EFFECT OF BIOACTIVE PROTEINS ON GAIT KINEMATICS AND SYSTEMIC INFLAMMATORY MARKERS IN MATURE HORSES

A Thesis

by

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ABSTRACT

Twenty-seven mature Quarter horses were used in a randomized design to determine the effects of bioactive protein supplementation on gait kinematics and systemic inflammatory markers in a 34-d trial. Treatments consisted of oral doses of 230 g/d of 0 g (CON; n=9), 40 g of bioactive proteins (40; n=9; LIFELINE, APC Inc.), and 80 g of bioactive protein (80; n=9) daily. Horses were fed a commercial concentrate at 0.5% BW (as-fed) daily and received *ad libitum* coastal bermudagrass (*Cynodon dactylon*) hay daily. On d 33, horses participated in a trailering and riding challenge.

Kinematic gait analysis was performed on d 0 for use as a covariate, and on d 14, 28, and 34 to allow for determination of potential time and dosage effects. Video footage was collected and analyzed using gait analysis software (EquineTec) for determination of stride length (SL) and range of motion (ROM). Blood was collected via jugular venipuncture on d 0, 14, 28, and 34 for determination of systemic expression of TNF-α and IL-1β. Data were analyzed using PROC MIXED of SAS.

A trend towards treatment x time interaction was observed in ROM of the knee at the walk ($P = 0.10$), due to the increasing ROM for 40 and 80 as time increased and decreasing ROM for CON. A treatment x time interaction was observed ($P < 0.01$) for hock ROM at a walk resulting from CON and 80 decreasing from d 14 to d 28 with CON increasing, while from d 28 to 34 ROM at a walk decreased for 40 and increased for 80. The main effect of treatment on hock ROM at the walk was quadratic ($P < 0.01$) and characterized by higher ROM values for 40 compared to 0 or 80. A significant

treatment x time interaction was observed in expression of IL-1β ($P < 0.01$), and can be explained by lower concentrations of IL-1β for 80 on d 34, with 40 being intermediate and CON being the highest. Increased articular ROM with decreased expression of IL-1β may indicate potential anti-inflammatory effects of dosage of 80 g/d of bioactive proteins.

DEDICATION

As with everything, for JAC

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Contributors

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TABLE OF CONTENTS

LIST OF FIGURES

Page

LIST OF TABLES

CHAPTER I

INTRODUCTION

Osteoarthritis affects all horses regardless of breed or discipline, and constitutes the greatest single economic loss to the equine industry (Frisbie et al., 2006). Therefore, developing nutritional strategies to mitigate the progression of degenerative joint disease may be beneficial to reduce the use of pharmaceuticals, which act only to alleviate the symptoms associated with the disease. One potential alternative is the use of dietary bioactive proteins to reduce the inflammatory effects associated with the degeneration of articular cartilage. These proteins are a diverse mixture of functional components with biological activity independent of their nutritional value, including immunoglobulins, growth factors, biologically active peptides, and other factors with biological activity within the intestine (Campbell et al., 2010b).

Spray dried plasma was found to be more successful than antibiotics in reducing the expression of pro-inflammatory cytokines in pigs challenged with *Escherichia coli* (E. coli) K88 (Bosi et al., 2004). Beski et al. (2016) observed that broiler chickens receiving spray-dried plasma protein (SDPP) supplementation in starter diets had decreased concentrations of immunoglobulins in serum, indicating that stimulation of the immune system was reduced and more resources could be allocated to maintenance and development. In weaned rats challenged with *Staphylococcus aureus* superantigen B, dietary supplementation with plasma proteins reduced immune activation of Peyer's patches and mesenteric lymph nodes (Perez-Bosque et al., 2004).

Later studies conducted in horses have shown that bovine serum improved gait kinematics in mature horses. Coverdale and Campbell (2014) observed that horses receiving a SDPP supplement showed a trend towards improved mean stride length (SL) for the front limb on d 14, and a significant increase in mean SL on d 28. Hind limb SL behaved similarly. As levels of bioactive proteins increased from 140 g to 210 g, mean SL increased in the front limb at the walk (Coverdale and Campbell, 2015). Hind limb SL showed a trend towards increasing with greater inclusion of SDPP. This suggests that SDPP acts through the common mucosal system to affect local sites of inflammation in joints to decrease inflammation and pain, leading to an increase in joint function.

During normal and pathological turnover of articular cartilage, degradation products and inflammatory exudate are released into the synovial fluid, and later can enter systemic circulation if inflammatory processes cannot be contained locally. Assays allow for specific detection of these fragments in body fluids (Lohmander, 1988). The ability to determine levels of biomarkers in synovial fluid, serum, and urine allows for their use as indicators of inflammatory processes (McIlwraith, 2005). Systemic levels of biomarkers in serum and urine give an indication of total metabolic activity originating from all joints in the entire body (Sumer et al., 2006). Reducing systemic inflammation while improving gait kinematics would indicate that joint disease progression may be inhibited, but this area of research is limited in horses, as no studies have been done to determine the anti-inflammatory effects of SDPP in mature horses. Therefore, the objective of this study was to determine the effect of SDPP

supplementation on gait kinematics and systemic inflammatory markers in mature horses.

CHAPTER II

REVIEW OF THE LITERATURE

Bioactive Proteins

Introduction

Dietary ingredients alter immune functions in multiple ways and affect resistance to disease (Perez-Bosque et al., 2008). One such ingredient is spray-dried plasma protein (SDPP), which is derived from porcine and bovine plasma and contains a diverse mixture of functional components with biological activity independent of their nutritional value, including immunoglobulins, growth factors, biologically active peptides, and other factors with biological activity within the intestine (Campbell et al., 2010b). This class of bioactive proteins has improved performance and reduced inflammation in several species, and are often supplemented to increase feed intake, improve nutrient digestibility, and preserve enteral barrier function (Jeong et al., 2016).

Bioactive proteins are comprised of peptide sequences that are hydrolyzed by proteolytic enzymes and released as individual peptides that can become physiologically active. Proteolysis can occur during *in vivo* enzymatic digestion in the gastrointestinal tract by endogenous and microbial enzymes, and during *in vitro* food processing (Martinez-Augustin et al., 2014). Globulin proteins make up approximately 38% of SDPP, adding immunological support to animal diets. Immunoglobulin G (IgG) is believed to be an integral component to the function of plasma derived bioactive proteins, but over 250 peptides have been identified in plasma. IgG contributes to SDPP

effects, but other peptides have been attributed to SDPP health modulation as well (Moreto and Perez-Bosque, 2009).

In the USA, SDPP is a by-product of livestock harvesting facilities, and is therefore available throughout the year, a desirable factor for a by-product used in the livestock industry. However, a major constraint to the use of SDPP is the cost of the product, as it is approximately 8 times more expensive than vegetable protein sources such as soybean meal (Beski et al., 2016, Jeong et al., 2016). Preferred traits for by-products used in livestock include availability in large quantities year-round, high nutritional value, and low cost. Spray-dried plasma protein meets 2 of these traits, but the cost is often offset by the benefits (Luzier and Summerfelt, 1995). Spray-dried plasma protein has an excellent amino acid (AA) profile, and close to 99% digestibility (Gisbert et al., 2015). Additionally, SDPP is a source of high-quality protein without anti-nutritional components such as antigens, trypsin inhibitors, or oligosaccharides which limit nutrient absorption. However, terrestrial animal by-product meals, such as non-ruminant blood and blood products, are the largest underutilized safe source of animal protein and lipid available on the international market (Gisbert et al., 2015).

Production of Bioactive Proteins

Spray-dried plasma protein is a feed ingredient that is produced by centrifuging hygienically collected blood from healthy animals approved for slaughter for human consumption following veterinary inspection. Blood is collected into stainless steel tanks or troughs containing an anti-coagulant. The liquid supernatant is obtained after centrifuging whole blood from bovine or porcine origins, and is concentrated through

membranes and chilled to 4-5°C before being transported to a facility for dehydration (Quigley and Wolfe, 2003). It is typically spray-dried into a light brown powder or formed into a pressed pellet. Spray-drying preserves the functional characteristics of the proteins, including albumin and IgG, due to its mild dehydration process (Rodriguez et al., 2016). Therefore, spray-dried blood products have the highest nutritional value, as the proteins undergo less denaturing and heat damage during mild dehydration compared to the traditional rendering process (Gisbert et al., 2015).

Borg et al. (2002) investigated differences between species of origin and location of blood collection on biological characteristic of SDPP. Antibody titers to specific antigens was quantified in order to compare bovine and porcine products from plants in the USA, Canada, Argentina, Ireland, UK, and Spain. No differences in SDPP protein content were identified due to geographical location of collection or specie of origin. Immunoglobulin content acted in a similar manner, with no differences observed due to location or specie of origin. However, regardless of species, SDPP collected in summer months demonstrated increased levels of antibody titers compared to that collected during winter months. It was hypothesized that differences between time of year may be due to a reduction in immune function associated with an increase in environmental stress in winter, as more immunoglobulins remained within the lymphatic tissue rather than in circulation (Borg et al., 2002).

Safety Protocols

As with any animal by-product, the greatest concern with production and usage is the spread of communicable disease. Commercially produced SDPP utilizes only blood

collected from healthy cattle and swine that have passed *ante mortem* veterinary inspection and have been approved as fit for slaughter for human consumption. By avoiding animals with clinical signs of disease, the risk of potential pathogen transmission is drastically reduced (Polo et al., 2013).

Additional factors that contribute to safety include the thermodynamics of commercial spray driers and the presence of neutralizing antibodies. The intense thermodynamics of commercial spray driers results in rapid dehydration and involves a minimum heat treatment of 80°C throughout the product. Gerber et al. (2014) discovered that the spray-drying process inactivated pathogens for porcine epidemic diarrhea virus, and SDPP was therefore not a potential method for transmission of the disease. Viruses such as swine vesicular disease and porcine respiratory and reproductive syndrome that are typically resistant to physical and chemical processing conditions are also neutralized by SDPP processing (Pujols et al., 2007). The short drying time followed by a rapid temperature increase does not allow time for microorganisms to adapt, and no live pathogens were found in processed samples of SDPP. A single batch of commercially produced SDPP contains pooled plasma from 600-1000 donor animals (Polo et al., 2013) resulting in a high likelihood that the SDPP contains antibodies and neutralizing antibodies against all pathogens present in circulation at time of collection. Therefore, the inherent neutralizing antibodies against specific pathogens of concern can be considered an effective biosafety component in the SDPP manufacturing process.

Proposed Mechanisms

Effects of SDPP in animal diets can be attributed to improvements in overall gastrointestinal tract (GIT) health, including modulation of the immune response, total antioxidant capacity, and gut morphology (Tran et al., 2014). Benefits may follow direct pathways by improving feed consumption or through indirect mechanisms at luminal, mucosal, or systemic levels. Luminal effects include improvements in immunocompetence or a reduction in mucosal binding of antigens (Moreto and Perez-Bosque, 2009). It was originally hypothesized by Kats et al. (1994) that supplemental SDPP improved palatability, as pigs fed SDPP consumed 95 g/d more feed than controls and had a higher average daily gain (ADG). Therefore, SDPP improved growth performance as a result of increased feed intake. However, SDPP contains approximately 20% IgG, so Tran et al. (2014) attributed increased growth performance to the biologically active IgG component of SDPP interacting with intestinal microbiota in the lumen. Immunoglobulins are relatively resistant to digestion due to the presence of protease inhibitors and protein conformation (Quigley et al., 2002). They are not absorbed from the intestinal lumen to systemic circulation, implying that surviving immunoglobulins remain biologically active (Petschow et al., 2014). For example, the predominant form of IgA, sIgA, is found in small amount in plasma but is protected from proteolytic degradation in the GIT. It is therefore suitable for secretion from the small intestine because it can withstand the antagonistic environment of the gastrointestinal tract and other mucosal sites (Martinez-Augustin et al., 2014). It is likely

that improved performance is due to the components of SDPP, and not just the resulting improved feed intake.

Increased digestibility of feedstuffs indicates an improvement in GIT function when animals are on a limit-fed diet. Rodriguez et al. (2016) observed improved digestion for DM (0.93 vs. 0.89), CF (0.96 vs. 0.86), ash (0.74 vs. 0.60), Ca (0.62 vs. 0.51), and P (0.69 vs. 0.58) in cats supplemented with SDPP compared to controls. Improved DM digestion indicates an improvement in overall GIT health and function. Spray-dried plasma proteins improved CF digestion not through addition of a source of digestible fiber to the diet, but rather by increasing the activity of intestinal flora that utilize the fiber. Greater apparent digestion of Ca, P, and ash indicates that these components are more readily available in SDPP, and are highly digestible and soluble.

Addition of SDPP to animal feed helps to maintain immune system integrity and support growth performance during physiological stress (Campbell et al., 2010a). This has been attributed to several potential mechanisms, including improving the immune function of the GIT. The exact mechanism used by SDPP to elicit anti-inflammatory responses remains unknown. Positive effects may be related to modulation of the inflammatory response through active components of SDPP interacting with gut associated lymphoid tissue (GALT) to alter systemic responses to stress (Campbell et al., 2010a). Immunoglobulins and glycoproteins present in SDPP may improve immunocompetence or reduce pathogen adhesion to the mucosa (Bosi et al., 2004). Rodriguez et al. (2007) observed that plasma immunoglobulins were partially resistant to digestion in adult dogs and cats, and retained part of their immunological function.

Serum IgG is comprised of nearly equal proportions of IgG1 and IgG2 isotypes, and lends to the idea that uniform distribution of IgG isotypes in SDPP contributes to improved utilization of this immunoglobulin (Arthington et al., 2000).

Modulation of the intestinal antioxidant defense system may also play a key role in SDPP's improvement of GIT health. Antioxidant enzymes are integral to the antioxidant system of the animal, as they protect cells from oxidative stress. In gilthead sea bream fed supplemental SDPP, changes in antioxidant enzyme activities were observed as well as increases in goblet cell numbers, indicating that SDPP promoted intestinal cell health and development (Gisbert et al., 2015). Additionally, SDPP lowers inflammatory cytokine expression in numerous tissues (Touchette et al., 2002). In a disease challenge trial, it was observed that performance-enhancing properties of SDPP were independent of growth-promoting properties of antimicrobials in the diet of pigs. This indicated that the activity of SDPP and antibiotics are regulated through separate mechanisms. If the same mechanism was used, an interaction would have been observed (Coffey and Cromwell, 1995).

Benefits Relating to Growth and Performance

Several growth factors found in SDPP have the potential to stimulate intestinal growth, protein synthesis, and repair following damage (Corl et al., 2007). These nutritive benefits support normal digestion, absorption, and metabolism by competitively inhibiting endotoxins, promoting a stable GIT microbiome, improving GIT barrier function, and maintaining GIT immune balance in mucosa (Petschow et al., 2014). Improved nutrient absorption may result from tissue specific responses to SDPP. Tran et

al. (2014) observed that duodenal tissue responded to SDPP more than ileal sections by increasing the number of newly synthesized enterocytes. Because more enzymatic degradation occurs in the duodenum, this may explain improved nutrient availability to the animal, and the subsequent improvements in growth performance. Larger numbers of enterocytes and goblet cells additionally influence the capability for enzymatic breakdown of nutrients. Stress events typically result in a reduction in feed intake, which leads to atrophy of villi and decreased surface area for nutrient absorption. Feeding SDPP increases viability and proliferation of enterocytes, increasing the potential for improved gut barrier function (Tran et al., 2014). Improved growth performance of animals receiving SDPP may also result from increased sodium-glucose cotransporter 1 (SGLT1) function. Moreto and Perez-Bosque (2009) attributed alterations in D-glucose transport in disease challenged mice to effects on SGLT1, a sodium dependent glucose transporter found in enterocytes of the small intestine. In challenged rats receiving SDPP, SGLT1 maximum capacity was increased, possibly due to a reduction in pro-inflammatory cytokines. If resources are limited, increased glucose availability to tissues allows more nutrients to be dedicated to maintenance and growth, therefore improving feed efficiency.

Improvements from supplementation of SDPP is most likely achieved through multiple pathways. In some cases, the effects may be due to absorbed nutrients acting on peripheral tissues, such as antioxidant molecules, that once absorbed from the GIT eventually reach a location where they reduce oxidative damage. Mechanisms may also be more indirect, involving activation of anti-inflammatory mediators that signal target

tissues. Additionally, luminal contents may interact with mucosal elements to modulate a local immune response which can in turn propagate the response in other lymphoid tissues due to the interconnection of the mucosal system.

Bioactive Proteins and Health Mitigation

Immune Modulation

To prevent tissue damage and intestinal barrier dysfunction, excessive inflammation from immune responses must be prevented. Healthy immune responses recognize antigens and enact an inflammatory response to prohibit damage from potential pathogens. The intestinal immune system is the first line of defense against ingested antigens. GALT comprises 80% of mucosal immune tissue and is distributed through the intestine in structured units. It consists of organized lymphoid tissue, as well as T cells and innate immune cells dispersed throughout the *lamina propria*. While isolated clusters of lymphoid tissue may be sites of induction of immune responses, it is most likely the *lamina propria* that function in immune effector activity as most T cells present are antigen experienced. The intestinal epithelial layer also contains T cells and is vital to intestinal immunity and barrier function. Intestinal epithelial cells are involved in recognition of antigens through use of pattern recognition receptors (PRRs), and secrete signaling molecules for communication with underlying cells (Coombes and Maloy, 2007). The epithelial cell layer T cells are called intraepithelial lymphocytes (IEL), and many of them are distinct from T cells found in lymphoid organs. Organized intestinal lymphoid tissue refers to Peyer's patches and isolated lymphoid follicles. Peyer's patches are comprised of both B and T cells, with microfold (M) cells that make

up the overlying epithelium and function in antigen transport. Intestinal barrier integrity is a key component for proper epithelial cell function and prevention of entry of pathogenic bacteria. SDPP supplementation is believed to reduce toxin-induced increases in intestinal permeability by preventing passage of antigens to interstitial space, effectively blocking a local inflammatory response at the cellular level (Santos et al., 2001).

Enterocytes are continuously shed from the tips of villi and replaced with new cells migrating up from crypts. Deeper crypts have been observed after SDPP supplementation, suggesting better maintenance of barrier integrity though increased enterocyte renewal. However, this effect has only been observed when the barrier is compromised (Bosi et al., 2004). Responses to SDPP are greater in challenged vs. clean environments, possibly from specific immunoglobulin proteins in the plasma that improve performance (Coffey and Cromwell, 1995). Pierce et al. (2005) attributed the enhanced performance in early weaned pigs receiving SDPP to IgG. They theorized that IgG prevented viruses and bacteria from colonizing and damaging the GI tract, resulting in a more functional intestinal wall. However, other benefits of SDPP, such as anti-inflammatory properties, cannot be credited to IgG alone. The inflammatory pathway is complex, and SDPP has many components. The many benefits of SDPP and the multiple pathways potentially affected creates some doubt that one constituent of SDPP could be the single key factor. A proposed mechanism for improved performance with SDPP inclusion suggests the level of immune cell activation limits energy reserves for growth (Perez-Bosque et al., 2004). Stimulation of the immune system shifts available

energy from productive functions and diverts them to support immune response (Moreto and Perez-Bosque, 2009). In this theory of immune system involvement, SDPP improves intestinal homeostasis and reduces basal activation of the immune system by preventing the increase in γδ-T lymphocytes in Peyer's patches and *lamina propria*. Following this theory, SDPP would improve performance and growth by providing immunoglobulins, therefore limiting immune cell activation in the intestine, and allowing available energy to be directed toward growth rather than preparing for inflammation or disease. Beski et al. (2016) observed that broiler chickens receiving SDPP supplementation in starter diets had decreased concentrations of immunoglobulins in serum, indicating that stimulation of the immune system was reduced and more resources were likely available for maintenance and growth. In weaned rats challenged with *S. aureus* superantigen B, dietary supplementation with plasma proteins reduced immune activation of Peyer's patches and mesenteric lymph nodes (Perez-Bosque, 2004). Nofrarias et al. (2006) found that SDPP supplemented pigs had improved feed efficiency, reduced percentages of immune cell subsets in blood and GALT, reduced intra-epithelial lymphocyte (IEL) numbers, and reduced density of *lamina propria* cells. These decreased levels of immune involved cells implicates a lower activation of the immune system in SDPP fed pigs compared to controls.

Stress events cause an increase in pro-inflammatory cytokines that reduce intestinal barrier health, as activation of T-helper lymphocytes instigates a cytokine release that increases the innate and acquired immune response (Mowat, 2003). An additional proposed mechanism involved regulation of GALT by reducing

overstimulation and subsequent expression of pro-inflammatory cytokines, explaining the reduction of mucosal inflammation by SDPP (Moreto and Perez-Bosque, 2009). SDPP mediates immune cell populations by reducing macrophage numbers in Peyer's patches while also reducing percentage of macrophages, B lymphocytes, and T cells in lymph nodes (Campbell et al., 2010a). Bioactive proteins may be equally effective at modulating non-intestinal mucosal tissue immune responses. Extensive communication between the mucosal tissues exists due to the common mucosal system for generation of T lymphocyte responses (Maijo et al., 2012). Because organized GALT is an inductor site, it connects with local and peripheral effector sites; this allows SDPP to have systemic effects and potentially reduce overstimulation of the common mucosal system (Moreto and Perez-Bosque, 2009).

Alteration of Cytokines

The bioactive proteins in SDPP can interact with intestinal mucosal immune cells and reduce GALT activity by changing the cytokine environment (Moreto and Perez-Bosque, 2009). Physical stress induces intestinal barrier dysfunction through reductions in GI blood flow, leading to tissue hypoxia, ATP depletion, acidosis, and oxidative stress. This causes opening of tight junctions and enterocyte damage, and eventually increased intestinal susceptibility to disease and inflammation (Lambert, 2009). Increased production of pro-inflammatory cytokines mediates the inflammatory response in response to infection. They function as cell signaling chemoattractants and lead to expression of adhesion molecules and localization and maturation of immune cells (Moreto and Perez-Bosque, 2009). Interleukin-8 (IL-8), tumor necrosis factor alpha

(TNF-α), and interferon gamma (IFN-γ) are expressed in high amounts in inflamed gut mucosa. IL-8 is expressed in enterocytes and macrophages, causing release of other inflammatory cells and neutrophils, inflammatory cells present in infectious disease. Cytokine TNF-α is a key component of tissue damage and can increase the ability of tissue to secrete IL-8, propagating the local or systemic inflammatory response. It has also been considered one of the major mediators of systemic progression and tissue damage in severe disease (Mazzon and Cuzzocrea, 2008). IFN-γ is produced by macrophages and other cells as an activating factor in cell signaling. These pro-inflammatory cytokines increase the expression of inducible nitric oxide synthase (iNOS) and contribute to compromised intestinal barrier integrity in inflammatory GI diseases (Perez-Bosque et al., 2010). Both IFN-γ and TNF-α reduce GI permeability by internalizing tight junction proteins (Utech et al., 2006).

It is believed that bioactive proteins modulate the immune response to pathogens or potential toxins by regulating the balance of pro-inflammatory and anti-inflammatory cytokines. Supplementation of SDPP led to a decrease in IL-8 and TNF-α in pathogen challenged weaned pigs, demonstrating that SDPP decreased intestinal inflammation by reducing inflammatory cytokine expression (Bosi et al., 2004). Decreased mucosal expression of TNFα, IFN-γ, and IL-6 with lower iNOS activity suggests a reduction in mucosal mediators involved in intestinal inflammation (Perez-Bosque et al., 2010). Additionally, TNFα reduces the effectiveness of growth factors promoting protein synthesis, so SDPP supplementation may improve performance by reducing TNFα and restoring epithelial cell response to insulin-like growth factor 1 (IGF-1) (Corl et al.,

2007). Because the improved growth effects of SDP are typically only observed in challenged environments compared to the relatively continual decrease in pro-inflammatory cytokines, it is believed that the mechanisms used to reduce intestinal inflammation are not tightly correlated with beneficial effects on barrier function (Peace et al., 2011).

In a mouse model of intestinal inflammation, SDPP supplementation prevented *S. aureus enterotoxin B* (SEB) induced expression of TNF and IL-1β in jejunum mucosa (Perez-Bosque et al., 2016). TNF-α, with other pro-inflammatory cytokines, induces expression of ligands vascular cell adhesion molecule 1 (VCAM-1) and intracellular adhesion molecule 1 (ICAM-1) as well as adhesion molecule receptors to perpetuate the propagation of the immune response and sustain inflammation. The SDPP also increased the expression of anti-inflammatory molecules IL-10 and TGF-β by 2-3 fold, altering the production of lymphocytes, phagocytes, and dendritic cells to affect the immune response. Percentage of activated neutrophils and relative number of activated Th lymphocytes was also reduced, indicating that bioactive proteins can attenuate both innate and acquired immunity.

Applications in Livestock

Campbell et al. (2010) suggested that improved intestinal integrity and subsequent growth performance in disease challenged animals was due to SDPP modulating the immune response to infection. Presence of pathogens is associated with a reduction in epithelial junction complex function and increased permeability. Pro-inflammatory cytokines produced during an inflammatory response affect the expression

of nutrient transport proteins and intestinal membrane permeability (Moreto and Perez-Bosque, 2009). SDPP has been shown to reduce the increase in flux, possibly by mediating pro-inflammatory cytokines that can lead to tight-junction dysfunction while increasing expression of anti-inflammatory cytokines. In early-weaned pigs challenged with enterotoxigenic *E. coli* K88 (ETEC K88), SDPP improved growth and reduced inflammation. Enhanced growth performance, reduced IgA secretion, decreased intestinal mucosal damage, and reduced pro-inflammatory cytokine expression in the gut was observed in SDPP treated animals. Pigs fed SDPP compared to control were equally susceptible to disease, indicating a strong efficacy for SDPP to prevent ETEC K88 pathogenesis (Bosi et al., 2004). It is possible that SDPP glycoproteins may have inhibited ETEC K88 adhesion by competing with intestinal glycoprotein receptors in the intestine, and SDPP immunoglobulins may have acted to decrease bacteria available for colonization.

Efficacy of SDPP was also tested in neonatal pigs infected with rotavirus. Pigs fed plasma protein had improvements in gut health, reductions in diarrhea, and maintained greater intestinal function during acute infection than control pigs. Most importantly, diarrhea of supplemented pigs was resolved. Rotavirus shedding scores were similar to control pigs, but no diarrhea was observed (Corl et al., 2007). This was again attributed to glycoproteins and immunoglobulins present in SDPP. A similar effect was observed by Quigley and Drew (2000) in dairy calves challenged with *E. coli*. Calves were treated either with antibiotics or SDPP, with treatments showing a 25 and 34% increase in BW gain compared to controls. Calves challenged with coronavirus

responded to oral SDPP supplementation in a comparable manner. Treated calves had increased feed intake more closely correlated with ADG compared to control calves, suggesting decreased variability in efficiency of treated animals to convert nutrients into BW (Arthington et al., 2002).

A common industry issue involves feedstuff contamination with mycotoxins. Mycotoxins decrease nutrient availability to the animal and negatively impact growth performance. Weaver et al. (2014) tested the mitigating effects of SDPP supplementation on pigs consuming corn naturally contaminated with mycotoxins. Pigs receiving bioactive proteins showed a 41.8% increase in ADG and a 35.5% increase in average daily feed intake (ADFI) immediately after weaning compared to controls. Multiple mycotoxins negatively impacted the performance of all animals, but the effect on ADG and ADFI was more significant in untreated pigs who did not receive SDPP prior to weaning, with ADG reduced by 15% and ADFI reduced by 13.6%. Addition of SDPP most likely caused improvements in gut health prior to the stress of weaning, preventing the decreases in feed intake and growth efficiency. Treated pigs also demonstrated lower blood urea nitrogen (BUN) levels, indicating that bioactive proteins in the intestine have antimicrobial effects and decrease catabolism of amino acids to ammonia and urea.

Bioactive Protein Use in Equines

Current literature indicates that SDPP promote repair of tight junctions and reduce permeability and inflammation in the GIT. Expression of pro-inflammatory cytokines is also reduced, suggesting that SDPP mediates the immune response by

reducing prolonged inflammation and subsequent tissue damage during stress. McClure et al. (2016) investigated the ability of SDPP to ameliorate and prevent squamous gastric ulcers in horses, and observed that daily dosage of 210 g prevented formation of squamous gastric ulcers. Significantly fewer horses on an exercise and training program supplemented with 210 g/d SDPP developed ulcers compared to control horses, demonstrated by 14.3% incidence in SDPP treated horses compared to 85.7% incidence in untreated horses. Horses on 80 g/d SDPP trend toward a decreased incidence of squamous ulcers compared to controls (35% vs. 67%, respectively), most likely the result of decreased inflammation in gastric mucosa. Spray-dried plasma proteins may also instigate gastric mucosal tissue repair. It is reasonable to conclude that growth factors present in plasma and serum such as IGF-1 and epidermal growth factor are present in SDPP and are biologically active. Gastric mucosa exhibits receptors for epidermal growth factor, with the majority in basal epithelial layers where cell proliferation occurs (Jeffrey et al., 2001). If damage has occurred, increasing the amount of growth factor present in a location dense with receptors may improve rate of tissue renewal and allow for improved gastric mucosal integrity.

Immunoglobulins and growth factors are components of serum, plasma, and colostrum. Bovine colostrum containing bioactive proteins was fed to racehorses, and improved racing performance and postrace recovery. Bloodstock Research Information Services (BRIS) speed figures were used to determine changes in performance ability. BRIS speed figures are determined by a computed algorithm which includes racetrack, surface, track condition, quality of competing horses, and other factors that affect racing

performance of a horse (Fenger et al., 2014). Horses receiving 100 g of bioactive protein responded with 5 BRIS speed figure improvement, which represents an approximate 5 length improvement in performance. Treated horses were able to return to racing 7.5 d sooner than control horses, possibly because of a reduction in skeletal muscle oxidative stress.

Spray-dried plasma proteins have been shown to improve gait kinematics in mature horses. Coverdale and Campbell (2014) observed that horses receiving a SDPP supplement trended towards greater mean SL for the front limb on d 14, and a significant increase in mean SL on d 28. Hind limb SL behaved similarly. As levels of SDPP increased from 140g to 210g, mean SL increased in the front limb at the walk (Coverdale and Campbell, 2015). Hind limb SL followed a similar trend with increasing inclusion of SDPP. This suggests that SDPP affects local sites of inflammation in joints to decrease inflammation and pain, leading to an increase in joint function.

Inflammation and Joint Disease

Implications

Joint disease is one of the most common causes of pain, disability, and economic loss in all populations (Loeser, 2010). In humans, physical impairment resulting from joint disease in a lower limb is equivalent to disability resulting from end stage kidney or heart failure (Saltzman et al., 2006). Degenerative joint disease in horses was first reported in 1938 in Army horses and mules in Panama (Callender and Kelser, 1938). Since then, it has been a widely studied area in man and equines, receiving clinical attention from the American Association of Equine Practitioners in 1966 where it was

discussed as a relationship between lameness and "use trauma". Currently, joint disease constitutes the greatest single economic loss to the equine industry and represents a significant welfare concern (Frisbie et al., 2006). Lameness resulting from joint disease is the most prevalent cause of decreased performance and wastage in racehorses (Goodrich and Nixon, 2006). Pain resulting from joint disease does not originate from cartilage itself because cartilage is strictly aneural. However, cartilage fragments created by degradative processes elicit synovitis and cause a release of inflammatory mediators that result in joint pain (Giant and Olah, 1980). Many local mediators released during inflammatory responses act on pain receptors to induce further inflammation or lower the threshold to other pro-inflammatory stimuli (Schaible et al., 2002).

Biomechanical derangements both predispose to and perpetuate inflammatory joint disease (Krasnokutsky et al., 2008). McIlwraith (2005) described equine joint disease as a group of disorders with a common end stage culminating in progressive destruction of articular cartilage in coordination with degradation of soft tissue and bone. Trauma resulting in joint damage can originate in the synovial membrane, joint capsule, subchondral bone, ligaments, or articular cartilage, or any combination of the above. Typically, joint disease results from normal loading on abnormal tissues, or abnormal loading on normal tissues. Abnormal loading on normal tissue commonly results from acute mechanical trauma, repetitive load, or overload, with the amount of damage related to the intensity and force of the impact.

Due to current training regimens, the time away from exercise required to permit recovery from an inflammatory response is often shortened or ignored. This perpetuates

production of inflammatory mechanisms and can be deleterious to tissue and joint components, leading to normal loading on abnormal tissue (Palmer and Bertone, 1994). Chronic joint inflammation results in an imbalance in normal cartilage metabolism due to an upregulation of matrix destruction with no compensatory increase in synthesis. Current therapy options often worsen the original insult, potentially contaminate the joint, or cause further degradation of the cartilage matrix. Continuing exercise will alter the biomechanical properties of the diarthrodal joint and can lead to degenerative joint disease, limiting the athletic capability and potential of the horse (Palmer and Bertone, 1994).

Local Response to Inflammation

The synovial membrane provides a selective barrier for filtration of plasma components in the synovial joint. In the horse, acutely inflamed joints show increased vascular permeability, edema, inflammatory cell influx, and increased synoviocytes compared to normal joints (Johansson and Rejno, 1976). Non-specific mediators also enter the inflamed joint due to disruption of the blood-synovial barrier, including kinin, histamine, plasminogen, and trypsin. These mediators are autocatalytic, and potentiate a cascade effect similar to the complement system that results in infiltration of leukocytes and monocytes. Leukocyte derived products stimulate macrophages, synoviocytes, and chondrocytes, resulting in an increase in required mediators like cytokines. Cytokines include a group of small inducible polypeptides, including interleukins, interferons, TNF, colony stimulating factors, and growth factors (Palmer and Bertone, 1994). Because cytokines regulate innate and acquired immune

functions, it is reasonable that intense effects from these signaling molecules in acute or chronic inflammatory responses is related to unregulated synthesis of pro-inflammatory cytokines or inadequate production of anti-inflammatory cytokines (McKay and Baird, 1999). Cytokines exert several effects on tissue function, including synergistic, pleiotropic, and inhibitory. Due to complex intracellular signaling, this often involves non-cytokine mediators. Immune mediated changes in tissue function are due to the pleiotropic effects of multiple mediators.

In general, cytokines induce expression of immune accessory molecules in tissue, which increases the capacity to influence an inflammatory response (McKay and Baird, 1999). A successful inflammatory response results in the host controlling inflammation to prevent tissue damage from excessive cytokine production while also stimulating antigen specific responses to eliminate the pathogen and protect itself from further exposures. A balance of pre-formed and acute inflammatory mediators in addition to naturally occurring inhibitors determines the extent, duration, and reversibility of tissue damage and membrane response (Palmer and Bertone, 1994). Most often, the inflammatory process begins in the synovium, cartilage, joint, or subchondral bone and initiates a cascade of inflammatory mediators from the original insult. These mediators propagate the inflammatory response into secondary tissues that release additional metabolites, including those of arachidonic acid which initiates pain through prostaglandin release. Arachidonic acid is then oxidized by either cyclooxygenase (COX) or 5-lipoxygenase, and subsequent formation of prostaglandins or leukotrienes, respectively (Goodrich and Nixon, 2006). All inflammatory processes are integrated in

COX pathways, with COX 1 constitutive and present in all cell types. It functions to provide prostaglandins in the stomach and intestine to maintain integrity of mucosal epithelium. Conversely, COX 2 is absent in normal tissue and must be induced. The majority of stimuli known to induce COX 2 are pro-inflammatory, such as IL-1 and TNF-α. During an inflammatory response, prostaglandins are produced at the site of incidence and cause hyperalgesia with production of pro-inflammatory cytokines and induction of COX 2 in inflammatory cells. Non-steroidal anti-inflammatory drugs (NSAIDS) inhibit prostaglandin production during inflammation, but also inhibit homeostatic maintenance of mucosa. Anti-inflammatory mediators such as IL-4, IL-10, and alpha-2-macroglobulin (α2M) will decrease induction of COX 2 in a manner similar to corticosteroids or COX 2 specific inhibitors (Vane et al., 1998).

Immunological response to infection or injury involves highly regulated interactions between pro- and anti-inflammatory mediators and multiple body systems. While intended to eliminate infection, pro-inflammatory reactions are often responsible for tissue damage if left unchecked. Extensive anti-inflammatory reactions are necessary to limit tissue damage, but can lead to increased susceptibility to secondary infection (Wong and Wilkins, 2015). When external influences exceed physiological capacity or when internal disruptions lower the ability for adaptive response, the homeostatic system breaks down and disease ensues (De Grauw, 2011). Under physiological conditions, a balance is maintained between synthesis and degradation. When pathological disease conditions persist, this balance is tipped towards net loss of tissue (Lohmander, 1988). Early damage is seen at the articular surface and in the upper mid zone of cartilage and is

accompanied by loss of large aggregating proteoglycans and small proteoglycans. Exposure to cytokines such as IL-1β increase proteolytic activity and matrix turnover (Hardingham and Bayliss, 1990). As disease progresses, large proteoglycans exhibit more degradative changes and eventually are lost and replaced with larger molecules with different side chains. Fibrillation of cartilage occurs, and collagen damage extends throughout cartilage near the end stage of joint disease. The organization of small proteoglycans is altered, and an overall reorganization of proteoglycans leads to net collagen destruction (Poole et al., 1992).

Generally, inflammation and angiogenesis accompany each other. Inflammation causes angiogenesis, and angiogenesis perpetuates inflammation. Inflammatory cells such as macrophages secrete pro-angiogenic factors as well as signals that cause release of other cells that produce additional angiogenic substances. Blood vessel permeability and upregulation of adhesion molecules increase as part of angiogenesis, and further perpetuate the immune response (Krasnokutsky et al., 2008). Vasodilation resulting from mediator release facilitates leukocyte margination to the affected area. In the initial stages of acute inflammation, inactive forms of non-specific mediators of inflammation enter the joint due to mechanical disruption of the blood-synovial barrier (Palmer and Bertone, 1994). Leukocyte infiltration is enhanced by formation of local edema, which creates an aqueous environment for migration through the extracellular matrix. Stimulation of macrophages, synoviocytes, and chondrocytes by leukocyte derived products upregulates production of mediators such as cytokines, eicosanoids, and matrix enzymes. Chemokines assist with leukocyte migration, and monocytes produce pro-

inflammatory cytokines which alter the effectiveness of phagocytosis of pathogens and removal of degraded host cells (Wong and Wilkins, 2015). Activated macrophages, synoviocytes, and connective tissue cells all have the capability to secrete cytokines, which in turn initiate further degradative processes. The major initial degradative step involves matrix metalloproteinases (MMP) released by invading cells that cause mechanical disruption and release of free radicals (Murphy et al., 1991). MMPs are Zn^{2+} dependent extracellular enzymes that function in tissue remodeling. Expression of MMPs is regulated by a number of cytokines, growth factors, and hormones. Many are products of macrophages, such as IL-1 and TNF-α. Inactivation of MMPs involves specific tissue inhibitors of metalloproteinases (TIMP) and the proteinase scavenger α2M. Because it is highly present in body fluid, it has been suggested that α2M plays an integral role in MMP inhibition. Although it is present in synovial fluid during inflammation, α2M has a limited function due to its large size. TIMPs are considered a major inhibitor of inflammation at tissue level, as high molecular weight α2M has difficulty penetrating tissues like articular cartilage. However, in body fluids, α2M is the major inhibitor of MMPs. In the presence of both TIMP and α2M, activated MMPs are entrapped by α2M almost exclusively (Tchetverikov et al., 2005). The balance of pre-formed and activated inflammatory mediators with naturally occurring inhibitors determines the extent, duration, and potential tissue damage from an inflammatory response (Palmer and Bertone, 1994).

Systemic Response to Inflammation

Systemic inflammation arises following local inflammation if pro-inflammatory mediators overwhelm the initial localized host defenses. The purpose of the local inflammatory response is to contain tissue damage at the site of the initiating insult. Tissue injury or pathogen introduction instigates the innate immune response as a first mechanism of defense, resulting in activation of endogenous mechanisms and release of mediators. If not successfully contained by local immune responses, pathogen-associated molecular patterns (PAMP), danger-associated molecular patterns (DAMP), and pro-inflammatory cytokines propagate signals to the system defenses after promoting vasoactive and phagocytic phases of inflammation (Vinther et al., 2016). Cell associated pattern-recognition receptors (PRR) recognize PAMP and DAMP and activate signal transduction pathways. Locally produced cytokines may be released into systemic circulation and further instigate fever, neutrophil recruitment, and acute phase protein responses. Cytokines may also be introduced into systemic circulation through an indirect method. Locally inflamed tissues release pro-inflammatory substances that activate endothelial cells and peripheral blood leukocytes (PBL) to prompt pro-inflammatory cytokine release. An additional proposed mechanism for initiation of systemic inflammatory response syndrome (SIRS) involves local inflammatory substances inducing transcriptional changes in inflammatory genes in circulating leukocytes. In horses and other mammals, systemic release of TNF-α is essential in immunological host defense, and TNF-α concentration directly correlates with disease severity and lethality (Rutten et al., 2016). Additionally it leads to production of other

pro-inflammatory cytokines that cause increased leukocyte extravasation and tissue damage, contributing to the development of organ damage and sepsis. Increased concentrations of cytokines in addition to high levels of prostaglandins and leukotrienes result in endothelial dysfunction, leading to vasodilation and increased capillary permeability (Wong and Wilkins, 2015). If left unchecked for an extended time, inflammatory mediators will eventually stimulate the sympathetic nervous system and result in activation of adrenergic receptors on macrophages. This increases release of cytokines and chemokines and intensifies the inflammatory reaction and SIRS.

It is unknown what mechanisms determine if a local inflammatory response will initiate a systemic response or be contained by local host defenses. The severity of the instigating insult is most likely a key component. Overall, the greater the insult, the larger the response from the host's immune defense. Limited insults typically result in a shorter inflammatory response that is reversed when the damage is controlled and eliminated. Likewise, a significant insult will result in a coordinating substantial response, eventually culminating in SIRS. Lack of information in this area may result from limited understanding of associations between non-specific clinical signs of systemic inflammation and the relationship between clinical signs and the immune response (Vinther et al., 2015). Systemic inflammatory response syndrome can be initiated from 2 pathways: one being products released from infection and other involving products from released from cells damaged by trauma or injury. Diseases and insults resulting in SIRS are widespread and include endotoxemia, septicemia,

hemorrhagic shock, burns, localized bacterial infections, and noninfectious conditions like ischemia and trauma (Hooijberg et al., 2014, Wong and Wilkins, 2015).

There are several anti-inflammatory mediators that function in systemic and local inflammation. Several cytokines have anti-inflammatory properties, but due to their short half-lives instigate effects near the site of incidence. Alpha-2-macroglobulin functions in a similar manner, but is ubiquitous in plasma and restricted in activity in joint due to its large size. It is a large glycoprotein produced mostly by hepatocytes and is present in high concentrations in plasma. It inhibits many proteases such as MMPs by acting as molecular trap, and is released with other plasma proteins into exudate. Once bound to a protease, α2M undergoes a conformational change that increases transport speed, allowing it to be rapidly cleared by mediated endocytosis in hepatocytes and macrophages. Unbound α2M is not recognized by receptors and has a prolonged half-life in circulation. In addition to functioning as a proteinase inhibitor, α2M inhibits macrophage production of superoxide anion, suppresses immune cell modulatory action, and inhibits activated T cell proliferation. It may also have regulatory action on cytokines. Conformation of α2M influences binding of cytokines such as IL-1 and TNF-α, creating a mediating effect on inflammatory responses (Cote et al., 1998).

Cytokines as Markers of Inflammation in the Equine

Markers are molecules that are normal products of metabolic signaling processes that are direct or indirect indicators of tissue turnover. The balance between anabolic and catabolic processes is disturbed during inflammation and disease, so concentrations of markers will change. Inflammatory molecules from cartilage, menisci, ligament, or

synovial membrane can enter the synovial fluid or systemic circulation if inflammatory processes cannot be contained locally. During normal and pathological turnover, degradation products and inflammatory exudate are released to circulation. Newly developed sensitive assays allow for specific detection of these fragments in body fluids (Lohmander, 1988). The ability to determine levels of biomarkers in synovial fluid, serum, and urine allows for their use as indicators of inflammatory processes (McIlwraith, 2005). Systemic levels of biomarkers in serum and urine give an indication of total metabolic activity originating from all joints in the entire body (Sumer et al., 2006). However, a complicating factor in analyzing systemic markers is the unknown influence of renal or liver disease on serum antigen concentrations.

Pro-inflammatory cytokines TNF-α and IL-1β are soluble cell signaling molecules that utilize many mechanisms to affect various cell types. They recruit circulating neutrophils to sites of inflammation after release from tissue macrophages in response to injury or infection and directly stimulate isolated neutrophil oxidative activity in the equine (Benbarek et al., 2008). Both are active at low level concentrations and are integral in maintaining oxidative neutrophil activity and contributing to perpetuation of disease. Interleukin one beta is considered the master cytokine in joint disease. It is formed when IL-1 is enzymatically cleaved into 2 lower weight forms, α and β, with IL-1β activity about 90 times more potent than IL-1α. Receptors are highly sensitive and only need small amounts of IL-1β to initiate destructive joint processes. Additionally, receptors of diseased or inflamed cells have increased numbers of receptors, increasing the susceptibility of degradation. Similar to other pro-

inflammatory cytokines, IL-1 induces synthesis of MMPs, eicosanoids, plasminogen activators, and acute phase proteins while inhibiting synthesis of macromolecules, collagen, and aggregating proteoglycan. However, IL-1 has a unique capacity to stimulate the oxidation of arachidonic acid through activation of membrane bound phospholipase A2 in chondrocytes, synoviocytes, and monocytes (Palmer and Bertone, 1994). TNF-α is the most prominent cytokine involved in acute inflammation, but IL-1β remains high through all stages (McIlwraith, 2012). Both TNF-α and IL-1β suppresses tissue synthesis and increase rate of degradation by stimulating proteinase production (Hardingham and Bayliss, 1990). IL-1 stimulates chondrocytes to increase metabolism of proteoglycans while also inhibiting synthesis of new proteoglycans, and TNF-α acts similarly (Lohmander, 1988). Although it is 10 times less potent than IL-1β, TNF-α acts synergistically with IL-1 to instigate production of MMPs and prostaglandins to inhibit tissue synthesis. Combined they are considered the major catabolic systems involved in inflammatory tissue damage (Martel-Pelletier, 1998). Autocrine and paracrine activities have been attributed to TNF-α and IL-1β, with both mechanisms functioning to amplify the original inflammation stimulus to either speed up homeostatic regulation or exacerbate tissue damage in a disease state (Westacott and Sharif, 1996). The effect of TNF-α on macrophages is self-limiting, as it binds to a different cell surface receptor than IL-1. In addition to synergistic efforts with IL-1, TNF-α also instigates production of IL-6, IL-8, and itself (Palmer and Bertone, 1994).

Regulation of pro-inflammatory mediators is integral to prevention of tissue damage. A variety of proteins are responsible for controlling expression of cytokines in

an attempt to prevent exaggerated secretion. Appropriate levels are necessary to remove potential pathogens, but self-destruction due to over-production of cytokines negates the basic role of the immune system as a defense mechanism. Pro-inflammatory TNF-α is expressed in two forms in different amounts at different proportions in different cell types. Type I receptors signal cytotoxic activities of TNF-α, while Type II receptors signal inflammatory and proliferative activities. Therefore TNF-α is controlled at the transcriptional and translational levels intracellularly, extracellularly by enzymes, the amount of receptor, and density of receptors on target cells (Murtaugh et al., 1996). Regulation of IL-1 is similarly complex for the same reasons. It also has 2 receptors, with the first functioning in signal transmission. However, receptor II binds to IL-1 with equal affinity but does not lead to cell signaling, and therefore acts as a competitive inhibitor to control the intensity of IL-1 expression at the surface of target cells. An additional regulatory mechanism for IL-1 is IL-1 receptor antagonist protein (IL-1ra). It is homologous to both forms of IL-1 (IL-1α and IL-1β), and binds the receptors with slightly less affinity, but does not induce signal transduction (Murtaugh et al., 1996).

Several biomarkers are currently utilized for use in equine research. In coordination with TNF-α, IL-1β is considered one of the most important catabolic cytokines in joint disease (Ross et al., 2012). Levels of IL-1β are increased in serum in inflammatory responses involving the respiratory tract, GIT, muscular system, and other body tissues (Ainsworth and Reyner, 2012). Ehrle et al. (2015) investigated the various levels of IL-1β in equine serum and synovial fluid in naturally occurring osteoarthritis and septic arthritis. Levels of IL-1β showed a positive correlation with inflammatory

score and were significantly different between mild joint disease, advanced joint disease, and septic arthritis groups. IL-1β in serum reflected the severity of intrasynovial inflammation, and was the only parameter which showed significantly different levels between control, aseptic, and septic groups, as well between the 2 aseptic groups. Horses with abnormal hematological findings not explained by joint disease were identified with a blood chemistry panel and excluded to ensure that levels of cytokines were reflective of joint disease and no other inflammatory processes. Blood chemistry panels measure indicators of internal organ disease such as total serum protein (TSP), albumin, phosphorus (P), blood urea nitrogen (BUN), creatinine, bilirubin, creatine kinase (CK), globulins, and gamma glutamyl transferase (GGT). Glucose is also measured to determine if normal levels are present, as some internal organ diseases can affect glucose uptake and utilization. Alterations in TSP, albumin, or globulins can indicate the presence of dehydration, inflammation, decreased liver function, blood loss, or kidney loss. Phosphorus, BUN, and creatinine levels are increased if kidney function is compromised, with total bilirubin and GGT increasing if liver damage is present (Bauer, 1990).

Kinematic Gait Analysis

Biomechanics of Movement

Locomotion is the ultimate expression of exercise activity, and involves moving all body and limb segments in rhythmic and automatic patterns that are defined as various gaits. Gaits are defined as complex and strictly coordinated movements of the limbs and entire body which result in progressive movement. The two types of gait are

distinguished by symmetry or asymmetry of limb movement sequence with respect to time and median plane of the horse, with symmetric gaits including the walk and trot and asymmetric the canter and gallop. The trot, canter, and gallop have a suspension phase when there is no hoof contact with the ground (Barrey, 1999). Clayton (1989) defined the walk as a natural 4 beat gait with no suspension time. It has 4 footfalls per stride with 8 periods of stance phase with the horse always having 2 feet on the ground. Progressive motion is achieved by the reach of the limbs, not suspension (Moore, 2010). The trot has 2 footfalls of diagonal pairs with 2 stance phases and 2 suspension phases. Ground is covered by the reach of limbs as well as during suspension phases; however, some horses have less reach and cover more ground during suspension. Conversely, some horses "sprawl" to increase forward motion and have an increased range of motion (ROM) of suspended limbs during prolonged stance phases (Moore, 2010).

A full cycle of limb motion is considered a stride. The beginning of a stride can be at any point in the pattern of movement and end of that stride at the same place when a new pattern is beginning. Therefore, SL corresponds to the distance between 2 successive hoof placements of the same limb, and is calculated as the average distance covered between 2 consecutive contacts of the forelimb or hindlimb (Galisteo et al., 2001; Barrey, 1999). Each stride includes a stance phase when the limb is in contact with the ground and a swing phase when the limb has no contact with the ground. Stance phase is comprised of restraint and propulsion stages. Restraint stage last from heel contact to mid-stance position, and propulsion includes the remainder of stance phase from mid-stance to lift off (Drevemo et al., 1980). Both fore and hindlegs can

adduct, abduct, and push against the ground during stance and swing phase. The hind legs also load the forelegs if required, and push backward to propel the horse forward to increase speed efficiency and create a rolling motion over the forelimbs (Moore, 2010).

High speeds elicit high cardiac and metabolic responses that are explained by a linear increase in SL (Barrey, 1999). Stride length and frequency are functions of the musculoskeletal system, with SL as the primary function in negative work mode. During negative work, muscles maintain tension in stretching tendons as load is added. Stride length is equivalent to amplitude and behaves according to a power law, where amplitude increases with constant or decreasing frequency (Rooney et al., 1991). It is the primary determinant of velocity and aerobic energy expenditure in sound horses (Peham et al., 2001). The relationship between velocity, SL, and stride frequency in horses was observed by Rooney et al. (1991). They determined that stride frequency increased as walking velocity increased, but SL was relatively unaffected. At the transition from walk to trot and thereafter, increased velocity was associated with variable alterations in increases of SL and stride frequency. Length of stride increases as velocity increases, and maximum stride frequencies occur before peak speed is reached. Horses with longer SL typically have higher peak speeds than shorter strided horses. However, factors such as conformation, cardiovascular and pulmonary function, energy metabolism, and innate desire to perform must be considered in predictions of speed or performance potential (Ratzlaff et al., 1995).

Variation and Consistency in Motion Pattern Analysis

The most constant kinematic components include SL, swing, step, and suspension duration with significant correlations between SL, duration, and swing phase duration (Leach, 1983). Intra-individual locomotor patterns are relatively stable and consistent, and repeated from one stride to the next with only minor deviations. This allows for short-term comparisons of the effect of therapeutic agents or treatments. A minimum of 3-5 strides is necessary for sufficient understanding of stride in an individual horse (Drevemo et al., 1980, Leach, 1983). Speed is an important influence on motion pattern consistency (Peham et al., 2001). Little variation is found for intra-individual stride characteristics using 2D methods if speed is controlled. Selection of speed depends on conformation, individual optimum trotting speed, potential presence of lameness, and analysis protocol, with the recommendation of trotting in hand at the slowest self-selected speed to facilitate evaluation (Peham et al., 2000). An optimum trotting speed where variation of motion is small exists for every horse, allowing for precise and reproducible gait analysis. On firm footing, horses will self-select speeds close to energetically optimum where energy consumption is minimized at the chosen stride frequency-stride length combination (Peham et al., 1998). Each individual develops a stable locomotor pattern that is repeated regularly with minor deviations compensating for internal and external disturbances (Drevemo et al., 1980; Leach, 1983).

Conformation, training, innate and learned motion patterns, and physical condition can affect the pattern of locomotion (Peham et al., 1998). Variability increases

for inter-individual stride parameters especially if differences in breed, conformation, height, or limb length are present (Drevemo et al., 1980; Hobbs et al., 2010). Training tends to improve coordination and creates more regular movements, but there is still a tendency toward increased variation within all horses. Differences in maximum extension of the limbs may also be involved, typically as longer restraint stages in the hindlimbs (Drevemo et al., 1980). Additionally, continuous variations exist within each gait; for example, in normal variations of the trot, speed increases from collected to extended (Barrey, 1999). This makes inter-individual comparisons difficult, and a stable motion pattern necessary for reproducible analysis of motion. Other factors such as the presence and weight of shoe can modify gait kinematics (Leach, 1983).

Effects of Lameness and Inflammation on Biomechanics

The extensive amount of injuries impacting the locomotor functions of performance horses justifies the investment in equine kinematic and kinetic research and methods for preventing lameness and injury (Barrey, 1999). While SL is understood to be the primary component affecting velocity and energy expenditure in sound horses, the relationship in lame horses is more complex. Horses with joint disease or injury and resulting lameness have an increased heart rate and respiratory rate due to pain associated with movement. Limb and body trunk movements also deviate from normal in the presence of lameness, making motion less efficient. Both of these components of lameness increase energy expenditure more than increased SL (Peham et al., 2001).

Stride length variability is higher in sound horses compared to lame horses. Variation in sound horses results from minor repeated bouts of acceleration and

deceleration as compensation for various forces. This motion variability does not lead to pain, making other factors decisive. However, in lame horses, any deviation from a normal locomotor pattern may cause increased pain, making minimum variation a sign of disease (Peham et al., 2001). Unloading of a painful limb leads to redistribution of corresponding body segments, with these changes closely related to changes in stride timing and coordination. In proximal joints, such as the shoulder and hock, flexion increases in the lame limb due to smooth limb loading controlled by extensor muscles (Weishaupt et al., 2004). Pain resulting from lameness is often apparent in both stance and swing phases. During stance phase, normal changes of weight bearing lameness occur; during swing phase, the flexion of affected and corresponding joints is decreased. Overall, maximal flexion and total ROM is restricted.

Application of Kinematic Gait Analysis in Equines

Kinematics is the study of changes in the position of body segments in space during a specified time. Motions are described by linear and angular variables referencing time, displacement, velocity, and acceleration. The geometry of movement is observed without considering the forces that cause the movement (Wieshaupt et al., 2004). The first experimental kinematic measures were documented by Marey (1873) who studied the timing of each gait with a chronographic method. Muybridge (1887) followed this by using a series of cameras to analyze the horse's locomotive pattern. Modern analysis utilizes markers attached to the body which are filmed by video camera, with the successive images analyzed for parameters of interest. Skin displacement over the skeleton can create variability especially in proximal joints (Van

Weeren et al., 1990a,b). Manual analysis is required because automatic detection of markers in distal segments can be difficult (Barrey, 1999).

Motion analysis utilizes reflective markers attached to the horse's body at predetermined landmarks that are used to calculate joint angles. At least 3 markers are necessary for each body segment to create a local coordinate system and are intended to represent the approximate instantaneous center of rotation in the joint (Barrey, 1999). Markers must be lightweight and easy to replace if dislodged, and can range from 1mm to 25 mm in size. Kinematic analysis uses 2D systems which are less expensive and exceptionally useful for studying sagittal plane motions like flexion and extension. Sagittal plane systems utilize lateral markers, but often simplify the lower limb due to the small size of pastern segments (Hobbs et al., 2010). The benefit of applied gait analysis is the reliable documentation of individual patterns, degree of lameness if present, and the results of treatments (Weishaupt et al., 2004).

Summary

The benefits of SDPP in growth and performance are evident in several livestock species. The ability of dietary bioactive proteins to modulate the immune response and reduce expression of pro-inflammatory cytokines has been demonstrated effectively through disease challenges in animals as well as in human medicine. The use of gait kinematic analysis has been used in horses to show improved performance characteristics following SDPP supplementation. However, a relationship between gait kinematics and inflammatory cytokines has yet to be established or related to SDPP supplementation. Therefore, the objective of the current study was to determine the

effect of dietary bioactive protein supplementation on gait kinematics and systemic inflammatory markers in mature horses.

CHAPTER III

MATERIALS AND METHODS

Horses and Dietary Treatments

Twenty-seven mature horses (n=25 geldings, n=2 mares) from an established herd at Parsons Mounted Cavalry (Texas A&M University, College Station, TX) were utilized in a randomized design for a 34-d trial. Horses were stratified by BW (560±52 kg), BCS (5.21±0.42), and age (13±3 yr). All procedures and handling of horses was approved by the Institutional Animal Care and Use Committee (AUP# 2015-0380).

Horses received a 14% CP pelleted concentrate (Cargill, Elk River, MN) offered at 0.5% BW daily (as-fed) at 12-h intervals. Horses were randomly assigned to 1 of 3 dietary treatments that included varying levels of Lifeline Equine Elite supplement (APC, Inc., Ankeny, IA), with the active ingredient BioThrive, a bovine serum isolate. Control horses (n=9) received 0-g BioThrive, and supplemented horses received one of two doses of BioThrive, either 40-g (**40**; n=9) or 80-g (**80**; n=9) per day. For investigators to remain blinded, treatments were coded alphabetically and pre-weighed to 230 g, mixed with concentrate immediately prior to feeding. All horses received treatments on d 1, with no significant refusals after d 3. All horses received *ad libitum* access to coastal Bermudagrass hay (*Cynodon dactylon*) with *ad libitum* access to water. All horses were fed individually in 3 × 3 m stalls, and allowed 1-h to consume concentrate before orts were collected. Intake and refusals were measured daily, with horses consuming an average of 2.79-kg of feed during the study. After feeding, horses

were turned out to a dry-lot track (9 m × 1600 m) for voluntary exercise. All horses were barefoot, and received care from the same professional farrier every 4-wk. Injury or lameness was recorded if present. From d 0-32, horses were exercised in accordance with Parsons Mounted Cavalry protocol, consisting of basic horsemanship maneuvers at the walk, trot, and canter for approximately 60 min/d with a single assigned cadet 5-d/wk. On d 33, horses with appropriate training (Control: n=6, 40: n=8, 80: n=7) participated in the Battle of Flowers Parade in San Antonio, TX. Horses were hauled for approximately 580-km round-trip on a stock trailer, and marched on concrete at the walk for 6.5 km during the parade.

Samples of concentrate and treatments were all analyzed by a commercial laboratory for nutrient content (Table 1).

Table 1. Nutrient analysis of the commercial concentrate and SDPP supplements.

	Concentrate[a]	CONTROL	40[b]	80[b]
Dry Matter, %	90.00	91.92	93.02	91.81
CP, % DM	17.91	32.58	34.36	38.78
NDF, % DM	28.70	32.91	32.44	32.72
ADF, % DM	16.64	22.97	22.43	21.42
Ca, % DM	1.17	1.58	1.68	1.74
P, % DM	0.99	1.09	0.99	0.86

[a]14% CP pelleted feed (Cargill, Elk River, MN)

[b]Lifeline Equine Elite supplement (APC, Inc., Ankeny, IA)

Sample Collection

Bodyweight was recorded weekly via a digital scale (Bastrop Scale, Inc., Bastrop, TX), with concentrate intake adjusted accordingly. Body condition score was also determined weekly, using the 1-9 scale described by Henneke et al. (1983). Plasma and serum samples were collected every 7-d from d 0 to d 28, and 24-h post-parade on d-34. Approximately 50 mL whole blood was collected via jugular venipuncture. Samples intended for plasma analysis were collected into 10-mL evacuated tubes containing 0.1 mL 15% buffering solution and 15 mg K_3 EDTA (BD Vacutainer, Franklin Lakes, NJ) and immediately placed in ice. Samples intended for serum analysis were collected into 10 mL evacuated non-additive tubes (BD Vacutainer, Franklin Lakes, NJ) and allowed to remain at body temperature for 30 min prior to processing to allow blood to appropriately clot. All samples were centrifuged at 2000 × *g* at 4°C for 20 min. Supernatant was removed to pour off tubes and placed into a freezer at -20°C for subsequent analysis.

Gait analysis was performed biweekly on d 0, 14, and 28, and 24-h post-parade on d-34. Horses were walked and trotted in hand with a handler assigned for the duration of the study for 3 passes over a 10-m flight path on solid dirt footing. Reflective markers were placed on major joints on the right front and right hind limb to allow for visibility and calibration for later analysis. Front limb markers included the greater tubercle of the humerus, lateral humeral epicondle, ulnar carpal bones, lateral metacarpal epicondyle, middle phalanx-proximal phalanx junction, and proximal phalanx-distal phalanx junction. Hind limb markers included distal phalanx-middle

phalanx junction, middle phalanx-proximal phalanx junction, proximal phalanx- third metacarpal junction, tarsal bones, and lateral femoral epicondle. Video footage was collected and recorded using EquineTec software (EquineTec, Monroe, GA) installed on an HP Pavilion m6 laptop with 2.3 GHz microprocessor and AMD Rodeon HD 7660G video graphics (Hewlett-Packard, Palo Alto, CA).

Sample Analysis

Serum collected was analyzed for TNF-α and IL-1β using commercially available ELISA kits previously validated for use in the equine (Kamm et al., 2010; Lavoie-Lamoureux et al., 2010). The TNF-α assay was a 96-well plate sandwich ELISA which measured natural and recombinant equine TNF-α (R&D Systems, Inc., Minneapolis, MN). The IL-1β assay was also a 96-well plate sandwich ELISA which measured natural and recombinant equine IL-1β (R&D Systems, Inc., Minneapolis, MN). Plates were prepared by adding diluted goat anti-equine TNF-α capture antibody for TNF-α or diluted goat anti-equine IL-1β capture antibody for IL-1β, followed by an overnight incubation at room temperature at 23˚C to allow the antibody to bind. Plates were then washed 3 times and blot dried after each wash. After washing, plates were blocked by addition of diluted reagent diluent and allowed to incubate for 1-h at room temperature. Plates were again washed 3 times, with blot drying following each wash. Samples and standards were then added, with standards prepared using a 2-fold serial dilution for a 7 point standard curve. Plates were then sealed and allowed to incubate at room temperature for 2-h. Following incubation, plates were washed 3 times and blotted dry. After washing, TNF-α plates received biotinylated goat anti-equine TNF-α

detection antibody and were allowed 2-h to incubate at room temperature. Plates for IL-1β received biotinylated goat anti-equine IL-1β detection antibody, diluted in reagent diluent and 2% heat inactivated goat serum, and were allowed 2-h to incubate at room temperature. Plates were then washed 3 times and blotted dry, and Streptavidin horseradish-peroxidase (Streptavidin-HRP) was added. Plates were then covered and allowed 20-min to incubate at room temperature out of direct light. Plates were again washed 3 times and blotted dry, then substrate solution was added before plates were allowed another 20-min incubation at room temperature out of direct light. After 20-min, 2 N sulfuric acid ($H_2S0_{4)}$ was added as a stop solution, and optical density was determined immediately using a microplate reader with optical density at 450 nm, with wavelength correction at 570 nm (BioRad 680 Microplate Reader, BioRad Laboratories, Hercules, CA). Inter- and intra-assay coefficients of variation varied from 7.1-8.7% and 1.2-6.1% respectively for TNF-α, with IL-1β values ranging from 6.5-7.3% and 2.3-4.2%.

Plasma samples were used to determine blood chemistry profiles to indicate if any horses presented abnormal hematology that would relate to internal organ disease not joint disease. Samples were analyzed by Texas Veterinary Medical Diagnostic Lab (College Station, TX) for TSP, albumin, P, glucose, BUN, creatinine, CK, total bilirubin (TB), bilirubin direct (BD), GGT, albumin:globulin (A/G) ratio, and globulins. Horses with values outside of normal ranges were excluded from cytokine analysis to ensure that expression of systemic inflammatory markers were reflective of joint disease and no other inflammatory processes or internal organ disease. One horse from control, 40 and

80 were removed from cytokine analysis due to abnormal blood chemistry parameters not explained by joint disease.

EquineTec software was used to determine SL of the right front and right hind limbs, as well as the ROM of the knee and hock at both the walk and trot. Stride length was measured as the distance the limb traveled during swing phase, and ROM was determined as the distance between minimum and maximum angles during swing phase.

Statistical Analysis

All data were analyzed as a randomized design using the PROC MIXED procedure of SAS (SAS Inst., Inc., Cary, NC) with effects for treatment, day, and treatment × day interaction as well as linear and quadratic contrast. The model uses RANDOM and REPEATED statements to account for variability between animals. Day 0 was used as a covariate for gait kinematics to account for inter-individual variability. Effects were considered significant if $P \leq 0.05$, with a trend towards significance if $P \leq 0.10$.

CHAPTER IV

RESULTS AND DISCUSSION

Body Weight and Body Condition Score

There was a treatment × time interaction for BCS ($P < 0.01$) with 80 SDPP having a greater BCS on d 14 when compared to both the 40 g and controls. Horses receiving 80 maintained BCS through d 28, while control and 40 increased BCS such that there was no significant difference between treatments on d 28. There was no effect of SDPP supplementation on BW ($P = 0.76$; Fig. 1); however, there was an effect of time on BW ($P < 0.01$). All horses, regardless of treatment, gained BW and BCS until d 28, and lost BW to d 34 while BCS remained constant. This may be explained by the change from group feeding to individual feeding at the initiation of the study to ensure that each horse was fed to meet 100% NRC requirements for mature horses undergoing light exercise. Weight loss on d 34 was likely due to the effect of trailering and participation in the parade challenge on d 33 of the study. The decrease in BW is related to body water lost through sweat and subsequent dehydration as horses were weighed 24- h following the transport.

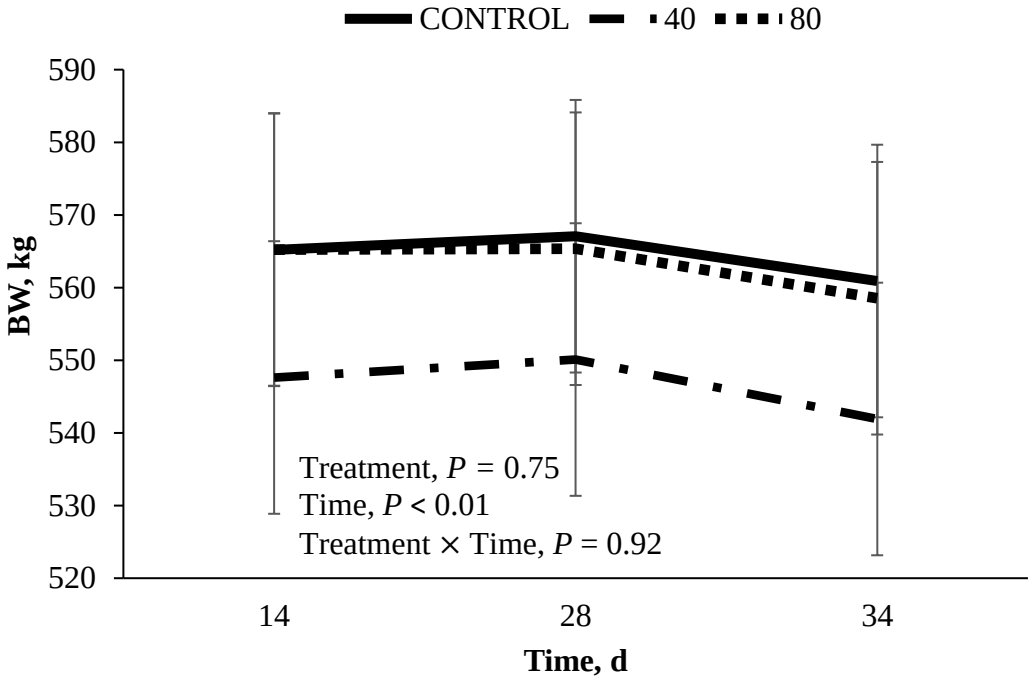

Figure 1. Body weight (kg) over time (d) in horses receiving a pelleted concentrate with control, 40, or 80 g/d of spray-dried plasma proteins.

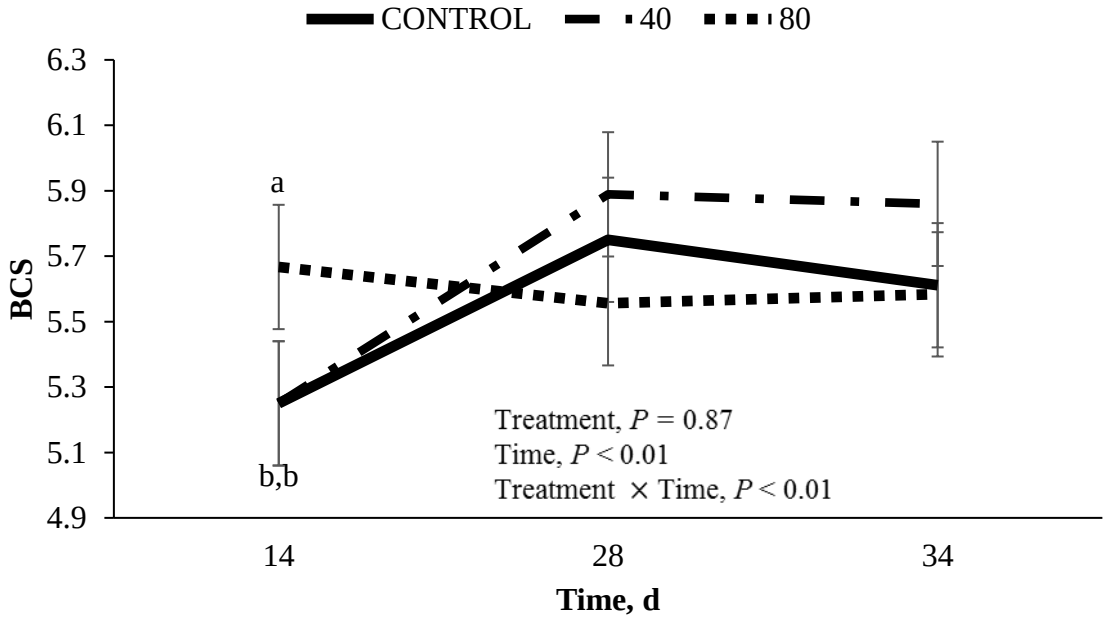

Figure 2. BCS over time (d) in horses receiving a pelleted concentrate with either control, 40, or 80 g/d of spray-dried plasma proteins. [a,b,c] superscripts denote a difference in treatments at each time.

Gait Kinematic Parameters

Stride Length

A trend towards a treatment × time interaction ($P = 0.10$, Fig. 3) was observed in SL of the fore limb at the walk, largely driven by an increase in SL in horses receiving 80 while horses receiving control and 40 decreased from d 14 to d 28. From d 28 to 34, control increased while 40 and 80 decreased. A trend towards a linear effect ($P = 0.09$) was also demonstrated with control SL at 155 cm on d 28, 40 at 168 cm, and 80 at 168.9 cm. No significant effects were observed for stride length of the fore limb at the trot ($P = 0.21$, Fig. 4).

A trend towards treatment × time interaction ($P = 0.07$, Fig. 5) was observed for hind limb SL at the walk, resulting from decreases in SL from d 14 to 28 in control and 40 while 80 increased. From d 28 to 34, 40 and 80 decreased while control increased. Stride length of the hind limb at the walk was represented by a linear contrast ($P = 0.05$) that was explained by a longer SL of treated horses compared to control. Treatment did not significantly ($P = 0.17$, Fig. 6) affect stride length of the hind limb at the trot.

Improvements in SL in the fore limb to d 28 are similar to those observed by Coverdale and Campbell (2014, 2015). The current study included a challenge component that included trailering and riding on d 33, leading to the overall trends being altered from previous studies with no challenge. Fore limb and hind limb SL at the walk had a trend toward significance in the current study compared to fore limb and hind limb SL at the trot. This may be explained by the varying biomechanics of movement for the different gaits. Because the walk has no suspension phase (Clayton, 1989), training and

coordination of the individual horse plays a smaller role. This would allow any potential benefits from supplementation of SDPP to be more evident and less reliant on the individual's athletic ability. Bioactive proteins typically modulate immune responses to physiological stress in a challenged environment (Bosi et al., 2004, Perez-Bosque et al., 2016). However, SL for both limbs at the walk decreased on d 34. The inconsistent response may be explained by other factors influencing SL after the challenge, such as muscle soreness and behavior during the challenge. Additionally, during gait kinematic analysis, horses were recorded at the walk before moving into the trot. This amount of movement may have acted as a warm-up to exercise, allowing horses to become more fluid in their movements.

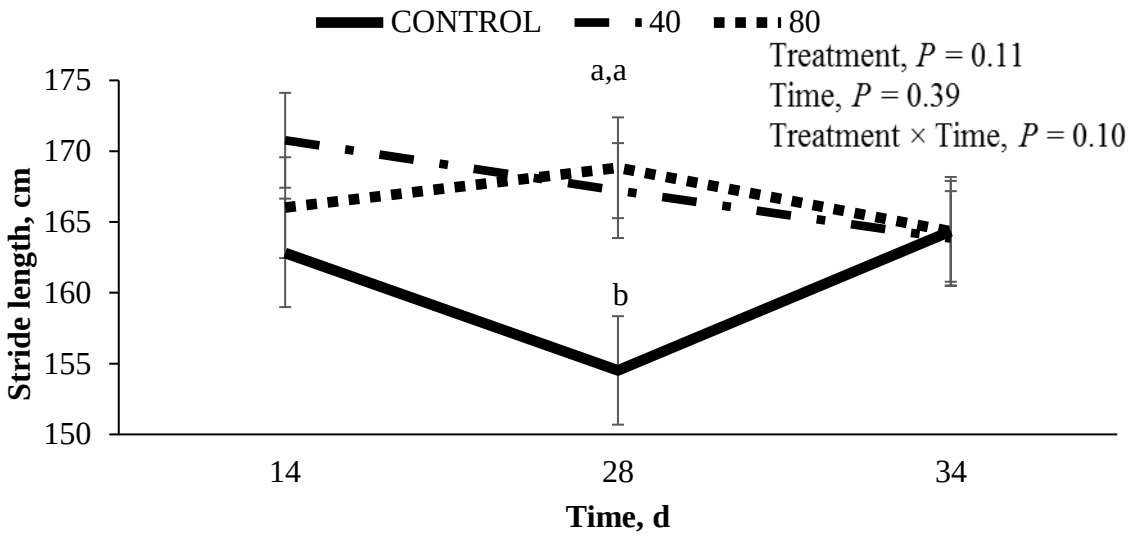

Figure 3. Stride length (cm) of the fore limb at the walk over time (d) in horses receiving a pelleted concentrate with either control, 40, or 80 g/d of spray-dried plasma proteins. [a,b,c] superscripts denote a difference in treatments at each time.

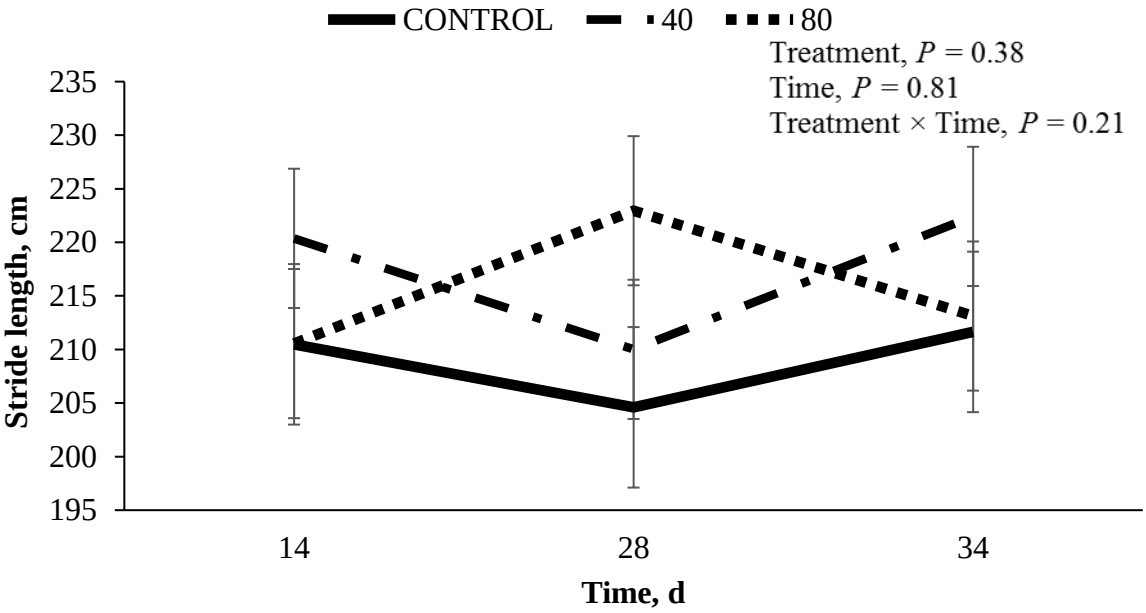

Figure 4. Stride length (cm) of the fore limb at the trot over time (d) in horses receiving a pelleted concentrate with either control, 40, or 80 g/d of spray-dried plasma proteins.

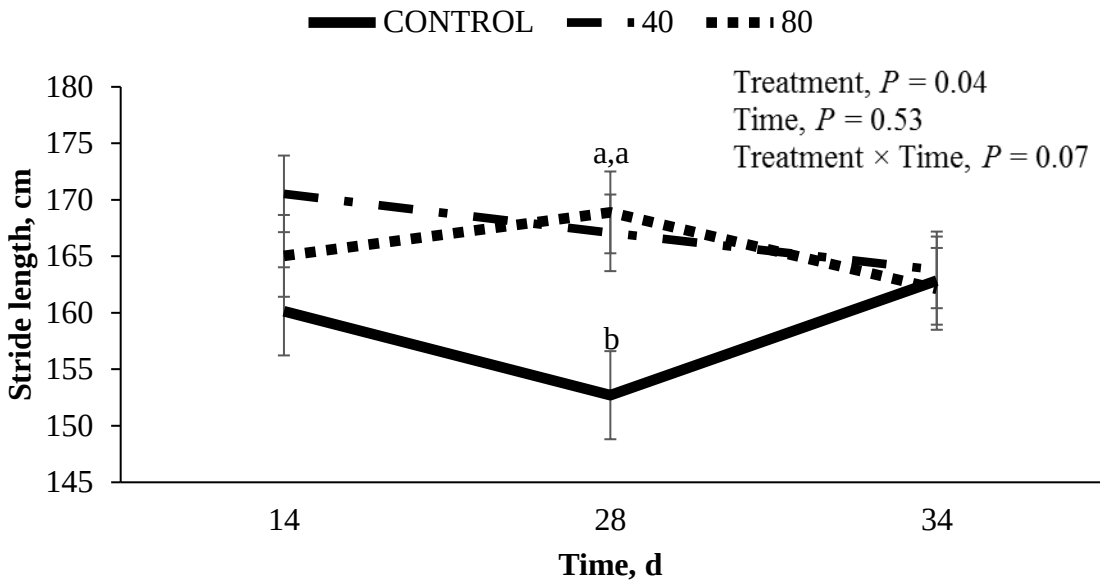

Figure 5. Stride length (cm) of the hind limb at the walk over time (d) in horses receiving a pelleted concentrate with either control, 40, or 80 g/d of spray-dried plasma proteins. [a,b,c] superscripts denote a difference in treatments at each time.

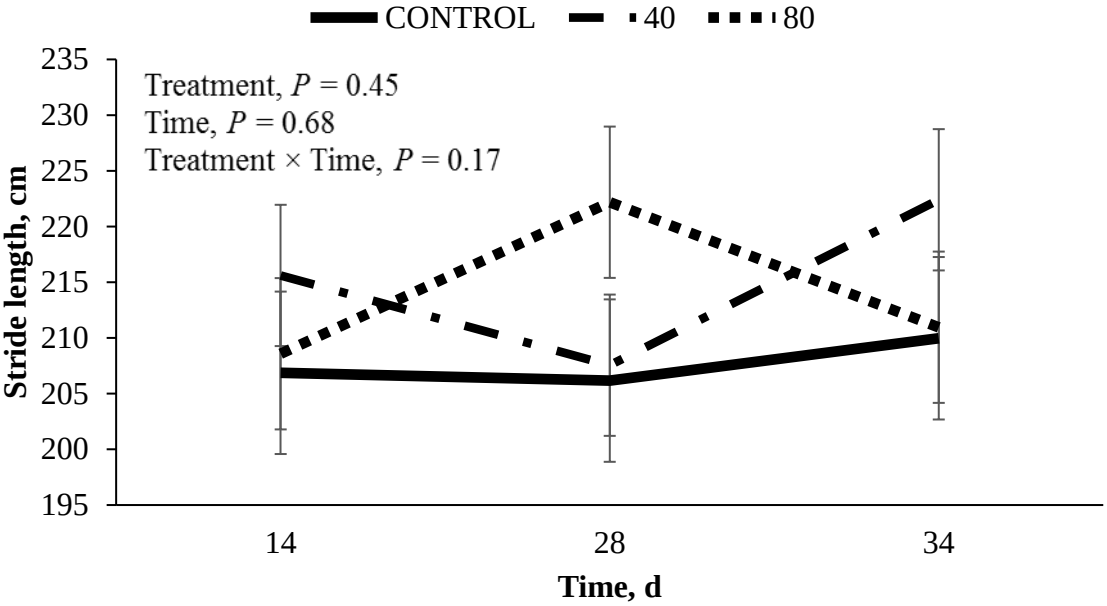

Figure 6. Stride length (cm) of the hind limb at the trot over time (d) in horses receiving a pelleted concentrate with control, 40, or 80 g/d of spray-dried plasma proteins.

Range of Motion

A trend towards treatment × day interaction was observed in the knee at the walk ($P = 0.10$, Fig. 7), due to the increased range of motion for treated horses compared to controls on d 34. Range of motion in control horses decreased from d 14 to d 34, while treated horses increased. Horses receiving 80 increased ROM through the study, with ROM reaching 32.5˚ on d 28 and 34.6˚ on d 34. Horses receiving 40 increased ROM to 34.4˚ on d 28 and decreased to 33.6˚ on d 34. Supplementation of SDPP did not affect knee range of motion at the walk or trot ($P = 0.56$, $P = 0.38$, respectively). No significant effects were observed in knee ROM at the trot ($P = 0.64$, Fig. 8).

The interaction observed for knee range of motion at the walk but not the trot may also be attributed to differing gait biomechanics as well as the individual horse's athletic ability. Previous studies (Coverdale and Campbell, 2014, 2015) did not observe trends or significance in knee ROM at either gait. The response observed in the current study for this parameter of gait kinematic analysis to the challenge on d 33 indicates that treated horses showed improvements in performance compared to control. Articular ROM relates more closely to joint comfort than SL, suggesting that bioactive protein supplementation was more effective in improving articular ROM than improving SL following a challenge.

A significant treatment × time interaction was observed ($P < 0.01$, Fig. 9) for hock ROM at the walk, which resulted from 80 increasing ROM from d 28 to d 34, 40 decreasing from d 28 to d 34, and control horse values remaining constant. Supplementation of SDPP significantly ($P < 0.01$) affected hock range of motion at the

walk best explained by a quadratic effect ($P < 0.01$) demonstrated by 40 having the highest ROM at 33.2˚, followed by control with 28.6˚, with 80 having the lowest ROM of 28.0˚. Hock range of motion at the trot was affected by time ($P = 0.05$, Fig. 10) with all horses decreasing in ROM over time. There was no effect of supplementation of SDPP on hock ROM at the trot ($P = 0.39$).

There is no current literature on gait kinematic analysis of hock ROM as it relates to SDPP supplementation. It is unknown why horses receiving 40 g/d showed a higher hock ROM at the walk to d 28 compared to horses receiving 80 g/d. However, hock ROM at the walk responded to the d 33 challenge similarly to knee ROM at the walk, with horses receiving 80 g/d SDPP showing improved articular ROM. This further suggests that SDPP are more effective at improving articular ROM than SL after a challenge.

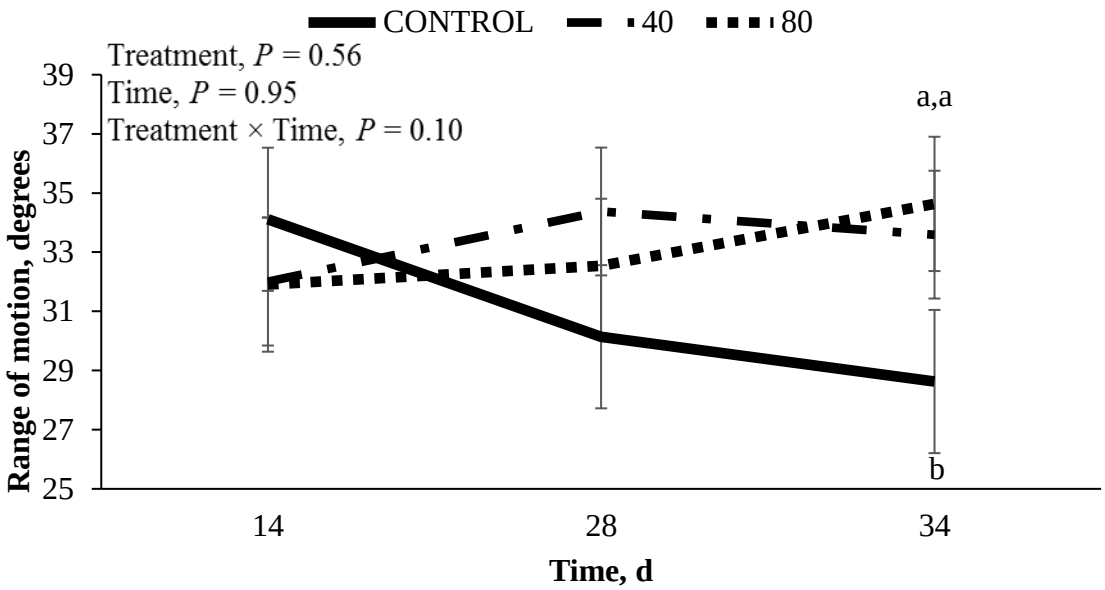

Figure 7. Range of motion (degrees) of the knee at the walk over time (d) in horses receiving a pelleted concentrate with either control, 40, or 80 g/d of spray-dried plasma proteins. [a,b,c] superscripts denote a difference in treatments at each time.

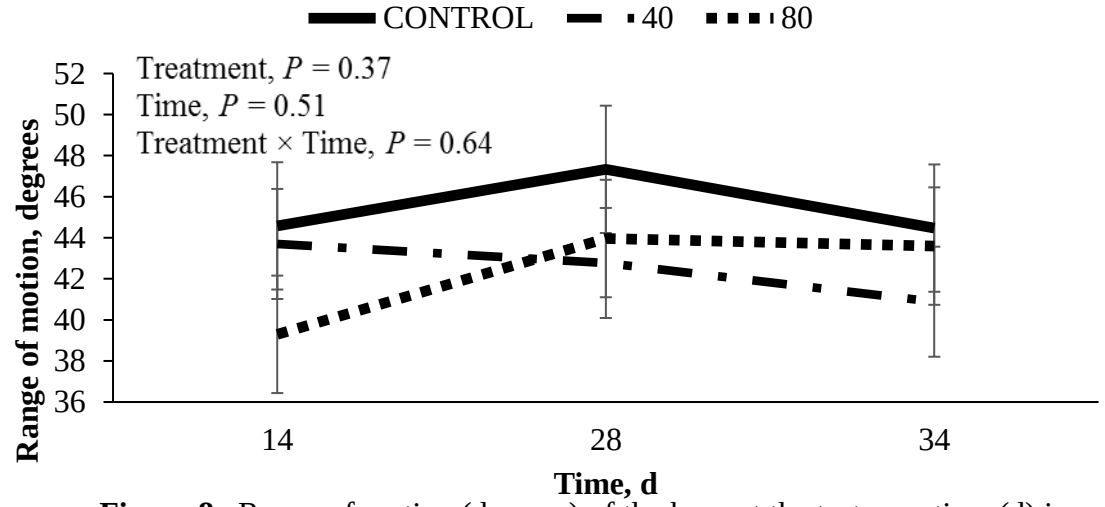

Figure 8. Range of motion (degrees) of the knee at the trot over time (d) in horses receiving a pelleted concentrate with either control, 40, or 80 g/d of spray-dried plasma proteins.

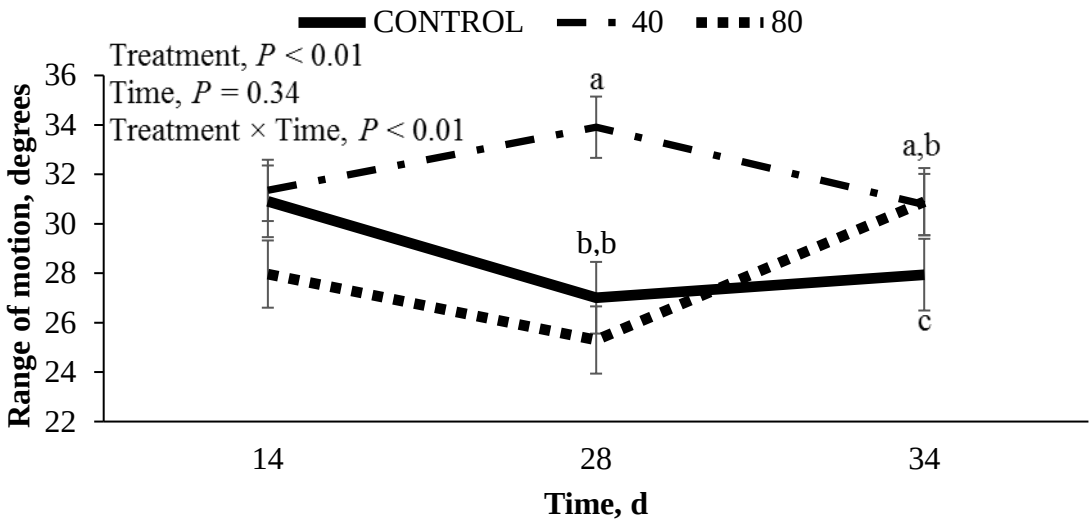

Figure 9. Range of motion (degrees) of the hock at the walk over time (d) in horses receiving a pelleted concentrate with either control, 40, or 80 g/d of spray-dried plasma proteins. [a,b,c] superscripts denote a difference in treatments at each time.

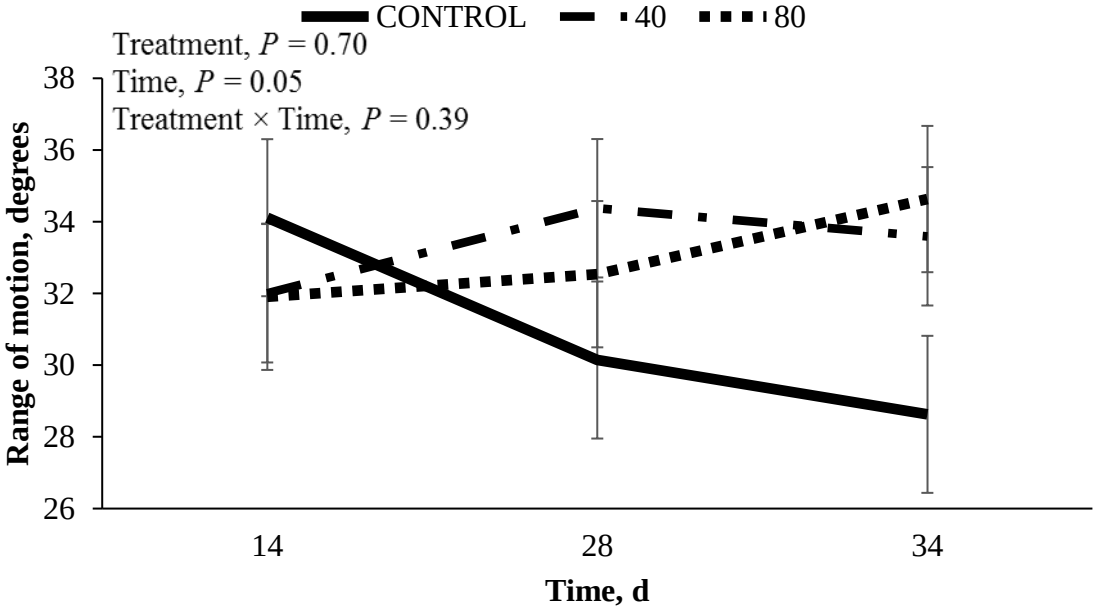

Figure 10. Range of motion (degrees) of the hock at the trot over time (d) in horses receiving a pelleted concentrate with either control, 40, or 80 g/d of spray-dried plasma proteins.

Blood Chemistry Parameters

Treatment × time interactions were observed for total serum protein, albumin, globulins, and glucose. Supplementation of SDPP did not affect blood chemistry parameters.

A significant treatment × time interaction for total serum protein (TSP) was observed ($P = 0.04$, Fig. 11), resulting from a decrease in TSP in control horses with a corresponding increase in TSP in horses fed SDPP to d 28. However, it is important to note that all treatment means remained within the normal range of 4.6 -6.9 g/dL throughout the study. A significant treatment × time interaction was for albumin observed ($P < 0.01$, Fig. 12) with a decrease in control horses to d 28 while albumin increased in treated horses. All treatment means remained in the normal range of 2.5-4.2 g/dL throughout the study. There was also a trend toward treatment × time interaction for globulins ($P = 0.09$, Fig. 13), driven by an increase to d 28 in horses receiving 40 and 80 g/d SDPP with a decrease in control horses. All treatment means remained within the normal range of 2.6–4.0 g/dL throughout the study.

Total serum protein is a measure of all the proteins in the blood, including albumin and globulins. Albumin is a blood protein formed in the liver and is integral for maintenance of osmotic pressure, while globulins include antibodies and proteins in blood clotting and inflammation and are a key component of the immune response. Because SDPP contains functional factors such as immunoglobulins, growth factors, and active peptides (Campbell et al., 2010b), it is reasonable to conclude that levels of total serum protein, albumin, and globulins would increase in treated horses. Although

resistant to digestion, these functional components may affect stimulation of the common mucosal system and subsequent production of immune related molecules including albumin and globulins. Decreasing levels of these parameters to d 34 in treated horses compared to controls, whose levels increased from d 28 to d 34 following the d 33 challenge, may indicate modulation of the immune response following physiological stress.

There was a significant treatment × time interaction for glucose ($P = 0.02$, Fig. 14), demonstrated by lower levels of plasma blood glucose on d 34 in horses receiving 80 g/d of SDPP compared to those receiving 0 or 40 g/d. All treatment means remained within the normal range of 72-114 mg/dL throughout the study.

Previous studies have observed modulatory effects of SDPP on plasma glucose resulting from alterations in SGLT1 expression in a challenged environment (Moreto and Perez-Bosque, 2009). The current study shows comparable results. Following the d 33 challenge, horses receiving 80 g/d of SDPP had lower plasma blood glucose than those on 0 or 40 g/d.

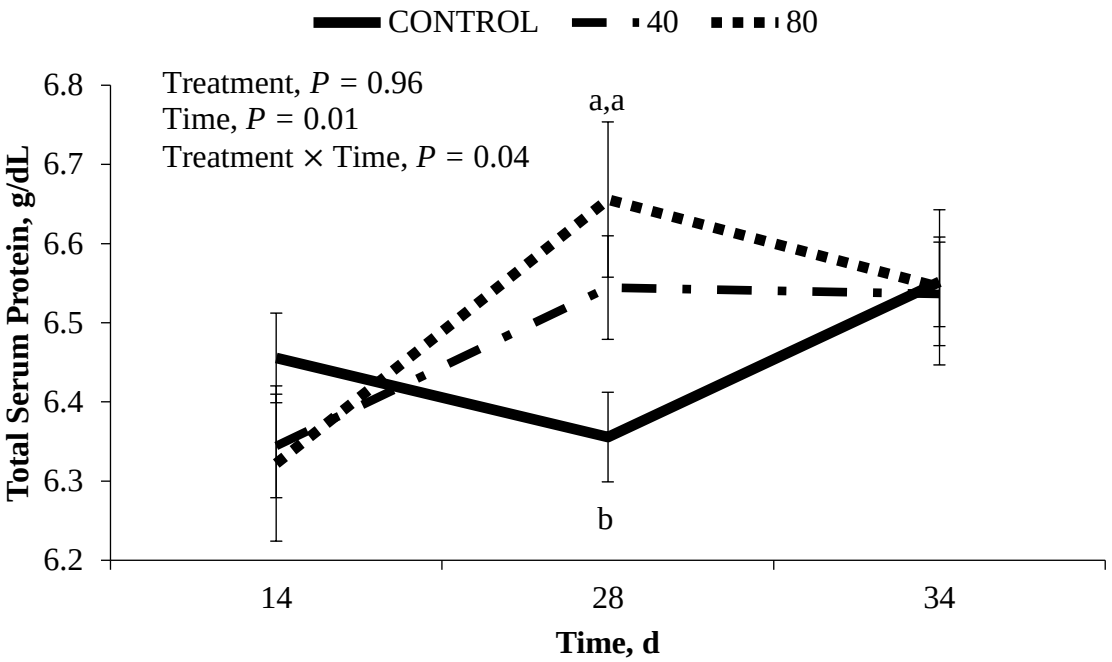

Figure 11. Total serum protein (g/dL) over time (d) in horses receiving a pelleted concentrate with either control, 40, or 80 g/d of spray-dried plasma proteins. [a,b,c] superscripts denote a difference in treatments at each time

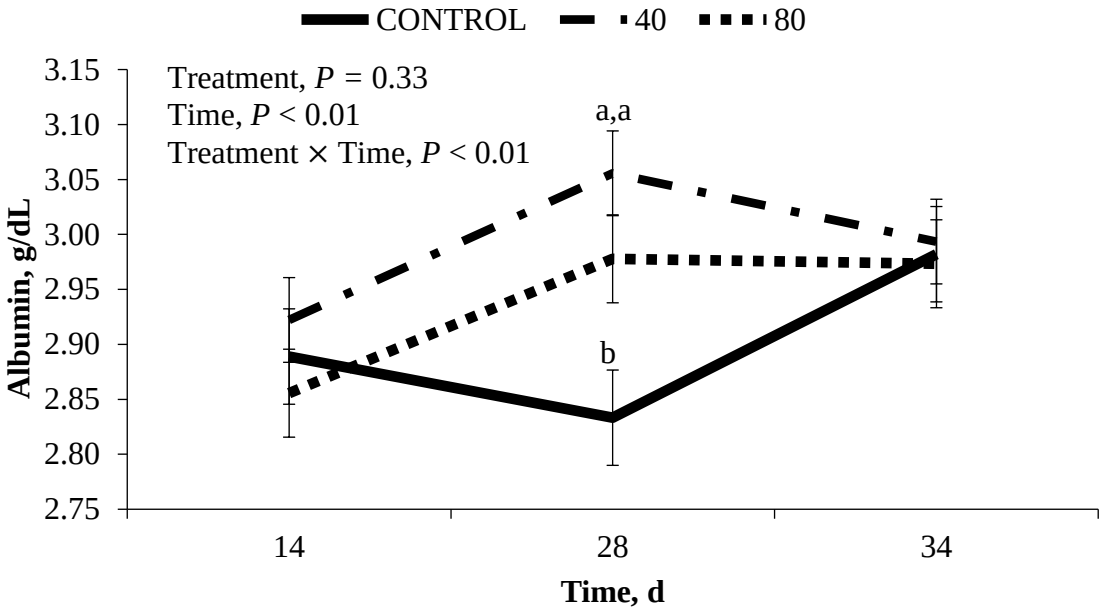

Figure 12. Albumin (g/dL) over time (d) in horses receiving a pelleted concentrate with either control, 40, or 80 g/d of spray-dried plasma proteins. [a,b,c] superscripts denote a difference in treatments at each time.

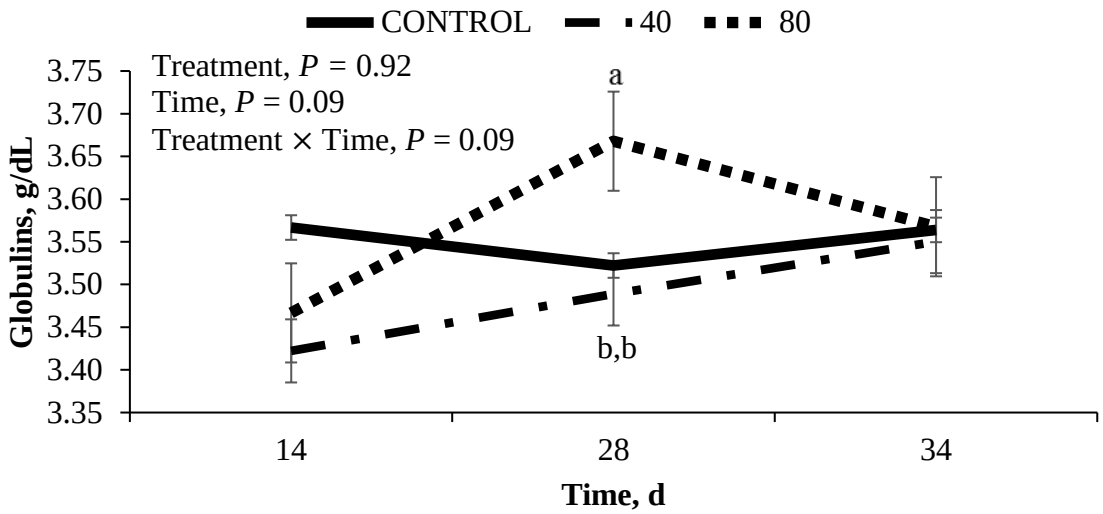

Figure 13. Globulins (g/dL) over time (d) in horses receiving a pelleted concentrate with either control, 40, or 80 g/d of spray-dried plasma proteins. [a,b,c] superscripts denote a difference in treatments at each time.

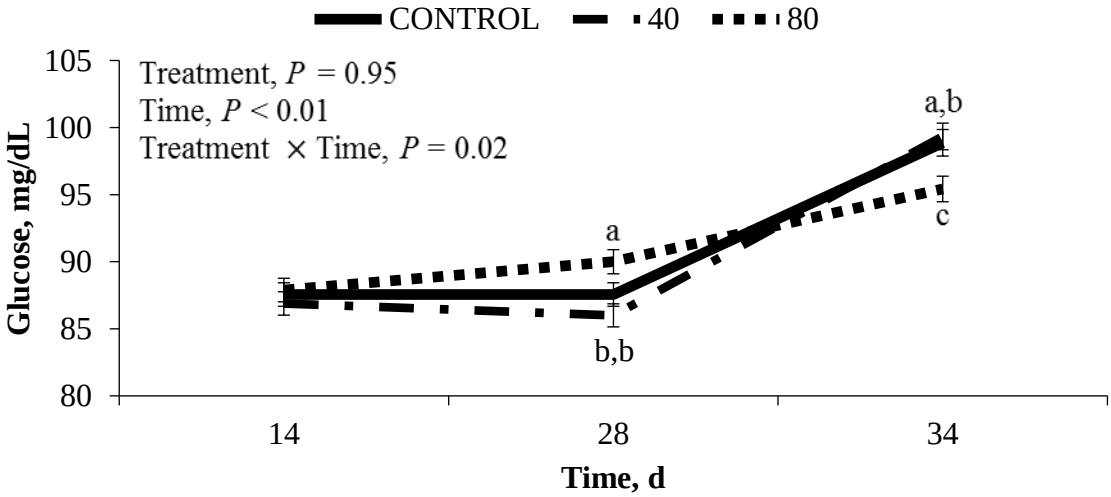

Figure 14. Blood glucose (mg/dL) over time (d) in horses receiving a pelleted concentrate with either control, 40, or 80 g/d of spray-dried plasma proteins. [a,b,c] superscripts denote a difference in treatments at each time.

Serum Inflammatory Markers

A significant treatment × time interaction was observed for IL-1β ($P < 0.01$, Fig. 15), explained by horses receiving 0 and 40 g/d SDPP increasing to d 34 while horses receiving 80 g/d demonstrated decreased expression of IL-1β. Dietary treatment of SDPP did not affect serum levels of TNF-α ($P = 0.51$). Concentrations of TNF-α increased over time ($P = 0.05$, Fig. 16), with all horses increasing from d 28 to d 34. Time also affected IL-1β concentration ($P < 0.01$) as all treatments decreased to d 28.

Previous studies in other species have demonstrated regulation of cytokine expression by SDPP (Bosi et al., 2004, Perez-Bosque et al., 2016). In a pathogen challenge in swine, SDPP decreased mucosal expression of TNF-α. However, in the current study TNF-α levels were not altered by dietary treatment. This most likely resulted from the use of a physical stressor rather than a pathogen challenge. Pathogen recognition functions through acquired immunity, while acute physical stress functions through innate immunity. This change could explain the difference in responses between the current study and the Bosi et al. (2004) swine study. Similarly to the Perez-Bosque et al. (2016) mouse model, the current study demonstrated decreased levels of IL-1β in horses receiving 80 g/d of SDPP following the d 33 challenge. Horses receiving 80 g/d SDPP had increasing values of TNF-α to d 28 and d 34 with decreasing values of IL-1β. This could indicate an increased ability to efficiently handle a stress related immune response before IL-1β is expressed. Because IL-1β is the major cytokine associated with joint disease, this also suggests that dietary SDPP improved articular range of motion through a reduction in the expression of pro-inflammatory IL-1β. An

additional theory for the modulation of IL-1β and not TNF-α on d 34 involves the timing of sampling. Blood samples were obtained 24-h after the d 33 challenge. Because TNF-α levels are highest during acute inflammation (McIlwraith et al., 2012), this time lapse may have allowed horses to begin resolution of any ongoing inflammatory response. Levels of IL-1β fluctuate between acute and chronic inflammation, but remain present, allowing for any modulatory effects of dietary SDPP to be observed 24 h after the challenge event.

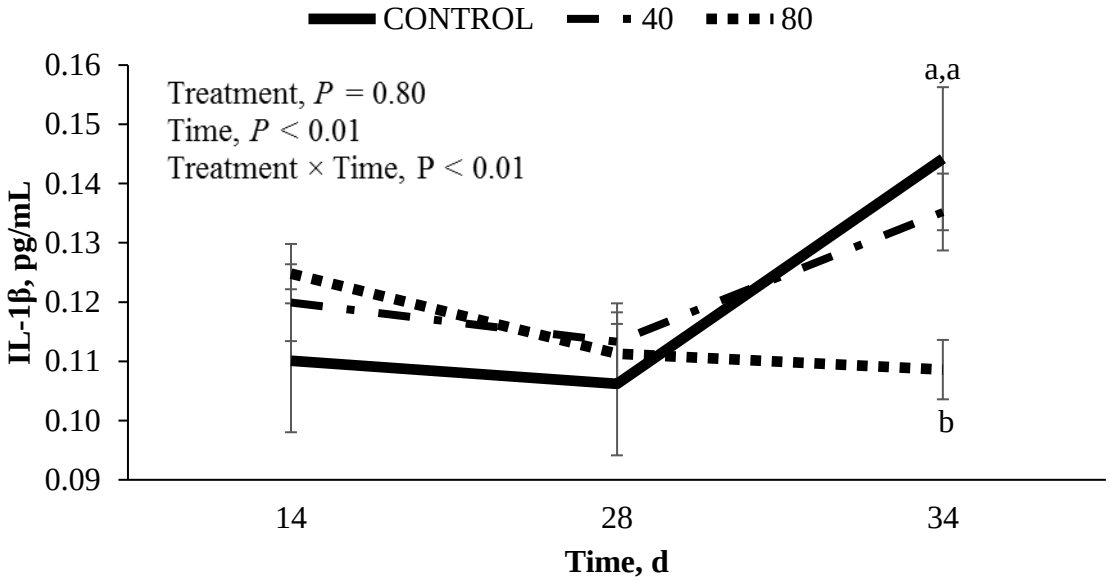

Figure 15. Serum concentrations of interleukin one beta (IL-1β) (pg/mL) over time (d) in horses receiving a pelleted concentrate with either control, 40, or 80 g/d of spray-dried plasma proteins. [a,b,c] superscripts denote a difference in treatments at each time.

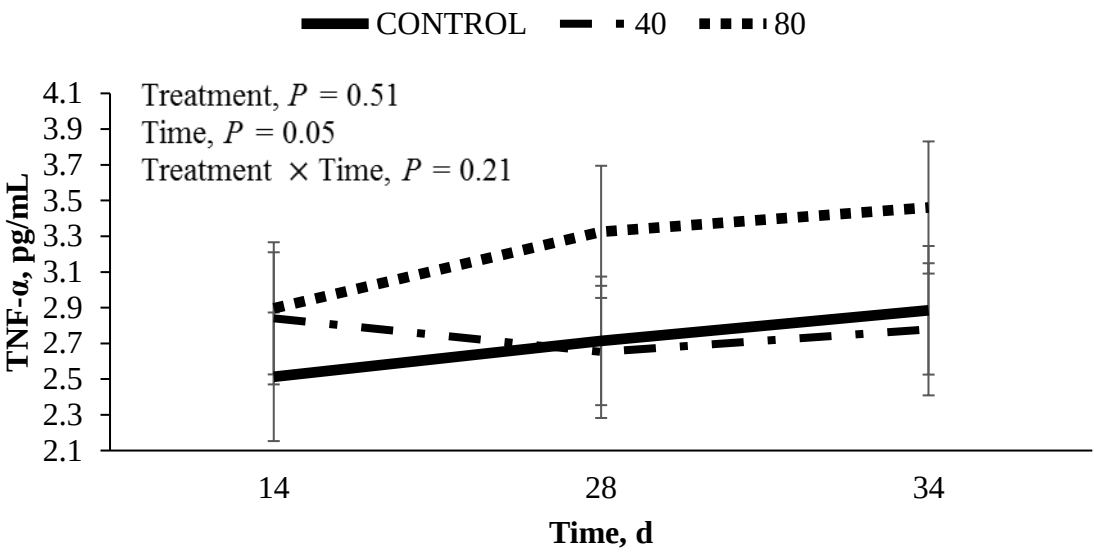

Figure 16. Serum concentrations of tumor necrosis factor alpha (TNF-α) (pg/mL) over time (d) in horses receiving a pelleted concentrate with either control, 40, or 80 g/d of spray-dried plasma proteins.

CHAPTER V

SUMMARY

Previous studies have indicated the benefits of dietary supplementation of SDPP to mature horses. However, limited to no data exists linking gait kinematics to the expression of systemic inflammatory cytokines that are related to joint health. As performance characteristics are difficult to measure objectively, the use of gait analysis software allows for on-farm parameters to be documented with the associated expression of inflammatory cytokines. In addition to the maintenance characteristics of dietary SDPP, information is limited in a challenged model as a means to evaluate these dietary additives while the horse is not at physiological homeostasis.

The current study indicated that dietary bioactive protein supplementation affected SL of the hind limb at the walk and hock ROM at the walk. All other gait kinematic parameters, blood chemistry, and cytokines were not affected by treatment. However, hock ROM at the trot tended to increase over time, as well as several blood chemistry parameters and inflammatory cytokine TNF-α. Levels of IL-1β decreased over time, with a significant treatment x time interaction following the d 33 challenge. Treatment x time interactions were observed for hock ROM at the walk, with trends toward interactions for SL of the fore and hind limb at the walk, knee ROM at the walk. Blood chemistry parameters TSP, albumin, and glucose demonstrated treatment x time interactions, with trends noted for TB and globulins. There was no effect of treatment on BW or BCS.

Based on these results, further studies are needed to determine the efficacy of dietary bioactive protein supplementation as a modulator for chronic inflammation and joint disease in mature horses. Current treatment for joint disease focuses on controlling pain without altering the source of pain and inflammation, therefore finding a preventative dietary supplement would be advantageous.

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