrite concentrations, and differences in markers of exercise intensity.

***Hypothesis:*** It was hypothesized that a higher intensity exercise test would increase cytokine gene expression in circulation, total nitrite concentration both in plasma and SF, as well as markers of exercise intensity to a greater degree than less intense exercise tests.

## **Journal Article 2**

***Rationale:*** High intensity exercise results in a pro-inflammatory response as well as an increase in reactive oxygen specie formation that may surpass the body's endogenous antioxidant defenses, including the scavenging capacity of superoxide dismutase (SOD). Oral SOD formulations have been shown to be bioavailable and effective in helping reduce exercise-induced oxidative stress and inflammation in various animal models although this has not been demonstrated in healthy horses.

**Objective:** To evaluate the effect of exogenous SOD supplementation on inflammatory responses and antioxidant status in horses undergoing intense exhaustive exercise.

**Hypothesis:** It was hypothesized that supplementation with exogenous SOD would elicit a protective effect against exercise-induced inflammation and oxidative stress by reducing markers of inflammation and improving antioxidant status in horses.

### **Journal Article 3**

**Rationale:** Inflammation and increased rate of cartilage turnover are indicated in adaptive responses to exercise and joint disease. Inflammation and cartilage metabolism are most commonly evaluated in a repetitive exercise model or in a joint pathology model, whereas changes relative to a single bout of exercise in healthy horses have not been explored. This insight could be useful in distinguishing between changes due to exercise or those that may be indicative of early signs of degenerative processes in equine athletes. Furthermore, benefits from oral supplementation of SOD, specifically anti-inflammatory and chondroprotective properties in joints of horses, have not been evaluated.

**Objective:** To evaluate 1) effects of repeated arthrocentesis within a 48 h time frame on markers of inflammation and cartilage metabolism in synovial fluid of healthy horses at rest and 2) the effects of a single bout of intense exhaustive treadmill exercise as well as 3) the effects of exogenous SOD supplementation on biomarkers of joint inflammation and cartilage metabolism in horses.

***Hypothesis:*** It was hypothesized that repeated arthrocentesis within a 48 h time frame would not influence biomarkers of inflammation (PGE<sub>2</sub>) and cartilage metabolism (CS-846 epitope) in synovial fluid of mature, healthy horses at rest. It was also hypothesized that a single bout of intense exhaustive treadmill exercise would elicit localized inflammation and an increase in anabolic cartilage metabolism within the carpus and hock joints of mature healthy horses. Lastly, it was hypothesized that exogenous SOD supplementation would elicit a protective effect in the carpus and hock joints by reducing markers of inflammation and maintaining homeostatic cartilage metabolism following intense exhaustive exercise in mature healthy horses.

## Journal Article 1

### **Inflammatory responses to three modes of intense exercise in Standardbred mares – A pilot study**

Lamprecht, E. L., C. A. Bagnell, and C. A. Williams. 2009. Inflammatory responses to three modes of intense exercise in Standardbred mares – A pilot study. *Comp. Ex. Physiol.* 5:115-125.

**ABSTRACT:** The objective of this study was to compare exercise-induced inflammatory responses in horses undergoing three different treadmill exercise tests. Mares completed a graded exercise test (GXT), an interval exercise test (IET), and a repeated sprint exercise test (RSET). Blood and synovial fluid samples were taken 24 h before (PRE) exercise, 20–30 min, 2, and 24 h following exercise (REC). Blood was analyzed for total protein, haematocrit, and total nitrite concentration. Pro-inflammatory and anti-inflammatory cytokine transcripts were measured in whole blood using quantitative real-time polymerase chain reaction. Synovial fluid was analyzed for total nitrite. Mares spent more time at greater than 90% of their maximum heart rate during the RSET than they did for either the GXT or IET. There was an effect of exercise test ( $P < 0.0001$ ) and exercise test by sample interaction ( $P = 0.010$ ) for plasma total nitrite. Pro-inflammatory cytokine transcripts and plasma total protein were elevated ( $P < 0.05$ ) after exercise and there was a higher ( $P < 0.0001$ ) nitrite concentration in tibiotarsal joints compared to radiocarpal joints. As hypothesized, the higher intensity exercise test (RSET) resulted in greater nitric oxide responses as well as markers of exercise intensity compared to less intense exercise tests (IET, GXT).

**Key Words:** cytokines, equine, exercise, inflammation, nitric oxide, synovial fluid

## INTRODUCTION

Local and systemic inflammation is an immune response to the presence of microorganisms or to injury which allows repair of damaged or infected tissue and a return to homeostatic conditions through a balance of local innate immune mechanisms and brain-derived immunoregulatory output via the autonomic nervous system (Higgins and Lees, 1984). Inflammation has been implicated in the pathogenesis of many debilitating chronic diseases such as arthritis, inflammatory bowel diseases, respiratory diseases, heart disease, and autoimmune diseases, just to mention a few (Han and Ulevitch, 2005). The immune response to exercise has also been investigated and similarities between exercise-induced and disease related immune changes have been documented (Cannon and Kluger, 1983; Moldoveanu et al., 2001). Several factors including the modality, intensity, and duration of exercise influence the inflammatory response (Brenner et al., 1999). Sub-clinical injury and the subsequent immune response to acute intense exercise may set the stage for a chronic inflammatory and immunosuppressed state resulting in a predisposition to infection, poor physical performance, and potentially the development of chronic diseases (Shek et al., 1995; Brenner et al., 1999).

Cytokines are hormone-like proteins that mediate inflammatory responses by autocrine, paracrine, and endocrine effects and can be considered markers of inflammation (Cannon, 2000). Strenuous exercise, energy crisis, stress hormones and

oxidative stress are examples of physiological stimuli that modulate cytokine production (Cannon, 2000). Cytokines, including tumor necrosis factor alpha (TNF $\alpha$ ), interleukin-1 beta (IL-1 $\beta$ ), interleukin-6 (IL-6), interferon gamma (IFN $\gamma$ ), and interleukin-10 (IL-10), are largely responsible for dictating profound physiological changes both locally and systemically. Dysregulation of the inflammatory response resulting in excessive production of pro-inflammatory cytokines or their production in the wrong biological context may lead to chronic inflammation which is detrimental to a horse's welfare and can be life-threatening in extreme cases (Han and Ulevitch, 2005).

Nitric oxide (NO) is another physiologic mediator of inflammation and its specific role both systemically and in the synovial joint needs further investigation. Nitric oxide is a highly reactive, cytotoxic free-radical and a by-product of the oxidation of L-arginine to citrulline catalyzed by nitric oxide synthase (NOS). Nitric oxide plays a critical role in vasodilation, and synaptic transmission in the central nervous system (Moncada and Higgs, 1993), and immune function (Sureda et al., 2006). It catalyses the IL-1-induced inhibition of proteoglycan synthesis (Bird et al., 1997), plays a role in chondrocyte apoptosis (Kim et al., 2003) and was demonstrated to activate matrix metalloproteinases (Murrell et al., 1995). It has been reported that in clinical cases of osteoarthritis, synovial fluid concentrations of NO were elevated (Farrell et al., 1992; Johnston and Fox, 1997) and that NO is quantifiable in synovial fluid during an inflammatory response (Dimock et al., 2000; Van den Boom et al., 2005). Furthermore, concurrent induction of cyclooxygenase-2 (COX-2) and inducible NOS by cytokines may occur in inflamed tissue where NO can stimulate COX-2 to produce more prostaglandins (Johnston and Fox, 1997). Although the specific role of NO in the pathogenesis of

degenerative joint disease has not been elucidated, production by synovial fibroblasts and chondrocytes has been documented (Johnston and Fox, 1997).

Equine athletes, like their human counterparts, suffer from challenges to the immune system related to exercise. Post-exercise problems associated with increased expression of inflammatory markers can range from the mild symptoms of delayed-onset muscle soreness to debilitating problems related to soft tissue, joint and bone damage (Auer et al., 1989). More specifically, exercise-induced inflammation is problematic in the equine athlete resulting in impaired health, lost training time and millions of dollars in veterinary expenses.

The objective of this study was to compare exercise-induced inflammatory responses in horses undergoing three different treadmill exercise tests of varying intensity, based on cytokine gene expression in peripheral blood, plasma and synovial fluid total nitrite concentrations, and differences in markers of exercise intensity. It was hypothesized that a higher intensity exercise test would increase cytokine gene expression in circulation, total nitrite concentration both in plasma and SF, as well as markers of exercise intensity to a greater degree than less intense exercise tests.

## **MATERIAL AND METHODS**

### ***Subjects***

Four healthy, unfit Standardbred mares aged  $8.5 \pm 1.7$  yrs, weighing  $472.3 \pm 13.6$  kg were used in this study. Mares were selected based on similar body condition (Henneke et al., 1983;  $5.3 \pm 0.14$ ), percent body fat (Westervelt et al., 1976;  $16.5 \pm 1.5$

%), and similar heart rate responses to a graded exercise test performed one month prior to the study. Mares were housed on 2-acre dry lots and were offered free choice water, salt, moderate-quality mixed grass hay and 2 kg of a 12 % crude protein pellet to meet daily maintenance needs. Mares were habituated to the treadmill laboratory and to running on the treadmill prior to an initial graded exercise test (GXT), and were not exercised in between the initial GXT and the start of the trial. The Rutgers University Institutional Animal Care and Use Review Board approved all methods and procedures used in this experiment.

### ***Exercise Tests***

Each mare completed three different treadmill exercise tests in a completely randomized order with 7 days between each test (Table 1). During each exercise test, the mares ran on a high speed equine treadmill (Sato I-Equine Dynamics, Yellow Springs, OH) that was fixed at a 6 % grade. Immediately before exercise each mare was fitted with a heart rate monitor (Polar Equine Heart Rate Monitor; FitMed Inc., Mill Valley, CA) and resting heart rate as well as heart rate during exercise was recorded during the last 15 s of each step, and was monitored after exercise until recovery. Maximal heart rate ( $HR_{max}$ ), as determined by a GXT one month prior to the start of the study, was used as a determinant of fitness to standardize the exercise protocols, since the treadmill speed at which  $HR_{max}$  is achieved during a stepwise test is correlated with maximal oxygen consumption. In each case horses ran to fatigue or until the test was finished. At time of fatigue or end of the test, horses completed 1 min of walking at  $1.5 \text{ m s}^{-1}$ .

The three different exercise tests consisted of the GXT, the interval exercise test (IET), and the repeated sprint exercise test (RSET). The GXT lasted an average of  $9.56 \pm 0.26$  min and began with 2 min walking at  $1.5 \text{ m s}^{-1}$ , 1 min trotting at  $4 \text{ m s}^{-1}$  and 1 min at  $6 \text{ m s}^{-1}$ . This was followed by incremental increases of  $1 \text{ m s}^{-1}$  every 60 s until fatigue (McKeever and Malinowski, 1997). The IET lasted an average of  $18.25 \pm 0.40$  min and began with a warm up of 2 min walking at  $1.5 \text{ m s}^{-1}$  and 8 min trotting at  $4 \text{ m s}^{-1}$ . The warm up was followed by 2 intervals each consisting of 2 min at 100 %  $\text{HR}_{\text{max}}$  ( $8\text{--}11 \text{ m s}^{-1}$ ) with 4 min at  $4 \text{ m s}^{-1}$  between (Williams and Carlucci, 2006). The RSET lasted an average of  $20.63 \pm 0.38$  min and began with 2.5 min of walking at  $1.5 \text{ m s}^{-1}$  followed by 4 min of trotting at  $4 \text{ m s}^{-1}$ . Following warm up, mares completed 2 min sprints at 7, 8, 9 and  $10 \text{ m s}^{-1}$  with 2 min of walk at  $1.5 \text{ m s}^{-1}$  between each sprint (Wilson et al., 1998; Graham-Thiers et al., 2003).

### ***Sampling Procedure***

Blood samples (20 ml) were taken via jugular venipuncture 24 h prior to the exercise test (PRE), 20-30 min (END), 2 h (2 h REC), and 24 h post-exercise (24 h REC). Samples were placed in pre-chilled sodium heparin and EDTA tubes (Vacutainer, Becton Dickson, Inc., Franklin Lakes, NJ), immediately placed on ice and analyzed for packed cell volume or haematocrit (Hct) using microhaematocrit technique (Spiracrit, Oxford Labware-Division of Sherwood Medical, St. Louis, MO). The blood collection tubes were then centrifuged at  $1500 \times g$  for 5 min and analyzed for plasma total protein by refractometry (Refractometer, Leica Microsystems, Buffalo, NY). The plasma supernatant was frozen at  $-80^{\circ} \text{C}$  for later analysis of plasma total nitrite as an indicator of

NO concentration (QuantiChrom™ NO Kit; BioAssay Systems, Hayward, CA; intraassay CV = 5.82 % [plasma], 5.04 % [SF]; interassay CV = 7.96 % [plasma]). Rectal temperature (RT) and body weight (BW) were also taken before and after each exercise test.

Synovial fluid samples were collected bilaterally via aseptic arthrocentesis from both radiocarpal joints and tibiotarsal joints at the same sampling times mentioned above. Samples from the same joint spaces were pooled from the right and left legs into pre-chilled EDTA vacutainer collection tubes and immediately placed on ice. Each pooled sample was centrifuged for 20 min at 1500 x g and the supernatant was aliquoted and stored at -80° C until analysis of total nitrite.

The use of quantitative real time polymerase chain reaction was employed to indirectly evaluate cytokine gene expression in the circulation during this study since commercially available kits for these equine cytokine proteins are either unavailable or were found to be unreliable. Peripheral blood (2.5 ml) was collected via jugular venipuncture into PAXgene® blood RNA collection tubes containing quaternary amine surfactants (Qiagen/Becton Dickinson, Valencia, CA) at each sample time. Total RNA was isolated from 2.5 ml of whole blood according to manufacturer's instructions and the RNA was quantified using a spectrophotometer (Biophotometer, Eppendorf, Westbury, NY). The RNA purity and quantity in each extraction were found to be sufficient for gene expression analysis. Optical density ratios at 260:280 nm were consistently greater than 1.9 and indicated an RNA yield of ~ 50 µg RNA ml<sup>-1</sup> blood. A detailed description of RNA preparation, reverse transcription, and amplification was described previously (Vick et al., 2007). Relative quantification ( $2^{-\Delta\Delta CT}$  method) was used to analyze the

changes in gene expression (Livak and Schmittgen, 2001; Ainsworth et al., 2003).

Cytokine (target) gene expression was normalized to that of the endogenous control gene beta-glucuronidase ( $\beta$ -GUS) and fold changes in target gene expression were calculated relative to a calibrator sample within the data set. The following cytokine transcripts were measured: TNF $\alpha$ , IL-1 $\beta$  (pro-inflammatory), IFN $\gamma$  (immunomodulatory), IL-6 (multifunctional), and IL-10 (anti-inflammatory). Equine-specific cytokine primer and probe sequences and PCR amplicon fragment sizes can be found in Table 2.

### ***Statistics***

Statistical significance was set at  $P < 0.05$  and trends were defined as  $P < 0.1$ .

Cytokine data are presented as relative mRNA transcript (RMT) or the mean fold changes in target gene expression in response to intense exercise  $\pm$  standard error. All other data are presented as the mean  $\pm$  SE. Changes were analyzed using a general linear model ANOVA using SAS 9.1. The model used exercise test and sampling time as main effects, horse was nested within exercise test, and interactions between exercise tests and time were tested. Effect of joint was also determined for total nitrite concentration in synovial fluid. Post-hoc analysis of all significant main effects was performed using the Ryan, Einot, Gabrielle, Welsch Multiple Range test.

## **RESULTS**

The average duration of each exercise test, and the amount of time spent at or above 60 or 90 % of heart rate max is depicted in Table 3. Compared to pre-exercise measurements, rectal temperature was higher ( $P < 0.0001$ ) following exercise for all three

exercise tests (Table 4). Similarly, a main effect of sampling time ( $P < 0.001$ ) for plasma total protein and Hct was detected (Table 5).

There was no main effect of exercise test for any cytokine transcript data however, for  $\text{TNF}\alpha$  ( $P = 0.042$ ),  $\text{IL-1}\beta$  ( $P < 0.0001$ ),  $\text{IFN}\gamma$  ( $P = 0.0057$ ), and  $\text{IL-6}$  ( $P = 0.005$ ) there was a main effect of sampling time (Figure 1). Gene expression for  $\text{TNF}\alpha$  at the PRE sample ( $1.02 \pm 0.08$  RMT) was lower ( $P < 0.05$ ) compared to all other sampling times (Figure 1A). Interferon- $\gamma$  peaked at the END sample ( $13.8 \pm 2.9$  RMT) where it was higher ( $P < 0.01$ ) when compared to all other sampling times across all exercise tests (Figure 1B). Interleukin-6 showed similar results as  $\text{IFN}\gamma$ , and was highest ( $P < 0.01$ ) at the END sample time ( $2.51 \pm 0.285$  RMT) when compared to all other sample times (Figure 1C). Interleukin- $1\beta$  transcript was higher ( $P < 0.001$ ) at the 2 h REC ( $4.98 \pm 0.50$ ) sample compared to the other sample times across all exercise tests (Figure 1D). There was no effect of exercise test for IL-10, although there was a trend ( $P = 0.072$ ) for a sample time effect where IL-10 transcript was highest ( $P < 0.05$ ) at the END sample time ( $2.12 \pm 0.26$  RMT) compared to all other samples times (Figure 1E).

There was a main effect of exercise test on plasma total nitrite concentration ( $P < 0.0001$ ; Figure 2). Higher plasma nitrite concentrations were associated with the RSET ( $0.085 \pm 0.006$  mg  $\text{dl}^{-1}$ ) when compared to the IET ( $0.067 \pm 0.007$  mg  $\text{dl}^{-1}$ ;  $P = 0.003$ ) and GXT ( $0.053 \pm 0.004$  mg  $\text{dl}^{-1}$ ;  $P < 0.0001$ ) across all sample times. There was also a sample time by exercise test interaction ( $P = 0.0097$ ), where plasma samples from the GXT showed no change in nitrite concentration, the IET samples showed an increase from PRE to the 2 h REC ( $P = 0.016$ ) and 24 h REC ( $P = 0.008$ ), and the RSET samples showed a decrease from the END sample to the 2 h REC ( $P = 0.021$ ) and 24 h REC

sample ( $P = 0.030$ ). Radiocarpal (carpus) and tibiotarsal (hock) synovial fluid nitrite concentrations showed no main effects of exercise test or sampling time, however, there was more nitrite in the hock joints ( $0.083 \pm 0.004 \text{ mg dl}^{-1}$ ) compared to the carpal joints ( $0.053 \pm 0.001 \text{ mg dl}^{-1}$ ; ( $P < 0.0001$ ) Figure 3).

## DISCUSSION

Despite numerous studies evaluating cytokine changes in response to different modes and intensities of exercise in humans, there has been limited work of this nature done in horses (Colahan et al., 2002; Ainsworth et al., 2003), and to the best of our knowledge this is the first study to investigate cytokine response to three different modes of intense exercise in the same horses. These types of data are often difficult to compare to other studies due to species differences, sample timing and the type (eccentric, concentric, isometric), duration, and intensity (submaximal vs. maximal) of exercise employed. Therefore, the following discussion does include a comparative approach, however similarities and differences must be considered carefully and in the proper context. It is also important to keep in mind that this study was a pilot designed to evaluate the logistics of the proposed experimental design and to determine if certain variables were quantifiable in our animal model and if they would be useful endpoints to be studied under different experimental conditions. The small sample size, variability in individual responses, and power to detect treatment differences was taken into consideration by the authors when assigning level of significance. In the present study, the RSET was found to be the most rigorous protocol (4 bouts of near maximal sprints),

closely followed by the IET (2 maximal bouts), indicated by NO responses and other markers of performance.

Normal increases in Hct, total protein and RT occurred after exercise. There was a 7.2 % increase in RT during the IET and RSET and only 4.5 % increase during the GXT, indicating that the IET and RSET are more rigorous, thereby generating more metabolic heat possibly due to longer test durations when compared to the GXT. Furthermore, maximal heart rates were achieved, sometimes multiple times throughout the duration of a test, an indication of the exercise intensity and physical effort of each horse. Mares spent more time at greater than 90 % of their  $HR_{max}$  during the RSET, when compared to the GXT and IET.

The cytokine transcript data in the present study mirror those reported in humans (Brenner et al., 1999; Ostrowski et al., 1999) by demonstrating an up-regulated pro-inflammatory cytokine or an acute phase response to intense exercise in horses and a small anti-inflammatory response accompanied by supporting performance data. It has been reported that longer duration exhaustive exercise can result in immunosuppression and increased susceptibility to infection (Shek et al., 1995), and that inflammatory vs. anti-inflammatory cytokine balance is an important factor. The pro-inflammatory response to exercise is most likely kept in balance by the counter response of anti-inflammatory cytokines and other cytokine soluble receptors and receptor antagonists restricting the magnitude and duration of the response, which has been noted in other exercise studies (Ostrowski et al., 1999). Furthermore, parallels between regulatory and counter regulatory responses to a single bout of exhaustive exercise or responses to prolonged systematic heavy training programs to clinical sepsis and surgical trauma have

been documented (Brenner et al., 1999). This makes sense since during strenuous physical activity, sub-clinical injury or micro-damage to tissue occurs priming the body for an inflammatory reaction and potentially a chronic inflammatory situation and immunosuppression (Cross, 1996; Brenner et al., 1999). Studies have shown that intensity, determined by stress hormone response and not just muscle micro-damage, influences the type and magnitude of an exercise-induced inflammatory response (Rhind et al., 1996; Brenner et al., 1999). This may indicate that other factors unique to exercise are responsible for the observed responses and clear differences between exercise-induced inflammation and that resulting from sepsis or acute injury. Furthermore, training adaptations have been found to reduce inflammatory responses in humans (Peake et al., 2005), thereby making comparisons in an untrained or unfit model to an entire population including trained athletes less relevant (Rhind et al., 1996). It is thought that the mechanism behind the training adaptation, or “anti-inflammatory” effects of exercise-training are related to a shift in Th1:Th2 cell response in favor of Th2 or anti-inflammatory processes (Flynn et al., 2007).

The increased expression of  $\text{TNF}\alpha$ ,  $\text{IL-1}\beta$ ,  $\text{IFN}\gamma$  and  $\text{IL-10}$  in the present study may indicate that the exercise tests may have altered TH1 and TH2 lymphocyte balance. A similar response has been documented in humans (Moyna et al., 1996), where effects of acute continuous incremental exercise on cytokine production were evaluated. Furthermore, Brenner et al. (1999) evaluated the effects of three modes of exercise, short duration high intensity (~ 5 min), circuit training (eccentric exercise), and long duration (2 h), on markers of inflammation in healthy moderately fit men. The long duration exercise elicited significant increases in plasma  $\text{TNF}\alpha$  and  $\text{IL-6}$ , although  $\text{IL-6}$  also

tended to increase following the other two exercise challenges but did not achieve statistical significance. Unlike the present study, IL-10 showed a decline following the short duration test and did not change for either of the other tests (Brenner et al., 1999).

Tumor necrosis factor- $\alpha$  was a target inflammatory mediator in the present study due to its central role in the initiation of the immune response to injury or infection. Its ability to augment its own production as well as that of IL-1 $\beta$ , eicosanoids, reactive oxygen species and nitrogen intermediates, makes it an obvious therapeutic target in efforts made to prevent or control inflammatory responses (Pavlov and Tracey, 2004). In the present study TNF $\alpha$  gene expression increased immediately after exercise and remained elevated until 24 h REC, unlike the other inflammatory cytokine transcripts which returned to baseline values by 24 h REC, suggesting an immediate and prolonged vs. a transient pro-inflammatory response to intense acute exercise. Human studies have shown that plasma TNF $\alpha$  protein peaked (2 - 3 fold increase) immediately following a marathon race, but only remained elevated for 3 h of the recovery phase (Ostrowski et al., 1999). Similar TNF $\alpha$  responses have also been seen in prolonged exercise studies in humans (Gannon et al., 1997; Brenner et al., 1999; Niess et al., 1999). For all of the mentioned human studies, the TNF $\alpha$  increases were resolved by 24 h of recovery. In the present study, the prolonged TNF $\alpha$  gene expression may indicate a more pronounced pro-inflammatory response to the exercise as well as exercise-induced tissue damage, more characteristic of sepsis or acute injury (Northoff et al., 1995; Bruunsgaard et al., 1997).

Interleukin-1 $\beta$  up-regulates a variety of genes including those that up-regulate its own expression and that of IL-6, and also induces enzymes necessary for the synthesis of leukotrienes, prostaglandins and NO (Moldoveanu et al., 2001). In the present study, IL-

1 $\beta$  transcript, showed a delayed peak when compared to the other cytokine responses. In the study conducted by Ostrowski et al. (1999), plasma IL-1 $\beta$  was elevated two-fold immediately following a marathon when compared to pre-race values, and had returned to pre-race levels by 1 h into recovery. Another study done by Mucci et al. (2000) showed a 17 % increase in IL-1 $\beta$  during and following an exhaustive incremental bicycle test in endurance athletes. An increase in IL-1 $\beta$  in blood components following exercise has primarily been reported in studies where the exercise is long term such as in a marathon or endurance race. In the present study, the increase in IL-1 $\beta$  gene expression after a short exhaustive bout (< 30 min duration), two hours into recovery is a novel finding in horses. Only Suzuki et al. (2002) saw the same delayed response in humans following a treadmill graded exercise test. Interleukin-1 $\beta$  is thought to be produced locally within tissue, therefore, it is suspected that the delayed response may reflect leakage into circulation upon tissue damage (Suzuki et al., 2002). Increased IL-1 $\beta$  in urine output following exercise has been documented in marathon runners immediately following the race (Suzuki et al., 2003; Sprenger et al., 1992), indicating that it has a fairly rapid clearance from circulation as does TNF $\alpha$  (Suzuki et al., 2002).

Prostaglandins and IL-6 up-regulate the production and secretion of IL-10 which in turn inhibits TNF $\alpha$ , IL-1 $\beta$ , and IFN $\gamma$  production. Therefore, IL-6 can be considered an inflammatory response cytokine since it does not induce an inflammatory response (Petersen and Pedersen, 2005). The up-regulation of IL-6 and IL-10 transcripts in the present study may have served to keep an exercise related inflammatory response in check, thereby preventing a chronic condition (Petersen and Pedersen, 2005). A similar response in humans has also been published, where IL-6 and IL-10 were significantly

elevated following a marathon race when compared to resting values and remained elevated out to 4 h of the recovery phase (Ostrowski et al., 1999). Another group reported significant increases in plasma IL-10 compared to pre-exercise values, immediately following, and 1 h into recovery after a high-intensity trial in trained male runners (Peake et al., 2005). Perhaps if more frequent samples following exercise had been taken for the present study, the IL-10 response could have been better defined. It is possible that the sample timing could have missed the true IL-10 peak or that clearance rates were faster than production.

Interleukin-6 is thought to be one of the most sensitive indicators of fatigue, skeletal muscle damage and metabolic turnover primarily due to the large increases that have been documented in human athletes following both dynamic eccentric exercise (Bruunsgaard et al., 1997; Ostrowski et al., 1998; Pedersen, 2000), and static resistance exercise (MacIntyre et al., 2001). Therefore, IL-6 response and possibly IFN $\gamma$  response to the exercise tests in the current study may indicate that the exercise tests were strenuous enough to induce skeletal muscle damage predominantly through eccentric contractions. It is evident from the literature that recruitment of blood mononuclear cells during physical activity may be due to changes in plasma catecholamine levels, however, this does not hold true for the cytokine response to exercise indicating that inflammatory responses in muscle may contribute to the exercise induced cytokine response systemically, with a more profound effect occurring after eccentric exercise in untrained individuals (Bruunsgaard et al., 1997). Furthermore, the mares in the present study ran on a 6 % incline on the treadmill, which compared to a flat grade, requires a higher degree of eccentric contraction during exercise.

Interferon- $\gamma$  has the ability to up-regulate reactive oxygen and nitrogen species production by priming macrophages as well as to influence cell-mediated cytotoxicity and exhibit anti-microbial properties (Ainsworth et al., 2003). The post-exercise increase in IFN $\gamma$  gene expression in the present study was unique compared to results in human literature, and may be due to an increase in natural killer (NK) cells during short intense bouts of exercise (Suzuki et al., 2002). Interferon- $\gamma$  is critical in the development of a Th-1 response and is primarily produced by CD4+, CD8+, CD16+, and NK cells. Fluctuations in IFN $\gamma$  seem to be related to changes in these cell types relative to exercise (Haahr et al., 1991; Ainsworth et al., 2003), therefore the unexpected increase in the present study may be due to an increase in lymphocytes and NK cells (Horohov et al., 1996; Horohov et al., 1999; Folsom et al., 2001), although direct evidence of this was not obtained. Furthermore, an exaggerated response of IFN $\gamma$  (as was seen in the present study) to exhaustive exercise may be related to the unfit status of the horses. In a study conducted by Ainsworth et al. (2003), changes in IFN $\gamma$  mRNA levels were not detected following (24 h REC) a maximal exercise test, similar to that of the GXT, in trained Standardbreds. This indicates that an adaptation to exercise might occur with training relative to cytokine response, or that cytokine fluctuation was not detected due to sample timing.

The authors would like to point out certain limitations regarding the use of qRT-PCR to quantify cytokine response. Often the assumption that mRNA levels directly relate to protein expression and/or biological activity is made and can be misleading; therefore caution should be used in order that over-interpretation of gene transcription

data does not occur. In any case, direct correlations between mRNA levels and protein levels should not be made.

It was expected that NO response would be similar to that of the pro-inflammatory cytokines, as it known that cytokines induce COX-2 and upregulate iNOS (Johnston and Fox, 1997). However, the higher total plasma nitrite concentration relative to the RSET remained constant during the PRE and END samples indicating that increased endogenous NO production via a concomitant increase NOS was able to maintain equilibrium by keeping up with increased demands for NO during and immediately following exercise. The need for NO is dependent on the degree of physical exertion, or how strenuous a physical activity is. This was shown by Kindig et al. (2000), where horses that were exercised at 50 and 80 % of their peak pulmonary oxygen uptake ( $VO_{2peak}$ ) and treated with an NOS inhibitor (N(G)-L-nitro-arginine methyl ester; L-NAME) were able to compensate for reduced body oxygen delivery via increased oxygen extraction and did not experience a significant decline in  $VO_2$ . However, horses treated with L-NAME exercising at their maximal capacity were not able to do this and did experience a reduced  $VO_2$ . Similar results from a comparable study done in Thoroughbreds were obtained by Manohar et al. (2006). In the present study, the strenuous nature of the RSET, when compared to the other tests, was demonstrated by the decline in circulating NO from the END to the 2h REC sample, remaining low out to 24 h REC, a response only seen following the RSET. The RSET NO data coupled with the heart rate data support what was seen in previous studies as our mares spent more time at greater than 90 % of their  $HR_{max}$  during the RSET, then they did for either the GXT or the IET. This may indicate that the stress or NO demand induced by the RSET required

that NO be used faster than it could be made, resulting in the decrease after exercise. In contrast, NO increased from the PRE sample to 2 h REC and remained elevated out to 24h REC for the IET and there was no change in plasma NO for the GXT. The IET appeared to be strenuous enough to elicit upregulation and production of NO, but not so strenuous that the body was unable to compensate for the increase in demand. It is apparent that the mares were able to increase NOS activity and subsequent NO production following the onset of exercise, something they could not achieve for the RSET which was clearly a more demanding test. The GXT appeared to be the least strenuous of the tests and apparently did not require anything beyond basal or normal resting NOS activity and NO production, which was sufficient to meet demands during and following the GXT, as evidence by no change in plasma NO concentration at each sample time.

It is suspected that the decline in plasma NO following the RSET in the present study resulted from one or more of the following reasons; increased utilization, increased excretion, and decreased production or bioavailability due to increased oxidative stress. It is well documented that NO is utilized by the endothelium to facilitate cardiorespiratory function under the stress of maximal exercise and recovery, ie. oxygen delivery, vaso- and bronchiodilation, increased cardiac output, and thermoregulation; without such would result in a profound hemodynamic impairment in maximally exercising horses (Kindig et al., 2000; Mills et al., 1997). The heart rate, TP, haematocrit, and temperature data in the present study all suggest the intense nature of this exercise test, and without increased utilization of NO would not have been possible. Furthermore, it is possible that the bioavailability of NO was reduced relative to the

RSET, resulting from decreased NOS expression (Sureda et al., 2006), NOS uncoupling (Cai and Harrison, 2000), reduced antioxidant enzyme activity (Tauler et al., 2002), neutrophil priming for ROS formation (Suzuki et al., 1999), or increased presence of superoxide anions (Clarkson and Thompson, 2000). Superoxide dismutase is considered by some (Cai and Harrison, 2000; Jonsson et al., 2002; Jung et al., 2003) to be the major limiting factor in NO bioavailability due to its role in protecting NO by scavenging superoxide anion with which NO is capable of reacting with to form peroxynitrite. Exercise-induced generation of superoxide and NO in a physiological state where SOD activity is impaired, lends to the formation of peroxynitrite, reduced NO bioavailability, and oxidative stress (Sureda et al., 2006).

Why there was more NO at the PRE and END samples for the RSET when compared to the GXT and IET is unknown. This might be a reflection of environmental influence(s) that were not able to be controlled for during the study and was an unexpected finding. However, an analogous decline in plasma NO following exhaustive treadmill exercise (RSET) was replicated in a similar sample population of twelve Standardbred mares (Lamprecht; unpublished data).

No effect of exercise or sample time on nitrite concentration in the joints was detected which may indicate that the mares were able to quickly adapt to the repeated arthrocentesis protocol and the rigorous exercise. Repetitive arthrocentesis has been previously reported (Van den Boom et al., 2005) to be a confounding variable when looking at certain inflammatory markers in SF. Conversely, other studies have demonstrated no effect of repeated SF sampling in inflammatory regulators such as prostaglandin E<sub>2</sub> (Lamprecht; unpublished data) and TNF $\alpha$  (Van den Boom et al., 2004).

The higher concentration of nitrite in the hock joints compared to the carpal joints may be attributable to conformational and biomechanical features unique to Standardbreds. A study by Grondahl and Dolvik (1993) reported that 14.3 % of 753 young Standardbred trotters were diagnosed with osteochondrosis in the tibiotarsal joint. In another equine study performed by Dutto et al. (2004), which compared forces generated from trotting on a level surface and on an incline in both fore- and hindlimbs, it was found that the horses trotting up an incline experienced more propulsive and vertical force on the hindlimb while the amount of force decreased for the forelimb. Perhaps the mechanical stress the chondrocytes within the hock joint undergo upregulates IL-1 $\beta$  which has been shown to increase NO synthesis (Bird et al., 1997; Fenton et al., 2000; Tung et al., 2002). Although NO is a known mediator of joint disease, its specific role as a pro-inflammatory agent or protective molecule remains controversial. Elevated basal levels of NO in the hock may serve as a protective mechanism. *In vitro* studies evaluating the role of NO in the joint have shown reduced proteoglycan depletion in an equine osteoarthritic explant culture model which was considered a protective effect (Stefanovic-Racic et al., 1994; Stefanovic-Racic et al., 1995). Furthermore chondrocytes, as compared to synoviocytes, have been demonstrated to have substantial basal levels of NO (Wiseman et al., 2003). Analysis of chondrocyte cytokine gene expression, and other markers of inflammation such as prostaglandin E2 and articular tissue turnover, would help elucidate the role of NO in joint inflammation and pathogenesis of joint disease in horses. Future studies including more frequent and/or additional sample times past 24 h REC in addition to measuring iNOS expression and evaluating urinary and respiratory output of nitrate as well as markers of oxidative stress and/or antioxidant status could provide valuable

information for future studies characterizing the NO response to exhaustive exercise in horses and its role in exercise induced inflammation and oxidative stress.

## CONCLUSION

In conclusion, the RSET was found to be the most rigorous exercise test when compared to the GXT and IET. The data presented here indicate that the  $\text{TNF}\alpha$ , IL-6, IL- $1\beta$  responses to acute intense exercise are indeed similar to the initiation of an acute phase response, however it is not characteristic of a full blown systemic response. It is unlikely that the mares experienced other biological effects of these pro-inflammatory mediators such as organ dysfunction, characteristic of acute phase inflammation. Furthermore, it appears that the RSET was the only treadmill test that rendered the horse incapable of mounting a sufficient response to meet an increased demand for NO due to the high intensity of the test. The higher concentration of NO in the hocks vs. the carpal joints was an interesting yet unexpected finding warranting further investigation and the use of gait and force plate analysis to evaluate potential differences in the stress placed on different joints during strenuous exercise in horses. By investigating physiological changes in relationship to pro-inflammatory cytokine transcripts and other markers of inflammation, imbalance in the physiological response can be recognized allowing for early detection of injury or a chronic inflammatory state, often caused by overtraining in elite athletes. These data may be useful in validating an exercise-induced inflammation model in horses providing a valuable tool for investigating environmental and physiological factors, novel therapies, and management practices that may be integral in inducing or inhibiting inflammatory processes in the equine athlete. This 'natural' model

may be a more practical alternative to pharmaceutical or surgical intervention to study inflammation in horses.

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## TABLES AND FIGURES

Horse	Week 1 test	Week 2 test	Week 3 test
1	GXT	RSET	IET
2	GXT	RSET	IET
3	GXT	IET	RSET
4	RSET	GXT	IET

*Table 1.* Order of treadmill exercise tests including the graded exercise test (GXT), interval exercise test (IET) and the repeated sprints exercise test (RSET), for each individual horse. Both order of run and order of test were fully randomized and there were 7 d between each exercise test.

<b><u>Primer Sequence:</u></b>			<b><u>PCR Amplification Fragment Size (bp)<sup>a</sup></u></b>
<b><u>Cytokine Amplicon</u></b>	<b><u>FORWARD PRIMER</u></b>	<b><u>REVERSE PRIMER</u></b>	
		<b><u>Probe Sequence</u></b>	
	GCTCATCTGGAACCTTGCTGATTTT		1563-1587
			1625-1647
$\beta$ -Gus	CTGACGAGTGAAGATCCCCTTT	FAM CTCTCTGCGGTGACTGG NFQ	85
	TTACCGAATGCCTTCCAGTCAAT		1073-1095
TNF $\alpha$	GGGCTACAGGCTTGTCACCT	FAM CCAGACACTCAGATCAT NFQ	1346-1325
	CCGACACCAGTGACATGATGA		274
			34-54
			98-74
IL-1 $\beta$	ATCCTCCTCAAAGAACAGGTCATTC	FAM ATTGCCGCTGCAGTAAG NFQ	65
	AGCAGCACCAGCAAGCT		319-335
			393-375
IFN $\gamma$	TTTGCGCTGGACCTTCAGA	FAM ATTCAGATTCCGGTAAATGA NFQ	75
	GGATGCTTCCAATCTGGGTTCAT		289-312
			353-331
IL-6	TCCGAAAGACCAGTGGTGATTTT	FAM ATCAGGCAGGTCTCCTG NFQ	65
	AGGACCAGCTGGACAACATG		173-232
			271-247
IL-10	GGTAAACTGGATCATCTCCGACAA	FAM CCAGGTAACCTTAAAGTC NFQ	99

*Table 2.* Equine cytokine primer and probe sequences and PCR amplification fragment size. <sup>a</sup>bp - base pairs

Exercise Test Comparisons	Exercise Test Duration (min)	Time (min) spent at or above 60 % $HR_{Max}$	Time (min) spent at or above 90 % $HR_{Max}$
<b>GXT</b>	9.6 ± 0.3	8.8 ± 0.8	5.0 ± 0.6
<b>IET</b>	18.3 ± 0.4	18.0 ± 0.6	6.0 ± 2.0
<b>RSET</b>	20.6 ± 0.4	17.0 ± 1.4	7.5 ± 0.5

*Table 3.* Comparison of exercise test duration (min), time (min) spent at or above either 60 or 90 % maximal heart rate for the graded exercise test (GXT), interval exercise test (IET) and the repeated sprints exercise test (RSET). Data are presented as means ± SE (n = 4).

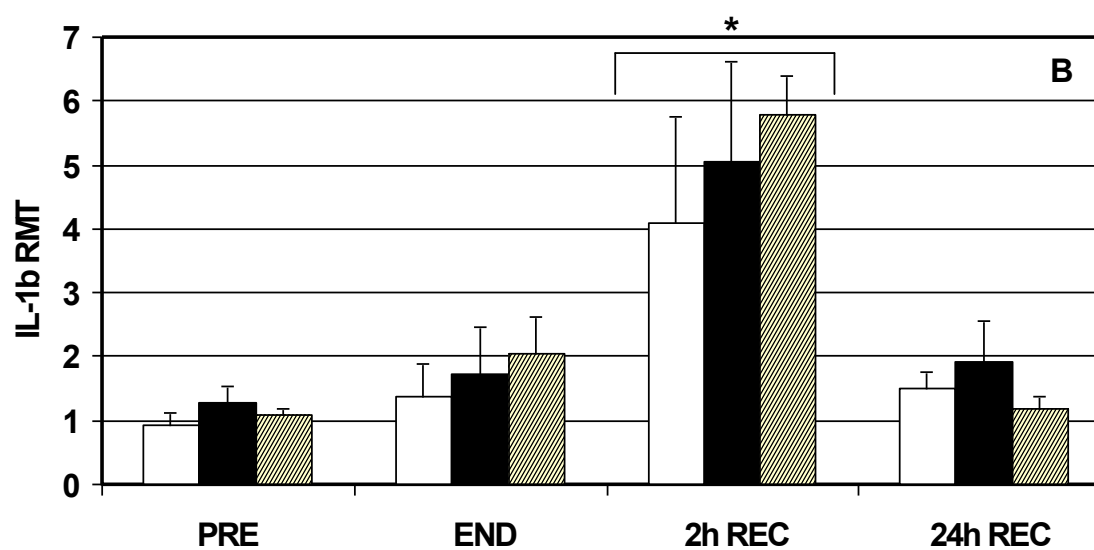
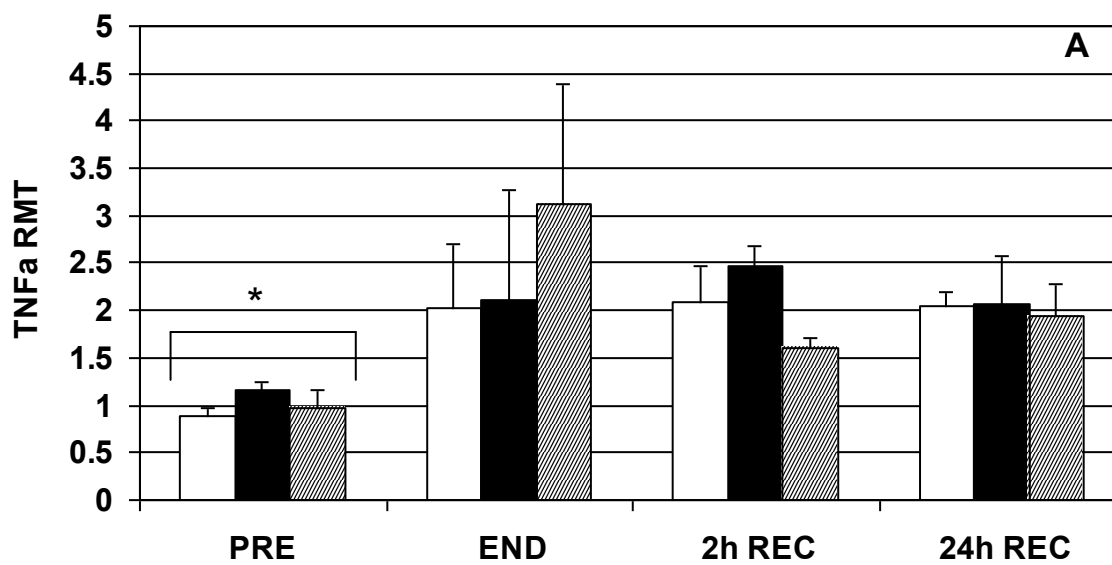
Rectal Temperature,		
°C	Before Exercise	After Exercise
<b>GXT</b>	37.2 ± 0.2 <sup>a</sup>	38.90 ± 0.2 <sup>b</sup>
<b>IET</b>	37.3 ± 0.1 <sup>a</sup>	40.1 ± 0.5 <sup>b</sup>
<b>RSET</b>	37.3 ± 0.1 <sup>a</sup>	40.0 ± 0.5 <sup>b</sup>

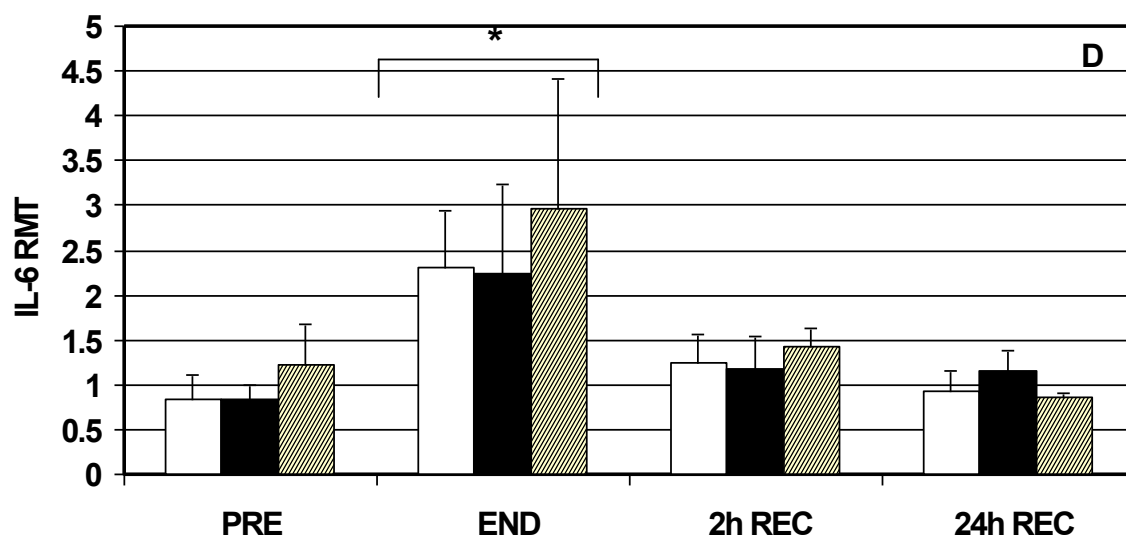
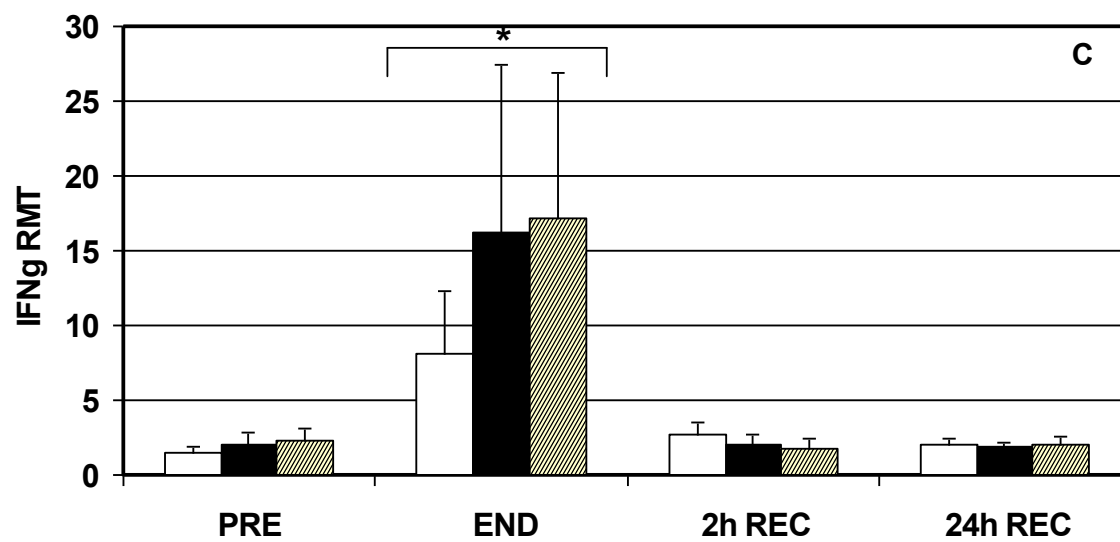
*Table 4.* Comparison of rectal temperature (°C), before and after exercise for a graded exercise test (GXT), interval exercise test (IET) and the repeated sprints exercise test (RSET). Data are presented as means ± SE (n = 4). Values with different superscripts are significant at  $P < 0.05$ .

<b>Haematocrit,</b>				
<b>%</b>	<b>PRE</b>	<b>END</b>	<b>2h REC</b>	<b>24h REC</b>
<b>GXT</b>	37.0 ± 1.8 <sup>a</sup>	46.5 ± 1.3 <sup>b</sup>	39.8 ± 1.2 <sup>a</sup>	37.6 ± 2.7 <sup>a</sup>
<b>IET</b>	37.0 ± 3.2 <sup>a</sup>	45.5 ± 2.0 <sup>b</sup>	37.5 ± 2.4 <sup>a</sup>	37.5 ± 1.8 <sup>a</sup>
<b>RSET</b>	37.0 ± 2.1 <sup>a</sup>	45.4 ± 1.8 <sup>b</sup>	40.6 ± 1.6 <sup>a</sup>	37.5 ± 2.0 <sup>a</sup>
<b>Total Plasma</b>				
<b>Protein, g/dl</b>	<b>PRE</b>	<b>END</b>	<b>2h REC</b>	<b>24h REC</b>
<b>GXT</b>	6.1 ± 0.2 <sup>a</sup>	6.7 ± 0.2 <sup>b</sup>	6.4 ± 0.1 <sup>ab</sup>	6.6 ± 0.1 <sup>b</sup>
<b>IET</b>	6.5 ± 0.1 <sup>a</sup>	7.0 ± 0.3 <sup>b</sup>	6.5 ± 0.1 <sup>a</sup>	6.5 ± 0.1 <sup>a</sup>
<b>RSET</b>	6.3 ± 0.1 <sup>a</sup>	6.5 ± 0.1 <sup>a</sup>	6.5 ± 0.2 <sup>a</sup>	6.5 ± 0.2 <sup>a</sup>

*Table 5.* Comparison of haematocrit (%) and plasma total protein (g dl<sup>-1</sup>), before and after exercise for a graded exercise test (GXT), interval exercise test (IET) and the repeated sprints exercise test (RSET). Data are presented as means ± SE (n = 4).

Different superscripts differ at  $P < 0.001$ .





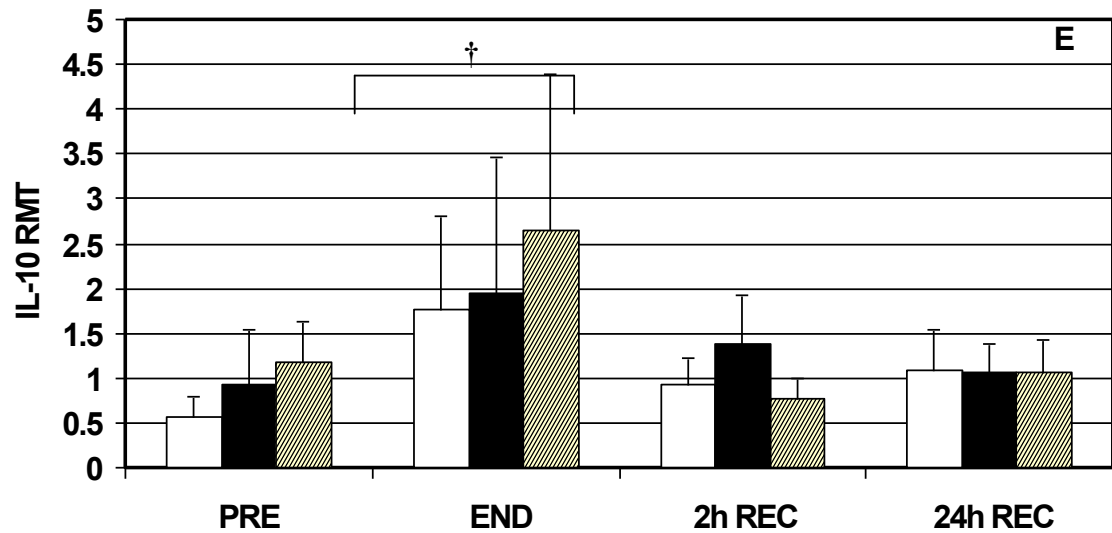
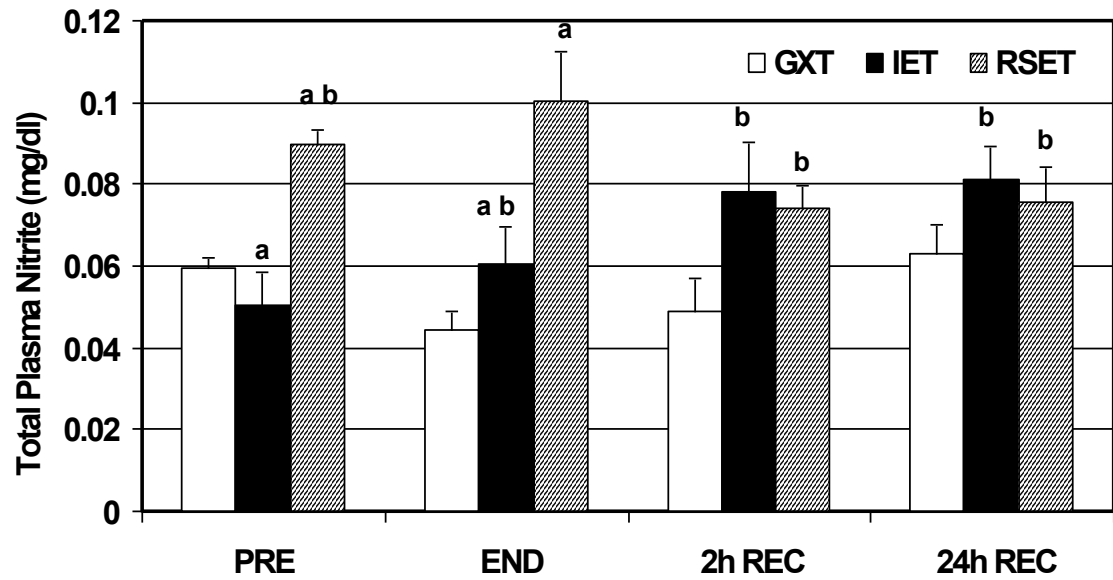
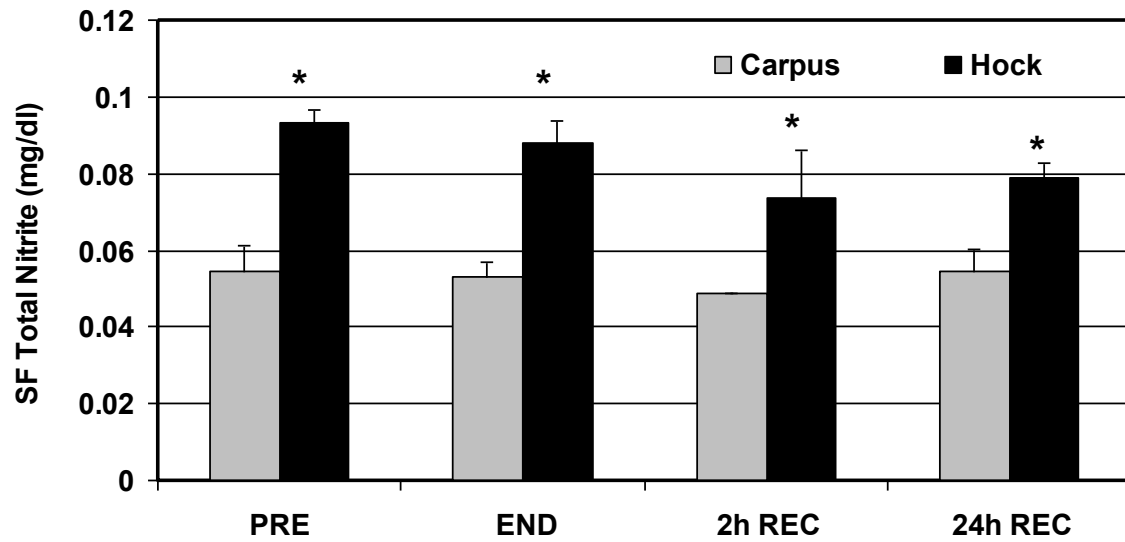


Figure 1 A - E. Tumor necrosis factor  $\alpha$  (TNF $\alpha$ ; A), Interleukin-1 $\beta$  (IL-1 $\beta$ ; B), Interferon  $\gamma$  (IFN $\gamma$ ; C), Interleukin-6 (IL-6; D), Interleukin-10 (IL-10; E) relative mRNA transcript (RMT) in peripheral blood of Standardbred mares before exercise (PRE), 20-30 min after exercise (END), and 2 h, 24 h of recovery (REC) for the graded exercise test (GXT; white bars), interval exercise test (IET; black bars) and the repeated sprints exercise test (RSET; hashed bars). qRT-PCR data are presented as the mean fold change in target gene expression. Bars represent mean  $\pm$  SE; an asterisk (\*) denotes differences between sample times at  $P < 0.05$  and an “†” denotes differences between sample times at  $P < 0.1$ .



*Figure 2.* Total plasma nitrite concentration ( $\text{mg dl}^{-1}$ ) in Standardbred mares before exercise (PRE), 20-30 min after exercise (END), and 2 h, 24 h of recovery (REC) for the graded exercise test (GXT), interval exercise test (IET) and the repeated sprints exercise test (RSET). Data are presented as the mean  $\pm$  SE ( $n = 4$ ). Different superscripts denote significant differences in sample time within exercise test at  $P < 0.05$ .



*Figure 3.* Synovial fluid total nitrite concentration ( $\text{mg dl}^{-1}$ ) in radiocarpal (carpus) and tibiotarsal (hock) joints of Standardbred mares before exercise (PRE), 20-30 min after exercise (END), 2 h, and 24 h of recovery (REC). Data are presented as the mean  $\pm$  SE of all three exercise tests combined. An asterisk (\*) denotes a significant difference between carpus and hock joint spaces within sample time ( $P < 0.0001$ ).

## Journal Article 2

### **Superoxide dismutase supplementation does not influence antioxidant status or systemic cytokine responses in an equine exercise model**

**ABSTRACT:** Both exercise and inflammatory processes have been shown to induce oxidative stress, which if left unresolved can result in chronic degenerative disorders. Supplementation of superoxide dismutase (SOD) has been shown to be beneficial in reducing oxidative stress and inflammation in various species however this has not been shown in horses. Objectives of this study were to evaluate effects of intense exhaustive exercise and exogenous SOD supplementation on markers of antioxidant status and inflammation in horses. Healthy unfit Standardbred mares ( $n = 12$ ) were used in this double blind, randomized crossover design. Horses received either treatment (TRT; 3 g/d of oral SOD powder with 3000 IU) or placebo (CON; 3 g/d cellulose powder) for 6 wks with a 6 wk wash-out between phases. Mares ran a repeated sprint exercise test on d 42 of each phase. Blood was collected before (PRE), at fatigue during exercise (PEAK), 30 min, 2 h, 4 h, 24 h, and 36 h post exercise (POST). Samples were analyzed for erythrocyte SOD activity, glutathione peroxidase activity (GPx), total glutathione (GSH-T), nitric oxide (NO), creatine kinase (CK) and cytokine transcripts (PRE, 30 min, 2 h, 24 h POST only) including tumor necrosis factor alpha (TNF $\alpha$ ), interferon gamma (IFN $\gamma$ ), interleukin-1 beta (IL-1 $\beta$ ), interleukin-6 (IL-6), and interleukin-10 (IL-10). Data were analyzed using ANOVA with repeated measures in SAS. No effect of SOD supplementation was detected. Exercise resulted in increased SOD ( $P = 0.002$ ), GSH-T ( $P < 0.01$ ), GPx ( $P < 0.01$ ), IFN $\gamma$  ( $P = 0.007$ ), IL-10 ( $P =$

0.007), and IL-1 $\beta$  ( $P = 0.0002$ ); however, NO ( $P < 0.01$ ) was decreased and there was no change in IL-6 or TNF $\alpha$  transcripts following exercise. Results suggest that the exercise did increase antioxidant defenses, and markers of inflammation, most likely due to an exercise-induced increase in tissue damage and oxidative stress. Additional research is needed to identify benefits of SOD supplementation in horses.

**Key Words:** Antioxidant, cytokines, equine, inflammation, oxidative stress, superoxide dismutase

## INTRODUCTION

Oxidative stress can be defined as an imbalance of reactive oxygen species (ROS) and antioxidant defenses in favor of oxidants (Urso and Clarkson, 2003). Reactive oxygen species can be defined as oxygen-containing molecules including oxygen-derived free radicals (molecules with an unpaired electron) and several non-radical derivatives, which are produced by oxidative reactions and are more reactive than atmospheric oxygen (Sjodin et al., 1990; Sen, 1995; Noguchi and Niki, 1999). The formation of ROS is not necessarily damaging or harmful, and in fact is necessary for several metabolic and immune functions. However, the overproduction of these ROS can result in damage to lipids, proteins, and genetic material which manifests itself as decreased physical performance (Clarkson, 1995), muscular fatigue (Friden and Lieber, 1992), muscle damage (Dekkers et al., 1996; Goldfarb, 1999), and overtraining syndrome (Tiidus, 1998). Physical activities, varying in intensity, have been shown to increase the

generation of ROS, including free radicals, in horses. This has been documented in horses engaging in various types and levels of exercise intensity including endurance horses (Marlin et al., 2002; Williams et al., 2004), Thoroughbred racehorses (Ishida et al., 1999; White et al., 2001), and Standardbred trotters (Kinnunen et al., 2005).

Several mechanisms are involved in the formation of ROS *in vivo*. With exercise comes a need for increased oxygen uptake to meet energy demands. Maximal oxygen uptake ( $\text{VO}_{2\text{max}}$ ) in horses can reach  $200 \text{ ml O}_2 \text{ kg}^{-1} \text{ min}^{-1}$  during exercise (Jones and Lindstedt, 1993; Young et al., 2002), an increase of about 30 times compared to that of oxygen intake (respiratory velocity) at rest (Butler et al., 1993). Although most of the oxygen consumed is converted to carbon dioxide and water, both products of the mitochondrial respiratory chain, an incomplete reduction of 1 to 2% of the oxygen results in the formation of ROS, specifically superoxide radicals ( $\text{O}_2^\bullet$ ) (Clarkson and Thompson, 2000). Prolonged exercise can result in ischemia and eventually a hypoxic state in athletes. Increased production of superoxide can also occur during ischemic muscular work as a result of xanthine oxidase-catalyzed degradation of hypoxanthine (Konig et al., 2001). Furthermore, exercise-induced muscle damage may result in the activation of polymorphoneutrophils (PMN) and subsequent oxidative burst which has been shown to increase ROS formation (Konig et al., 2001). Other mechanisms thought to result in the formation of ROS include the loss of calcium homeostasis in stressed myocytes, catecholamine auto-oxidation, and enhanced cytokine production with activation of nuclear factor- $\kappa\text{B}$  (NF- $\kappa\text{B}$ ; Konig et al., 2001; Urso and Clarkson, 2003).

Intense physical activity has also been shown to induce sub-clinical tissue damage and a subsequent immune response involving the up-regulation of inflammatory

mediators termed cytokines. Exercise-induced increases in cytokine transcripts are similar to that of an acute phase immune response and have been previously demonstrated in horses (Streltsova et al., 2006; Lamprecht et al., 2009). This immune response to intense exhaustive exercise may set the stage for a chronic inflammatory and/or an immunosuppressed state and a predisposition to infection, poor physical performance, and/or onset of chronic diseases (Shek et al., 1995; Brenner et al., 1999). Furthermore, inflammatory processes have also been associated with redox imbalances favoring pro-oxidants, resulting in oxidative stress (Lang et al., 2002). Consequently, efforts have been made to identify nutritional interventions which are effective in helping reduce exercise-induced oxidative stress, inflammation, and restoration of homeostatic conditions. The use of orally available antioxidant enzymes seems logical because they catalyze the detoxification of their ROS substrates in a disproportionate manner, instead of getting stoichiometrically consumed like exogenous antioxidant substrates, providing they can be easily and effectively administered and delivered to the target tissue without loss of enzymatic activity (Nelson et al., 2006).

Superoxide dismutases (SOD) are enzymatic antioxidant defenses which catalyze the dismutation of superoxide ions into oxygen and hydrogen peroxide. In addition to SOD antioxidant enzymes including glutathione peroxidase (GPx) and catalase help facilitate the breakdown of hydrogen peroxide into singlet oxygen and water preventing the formation of the damaging hydroxyl radical (Fridovich, 1975). It has been widely recognized that such enzymes provide a defense system, essential for the survival of aerobic organisms (Beyer, 1991). Both in chronic and acute inflammatory states, the increased production of the superoxide anion can surpass the endogenous SOD

scavenging capacity resulting in oxidative stress. Studies reporting the successful use of antioxidant enzymes as dietary interventions are increasing. Furthermore, there are several reports indicating that supplementation with exogenous SOD is effective in reducing pro-inflammatory cytokines and inhibiting neutrophil infiltration to sites of tissue damage in several inflammation models (Salvemini et al., 1999; Salvemini et al., 2001; Cuzzocrea et al., 2001; Masini et al., 2002). Oral formulations of SOD have been shown to be bioavailable and beneficial in other species (Vouldoukis et al., 2004b; Kick et al., 2007), however this has not been demonstrated in horses. Therefore the purpose of this study was to evaluate the effect(s) of oral SOD supplementation on inflammatory responses and antioxidant status in horses undergoing intense exhaustive exercise. It was hypothesized that supplementation with exogenous SOD would elicit a protective effect against exercise-induced inflammation and oxidative stress by reducing markers of inflammation and improving antioxidant status in horses.

## MATERIALS AND METHODS

### *Subjects*

A sample of 12 healthy, unfit Standardbred mares aged  $8 \pm 1$  yr, weighing  $513.8 \pm 16.7$  kg, with lameness scores of  $0.3 \pm 0.1$  (12), body condition scores of  $5.3 \pm 0.1$  (13) and per cent body fat at  $20.7 \pm 1.8$  % (14), were used in this study. Mares were housed in New Jersey at the Rutgers University Equine Agricultural Experiment Station Research Facility on 2 acre exercise lots and were provide *ad libitum* access to moderate quality grass hay, water and salt. They also received 1 kg of a 10 % crude protein sweet feed (Nutrena Vitality, Cargill Inc., Minneapolis, MN) twice daily to meet maintenance

requirements. The Rutgers University Institutional Animal Care and Use Review Board approved all methods and procedures used in this study.

### ***Design***

This study was conducted as a placebo controlled randomized crossover design. Mares were randomly assigned to either a treatment group (TRT) which received 3 g/d or 3000 IU of a proprietary oral formulation of SOD powder extracted from cantaloupe melon and chemically combined with wheat gliadin (vegetal hydrophobic biopolymer), or a placebo control group (CON) which received 3 g/d of an microcrystalline cellulose powder. Both TRT and CON groups received their supplement top dressed on their morning grain ration and all investigators were blind to the treatments. The duration of the study was 18 wks and started the last week in June and ended the first week in November, 2007. Phase-1 of the study consisted of a 42 d supplementation period which concluded with a repeated sprint exercise test (RSET) on d 42 previously determined to elicit an inflammatory and oxidative stress response in horses (Lamprecht et al., 2009). Mares then completed a 42 d washout period, the experimental groups were crossed over and an identical 42 d supplementation period (Phase-2) and RSET was completed. Before each phase of the study, standardized methods were used to evaluate each mare for body condition (Henneke et al., 1983), orthopedic soundness (AAEP, 1991), and percent body fat (Westervelt et al., 1976).

### ***Exercise test protocol***

All mares were habituated to Rutgers Equine Exercise Physiology Laboratory and

to running on the treadmill fixed at a 6 % grade (Sato II, Sato Treadmill, Knivsta, Sweden) prior to the start of the study. The RSETs were conducted on d 42 of Phase 1 and 2 of the study and on the day of the RSET, horses were fed their respective supplement 60 min before the start of the exercise test. Intravenous catheters (Angiocath™, Becton Dickinson, Franklin Lakes, NJ, USA) were also inserted percutaneously in to the left jugular using aseptic technique and local anesthesia (2 % lidocaine HCl inj., usp 20 mg/ml, Abbott Laboratories, Chicago, IL, USA). The catheters were kept patent with sterile heparinized (Heparin sodium, inj., USP 5000U/ml, American Pharmaceutical Partners Inc., Schaumburg, IL, USA) physiological saline solution at a working concentration of 10 U/ml. Immediately before exercise mares were fitted with a heart rate monitor (Polar Equine Heart Rate Monitor; FitMed Inc., Mill Valley, CA, USA), weighed on an electronic scale, and rectal temperature (RT) was taken. Resting heart rate (HR) as well as HR during exercise was recorded during the last 15 s of each step, and was monitored after exercise until recovery, and post exercise body weight and RT was determined. The RSET lasted an average of  $18.4 \pm 0.7$  min and began with 2.5 min of walking at 1.5 m/s followed by 4 min of trotting at 4 m/s. Following warm up, mares completed 2 min sprints at 7, 8, 9 and 10 m/s with 2 min of walk at 1.5 m/s between each sprint (Graham-Tiers et al., 2003; Wilson et al., 1998). Mares ran to fatigue or until the test was completed, at which time they spent 1 min walking at 1.5 m/s. Fatigue was defined as an inability of the mares to keep up with the treadmill despite humane encouragement.

### ***Sampling and analysis***

Venous blood samples were collected relative to the RSET via an indwelling jugular catheter and placed into pre-chilled 10 ml tubes containing sodium heparin, another 10 ml tube containing 10 ml tube containing ethylenediaminetetraacetic acid (EDTA), and serum separator tubes containing clot activator and gel for serum separation and serum separator tubes containing clot activator and gel for serum separation (Vacutainer, Becton Dickinson, Franklin Lakes, NJ, USA). Samples were taken before exercise (PRE), at maximal effort when the mares began to fatigue at the culmination of the RSET (PEAK), 30 min, 2 h, 4 h, 24 h, and 36 h post exercise (POST). Heparinized tubes were placed back on ice and immediately analyzed for packed cell volume (Hct) using microhematocrit technique (CritSpin<sup>®</sup> S-120, Iris Sample Processing, Westwood, MA, USA) and were centrifuged for 10 min at 1500 x g at 4 °C. Serum tubes sat at room temperature for approximately 1 h to allow for blood clotting, and were centrifuged for 20 min at 1500 x g at 10 °C. The plasma fraction was then analyzed for total protein (TP) using digital refractometry (Palm Abbe Veterinary Refractometer, MISCO Inc., Cleveland, Ohio, USA) and then plasma and serum were aliquoted and frozen at -80 °C for later analysis. Blood components were analyzed for plasma lactate (LA; YSI Sport<sup>™</sup> 1500, YSI Inc. Life Sciences, Yellow Springs, Ohio, USA; intraassay CV = 0.79 %; interassay CV = 0.72%), serum creatine kinase (CK; VetTest CK Slides; VetTest 8008 analyzer, IDEXX Laboratories Inc., Westbrook, MA, USA; intraassay CV = 1.39%; interassay CV = 1.29%), erythrocyte SOD activity (SOD Assay Kit–WST, Dojindo Molecular Technologies Inc., Rockville, MD, USA; interassay CV = 13.7%; Cybulski et al., 2009) relative to that of erythrocyte hemoglobin concentration (QuantiChrom<sup>™</sup>

Hemoglobin Assay Kit, BioAssay Systems, Hayward, CA, USA; Qin et al., 2007), erythrocyte GPx activity (OxisResearch™, Foster City, CA, USA; intraassay CV = 0.96%; Ursini et al., 1995), total erythrocyte glutathione (GSH-T; OxisResearch™, Foster City, CA, USA; intraassay CV = 1.12%; interassay CV = 1.17%; Richie et al., 1996), and plasma nitric oxide (NO; BioAssay Systems, Hayward, CA, USA; intraassay CV = 4.7%; interassay CV = 7.96%; Hasegawa et al., 2007). All assays were run according to manufacturer's instructions.

For assays utilizing erythrocyte lysate, 500 µl whole blood from a sodium heparin collection tube, was transferred 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 0.9% sodium chloride solution, thoroughly vortexed and centrifuged a second time as described above. The saline supernatant was carefully removed and discarded from the sample, and the remaining erythrocytes were lysed with 1 ml of ice-cold distilled deionized water. Erythrocyte lysate was aliquoted and immediately stored at -80°C for later analysis.

Blood was also collected by means of jugular puncture into PAXgene blood RNA collection tubes (Qiagen/Becton Dickinson, Valencia, CA, USA) containing quaternary amine surfactants at PRE, 30 min, 2 h, and 24 h POST sample times. Total RNA was collected from 2.5 ml of whole blood according to manufacturer's instructions and the RNA was quantified using a spectrophotometer (Biophotometer, Eppendorf, Westbury, NY, USA). The RNA purity and quantity in each extraction were found to be sufficient for gene expression analysis and amplification efficiencies were between 1.8 and 2.2. A detailed description of RNA preparation, reverse transcription, and amplification was

described previously (Vick et al., 2007). Relative quantification ( $2^{-\Delta\Delta CT}$  method) was used to analyze the changes in gene expression (Livak and Schmittgen, 2001; Ainsworth et al., 2003). Cytokine (target) gene expression was normalized to that of the endogenous control gene beta-glucuronidase ( $\beta$ -GUS) and fold changes in target gene expression were calculated relative to a calibrator sample (mean resting baseline) within each data set. Amplification efficiency variation was taken into account and corrected for using the LinReg 7.0 software (Ramakers et al., 2003). The following cytokine transcripts were measured: tumor necrosis factor alpha (TNF $\alpha$ ), interleukin-1 beta (IL-1 $\beta$ ; pro-inflammatory), interferon gamma (IFN $\gamma$ ; immunomodulatory), interleukin-6 (IL-6; multifunctional), and interleukin-10 (IL-10 anti-inflammatory). Equine cytokine primer and probe sequences and PCR amplicon fragment sizes were previously published (Lamprecht et al., 2009).

### ***Statistics***

Cytokine data are presented as relative mRNA transcripts (RMT) or the mean fold changes in target gene expression normalized to an endogenous control gene ( $\beta$ -GUS) and relative to that of a calibrator sample (resting, pre-supplementation baseline) in response to intense exercise  $\pm$  SE. All other data are summarized as the mean  $\pm$  SE. Data were analyzed using a MIXED model ANOVA with repeated measures in SAS 9.1 to evaluate effects of SOD treatment, acute exercise (as sample time), experimental phase, and their interactions. Horse was nested within treatment as the subject, and sample time was designated as the repeated effect. The Satterthwaite approximation of standard errors was utilized to account for any unequal variances. Significant main

effects were further analyzed using Tukey-Kramer post hoc analysis to further elucidate significant changes as inferred when  $P < 0.05$ . Pearson's product moment correlation was used to test for associations between the variables measured and only significant associations were reported.

## RESULTS

A main effect of exercise ( $P < 0.0001$ ) was detected for Hct, TP, LA, and CK, where maximal values occurred at PEAK during exercise (Table 1) however, there were no differences ( $P > 0.05$ ) between treatment groups for any of these parameters. Time to fatigue during the RSET was  $17.5 \pm 0.8$  min for CON and  $17.3 \pm 0.6$  min for TRT. There was a  $5.6 \pm 0.4$  % and a  $6.1 \pm 0.2$  % increase in RT as well as a  $1.9 \pm 0.2$  % and a  $1.6 \pm 0.2$  % decrease in body weight from pre- to post-exercise for CON and TRT groups, respectively.

There were no differences between the TRT and CON groups for any of the parameters measured. The RSET did influence SOD activity ( $P = 0.002$ ), GPx activity ( $P < 0.0001$ ), GSH-T ( $P < 0.0001$ ), NO ( $P = 0.0002$ ), IFN $\gamma$  ( $P = 0.007$ ), IL-10 ( $P = 0.007$ ), and IL-1 $\beta$  ( $P = 0.0002$ ) transcripts; however, there was no effect of sample time on IL-6 or TNF $\alpha$  gene expression ( $P > 0.05$ ).

Erythrocyte SOD activity increased from PRE to PEAK ( $P = 0.001$ ) followed by a decrease ( $P = 0.006$ ) to pre-exercise activity at 30 min POST (Figure 1-A). Erythrocyte GPx activity was higher ( $P < 0.01$ ; Figure 1-B) at PEAK when compared to other time points, and returned to pre-exercise activity by 30 min POST. Similar to GPx, erythrocyte GSH-T was also highest ( $P < 0.001$ ; Figure 1-C) at PEAK compared to other

samples times, and gradually returned to pre-exercise values by 4 h POST. Plasma NO decreased ( $P = 0.002$ ; Figure 1-D) from 30 min POST to 24 h POST and returned to PRE values at 36 h POST.

Interferon gamma transcripts increased ( $P = 0.03$ ; Figure 2-A) from PRE to PEAK and decreased ( $P = 0.01$ ) to pre-exercise levels at 2 h POST. Gene expression for IL-1 $\beta$  was higher at 2 h POST ( $P < 0.005$ ; Figure 2-B) when compared to PRE, 30 min and 24 h POST samples. Transcripts for IL-10 also tended to increase ( $P = 0.08$ ; Figure 2-C) from PRE to PEAK and then decreased ( $P = 0.005$ ) from PEAK to 24 h POST returning to pre-exercise values.

Main effect of experimental phase was also detected for total protein ( $P = 0.0001$ ), GPx ( $P = 0.003$ ), GSH-T ( $P = 0.01$ ), NO ( $P = 0.0043$ ), IFN $\gamma$  ( $P = 0.02$ ), and IL-10 ( $P = 0.02$ ). When comparing Phase 1 to Phase 2, TP was higher ( $P = 0.0001$ ), GPx activity was lower ( $P = 0.003$ ; Figure 3-B), GSH-T was higher ( $P = 0.01$ ; Figure 3-C), NO was higher ( $P = 0.004$ ; Figure 3-D), IFN $\gamma$  was higher ( $P = 0.02$ ; Figure 4-A), and IL-10 was higher ( $P = 0.02$ ; Figure 4-C) during Phase 1. Correlations were detected for each parameter measured and can be found in Table 2.

## DISCUSSION

While there have been several studies that evaluated the efficacy of SOD supplementation in other species, this is the first study to investigate the effects of SOD supplementation on cytokine transcripts and antioxidant status in an equine exercise model.

### ***Effect of treatment***

It was hypothesized that SOD supplementation in horses would mirror the beneficial findings reported in the literature demonstrating oral bioavailability (Vouldoukis et al., 2004b; Arent et al., 2009) and subsequent benefits including anti-inflammatory (Vouldoukis et al., 2004a; Arent et al., 2007), and antioxidant effects (Chenal et al., 2006; Kick et al., 2007) reported in several other animal models. Data from a porcine ischemia reperfusion injury model (Kick et al., 2007) and a human hyperbaric oxygen oxidative stress model (Muth et al., 2004) suggested that supplementation of a vegetal preparation of SOD combined with a wheat-gliadin biopolymer (SOD/gliadin) is protective against oxidative DNA damage and decreases markers of lipid peroxidation in circulation.

Mice supplemented with SOD/gliadin for 28 d showed a rise in circulating antioxidant enzyme activity which was positively correlated with an increased erythrocyte resistance to oxidative induced hemolysis (Vouldoukis et al., 2004b). Supplemented mice also exhibited increases in hepatic antioxidant defenses which were correlated with a significant decrease in hepatocyte apoptosis in the presence of Sin-1 (peroxynitrite chemical donor) when compared to controls (Vouldoukis et al., 2004b). In another study, peritoneal macrophages activated by an intra-peritoneal injection of IFN $\gamma$ , collected from mice after a 28 day supplementation of SOD/gliadin, and stimulated with IgG<sub>1</sub>IC *ex vivo*, exhibited a reduction in TNF $\alpha$  production and elevated IL-10 production as well as decreased superoxide, NO, and peroxynitrite concentrations (Vouldoukis et al., 2004a). However, the present study failed to demonstrate similar cytokine modulation in horses following SOD supplementation.

Supplementation of gliadin/SOD in human soccer athletes has been shown to reduce oxidative stress. Specifically, a decrease in the magnitude of 8-iso PGF<sub>2</sub> $\alpha$  response was observed in the experimental group following a graded step-wise exhaustive treadmill exercise test (Arent et al., 2009). Similarly, college football athletes supplemented with a proprietary nutraceutical drink blend containing SOD, coenzyme Q10 and beta glucans during a 7 wk training period demonstrated anti-inflammatory (reduced IL-6), antioxidant (reduced 8-iso PGF<sub>2</sub> $\alpha$ ), and less muscle damage (decreased CK) both after acute and chronic bouts of exercise compared to unsupplemented controls (Arent et al., 2007). In patients with HIV or AIDs also demonstrating compromised antioxidant status, it was determined that SOD/Gliadin (1000 IU d<sup>-1</sup>) supplementation for 3 wks normalized the patients circulating SOD activity and total antioxidant status (TAS), demonstrating that oral supplementation with vegetal SOD supplement can improve systemic antioxidant defenses (Chenal et al., 2006). However, SOD supplementation failed to modulate any of the inflammatory, antioxidant, or performance parameters measured either before or after exercise in the present study.

Several factors may have contributed to the ineffectiveness of SOD supplementation on markers of inflammation and antioxidant status in the present study including length of supplementation period preceding the RSETs, bioavailability of the oral SOD formulation or failure of the gliadin delivery technology, SOD dosage rate, the unfit status of the experimental animals, the exercise modality employed, and the normal endogenous antioxidant status of the healthy mares.

The 42 d supplementation period may have been insufficient to detect increased erythrocyte SOD activity as a result of oral SOD supplementation. The average lifespan

of an erythrocyte is 155 d in horses (Carter et al., 1974) compared to 120 days in humans (Nelson et al., 2006) and 40 days in mice (VanPutten, 1958). Mature erythrocytes lack a nucleus and thus the ability to synthesize new enzymes once in circulation. Relative to the present study, any erythrocytes in circulation 113 days prior to supplementation would have been unaffected by SOD supplementation, therefore diluting out any treatment-related increases in enzyme activity in erythrocytes synthesized during the supplementation period in the current study. As SOD is found primarily in erythrocytes (Jadot et al., 1995), in order to have maximized the potential for 100% of circulating and stored erythrocytes to have elevated SOD activity resulting from supplementation, the supplementation duration would have to of been at least 155 days in horses, theoretically.

The current study failed to provide evidence that the oral SOD was bioavailable. The SOD may not have been effectively delivered to the target tissue, or the enzyme activity may have been compromised at some point in the digestive and or absorption processes. Historically speaking, intravenous administration of SOD was the most common route of delivery, but its therapeutic capabilities were limited due to its short half life of less than 30 min (Baret et al., 1984). Oral administration of SOD is a fairly new practice and the use of delivery technologies is a growing area of interest. The gliadin coating used in the present study is a hydrophobic gliadin-biopolymer demonstrated to be an effective carrier for oral delivery of active food ingredients, specifically lipophilic molecules (Arangoa et al., 2001). Gliadin biopolymer has been shown to enhance oral pharmacology of SOD (Dugas, 2002) delaying enzyme release thereby preserving the enzyme activity throughout the gastrointestinal (GI) tract as well as purportedly increasing intestinal permeability via increased zonulin release which

opens the enterocyte tight junctions (Clemente et al., 2003; Drago et al., 2006). It has been demonstrated that the SOD enzyme requires protection during GI transit (Vouldoukis et al., 2004b). Failure of SOD efficacy in the present study may be due to the gliadin biopolymer releasing the active ingredient at an inappropriate time during GI transit, rendering it incapable of being absorbed and inducing changes in circulating redox status. Arangoa (1999), demonstrated that Gliadin nanoparticles, rich in neutral and lipophilic residues which promote hydrogen bonding with mucosa and increased hydrophobic interactions with the biologic tissue (Arangoa et al., 2000), showed a high affinity of gliadin for upper GI (gastric) mucosa in an animal model. It was found that 60 min following an oral dose of Gliadin nanoparticles, 15% of the dose remained in the gastric portion of the GI tract and there was little evidence of the nanoparticles in lower portions of the GI tract (Arangoa et al., 2001). Therefore, it is speculated that the Glisodin/SOD did not reach the target site of absorption in the small intestine but instead was degraded in the stomach due to bioadhesion of the gliadin to the gastric mucosa in the present study. Unlike lipophilic molecules, enzyme activity (SOD) would be compromised if released in the gastric portion of the GI tract, and therefore would not be able to exert any biological effects. As usefulness of the gliadin delivery technology has been shown for delivery and absorption specifically of lipophilic molecules, it may not be appropriate for the delivery of enzymes at all, or enzyme supplementation specifically in horses. Another group looked at controlled drug delivery or the biodegradation of Gliadin in an *in vitro* dissolution test apparatus at 37°C and 0.1 M hydrochloric acid media. It was determined that after about 1 h in this environment, 50 % of the active ingredient had been released and after about 3.3 h, 85% of the active ingredient had been

released from the Gliadin, and so it was concluded that the Gliadin delivery technology had application in cases where the active ingredient requires protection from acidic gastric juices and slow release over time (Stella et al., 1995).

Subsequent studies evaluating the bioavailability of oral SOD in horses should include an investigation of the usefulness of gliadin biopolymer delivery technology and its bioadhesion and pharmacokinetic properties in horses. Alternative technologies designed specifically for the unique characteristics of the active supplement ingredient as well as unique features of the equine GI tract should be considered in order to avoid supplement degradation and to ensure the absorption of oral supplement formulations.

Failure of the oral SOD formulation to elicit increases in erythrocyte SOD activity may have been due to an insufficient dosing rate. In the present study 3000 IU d<sup>-1</sup> SOD was fed at an average rate of  $5.84 \pm 0.18$  IU kg bw<sup>-1</sup> d<sup>-1</sup> to the research mares. A similar SOD preparation was fed to pigs at rates of 0.316 and 1.2 IU kg bw<sup>-1</sup> d<sup>-1</sup> which allegedly resulted in 25% and 41% increases in plasma SOD activity, respectively (Dr. David Griffin, personal communication; Promutase 200 website). In dogs, SOD has been supplemented at the rate of 200 IU d<sup>-1</sup> (10 IU kg<sup>-1</sup>) for dogs weighing 20 kg or less, and 400 IU d<sup>-1</sup> in dogs weighing more than 20 kg (Dr. David Griffin, personal communication) and mice have received SOD supplement at the rate of 0.003 IU kg bw<sup>-1</sup> d<sup>-1</sup> (Vouldoukis et al., 2004b). Although the horses in the present study were fed at a substantially higher rate than the other species, this dose could be insufficient and future research in horses should investigate different supplementation rates in excess of 3000 IU d<sup>-1</sup>.

Effects of SOD on inflammation and antioxidant status following a single bout of exhaustive exercise in an unfit horse was a point of interest in the current study in order to avoid confounding factors associated with repeated or chronic exercise. Furthermore, this unfit model was thought to be more susceptible to tissue microdamage, inflammation, and oxidative stress resulting from the RSET compared to that of a physically fit model. This unfit model theoretically would have experienced the greatest benefit from SOD supplementation when compared to a fit, conditioned individual who might be better able to cope with exercise-induced ROS formation and inflammation, having experienced physiological adaptations to exercise training, as has been previously demonstrated in horses and humans (Powers et al., 1999; Kasapis and Thompson, 2005; Williams et al., 2008; Arent et al., 2009). Although an antioxidant drink blend containing SOD did result in a blunted oxidative stress response to maximal exercise in human soccer athletes following 20 d of primarily aerobic conditioning, this may not hold true for horses. Chronic exercise training may result in higher degree of oxidative stress and a greater need for antioxidant defenses when compared to acute exercise. Therefore, benefits from antioxidant supplementation may be more easily detected in a chronic exercise model. Endogenous antioxidant defenses may be sufficient to handle the magnitude of oxidant stress relative to a single intense bout of exercise therefore, exogenous antioxidant supplementation may be superfluous and supplemental benefits may be difficult to quantify in this exercise model. Furthermore, differences in degree of oxidant response have been demonstrated between aerobic and anaerobic exercise training models without regard to antioxidant supplementation. In one study, exercise modality did not have an effect on SOD activity although GPx activity was increased by

aerobic training (Selamoglu et al., 2000). Lipid peroxidation was lower in aerobically trained individuals compared to non-active controls and was similar to anaerobically trained individuals. Therefore, it was concluded that aerobic, and not anaerobic training, influences oxidant stress and antioxidant status to a greater degree. Similarly, no changes in erythrocyte SOD and GPx following an intense acute exercise bout were reported in a primarily anaerobic equine exercise model (12 min at 4 m s<sup>-1</sup> at an 11 % grade with 17 kg added weight; Ji et al., 1989). However, the horses did undergo extensive treadmill conditioning prior to the exercise test, which could have resulted in adaptation of the antioxidant system and no observable changes in these enzymes. The extent of exercise-induced tissue damage and inflammation may also influence the effectiveness of antioxidant supplements. One study demonstrated that antioxidant supplementation for 6 wks did not alter plasma levels of cytokines and C-reactive protein following marathon exercise where muscle damage was induced (Mastaloudis et al., 2004), whereas in a study using a human exercise model where exercise-induced muscle damage was not detected, antioxidant supplementation for 4 wks did attenuate elevated plasma levels of cytokines, C-reactive protein and cortisol following exercise (Fischer et al., 2004). It is possible that the unfit animals, having performed a considerable amount of anaerobic exercise during the RSET without having undergone exercise training, may not have been the best model to evaluate effects of exogenous SOD supplementation for the present study. Exercise modality, fitness status, and degree of exercise-induced tissue damage could influence the necessity and ability of exogenous SOD to exert clinical benefits, and should be taken into consideration for future studies evaluating SOD supplementation in horses.

### *Effect of exercise*

The RSET was strenuous enough to elicit increases in muscle enzymes, pro- and anti-inflammatory cytokine gene expression, SOD activity, GPx activity, GSH-T, and a reduction in NO concentration indicating that this exercise test was effective in eliciting inflammation, increases in antioxidant defenses, which also suggests increases in oxidative stress, although direct analysis of oxidative stress biomarkers was not performed. Normal increases in Hct and TP from PRE to PEAK samples were expected findings in the present study and are consistent with previous studies evaluating hematologic and plasma biochemical measurements in exercising horses (Szucsik et al., 2006; Williams et al., 2008). Compared to PRE values, Hct and TP concentration were higher at 24 h and 36 h POST indicating that the RSET was strenuous enough to negatively impact hydration status well after the cessation of exercise. The LA increase during and after exercise in the present study indicates that mares were working at a velocity consistently above their anaerobic threshold and in a state where the lactate efflux mechanism was saturated. This saturation may have resulted in an increased accumulation of lactic acid within skeletal muscle and a subsequent drop in pH (or increase in hydrogen ions) associated with the dissociation of lactic acid into lactate and hydrogen ions. This muscle acidosis has been reported to have detrimental effects on performance in humans (Roth, 1991) and may be associated with a decline in muscle ATP and delayed onset muscle soreness (Snow and Valberg, 1994). Supplementation with a nutraceutical blend containing SOD has been shown to be beneficial in humans, resulting in an improvement in the velocity at which lactate threshold is reached during intense exercise (Arent et al., 2009), however this was not seen in the present study.

High PEAK CK values in the present study are comparable to those of horses having engaged in racing and training regimes intense enough to induce overtraining syndrome (Padalino et al., 2007). Exercise-induced increases in CK in the present study may indicate increased myocyte membrane permeability (Harris, 1998), which was unattenuated by SOD supplementation. These data are similar to findings by Piercy and colleagues (2001), where supplementation of an antioxidant mixture for 3 wks in sled dogs did not attenuate increased plasma CK following exercise. Increases in CK have also been positively correlated with increases in ROS formation and subsequent increases in lipid peroxidation both in horses and sled dogs (Williams et al., 2004; Williams et al., 2005; Frankiewicz-Jozko and Szarska, 2000; Hinchcliff et al., 2000). Therefore, it is plausible that exercise-induced increases in ROS may influence myocyte membrane permeability permitting the muscle enzymes to enter circulation (McBride and Kraemer, 1999). The positive correlation between CK, an indicator of muscle membrane leakage and in extreme cases muscle damage, and pro-inflammatory cytokines may also be playing a role in the formation of ROS. It has been documented that pro-inflammatory cytokines can prime neutrophils to migrate to sites of inflammation where they initiate the release of ROS (Zhang et al., 2002) and therefore are considered important mediators of oxidative stress (Haddad and Harb, 2005). Creatine kinase was positively correlated with IFN $\gamma$  and IL-10 in the current study, suggesting that muscle damage resulting from exercise and increased ROS production coincides with a pro-inflammatory immune response to the tissue damage.

The RSET exercise modality did elicit an inflammatory and antioxidant response in the mares as evidenced by increases in antioxidant enzymes, GSH-T, NO and cytokine

transcripts. These physiological changes are most likely in response to incurred tissue microdamage and increased ROS generation during and following the intense, exhaustive exercise test. Several studies have demonstrated similar exercise-induced increases in antioxidant status or oxidative stress after a single intense bout of exercise both in humans (Hong et al., 2004; reviewed in Bloomer, 2008; Andersson et al., 2009) and horses (Mills et al., 1996; Chiaradia et al., 1998; White et al., 2001; Kinnunen et al., 2005; Williams et al., 2006; Donovan et al., 2007b; Williams et al., 2008), and following prolonged intense exercise in horses (Marlin et al., 2002; Williams et al., 2005). Unlike a graded exercise test or interval exercise, which are common exercise modalities used to evaluate physiological changes in horses, the RSET is longer in duration and resulted in mares spending  $36.1 \pm 4.6\%$  of the total test time or  $6.6 \pm 0.84$  min out of the 18.4 min total test time at or above 90% of their peak HR in the present study. One study demonstrated a weak correlation between LA and lipid hydroperoxide which may indicate that exercise intensity, in addition to the duration of exercise is a key determinant of oxidative stress (Williams et al., 2008). Furthermore, LA in the present study was positively correlated with CK, pro-inflammatory cytokine IFN $\gamma$  and TNF $\alpha$  transcripts, as well as the anti-inflammatory cytokine IL-10, which further demonstrates that type and intensity of exercise is a determinant of systemic inflammatory response. In contrast, longer duration submaximal exercise has not been reported to elicit exercise-induced oxidative stress in horses (Williams et al., 2004) and sled dogs (Hinchcliff et al., 2003). Furthermore, exercise intensity has been shown to influence oxidant responses and efficacy of SOD supplementation in exercising humans. A study by Hong et al. (2004), compared effects of SOD supplementation ( $1500 \text{ IU d}^{-1}$ ) for 4 wks on exercise-induced

LA release and total antioxidant status (TAS) in healthy human volunteers undergoing either moderate or severe exercise. Improvements in TAS and magnitude of LA response were only observed in the severe exercise group. These data reinforce the idea that exercise intensity in addition to exercise duration, has significant impact on the extent of oxidant stress induction, such that higher intensity results in more oxidant stress, which could also have a direct impact on the efficacy of an exogenous antioxidant supplement.

*Antioxidant response to exercise.* The authors speculate that increases in SOD, GPx, and GSH-T at PEAK are compensatory responses to exercise-induced oxidative stress in the current study. When anti-oxidant and pro-oxidant signals indicate that there is disequilibrium in the redox status in favor of pro-oxidants, this augments an inflammatory response (Haddad and Harb, 2005). Cytokines can in turn influence the redox equilibrium via upregulation of antioxidant enzymes and changes in reduced to oxidized glutathione (GSH/GSSH) shuttling and recycling (Chen et al., 1998). One of the mechanisms through which ROS are thought to incur tissue damage is in an ischemic state where cytokine-induced activation of xanthine oxidase results in neutrophil activation and adhesion through superoxide formation, resulting in the release of proteases and other ROS which in turn cause damage to tissues (Niess et al., 1999). Specifically, cytokines have been shown to increase Mn-SOD activity in treadmill exercised rats which was considered cardio-protective within the context of ischemic injury (Yamashita et al., 1999). Surprisingly, there were no correlations between SOD, cytokines or any other parameter in the current study which indicates upregulation of endogenous SOD is being mediated by an alternative mechanism in this case.

Alterations in SOD, GPx and GSH-T in response to exercise vary within the literature. With regard to the current study, GPx and GSH-T were positively correlated and increased as the mares were fatiguing at the end of the RSET. This increase in the antioxidant enzyme and its thiol substrate is in accordance with previous studies reporting similar increases in SOD, GPx, and GSH-T in horses (Frankiewicz-Jozko and Szarska, 2000; Williams et al., 2006), GSH-T and GPx in rat muscle (Ji and Fu, 1992) and GSH-T in human plasma (Sahlin et al., 1991) either during or following strenuous exercise. These elevations in antioxidant defenses indicate that the exercise stress increased ROS production. Specifically, the increase in SOD activity suggests increased generation of superoxide anions, and the increases in GPx and GSH-T indicate an increased production of hydrogen peroxide, most likely from the metabolism of superoxide by SOD, and therefore a need to eliminate these damaging peroxides via oxygenation of reduced glutathione, in the present study. Furthermore, GSH-T and CK were positively correlated in the present study, as has been reported previously by Williams et al. (2005), suggesting that increasing ROS formation may be altering muscle membrane integrity allowing for increases in circulating muscle enzymes and antioxidant defenses.

In contrast to the present study's findings, several other studies reported no changes in erythrocyte GPx or GSH-T (Kinnunen et al., 2005) relative to an acute exercise bout in horses, reduced erythrocyte GPx activity, and no changes in SOD activity in trained Thoroughbred horses following intense exercise on a race track (1000 m at  $15 \text{ m s}^{-1}$ ; Ono et al., 1990). Decreases in GPx and GSH-T in endurance horses following an 80 km race (Hargreaves et al., 2002) and a 140 km race (Marlin et al., 2002) have also been reported as well as decreases in erythrocyte GPx with no changes in SOD

activity in conditioned sled dogs following repeated sub-maximal exercise bouts during a long distance race (Hinchcliff et al., 2003). It should be noted that the exercise modalities, exercise intensities, duration, and fitness status of the experimental models differ from study to study, and comparison between studies should be made with this in mind. Interestingly, in a majority of the studies that did not report changes in antioxidant defenses, the experimental subjects were exercise trained for their respective exercise test, indicating that antioxidant systems most likely adapt under conditions of regular training (Niess et al., 1999).

*Nitric oxide response to exercise.* The decrease in plasma NO concentration and up-regulation of cytokine transcript following exercise were similar to that of previous reports in horses undergoing a single bout of intense exhaustive exercise, with the exception of TNF $\alpha$  and IL-6 transcripts remaining unchanged in the present study (Donovan et al., 2007a; Lamprecht et al., 2009). The decline in NO is thought to be due to increased utilization, increased excretion, and decreased production or bioavailability due to increased oxidative stress. Plasma NO was positively correlated with pro-inflammatory cytokines IFN $\gamma$  and TNF $\alpha$ , and negatively correlated with GPx in the present study. The inverse relationship of NO to GPx may reflect the increase in ROS and subsequent decrease in NO bioavailability and an increase in GPx to counter the oxidative stress. Exercise-induced generation of superoxide and NO lends to the formation of peroxynitrite, an extremely damaging reactive nitrogen specie, therefore, reducing NO bioavailability (Sureida et al., 2006). The positive association of NO with pro-inflammatory cytokines most likely reflects the post-exercise decline in IFN $\gamma$  and NO

in the present study, indicating recovery from exercise-induced inflammation and increased utilization or depletion of NO. Evidence in the literature suggests that intense and prolonged exercise results in microdamage to tissues and a subsequent cytokine mediated neutrophilia, resulting in increased circulating neutrophils recruited from the marginated pool (Cannon et al., 1990; Cannon et al., 1991). This results in an increased capacity for ROS generation and subsequent tissue damage and repair (Tidball, 1995; Suzuki et al., 1996a; Suzuki et al., 1996b; Suzuki et al., 1999). Nitric oxide is considered an important mediator of inflammation and is thought to be integral in protecting muscle cells from inflammatory damage (Tidball, 2005). Specifically, muscle derived NO has been shown to decrease cytotoxicity by inhibiting NADPH oxidase (Clancy et al., 1992; Fujii et al., 1997), reacting with hydrogen peroxide (Gupta et al., 1997), and inhibiting neutrophil lysis of myocytes (Nguyen and Tidball, 2003). Nitric oxide has also been shown to reduce inflammation by inhibiting the expression of intracellular adhesion molecule (ICAM; Aljada et al., 2000), reducing AP-1 binding to ICAM promoter (Berendji et al., 2001), inhibiting expression of E-selectin (Liu et al., 1998), and P-selectin (Kosonen et al., 2000), as well as increasing inflammatory cell apoptosis (Albina et al., 1993; Blaylock et al., 1998; Ward et al., 2000). Nitric oxide is also a potent vasodilator which facilitates an increased blood supply to damaged tissues, enabling repair mechanisms (Wilson and Kapoor, 1993; Hirai et al., 1994; Hickner et al., 1997).

*Cytokine response to exercise.* Increases in cytokine transcripts at PEAK and following the RSET indicate a pro-inflammatory response to exercise and a counter anti-inflammatory (IL-10) response to prevent an exaggerated or chronic inflammatory state.

Similar increases in circulating cytokine transcript have been documented and attributed to post exercise endotoxemia (Baker et al., 1988; Barton et al., 2003). This response is similar, although generally not as severe, as to that seen in sepsis, again resulting in higher levels of circulating endotoxin, cytokines, leukocytes, and ROS (Brock-Utne et al., 1988; McCarthy and Dale, 1988; Maughn et al., 1989). It is probable that the horses in the current study did experience mild endotoxemia following the RSET, resulting in an acute phase response, as evidenced by the increase in cytokine transcripts although direct measurements of neutrophilia or circulating endotoxin were not directly quantified.

Positive correlations detected between the cytokines evaluated in the present study further illustrate the close relationships between these cytokines. For example, IFN $\gamma$  is considered a pro-inflammatory cytokine that serves to activate macrophages, augment the synthesis of other pro-inflammatory cytokines, and induce nitric oxide synthase and ROS formation (Moldoveanu et al., 2001; Ainsworth et al., 2003). The pro-inflammatory response also signals anti-inflammatory cytokines, such as IL-10, which is also thought to possess antioxidant properties. Interleukin-10 inhibits several cytokines including IFN $\gamma$ , IL-1 $\beta$ , TNF $\alpha$ , and IL-6, mainly by initiating the degradation of mRNA for these cytokines as well as the inhibition of antigen presentation (Haddad and Fahlman, 2002; Oberholzer et al., 2002) and release of ROS (Pezzilli et al., 1997). The inhibition of pro-inflammatory cytokines, NO production, and superoxide anion formation in endothelial tissue (Gunnnett et al., 2000), can have an impact on neutrophil priming for oxidative burst (Gougerot-Podicalo et al., 1996). Furthermore, antioxidants have been shown to upregulate IL-10 in antigen-IgE activated mast cells *in vitro* (Chen et al., 2000).

### *Effect of experimental phase*

Differences between experimental Phases for TP, GPx, GSH-T, NO, IFN $\gamma$ , and IL-10 were unexpected findings, and there are possible confounding variables that may have contributed to this finding. Great care was taken to control for all possible variables in this study, however the cross-over design resulted in an 18 wk study duration which started during the summer month of July and continued through the fall ending in November. The RSETs were conducted in an indoor climate controlled treadmill laboratory, however it was determined that the mean ambient temperatures during the RSETs for each Phase did vary (Phase 1,  $22.7 \pm 0.37$  °C with  $72.5 \pm 2.1$  % humidity vs. Phase 2,  $15.8 \pm 1.0$  °C with  $57.9 \pm 2.7$  % humidity) and could have influenced the inflammatory and antioxidant responses. A couple of studies have shown that higher ambient temperatures contribute to increased oxidative stress and lower antioxidant status in endurance horses immediately following exercise (Mills et al., 1996; Hargreaves et al., 2002). This was not however reflected in mean percent increase in RT from pre- to post-exercise samples for each Phase (Phase 1,  $6.0 \pm 0.4$  %; vs. Phase 2,  $5.8 \pm 0.3$  %). Differences in humidity may have resulted in greater sweat losses as is indirectly indicated by percent loss in body weight from pre- to post-exercise samples for each Phase. There was greater weight loss occurring in the summer (Phase 1) when it was warmer and more humid compared to Phase 2 (Phase 1,  $2.0 \pm 0.2$  % vs. Phase 2,  $1.5 \pm 0.1$  %). Increased sweat loss due to higher temperatures can result in higher plasma TP concentrations resulting from the fluid shift out of the vasculature into the interstitial space to facilitate evaporative cooling. Lastly, all experimental horses were mares, and

shifting hormonal profiles may have influenced biomarkers in the present study. Since the study spanned both estrous (summer) and senescent (fall) reproductive periods, differences due predominating hormones in the luteal (progesterone) and follicular (estradiole) phases of their reproductive cycle may have influenced the inflammatory and subsequently the antioxidant parameters measured during Phase 1 of the study and not during Phase 2. Future studies utilizing cycling mares should consider estrus synchronization or evaluating samples for reproductive hormones to account for potential confounding factors.

## CONCLUSION

In conclusion, fluctuations in markers of hydration status, muscle membrane integrity, inflammation, and antioxidant status relative to intense exhaustive exercise, were not altered by SOD supplementation in Standardbred mares in the present study. The RSET did result in a pro-inflammatory response as well as upregulated antioxidant defenses, which suggests that it is an appropriate exercise modality for investigating systemic inflammatory response syndrome and subsequent oxidative stress in unfit horses. The increase in cytokine transcripts, including  $\text{IFN}\gamma$ ,  $\text{IL-1}\beta$ , and  $\text{IL-10}$ , is a component of the exercise-induced inflammatory response believed to be intimately involved in the formation of ROS, although the possibility that ROS initiate cytokine responses must also be considered as these two pathways are not mutually exclusive. Further research is needed to elucidate benefit(s) of SOD supplementation and effective supplement delivery technologies in horses.

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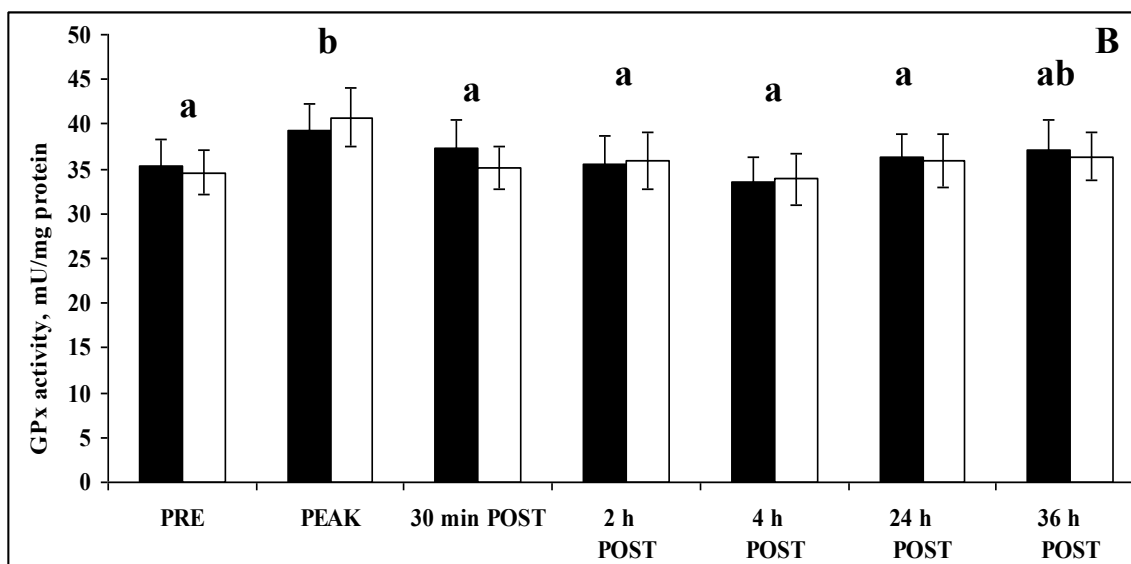
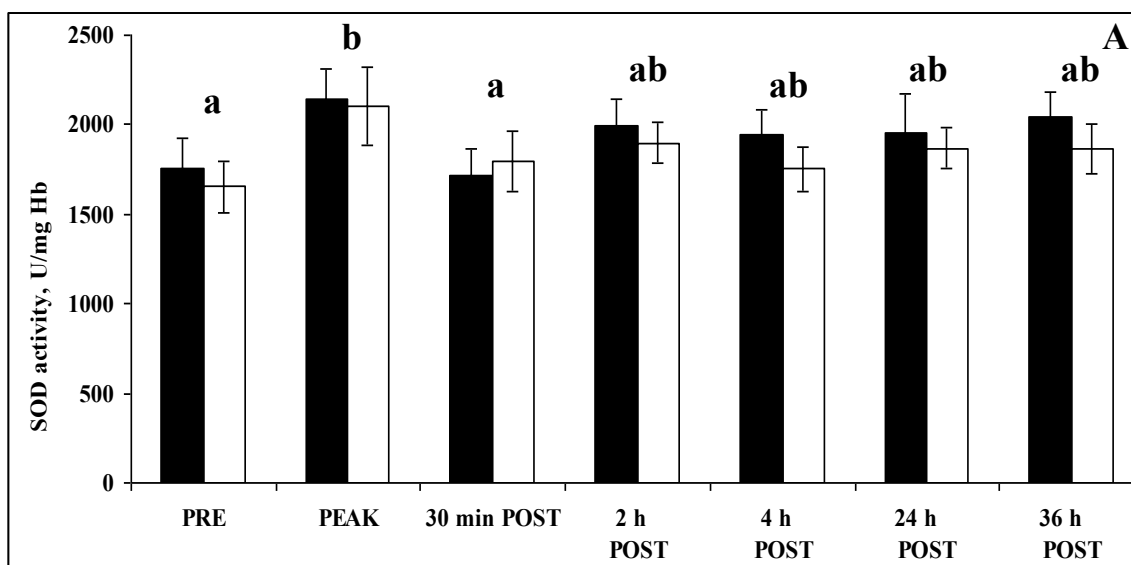
## TABLES AND FIGURES

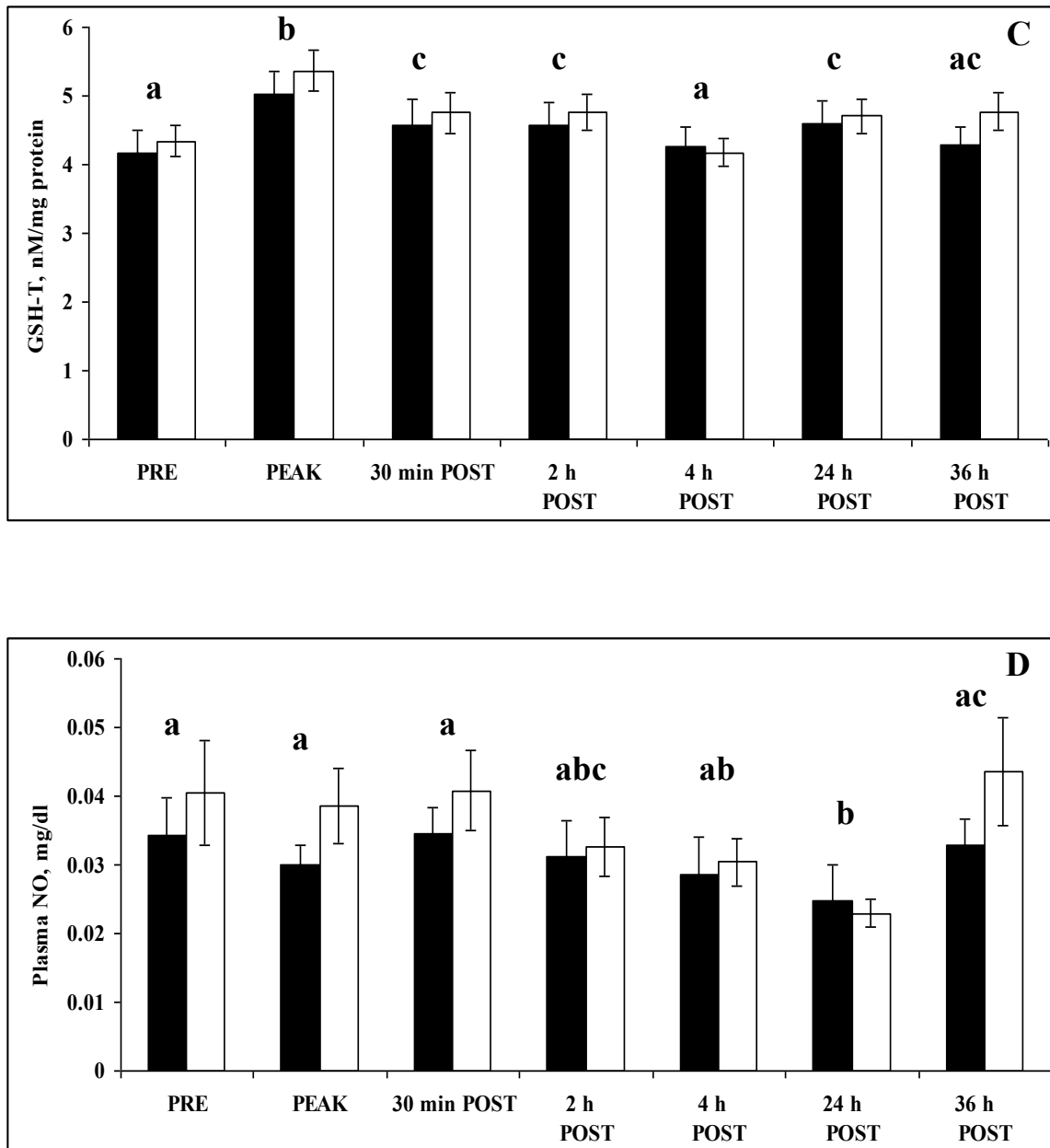
	<b>Pre</b>	<b>Peak</b>	<b>30 min Post</b>	<b>2 h Post</b>	<b>4 h Post</b>	<b>24 h Post</b>	<b>36 h Post</b>
<b>Hct, %</b>	36.5 ± 0.9 <sup>a</sup>	54.1 ± 1.2 <sup>b</sup>	45.2 ± 1.2 <sup>c</sup>	40.4 ± 1.1 <sup>d</sup>	35.4 ± 1.1 <sup>a</sup>	40.1 ± 1.2 <sup>d</sup>	40.1 ± 1.0 <sup>d</sup>
<b>TP, g/dl</b>	6.3 ± 0.1 <sup>a</sup>	7.5 ± 0.2 <sup>b</sup>	6.5 ± 0.1 <sup>c</sup>	6.3 ± 0.1 <sup>a</sup>	6.2 ± 0.1 <sup>a</sup>	6.6 ± 0.1 <sup>c</sup>	6.6 ± 0.1 <sup>c</sup>
<b>LA, mmol/L</b>	0.7 ± 0.1 <sup>a</sup>	23.4 ± 2.1 <sup>b</sup>	14.0 ± 2.1 <sup>c</sup>	2.2 ± 0.3 <sup>a</sup>	1.0 ± 0.1 <sup>a</sup>	0.7 ± 0.1 <sup>a</sup>	0.8 ± 0.1 <sup>a</sup>
<b>CK, U/L</b>	205.6 ± 15.0 <sup>a</sup>	276.7 ± 21.9 <sup>b</sup>	255.8 ± 20.6 <sup>c</sup>	254.3 ± 21.2 <sup>c</sup>	255.7 ± 22.3 <sup>c</sup>	206.3 ± 14.3 <sup>a</sup>	228.4 ± 18.1 <sup>d</sup>

*Table 1.* Hematocrit (Hct), plasma total protein (TP), plasma lactate (LA), serum creatine kinase (CK) concentrations before (PRE), during (PEAK), 30 min, 2 h, 4 h, 24 h, and 36 h after exercise (POST). There were no differences between treatment groups, therefore data have been combined and are presented as mean ± SE; different superscripts denote differences within rows at  $P < 0.05$ .

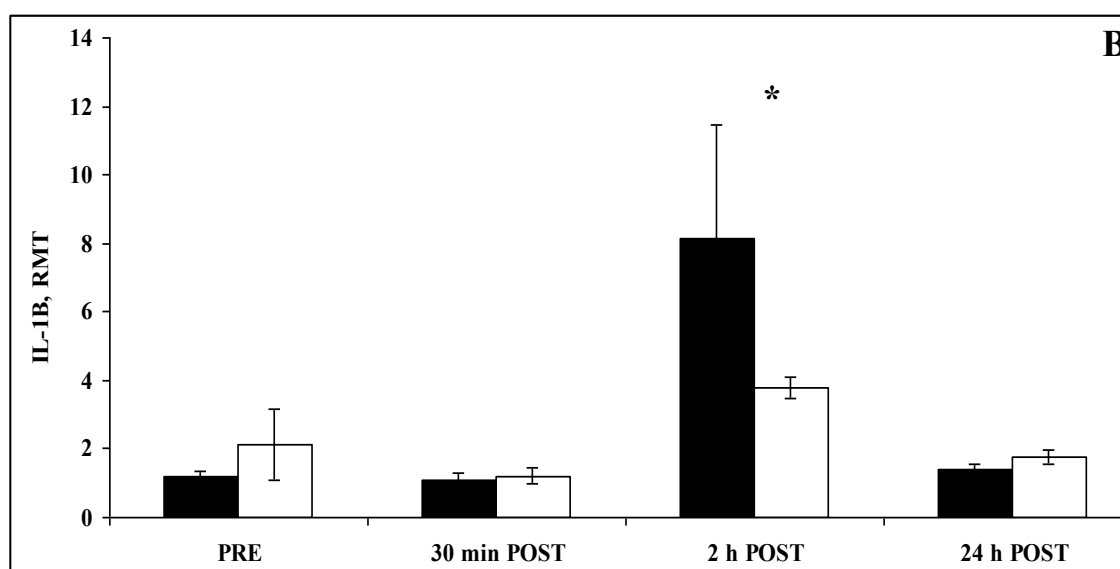
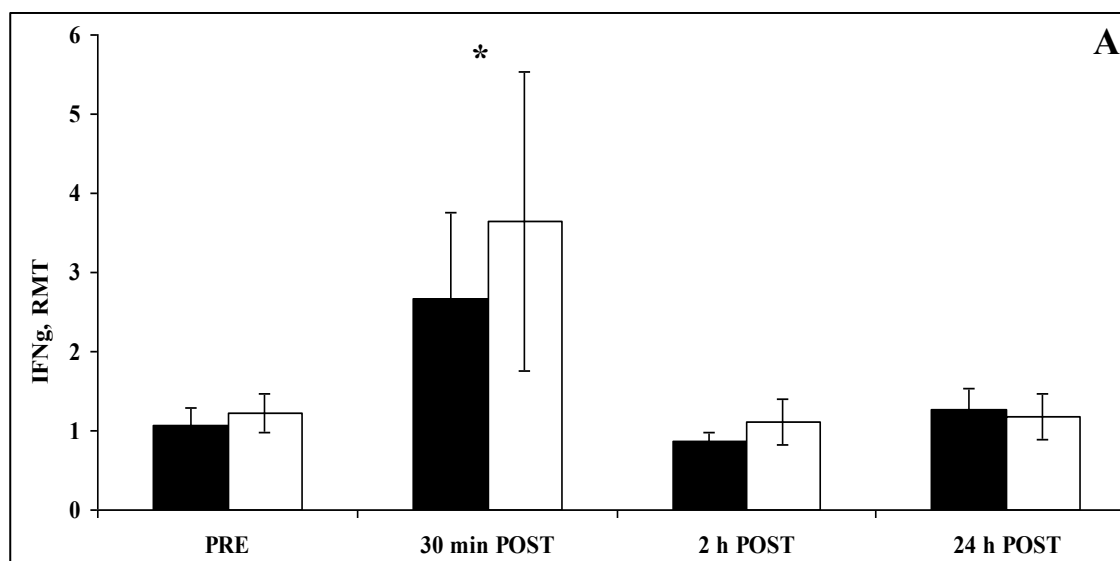
<b>Hct<sup>a</sup></b>	<b>TP<sup>a</sup></b>	<b>LA<sup>a</sup></b>	<b>CK<sup>b</sup></b>	<b>GPx<sup>c</sup></b>	<b>GSH-T<sup>c</sup></b>	<b>NO<sup>d,e</sup></b>	<b>IFN<math>\gamma</math><sup>e</sup></b>	<b>IL-1<math>\beta</math><sup>e</sup></b>	<b>IL-10<sup>e</sup></b>	<b>TNF<math>\alpha</math><sup>e</sup></b>	<b>IL-6<sup>e</sup></b>
TP	LA	Hct	Hct	TP	Hct	TP	Hct	IL-6	Hct	Hct	IL-1 $\beta$
r = 0.40	r = 0.24	r = 0.61	r = 0.48	r = -0.38	r = 0.29	r = 0.22	r = 0.50	r = 0.25	r = 0.37	r = 0.37	r = 0.25
<i>P</i> < 0.0001	<i>P</i> = 0.02	<i>P</i> < 0.0001	<i>P</i> < 0.0001	<i>P</i> = 0.0002	<i>P</i> = 0.004	<i>P</i> = 0.03	<i>P</i> < 0.0001	<i>P</i> = 0.02	<i>P</i> = 0.0002	<i>P</i> = 0.0002	<i>P</i> = 0.02
LA	GPx	TP	LA	NO	TP	GPx	TP	TNF $\alpha$	LA	LA	TNF $\alpha$
r = 0.61	R = -0.38	r = 0.24	r = 0.39	r = -0.22	r = 0.29	r = -0.22	r = 0.23	r = 0.33	r = 0.37	r = 0.29	r = 0.59
<i>P</i> < 0.0001	<i>P</i> = 0.0002	<i>P</i> = 0.02	<i>P</i> < 0.0001	<i>P</i> = 0.04	<i>P</i> = 0.004	<i>P</i> = 0.04	<i>P</i> = 0.03	<i>P</i> = 0.001	<i>P</i> = 0.0002	<i>P</i> = 0.004	<i>P</i> < 0.0001
CK	GSH	CK	GSH	GSH	CK	IFN $\gamma$	LA		CK	CK	
r = 0.48	R = 0.29	r = 0.39	r = 0.28	r = 0.21	r = 0.28	r = 0.31	r = 0.50		r = 0.33	r = 0.28	
<i>P</i> < 0.0001	<i>P</i> = 0.004	<i>P</i> < 0.0001	<i>P</i> = 0.006	<i>P</i> = 0.04	<i>P</i> = 0.006	<i>P</i> = 0.003	<i>P</i> < 0.0001		<i>P</i> = 0.001	<i>P</i> = 0.005	
GSH	IFN $\gamma$	IFN $\gamma$	IFN $\gamma$		GPx	TNF $\alpha$	CK		IFN $\gamma$	NO	
r = 0.29	r = 0.23	r = 0.50	r = 0.31		r = 0.21	r = 0.20	r = 0.31		r = 0.58	r = 0.20	
<i>P</i> = 0.004	<i>P</i> = 0.03	<i>P</i> < 0.001	<i>P</i> = 0.003		<i>P</i> = 0.04	<i>P</i> = 0.05	<i>P</i> = 0.003		<i>P</i> < 0.0001	<i>P</i> = 0.05	
IFN $\gamma$		IL-10	IL-10				NO		TNF $\alpha$	IFN $\gamma$	
r = 0.50		r = 0.37	r = 0.33				r = 0.31		r = 0.57	r = 0.57	
<i>P</i> < 0.0001		<i>P</i> = 0.0002	<i>P</i> = 0.001				<i>P</i> = 0.003		<i>P</i> < 0.0001	<i>P</i> < 0.0001	
IL-10		TNF $\alpha$	TNF $\alpha$				IL-10			IL-1 $\beta$	
r = 0.37		r = 0.29	r = 0.28				r = 0.58			r = 0.33	
<i>P</i> = 0.0002		<i>P</i> = 0.004	<i>P</i> = 0.005				<i>P</i> < 0.0001			<i>P</i> = 0.001	
TNF $\alpha$							TNF $\alpha$			IL-10	
r = 0.37							r = 0.57			r = 0.57	
<i>P</i> = 0.0002							<i>P</i> < 0.0001			<i>P</i> < 0.0001	
									IL-6		
									r = 0.59		
									<i>P</i> < 0.0001		

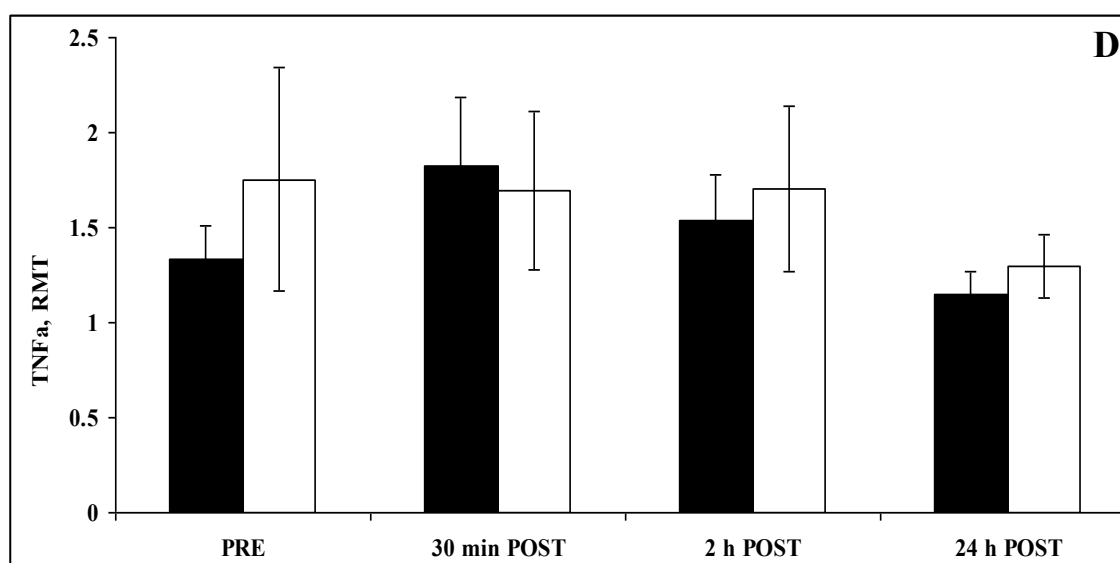
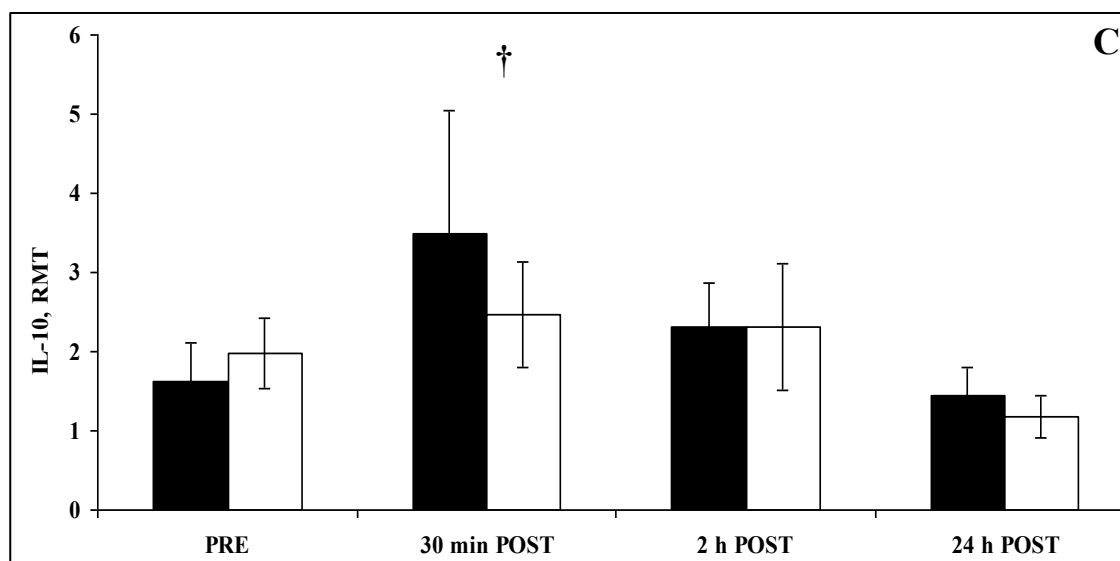
*Table 2.* Correlations of indices of performance<sup>a</sup>, muscle membrane leakage<sup>b</sup>, antioxidant status<sup>c</sup>, oxidative stress<sup>d</sup>, and inflammation<sup>e</sup> for hematocrit (Hct), plasma total protein (TP), plasma lactate (LA), serum creatine kinase (CK), erythrocyte glutathione peroxidase (GPx), erythrocyte total glutathione (GSH-T), plasma total nitrite (NO), interferon gamma (IFN $\gamma$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ), interleukin-10 (IL-10), tumor necrosis factor- $\alpha$  (TNF $\alpha$ ), and interleukin-6 (IL-6) transcripts. Associations were detected between each biomarker heading (bolded) and those indices within their respective columns.





*Figure 1, A - D.* Erythrocyte superoxide dismutase activity (SOD; A), glutathione peroxidase activity (GPx; B), total glutathione (GSH-T; C), and plasma nitric oxide (NO; D) before (PRE), during (PEAK), 30 min, 2 h, 4 h, 24 h, and 36 h after exercise (POST). Solid bars represent TRT, and clear bars CON groups. Data are presented as mean  $\pm$  SE; means without a common superscript differ ( $P < 0.05$ ) between sample times.





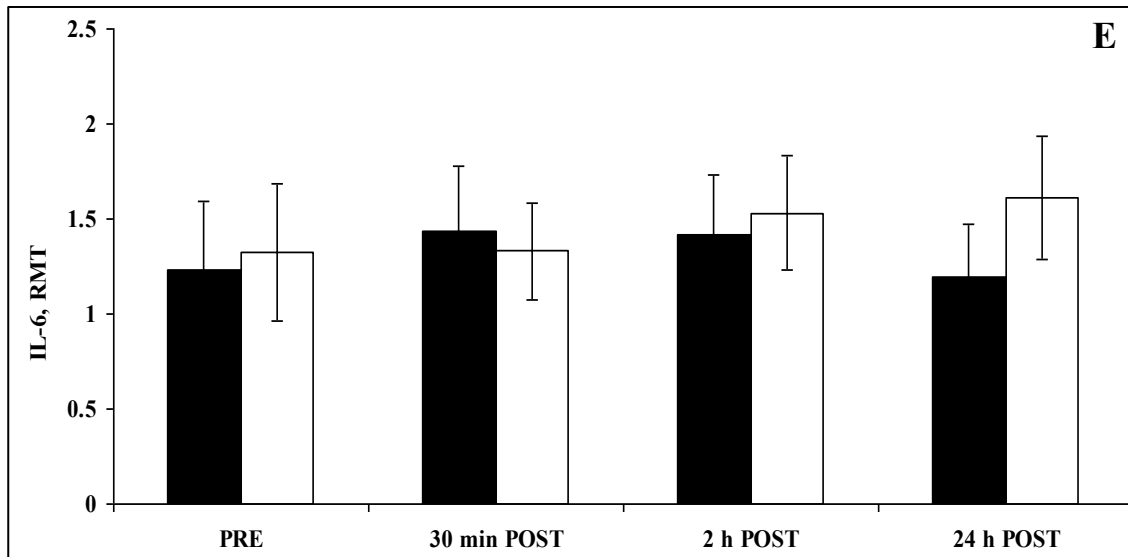
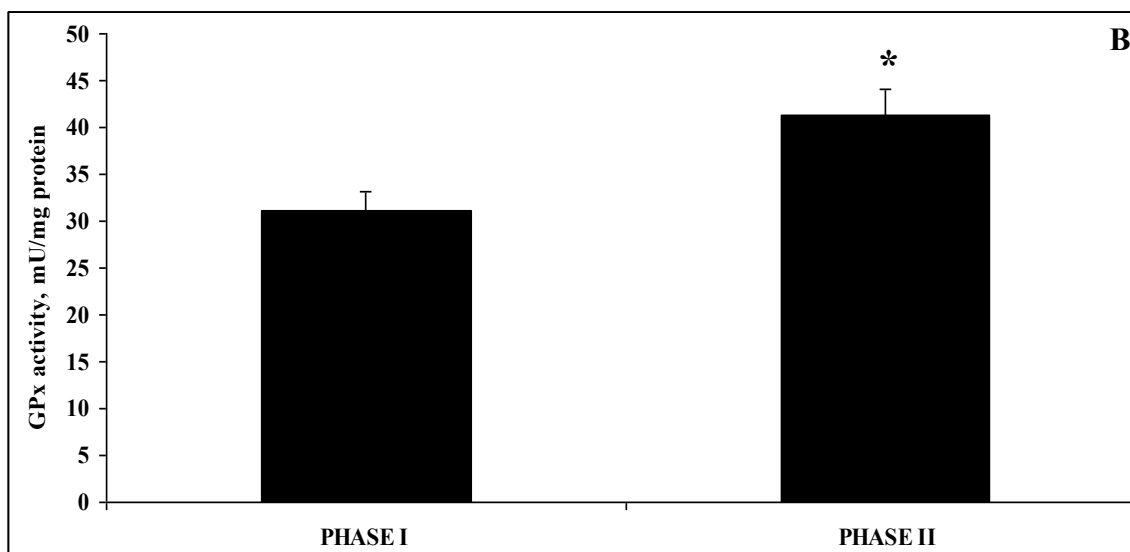
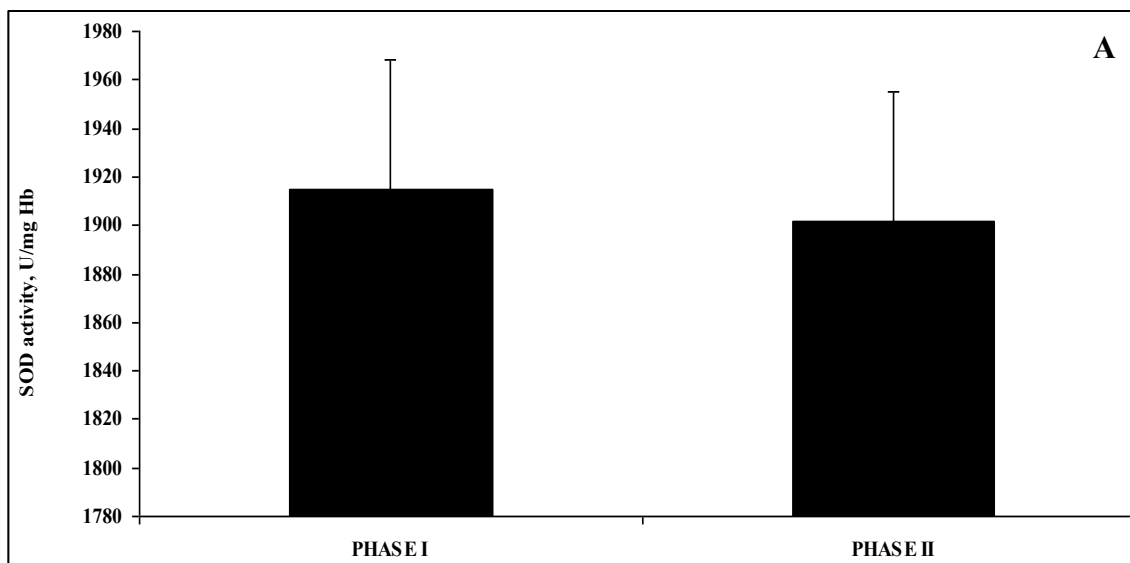


Figure 2, A - E. Interferon  $\gamma$  (IFN $\gamma$ ; A), interleukin-1 $\beta$  (IL-1 $\beta$ ; B), interleukin-10 (IL-10; C), tumor necrosis factor  $\alpha$  (TNF $\alpha$ ; D), interleukin-6 (IL-6; E), relative mRNA transcript (RMT) in peripheral blood of Standardbred mares before exercise (PRE), 30 min after exercise (POST), 2 h, and 24 h POST. Solid bars represent TRT, and clear bars represent CON groups. qRT-PCR data are presented as the mean fold change in target gene expression. Data are presented as mean  $\pm$  SE; an asterisk (\*) denotes differences between sample times at  $P < 0.05$  and an “†” denotes differences between sample times at  $P < 0.1$ .



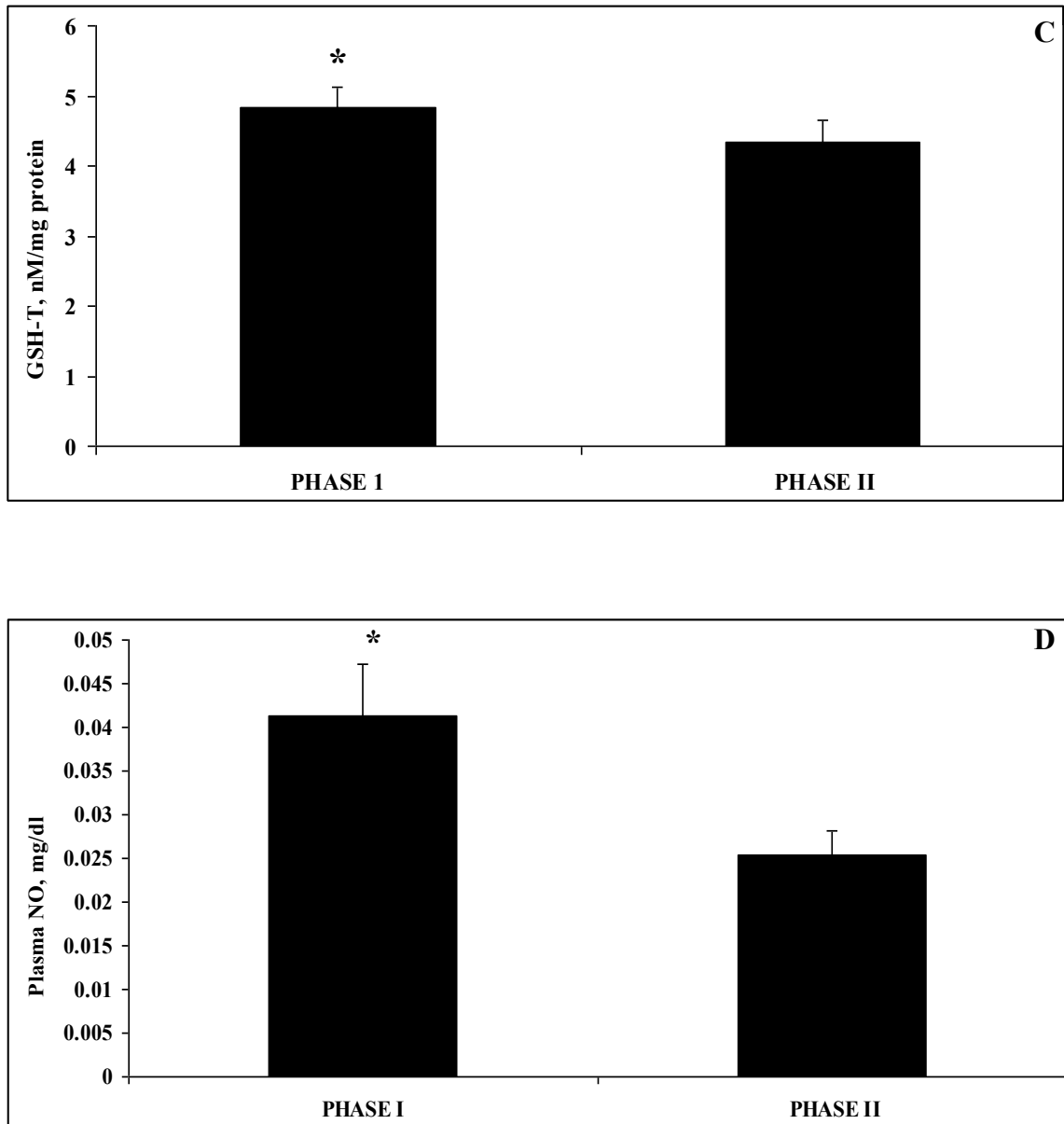
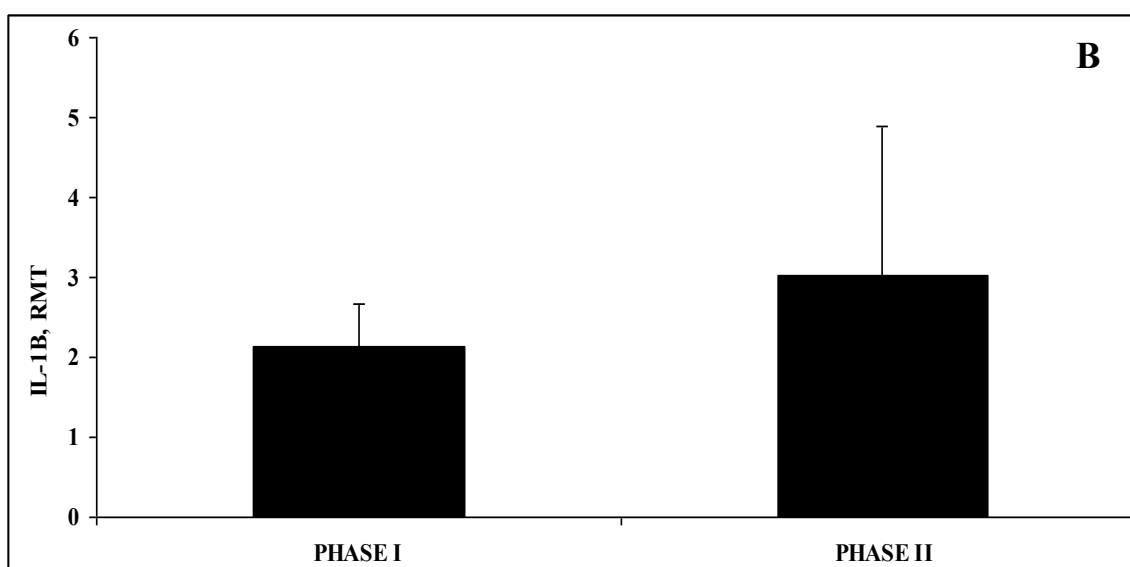
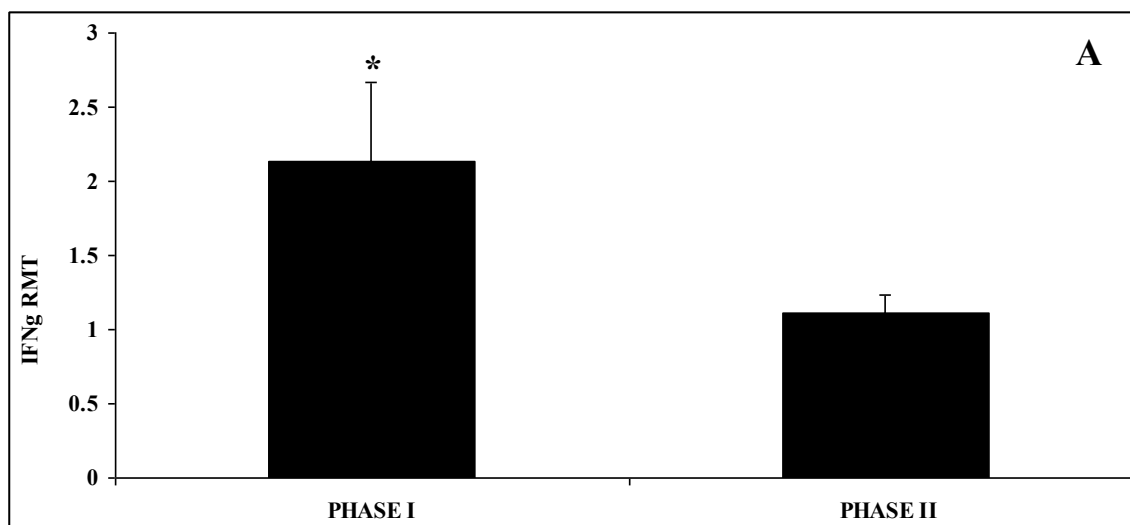
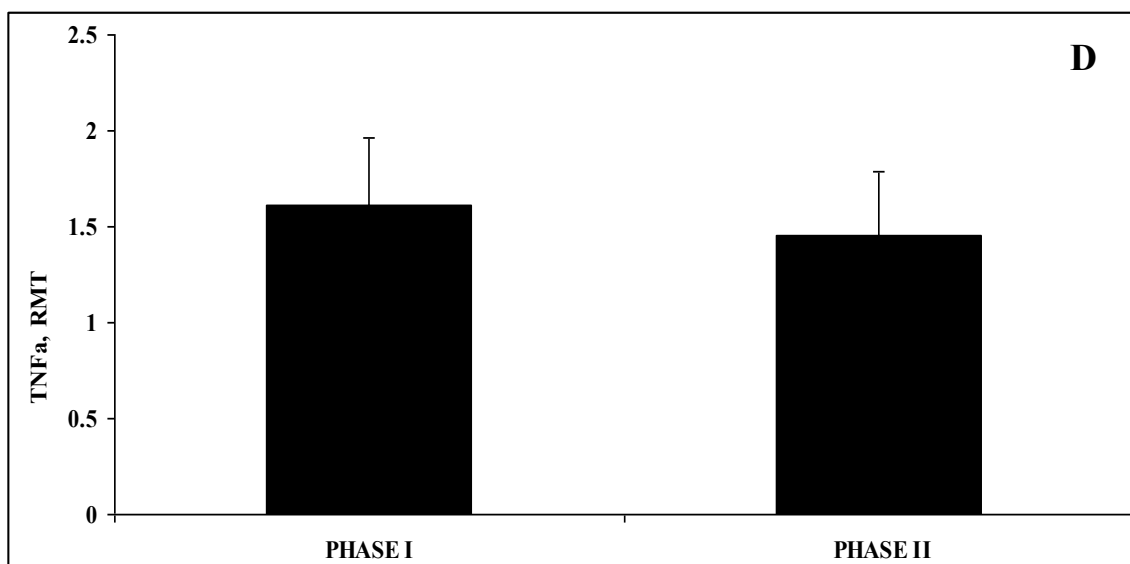
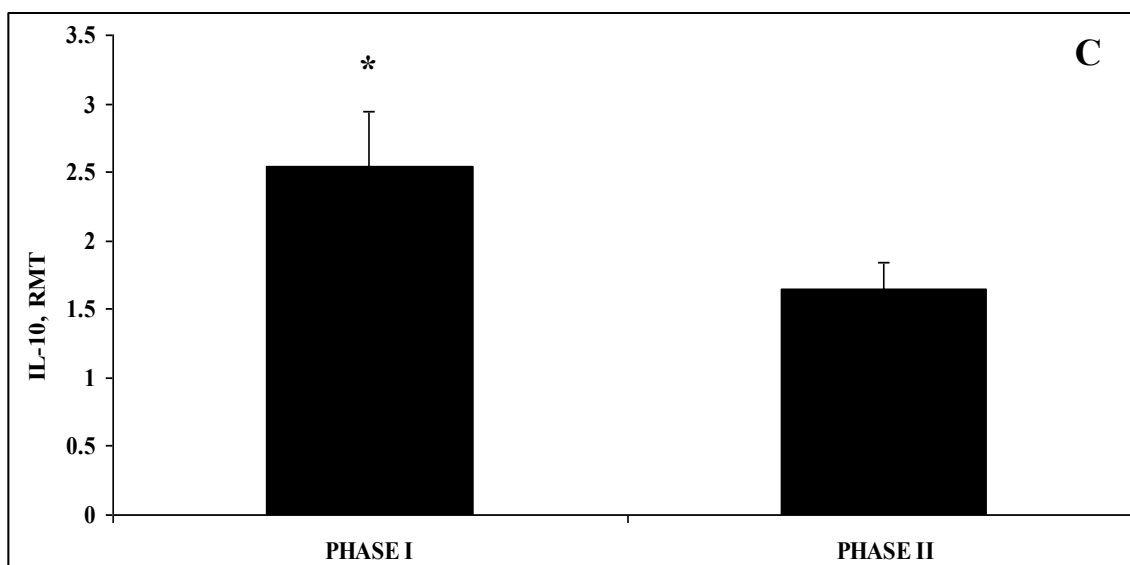
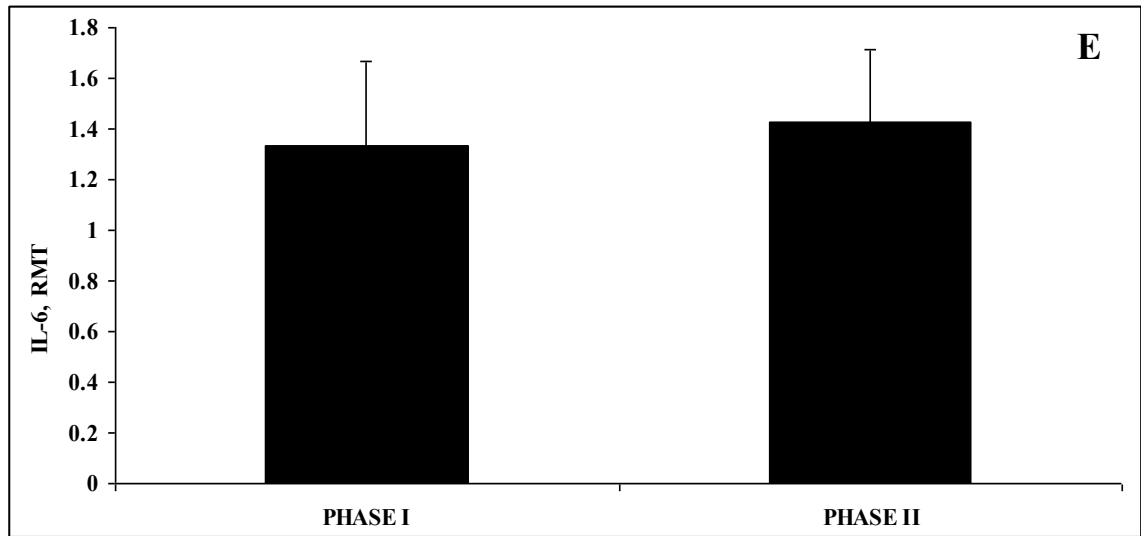


Figure 3 A – D. Erythrocyte superoxide dismutase activity (SOD; A), glutathione peroxidase activity (GPx; B), glutathione (GSH-T; C), and plasma nitric oxide (NO; D) during experimental Phase 1 vs. Phase 2. An asterisk (\*) denotes differences at  $P < 0.05$ .







*Figure 4, A - E* . Interferon  $\gamma$  (IFN $\gamma$ ; A), interleukin-1 $\beta$  (IL-1 $\beta$ ; B), interleukin-10 (IL-10; C), tumor necrosis factor  $\alpha$  (TNF $\alpha$ ; D), interleukin-6 (IL-6; E), relative mRNA transcripts (RMT) in peripheral blood of Standardbred mares during Phase 1 and 2 of the experimental trial. qRT-PCR data are presented as the mean fold change in target gene expression. Bars represent mean  $\pm$  SE; an asterisk (\*) denotes differences between experimental Phases 1 and 2 at  $P < 0.05$ .

### Journal Article 3

#### **Repeated arthrocentesis and intense exhaustive exercise influence biomarkers of inflammation and cartilage metabolism in equine synovial joints**

**ABSTRACT:** Supplemental superoxide dismutase (SOD) has been shown to exert anti-inflammatory and chondroprotective properties in various species. However, this has not been shown in horses following oral administration of SOD. Objectives of the present study were to evaluate effects of repeated arthrocentesis, intense exhaustive exercise, and exogenous SOD supplementation on markers of inflammation and cartilage metabolism in healthy mature horses.

**Pilot Study:** Six healthy mature Standardbred mares underwent four aseptic arthrocentesis procedures (T-1 – T-4) in the radiocarpal joint space within a 48 h time frame. Samples were analyzed for epitope of chondroitin sulfate-846 (CS) and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>). Data were analyzed using a MIXED model ANOVA with repeated measures in SAS 9.1. Repeated sampling resulted in an increase ( $P = 0.04$ ) in CS at the last sample time (T-4), and no changes were detected for PGE<sub>2</sub>.

**Exercise Trial:** Healthy mature unfit Standardbred mares were used in this double blind, randomized crossover design. Horses received either treatment (TRT; 3 g/d of oral SOD powder with 3000 IU) or placebo (CON; 3 g/d cellulose powder) for 6 wks (Phase 1 and 2) with a 6 wk wash-out between phases. Mares ran a repeated sprint exercise test on d 42 of each phase. Blood and synovial fluid were collected before (PRE), 30 min, 2 h, and 24 h after exercise (POST) and were analyzed for CS and PGE<sub>2</sub> (synovial fluid only).

Data were analyzed using a MIXED model ANOVA with repeated measures in SAS 9.1. Data analysis revealed lower ( $P \leq 0.05$ ) serum concentrations of CS at 30 min and 2 h POST compared to 24 h POST. Serum CS was also higher ( $P = 0.001$ ) during experimental Phase 2 when compared to Phase 1. Synovial fluid CS was elevated ( $P < 0.01$ ) at 30 min POST compared to PRE and 2 h POST, and compared to all other sample times was higher ( $P \leq 0.0001$ ) at 24 h POST. Higher ( $P = 0.03$ ) CS-846 synovial fluid concentrations were detected during experimental Phase 1 compared to Phase 2, and hock joints contained higher ( $P < 0.0001$ ) concentrations of CS compared to carpus joints. Adjusted PGE<sub>2</sub> concentrations relative to PRE were elevated ( $P = 0.03$ ) at 24 h POST and demonstrated a trend ( $P = 0.08$ ) for being elevated above 30 min POST samples. There were also greater ( $P = 0.04$ ) increases in PGE<sub>2</sub> concentrations in hock joints when compared to carpus joints.

Key Words: Arthrocentesis, Equine, Inflammation, Superoxide dismutase, Synovial fluid, Treadmill exercise

## INTRODUCTION

Horses begin exercise training at a young age, and many are engaged in several different athletic pursuits throughout their lifetime. Consequently, there is a high rate of morbidity and mortality associated with these athletic endeavors, which has propagated a much needed area of research investigating the effects of exercise on the musculoskeletal systems in horses (reviewed in Firth, 2006). Inflammation is a fundamental response to

infection or trauma, and is manifested in a number of common equine diseases such as osteoarthritis and tendonitis. Sub-clinical injury and the subsequent immune response to acute intense exercise may set the stage for a chronic inflammatory and immunosuppressed state resulting in a predisposition to infection, poor physical performance, and potentially the development of chronic diseases (Shek et al., 1995; Brenner et al., 1999). Post-exercise problems associated with increased expression of inflammatory markers can range from the mild symptoms of delayed-onset muscle soreness to debilitating problems related to soft tissue injury and joint and bone damage (Auer et al., 1989). Chronic inflammation, associated with the aforementioned, can lead to tissue destruction, subsequent poor performance and a likely end to the athletic career of a horse (Petersen et al., 2004).

Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) is a prostanoid, or biologically active lipid product of the cyclooxygenase-2 (COX-2) catalyzed metabolism of arachidonic acid. Prostaglandin E<sub>2</sub> serves predominantly as an inflammatory mediator and smooth muscle relaxant. In the joint PGE<sub>2</sub> most likely originates from articular tissues and is important for the maintenance of local cartilage homeostasis (Amin et al., 1997; Murakami et al., 1998; von Rechenberg et al., 2000; Tchetina et al., 2007). It also mediates local inflammation and sensitivity to pain, and is considered an indirect biomarker of joint disease. Increased PGE<sub>2</sub> concentrations in synovial fluid at concentrations of 22.5 pg ml<sup>-1</sup> or greater, are considered an excellent indicator of synovitis (McIlwraith, 2005; Frisbie et al., 2008) and predictor of equine joint disease (Kirker-Head, et al., 2000; Bertone et al., 2001). Regular treadmill exercise training has been shown to induce increases in synovial fluid PGE<sub>2</sub> concentrations in healthy horses (Frisbie et al., 2008), however changes relative to

a single bout of intense exhaustive treadmill exercise have not been documented in the joints of healthy horses.

More recently, efforts have been made to identify and characterize biomarkers that would serve as early warning signs of joint disease, or characterize pre-clinical changes that could predispose an animal to the development of arthropathies later on (Billinghurst et al., 1997; Frisbie et al., 1999; Robion et al., 2001; McIlwraith, 2005; Frisbie et al., 2008). Chondroitin sulfate along with keratan sulfate, are primary glycosaminoglycan side chains for the proteoglycan monomer embedded within the collagen framework of articular cartilage (Ray et al., 1996). The CS-846 epitope (CS) is found on fragments of aggrecan that have been released from the cartilage matrix. Monoclonal antibodies against this epitope have been produced in order that it can be quantified using either a radioimmunoassay or enzyme immunoassay (Poole et al., 1994; Ray et al., 1996). Recent literature has found this epitope to be a good indicator of cartilage metabolism, more specifically aggrecan synthesis, primarily because it is attached almost exclusively to the most intact and newly synthesized aggrecan molecules (Rizkalla et al., 1992; Poole et al., 1994). Along with another biomarker for type II collagen synthesis, CS has been useful in detecting osteochondral change in equine joints (Frisbie et al., 1999; McIlwraith, 2005; Frisbie et al., 2008).

Many of the existing intra-articular treatments intended to treat symptoms of inflammatory joint diseases are invasive, expensive, and controversial as to whether or not they augment catabolic tissue metabolism in the joint with prolonged or chronic use (Behrens et al., 1975; Chunekamrai et al., 1989; Robion et al., 2001; Celeste et al., 2005). Furthermore, due to the poor long term prognosis associated with arthropathies

attributable to a limited capacity for cartilage regeneration (Hunter, 1743), there should be a focus on development of preventative strategies. Nutritional supplements are popular and easily obtainable. Unfortunately, the nutraceutical industry is widely unregulated and products do not undergo the same scrutiny, nor are they subject to the regulatory standards that the pharmaceutical industry is held to. Most nutritional supplements designed to promote joint health or decrease symptoms of disease have many testimonials claiming benefits, but lack controlled research to establish their safety and efficacy (Fortier, 2005). These systemic therapies are non-invasive, easy to administer and have the potential to provide effective, practical, affordable options for the prevention of and management of inflammatory joint disease in horses; however, their safety and efficacy must be verified with controlled science-based inquiry.

Exogenous superoxide dismutase (SOD) has been used as an oral supplement (Vouldoukis et al., 2004b; Arent et al., 2007; Arent et al., 2009), topical treatment (Petelin et al., 2000) and injectable treatment (Huber, 1981; Auer et al., 1990), however, benefits from oral supplementation of SOD, specifically anti-inflammatory and chondroprotective properties in synovial joints of horses, have not been evaluated. Identification of inflammatory and metabolic changes in synovial joints, associated with one bout of exhaustive exercise, would provide insight into changes exclusively associated with acute exercise providing better tools with which to detect and distinguish between adaptive responses and changes indicative of early degenerative processes which could lead to diseases such as osteoarthritis. Early detection provides an opportunity to employ preventative measures in order to avoid the progression joint disease.

The objectives of this study were two-fold: 1) to evaluate the effects of repeated arthrocentesis within a 48 h time frame, on markers of inflammation and cartilage metabolism, in synovial fluid of healthy horses at rest, and 2) to evaluate the effects of a single bout of intense exhaustive treadmill exercise and exogenous SOD supplementation on biomarkers of joint inflammation and cartilage metabolism in horses. It was hypothesized that repeated arthrocentesis within a 48 h time frame would not influence biomarkers of inflammation (PGE<sub>2</sub>) and cartilage metabolism (CS) in synovial fluid of mature, healthy horses at rest. It was also hypothesized that a single bout of intense, exhaustive treadmill exercise would elicit localized inflammation and an increase in anabolic cartilage metabolism within the carpus and hock joints of mature healthy horses. Lastly, it was hypothesized that exogenous SOD supplementation would elicit a protective effect in the carpus and hock joints by reducing markers of inflammation and maintaining steady cartilage metabolism following intense exhaustive exercise in mature healthy horses.

## **MATERIALS AND METHODS**

### **Pilot study**

#### ***Subjects***

A sample of 6 healthy, sound, unfit Standardbred mares aged  $8 \pm 1$  yr, weighing  $550.0 \pm 23.5$  kg, with body condition scores of  $5.7 \pm 0.1$  (Henneke et al., 1983) and per cent body fat at  $19.2 \pm 0.7\%$  (Westervelt et al., 1976), were used in this preliminary study. Mares were housed in New Jersey at the Rutgers University Equine Agricultural Experiment Station Research Facility on 2 acre exercise lots and were provide *ad libitum*

access to moderate quality grass hay, water and salt. They also received 2 kg of a 12% crude protein pellet to meet maintenance requirements. The Rutgers University Institutional Animal Care and Use Review Board approved all methods and procedures used in this study.

### ***Sampling procedure***

Synovial fluid samples were collected via aseptic arthrocentesis from one radiocarpal joint in each horse. Mares underwent four arthrocentesis procedures in the same joint space within a 48 h time frame at the following intervals; initial joint tap (T1), second joint tap 24 h following the initial tap (T2), third tap 26 h following the first tap (T3), and a fourth tap 48 h following the initial tap (T4). Mares were mildly sedated with 0.3 – 0.5 ml of detomidine hydrochloride (10 mg ml<sup>-1</sup>; Dormosedan, Pfizer Inc., New York, NJ, USA) intravenously 10 min prior to each arthrocentesis procedure. The synovial fluid was initially collected into sterile syringes using sterile 20 gauge one inch hypodermic needles, and then immediately transferred to pre-chilled 10 ml tubes containing ethylenediaminetetraacetic acid (EDTA; Vacutainer, Becton Dickinson, Franklin Lakes, NJ, USA) and placed on ice. Samples were centrifuged for 20 min at 1500 x g to remove any cellular debris, aliquoted and stored at -80°C for later analysis.

### ***Sample Analysis***

For both the Pilot and Exercise Studies, concentrations of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>; R&D Systems Parameter High Sensitivity PGE<sub>2</sub> Assay, Minneapolis, MN, USA; Au et al., 2007; Intra-CV = 18.0% Inter-CV = 13.9%) were measured in synovial fluid

according to manufacturer's instructions. The epitope of chondroitin sulfate 846 (AggreCan CS-846 Epitope ELISA; IBEX Pharmaceuticals Inc., Montreal, Quebec, Canada; Frisbie et al., 1999; Intra-CV = 3.1%; Inter-CV = 8.1%) was measured in synovial fluid and serum samples. Chondroitin sulfate-846 samples were analyzed at the Michigan State University Equine Nutrition Laboratory, East Lansing, MI.

### ***Statistics***

Data were analyzed using a MIXED model ANOVA with repeated measures in SAS 9.1 to evaluate effects of repeated arthrocentesis sampling. Significant main effects were further analyzed using Tukey-Kramer post hoc analysis to further elucidate significant changes as inferred when  $P < 0.05$ . Data are presented as the mean  $\pm$  SE and significance was set at  $P < 0.05$ .

### **Exercise Trial**

#### ***Subjects***

A sample of 12 healthy, unfit Standardbred mares aged  $8 \pm 1$  yr, weighing  $513.8 \pm 16.7$  kg, with lameness scores of  $0.3 \pm 0.1$  (AAEP, 1991), body condition scores of  $5.3 \pm 0.1$  (Henneke et al., 1983) and per cent body fat at  $20.7 \pm 1.8$  % (Westervelt et al., 1976), were used in this study. Mares were housed in New Jersey at the Rutgers University Equine Agricultural Experiment Station Research Facility on 2 acre exercise lots and were provide *ad libitum* access to moderate quality grass hay, water and salt. They also received 1 kg of a 10 % crude protein sweet feed (Nutrena Vitality, Cargill Inc., Minneapolis, MN) twice daily to meet maintenance requirements. The Rutgers

University Institutional Animal Care and Use Review Board approved all methods and procedures used in this study.

### ***Experimental design***

This study was conducted as a placebo-controlled, randomized crossover design where investigators were blind to treatments. Mares were randomly assigned to either a treatment group (TRT) which received 3 g/d (3000 IU) of a proprietary oral formulation of SOD powder, extracted from cantaloupe melon and chemically combined with wheat gliadin (vegetal hydrophobic biopolymer), or a placebo control group (CON) which received 3 g/d of an microcrystalline cellulose powder. Both TRT and CON groups received their supplement top dressed on their morning grain ration. The duration of the study was 18 wks and started the last week in June and ended the first week in November, 2007. Phase-1 of the study consisted of a 42 d supplementation period which concluded with a repeated sprint exercise test (RSET) on d 42, previously determined to elicit inflammatory and oxidative stress responses in horses (Lamprecht et al., 2009). Mares then completed a 42 d washout period, the experimental groups were crossed over, and an identical 42 d supplementation period (Phase-2) and exercise test (RSET) was completed. Before each Phase of the study, standardized methods were used to evaluate each mare for body condition (Henneke et al., 1983), orthopedic soundness (AAEP, 1991), and per cent body fat (Westervelt et al., 1976).

All mares were habituated to Rutgers Equine Exercise Physiology Laboratory and to running on the treadmill fixed at a 6 % grade (Sato II, Sato Treadmill, Knivsta, Sweden) prior to the start of the study. The RSETs were conducted on d 42 of Phase 1

and 2 of the study and on the day of the RSET, horses were fed their respective supplement 60 min before the start of the exercise test. Intravenous catheters (Angiocath™, Becton Dickinson, Franklin Lakes, NJ, USA) were also inserted percutaneously in to the left jugular using aseptic technique and local anesthesia (2 % lidocaine HCl inj., usp 20 mg/ml, Abbott Laboratories, Chicago, IL, USA). The catheters were kept patent with sterile heparinized (Heparin sodium, inj., USP 5000U/ml, American Pharmaceutical Partners Inc., Schaumburg, IL, USA) physiological saline solution at a working concentration of 10 U/ml.

### ***Exercise test***

The RSET lasted an average of  $18.4 \pm 0.7$  min and began with 2.5 min of walking at 1.5 m/s followed by 4 min of trotting at 4 m/s. Following warm up, mares completed 2 min sprints at 7, 8, 9 and 10 m/s with 2 min of walk at 1.5 m/s between each sprint (Graham-Tiers et al., 2003; Wilson et al., 1998). Mares ran to fatigue or until the test was completed, at which time they spent 1 min walking at 1.5 m/s. Fatigue was defined as an inability of the mares to keep up with the treadmill despite humane encouragement.

### ***Sampling***

Venous blood samples were collected relative to the RSET via an indwelling jugular catheter and placed into 10 ml serum separator tubes containing clot activator and gel for serum separation (Vacutainer, Becton Dickinson, Franklin Lakes, NJ, USA). Blood samples were collected 10 min prior to exercise (PRE), 30 min, 2 h, and 24 h post-exercise (POST). Serum tubes sat at room temperature for approximately 1 h to allow for

blood clotting, and were centrifuged for 20 min at 1500 x *g* at 10 °C. Serum was aliquoted and stored at -80°C for later analysis.

Synovial fluid was collected via aseptic arthrocentesis from one radiocarpal and one tibiotarsal joint space as described above for the Pilot Study, placed into pre-chilled 10 ml tubes containing EDTA (Vacutainer, Becton Dickinson, Franklin Lakes, NJ, USA), and immediately placed back on ice. Samples were taken 24 h before exercise (PRE), 30 min, 2 h, and 24 h post exercise (POST). The SF was centrifuged for 20 min at 1500 x *g* to remove any cellular debris, aliquoted and stored at -80°C for later analysis. Sample timing in the Exercise trial mirrors that of the Pilot study with the added bout exercise occurring before the second sample (30 min POST). Serum and synovial fluid were analyzed for CS and PGE<sub>2</sub> using commercial ELISA kits according to manufacturer's instructions, as described above.

### ***Statistics***

Data were analyzed using a MIXED model ANOVA with repeated measures in SAS 9.1 to evaluate effects of SOD supplementation as (TRT), acute exercise (as sample time), joint space (as carpus and hock), experimental phase (as Phase 1 and 2), and their interactions. Horse was nested within Treatment as the subject, and Sample time was designated as the repeated effect. The Satterthwaite approximation of standard errors was utilized to account for any unequal variances. Significant main effects were further analyzed using Tukey-Kramer post hoc analysis to further elucidate significant changes as inferred when  $P < 0.05$ . Pearson's product moment correlation was used to test for associations between the variables measured.

## RESULTS

### Pilot Study

Main effect of repeated arthrocentesis was detected for CS-846 ( $P = 0.04$ ; Figure 1 A) where CS-846 synovial fluid concentrations were higher ( $P \leq 0.05$ ) at T4 when compared to T1 and T3. Differences between sampling times for PGE<sub>2</sub> ( $P > 0.05$ ; Figure 1 B) were not detected.

### Exercise Trial

There were no TRT effects detected for any parameter measured. For CS concentrations in serum, there were main effects of exercise ( $P = 0.009$ ) and experimental phase ( $P = 0.001$ ). There were also main effects of exercise ( $P < 0.0001$ ), joint ( $P < 0.0001$ ), and experimental phase ( $P = 0.03$ ) for CS in synovial fluid concentrations of CS.

Further data analysis revealed lower ( $P \leq 0.05$ ) serum concentrations of CS at 30 min and 2 h POST samples when compared to 24 h POST (Figure 2 A). Serum concentrations of CS were also higher ( $P = 0.001$ ) during experimental Phase 2 when compared to Phase 1 (Figure 3 A). Synovial fluid concentrations of CS were elevated ( $P < 0.01$ ) at 30 min POST compared to PRE and 2 h POST samples, and when compared to all other sample times, synovial fluid CS was higher ( $P \leq 0.0001$ ) at 24 h POST (Figure 2 B). There were higher ( $P = 0.03$ ) CS synovial fluid concentrations during experimental Phase 1 compared to Phase 2 (Figure 3 B), and hock joints contained higher ( $P < 0.0001$ ) concentrations of CS compared to carpus joints (Figure 4).

Main effects for PGE<sub>2</sub> concentrations in synovial fluid were not detected ( $P > 0.05$ ; Figure 5); however, after adjusting the data by subtracting the PRE value from each POST value, and evaluating changes relative to pre-exercise values, effects of exercise ( $P = 0.03$ ) and joint ( $P = 0.04$ ) were detected. Prostaglandin E<sub>2</sub> was elevated ( $P = 0.03$ ) at 24 h POST relative to PRE values, and demonstrated a trend ( $P = 0.08$ ) for being elevated above 30 min POST samples as well (Figure 7). Similar to synovial fluid CS concentrations, there were greater ( $P = 0.04$ ) increases in PGE<sub>2</sub> concentrations in hock joints when compared to carpus joints.

## DISCUSSION

These studies were designed to identify changes in biomarkers of inflammation and cartilage metabolism resulting from repeated arthrocentesis (Pilot study), and one intense exhaustive bout of treadmill exercise both with and without exogenous SOD supplementation (Exercise trial) in mature, healthy, unfit mares.

### *Effect of repeated arthrocentesis*

The Pilot study was conducted in order to identify possible confounding factors arising from repeated arthrocentesis in the same joint space over a relatively short time-frame in mature healthy horses. Data from this study indicated that repeated synovial fluid sampling did not elicit a detectable inflammatory response based upon statistically similar PGE<sub>2</sub> concentrations, but did seem to influence anabolic joint metabolism as evidenced by elevated CS at the last sample time. These data are in partial contrast to a

previous study which demonstrated increases in synovial fluid concentrations of PGE<sub>2</sub> and nitric oxide following 2 consecutive joint taps with a 12 hour interval between taps, when compared to baseline values (Van Den Boom et al., 2005). Increases in glycosaminoglycans were also detected after 2 consecutive taps with a 60 hour interval between taps, in radiocarpal and tarsocrural joints of mature healthy horses. Another study reported increased matrix metalloproteinase-1, a biomarker for tissue remodeling, in equine synovial fluid following repeated arthrocentesis within a 60 h time-frame (Brama et al., 2004). These findings are in partial contrast to additional reports in the literature. A study by Lamprecht et al. (2009), did not find a repetitive arthrocentesis protocol, similar to the present study's, to modulate synovial fluid concentrations of nitric oxide, a marker of inflammation, in radiocarpal and tibiotarsal joints of mature, healthy mares following intense exhaustive treadmill exercise. Another study reported no effect of repeated synovial fluid sampling (4 taps over 10 days with intervals ranging from 12 h to 168 h between taps) on the pro-inflammatory regulator tumor necrosis factor alpha, in synovial fluid of normal equine joints (van den Boom et al., 2004). Furthermore, a study where healthy horses underwent weekly synovial fluid aspiration for a total of 13 wks did not report evidence of repeated sampling effects on keratin sulfate or CS in control radiocarpal joints contralateral to experimentally injected joints (Robion et al., 2001). Authors of a similar study, during which healthy young horses underwent repeated arthrocentesis for 13 wks, concluded that any effects of the repeated synovial fluid sampling were minor and most likely did not confound experimental data (Frisbie et al., 2008). Relative to the Pilot study, there were no differences in radiocarpal synovial fluid concentrations of PGE<sub>2</sub> between sample times indicating that repeated arthrocentesis

within a 48 h time-frame at these intervals, does not appear to influence biomarkers of inflammation. However, repeated synovial fluid aspiration within a 24 h time-frame may influence biomarkers of joint metabolism, as was seen with elevated CS concentrations at the last sampling time out of four, in the Pilot study, and should be taken into consideration when interpreting related data.

### ***Effect of Exercise***

Most *in vivo* studies investigating joint health and disease involve either animals with clinical conditions, or an artificially induced disease model attained through surgically created osteochondral defects, or chemically induced synovitis (McIlwraith and Van Sickle, 1981; Smith et al., 1998; Frisbie et al., 2008). Many studies also incorporate a prolonged or submaximal exercise regimen into their experimental design (Murray et al., 2001; Caron et al., 2002). These study designs are useful within a limited context and many are focused on treatment strategies for existing conditions, rather than early detection and preventative strategies. The use of intense treadmill exercise to induce inflammation provides a realistic, repeatable, and non-invasive model of inflammation and joint metabolism under dynamic physiologic stress.

The relative increase in PGE<sub>2</sub> from PRE to 24 h POST in the Exercise trial suggests a slightly delayed onset of inflammation in response to the RSET. Elevated concentrations of PGE<sub>2</sub> have previously been shown to occur in inflamed and osteoarthritic joint tissues of horses (May et al., 1994; Tung et al., 2002; Chan et al., 2005; van den Boom et al., 2005; Au et al., 2007; Frisbie et al., 2008). Furthermore, these elevated prostaglandins may compromise the cartilage matrix by decreasing

proteoglycan content (Tietz and Chrisman, 1975; Lipiello et al., 1978) as well as initiating a localized pain response via sensitization of peripheral nociceptor terminals (Tchetina et al., 2007). In a previous study, increases in circulating pro-inflammatory cytokine transcripts (interleukin-1 beta) were detected 2 h following intense exhaustive treadmill exercise (Lamprecht et al., 2009). Interleukin-1 beta is a regulator of PGE<sub>2</sub> release from joint tissues, which could help explain why the increase in PGE<sub>2</sub> is delayed past the 2 h POST sample following the RSET in the current study. It is unknown if this was a transient increase, or if PGE<sub>2</sub> levels remained elevated for a sustained period of time as sampling was not carried out past 24 h POST. The resting and post-exercise PGE<sub>2</sub> concentrations were higher in the current study compared to previous reports in horses (Frisbie et al., 2008), which might be attributable to different analytical techniques (extraction of PGE<sub>2</sub> from SF vs. evaluation of concentrations in diluted synovial fluid). Circulating concentrations of PGE<sub>2</sub> were not measured in the current study as unsuccessful attempts at this have been made in both intensely and extended sub-maximally exercised horses (Hinchcliff et al., 1994; Mitten et al., 1995).

Healthy articular cartilage serves to distribute load in a pain and friction-free manner. Cartilage consists of specialized cells (chondrocytes) distributed in three distinct layers within an intracellular matrix consisting primarily of proteoglycans and type II collagen (Smith et al., 2000). The proteoglycans can further be subcategorized into aggregating proteoglycans, and non-aggregating large and small proteoglycans. Specifically, aggrecan interacts with hyaluronic acid through non-covalent bonds that serve to resist compressive strain, whereas the fibrous collagen network contributes the tensile properties of cartilage (Hardingham and Muir, 1972; Askew and Mow, 1978).

The integrity of the matrix is dependent on chondrocyte metabolism, which has been shown to be influenced by stressors, including shear and compressive strain, which occur with exercise (reviewed by Smith et al., 2000).

Normal joint loading is important for the maintenance of articular health however, loads that exceed the physical threshold can cause damage and a loss of cartilage homeostasis in favor of matrix degradation. According to the Donnan equilibrium theory (Schneiderman et al., 1986; Gray et al., 1988), exercise results in compression and deformation of the chondrocytes within their matrix resulting in changes in ionic concentrations, osmolarity, and pH of the cellular environment (Guilak et al., 1999). Joint loading also results hydrostatic pressure gradients, changes in intratissue fluid flow and electrokinetic effects altering the transport of nutrients and macromolecules such as growth factors and cytokines (Bhakta et al., 2000; Kerin et al., 2002). It has been shown that different types of joint loading influence chondrocyte metabolism (Guilak et al., 1995; Kim et al., 1995) and furthermore, associations between the type of athletic sport, performance level and specific orthopaedic injuries have been documented in horses (Murray et al., 2006).

Studies have shown that shear stress modulates inflammatory and matrix macromolecule metabolism in chondrocytes. One study, using normal human and bovine articular chondrocyte culture, demonstrated that fluid-induced shear stimulated GAG synthesis, and after 48 h of shear stress PGE<sub>2</sub> production was increased when compared to controls (Smith et al., 1995). Additionally, a study using normal human and bovine articular chondrocyte cultures demonstrated that intermittent hydrostatic pressure increased aggrecan mRNA signal, whereas constant pressure had no effect (Smith et al.,

1996). Both intermittent and constant pressures resulted in increased GAG production suggesting that hydrostatic pressure can influence chondrocyte extracellular matrix metabolism. Furthermore, several *in vitro* cartilage explant culture models have shown that lower frequency and low compression joint loading results in an inhibition of matrix synthesis whereas higher compressive loading resulted in increased chondrocyte metabolism (Knight et al., 1998; Lee and Bader, 1997; Lee et al., 1998). It is plausible that the type of joint loading resulting from the RSET in the current study induced enough fluid-induced shear and change in hydrostatic pressure gradient, to induce a delayed increase in PGE<sub>2</sub> relative to PRE concentrations.

The increase in CS at 30 min POST, which had declined back to pre-exercise levels by 2 h POST in the Exercise trial, suggests a quick and transient increase in aggrecan synthesis. The magnitude of change from pre- to post-exercise samples in the Exercise trial was similar to that reported in chronically exercised horses (Frisbie et al., 2008). Furthermore, absolute concentrations of CS in the present study were lower than those reported in the same chronically exercised healthy horses, and in horses with clinical osteoarthritis (Frisbie et al., 2008). When magnitude of change from T2 to T4 and 2 h POST to 24 h POST were compared between the Pilot and Exercise studies, respectively, the increase in CS was 2.6 times greater in the Exercise study. Furthermore, absolute concentrations of CS were approximately 2057 ng/ml higher for the 2 h POST sample in the Exercise study compared to the T2 sample in the Pilot study. This data suggests that the RSET did elicit immediate changes in cartilage turnover, most likely in response to cyclic high compressive loading possibly above their threshold frequency (Palmoski and Brandt, 1984; Sah et al., 1989; Parkkinen et al., 1993; Bushmann et al.,

1999; Frank et al., 2000). Several other studies have reported exercise-induced changes in cartilage metabolism. The influence of repetitive exercise on glycosaminoglycan and type II collagen metabolism in the joints of foals resulted in an increased maturation of the cartilage when compared to spontaneous pasture-exercised controls (van Weeren et al., 2008). At the end of a 6 wk training study in horses, investigators reported an increase in newly synthesized proteoglycan from an *ex vivo* cartilage culture when compared to cartilage from non-exercised controls (Palmer et al., 1995). Another study reported a decrease in aggrecan synthesis and concomitant increase in decorin synthesis in equine cartilage explants that persisted for 16 wks of rest following 17 wks of strenuous exercise (Little et al., 1997). Increases in CS and keratan sulfate (catabolic marker of cartilage metabolism) have been associated with cases of osteochondral fragmentation in horses (Frisbie et al., 1999) and repetitive exercise over time (Okumura et al., 2002; Frisbie et al., 2008). Healthy horses seem to be able to return to homeostatic conditions in joint tissues fairly quickly as was the case in the present study; however, if this modality and intensity of exercise were repeated without sufficient recovery over time, it could result in a chronic degenerative state, ultimately compromising the stability and functionality of the articular cartilage. The increase in CS synovial fluid concentrations at 24 h POST in the Exercise trial is thought to be an artifact of repeated arthrocentesis, as was detected in the Pilot study. It is possible that the delayed increase in PGE<sub>2</sub> may have prompted a compensatory increase in aggrecan synthesis as indicated by the increase in CS, although this cannot be verified in the present study.

These results also provide insight into sample timing when investigating changes in joint metabolism. When evaluating horses for changes in cartilage metabolism

indicative of early pathology, it could be misleading to take a sample within 30 min of intense exhaustive exercise, as elevated concentrations of cartilage metabolic biomarkers could be falsely interpreted as early pathology rather than exercise-induced changes. If biomarkers remain elevated greater than 2 h to 24 h following exercise, this might be indicative of a predisposing condition for joint disease and further investigation should be conducted. Therefore, samples should be taken well after the cessation of exercise to obtain clinically accurate or relevant data regarding joint disease etiologies.

Increases in serum CS concentrations were not detected as an effect of the RSET in the Exercise trial. Additionally, concentrations of CS were 96.8 % higher in synovial fluid when compared to serum concentrations. Significant associations between serum and synovial fluid biomarkers of cartilage metabolism have been reported (Frisbie et al., 2008); however, associations between serum and synovial fluid CS concentrations were not detected in the Exercise trial. This suggests that biomarkers in serum may not be sensitive or accurate indicators of adaptive physiological changes occurring within healthy joints. Serum markers may be more useful in evaluating existing clinical cases of degenerative joint disease (Poole et al., 1994), and based on data from the present study, it is not recommended that they be used as a tool for detecting exercise-induced patterns of change in healthy diarthroidial joints.

For both PGE<sub>2</sub> and CS, higher concentrations were detected in hocks when compared to carpus joints in the Exercise trial. This finding is in accord with a previous study which found higher concentration of nitric oxide in hock joints compared to that of carpus samples from mature mature healthy Standardbred mares (Lamprecht et al., 2009). Another study also demonstrated increased nitric oxide synthesis in response to shear

stress in bovine articular chondrocytes in culture, which in turn mediated an increase in GAG synthesis (Das et al., 1997). The differences between hock and carpus joints in the present study may be attributable to conformational and biomechanical features unique to Standardbred trotters or pacers, with a higher degree of dynamic compressive and shear stress occurring in the hock joints. A study by Grondahl and Dolvik (1993) reported that 14.3% of 753 young Standardbred trotters sampled were diagnosed with osteochondrosis in the tibiotarsal joint and 11.8% were diagnosed with bony fragments in the palmar/plantar portion of the metacarpo- and metatarsophalangeal joints. These data provide further evidence that Standardbreds experience more exercise-induced stress in the hock joints compared to joints in the forelimbs, and should be considered in light of athletic conditioning, rehabilitation from injury, and animal management practices.

### ***Effect of nutritional supplementation***

Exogenous SOD has been shown to be bioavailable when administered orally (Vouldoukis et al., 2004b; Arent et al., 2009), and subsequent benefits including anti-inflammatory (Vouldoukis et al., 2004a; Arent et al., 2007), antioxidant (Chenal et al., 2006; Kick et al., 2007), and chondroprotective (McCord, 1974; Betts and Cleland, 1982; Auer et al., 1990) properties, have been reported in several species. Furthermore, evidence of reactive oxygen species (ROS; specifically superoxide anion) in the joint has been reported (Dimock et al., 2000). Presence of ROS in joints can lead to proteoglycan cleavage (Greenwald and Moy, 1979), compromising the integrity of the cartilage matrix. It was hypothesized that exogenous SOD would be bioavailable and capable of exerting anti-inflammatory and chondroprotective properties in equine synovial joints following

intense exhaustive treadmill exercise however, this was not demonstrated in the present study. Data from the present study suggest that supplemental SOD has no effect on PGE<sub>2</sub> or CS in the joints of exercising horses.

A previous study demonstrated that addition of SOD to bovine synovial fluid was protective against hyaluronic acid depolymerization by superoxide derived hydroxyl radicals *in vitro*, thereby preserving the viscoelastic properties characteristic of healthy synovial fluid (McCord, 1974). Palosein (generic name Orgotein), an FDA-approved, injectable form of a Cu-Zn protein with high SOD enzyme activity derived from bovine liver (Huber, 1981), has also been shown to ameliorate free radical-induced (superoxide) loss in synovial fluid viscosity of horses (Auer et al., 1990). Hypoxanthine-induced hyaluronic acid degradation in human synovial fluid *in vitro* has also been demonstrated (Betts and Cleland, 1982). However, exogenous SOD supplementation in the Exercise trial failed to exert any anti-inflammatory or chondroprotective effects. Several factors may have contributed to the ineffectiveness of SOD supplementation in the current study. Contributing factors to this may include, but are not limited to, the length of supplementation period preceding the RSETs, bioavailability of the SOD preparation or failure of the gliadin delivery technology, the supplemental dosage rate, the unfit status of the experimental animals, and the experimental exercise modality employed. In light of this data, SOD supplementation does not appear to be a viable solution for reducing exercise-induced inflammation, or influencing joint metabolism in horses.

## CONCLUSION

Repeated arthrocentesis within a 48 h time frame does not seem to influence PGE<sub>2</sub> concentrations in joints of healthy mature horses, although changes in cartilage metabolism (CS) seem to be influenced by repetitive synovial fluid sampling, and should be considered a possible confounding factor when evaluating biomarkers of joint health. Exogenous SOD did not appear to exert any effect on joint inflammation or anabolic cartilage metabolism, therefore, benefits or detriments associated with SOD supplementation in horses remains to be elucidated. The inflammatory and metabolic changes detected in synovial fluid in the Exercise trial are thought to be normal physiological responses to a single bout of intense exercise. These data suggest that joint health in mature healthy horses is not compromised following a single intense bout of exhaustive exercise. However, repetitive exercise of this nature, without sufficient recovery periods, could result in sustained localized joint inflammation resulting in a chronic degenerative state. The transient increase in aggrecan synthesis may be part of an adaptive response to intense exercise, helping to maintain the integrity of articular joint tissues upon subsequent bouts of exercise. Standardbreds most likely experience higher loading in hock joints relative to carpus joints, therefore, efforts should be made to help minimize this localized stress, and hock joints should be monitored closely following intense exercise. This data could be useful when attempting to distinguish between normal adaptive responses to exercise and early clinical signs of joint disease in horses.

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## FIGURES

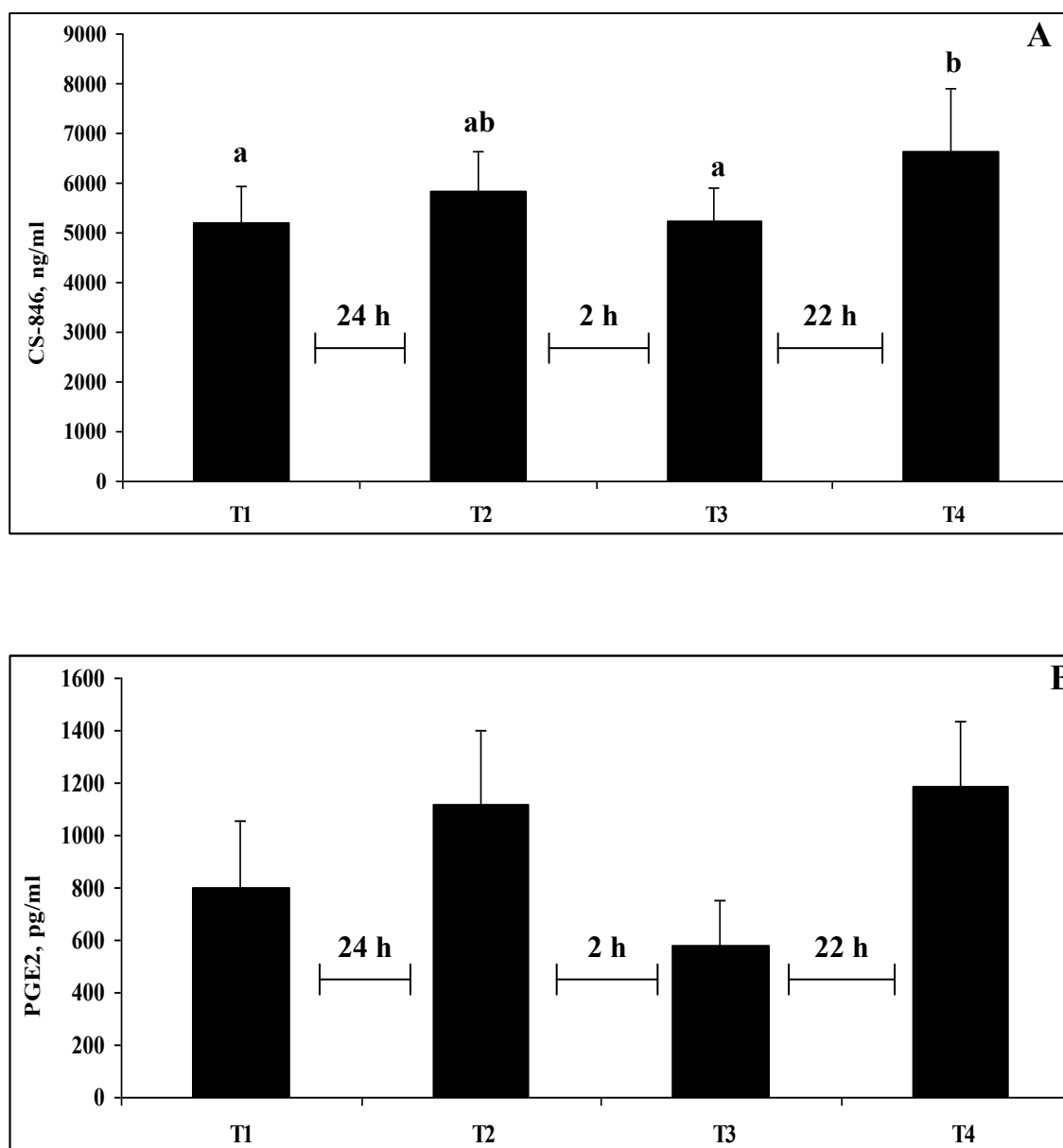


Figure 1, A – B. Pilot study: Synovial fluid concentrations of chondroitin sulfate-846 (CS-846; A) and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>; B) during 4 repeated arthrocentesis procedures in a 48 h time-frame at the following intervals: initial joint tap (T1), 24 h following T1 (T2), 26 h after T1 (T3), 48 h following T1 (T4), in carpus

joints. Specific time intervals between sample times are indicated by brackets between the bars. Data are presented as mean  $\pm$  SE. Means without a common superscript differ ( $P < 0.05$ ).

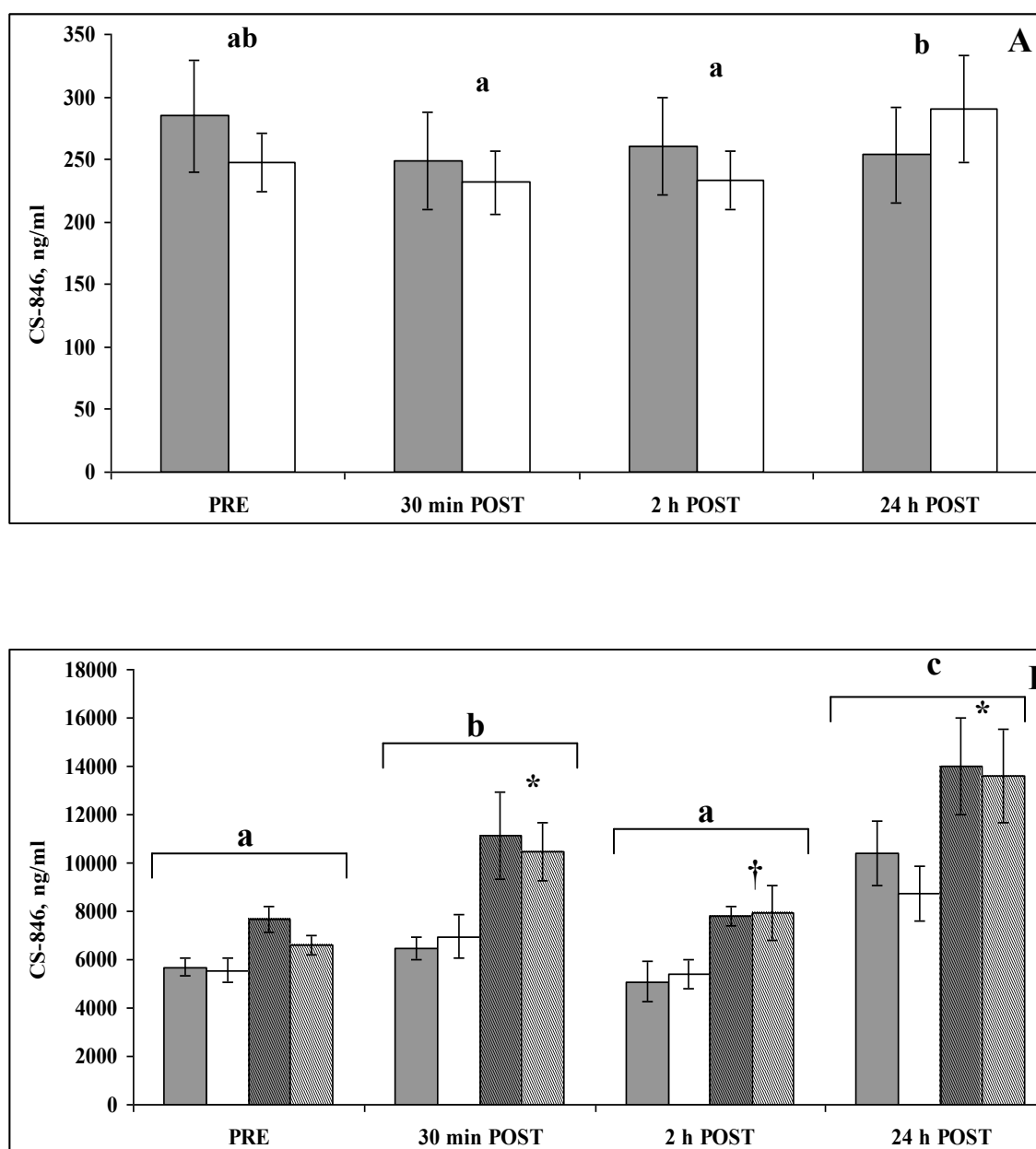


Figure 2, A - B. Exercise trial: Serum concentrations (A) and synovial fluid concentrations (B) of chondroitin sulfate-846 epitope (CS-846) before exercise

(PRE), 30 min, 2 h, and 24 h following exercise (POST). For Figure 2-B, open bars represent carpus samples, hashed bars represent hock samples, grey bars represent TRT groups, and white bars represent CON groups. Data are presented as mean  $\pm$  SE. Means without a common superscript differ ( $P < 0.05$ ) between sample times and an asterix (\*) infers significant differences ( $P < 0.05$ ) and a cross (†) infers a trend towards significant differences ( $P < 0.1$ ) between joint spaces within a sample time.

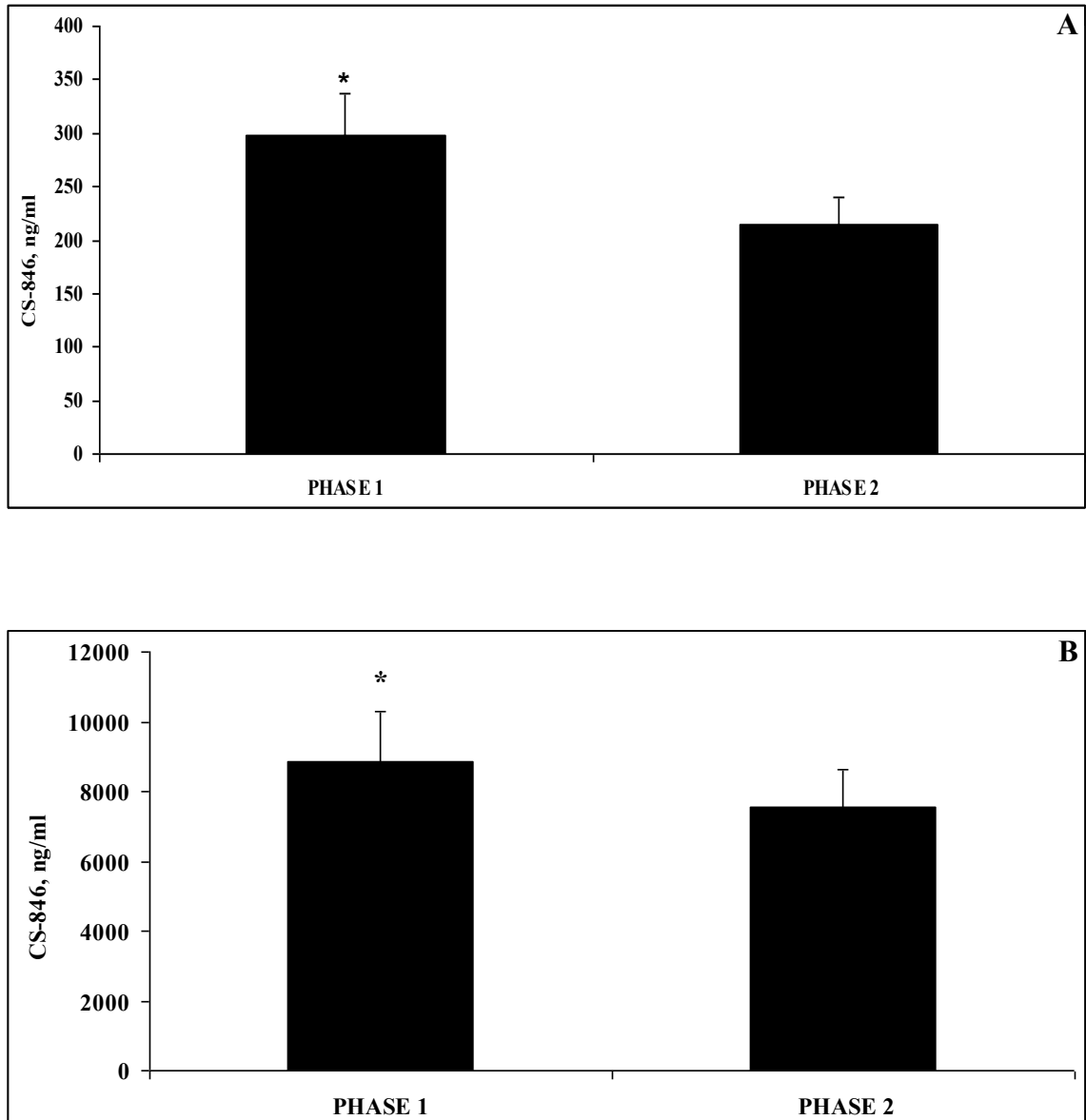
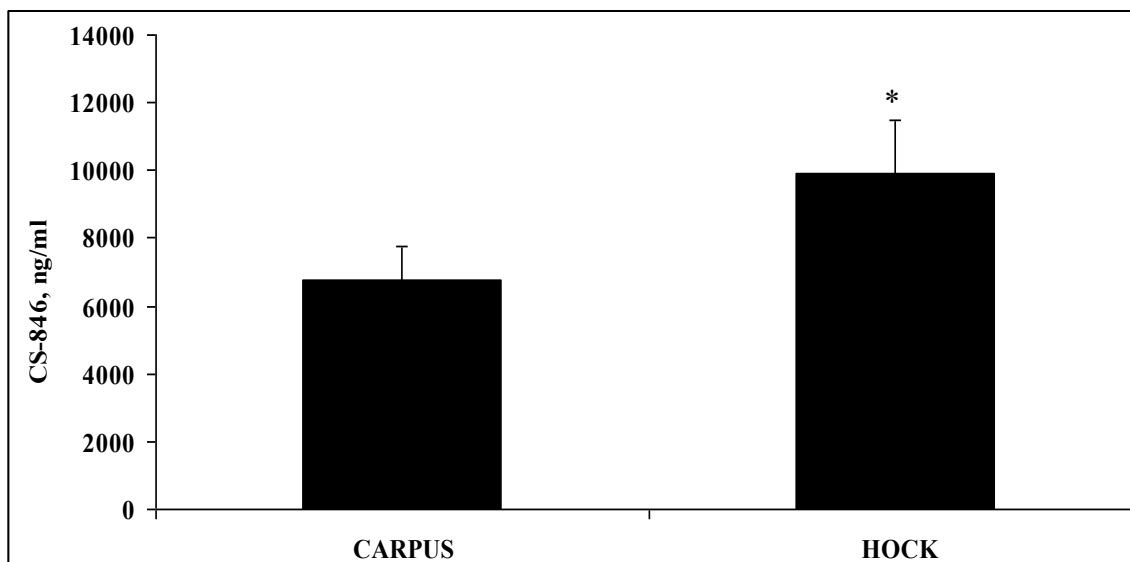
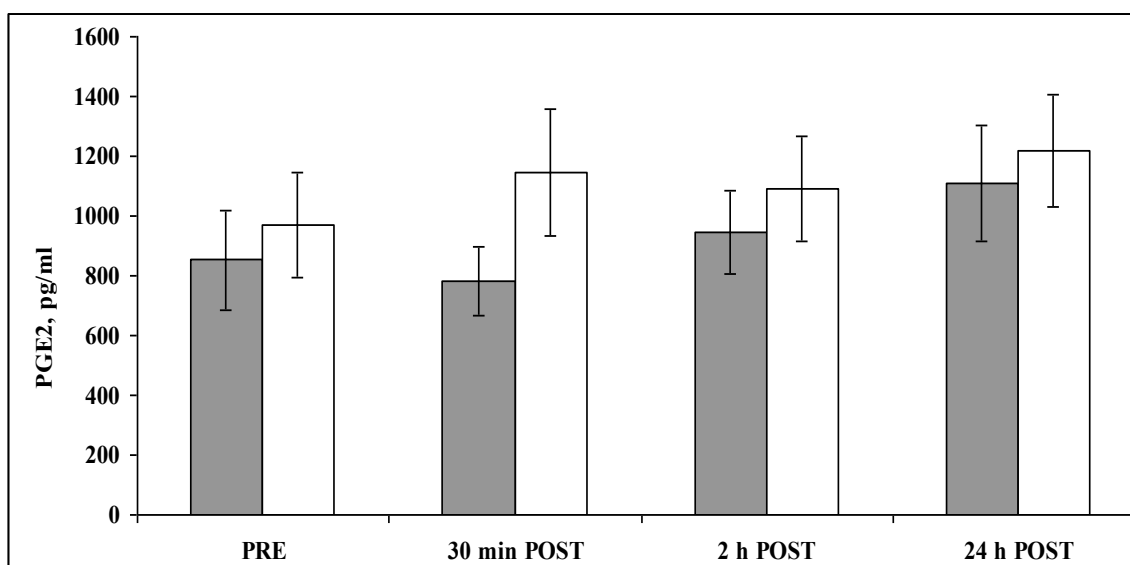


Figure 3, A – B. Exercise trial. Serum (A) and synovial fluid (B) concentrations of chondroitin sulfate-846 epitope (CS-846) during experimental Phases 1 and 2. Data are presented as mean  $\pm$  SE. An asterisk (\*) denotes differences at  $P < 0.05$ .

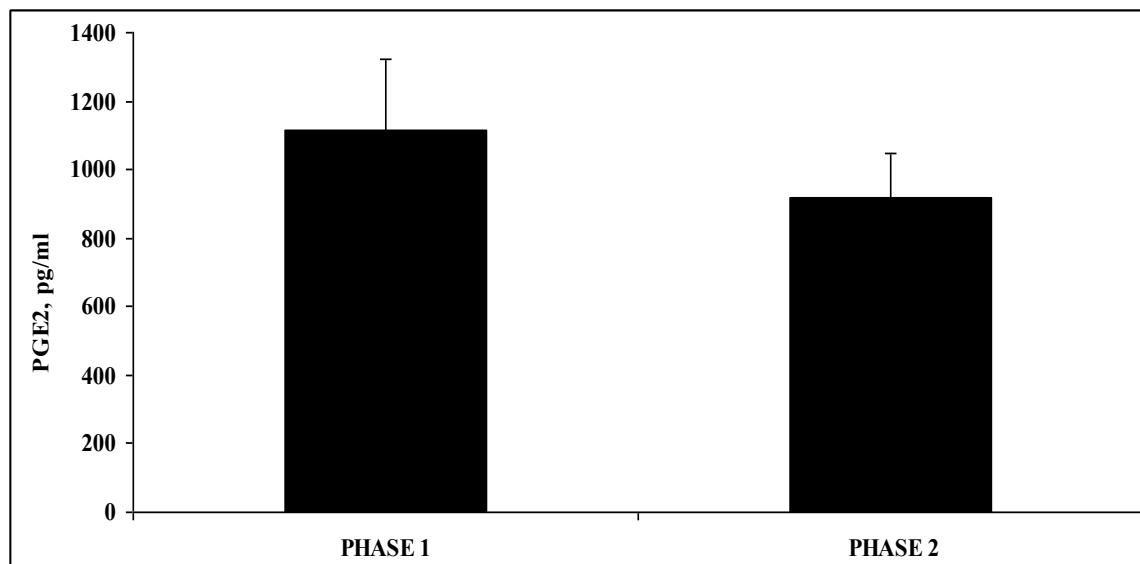


*Figure 4.* Exercise trial. Synovial fluid concentrations of chondroitin sulfate-846 epitope (CS-846) in carpus and hock joints. Data are presented as mean  $\pm$  SE. An asterix (\*) denotes differences at  $P < 0.0001$ .

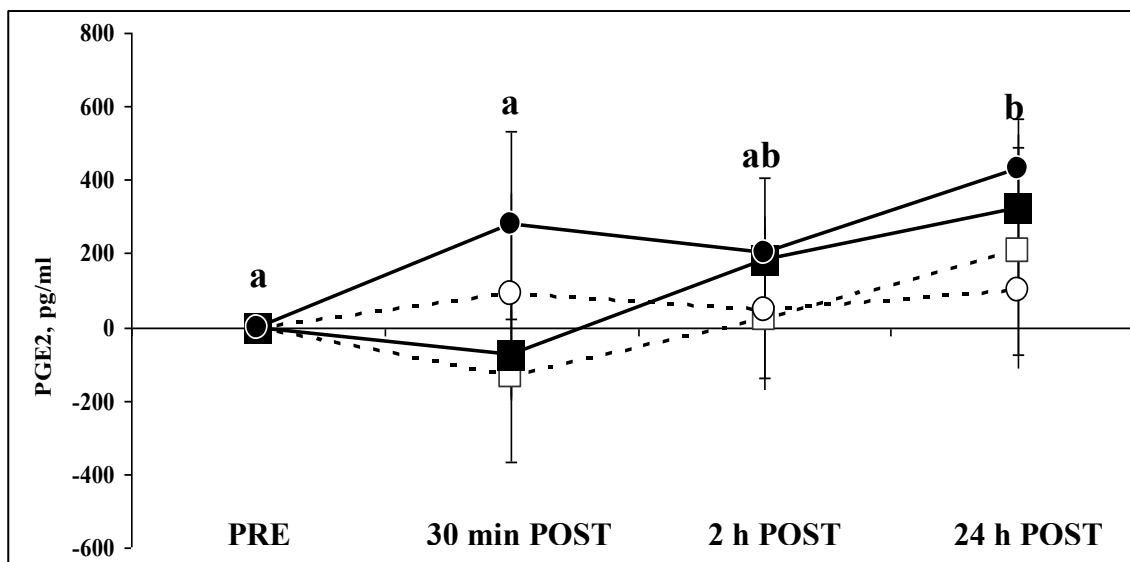


*Figure 5.* Exercise trial. Synovial fluid concentrations of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) before exercise (PRE), 30 min, 2 h, and 24 h following exercise (POST). Grey bars

represent TRT group and open bars represent CON group. Data are presented as mean  $\pm$  SE. Significant differences were not detected ( $P > 0.05$ ).



*Figure 6.* Exercise trial. Synovial fluid concentrations of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) during experimental Phases 1 and 2. Data are presented as mean  $\pm$  SE;  $P = 0.1$ .



*Figure 7.* Exercise trial. Relative synovial fluid concentrations of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), 30 min, 2 h, and 24 h following exercise (POST) compared to samples collected 24 h prior to exercise (PRE). Data were adjusted by subtracting PRE values from each sample time, and are presented as mean relative change from PRE  $\pm$  SE. Squares indicate TRT group and circles indicate CON group, no differences ( $P > 0.05$ ) were detected between these groups. Greater ( $P = 0.04$ ) increases in PGE<sub>2</sub> in hock joints (solid shapes) compared to carpus joints (open shapes) were detected. Differences in superscripts indicate changes within sample time. Statistical significance was inferred at  $P < 0.05$ , and trends were defined as  $P < 0.1$ . Differences in PGE<sub>2</sub> concentrations were detected between PRE and 24 h POST ( $P = 0.03$ ) as well as 30 min POST and 24 h POST ( $P = 0.08$ ).

## Overall Discussion

Along with the transitioning of the horse's role in modern society from farming and agriculture to sport and recreation, came new physical demands and stressors. Despite this changed societal role, equine-related economic impact remains significant, and warrants new and innovative technologies to keep our equine athletes and companions healthy and performing at their genetic potential. Chronic diseases, with etiologies embedded in inflammation and oxidant stress, are prevalent among all breeds of horses and are influenced by many factors. Due to breed-specific, conformational, biomechanical, and individual physiological variation as well as the wide variety of sport and recreational activities horses participate in, there exists a multitude of unique physiological responses to each of these activities, many of which have yet to be characterized. Efforts have been made to understand these responses and the implications they have on the overall performance, health and well-being of the horse, however, inconsistent methodologies and limited experimental control have resulted in a disjointed body of knowledge from which we have to reflect upon. Furthermore, a focus on identifying treatments for clinical conditions, rather than preventative measures, has been common practice. A shift in this paradigm, from treatment to preventative strategies and early disease detection, could be useful and economically advantageous to the equine community. The purpose of the research presented herein was to build upon existing knowledge, with the intent to provide useful tools and references with which anyone could use to evaluate interventions intended to manage inflammation and antioxidant status in horses undergoing intense exercise.

Standardized models and methodologies lend researchers an equal playing field on which to compare research, analyze data, draw conclusions, and raise questions that further advance our knowledge with increased efficiency. Overall, this research provides a snapshot of the physiological response to different exercise modalities in healthy mature unfit horses. Understanding the differences, or lack thereof, with different types of exercise can be useful in building management and training programs designed to maximize the athlete's potential, while preserving ideal health status and increasing the longevity of an athletic career.

Overall, the data presented indicates that a single bout of exhaustive exercise does elicit an inflammatory response as well as changes in antioxidant status in healthy mature Standardbreds. Increases in biomarkers of inflammation and anabolic cartilage metabolism in synovial joints were also described following intense exercise in the same horses. These physiological changes during and following exercise are most likely integral in the adaptive response to exercise necessary to improve fitness status and prevent injury upon subsequent athletic endeavors. Furthermore, it is suspected that differences in breed, biomechanics, and conformation have a significant impact on inflammatory processes, specifically in joints, which should be taken into consideration and monitored closely.

Probably the most significant contribution that the present body of work has made to the literature, is a partial characterization of a healthy response to intense exhaustive exercise in healthy mature Standardbreds. "Normal parameters" for inflammatory cytokine response and antioxidant status following a single bout of intense exercise have not been fully outlined in healthy horses, which makes identifying normal vs. abnormal

responses difficult. An inability to distinguish between adaptive and pathological changes leaves the door open for the onset of chronic conditions and the subsequent onset of disease. This is particularly true for joint disease, which remains a high concern for equine professionals and enthusiasts alike. Early detection and preventative action seems to be the best strategy for reducing the incidence of arthropathies in horses. Therefore, the present work provides a reference point when assessing exercise-induced changes in healthy horses. This work is not intended to be extrapolated to other species and repetition of these methodologies is necessary to further validate findings.

As with most research, many more questions have been raised than have been answered. It would be useful to have a set of data detailing the same subset of inflammatory, antioxidant, and performance parameters in a fit versus unfit equine model to evaluate the effects of exercise training, as well as exploring differences related to aerobic versus anaerobic types of exercise. One could even go a step further and look at differences relevant to breed, age, or sex.

Oral supplementation of superoxide dismutase (SOD) was found to be a safe, although ineffective antioxidant, anti-inflammatory, and chondroprotective agent in horses. The failure of SOD to exert any effects in the present work warrants investigation into technology development that would facilitate successful delivery of oral supplements to the intended site of action. The convenience, and in many cases cost effectiveness of oral formulations versus injectable or physical therapies, demands this type of inquiry, and drives the nutraceutical market despite a lack of safety and efficacy validation, and regulatory oversight. Not surprisingly, SOD has been included as an active ingredient in several nutraceutical blends and is currently available on the market, for horses. This is

not a unique situation, as most supplements hit the market with little or no research to validate label claims, leaving consumers with lighter pocket books, difficult decisions, and often ineffective solutions for their horse. The type of research presented here, provides a framework with which investigators could use to ultimately save consumers money, provide regulatory criteria, and develop safe, effective interventions for exercise-induced maladies for the equine community. The nutraceutical market is a vast and influential industry that needs tighter regulation founded upon sound scientific evidence of basic safety and efficacy in the target species to validate product claims.

## Curriculum Vita

### Emily Dawn Lamprecht

#### I. DEGREES EARNED

- December, 2003     University of Missouri-Columbia, Columbia, MO, College of Agriculture, Food, and Natural Resources, B.S. Animal Science.
- May, 2004           University of Missouri-Columbia, Columbia, MO, College of Arts and Sciences, B.A. Psychology.
- January, 2010       Rutgers, The State University of New Jersey, New Brunswick, NJ, School of Environmental and Biological Sciences, Ph.D. Graduate Program in Endocrinology and Animal Biosciences

#### II. PRINCIPLE OCCUPATIONS

- August, 2004 – June, 2005     Teaching Assistant, Rutgers University - *Biology (01:119:101/102)*
- August, 2005 – June, 2006     Graduate Fellow - *National Science Foundation*
- August, 2006 – December, 2006   Teaching Assistant, Rutgers University - *Animal Nutrition Laboratory (11:067:331)*
- January, 2007 – June, 2007       Teaching Assistant, Rutgers University - *Animal Handling and Fitting (11:067:175)*
- August, 2007 – December, 2007   Teaching Assistant, Rutgers University - *Animal Nutrition Laboratory (11:067:331)*
- January, 2008 – June, 2008       Teaching Assistant, Rutgers University - *Animal Microtechniques and Tissue Culture (11:067:430)*

#### III. PUBLICATIONS

- Lamprecht, E. D.**, C. A. Bagnell, C. A. Williams. 2009. Inflammatory responses to three modes of intense exercise in Standardbred mares – A pilot study. *Comp. Ex. Phys.* 5:115-125.
- Fiorellino, N. M., **E. D. Lamprecht**, C. A. Williams. 2009. Absorption of different oral formulations of natural vitamin E in horses. *J. Equine Vet. Sci.* 29:100-104.
- Williams, C. A. and **E. D. Lamprecht**. 2008. Some commonly fed herbs and other functional foods in equine nutrition: A review. *Vet. J.* 178: 21-31.