INFLUENCE OF SUPPLEMENTATION OF *SACCHAROMYCES CEREVISIAE* FERMENTATION PRODUCT ON INFLAMMATION IN YOUNG HORSES

A Thesis

by

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ABSTRACT

Mitigation of exercise-induced stress is of key interest in determining means by which to optimize performance horse health. To this end, dietary interventions that may attenuate damaging responses to stress in performance horses have received much attention but have yet to be clearly elucidated. The objective of this study was to test the hypothesis that dietary supplementation with a *Saccharomyces cerevisiae* fermentation product (Original XPC, Diamond V Mills, Inc., Cedar Rapids, IA) would decrease markers of exercise-induced stress in young horses. Quarter Horse yearlings (mean ± SD; 9 ± 1 mo) were fed a basal diet consisting of 1.25% BW/d (DM basis) custom-formulated commercial grain split into 2 equal feedings per day plus coastal bermudagrass hay *ad libitum.* Yearlings were balanced by gender, age, BW, and farm of origin and randomly assigned to one of two treatment groups: 1) no supplementation (CON; n=9), or 2) 21 g/d *Saccharomyces cerevisiae* fermentation product (10.5 g/feeding; SCFP; n=10). After 8 wk of treatment, horses underwent a 2-hr standardized submaximal exercise test (SET) on a free stall mechanical exerciser. Serum was collected before supplementation (wk 0), and at wk 8 pre-SET, and 0, 1, and 6 hr post-SET, and evaluated for serum amyloid A (SAA), cortisol, and cytokine concentrations by commercial ELISA. Data were analyzed using the mixed procedure in SAS v9.4 with repeated measures (time). Diet, time, and the diet × time interaction were included as fixed effects and horse(diet) was a random effect. In response to the SET at wk 8, SAA increased at 6 hr post-SET in CON ($P < 0.0001$) but was unchanged through 6 hr post-SET in SCFP. Serum cortisol increased in both groups immediately after the SET (0 hr post-SET; $P \leq 0.0005$) but returned to pre-SET levels in SCFP by 1 hr post-SET. At 6 hr post-SET, cortisol in CON had returned to pre-SET concentrations, while SCFP declined to be lower than pre-SET ($P = 0.0001$) and lower than CON ($P = 0.01$) at that time point. Eight weeks of

dietary supplementation with 21 g/d of SCFP may mitigate stress following prolonged exercise in young horses.

DEDICATION

I dedicate this thesis to my father, who has been my biggest supporter and my rock through this whole journey.

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CHAPTER I

INTRODUCTION

Performance horse health, particularly health of young horses entering exercise training programs, is of utmost importance to maintaining quality of life and performance longevity in horses. Various mechanisms to improve the longevity of performance horses have been investigated, including the role of stress-induced immune and inflammatory responses. Following a stressor such as exercise, pro- and anti-inflammatory cytokines, acute phase proteins (APPs), and stress hormones are released, which are responsible for many of the cardinal signs of inflammation, such as pyrexia (Pedersen et al., 2000; Crisman et al., 2008). Inflammation is the body's protective response to deviations or challenges to tissue homeostasis. This protective response is necessary for stress-induced cellular adaptations but can manifest as swelling, heat, pain, and loss of function (Chovatiya et al., 2014), and the resolution of inflammation is essential to prevent unnecessary cell damage (Landskron et al., 2014). Dietary additives, such as byproducts of yeast fermentation, have been the focus of several animal and human studies because of their potential immune boosting and anti-inflammatory effects (Evans et al., 2012, Glade et al., 1990, Morgan et al., 2007, Moyad et al., 2010, Paulsen et al., 2010, Zhu et al., 2017). Supplementation with a *Saccharomyces cerevisiae* fermentation product (SCFP) in rats appeared to provide both moderate immune-enhancing and anti-inflammatory benefits, demonstrating its potential to favorably modulate immune responses without excessive suppression or stimulation of overall immune activity (Evans et al., 2012). In addition, supplementation with SCFP decreased heat stress in rats and broiler chickens (Ducray et al., 2016, Price et al., 2018). In horses, Saccharomyces cerevisiae fermentation product supplementation enhanced submaximal exercise performance in endurance Arabian horses

(Wickler 2002) and increased the digestibility of low-quality forage (Morgan et al., 2007). The objective of this study was to evaluate the effects of dietary supplementation of a Saccharomyces cerevisiae fermentation product on systemic inflammation and the cellular stress response following exercise in young horses. It was hypothesized that horses receiving the Saccharomyces cerevisiae fermentation product would have lower systemic concentrations of stress markers and inflammation than control horses following a prolonged exercise stressor.

CHAPTER II

REVIEW OF THE LITERATURE

The Immune Response

Multiple systems in the body that work cohesively to promote and sustain the viability of overall function. One mechanism the body employs to detect, prevent, and limit infection is the immune system. The immune system is the body's defense to prevent or limit infection and tissue damage. Beisel (1999) broadly defined the immune system as all mechanisms and responses used by the body to defend itself against foreign substances, microorganisms, toxins, and noncompatible living cells. This system can detect when the body is no longer in equilibrium by recognizing and reacting to a variety of signals known as danger-associated molecular patterns (DAMPs). Disturbances to homeostasis are due to unhealthy cells, which can be affected by a range of issues such as infection or cellular damage caused by non-infectious agents like acute exercise (Chaplin 2010). When the immune system first recognizes these signals, it responds to address the problem in what is called the immune response, which can be broken down into two general categories: the innate immune response and the adaptive immune response. The innate immune system is the dominant immune system response and includes all facets of the host's immune defense mechanisms. Innate immunity is expressed in a variety of ways, from the initial response to physical and chemical stimuli to the coordinated recruitment and action of a series of specialized cell populations (Madsen et al., 2014). Because this system makes up the initial host response and the molecules used are expressed broadly on a large number of cells, the response activates rapidly after an invading pathogen or toxin is encountered. One part of the innate immune response includes contributions from many subsets

of leukocytes (white blood cells), including lymphocytes, granulocytes, monocytes, and macrophages. These cells release many different effector molecules, including cytokines that regulate the function of other cells, chemokines that attract inflammatory leukocytes, lipid mediators of inflammation, reactive free radical species, and bioactive amines and enzymes that also contribute to tissue inflammation (Chaplin 2010). Local and systemic inflammation is an immune response to deviations or challenges to tissue health such as infection, injury, or exercise, which allows for the repair of damaged tissue and a return to homeostatic conditions (Lamprecht et al., 2008).

The adaptive immune response is the second set of immune reactions and is a smaller response compared to the innate immune response. It is usually more prominent several days after the innate response and is generally expressed temporally. The adaptive immune response is composed of small numbers of cells that have specificity for a particular pathogen, toxin, or allergen, and proliferate after encountering their specific noxious stimuli. These long-lived cells can persist in a dormant state but will re-express effector functions rapidly after another encounter with their specific antigen (Chaplin 2010). These adaptive responses are primarily located on antigen-specific receptors expressed on T and B lymphocyte surfaces. In summary, the adaptive response manifests an "immune memory," allowing it to mobilize a more effective host response against specific pathogens when they are encountered a second time, even decades after the initial sensitizing encounter (Chaplin 2010).

The innate and adaptive immune systems have often been described as separate systems of the host response (Chaplin 2010). However, they usually act together, with the innate response representing the first line of host defense and the adaptive response following after several days, as antigen-specific cells proliferate. Additionally, many aspects of both systems act cohesively.

Components of the innate response contribute to the activation of the adaptive response's antigen-specific cells, and in turn, antigen-specific cells amplify their responses by recruiting innate mechanisms to greater control of invading microbes. In summary, the innate and adaptive immune responses are fundamentally different in their mechanisms of action; however, cooperation between them is essential for a fully effective immune response.

Inflammation and the Acute Phase Response

Inflammation is a biological reaction to a physical stressor which possibly evolved as an adaptive response for restoring tissue homeostasis (Medzhitov 2008, Ashley et al., 2012). Acute inflammation is the early (almost immediate) response that is triggered by noxious stimuli and conditions, such as infection and tissue injury (Chandrasoma et al., 1998, Medzhitov 2008). Acute inflammation may be regarded as the first line of defense against damage and is characterized by changes in the microcirculation including exudation of fluid and migration of leukocytes from blood vessels to the area of injury (Chandrasoma et al., 1998). Inflammatory reactions involve many pathophysiological processes and cell types. The primary function of localized inflammation is to isolate the underlying source of the disturbance and attract specialized cells and their products to the affected area so they can destroy potential pathogens, remove damaged tissue, and restore tissue health (Beisel 1999, Ashley et al., 2012). The healing process of inflammation involves the proliferation of fibroblasts, synthesis of collagen, phagocytic removal of inflammatory debris, and eventual disappearance of vascular congestion and edema (a common physical sign of inflammation; Medzhitov 2008). The local inflammatory response is accompanied by a systemic reaction and dramatic change in liver function known as the acute phase response (APR; Ostrowski et al., 1998, Nieman et al., 1999, Pedersen 2000). This response is a nonspecific and complex inflammatory response induced by a stressor and is

intended to bring defense cells (immune cells) to an affected area, inactivate, and destroy invaders. The APR is also designed to minimize tissue damage, enhance the repair process, and restore equilibrium after infection, trauma, or stress and is stimulated when injured cells release arachidonic acid metabolites and products of oxidative stress (Chisman et al., 2008). The APR can occur in response to many different types of stressors, such as generalized acute infectious illnesses, trauma, severe inflammatory processes, tissue injury, and other medical and surgical diseases (Beisel 1995; Cunningham-Rundles et al., 1993; Forse et al., 1994). The initial recognition of infection or injury is mediated by tissue-resident macrophages and mast cells, leading to the production of a variety of inflammatory mediators, including cytokines, which are released into the circulation by lymphocytes, monocytes, macrophages, and endothelial cells and also produced locally in tissues by resident macrophages and other types of cells (Nieman 1999). One of the purposes of these mediators is to elicit an inflammatory response locally, allowing polymorphonuclear leukocytes (PMN), neutrophils, monocytes, and other cells that are usually restricted to the blood vessels to penetrate blood vessel endothelium and enter the area of inflammation; this penetration is abetted by the release of chemoattractants (Beisel 1999, Medzhitov 2008). When neutrophils reach the damaged tissue site, they become activated, either by direct contact with pathogens or through the actions of cytokines secreted by tissue-resident cells and they attempt to kill invading agents by releasing the toxic contents of their granules. A successful acute inflammatory response results in the elimination of the infectious agents followed by a resolution and repair phase, which is mediated mainly by tissue-resident and recruited macrophages (Medzhitov 2008). However, an ongoing APR or chronic inflammation can lead to unnecessary cellular damage and even trigger cellular events that can promote malignant transformation of cells and carcinogenesis (Landskron et al., 2014).

Systemic Markers of Stress and Inflammation

Cytokines

The acute phase response and inflammation involve a tightly regulated cascade of immunological and physiological processes that are coordinated and controlled by immune signaling molecules called *cytokines* which are released both in the blood and locally at the site of inflammation (Ostrowski et al., 1998, Beisel 1999, Ashley et al., 2012). Cytokines facilitate an influx of specialized cells which participate in the clearing of antigens and healing of tissue. Injection of certain cytokines, such as tumor necrosis factor-α (TNFα), interleukin-1 (IL-1) and IL-6, into laboratory animals or humans will produce most, if not all, aspects of the acute phase response (Dinarello 1992, Dinarello 1997, Richards et al., 1998, Ostrowski et al., 1999, Pedersen et al., 2000). Although research investigating cytokines in horses is relatively new, the fundamental importance of their diverse functions is recognized and acknowledged in human studies.

Cytokines are small peptides that function as intercellular signals and mediators and are released from immune cells throughout the body (Beisel 1999). Cytokines come in numerous cell types, such as interferons, ILs, and growth factors, and can be either pro- or anti-inflammatory in function. When aseptic and septic inflammatory stimuli activate macrophages or monocytes, they quickly respond by producing pro-inflammatory cytokines, which travel via the blood to interact with specific receptor proteins located on the walls of target cells throughout the body (Beisel, 1995, Jacobsen et al., 2007, Chaplin 2010). Most cytokines work as monomers (a molecule that can be bonded to other identical molecules to form a polymer), although some exist as homodimers (IL-10), heterodimers, and trimers (TNFα; Rossio, 1999). Cytokines act on cells through transmembrane cell-surface receptors, meaning that the binding of the cytokine to

the receptor causes the cellular response by activating an intracellular signal transduction pathway. The activation of the signal pathway ultimately leads to induction of new gene transcription and acute phase protein (APP) synthesis (Rossio, 1999, Jacobsen et al., 2007, Chaplin 2010). Cytokines have diverse functions essential for immune cell growth, activation, and function. They promote, sustain, and terminate specific immunological responses that are appropriate for the pathogen or antigen toward which the response is directed (Rossio, 1999). In addition to their multiplicity of actions, cytokines tend to have high redundancy, with overlapping effects being common, and some cytokines even stimulate the synthesis and release of other cytokines (Beisel 1999). Since most cytokines have multiple functions, and some features overlap between different cytokines, they are considered to be highly synergistic and highly potent (Nieman 1999).

Cytokines are frequently divided into two groups and defined as either pro or anti-inflammatory cytokines. The 'pro-inflammatory' group is broad and includes cytokines such as IL-1 and TNF, and the 'anti-inflammatory' group includes IL-10. The pro-inflammatory group controls the growth and differentiation of T- and B-lymphocytes (cells that recognize foreign materials), initiate APRs, launch immune system activities, trigger central nervous system (CNS) responses, and stimulate the release or suppression of hormones (Beisel 1999). Previous work investigating pro-inflammatory cytokines has shown that pro-inflammatory cytokines also participate in inflammatory reactions. Cassatella et al. (1994) reported that TNFα and IL-1β were produced by PMN after stimulation with a noxious agonist such as lipopolysaccharide (LPS). Many studies have also shown that injection of TNFα, IL-1β, and IL-6 into laboratory animals or humans will produce most, if not all aspects of the acute phase response (Ostrowski et al., 1998).

IL-1

Interleukin-1 is part of the pro-inflammatory group of cytokines, which means it helps induce the acute phase response (APR) and inflammation. Interleukin-1 is a polypeptide produced by activated macrophages, which facilitates many host defense adaptations to environmental and infectious stresses (Cannon et al., 1986). The biological activities of the two isoforms of IL-1, IL-1β and IL-1α, are indistinguishable. Interleukin-1 upregulates a variety of genes, including those that upregulate both its own expression and IL-6, and induces enzymes necessary for the synthesis of leukotrienes and prostaglandins (Lamprecht et al., 2008). Interleukin-1 affects nearly every cell type, often in concert with another pro-inflammatory cytokine, TNFα. Although IL-1 can upregulate host defenses and function as an immunoadjuvant, IL-1 is a highly inflammatory cytokine, and the induction of cyclooxygenase-2 (PTGS2/COX2) by IL-1 in the central nervous system (CNS) is found to contribute to inflammatory pain hypersensitivity (Ren et al., 2009). Interleukin-1 receptor antagonist (IL-1ra) and IL-10 have been found to inhibit and regulate IL-1.

TNFα

Tumor necrosis factor alpha (also known as cachectin) is a multifunctional trimer cytokine that is mainly secreted by neutrophils, activated T and B lymphocytes, NK cells, astrocytes, endothelial cells, and smooth muscle cells. Tumor necrosis factor α is classified as pro-inflammatory and is a component of the acute phase response (APR). Tumor necrosis factor cytokines are part of the family of cytokines that stimulate immune-cell proliferation and activation and are critical for activating inflammatory responses (Rossio, 1999). Tumor necrosis factors have a direct or indirect effect that results in the killing of cells bearing foreign antigens, such as viral-infected cells, cells infected with intracellular parasites, or cancer cells (Rossio,

1999). Tumor necrosis factor is also involved in the regulation of a broad spectrum of biological processes, including cell proliferation, differentiation, apoptosis, lipid metabolism, and coagulation. Many of the actions produced by TNFα are functionally similar to the effects produced by IL-1and has been a target inflammatory mediator investigated in several studies due to its central role in the initiation of the immune response to injury or infection (Lamprecht et al., 2008). The over-production of TNFα has been implicated in several pathological conditions, including fever, cachexia (progressive wasting), septic shock following infection with Gram-negative bacteria, autoimmune disorders, and meningococcal septicemia (Beisel, 1999, Parameswaran et al., 2010).

IL-6

Interleukin-6 a cytokine that functions in inflammation and the maturation of B cells and is primarily produced at sites of acute and chronic inflammation, where it contributes to host defense through the stimulation of acute phase responses, hematopoiesis, and immune reactions. After IL-6 is synthesized in the initial stage of inflammation, it moves to the liver through the bloodstream, resulting in the rapid induction of the APR and an extensive range of acute phase proteins such as C-reactive protein (CRP) and serum amyloid A (SAA; Tanaka et al., 2014). Interleukin-6 has been classified as both a pro- and an anti-inflammatory cytokine, as it has demonstrated both effects in different studies, but more recent literature suggests that IL-6 has mostly regulatory effects during the APR (Pedersen 2000). Some of the misinterpretations of IL-6 may stem from the fact that assays used in early studies could not distinguish between IL-1 and IL-6 and were thought to have similar functions (Bagby et al., 1996, Ostrowski et al., 1998, Ostrowski et al., 1999). It is now known that IL-6 directly inhibits the expression of TNFα and IL-1 as well as upregulates the production and secretion of IL-10 which, in turn, inhibits TNFα

and IL-1β production. Due to its sometimes conflicting effects, many investigators have found it more reasonable to classify IL-6 as an inflammation-*responsive* cytokine rather than a pro- or anti-inflammatory cytokine, since IL-6 does not directly induce inflammation, but increases in response to it (Ostrowski et al., 1998, Pedersen 2000, Pedersen et al., 2000, Lamprecht et al., 2008). However, similar to IL-1 and TNFα, dysregulated or continual synthesis of IL-6 has a pathological effect on chronic inflammation and autoimmunity. Some of the pathological effects of excess IL-6 include, but are not limited to, amyloid A amyloidosis, hypozincemia seen in inflammation, and serum iron hypoferremia and anemia associated with chronic inflammation. IL-6 can also induce the excess production of vascular endothelial growth factor (VEGF) which can lead to enhanced angiogenesis and increased vascular permeability, which are pathological features of inflammatory lesions seen in synovial tissues of rheumatoid arthritis (RA; Tanaka et al., 2014).

IL-10

A number of different mechanisms can also inhibit the effects of cytokines, including anti-inflammatory cytokines. These cytokines are vital in the control of immune reactions by limiting the extent of the inflammatory response. For example, anti-inflammatory cytokines may terminate the immune and inflammatory response when the noxious stimuli have been removed (Rossio, 1999). Cytokines with inhibitory actions can also block the synthesis and release of other cytokines. Target cells release cytokine receptors into the plasma, and these soluble free receptors will intercept and inactivate the cytokine before it reaches the target cell. Additionally, completely blocking proteins will obstruct cell wall receptors so that cytokines cannot exert their cellular effects.

Interleukin-10 is a homodimer and is classified as an anti-inflammatory cytokine (Rossio, 1999). Interleukin-10 has been shown to inhibit the release of TNFα and IL-1 and induces the production of IL-1ra (Jenkins et al., 1994; Cassatella et al., 1994, Chernoff et al., 1995, Ostrowski et al., 1999). Chernoff et al. (1995) demonstrated that a single intravenous injection of IL-10 was safe in humans, had an inhibitory effect on T cells, and it suppressed the production of the pro-inflammatory cytokines, TNFα and IL-1β. Furthermore, the half-life of *IL-1ra* mRNA was prolonged in the presence of IL-10 (Chernoff et al., 1995). A study conducted by Cassatella et al. (1994) showed that IL-10 selectively upregulated IL-1ra production in LPS-activated PMN, while it inhibited the production of IL-1β and TNFα. This study suggested that IL-10 may be an important physiologic regulator of cytokine production from PMN and emphasizes the potential role of IL-10 in inflammatory responses. Another study by Jenkins et al. (1994) showed similar results and concluded that the net effects of IL-10 on inflammatory cells might have a substantial role in the modulation of biologic responses to IL-1.

Even though little research exists investigating the role of cytokines in horses, human studies have shown that inflammatory cytokines play a crucial role in the development of inflammatory responses. There have been many studies investigating the activities of cytokines due to the desire to mitigate/control inflammation following a stressor (Rossio, 1999). Even though cytokine-induced responses are generally protective in nature, excess production or activity of cytokines can be harmful. In fact, 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 (Lamprecht et al., 2008). Because most studies studying the role of cytokines are in humans, more studies investigating the role of cytokines in performance horses are warranted.

Cortisol

A vital component of the APR includes the release of hormones. During injury or illness, as well as other forms of stress, communication between the brain and immune system is crucial (Smith 2003). By definition, a hormone is a signaling molecule that regulates and coordinates physiologic and metabolic functions by acting on receptors located on or in target tissues (McKeever et al., 2014). Hormones play an essential role in the immune and inflammatory responses that make up the APR and can act as biomarkers that can quantitatively characterize the APR. One hormonal biomarker typically measured is the hormone, cortisol, which is the primary glucocorticoid produced and secreted by the adrenal cortex.

Cortisol is a well-documented marker of stress in both humans and horses, and it is often referred to as the "stress hormone" due to its involvement in the stress response (*e.g.,* affecting blood pressure, blood sugar, and other stress adaptations; Hannibal et al., 2014). The biological roles of cortisol fall into two major categories: substrate mobilization and immune modulation. Immunologically, cortisol functions as an anti-inflammatory mediator and plays a role in hypersensitivity, immunosuppression, and disease resistance (Hannibal et al., 2014). Furthermore, the release of glucocorticoids like cortisol modulates the intensity of the acute phase response, virtually all of whose components are inhibited by glucocorticoids, including cytokine production. The secretion of many inflammatory mediators by activated lymphocytes and macrophages is also inhibited (Nieman 1999). Once released, cortisol is taken up by a variety of tissues throughout the body, such as skeletal muscle, adipose tissue, and the liver. At these different tissues, the presence of cortisol mediates critical physiological processes which aid in stress recovery. Nieman (1999) described the pituitary-adrenal response to stress (*i.e.,* the release of cortisol) as a "mechanism to suppress and modulate an overexuberant inflammatory

response to toxins, antigens, injury, and invading organisms." Nieman explained that this is an essential biological function that serves as a balance to inflammatory reactions, as intense mobilization of cytokines and inflammatory mediators can potentially be fatal (Nieman, 1999).

The anti-inflammatory and immunosuppressive effects of cortisol have been well documented in both humans and horses (Smith 2003, Hannibal et al., 2014). A study in humans demonstrated that cortisol treatment decreased pro-inflammatory cytokine levels in the blood (TNFα IL-1β, and IL-6; Derijk et al., 1997, Pedersen 2000, Starkie et al., 2005, Edwards et al., 2006) as well as suppressed their actions (Smith, 2003). The immunosuppressive/anti-inflammatory effects of cortisol have been also shown to prevent the migration of leucocytes from circulation into extra-vascular fluid spaces, reduce the accumulation of monocytes and granulocytes at inflammatory sites, and inhibiting lymphocyte and leucocyte proliferation, migration, and cytotoxicity (Pedersen et al., 2000, Smith, 2003, Hannibal et al., 2014). Pedersen et al. (2000) also described a study in which corticosteroids given intravenously to humans caused lymphocytopenia, monocytopenia, eosinopenia, and neutrophilia that reach their maximum 4 h after administration.

Cortisol serves as a signal for cellular repair following a stressor, such as tissue damage after exercise (Davitt et al., 2014), which may elicit a permissive effect that is beneficial for training adaptation by preventing the immune system from stimulating an excessive inflammatory and immune reaction to acute exercise (Smith 2003, Edwards et al., 2006, McKeever et al., 2014). However, stress hormones such as cortisol are also shown to suppress macrophage and NK cell function, both of which are intimately involved in cell-mediated immunity (CMI; Smith, 2003). Because of the immunosuppressive effects of cortisol,

unresolved increases in cortisol in the blood may lead to decreased protection against intracellular pathogens, resulting in an increased incidence of infection (Smith, 2003).

Serum Amyloid A

The APR also includes the release of acute phase proteins (APPs) such as serum amyloid A (SAA). Much interest has been concentrated on APPs as potential indicators of the presence, degree, and time course of inflammation in recent decades. The production of APPs occurs in the liver and is mediated by the pro-inflammatory cytokines IL-1, IL-6, and TNFα, and secreted by the phagocytic cells in response to various types of tissue damage and the induction of the APR (Tarto et al., 2015). The key benefit to APPs as a marker of inflammation is that they have very low or undetectable levels in the plasma of healthy individuals. However, during the APR, plasma concentrations of APPs increase more than 10x, and sometimes 100x or 1000x, but a short half-life allows APPs to very quickly return to base levels as the acute stress recedes (Badolato et al., 1994, Jacobsen et al., 2007, Crisman et al., 2008, Turło et al., 2015a, Turło et al., 2015b). Serum amyloid A is one of the only identified and main major APP in the horse to date (Jacobsen et al., 2007). Serum Amyloid A is considered one of the more sensitive APPs and the literature has shown it to be a relatively accurate potential marker of subclinical inflammation or even microtrauma (McGowan et al., 2014, Turło et al., 2015a, Turło et al., 2015b). Serum amyloid A is an acute phase protein that is bound to high-density lipoproteins in the blood and secreted mainly by hepatocytes during the APR (Badolato et al., 1994). Modest, but noteworthy increases of SAA have been detected after a stressor such as strenuous exercise, heat stroke, and parturition, with a response time starting around 6 to 12 h after the stimulus and a peak at 48 h (Jacobsen et al., 2007, Crisman et al., 2008). The short half-life causes plasma SAA levels to decrease in close parallel with successful treatment and resolution of inflammation (Jacobsen et

al., 2007, Turło et al., 2015a). The characteristics of APPs mean that their concentrations are directly related to the severity of the underlying condition and can provide an objective measure of the severity and extent of the underlying condition (Chrisman et al., 2008, Turło et al., 2015a).

The physiologic roles of SAA are not entirely understood (Badolato et al., 1994), because various effects have been reported. The effects seen include enhancement or inhibition of leukocyte functions, chemotactic recruitment of inflammatory cells to the site of infection, inhibition of lymphocyte and endothelial cell proliferation, and inhibition of platelet aggregation. Other effects seen are phagocytosis (the ingestion of bacteria or other materials by phagocytes and amoeboid protozoans), lipid transportation to inflamed tissues, and the induction of enzymes involved in degradation of extracellular matrix (*i.e.,* metalloproteinases, collagenases; Badolato et al., 1994). When subcutaneously injected into mice, SAA recruited PMN cells and monocytes at the injection site, which suggested that SAA may participate in enhancing the migration of monocytes and PMN to inflamed tissues during an APR (Badolato et al., 1994). Serum amyloid A was also shown to inhibit myeloperoxidase release and modulate connective tissue breakdown in normal remodeling. These various effects observed suggest a 'housekeeping' role for the protein by providing a rapid defense against tissue injury from inflammatory conditions (Jacobsen et al., 2007, Crisman et al., 2008). However, when high-level concentrations of SAA persist for an extended period, serious complications and chronic inflammatory diseases may generate amyloid A amyloidosis, causing amyloid fibril deposition, which in turn causes progressive deterioration in various organs (Tanaka et al., 2014).

Acute phase proteins are being increasingly used as markers for identifying inflammation and monitoring response to treatment in horses. Use of APPs in veterinary medicine is becoming more widespread as more commercial diagnostic kits are validated (Crisman et al., 2008).

Serum amyloid A is the main acute phase protein (APP) in horses and is released into the bloodstream in the initial phase of inflammation and the APR (Tarto et al., 2015). The literature shows that in horses affected by inflammatory disorders of the gastrointestinal, respiratory and reproductive systems or in horses that develop complications after surgical procedures, SAA levels can exceed the physiological range from 10 to 1000 times which may be useful in disease diagnostics and prognostication (Jacobsen et al., 2005, Jacobsen et al., 2007, Canisso et al., 2014). The low or undetectable SAA levels in healthy horses, rapid and significant increase in plasma levels after an inflammatory stimulus, and the short half-life allow the course of inflammation to be monitored efficiently (Jacobsen et al., 2007).

Exercise as a Stressor

The immune response to exercise has been investigated in numerous studies, and similarities between exercise-induced and disease-related immune changes have been documented (Lamprecht et al., 2008). There is a great deal of research on the inflammatory response to exercise in human athletes because stress and inflammation have traditionally been associated with fatigue and impaired recovery from exercise (Liburt et al., 2010). However, less information is available regarding the inflammatory response to exercise in horses undergoing training. In humans and dog trials, a reaction analogous to the APR has been described after prolonged or strenuous exercise (Cywińska et al., 2012).

When at rest, the horse's internal environment can maintain homeostasis relatively easily. However, the introduction of work or exercise has the potential to disrupt internal balance and become a physiologic challenge that invokes an integrative response from multiple organ systems (Pedersen et al., 2000, McKeever, 2002, McKeever et al., 2014). Exercise induces numerous changes in the immune system, and many mechanisms appear to be involved in the

acute immune response to exercise, including changes in stress hormones and cytokine concentrations, body temperature changes, increases in blood flow, APP mobilization, and dehydration (Nieman 1999, Pedersen et al., 2000). Exercise, even in healthy individuals, is shown to lead to a robust inflammatory response characterized by mobilization of leukocytes and an increase in their numbers in the central circulation, an increase in circulating potent inflammatory mediators like IL-6, production of stress hormones such as cortisol, and the accumulation of APPs (Cooper et al., 2007, Crisman et al., 2008, Horohov et al., 2012). Even brief exercise can elicit an immune and inflammatory response (Cooper et al., 2007), but longer or progressively more intense work causes more systemwide alterations, which require integrated whole-body responses that involve neural and endocrine mediation (McKeever et al., 2014). Exercise can elicit an immunological "danger" type of stress and inflammatory response (Matzinger et al., 2001, Cooper et al., 2007) that is quantifiable and reproducible, can be modified experimentally, and thus considered as a prototype of stress. The immune system's response to strenuous exercise in humans is thought to mirror the immunological changes seen in response to clinical stressors such as surgery, trauma, and sepsis. Therefore, inducing exercise stress gives the ability to observe the different immune and inflammatory responses to stressors (Gleeson 2000, Pedersen, 2000, Edwards et al., 2006). Several factors, including the modality, intensity, and duration of exercise, influence the inflammatory response (Lamprecht et al., 2008). Even brief exercise can elicit an immune and inflammatory reaction that can induce immune changes for several hours, but longer or progressively more intense work causes more systemwide alterations that may last at least 24 h (Pedersen 2000, Cooper et al., 2007, McKeever et al., 2014).

The concept of immunosuppression in athletes following exercise has been investigated in many contemporary studies. Overtraining syndrome (OTS) is a term for when an athlete is training vigorously, yet performance deteriorates. One sign of OTS is suppressed immune function, with an increased incidence of upper respiratory tract infection (URTI; Smith, 2003). An increased incidence of URTIs has been associated with long duration or high-intensity training, such as a marathon, as well as with excessive/eccentric exercise (Smith, 2003). Nieman (1999) proposed that the relationship between exercise and URTI may be modeled in the form of a J-curve. This "J"-shaped model of relationship between varying amounts of exercise and risk of URTI. This model suggests that moderate exercise may lower risk of respiratory infection while excessive amounts may increase the risk (Nieman 1999). This model suggests that although moderate exercise training may decrease the risk of infection or illness when compared to a sedentary lifestyle, chance may rise above average during periods of excessive amounts of high-intensity exercise. However, it should be noted that more research using larger subject pools with improved research designs is necessary before this model can be accepted or rejected.

Recent studies have suggested that the exercise-associated induction of apoptosis (controlled cell death) may contribute to lymphocytopenia (abnormally low levels of lymphocytes in the blood) which could contribute to reduced immunity after intense exercise (Pedersen et al., 2000). Additionally, the lengthier and more intense the exercise bout (*e.g.,* marathon) the more extensive and prolonged the immune and inflammatory response. This is supported with studies showing moderate exercise bouts (< 60% maximal aerobic power and < 60 min duration) evoking little change from resting levels (Nieman 1999). However, training sessions in horses can sometimes last over 60 min, especially in competitions, and therefore may qualify as more than moderate exercise. The literature, in summary, suggests that the immune

system is suppressed following prolonged endurance exercise or high-intensity exercise, which decreases host protection (Nieman 1999).

Markers of Stress and Inflammation in Response to Exercise

Identifying markers of systemic and cellular stress and inflammation remains a popular topic in both human and animal studies because of a desire to control post-exercise stress and mitigate the associated inflammation. In human athletes, consequences related to increased expression of inflammatory mediators post-exercise can range from mild symptoms of delayed-onset muscle soreness to more debilitating problems related to soft tissue, joint, and bone damage (MacIntrye et al., 1995, Smith 2004, Lamprecht et al., 2008, Liburt et al., 2010, Horohov et al., 2012). Equine athletes, like their human counterparts, suffer from challenges related to exercise. Although the causes of lameness are varied, increased attention has been focused on the role of inflammatory mediators in this process (Horohov et al., 2012). Exercise-induced inflammation is a common problem in the equine athlete, resulting in impaired health, lost training time, and millions of dollars in veterinary expenses in the equine industry per year (Lamprecht et al., 2008). Injuries of bone, muscle, and tendon resulting from repetitive mechanical overload are some of the primary health issues in performance horses and are responsible for a considerable number of lost training days in racehorses (Dyson et al., 2008).

Early recognition of horses at risk of suffering the injury is crucial and the range of blood biomarkers have been examined in that context (Turło et al., 2015a) A study conducted with 2- and 3-year-old racing Thoroughbreds by Frisbie et al. (2010) investigated seven serum biomarkers for the detection of musculoskeletal injuries. A unique biomarker pattern occurred before each type of injury investigated and was beneficial in classifying horses as injured or uninjured. The researchers concluded that biomarkers have the potential to be used as a

screening aid prior to a musculoskeletal injury (Frisbie et al., 2010). Work with biomarkers has shown promise both in man and in horses for assessing musculoskeletal stress, but further study to evaluate biomarker levels horses is required as most work has mainly focused on bone and joint tissues (Ray et al., 1996; Poole 1997; Frisbie et al., 2008, Frisbie et al., 2010). Properly understood biomarkers could lead to early recognition of injury, identification of overtraining, and easier monitoring of recovery progression. Identified systemic biomarkers of stress and inflammation in horses include, but are not limited to, cortisol, SAA, pro-inflammatory cytokines (IL-1α/β and TNFα), inflammatory responsive cytokine IL-6, and anti-inflammatory cytokines (IL-10).

Cytokines

Exercise, even in healthy individuals, leads to a robust inflammatory response characterized by mobilization of cytokines and an increase in their concentrations in the central circulation (Cooper et al., 2007). Exercise-induced microdamage and stress stimulate a complex cascade of non-specific events known as the inflammatory response, which allows the body to restrict tissue damage to the site of the damage. This local response involves the production of cytokines that are released at the site of inflammation, including IL-1, IL-6, and TNFα, which are the cytokines that have been most extensively studied in response to exercise. The first study suggesting that exercise provoked a cytokine response reported that plasma obtained from human subjects after exercise, and injected intraperitoneally into rats, elevated rectal temperature (Cannon et al., 1983, Pedersen et al., 2000). Human studies then showed an upregulated pro-inflammatory cytokine or APR to intense exercise along with a small anti-inflammatory response. Contemporary studies have shown that even local musculoskeletal (work-related) injuries can lead to increases in systemic levels of mediators like TNFα and IL-1β that indicate

inflammation (Cooper et al., 2007). It should be noted that even though many studies have and still are investigating the response of cytokines after exercise, the results of those studies are inconsistent and sometimes conflicting, only agreeing that cytokine levels increase in some way after eccentric exercise (Pedersen 2000).

IL-1

Increases in IL-1(α/β) in the blood following exercise have been reported primarily in studies where the exercise is long term, such as in a marathon or endurance race. Interleukin-1β is thought to be produced locally within tissue, and therefore, it is suspected that the delayed response may reflect leakage into circulation upon tissue damage (Lamprecht et al., 2008). Early studies demonstrated that exercise induced an increase in IL-1 (Cannon et al., 1986; Evans et al., 1986); however, later it was realized that the biological assay used in early studies could not distinguish between IL-1 and IL-6 (Bagby et al., 1996, Ostrowski et al., 1998, Ostrowski et al., 1999). A study by Cannon et al. (1986) investigated the possibility that IL-1 secretion might be mediated by stress hormones associated with exercise. The study showed that IL-1 secretion by monocytes increased by the addition of physiological concentrations of epinephrine in vitro as well as low levels of hydrocortisone. However, higher concentrations in the physiological range did not affect, while combinations of epinephrine and hydrocortisone suppressed IL-1 secretion (Cannon et al., 1986). Cannon et al. (1986) also reported that IL-1 activity appeared in plasma several hours after exercise on a cycle ergometer (1 h at 60% of aerobic capacity). Similarly, urine levels of IL-1β increased until 12 h after long-distance running (Sprenger et al., 1992). One study investigated IL-1 levels after a 45-min bout of high-intensity eccentric exercise in trained vs. untrained men (Evans et al., 1986). In the trained men, plasma IL-1 was higher than in untrained men before exercise but did not significantly increase after exercise. In comparison,

IL-1 was significantly elevated 3 h after exercise in the untrained men. The results of this study suggest that training may affect IL-1β levels after exercise because eccentric exercise may lead to delayed muscle damage in untrained individuals (Evans et al., 1986). Even though many studies have demonstrated elevated levels of IL-1 after exercise, there are other studies that found no changes or only slightly elevated levels of IL-1 in plasma post-exercise (Sprenger et al., 1992; Ullum et al., 1994a; Drenth et al., 1995; Bruunsgaard et al., 1997; Nehlsen-Cannarella et al., 1997; Ostrowski et al., 1998). These conflicting results are likely due to inconsistent variables in each study, such as mode and duration of exercise, as well as different gender and ages of participants.

TNFα

Inconsistent findings have been reported for TNFα responses to exercise (Ostrowski et al., 1998). Dufau et al. (1989) and Espersen et al. (1990) reported increased plasma TNFα 2 h after completing a 2.5-h run and 1 h after a 5-km run, respectively, but other studies have failed to detect systemic TNFα after exercise (River et al., 1994, Ullum et al., 1994a, Ullum et al., 1994b). A study by Sprenger et al. (1992) showed elevated urine levels of TNFα immediately after 2 h of running, but a subsequent rapid decline to pre-exercise levels. Conversely, Ostowski et al. (1998) was not able to detect TNFα mRNA in blood or muscle samples 2 h after a marathon race; similarly, Drenth et al. (1995) did not detect an effect of exercise on plasma concentrations of TNFα after a 6-h endurance run. One of the few studies investigating TNFα in horses showed that exercise prompted significant increases in IL-1 and TNFα in blood and IL-6 and TNFα in muscle following an incremental graded exercise test (GXT) on a treadmill (1 m/s increases each min until fatigue, 6% grade; citation). There were no changes in muscle IL-1

or blood IL-6 following the GXT, and all pro-inflammatory cytokine levels in both blood and muscle returned to pre-GXT levels by 24 h post exercise. This study concluded that high-intensity exercise resulted in increases in the expression of inflammatory cytokines in both muscle and blood (Liburt et al., 2010).

IL-6

Interleukin-6 was initially thought to be a pro-inflammatory cytokine similar to IL-1, which may have resulted from the fact that early assays could not distinguish between IL-1 and IL-6. Therefore, IL-1 and IL-6 were assumed to have similar functions. However, more recent studies have demonstrated that IL-6 is an inflammation-*responsive* cytokine rather than a pro- or anti-inflammatory cytokine, since IL-6 does not directly induce inflammation but increases in response to inflammation (Ostrowski et al., 1998, Pedersen 2000, Pedersen et al., 2000, Lamprecht et al., 2008). Most of the literature agrees that IL-6 is produced both systemically and locally in the muscle, stimulated by muscle contractions (Pedersen 2000, Febbraio 2007). Contracting muscle releases IL-6 into circulation and affects fuel metabolism during exercise, contributing to glucose homeostasis by inducing hepatic glucose output and mediating lipolysis (Edwards et al., 2006, Febbraio 2007). Interleukin-6 also helps regulate immune responses to exercise by directly inhibiting the expression of TNFα and IL-1 as well as up-regulating production and secretion of IL-10, which also inhibits TNFα and IL-1β production. Interleukin-6 may also have a stimulatory effect on the secretion of APPs, which are a critical factor in the APR (*e.g.,* SAA; Heinrich 1990, Gleeson 2000). In a review of exercise and cytokines by Pedersen (2000), the increase in IL-6 was described as closely related to the duration as well as the intensity of the exercise, increasing up to 100-fold in humans after a marathon race.

Given that IL-6 is produced in large amounts in response to exercise, is produced locally in the skeletal muscle in response to contraction during exercise, and has growth factor abilities, IL-6 may likely be involved in and play a beneficial role in mediating exercise-related metabolic changes (Pedersen 2000). The literature also suggests that muscle IL-6 output may act as a mechanism contributing to the development of fatigue in prolonged exercise (Gleeson 2000). Ostrowski et al. (1998) conducted a study in humans examining IL-6 concentrations in muscle and blood mononuclear cells (BMNC) before and after exercise to investigate the role of exercise-induced muscle damage and IL-6 production. Before exercise, mRNA for *IL-6* was not detected either in muscle or in BMNC but was detectable in muscle biopsies after exercise (not in BMNC), suggesting exercise-induced damage of skeletal muscle fibers may trigger local production of IL-6 (Ostrowski et al., 1998). Supporting this finding, Gleeson (2000) reported that an active upper leg of humans released IL-6 into the circulation during prolonged single-limb exercise, and not noted at all in the resting leg. Based on this evidence, IL-6 is thought to be an indicator of skeletal muscle damage, primarily due to the significant increases in IL-6 concentrations in blood and muscle that have been documented in human athletes following both dynamic eccentric and static resistance exercise.

Even though IL-6 is one of the more commonly investigated cytokines when studying exercise stress, there are still conflicting results among the literature on its specific response to exercise, much like IL-1 and TNFα. Interleukin-6 is commonly shown to be produced in more copious amounts than any other cytokine in relation to exercise in humans and is most commonly seen after strenuous exercise (Pedersen 2000, Edwards et al., 2006). An early study by Northof et al. (1991) showed increased levels of IL-6 after a marathon. This finding of markedly elevated levels of IL-6 after exercise is consistent with many studies since then (Ullum

et al., 1994a; Drenth et al., 1995; Nehlsen-Cannarella et al., 1997; Castell et al., 1997; Rohde et al., 1997; Hellsten et al., 1997; Bruunsgaard et al., 1997; Ostrowski et al., 1998). Sprenger et al. (1992) conducted a study investigating cytokine levels in trained runners after a 20-km run. The study did not detect most of the investigated cytokines in plasma except for IL-6; however, only urine samples were analyzed. The IL-6 levels in urine were shown to increase until 7 h after the long-distance run (Sprenger et al., 1992). A study testing the hypothesis that the exercise-induced increase in circulating cytokine levels was associated with muscle damage showed that serum IL-6 increased after eccentric exercise and was correlated significantly to creatine kinase (CK; a marker of muscle damage) concentration in the following days, but changes were not noted after concentric exercise (Bruunsgaard et al., 1997). A slightly different result was reported by Edwards et al. (2006) in which the IL-6 response to two different intensities of exercise was investigated. Interleukin-6 increased above baseline immediately after the maximal exercise bout but not the submaximal exercise. However, IL-6 levels did increase at 30 and 60 min after exercise of both intensities, showing that 45 min of moderate-intensity exercise can increase IL-6, however, the inclusion of maximal effort may accelerate this response (Edwards et al., 2006).

Given the facts that IL-6, more than any other cytokine, is produced in large amounts in response to exercise, that IL-6 is produced locally in the skeletal muscle in response to exercise and that IL-6 is known to have growth factor abilities, it is likely that IL-6 plays a beneficial role and may be involved in mediating exercise-related metabolic changes (Pedersen 2000). However, similar to IL-1β and TNFα, dysregulated or continual synthesis of IL-6 has the potential to have a pathological effect on chronic inflammation and autoimmunity.

IL-10

Interleukin-10 is an anti-inflammatory cytokine that has been shown to inhibit the release of TNFα and IL-1 and induce the production of IL-1ra (Jenkins et al., 1994; Cassatella et al., 1994, Chernoff et al., 1995, Ostrowski et al., 1999, Rossio, 1999). It is suggested that IL-10 increases after strenuous exercise to mediate the resulting inflammatory response. A study investigating plasma levels of IL-10 after a 3-h marathon showed a 27-fold increase immediately post-exercise (Ostrowski et al., 1999). Similar results by Nieman et al. (2005) reported a 160-km run induced a 24x increase in IL-10 and 125x increase in IL-6, along with correlated levels of creatine phosphokinase (CPK). This study suggested that the increases of IL-6 and IL-10 post-exercise might serve as a check to exercise-related inflammatory responses to prevent chronic conditions. A similar response was also seen noted by Lamprecht et al. (2009), where IL-6 and IL-10 were significantly elevated following a marathon race when compared with resting values and remained elevated up to 4 h into the recovery phase. A majority of studies investigating IL-10 used long duration or eccentric exercise under the assumption that IL-10 raises in response to stress or inflammation triggered by exercise-induced tissue damage. However, a study by Conroy et al. (2016) determined that regular aerobic exercise (submaximal) did not alter basal levels of interleukin IL-10. Another study focused on assessing the effects of exercise training on biomarkers of inflammation in postinfarction patients and found that exercise-training moderately improved IL-10 levels when compared to a sedentary group of patients (Ribeiro et al., 2012). It should be noted that even though the difference was statistical, the change in values was numerically small and, therefore, may not have had physiological implications. However, the study still concluded that exercise training somewhat improved the inflammatory profile in post-myocardial infarction patients by enhancing the anti-inflammatory cytokine IL-10 (Ribeiro et al., 2012). Compared to IL-1 and IL-6, much less is known about the response of IL-10 to

exercise in humans, and even less research has been conducted in horses. Much more work is needed to investigate differences in IL-10 response to various exercise modalities and intensities, as well as more thoroughly investigating the role of IL-10 in performance horses. Because it is an anti-inflammatory cytokine, increasing IL-10 production could be beneficial in helping regulate inflammation, especially after exercise.

In conclusion, previous literature has primarily focused on cytokine responses to eccentric exercise. Overall, increases in the pro-inflammatory cytokines, TNFα and IL-1, and a dramatic increase in the inflammation responsive cytokine, IL-6, were noted after strenuous aerobic exercise, such as a marathon (Ostrowski et al., 1999, Nieman et al., 2005, Gleeson 2007). This influx of cytokines was balanced by a release of cytokine inhibitors (IL-1ra) and the anti-inflammatory cytokine, IL-10 (Ostrowski et al., 1999, Gleeson 2007). However, many studies are still in disagreement on how pro and anti-inflammatory cytokines specifically respond to exercise. There are several possible explanations for the variability in response to exercise in cytokines. One difficulty in interpreting the literature is the varying types of physical activity, as well as the intensity and duration of the exercise between studies. In addition, there is increasing recognition that individual difference factors, such as training status, age, and sex, may affect the inflammatory response to exercise (Edwards et al., 2006). Currently, the effect of age on cytokine expression after exercise has not been thoroughly investigated and could play a role in the differences reported between studies, as growth may contribute to systemic inflammation and stress.

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 completed in horses. In equine athletes, the limited studies have exhibited evidence of increased inflammation

subsequent to exercise as characterized by increased expression of mRNA for *TNFα*, *IL-1β*, and *IL-6* in peripheral blood mononuclear cells, as well as *IL-6* in muscle (Nieman et al., 2005, Liburt et al., 2010, Horohov et al., 2012). Another study conducted by Lamprecht et al. (2008) measured pro-inflammatory and anti-inflammatory cytokine transcripts in the whole blood of Standardbred mares after exercise and noted that pro-inflammatory cytokine transcripts were elevated after exercise. Another study in horses demonstrated that mRNA expression for inflammatory cytokines increased following exercise and suggested that those markers of inflammation may play a role in delayed onset muscle soreness (Liburt et al., 2010). More research is needed investigating the role of pro- and anti-inflammatory cytokines as markers of systemic inflammation and stress and their response to exercise to be able to manage and maintain working horses properly.

Cortisol

Exercise has been shown to increase the concentrations of hormones in the blood, including (but not limited to) epinephrine, norepinephrine, and cortisol (Pedersen et al., 2000). Exercise-induced plasma cortisol concentrations have been extensively documented in both humans and horses; however, it should be noted that some of the literature conflicts or disagrees with what types of exercise cause increases in serum cortisol. In most studies, increased serum cortisol is reported after excessive or intense exercise (Smith 2003). Other studies show that cortisol mostly increases during long-duration exercise (Galbo 1983, Pedersen et al., 2000). Another study in humans showed that serum cortisol concentrations were elevated above control levels for several hours following prolonged running at high-intensity (Nieman, 1999). However, the most commonly accepted relation of cortisol level and exercise is that cortisol appears to be affected by both intensity and duration of exercise (Marc et al., 2000, McKeever

2002, Starkie et al., 2005, Gordon et al., 2006). Moreover, levels of cortisol increase at a rate relatively proportional to the exercise intensity, but the maximum level is dependent upon the total duration (time) of the exercise session (Marc et al., 2000, McKeever 2002, Starkie et al., 2005, Gordon et al., 2006).

These studies observed that substantial increases in plasma cortisol occur in response to high-intensity exercise; however, moderate increases were still evident during submaximal or moderate exercise (Marc et al., 2000, Smith, 2003, Gordon et al., 2006). In a study investigating both submaximal and maximal exercise, cortisol increased in both compared to the control condition, but the increase was greater after maximal exercise than submaximal exercise. Further results investigating cortisol in response to different exercise intensities in humans supported the view that moderate to high-intensity exercise provokes increases in circulating cortisol levels (Hill et al., 2008). However, this study is one of the few looking at the response of cortisol to low intensity exercise (40% VO_2max). After corrections for plasma volume reduction and circadian factors, researchers reported that low-intensity exercise resulted in a reduction in circulating cortisol levels (Hill et al., 2008). Lastly, a study comparing young and old horses reported that, while cortisol concentrations increased in both young and adult horses after exercise, younger horses had higher concentrations after exercise than older horses. This finding may be a result of the stress of natural growth and might affect the overall levels of cortisol in young horses after exercise.

Reliable physiological markers for performance evaluation in sport horses are missing. However, cortisol has been investigated as a potential biomarker for stress in horses after exercise (Marc et al., 2000). Cortisol likely serves as a signal for cellular repair following a stressor, such as tissue damage after exercise (Davitt et al., 2014). Further, cortisol may elicit a

permissive effect that is beneficial for training adaptation by preventing the immune system from eliciting an excessive inflammatory and immune reaction to acute exercise (Smith, 2003, Edwards et al., 2006, McKeever et al., 2014). Further, excessive increases in plasma cortisol concentrations immediately after exercise followed by disproportionately low plasma cortisol may be a marker of overtraining in horses (Davitt et al., 2014). Additionally, cortisol has been linked to some of the immunosuppressive changes experienced during recovery. Glucocorticoids (*i.e.,* cortisol) administered to humans *in vivo* have been reported to cause neutrophilia, eosinopenia, lymphocytopenia, and suppression of both NK and T-cell function, all of which occur during recovery from prolonged, high- intensity, cardiorespiratory exercise (Nieman, 1999). Therefore, studies have suggested that high levels of cortisol may play a role in immunosuppression sometimes seen in athletes (Nieman, 1999).

Serum Amyloid A

Acute phase proteins have been described in human and animal patient studies as a useful tool for assessing health, as they closely reflect the APR (Cywińska et al., 2012). In horses, SAA level has been shown to increase from baseline levels from 10 to 1000 times in response to stressors such as inflammatory disorders or developing complications after surgical procedures, and may be useful in disease diagnostics and prognostication (Jacobsen et al., 2005, Jacobsen et al., 2007, Canisso et al., 2014). However, there is less known about the relation between APPs like SAA and less severe stressors such as exercise or musculoskeletal injury. Serum amyloid A is considered a sensitive APP and may be an accurate marker of subclinical inflammation or even repetitive microtrauma that may play a role in overtraining syndromes. A study in Thoroughbred racehorses showed that training-induced muscle, skeletal and joint trauma might result in acute phase response reflected by the changes in the blood concentration of serum

amyloid A (SAA) (Turło et al., 2015a). Results showed that mean levels of SAA within the first 4 days of muscle and tendon injuries were significantly higher when compared to bone fractures, dorsal metacarpal disease, joint trauma, and healthy horses (control; Turło et al., 2015a). Researchers concluded that strain injuries of muscle and tendons could cause a moderate increase in SAA blood concentration in racehorses, reflecting the occurrence of the acute phase response. In comparison, bone, metacarpal, and joint injuries did not show as substantial of an increase in SAA (Turło et al., 2015a). Even though the cause of this difference has not yet been determined, it is assumed the lower mass of affected hard tissues and their reduced vasculature may be a limiting factor for the release of locally produced pro-inflammatory mediators, like IL-6, to the bloodstream. Injuries of muscle and tendon related to mechanical stress associated with race training can affect the serum level of SAA and should be considered in the interpretation of this biomarker in racehorses (Turło et al., 2015a). Another study conducted by Turło et al. (2015b) reported that racehorses with stress-related injuries of the musculoskeletal system showed higher SAA levels than non-injured horses on the 3rd and 4th day after the race and that SAA concentration correlated positively with white blood cell count. Once again, it was suggested that racing effort might cause an increase in SAA level, but may be more pronounced in horses showing clinical signs of orthopedic injury after the race (Turło et al., 2015b).

Regardless of injury, small, but noteworthy increases in SAA have been detected post-intense exercise in horses (McGowan et al., 2014). A study in endurance Arabians showed that after long-distance rides, the level of SAA markedly increased (Cywińska et al., 2012). Another study in Arabians by Cywińska et al. (2013) investigated the difference in SAA response to endurance training. The study showed that experienced (conditioned) horses, trained and prepared for rides, did not show any changes in SAA level (compared to resting level) post-

endurance ride. However, in contrast, there was a significant increase in SAA concentration (compared to resting levels) in horses in early training (only prepared for moderate distance rides) that underwent the same effort. It was noted that more research is needed to deduce whether the difference in SAA seen indicated over-training or an adaptation to an increasing workload (Cywińska et al., 2013).

In conclusion, SAA could be used as a sensitive biomarker for detecting levels of inflammation in horses after exercise and may even play a role in accurately detecting musculoskeletal injuries. More research needs to identify the influence of differing degrees of exercise on SAA levels, as most of the research reported is in intense exercise (racehorses). There is also little information regarding the influence of training on SAA levels post-exercise, which could be beneficial in identifying SAA's potential role in exercise-induced adaptations.

Biomarkers in Response to Exercise

In response to acute exercise (the most frequently studied area of exercise immunology), a rapid interchange of immune cells between peripheral lymphoid tissues and the circulation occurs (Nieman, 1999). The extent of the response depends on many factors, including the intensity, duration, and mode of exercise, which can each affect concentrations of hormones and cytokines. In general, acute exercise bouts of moderate length (< 60 min) and intensity (< 60% VO_2max) are associated with fewer perturbations and less stress to the immune system than prolonged, high-intensity sessions. All previously described biomarkers (cortisol, SAA, IL-1, IL-6, IL-10, and TNFα) are described extensively in the literature as markers of stress and inflammation appear to at least increase after high-intensity or long duration exercise, and some studies show increases after moderate exercise in some of the markers.

Dietary Interventions

Human literature suggests that a heavy schedule of training and competition may lead to immunosuppression in athletes, placing them at a higher risk for opportunistic infection and overall immune system dysfunction (Bishop et al., 1999). As a part of the immune response, inflammation is beneficial because disruptions of homeostasis to repair tissue injury permit stress-induced cellular adaptations. It is generally accepted that an inflammatory response is beneficial when moderate and controlled (*e.g.,* protecting against infection), but can become detrimental if dysregulated (Medzhitov, 2008). Prolonged or exaggerated stress responses has been shown to potentially perpetuate hormonal dysfunction, widespread inflammation, and chronic pain (Hannibal et al., 2014). Additionally, an unresolved inflammatory response can become counterproductive and exacerbate, rather than amend, the underlying problem, as in instances of overuse syndromes or repeated injuries (Cooper et al., 2007). Unregulated inflammation can manifest as swelling, pain, and loss of function and chronic inflammation can lead to excessive collateral damage and unnecessary cellular damage (Chandrasoma et al., 1998, Ashley et al., 2012., Chovatiya et al., 2014). Severe chronic inflammation has even been shown to induce symptoms such as malignant cell transformation in surrounding tissue, and septic shock (Landskron et al., 2014). Thus, the timely resolution of inflammation is imperative. Due to the interplay of inflammation and the immune system, the immune-suppressing effects of long-duration or intense exercise, and the need for the timely resolution of inflammation, immune boosting and anti-inflammatory dietary additives have been a topic of interest in both human and equine research (Paulsen et al., 2010).

Strenuous exercise is known to suppress the immune system, and nutritional supplements have been proposed to boost post-exercise immunity, as nutrition is a critical determinant of immune responses (Bishop et al., 1999). However, to date, few additives are known to be

effective (Carpenter et al., 2012). Nutrient availability has the potential to affect almost all aspects of the immune system because the majority of immune responses involve cell replication or the production of proteins with specific functions (*e.g.,* cytokines) and most nutrients are involved in protein synthesis (Kubena et al., 1996, Bishop et al., 1999). Horse owners and trainers often look to dietary supplements to enhance the performance of their horses (Pratt-Phillips et al., 2014). However, anecdotal reports and media have often misled well-meaning equestrians by promoting various vitamins and minerals for their claimed performance benefits. However, people are often unaware that micronutrient supplementation is only beneficial when correcting a *deficiency*. Currently, there is little scientific evidence to substantiate claims of micronutrients acting as an ergogenic aid (a substance that increases or improves work performance) outside of correcting an already existing deficiency. However, there is thorough research demonstrating that excessive intakes of specific vitamins and minerals can be toxic (*e.g.,* selenium in horses; Bishop et al., 1999). Therefore, current research has turned to the investigation of other potential dietary additives.

Saccharomyces cerevisiae

Dietary beta-glucans (BG) have received significant attention as a nutritional additive because of their suggested modulatory properties of the immune system and inflammation. Beta-glucans are carbohydrates composed of glucose molecules linked together by several different types of chemical linkages, resulting in either a linear or branched structure. Cereal grain BG (oats and barley) have a linear structure, while BG from fungal sources (mushrooms and yeast) have a 1,3/1,6 linkage pattern with varying degrees of side-chain branches. The frequency and length of side-chain branches have important implications for biological activity, with the higher the degree of branching, the more "biologically active" the BG. One such BG is *Saccharomyces*

cerevisiae (SC), a type of yeast, which is shown to have a significant degree of side-chain branching and high antioxidant effects (Carpenter et al., 2012, Evans et al., 2012). The addition of direct-fed microbes (like *SC*) to manipulate the activities of the microbial population in the intestinal ecosystem has been investigated in both human and animal trials to investigate its ability to boost the immune system and subsequently reduce inflammation. Studies reported that the number of symptomatic illness or infection days were reduced in human adults supplemented with *SC* when compared with the placebo group (Moyad et al., 2010, Carpenter et al., 2012). However, it should be noted that some studies have mixed results due to the type of BG used, which may have structural differences when derived from different sources.

Medina et al. (2002) investigated the effect of supplementation of *SC* on the response to high-fiber (HF) and high-starch (HS) diets in horses. Supplementation of *SC* appeared to modify pH, concentrations of lactic acid and ammonia, molar percentages of acetate and butyrate with the HS diet and [(acetate + butyrate)/propionate] ratio when the HF diet was fed. Also, when the digestion of starch in the small intestine was at capacity, *SC* supplementation appeared to limit the extent of undesirable changes in the intestinal ecosystem of the horse (Medina et al., 2002). Additionally, other studies in horses showed that yeast supplementation improved cell-wall digestibility (Jouany et al., 2008) and limited hindgut dysbiosis (microbial imbalance; Jouany et al., 2009).

Many of the studies investigating *SC* fed to horses have focused primarily on digestion, however, work in humans and other species have demonstrated immune effects of *SC* supplementation. Carpenter et al. (2012) evaluated whether 10 days of supplementation with a defined source of baker's yeast BG could minimize post-exercise immunosuppression. The key findings were that supplementation before a bout of cycling in the heat increased total and pro-

inflammatory monocyte concentrations after exercise, increased LPS-stimulated production for certain cytokines before exercise, and increased plasma cytokine concentrations after exercise. It was concluded that BG supplementation maintained blood monocyte circulation and countered the monocytopenia (low levels of monocytes) observed in the placebo group post-exercise (Carpenter et al. 2012). It should be noted here that no notable differences were found between supplemented and non-supplemented groups for the LPS-stimulated production of IL-1β, IL-6, IL-10, or TNFα (Carpenter et al., 2012).

Yeast Fermentation Products

Byproducts of yeast fermentation have been the focus of many studies (Glade et al., 1990, Morgan et al., 2007, Moyad et al., 2010, Evans et al.2012, Zhang et al., 2013, Zhu et al., 2017) because of their potential to have similar effects as the yeast itself. Previous work has shown minimal to no adverse side effects with supplementation of an *SC* fermentation product (Glade et al., 1990, Medina et al., 2002, Morgan et al., 2007, Moyad et al., 2010, Zhang et al., 2013, Zhu et al., 2017), indicating its safety as a dietary supplement. Further, supplementation with a *Saccharomyces cerevisiae* (type of yeast) fermentation product (SCFP) appeared to provide both moderate immune-enhancing and anti-inflammatory benefits, demonstrating the potential to favorably modulate immune responses without excessive suppression or stimulation of overall immune activity (Carpenter et al., 2012, Evans et al., 2012).

In humans, supplementing with a dried fermentation product (DF) notably reduced the incidence and duration of cold- and flu-like symptoms during fall and winter months, regardless of influenza vaccination status (Moyad et al., 2010). It was suggested that a potential mechanism of action was enhanced salivary IgA production, NK cell activation, and increased antioxidant capacity (Moyad et al., 2010). Work by Evans et al. (2012) in rats had two main objectives: 1) to

examine the ability of DF (EpiCor) supplementation to prevent or reduce inflammation in rats after receiving a 1% carrageenan injection in a paw (localized inflammation model) and 2) to examine the ability of DF to treat established inflammation induced by type-2 collagen in mice over 4 weeks (autoimmune arthritis model). After all the rats got the carrageenan injection, the DF supplemented group showed significantly reduced swelling at all time points post-injection (1, 2, 3, 6, 12, and 24 h) when compared to the non-supplemented control group. Additionally, edema severity and prostaglandin E_2 (PGE_2) levels were reduced by approximately 50% and 25%, respectively. In the second half of the study, DF supplementation significantly reduced arthritis scores, antibody response to type-2 collagen, and interferon-gamma levels compared to controls. These results suggested that DF favorably impacted multiple acute and potentially chronic immunologic inflammatory control mechanisms and should be further tested in clinical trials (Evans et al., 2012). Another study in rats focused on the ability of a yeast fermentation product (EpiCor) to mitigate heat stress. Heat stress can result in a multitude of biological and physiological responses that can become lethal if not properly managed (Ducray et al., 2016). The trial exhibited that both villi height and total mucosal thickness in the small intestine decreased in heat-stressed rats after subjection to heat stress by more than 200µl in un-supplemented rats. However, oral treatment of rats with the fermentation product before heat stress prevented the traumatic effects on the intestine, indicated by no change in villi height or mucosal thickness after subjection to heat stress (Ducray et al., 2016). Treatment with the supplement was also successful in the prevention of LPS release into the bloodstream of the heat-stressed rats (Ducray et al., 2016). Elevation of body temperature also increased the concentration of vesicles released by the shedding of erythrocyte membranes, an indication of a pathological impact of heat on the erythrocyte structure. However, treatment of rats with the

yeast fermentation product protected erythrocytes from the heat-induced pathology (Ducray et al., 2016). Finally, exposure to heat stress conditions resulted in considerable increases of white blood cells in rats, except in those treated with the supplement before heat stress, whose white blood cell count remained the same as in non-heated controls. The results of this study showed the protective effect of yeast fermentation product in the prevention of complications, caused by heat stress (Ducray et al., 2016).

Research in chickens has also shown favorable effects of supplementation of yeast fermentation products. Functional metabolites of Diamond V Original XPC™ (Diamond V Mills, Cedar Rapids, IA), an *SC* fermentation product, helped balance the immune response and stress hormone levels in production poultry (Firman et al., 2013, Price et al., 2018). Heat stress in commercial broiler chickens can lead to cellular oxidative stress, which increases susceptibility to infectious diseases (Price et al., 2018). Price et al. (2018) demonstrated that birds fed XPC had a reduced negative response to heat stress as indicated by both lower plasma corticosterone concentrations and lower composite physical asymmetry scores (Price et al., 2018). The reduction in stress susceptibility observed by Price et al. (2018) combined with immune modulation observed by Chou et al. (2017) may explain why other studies have shown increased growth and feed conversion in birds fed XPC (Gao et al., 2008, 2009). Chickens supplemented with XPC had increased secretory IgA and intestinal IgM, and there was a positive impact of XPC supplementation on weight gain, feed conversion, and mortality (Gao et al., 2008, 2009). Similar results were noted by Al-Mansour et al. (2011), who showed that XPC decreased heterophil/lymphocyte ratios considerably in supplemented broiler chickens. Guo et al. (2013) also investigated the effects of an *SC* fermentation product on dairy cattle performance. Results showed that cows experiencing sub-acute ruminal acidosis (SARA) had increased levels of SAA.

However, cows receiving the XPC supplement had reduced levels of SAA (Guo et al., 2013), demonstrating that SCFP attenuated the inflammatory effect of the SARA challenge. Another study in dairy cattle showed that supplementation increased lactation performance of dairy cows and tended to reduce LPS concentration in plasma on two different farms (Zhang et al., 2013).

In horses, yeast fermentation supplementation has been investigated for both increased digestibility of foodstuffs and athletic performance enhancement (Glade et al., 1990, Medina et al., 2002, Wickler, 2002, Morgan et al., 2007, Grimm et al., 2016). Previous research has shown that yeast fermentation products may be stimulatory to equine hindgut digestion and can beneficially alter microbial population (Medina et al., 2002, Morgan et al., 2007, Grimm et al., 2016), which may help improve nutrient digestibility, increase microbial populations, and maintain cecal pH. Improving the digestibility of lower-quality forages could be advantageous both for the horse's health as well as for the producer. In a study by Morgan et al., dry matter, crude protein, and NDF digestibilities of low-quality hay were greater in horses fed a yeast culture supplement than un-supplemented horses, demonstrating improved digestibility of lower-quality Bermudagrass hay in mature horses. Glade et al. (1990) investigated the same supplement related to athletic performance in Arabian horses. The study demonstrated that horses supplemented with a yeast culture had significantly lower heart rates during the first 5 and the last 10 min of 35-min exercise bouts as well as numerically lower plasma lactate levels than non-supplemented horses, suggesting an enhanced state of athletic fitness (Glade et al., 1990). Also, initial increases in plasma triglyceride concentrations tended to be slower in the supplemented group than in the non-supplemented group, suggesting that fatty acid clearance from the plasma may have become more efficient, potentially via uptake by working muscle (Glade et al., 1990). Wickler (2002) investigated the effect of Diamond V's XPC supplement

(an *SC* fermentation product) on submaximal performance in Arabian endurance horses. Results showed a substantial increase in blood FFA, glucose, hemoglobin, and packed cell volume, demonstrating the potential ability of XPC to improve submaximal performance in horses (Wickler 2002).

Dietary Intervention of Yeast Products

In conclusion, yeast fermentation products favorably impact multiple acute and potentially chronic immunologic inflammatory control mechanisms and should be further tested in future trials. Although the mechanisms of action for yeast and yeast fermentation products are unknown, some suggest effects are due to its high antioxidant content. Evans et al. (2012) suggested that the yeast fermentation product used in his study contained a high concentration of metabolites and numerous and diverse free radical scavenger compounds, exemplified by its high antioxidant content as shown in an oxygen radical absorbance capacity (ORAC) assay. Supplementing with a fermentation product (*i.e.*, Epicor in humans and XPC in animals) has demonstrated the potential to favorably modulate immune responses without excessive suppression or stimulation of overall immune activity while having minimal adverse events. The interplay of the various compounds involved in immune activity suggests that modulation rather than excessive reduction or stimulation would explain some of the clinical findings with yeast product supplementation. Given this information, further investigations of yeast products should be conducted to better understand the areas where *SC* may continue to provide beneficial results (Evans et al., 2012), specifically research investigating *SC* fermentation products in horses and its effect on the stress response after exercise is needed.

CHAPTER III

MATERIALS AND METHODS

This study was reviewed and approved by the Institutional Animal Care and Use Committee at Texas A&M University (2016-0294).

Horses

Nineteen previously untrained Quarter Horses (11 fillies, 8 colts) entering their yearling year (mean ± SD; age: 9 ± 1 mo) with a starting BW of 266 ± 32 kg were enrolled in the 8-wk study. Horses originated from two sources: 8 were from the Texas A&M University (TAMU) horse center herd (College Station, TX) and 11 were leased from Birdsong Farms (Hearne, TX). Horses originating from Birdsong Farms were relocated to Freeman Arena prior to the beginning of the trial, and all horses remained there throughout the 8-wk trial. Horses were group-housed by gender and farm of origin (TAMU colts/TAMU fillies/Birdsong colts/Birdsong fillies) at Texas A&M University's Freeman Arena (College Station, TX) in approximately 0.5-hectare dry lots. All horses received a basal diet consisting of a commercial grain mix custom-formulated with no yeast fermentation products at 1.25% BW (DM basis) split into 2 equal meals per day, and Coastal bermudagrass hay fed *ad libitum* in dry lots devoid of fresh forage. The basal diet was formulated to meet or exceed the requirements of growing horses (NRC, 2007). Composited hay and grain samples were analyzed for nutrient composition prior to the initiation of the trial and are presented in Table 1. All horses received the basal diet (hay + grain) for 4 wk prior to the start of the trial. At wk 0, horses were stratified by age, sex, BW, and farm of origin and randomly assigned to one of two groups: 1) no supplementation (CON; n = 9) or 2) supplemented with 21 g/d of *Saccharomyces cerevisiae* fermentation product (Original XPC,

Diamond V Mills, Inc.; SCFP; n = 10) split into 2 equal feedings. The supplement was top-dressed on the SCFP horses' grain at each feeding (10.5 g/feeding).

Dietary Treatment

During the study, horses were hand walked into individually assigned 3.2 x 3.2 m stalls twice daily to receive their concentrate grain meal. Concentrate grain was split equally into AM and PM meals at 0600 and 1700, respectively. Any refused grain was weighed and recorded daily and subtracted from the total grain offered to obtain accurate grain consumption of each horse. All horses received *ad libitum* access to Coastal bermudagrass hay which was supplied in the form of a round bale. Individual hay intake was calculated from estimated intake while group-housed. Group-housed estimates were obtained from the disappearance of known quantities in each dry lot, were assumed to be equal among all horses in each group, and resulted in an average hay intake of 1.5% BW (DM basis) for each horse. Throughout the study, BW was monitored every 2 wk using a livestock scale accurate to 1 kg (Cardinal scales, Webb City, MO). Body condition score was also evaluated on the same day BW was measured by three independent investigators using the 1 to 9 scale described by Henneke et al. (1983). Grain offered was adjusted for each horse every 2 wk based on changes in BW due to growth and to maintain a BCS of 5 to 6.

Table 1. Nutrient composition of custom-formulated concentrate and Coastal bermudagrass hay offered to yearling horses.

Nutrient[1]	Concentrate[2]	Coastal Hay[3]
DE, Mcal/kg	0.61	0.39
CP, %	18.0	13.0
ADF, %	15.2	40.3
NDF, %	30.4	71.6
Starch, %	18.0	1.00
Crude Fat, %	8.40	1.70
Ca, %	1.40	0.44
P, %	1.06	0.22
Mg, %	0.57	0.17
K, %	1.40	0.88
Na, %	0.62	0.30
Cl, %	1.08	0.52
S, %	0.30	0.24
Fe, ppm	813	184
Zn, ppm	217	34.0
Cu, ppm	56.0	7.00
Mn, ppm	189	241
Co, ppm	1.97	0.47

[1] Values presented on a 100% DM basis.
[2] Concentrate = basal grain diet fed to all horses at 1.25% of Body Weight (BW) in Dry Matter (DM) per Day
[3] Coastal bermudagrass hay fed ad libitum to all horses

Submaximal Exercise Test

Following 8 wk on dietary treatments, all horses underwent a 2-hr submaximal exercise test (SET) on an 8-horse Panel Walker, measuring approximately 21 m in diameter (Priefert Manufacturing, Mount Pleasant, TX). The exercise test consisted of 8 replications of walking at 1.3 m/s for 3 min (24 min total), trotting at 3.1 m/s for 7 min (56 min total), and cantering at 5.4 m/s for 5 min (40 min total). Midway through the SET, horses reversed direction, and the second half of the SET was completed traveling in the opposite direction (1 hr per direction). To allow for accurate sampling, horses were balanced by treatment group, sex, and barn of origin and randomly assigned to one of three exercise groups. The SET was performed over three consecutive days and started at 0800 each day. Average temperature over the three days was 11.7°C with 83% relative humidity.

Sample Collection

Blood samples were collected at wk 0 and 8 pre-SET at 0500 prior to receiving any concentrate. Blood was also collected immediately after the SET (0 hr), and 1 and 6 hr after the SET at 1000, 1100, and 1600, respectively. Approximately 30 mL of blood was collected from each horse via venipuncture from the jugular vein into evacuated tubes (Vacutainer; Becton, Dickson and Co., Franklin Lanes, NJ). Tubes contained either no anticoagulant for serum collection or sodium heparin for plasma collection. Serum and plasma were isolated within 2 hr of collection, aliquoted, and stored at -80°C until analysis. Serum samples were analyzed for serum cortisol and serum amyloid A (SAA) concentrations using commercially available kits (cortisol: DetectX® Cortisol Immunoassay Kit, Arbor Assays, Ann Arbor, MI; SAA: Horse Serum Amyloid A (SAA) ELISA Kit, Innovative Research, Inc., Novi, MI). Each kit provided a pre-prepared 96-well microplate for use with the assay, and standards for each assay were diluted

according to the manufacturer's recommendations. For cortisol, serum was diluted 1:100 with the diluent provided in the kit. Samples with very low cortisol concentrations were diluted 1:25 with kit diluent and values were then divided by 4. For SAA serum was diluted 1:200 with the provided diluent. For both assays, the absorbance (450 nm) of the contents of each well was immediately determined using a plate reader. All samples were analyzed in triplicate. The interassay and intraassay CV were 8.5% and 5.3%, respectively, for cortisol and 2.2% and 4.5%, respectively, for SAA.

Statistical Analysis

One CON horse was dismissed from the SET due to lameness unrelated to the study and was not included in statistical analyses. Data were analyzed using PROC MIXED in SAS v9.4 with repeated measures (time). Diet, time, and the diet × time interaction were included as fixed effects and horse within diet was included as a random effect. Data were tested for normality and log-transformed prior to analysis if not normally distributed. The responses to diet (wk 0 to wk 8 pre-SET) were analyzed separately from the response to the wk 8 SET. All data are expressed as least squares means ± SEM. Significance was declared at $P \leq 0.05$, and trends declared at $P \leq 0.10$.

CHAPTER IV

RESULTS

Serum cortisol decreased from wk 0 to 8 ($P = 0.014$) in both groups but was unaffected by diet (Fig. 1A). Serum amyloid A was also similar between treatment groups but did not change between wk 0 and wk 8 pre-SET (Fig. 1B).

1A.

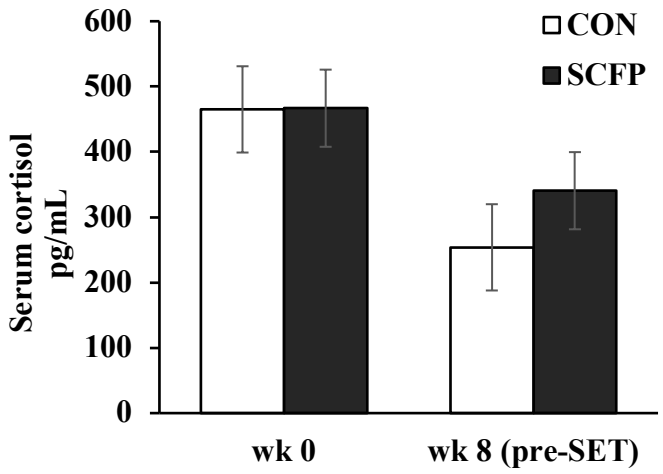

Figure 1A. Serum cortisol concentrations of yearling Quarter Horses before (wk 0) and after (wk 8 pre-SET) 8 wk of receiving basal grain diet only (CON; n = 8) or basal grain diet plus 21 g *Saccharomyces cerevisiae* fermentation product (SCFP; n = 10) per day. Overall effects of dietary treatment ($P = 0.500$), time ($P = 0.014$), and treatment × time ($P = 0.498$).

1B.

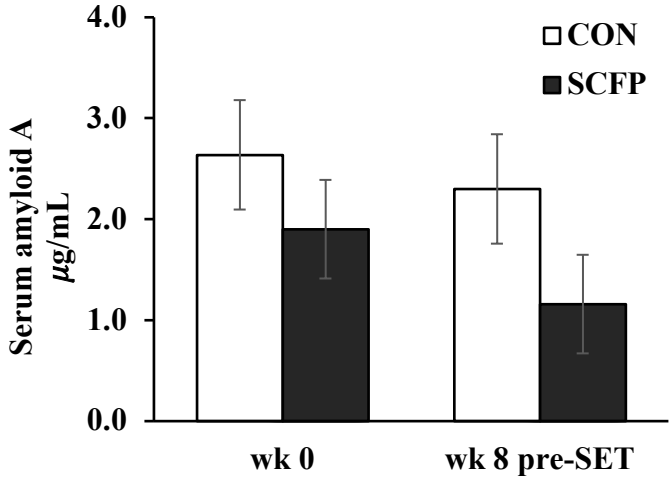

Figure 1B. Concentrations of serum amyloid A (SAA) of yearling Quarter Horses before (wk 0) and after (wk 8 pre-SET) 8 wk of receiving basal grain diet only (CON; n = 8) or basal grain diet plus 21 g *Saccharomyces cerevisiae* fermentation product (SCFP; n = 10) per day. Overall effects of dietary treatment ($P = 0.217$), time ($P = 0.223$), and treatment × time ($P = 0.640$).

In response to the wk 8 SET, serum cortisol increased in both groups immediately after the SET (0 hr post-SET; $P \leq 0.0005$) but returned to pre-SET levels in SCFP by 1 hr post-SET (Fig. 2A). At 6 hr post-SET, cortisol in CON had returned to pre-SET concentrations, while SCFP declined to be lower than pre-SET ($P = 0.0001$) and lower than CON ($P = 0.01$) at that time point (Fig. 2A). Serum amyloid A increased at 6 hr post-SET in CON ($P < 0.0001$) but was unchanged through 6 hr post-SET in SCFP (Fig. 2B). At 6 hr post-SET, SAA levels were significantly higher in CON than SCFP ($P < 0.0001$; Fig 2B).

2A.

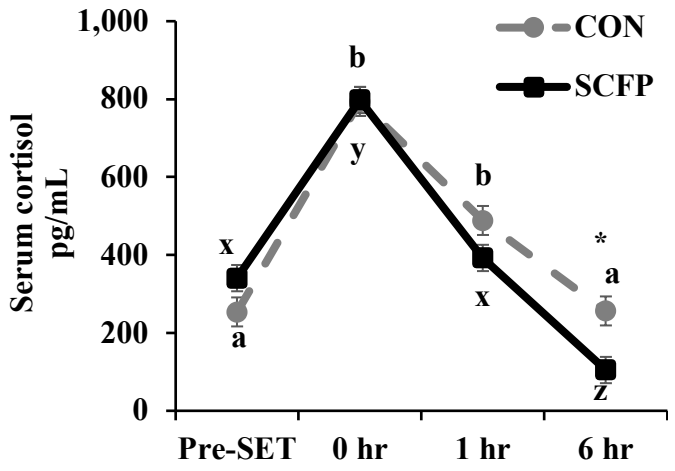

Figure 2A. Serum cortisol concentrations of yearling Quarter Horses before a 2-hr submaximal exercise test (Pre-SET), immediately post-SET (0 hr), and 1 hr, and 6 hr post SET following 8 wk of receiving either a basal grain diet only (CON; n = 9) or basal grain diet plus 21 g *Saccharomyces cerevisiae* fermentation product (SCFP; n = 10) per day. Overall effects of dietary treatment ($P = 0.316$), time ($P < 0.0001$), and treatment × time ($P = 0.057$). [a,b,c,x,y,z] Within diet, different letters differ ($P < 0.05$). * Within time, CON differs from SCFP ($P < 0.05$).

2B.

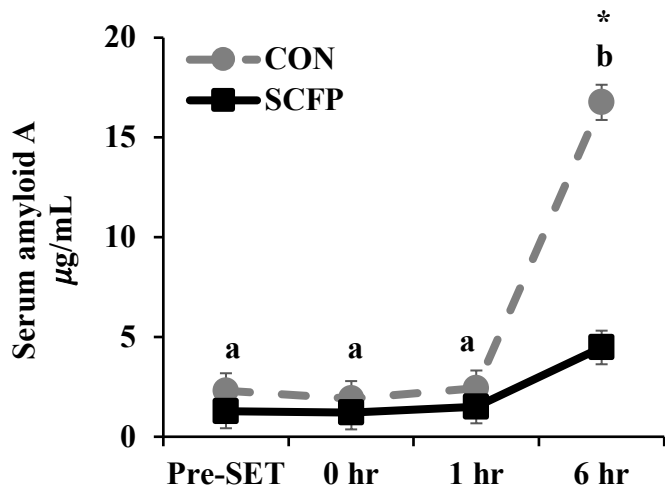

Figure 2B. Concentrations of serum amyloid A (SAA) of yearling Quarter Horses before a 2-hr submaximal exercise test (Pre-SET), immediately post-SET (0 hr), and 1 hr, and 6 hr post SET following 8 wk of receiving either CON or SCFP diet. Overall effects of dietary treatment (P = 0.316), time ($P < 0.0001$), and treatment × time ($P = 0.057$). [a,b,c,x,y,z] Within diet, different letters differ ($P < 0.05$). * Within time, CON differs from SCFP ($P < 0.05$).

CHAPTER V

DISCUSSION

Research exploring the inflammatory response to exercise in human athletes is prevalent. However, there is significantly less research investigating stress and inflammation in response to exercise in performance horses, especially young horses entering a training program. Inflammation is the body's protective response to deviations or challenges to tissue homeostasis and is necessary for stress-induced cellular adaptations. However, the resolution of inflammation is imperative to prevent unnecessary cell damage (Landskron et al., 2014). Unresolved inflammation may manifest as swelling, heat, pain, and loss of function (Chovatiya et al., 2014) and result in symptoms as mild as delayed-onset muscle soreness or as severe as debilitating injuries affecting soft tissue, joint, and bone (MacIntrye et al., 1995, Smith 2004, Horohov et al., 2012). Due to the interplay of inflammation and the immune system and the immune-suppressing effects of long-term or intense exercise, this study aimed to investigate a potential immune boosting and anti-inflammatory dietary additive for the preventative maintenance of young horses entering into an exercise training program.

The present study demonstrated that horses receiving dietary supplementation with a *Saccharomyces cerevisiae* fermentation product (Original XPC; Diamond V Mills, Inc.) for eight weeks responded more favorably to a prolonged exercise bout than non-supplemented horses. Serum cortisol in SCFP horses returned to pre-SET concentrations more quickly after exercise compared to CON horses. Additionally, serum amyloid A, a sensitive marker of inflammation, was unaffected by exercise in supplemented horses, while increasing significantly 6 hr after exercise in control horses.

Various mechanisms to improve the longevity of performance horses have been investigated, with an increased focus on the role of stress-induced immune and inflammatory responses (Horohov et al., 2012). In human and animal studies, yeast fermentation products have been shown to have potential benefits from reducing heat stress to enhancing the immune response (Moyad et al., 2010, Evans et al., 2012, Price et al., 2018). While less research is available documenting the effects of yeast byproduct supplementation in horses, some work has indicated positive outcomes of supplementation. In untrained, young horses, supplementation with a yeast culture decreased plasma lactate levels and delayed increases of plasma triglyceride levels following a treadmill exercise test compared to non-supplemented control horses (Glade et al., 1990). Supplemented horses also showed lower heart rates during the first 5 and last 10 min of the exercise test, suggesting enhanced athletic fitness in young horses prior to entering an exercise training program (Glade et al., 1990). Arabian endurance horses receiving a *Saccharomyces cerevisiae* fermentation product (Original XPC; Diamond V Mills, Inc.) for 8 wk showed improvement in submaximal performance as evidenced by a 15-80% increase in blood free fatty acids at all time points and an 8-10% higher level and less fluctuation of blood glucose during and after the exercise test when compared to the control group. Additionally, supplemented horses had a 5-9% increase in blood hemoglobin at all time points and a 3-9% increase in packed cell volume at all time points when compared to the non-supplemented group (Wickler 2002). Many trials have demonstrated favorable effects of supplementing with a yeast fermentation product; however, the impact of a yeast fermentation product on markers of stress and systemic inflammation in horses has not been thoroughly investigated.

There are few known biomarkers that can quantitatively capture acute responses to stress. Referred to as the “stress hormone,” cortisol is involved in many biological systems following a

stressor and, importantly, serves as a signal for cellular repair following exercise (McKeever et al., 2014). High levels of cortisol in the blood (hypercortisolism) can result in symptoms such as high blood pressure (Griffing 2014), potentially putting extra strain on the heart and blood vessels, which over time may increase the risk of heart attack or stroke (DeMarco et al., 2014). Long-term high blood pressure is also linked to heart and kidney disease (DeMarco et al., 2014). Excess cortisol is also associated with excessive tissue breakdown and increased blood glucose concentration (hyperglycemia; Griffing 2014), which if frequent or ongoing, can cause damage to nerves, blood vessels, and organs. Therefore, the ability of tissues to remove cortisol from circulation is essential to reduce the risk of bodily damage. In the current study, all horses exhibited an increase in serum cortisol immediately following the SET (0 hr). However, SCFP returned to pre-SET levels more quickly and had significantly lower levels of serum cortisol at 6 hr post-SET than CON horses. These results suggest that horses receiving the *Saccharomyces cerevisiae* fermentation product may have been more efficient at removing cortisol from circulation than non-supplemented horses, which may lead to a decreased degree of damage following exercise.

The use of APPs, especially serum amyloid A (SAA), to monitor acute immune responses is becoming more common in equine research as commercial diagnostic kits are being validated (Satoh et al., 1995, Crisman et al., 2008). Low or undetectable SAA levels in healthy horses combined with the sizable, rapid increase in plasma SAA after an inflammatory stimulus and a short half-life allows SAA to be efficiently used to closely monitor the course and treatment of inflammation (Jacobsen et al., 2007). In the current study, all horses had similar levels of SAA through 1 hr post-exercise. However, at 6 hr post-SET, CON horses had a significant increase in SAA while SCFP horses remained unchanged. The level of SAA was also

significantly higher at 6 hr post-SET in CON than SCFP. The difference in SAA levels likely indicates that horses receiving supplement had a diminished inflammatory response after exercise when compared to control horses, suggesting that the *Saccharomyces cerevisiae* fermentation product mitigated inflammation after exercise.

Systemic cytokine levels are an emerging area of interest when investigating exercise-induced inflammation. Cytokines are activated by inflammatory stimuli and they affect many different cells and tissues throughout the body, and combinations of various cytokines on target cells may have a stimulatory or suppressive effect. As an example, the accumulation of SAA generally requires IL-6 and IL-1β or TNFα production, so multiple cytokines and inflammatory mediators must be observed simultaneously to obtain a complete picture of the overall inflammatory response (Crisman et al., 2008). In humans, strenuous exercise results in an increase in a concentration of cytokines, including TNFα, IL-1β, IL-6 (pro-inflammatory), and IL-10 (anti-inflammatory; Pedersen et al., 2000). However, the type, intensity, and duration of physical activity have been shown to affect the cytokine profile differentially (Edwards et al., 2006, Crisman et al., 2008). Cytokine profiles of the horses in the current study have yet to be determined but may assist in identifying the mechanism by which SCFP affects inflammation in the horse.

CHAPTER VI

SUMMARY

The current study demonstrated that eight weeks of dietary supplementation with SCFP may mitigate stress following prolonged exercise in young horses. Decreased levels of SAA in the supplemented group of young horses suggests a lower level of systemic inflammation when compared to non-supplemented horses. Additionally, supplemented horses returned to baseline levels of cortisol quicker than control horses, suggesting that the supplemented horses may have been more efficient at recovering from the exercise stressor and clearing cortisol out of circulation than the control horses. Given the results, the effects of SCFP on the exercise-induced stress response in horses should be investigated further.

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