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INFLAMMATION AND INSULIN DYSREGULATION IN THE HORSE

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INFLAMMATION AND INSULIN DYSREGULATION IN THE HORSE

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Agriculture, Food and Environment
at the University of Kentucky

By

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2017

ABSTRACT OF DISSERTATION

INFLAMMATION AND INSULIN DYSREGULATION IN THE HORSE

Insulin dysregulation (ID) in the horse is receiving increasing attention as a serious health concern, in particular due to its association with the potentially career or life ending condition, laminitis. The role of inflammation and the immune system in ID as well as its associated health concerns has also been raised. However, the involvement of inflammation in and mechanisms behind ID in the horse remain unclear. Therefore, our overall hypothesis was that due in part to changes in their gut microbiota and plasma lipidome, horses with ID have changes in circulating proinflammatory markers, in particular in response to glycemic challenge, that further drive metabolic dysfunction. This work focuses on 7 potential associations between ID and inflammation to test this hypothesis; (H1) horses with ID will have an abnormal inflammatory response to glycemic challenge, (H2) ID horses will have differences in their gut microbiota compared to metabolically normal controls, (H3) these horses will likewise have differences in their plasma lipidome, (H4) response to routine vaccination will be reduced in horses with ID compared to metabolically normal controls, (H5) circulating endotoxin concentrations will be elevated in horses with ID, in particular in response to glycemic challenge, and their inflammatory and metabolic responses will be improved following supplementation with a gut modulating mannan rich fraction of the yeast cell wall, (H6) whole blood stimulation with endotoxin will induce TLR4 mediated inflammatory gene expression, and (H7) changing circulating lipid concentrations will improve both glycemic and inflammatory parameters in ID horses. Overall this work provides insight into contributing factors to ID in the horse, in particularly as they relate to inflammation.

KEYWORDS: Insulin dysregulation (ID), inflammation, gut microbiota, plasma lipidome, immune response to vaccination, and supplementation.

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May 16, 2017
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INFLAMMATION AND INSULIN DYSREGULATION IN THE HORSE

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CHAPTER 1 REVIEW OF THE LITERATURE

1.1. Phenotype

The percentage of adults in the United States considered to be overweight or obese (as measured by body mass index, or BMI) continues to increase, with some stating that up to 68.8% of the population falls into one these categories [1, 2]. The percentage of overweight or obese horses is likewise increasing, with reports ranging from 20.6-51% of U.S. or abroad populations affected [3-7]. There are many negative health concerns that have been associated with obesity in various species, including the horse. While it is unclear if obesity precedes these health concerns or if it is a resulting side effect, it is often considered a risk factor for many conditions. In particular, it is associated with insulin dysregulation and other endocrinopathic disorders in the horse [8-11].

The term insulin dysregulation (ID) [12] has recently been used in the literature to describe most forms of abnormal insulin concentrations or signaling in the horse, whether that be high resting insulin concentrations (hyperinsulinemia), an abnormal response to glycemic or insulinemic challenge (such as the oral sugar test, or OST [13]), or tissue insulin insensitivity (insulin resistance, or IR). Arguably of greatest concern is its association with the potentially fatal condition, laminitis [14-16]. While the mechanisms behind laminitis remain unclear, it is ultimately a failure of the hoof lamellar structures that can lead to rotation or sinking of the distal phalanx [17]. This failure typically involves inflammation, impedes mechanical function, and is extremely painful for the animal [18]. Further support of the connection between ID and laminitis has been demonstrated as laminitis can be induced with administration of exogenous insulin [19, 20]. While the prevalence of endocrine associated laminitis (or endocrinopathic laminitis) is not well known, incidence of laminitis in the equine population has been reported from as little as 1.5% to as much as 34% [21]. Additionally, one study reported that over a period of 16 mo in an equine referral hospital, 89% of laminitic cases had underlying endocrinopathies [22].

A history of or predisposition to laminitis, increased general or regional adiposity (fatty deposits around the neck, tail head, behind the shoulder, or in the mammary gland region), and the presence of ID are the three main defining characteristics of Equine Metabolic Syndrome (EMS) [23]. Other proposed characteristics for diagnosis of EMS

include lipid dysregulation such as hypertriglyceridemia or dyslipidemia, hyperleptinemia, arterial hypertension, and systemic inflammation [23]. EMS is considered to be similar to metabolic syndrome (MetS) [24, 25] in humans [26]. An individual will qualify as MetS if he or she exhibits 3 out of the 5 following risk factors: elevated fasting glucose, elevated systolic and/or diastolic blood pressure, hypertriglyceridemia, low high-density lipoprotein cholesterol (HDL) levels, and increased central adiposity [25]. Also, individuals with MetS often have increases in both circulating and adipose tissue levels of inflammation [27].

1.2. Inflammation

Research regarding EMS has concentrated on a better definition of the phenotype as well as potential underlying mechanisms [23, 28-31]. However, there have been conflicting results, particularly regarding inflammation. This may be due to several factors. Often, studies compare ponies rather than horses, or induce hyperinsulinemia with models such as dexamethasone administration [32] or diet-induced weight gain [33], rather than utilizing horses with naturally occurring ID. In many instances horses were not tested for potentially confounding factors, such as pituitary pars intermedia dysfunction (PPID), which can influence insulin and glucose dynamics [10, 11]. In addition, data regarding fasted versus fed states is in some instances not reported, assays used to determine insulin concentrations vary, and cut-off values used for diagnosis of ID are not established for these different assays in the horse [34-36].

Data concerning fed versus fasted states becomes important not only concerning insulin and glucose dynamics [37, 38], but also in regards to other parameters such as markers of inflammation. When indicated, much of the work in the horse has focused on fasting circulating or fasting tissue markers of inflammation. These results has been somewhat contradictory, with some reporting that horses with ID have an increase in inflammatory markers such as circulating tumor necrosis factor alpha (TNF-α) protein and TNF-α gene expression in adipose tissue [28, 39], as well as positive correlations between plasma concentrations of serum amyloid A and insulin [40]. Others have seen differences in inflammatory cytokine gene expression in nuchal adipose tissue between EMS horses and controls, with EMS horses having increases in IL-1β and IL-6 [41]. However, others found no differences or lower IL-1 and IL-6 peripheral blood mononuclear cell (PBMC)

gene expression and a trend for lower TNF-α plasma concentrations in obese horses, and a trend for a decrease in EMS horses PBMC TNF-α and IL-1β gene expression compared to metabolically normal controls [31, 42]. These contradicting results may be partly explained as humans and mice with or without metabolic dysfunction have been observed to have postprandial increases in inflammatory markers. TNF-α, IL-6, and IL-1β as well as increases in systemic soluble adhesion molecules, such as Intercellular Adhesion Molecule-1 (ICAM-1), have been observed in response to a high fat or combination high fat, high sugar/starch meal [43-45]. There are indications that this may be true in horses as well, with postprandial increases seen in IL-1β, TNF-α, IFN-γ, and IL-6 in response to oral feeding or oral administration of sugar/starch [46, 47].

Horses are as hind-gut fermenters able to utilize sugars and starches as an energy source [48] and easily break down these structural carbohydrates into digestible volatile fatty acids. Diets rich in these energy sources, particularly in large quantities, have been shown to promote insulin insensitivity in the horse [49], further associating the role of the gut in inflammation and ID. In fact, in equine induction models of laminitis utilizing a carbohydrate polymer made up of inulin-type fructans (oligo-fructose) given orally [50], horses had an increase in whole blood cytokine gene expression of IL-1β, IL-8, and IL-10 8 h post laminitis induction [51] as well as increases in lamellar IL-2, IL-6, IL-8, and IFN-γ at the onset of laminitis [18].

Postprandial increases in inflammatory markers have several possible explanations. They may be due in part to proposed differences in oxidative stress in humans, mice, and horses with metabolic dysfunction [31, 52-55]. Similar to inflammation, it has been suggested that oxidative stress increases postprandially, in particular in individuals with hyperglycemia and hypertriglyceridemia [56-58]. Increases in inflammatory markers following administration of a meal may also be due to increases in bacterial endotoxin concentrations that have been observed in humans in response to dietary fat [59, 60]. Likewise in high fat fed mice, endotoxemia has been shown to induce inflammation and insulin resistance [61]. Indeed, postprandial changes in bacterial endotoxin concentrations are not surprising due to the suspected involvement of the gut and its associated microbiota in both metabolic and inflammatory mechanisms [62, 63].

1.3. Microbiota

In horses and humans, the gut microbiota is both highly diverse and variable. Variation can be affected by, among other factors: genetics, age, diet, and disease states [64-66]. For example, in the healthy horse, the predominant gut bacterial phyla is *Firmicutes* [64, 67] and bacteria from the Clostridiales order, in particular the butyrate producing bacteria Ruminococcaceae and Lachnospiraceae, are positively correlated with intestinal health [68, 69]. However, following feeding of a high starch diet, horses have been shown to have an increase in the abundance of the one of the taxa from the Firmicutes phyla, Veillonellaceae [70]. Veillonellaceae, a gram-negative propionate/acetate producer, has been positively correlated with markers of metabolic dysfunction in high fat fed rats [71]. Horses with a history of laminitis have also been shown to have alterations in their gut microbiota, with previously laminitic horses having increased abundance of two unassigned members of the Clostridiales genera as well as differences in community structure, notably having a lower representation of Firmicutes and greater representation of Verrucomicrobia compared to controls [72]. Changes in bacterial abundance have likewise been shown in the horse at the onset of laminitis [73, 74]. In addition, decreases in Clostridiales have been documented in horses with colitis and in mares prior to the onset of colic [75, 76]. Metabolic dysfunction and obesity have also been associated with intestinal microbiota changes [77]. For example, an overabundance of the phyla Verrucomicrobia was observed in diet-induced obese minipigs [78]. Additionally, obesity has been linked with an overall decrease in microbial diversity in humans [79] and in dogs [80]. In horses, a decrease in diversity has been observed following antimicrobial administration [81] and in response to a change in diet [69], as well as in horses with EMS compared to metabolically normal controls [82]. These same EMS horses were also found to have differences in overall community structure, suggesting changes in composition due to overgrowth or decreased growth of specific community members. Given that in mice changes in the gut microbiota related to age have increased circulating concentrations of a component gram-negative bacteria cell wall, lipopolysaccharide (LPS), which subsequently resulted in an increase of inflammatory markers [83], these and other studies highlight the potential influence of the gut microbiota on metabolic dysfunction and its role in the induction of systemic, low grade inflammation.

In humans, diets or supplements that reduce the population of bacteria that produce LPS, for example the so called Mediterranean diet [84], supplementation with polyphenols [85], or probiotics [86], are suggested as possible therapeutic targets for individuals with metabolic dysfunction. Supplementation with pre or probiotics is thought to be particularly beneficial as they positively influence intestinal health by improving the balance of the microbiota population by the addition of live favorable bacteria (typically lactic acid producers) in the case of probiotics, or by promoting the growth of specific members of the existing bacterial community in the case of prebiotics [87]. One suggested method to influence the gut microbiota in the horse has been supplementation with the prebiotic, fructooligosaccharides [88, 89]. Fructooligosaccharides (FOS) exhibit prebiotic properties as they promote growth of certain intestinal bacteria. They are converted to short chain fatty acids following fermentation by the gut microbiota [90] and subsequently serve as a source of energy for intestinal epithelial cells, have a major role in communication between the gut microbiota and the host, and are thought to have beneficial effects on host metabolism and even inflammation [91, 92]. Indeed, supplementation with FOS has been shown to positively impact metabolic parameters in horses [93] as well as in obese dogs [94]. In addition, it has been shown that utilizing another dietary prebiotic, in the form of yeast or yeast cell wall, can likewise effect inflammation in dairy cows [95], poultry [96], humans [97-99], and horses [100, 101]. Yeast cell wall supplementation has in cattle been shown to influence glucose and insulin concentrations following supplementation and LPS challenge [102], and reduced circulating triglyceride and cholesterol levels in poultry post supplementation [103, 104]. Mannan oligosaccharides, thought to be one of the main effectors found in the yeast cell wall, reduced circulating LPS concentrations in pigs [105], primarily mediated by positive changes in the gut microbiota.

1.4. Lipids

Not only have increases in LPS been associated with changes in the gut microbiota, but they have also been associated with inflammation and alterations in lipid metabolism [106]. EMS and ID horses have abnormal metabolic function [23, 29, 107] as well as abnormal circulating lipid concentrations [40, 42, 107]. Lipidomic analysis has allowed for further exploration of lipids and lipid profiles (or lipidome) as well as their relationship to

metabolism and inflammatory processes [108]. Lipids, in particular the sphingolipid sphingomyelin as well as the glycerophospholipids ethanolamine or choline plasmalogens, have a major role in mammalian cellular membrane health [109]. Therefore, they have a direct impact on cellular function, including cell signaling [110, 111]. Cell signaling, such as activation of inflammatory pathways in multiple cell types (in particular macrophages, adipocytes, and hepatocytes) and gene expression of metabolic and inflammatory proteins (for example SOCS-3 and MCP-1) can be influenced in many species by changes in circulating lipid concentrations [112-114]. In particular, changes in ceramide concentrations [113, 115-117] strongly impact cell signaling and metabolism. Bioactive lipids, such as ceramide, in addition to their influence on inflammatory and insulin signaling, are able to act as chemoattractants and second messengers for multiple cell types, including immune cells [118-120]. Further evidence of the role of the lipidome in metabolic dysfunction is demonstrated considering that changes in nutrition and nutritional supplementation are able to effect change in the plasma lipidome, resulting in the decrease of inflammatory markers and an increase in insulin sensitivity in humans [121, 122]. In one study, phosphatidylcholine and sphingomyelin containing linoleic and docosahexaenoic acid (thought to be anti-inflammatory lipids [123]) were found to be increased in exercised rats whereas phosphatidylcholines, phosphatidylethanolamine, and phosphatidylinositol containing arachidonic acid (thought to be a pro-inflammatory lipid [124]) were increased in rats with metabolic dysfunction following feeding of a high fat diet [125].

Lipids not only influence cell signaling and impact cellular membrane health and function, but in humans and in mice there is evidence that they also play a role in the immune response. For example, the immune system can recognize both foreign lipids (for example KOD2-lipid A from Escherichia coli) [126], and self-lipids (such as gangliosides and glycosphingolipids) [127-129] as antigens. Lipids are also, as stated previously, able to act as lymphocyte chemoattractants [118]. Given associations between obesity, insulin resistance, systemic inflammation, and the immune response [31, 130, 131], questions have been raised as to the response to vaccination of those with metabolic dysfunction or obesity.

1.5. Immune responses

Following vaccination, the immune system initially mounts the rapid non-specific innate immune response [132]. This innate response involves the release of cytokines and chemokines which promote the activation of the second arm of the immune response, the adaptive immune response [133], which provides the host with long lasting specific protection from disease. There have been indications that in response to influenza vaccination, diet-induced obese mice have a decrease in both their antibody response and neutralizing capacity compared to metabolically normal controls [134]. Diet-induced obese mice have likewise been shown to have a decrease in hepatitis specific antibody and T cell activation, but an increase in T and B cell proliferation as well as increases in levels of IFN-γ and TNF-α in response to a hepatitis B vaccine [135]. Humans with type 1 diabetes are considered to be at risk for non-responsiveness to vaccination [136], and in a group of 245 hospital employees >3 yrs post hepatitis vaccination there was a correlation between increased adiposity and lower levels of circulating antibody [137]. Further, although body mass index was positively associated with an initially higher IgG antibody response, 12 months following influenza vaccination individuals with a higher body mass index exhibited a greater decline in antibody titers [138]. In the same study, obese individuals' virally challenged PBMCs displayed a lower functional protein expression and decreased $CD8^+$ T-cell activation ex vivo. There is however, no published work to date on the response to vaccination in the ID or obese horse.

1.6. Inflammatory mechanisms and signaling

While multiple studies have shown probable links between the immune system, inflammation, lipids, and metabolic dysfunction [127, 139, 140], the underlying mechanisms of ID and contributing factors to laminitis in the horse remain unclear. Genetics appear to influence the prevalence of ID in the horse as certain breeds, such as ponies and Andalusians, are predisposed to its development [23, 141]. Additionally environmental factors, including nutrition and exercise, play a role in the development of metabolic dysfunction [142]. There has also been recent discussion as to the role of corticosteroids in the pathogenesis or severity of ID and laminitis, although at present supporting evidence is weak [143, 144]. In obese or insulin resistant humans and mice,

there has been data showing that adipose tissue macrophages, as opposed to adipose tissue, are the primary producers of inflammatory cytokines, in particular TNF-α [145, 146]. In the adipose tissue of individuals with obesity and ID, macrophages accumulate in greater numbers and size, and favor M1 (thought to be pro-inflammatory) macrophages as opposed to M2 (thought to be anti-inflammatory) macrophages [131, 147]. Macrophages recognize pathogens principally through pattern recognition receptors (PRR), in particular toll-like receptors (TLRs) such as TLR-4. TLR-4 has been implicated in ID and obesity as the primary receptor for recognition of LPS [148]. LPS stimulated signaling through TLR-4 and its co-receptors, CD14 (cluster of differentiation 14) and MD-2 (Lymphocyte antigen 96), activate signaling cascades which ultimately result in activation of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), which in turn promotes pro-inflammatory gene expression [149]. This receptor complex has also been shown to recognize free fatty acids (FFA) as antigens similarly resulting in the production of inflammatory cytokines, such as TNF-α and IL-6 [148, 150]. There is cross talk between the TLR-4 and insulin signaling cascades, and it appears that humans with type 2 diabetes have additional insulin signaling intermediates within their insulin signaling cascade [151]. However, underlying mechanisms of abnormal inflammatory signaling remain unclear. In particular, it remains to be seen if changes in inflammatory responses or concentrations, possibly due to increases in circulating LPS concentrations, initiates abnormal insulin signaling or if the reverse is true.

1.7. Treatment and therapeutic strategies

Considering the large number of horses affected by obesity or ID, there have been an increasing number of studies examining potential therapies for metabolic dysfunction in the horse. Classically, horses with ID are treated with exercise and dietary restriction, which have been shown to decrease insulin resistance as well as markers of inflammation [29]. However, these changes often over a period of several months and may not be a viable treatment option in horses with complicating health concerns, such as chronic laminitis. There are two off-label pharmaceutical drugs commonly administered to horses with ID. One of which, (Metformin) works in the liver by suppressing glucose production [152], and the other (levothyroxine sodium) as a synthetic thyroid hormone (T4) upregulates

metabolic processes by stimulation of the thyroid [153]. However, as stated prior, metformin and levothyroxine sodium are used off label in the horse and their mechanism of action is not well established. In addition, published results in the horse are conflicting regarding their efficacy and long term safety [9, 154-156].

In humans and in other species, omega-3 fatty acid supplementation has been proposed as a treatment strategy for individuals with metabolic dysfunction [157]. Much of the research has focused on supplementation with the long-chain polyunsaturated omega-3 fatty acids docosahexaenoic acid (DHA) and/or eicosapentaenoic acid (EPA). Supplementation with these fatty acids appear to be dose responsive, as demonstrated in people and in horses [158, 159]. These fatty acids are readily incorporated and absorbed as increased concentrations have not only been observed in circulation following supplementation but in tissue as well; for example in skeletal muscle in horses [160] and adipose tissue in humans [161]. Supplementation with omega-3 fatty acids is thought to be beneficial for several reasons. Insulin sensitivity has been shown to be improved in rats, pigs, and humans [162-166]. In humans it has also been shown to promote body fat oxidation and reduce lipogenesis [167, 168] and has a positive effect on the plasma lipidome [169, 170]. In metabolically normal horses, supplementation with omega-3 fatty acids has indicated a positive effect on glucose clearance [171], improvement in markers of insulin sensitivity [172], and following dexamethasone administration has reduced baseline glucose and insulin concentrations and improved the modified insulin to glucose ratio [173]. It has also been shown in metabolically normal horses to reduce inflammatory mediators [174-176] and has an effect on circulating DHA, triglyceride, and phospholipid concentrations [159, 177, 178]. However, no work to date has been carried out regarding omega-3 fatty acid supplementation in the ID horse.

1.8. Overall hypothesis and specific aims

Overall, data is still limited regarding the pathophysiology of ID in the equine. It does appear that many biological processes are influenced in horses affected by ID, in particular the inflammatory process. However, more work needs to be done regarding the underlying mechanisms of this common and potentially career or life ending disorder. Therefore, we proposed with this work to investigate the role of inflammation in the horse

with ID with the hypothesis that due in part to changes in their gut microbiota and plasma lipidome, horses with ID have changes in circulating proinflammatory markers, in particular in response to glycemic challenge, which further drive metabolic dysfunction. In order to assess this overall hypothesis, 7 specific hypotheses (H) were examined.

(H1) Horses with ID will have an abnormal inflammatory response to glycemic challenge.

This hypothesis was generated to investigate the inflammatory and metabolic responses of the ID horse to an oral glycemic challenge utilizing a high sugar/starch challenge as well as a commonly used glycoside.

(H2) ID horses will have differences in their gut microbiota compared to metabolically normal controls.

Fecal DNA was used to determine differences in the composition of the gut microbiota in a group of ID horses compared to metabolically normal controls for this hypothesis.

(H3) These horses will likewise have differences in their plasma lipidome.

Differences in circulating plasma lipid concentration in ID horses for this hypothesis were determined by lipidomics analysis, as well as an analysis of baseline inflammatory markers.

(H4) Response to routine vaccination will be reduced in horses with ID compared to metabolically normal controls.

This hypothesis was tested by administration of routine influenza vaccination in a group of ID as well as metabolically normal control horses to examine humoral and cell mediated immune responses to vaccination.

(H5) Circulating endotoxin concentrations will be elevated in horses with ID, in particular in response to glycemic challenge, and their inflammatory and metabolic responses will be improved following supplementation with a gut modulating mannan rich fraction of the yeast cell wall.

Markers of inflammation, circulating endotoxin concentrations, and metabolic parameters and responses were measured in horses both with and without ID prior to and following supplementation with a gut modulating yeast cell wall product to test this hypothesis.

(H6) Inflammatory gene expression in response to a TLR4 agonist, LPS as well as in response to an oral sugar test will be different in horses with ID.

Whole blood was stimulated with endotoxin in ID and metabolically normal horses as baseline and following OST to determine inflammatory responses for this hypothesis.

(H7) Changing circulating lipid concentrations will improve both glycemic and inflammatory parameters in ID horses.

To test this hypothesis, ID horses were utilized for inflammatory, metabolic, and lipid concentrations and responses which were assessed both prior to and following supplementation with omega 3 fatty acids.

CHAPTER 2 METABOLIC AND INFLAMMATORY RESPONSES TO THE COMMON SWEETENER STEVIOSIDE AND A GLYCEMIC CHALLENGE IN HORSES WITH EQUINE METABOLIC SYNDROME

Domestic Animal Endocrinology, 2017: 60, 1-8

Extracts derived from the leaves of the stevia plant (stevioside) are commonly used as sweeteners for humans and horses. Stevioside appears to be safe for human consumption, including for individuals with insulin dysregulation. In the horse, the safety or metabolic effects of stevioside on normal animals or on those with metabolic dysfunction are unknown. Furthermore, the inflammatory response to a glycemic challenge or to stevioside in horses is not well defined. Therefore, the objective of this study was to measure the effects of stevioside and a glycemic challenge on insulin, glucose, and inflammatory responses in horses with a common metabolic dysfunction (equine metabolic syndrome or EMS) compared with non-EMS controls. To accomplish this, 15 horses were selected; 8 EMS and 7 age-matched controls. An oral sugar test was performed using Karo corn syrup (karo) or stevioside in a random crossover design. Horses were given 0.15 mL/kg body weight of karo or its equivalent grams of sugar in stevia dissolved in water. Blood samples were collected by jugular venipuncture before administration of either stevia or karo and at 60 and 240 min after administration. Serum was used for glucose and insulin determination and plasma for isolation of peripheral blood mononuclear cells (PBMCs) for inflammatory cytokine analysis via flow cytometry and reverse transcription PCR (RT-PCR). Stevia appeared to stimulate lower glycemic and insulinemic responses when compared to karo, in particular in EMS horses. EMS and control horses had inverse inflammatory responses to administration of either stevia or karo with EMS horses having a proinflammatory response ($P \leq 0.05$). These data provide evidence as to why horses with EMS may be predisposed to developing laminitis, potentially as a result of an exaggerated inflammatory response to glycemic and insulinemic responses. Furthermore, the data provide new avenues for exploring mechanisms behind the syndrome, in particular when using a glycemic challenge.

2.1. Introduction

Stevioside, a glycoside derived from the leaves of the stevia plant, has been increasingly used as a sweetener for both horses and humans [179]. It appears in a range of equine products, such as veterinary pastes, supplements, and anthelmintics. Stevioside can even be found in equine products intended for use in the insulin resistant animal. However, the safety and efficacy of stevioside in the metabolically normal or metabolically dysfunctional animal is unknown. For humans, stevioside is primarily used as a non-glycemic sugar replacer and is classified as Generally Recognized as Safe by the United States food and drug administration (Generally Recognized as Safe). Research indicates that stevioside is safe for human consumption, including for individuals with hypertension and metabolic dysfunction [180]. Stevioside may even be metabolically beneficial in individuals with metabolic disorders as it has been shown to increase insulin sensitivity in insulin resistant rats [181] and to positively influence glucose and insulin dynamics in people [182-185]. Humans with metabolic dysfunction typically suffer from hyperglycemia and hyperinsulinemia. Considering that stevioside stimulates insulin release from the pancreas [184], it can therefore have a potentially positive effect in humans by lowering circulating glucose concentrations. However, horses with metabolic dysfunction, unlike humans with metabolic syndrome, typically have circulating glucose concentrations within the normal range, but are hyperinsulinemic. Thus, increasing the amount of circulating insulin in the horse may actually exacerbate existing insulin dysregulation (ID) [12] in the horse with metabolic syndrome (equine metabolic syndrome, or EMS). Even with the increasing use of stevia in equine products, the effects of stevioside on glucose and insulin metabolism in the horse have yet to be determined. The oral sugar test (OST), a common dynamic test used to determine glycemic and insulinemic responses to a simulated high carbohydrate meal, is typically carried out using a bolus of Karo corn syrup (karo) as a sugar source. However, no research has characterized the inflammatory response to a glycemic challenge with either karo or stevia. Considering that increases in inflammatory markers such as tumor necrosis factor (TNF-α), interleukin-6 (IL-6), and interleukin-1β (IL-1β) have been observed postprandially in people with metabolic dysfunction [43] and in horses [46], as well as the prevalence of stevioside in the equine market, it is of importance to understand its metabolic and inflammatory effects on normal

and metabolic syndrome horses. Therefore, the experiment was designed not only to measure the effects of the OST using both stevioside and karo on insulin and glucose responses but also inflammatory responses in horses with EMS compared with non-EMS controls.

2.2. Materials and methods

2.2.1. Horse selection and EMS determination

Eight EMS horses and 7 controls were selected from the University of Kentucky, Department of Veterinary Science herd. EMS was defined by the criteria established in the 2010 American College of Veterinary Internal medicine consensus statement [23]. In brief, EMS was characterized by ID [12], general or regional adiposity, and a history of or predisposition to laminitis. All EMS horses were predisposed to laminitis given their increased adiposity and ID status, with 4 of the 8 selected EMS horses having a documented history of laminitis. A portable agriculture scale (model 700, Tru Test Inc., Mineral Wells, TX) was used to establish body weight, which was used to determine the amounts of stevia and karo to administer to each horse for the OST. Body condition score was determined and averaged between 3 trained investigators (S. E., M. M., and B. R.) using the 1 to 9 Henneke scoring system [186] with 1 representing an extremely emaciated animal and 9 representing an extremely obese animal. The same 3 trained individuals scored and averaged neck crests via the 0 to 5 cresty neck score (CNS) system established by Carter et al [187] with 0 representing no neck crest and 5 representing a crest so large it permanently droops to one side of the neck. Blood collection for serum and plasma samples was carried out via jugular venipuncture. To ascertain the presence of ID, the OST was performed similar to previously described [13]. Briefly, morning serum samples were collected, and oral sugar was administered in the form of 0.15 mL/kg body weight of karo. A second serum sample was collected 60 min post karo administration. A fasting insulin level of > 20 μIU/mL was considered indicative of hyperinsulinemia, and an increased insulin (> 60μU/mL) 60 min post administration of karo was considered diagnostic of ID [188]. All OST sampling was performed between 8 AM and 12 AM for all sampling time points. Horses were not fasted before sampling. Screening results from the OST along with phenotypic data are presented in Tables 2.1 and 2.2. Of the 8 EMS horses, 3 were of mixed

breed, 1 was a Thoroughbred cross, 1 horse was a Paint, 1 a Morgan, 1 a Warmblood, and 1 a Standardbred cross. Of the 7 control horses, 4 were Thoroughbreds, 2 were of mixed breed, and 1 was a Quarter Horse. Of the 7 control horses, 2 were of mixed breed, 4 were Thoroughbreds, and 1 was a Quarter Horse.

Table 2.1. Characterization of EMS and non-EMS control horses.

Group	(Insulin; μIU/mL) 60 min post oral sugar administration	BCS	CNS	Wt (Kg)
EMS	75 ± 6	6.8 ± 0.3	3.0 ± 1.1	569 ± 88
Control	31 ± 4	6.4 ± 0.1	1.2 ± 1.0	610 ± 56
P-value	*<0.001*	*0.278*	*0.009*	*0.307*

Abbreviations: BCS, body condition score; CNS, cresty neck score; EMS, equine metabolic syndrome; Wt, weight.
Italicized values represent statistical significance in the form of *P* values.

Table 2.2. Characterization of EMS and non-EMS control horses analysis on ranks.

Group	Baseline Serum (Insulin; μIU/mL)	Age
EMS	44 (95% CI: 26-51)	12 (95% CI: 10-13)
Control	16 (95% CI: 13-18)	14 (95% CI: 12-15)
P-value	*<0.001*	*0.054*

Abbreviations: EMS, equine metabolic syndrome.
Italicized values represent statistical significance in the form of P values.

Horses were also screened 1 mo before the start of study via thyrotropin releasing hormone (TRH) stimulation and low-dose dexamethasone suppression testing [189, 190] to ensure that none were affected by pituitary pars intermedia dysfunction (PPID). TRH stimulation testing was carried out as previously described [191, 192] at least 2-wk before the first sampling time point, but no further than 2 mo before sampling. In brief, a baseline blood sample (0800, EST) was taken via jugular venipuncture into EDTA containing tubes. Following this, a 1 mL dose of TRH dissolved to 1 mg/mL in 0.9% saline (Sigma-Aldrich, St. Louis, MO) was administered intravenously (IV). Ten min post TRH injection, a second blood sample was taken. Tubes were kept on ice for transport to the laboratory and immediately centrifuged and plasma isolated within 3 h after collection [193]. Samples were stored at -20^{o} C until shipped on dry ice to Cornell University for analysis of ACTH concentrations. Low-dose dexamethasone suppression testing was also performed 2 days

following TRH stimulation testing and was performed as previously described [194] and within the same time frame relative to the start of the study as TRH stimulation testing. In brief, a baseline jugular blood sample was taken (3:50 PM–6:00 PM), and an intramuscular dexamethasone (Henry Schein, Dublin, OH) bolus (0.04 mg/kg BW) was administered with a second blood sample taken 19 h later. A serum separator tube was used to collect samples. Tubes were centrifuged, serum was isolated and frozen at -20^{o} C until cortisol analysis. Concentrations of ACTH 10 min after TRH injection in excess of 100 pg/mL or concentrations of cortisol exceeding 1.0 µg/dL 19 h after dexamethasone injection were considered positive for PPID. Any animals meeting these criteria were excluded.

Analysis of ACTH, cortisol, and insulin were performed by Cornell University's Animal Health Diagnostic Center Endocrinology Laboratory (Ithaca, NY). Serum insulin concentrations were measured with a commercially available porcine insulin RIA (EMD Millipore Corp, Billerica, MA) validated by Cornell University's Animal Health Diagnostic Center Endocrinology Laboratory for use on equine serum samples. Serial dilutions of 3 equine samples with assay buffer were parallel to the standard curve, and samples that were spiked with 4 different quantities of porcine insulin (Sigma-Aldrich, St. Louis, MO, USA) had observed concentrations that averaged 91% of expected. The manufacturer did not report the cross-reactivity of the RIA antibody for equine insulin. However, the cross-reactivity for bovine insulin was reported as 90%, and the homology between equine and porcine insulins (98%) is greater than the homology between bovine and porcine insulins (96%). The sensitivity of the assay, as reported by the manufacturer, is 1.61 µIU/mL. The mean intra-assay and interassay coefficients of variation were 3.9% and 5.3%, respectively. ACTH concentrations were measured via an automated chemiluminescent enzyme immunoassay system (Immulite, Erlangen, Germany [195], and cortisol concentrations were determined using the Siemens Immulite Cortisol kit (Siemens, Erlangen, Germany) [196].

Glucose analysis was performed by Cornell University's Animal Health Diagnostic Center Clinical Pathology Laboratory (Ithaca, NY) using a hexokinase kinetic method with Roche reagents and the Hitachi P Modular from Roche (Roche Diagnostics, Indianapolis, IN), with a 2 control intra-assay variation of 0.6% to 0.7% and interassay variation of 2.04% and 2.28%.

2.2.2. Study design

To determine the effect of stevioside on the equine, 15 mixed breed and mixed sex animals (8 EMS and 7 age-matched controls, all non-PPID) were selected. Horse demographics are presented in Tables 2.1 and 2.2. Horses were maintained on an all forage diet with access to minimal mixed-grass pasture and ad libitum access to mixed-grass hay, a trace mineralized salt block, and water. They were housed at the University of Kentucky's Department of Veterinary Science's facilities. An OST was performed in July of 2015 on all horses, as previously described, but with the addition of a third blood sample taken 240 min after oral administration of either Karo corn syrup or stevioside (stevia). Blood samples for metabolic and inflammatory parameters (as described in the following) were collected concurrently at the 0, 60, and 240 min time points. Horses were provided either 0.15 mL/kg of karo or its stevioside equivalent in g stevia. For example, a 500 kg horse would be administered 75 mL karo, which contains 25 g sugar. Therefore, a 500 kg horse would receive 25 g of stevioside. The study was designed as a crossover with a 1-wk washout period; therefore, each horse received both Karo corn syrup and stevioside. Horses were randomly assigned to receive either karo or stevioside on week 1 vs week 2.

2.2.3. Peripheral blood processing for inflammatory cytokine production measured by flow cytometry

Heparinized blood was used to isolate peripheral blood mononuclear cells (PBMCs) by Ficoll-Paque PlusTM (Amersham Biosciences, Piscataway, NJ) gradient centrifugation [197]. For in vitro stimulation, 4 x 10^6 PBMC in 1 mL c-RPMI (RPMI-1640; Gibco, Grand Island, NY) supplemented with 2.5% fetal equine serum (FES; Sigma-Aldrich, St. Louis, MO), 100-U/mL penicillin/streptomycin (Sigma), and 55-mM 2-mercaptoethanol (Gibco) media was incubated at 37° C, 5% CO_2 with Brefeldin A (10 mg/mL; Sigma) and select wells with the positive control phorbol 12-myristate 13-acetate (PMA; 25 ng/mL; Sigma) and ionomycin (1 mM; Sigma) for 4 h. Following this, determination of cytokine gene expression was carried out by separating aliquots of the cells and placing them into Trizol (Ambion) to isolate RNA (described in the following). Remaining cells were assayed by flow cytometry for IFN-γ and TNF-α production by the following: cells were fixed in 2% paraformaldehyde (Sigma) and stored overnight at 4° C. After overnight storage, IFN-γ and TNF-α intracellular staining were carried out as

previously described [198]. After the cells were stained, aliquots of cells were resuspended in FACS flow, and flow cytometric acquisition was performed on a FACSCalibur (Becton Dickinson, San Jose, CA). Determination of IFN-γ and TNF-α protein production by lymphocytes was accomplished with the use of CELL QUEST (Becton Dickinson).

2.2.4. Inflammatory cytokine gene expression measured by real time-polymerase chain reaction (RT-PCR)

A modified Trizol method was used to extract total RNA. One microgram of RNA was reverse transcribed into cDNA as previously described [197]. In brief, 0.5 µg of each RNA sample and a reverse transcription master mix (Promega, Madison, WI) were incubated for 15 min at 42° C and for 5 min at 95° C. Five microliters of Master Mix (SensiMix HI-ROX 2x, Bioline) and 4.5 µL of cDNA were combined with 0.5 µL of the primer-probe of interest (TaqMan, Equine-specific, intron-spanning primers and probes; Applied Biosystems). Samples were run using Applied Biosystems Real-Time PCR (ABI Viia7) against the following genes: Housekeeping gene *beta*-glucuronidase (β-gus; Ec0347-0630_m1), interferon gamma (IFN-γ; Ec03468606_m1), IL-6 (Ec03468678_m1), IL-1β (Ec04260298_s1), TNF-α (Ec03467871_m1), and TLR4 (Ec03468994_m1) [199]. Samples were processed in duplicate and incubated for 95° C for 10 min. They then underwent 10 cycles at 95° C for 15 s and 60° C for 60 s. Relative changes in gene expression were determined by the $\Delta\Delta C_T$ method [200] with mean ΔC_T for time 0 set as the calibrator for all samples. Relative quantity (RQ) was calculated as $2^{-\Delta\Delta CT}$ and used to express results.

2.2.5. Data analysis

Study data were analyzed using a 2-period crossover design with repeated measures ANOVA. Analyses were completed using PROC MIXED, SAS 9.4 (SAS Institute Inc, Cary, NC, USA). In a typical crossover design, the group is determined by the sequence in which the treatment is applied. In this study, both EMS and control horses were in both sequences. To accurately test sequence and EMS vs control, a group variable was created. There were 4 groups based on period (week = 1 or 2), Stevia vs Karo, and EMS vs control. The main effects of sequence (if the horse received karo vs stevia week 1) and EMS vs control, as well as, the interaction of sequence x EMS vs control were tested using the error term of Horse ID (Group). This model also included the main effects of period, Stevia vs

Karo, and time (0, 60, and 240 min). The interactions included Stevia vs Karo x EMS vs Control, time x EMS vs Control, time x Stevia vs Karo, and time x Stevia vs Karo x EMS vs Control. Horse ID was the subject, and the repeated measure was set as time. Mauchly's Sphericity Test was used to test the form of the common covariance matrix. The sphericity assumption was not violated; therefore, compound symmetry covariance structure was selected. Pairwise comparisons were made using protected LSD. Only variables that found significant in the ANOVA were selected to apply pairwise comparisons. Screening data were analyzed with Sigma Plot, version 12 (Systat Software, San Jose, CA) with a 1-way ANOVA to compare EMS vs controls. For data not normal per Shapiro-Wilk testing, a Kruskal-Wallis 1-way ANOVA on ranks was performed. Differences were considered statistically significant when $P < 0.05$.

2.3. Results

2.3.1. Metabolic responses

There was an observed EMS vs Control by time point interaction ($P < 0.0001$) for serum glucose (Figure 2.1) with control horses at time point 60 higher compared with control horses at time points 0 and 240. Control horses at time points 0 and 240 were lower compared with EMS horses at all 3 time points. Control horses at time point 60 were not different from EMS horses at time point 60. There was also a stevia vs karo by time point interaction ($P = 0.0001$) with horses given karo lower at time points 0 and 240 compared with time point 60 in horses given karo. Horses given karo were higher at time point 60 compared with all 3 time points in horses given stevia. Horses given stevia were not different regardless of time point. There were also several overall differences observed. EMS horses were higher compared with controls ($P = 0.0033$), horses given karo were higher compared with those given Stevia ($P = 0.0002$), horses were higher at the 60 min time point compared with time points 0 and 240 ($P = 0.007$), and horses were higher in serum glucose concentrations during period 2 compared with period 1 ($P = 0.0015$).

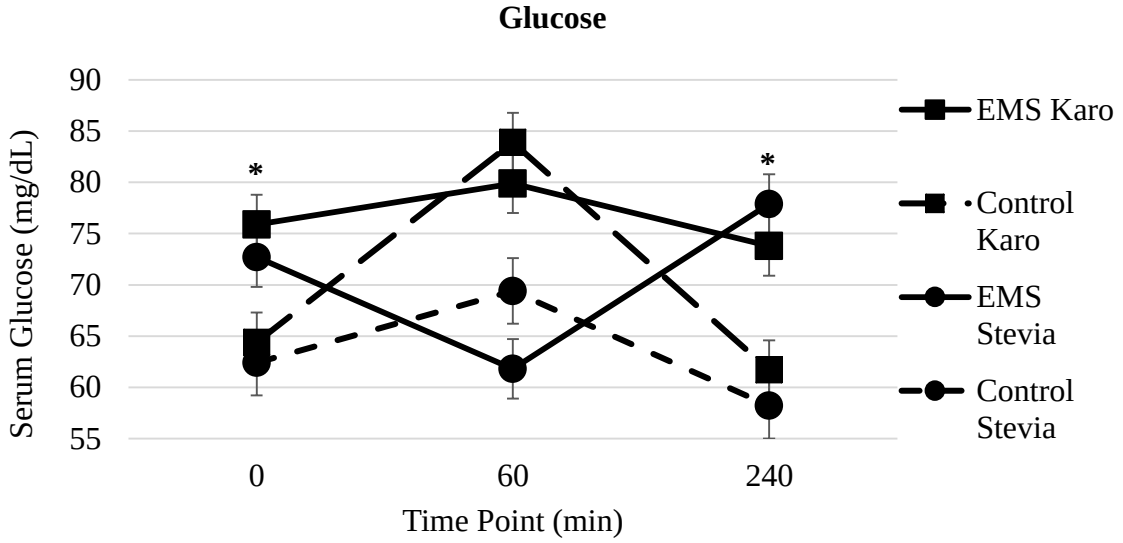

Figure 2.1. Serum glucose concentrations in EMS (n=8) and control (n=7) horses given Karo corn syrup and stevioside at baseline (0) and after administration (60 and 240 min). Differences ($P < 0.05$) within time point between EMS and non-EMS controls are represented by (*). EMS, equine metabolic syndrome.

There was an observed 3-way EMS vs control by stevia vs karo by time point interaction ($P = 0.0073$; Figure 2.2) for serum insulin. At time 0 control, horses given karo were lower compared with time point 60 for control horses given karo, EMS horses given karo, EMS horses given stevia, and time point 240 for EMS horses given stevia. At time point 60, control horses given karo were higher compared with time point 240 for control horses given karo, control horses given stevia at all time points, and time points 0 and 240 for EMS horses given karo. However, they were lower compared with time point 60 for EMS horses given karo. At time point 240, control horses given karo were lower compared with time point 60 for EMS horses given karo, and time points 60 and 240 for EMS horses given stevia. Control horses given stevia at all time points were lower compared with time point 60 for EMS horses given karo. At time point 240, control horses given stevia were also lower compared with time points 60 and 240 for EMS horses given stevia. EMS horses given karo were lower at time point 0 compared with time point 60 for EMS horses given karo. At time point 60, EMS horses given karo were higher compared with time point 240 for EMS horses given and EMS horses given stevia for all 3 time points. No time point differences were observed between control or EMS horses given stevia. Overall, horses

given karo had higher serum insulin concentrations compared with those given stevia ($P < 0.0001$), and insulin was higher at the 60 min time point compared with time points 0 and 240 ($P < 0.0001$).

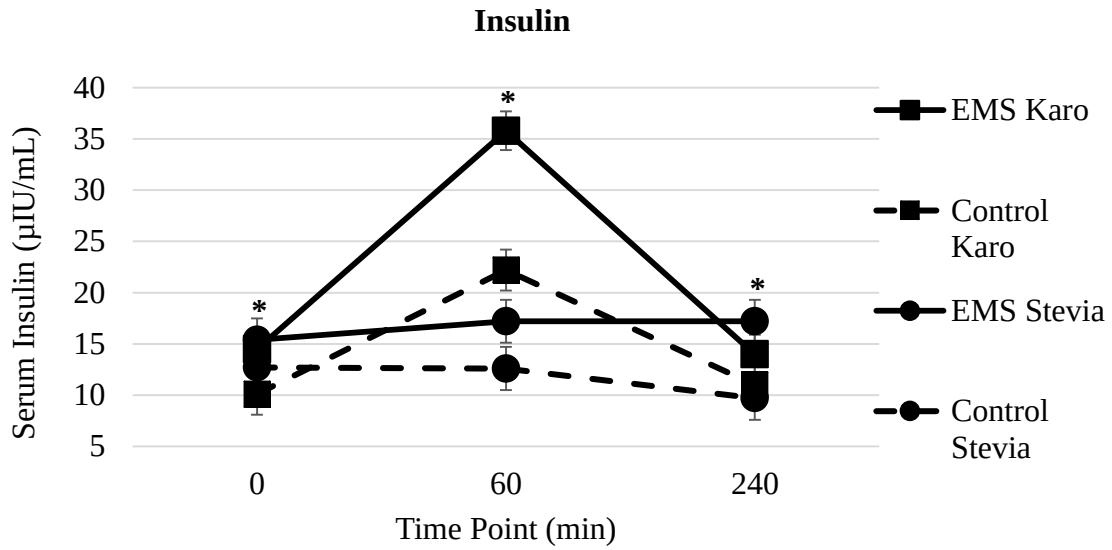

Figure 2.2 Serum insulin concentrations in EMS (n=8) and control (n=7) horses given Karo corn syrup and stevioside at baseline (0) and after administration (60 and 240 min). Differences ($P < 0.05$) within time point are represented by (*). EMS, equine metabolic syndrome.

2.3.2. Inflammatory cytokine production measured by flow cytometry

Flow cytometry results are presented in Figure 2.3. PBMCs stained intracellularly for IFN-γ had several differences in both the percentage of lymphocytes producing IFN-γ (% gated) and the mean fluorescence produced per lymphocyte (MFI). There was an EMS vs control by time point interaction ($P = 0.0016$) for IFN-γ % gated. Regardless of administration of karo or stevia, control horses had a higher percent gated at time point 0 compared with control horses at time points 60 and 240. Control horses at time point 0 were also higher than EMS horses at time point 240. However, control horses at time points 60 and 240 were lower compared with EMS horses at time point 60 for IFN-γ % gated. EMS horses at time point 60 were higher compared with EMS horses at time points 0 and 240. There was also an overall effect of period for IFN-γ % gated with horses higher period 1 compared with period 2 ($P = 0.0346$). IFN-γ MFI exhibited interactions between EMS vs control by Stevia vs karo ($P = 0.0355$) and EMS vs control by time point ($P = 0.0003$).

Regardless of administration of karo or stevia, control horses had a higher IFN-γ MFI at time point 0 compared with control horses at all other time points as well as compared with EMS horses at time points 0 and 240. Control horses at time points 60 and 240, however, were lower compared with EMS horses at time point 60. EMS horses were higher at time point 60 compared with EMS horses at time points 0 and 240 for IFN-γ MFI. No differences were observed between EMS and control horses at time point 240. Overall, IFN-γ MFI was lower in control horses given karo vs control horses given stevia.

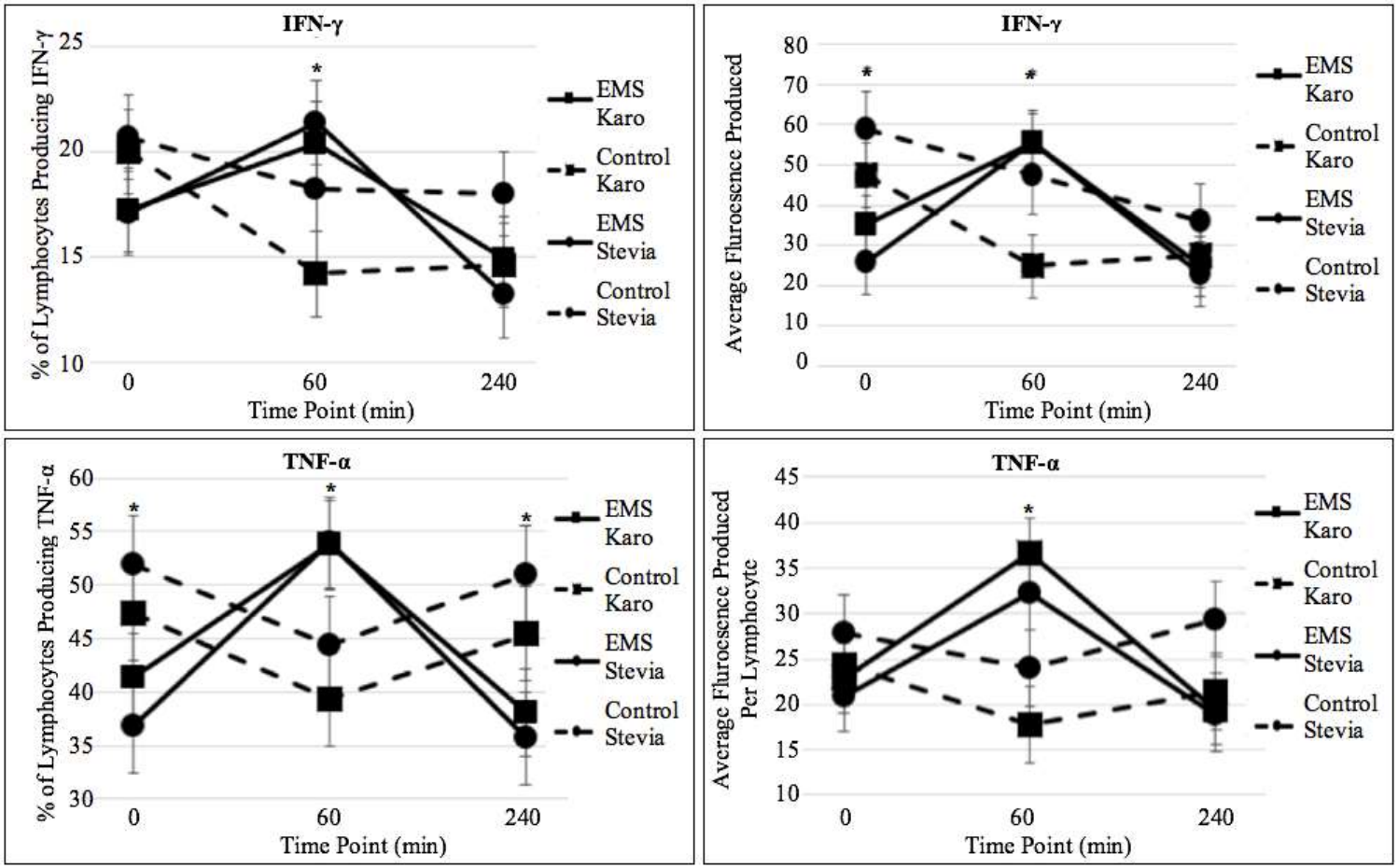

Figure 2.3. Flow cytometry results for PBMCs stained for IFN-γ and TNF-α in EMS (n=8) and control (n=7) horses given Karo corn syrup and stevioside at baseline (0) and after administration (60 and 240 min). Differences ($P < 0.05$) within time point between EMS and non-EMS controls are represented by (*). EMS, equine metabolic syndrome; PBMCs, peripheral blood mononuclear cells.

Results for TNF-α % gated and MFI were similar to those seen for IFN-γ. There was an interaction between EMS vs control and time point ($P < 0.0001$) for TNF-α % gated. Control horses given either karo or stevia were higher at time point 0 compared with control horses at time point 60 and EMS horses at time points 0 and 240. Control horses at time point 240 were also higher than EMS horses at time point 240. However, EMS horses at time point 60 were higher compared with control horses at time point 60 as well as EMS horses at time points 0 and 240. TNF-α MFI exhibited an EMS vs control by Stevia vs karo interaction ($P = 0.0402$) with control horses given karo lower compared with controls given stevia. In addition, there was an EMS vs control by time point interaction ($P = 0.0001$) with control horses at time points 60 and 240 lower compared with EMS horses at time point 60 and EMS horses at time point 60 higher compared with EMS horses at time points 0 and 240. In addition, there was an overall effect of period, with horses higher in TNF-α % gated and MFI in period 2 compared with period 1 ($P = 0.0496$).

2.3.3. Inflammatory cytokine gene expression measured by RT-PCR

PBMC gene expression results expressed as RQs and as measured by RT-PCR are presented in Figure 2.4. Gene expression results followed a similar pattern as flow cytometry results. There were several interactions for IFN-γ RQ as well; EMS vs control by time point ($P < 0.0001$), and EMS vs control by stevia vs karo ($P = 0.0021$). Control horses at time point 0 had a higher IFN-γ RQ compared with control horses at time point 60 and EMS horses at time points 0 and 240 regardless of whether horses were given karo or stevia. Control horses at time point 240 were higher compared with EMS horses at all time points. However, control horses at time point 60 were lower compared with control horses at time point 240 and EMS horses at time point 60. EMS horses at time point 60 were higher for IFN-γ RQ than EMS horses at time points 0 and 240. In addition, there was an overall difference of IFN-γ RQ for EMS vs control ($P = 0.0028$) with EMS horses higher compared with controls, and an overall stevia vs karo ($P = 0.017$) difference with horses given stevia higher compared with those given karo.

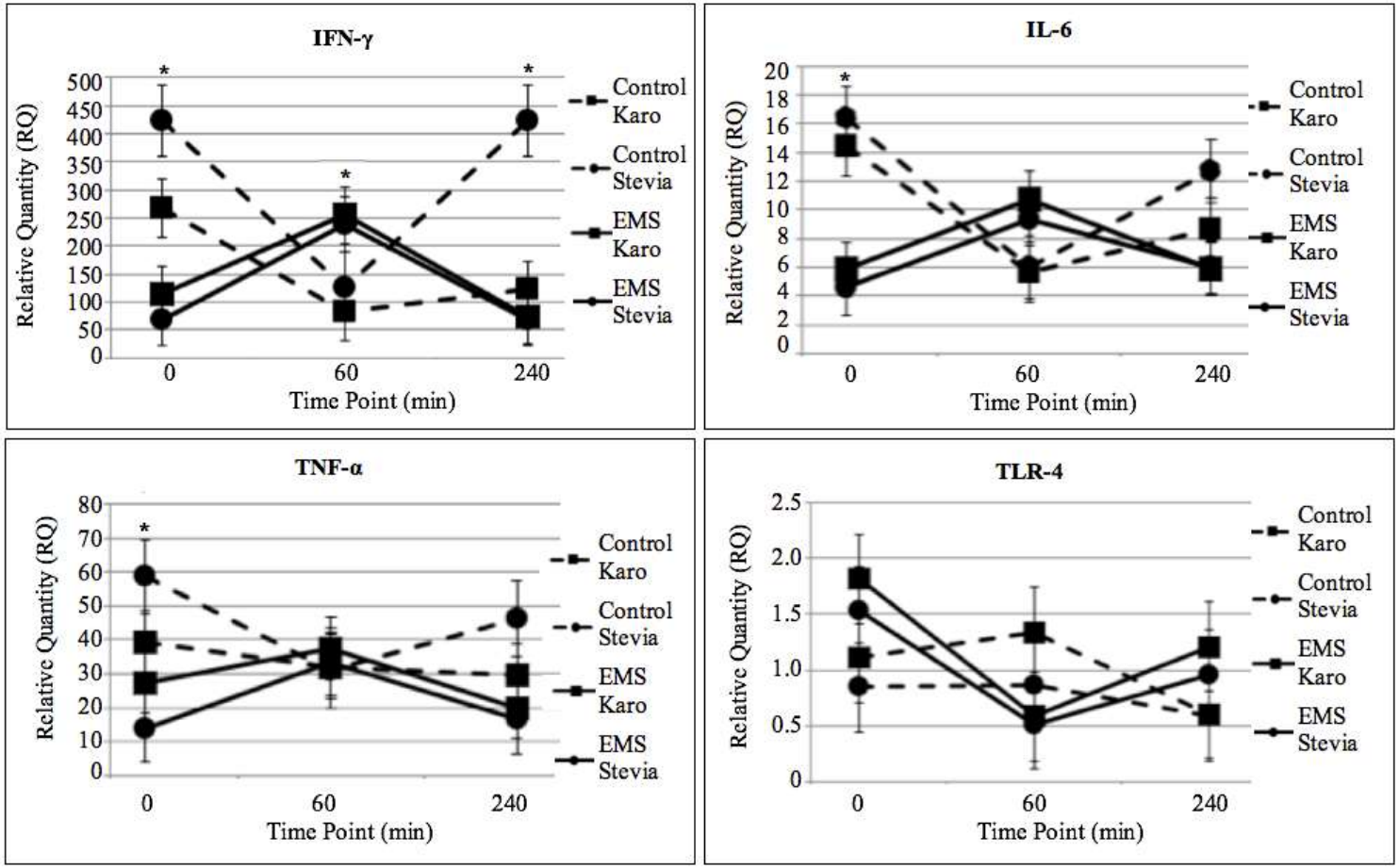

Figure 2.4. RT-PCR PBMC gene expression results in EMS (n=8) and control (n=7) horses given Karo corn syrup and stevioside at baseline (0) and after administration (60 and 240 min). Differences ($P < 0.05$) within time point between EMS and non-EMS controls are represented by (*). EMS, equine metabolic syndrome; PBMCs, peripheral blood mononuclear cells; RT-PCR, reverse transcription PCR.

For IL-6 RQ, there was an interaction between EMS vs control by time point ($P < 0.0001$). Control horses given either karo or stevia were higher at time point 0 compared with control horses at time points 60 and 240 and EMS horses at all time points. Control horses at time point 60 were lower compared with control horses at time point 240. Control horses at time point 240 were higher compared with EMS horses at time point 0. EMS horses at time point 60 were higher compared with EMS horses at time points 0 and 240. Overall, horses during period 1 had a higher IL-6 RQ compared with period 2 ($P = 0.0117$). TLR-4 RQ results were similar to those for IL-6 RQ with an interaction between EMS vs control by time point ($P = 0.0282$). Regardless of administration of stevia or karo, control horses at time point 240 were lower compared with EMS horse at time point 0. EMS horses at time point 0 were higher compared with EMS horses at time point 60. An overall period effect was also observed for TLR-4 RQ, with horses higher in period with compared with period 1 ($P = 0.0277$).

There was an interaction between EMS vs control and time point ($P = 0.0231$) for TNF-α RQ. There were no differences in TNF-α RQ between horses given stevia or those given karo. Control horses at time point 0 were higher compared with control horses at time point 60 and EMS horses at time points 0 and 240. EMS horses at time point 60 were higher compared with EMS horses at time point 240.

2.4. Discussion

Horses had expected glycemic and insulinemic responses to karo corn syrup with an increase in insulin and glucose at time point 60 and with EMS horses having a greater increase in insulin at time point 60 compared with controls.

Stevia is generally recognized as safe in humans [201] and is thought to decrease circulating glucose concentrations by stimulating the pancreatic beta cells to release insulin [184]. In this study, an oral bolus of stevioside produced a lower glycemic response when compared with karo corn syrup. In horses given stevioside, there was no significant increase in serum glucose at time point 60 compared with time points 0 or 240 in contrast to the marked rise in serum glucose in horses given karo. Although EMS horses had a decrease in serum glucose at time point 60 when given stevia compared with an increase when given karo, there was no significant interaction between EMS vs controls and karo

vs stevia. In addition, at time point 60, EMS horses given karo had higher serum insulin concentrations compared with EMS horses given stevia at any time point. At time point 60, EMS horses given stevia had similar serum insulin concentrations compared with control horses given karo, although control horses given karo were higher compared with control horses given stevia. Glucose and insulin responses in horses to stevia at time 60 indicate that in horses, in particular those with metabolic dysfunction, stevia may provide a protective effect on postprandial increases in these metabolic parameters. It is unlikely in horses that stevia stimulates insulin release from the pancreas to the extent that it does in rats [184], as increases in insulin at time point 60 in horses given stevia were significantly lower than those give karo. It is possible that stevia may induce an increased insulin receptor sensitivity in horses with EMS; however, more work needs to be done to discover possible explanations for differences in glucose and insulin responses to a glycemic challenge with karo or stevia.

There were observed differences for the flow cytometry analysis of IFN-γ and TNF-α intracellularly stained PBMCs. Regardless of administration of either karo or stevia, EMS horses had an increase in markers of inflammation at time 60, whereas control horses had a decrease in these same markers of inflammation. Similar results were observed for PBMC gene expression of inflammatory cytokines with EMS and control horses having contrasting inflammatory responses to an OST. These data suggest that EMS horses have an abnormal proinflammatory response to glycemic challenge when given karo or stevia. Data on postprandial inflammation in humans and other species, in particular in those with metabolic dysfunction are scarce. Most report an increase in systemic soluble adhesion molecules, such as Intercellular Adhesion Molecule-1 (ICAM-1) in response to either a high-fat meal or high-fat meal combined with glucose [43, 44]. Soluble adhesion molecules are involved in the immune and inflammatory responses [202] and have been linked to cardiovascular disease in humans [203, 204]. Some report an increase in adhesion molecules or proinflammatory cytokines in both normal and diabetic subjects in response to a meal with diabetic subjects having a significantly greater increase compared with normal controls [43, 45]. Others did not see this same increase [205], however, this may be due to differences in meal type or carbohydrate complexity [206].

In horses, most of the data regarding inflammation and glycemic and insulinemic responses have been focused on either basal circulating or tissue inflammatory markers at rest. Data have been somewhat conflicting with some groups showing increases in inflammatory markers such as TNF-α [28] and serum amyloid A [40] and others showing no differences between horses with ID and normal controls [31]. There has also been work in the horse focused on oligofructose-induced laminitis models with whole-blood inflammatory markers IL-1β, IL-8, and IL-10 increasing 8 h post induction of laminitis [51] in 1 study and increases in IL-2, IL-6, IL-8, and IFN-γ noted at the onset of laminitis in another study [18]. The available data regarding postprandial inflammation in the horse are scarce but have shown postprandial rises in IL-1β in response to feeding a high sugar high starch meal [46]. Increases in inflammatory markers following a meal may in part be due to oxidative stress or, to increases in bacterial endotoxin concentrations. These increases in inflammation appear to mirror the higher insulinemic responses in EMS horses and may be a possible mechanism that predisposes these animals to the development of laminitis. However, this remains to be further characterized.

Given these data, stevia appears to be well tolerated in EMS horses, considering that stevia did not induce the same metabolic responses as karo corn syrup on challenge. Thus, stevia may be a possible candidate as a non-glycemic sugar replacer in horses with ID. Further work is needed to explore inflammatory effects of stevia in metabolically normal animals and possible metabolically beneficial effects in horses with EMS. Also, these data reveal new questions about the response of EMS horses to an oral sugar challenge, in particular with regard to postprandial inflammation.

CHAPTER 3 COMPARISON OF THE FECAL MICROBIOTA IN HORSES WITH EQUINE METABOLIC SYNDROME (EMS) AND METABOLICALLY NORMAL CONTROLS FED A SIMILAR ALL FORAGE DIET

Journal of Equine Veterinary Science, 2016: 44, 9-16

Equine metabolic syndrome (EMS) is an ever-increasing problem in the equine industry, especially considering that it is a risk factor for the development of laminitis. Equine metabolic syndrome is similar to metabolic syndrome in humans, which has been associated with alterations in intestinal microbiota. However, no work to date has been published to characterize the fecal microbiota in the EMS horse to determine differences, if any, from the metabolically normal animal. Therefore, our objective was to characterize the fecal microbiota of horses with EMS compared with non-EMS controls. Ten horses were classified as having EMS, and 10 non-EMS controls were selected for this work. Equine metabolic syndrome was determined as: insulin resistance, general or regional adiposity, and a history of or predisposition to laminitis. Blood collection via jugular venipuncture was performed, along with an oral sugar challenge. Concurrent with blood collection, phenotypic measurements and fecal grab samples were taken. Fecal samples were used to extract DNA for next generation sequence-based analysis of the bacterial microbiota. Equine metabolic syndrome horses exhibited a decrease in fecal microbial diversity, and there were differences in overall community structure between EMS horses and controls. The operational taxonomic unit with the highest linear discriminant analysis effect size in association with EMS horses was a member of subdivision 5 of Verrucomicrobia incertae sedis (uncertain classification). Interestingly, the control group had an overrepresentation of genus Fibrobacter. These data demonstrate that EMS horses have differences in their fecal microbiota compared with controls.

3.1. Introduction

Horse are hindgut fermenters and as such, their gut microbiota is predominantly populated by bacteria from the phyla Firmicutes [64, 67]. However, even in healthy horses, the gut microbiota is naturally highly diverse and highly variable. It is influenced by factors such as diet, age, and individual variation [64-66]. Disease states in particular can have a significant impact on the gut bacterial population in the animal. For example, there is

evidence that a high-starch diet may increase the abundance of certain taxa from the family Veillonellaceae [70]. In addition, horses with chronic laminitis have differences in both microbiota abundance and diversity compared with controls [72], and the microbiota has significant changes in abundance during the onset of laminitis [73, 74].

Insulin resistance, general or regional adiposity, and a history of or predisposition to laminitis are the three main characteristics associated with equine metabolic syndrome (EMS) [23]. Equine metabolic syndrome is considered to be especially concerning to the equine population considering its connection to laminitis, which can result in inability for the animal to resume normal work or even euthanasia. Equine metabolic syndrome is comparable to metabolic syndrome (MetS) in humans, which is associated with similar factors, such as visceral obesity, glucose intolerance, and dyslipidemia [207-209].

In humans and mice, MetS and obesity have been linked to alterations in the intestinal microbiota [77]. It has been shown in high fat–fed mice that bacterial lipopolysaccharide (LPS) endotoxemia can induce inflammation and insulin resistance [61], both of which are considered to be influential in the pathophysiology of MetS [210]. Furthermore, age-related changes in the gut microbiota of mice have resulted in increased plasma LPS concentrations and a subsequent increase in markers of inflammation, indicating that shifts in the microbiota may play a major role in inducing low-grade systemic inflammation [83]. Studies in humans have shown that dietary changes can have a positive impact on the gut microbiota by reducing LPS producing bacteria and are proposed as a possible therapeutic target for individuals with metabolic dysfunction [84, 85]. However, no data to date have been presented exploring possible differences in the gut microbiota of horses with EMS compared with metabolically normal controls, nor what impact these differences may have on the pathophysiology of the syndrome.

Investigation into the mechanisms and factors contributing to EMS is becoming increasingly important considering its negative health consequences in these horses, in particular, the increased risk of laminitis. Therefore, the objective of this study was to investigate and characterize the fecal microbiota of horses with naturally occurring EMS compared with metabolically normal non-EMS controls.

3.2. Materials and Methods

3.2.1. Horse Selection and Sample Collection:

Twenty horses of mixed gender and breed were selected on the basis of EMS criteria as defined in the following section from the University of Kentucky's Department of Veterinary Science herd in the spring of 2014. Of these horses, 10 were classified as EMS, and 10 were classified as non-EMS controls. There was no significant difference in age between EMS and control horses (P = .903). All horses were housed at the University of Kentucky's Main Chance or Woodford farm facilities, maintained on a similar all forage diet of free choice mixed bluegrass, orchard and fescue hay (Table 3.1), and minimal pasture of a similar mixed grass type, and had access to water and a mineral block *ad libitum*. Horses were acclimated to their respective pastures for at least 2 months before sampling. All materials and methods were approved by the Institutional Care and Usage Committee of the University of Kentucky.

Table 3.1. Nutrient composition of mixed grass hay.

Component	As fed	Dry Matter
% Moisture	7.8	
% Dry Matter	92.2	
% Crude Protein	7.6	8.3
% Adjusted Crude Protein	7.6	8.3
% Acid Detergent Fiber	43.0	46.6
% Neutral Detergent Fiber	63.9	69.3
% NFC	15.8	17.1
% TDN	52.0	56.0
NFL, Mcal/Lb	0.41	0.44
NEM, Mcal/Lb	0.45	0.48
NEG, Mcal/Lb	0.21	0.23
Relative Feed Value		71
% Calcium	0.39	0.43
% Phosphorus	0.26	0.28
% Magnesium	0.13	0.14
% Potassium	1.65	1.79
% Sodium	0.0005	0.005
PPM Iron	427	463
PPM Zinc	17	18
PPM Copper	7	7
PPM Manganese	92	100
PPM Molybdenum	0.5	0.6
Horse DE, Mcal/Lb	0.80	0.86

Nutrient composition of mixed grass hay fed to all horses *ad libitum*. Analysis provided by Dairy One Forage Testing Laboratories (Ithaca, NY).

Equine metabolic syndrome was determined by the following criteria from the 2010 ACVIM consensus statement by Frank et al [23]: insulin dysregulation, general or regional adiposity, and a history of or predisposition to laminitis. To ascertain the presence of insulin dysregulation, an oral sugar test was performed after overnight fasting as previously described [13]. A fasting insulin level of >20 μIU/mL was considered indicative of hyperinsulinemia, and an increased insulin (>60 μIU/mL) 60 minutes after administration of oral sugar (Karo corn syrup) was defined as insulin resistance [13, 23]. The presence of either hyperinsulinemia or insulin resistance was considered positive for insulin dysregulation. Obesity and adiposity were determined by body condition score (BCS) and cresty neck score (CNS). Body condition score was established using the 1 to 9 Henneke scoring system [186], and CNS was determined via the 0-5 system established by Carter et al [187]. For both BCS and CNS, three trained investigators scored the animals, and theses scores were averaged to determine final BCS and CNS. Individuals scoring were blinded to which horses were positive for insulin dysregulation. Equine metabolic syndrome was defined as having increased adiposity with a BCS of 6.5 or above or a CNS greater than 2.5. All EMS horses had a history of or predisposition to laminitis. Insulin, BCS, and CNS data are presented in Table 3.2.

Table 3.2. Characterization of phenotypic measures in equine metabolic syndrome (EMS) versus non-EMS control horses.

Group	BCS	CNS	Basal Insulin (μIU/mL)	Post oral sugar Insulin (μIU/mL)
EMS	7.13 ± 0.15	3.57 ± 0.27	33.85 ± 2.66	63.26 ± 5.29
Control	5.85 ± 0.23	1.95 ± 0.18	13.03 ± 1.46	26.11 ± 3.77
P value	<.001	<.001	<.001	<.001

Average body condition score (BCS) and cresty neck score (CNS), and basal insulin and insulin 60 min post oral sugar administration in n = 10 EMS and n = 10 non-EMS control horses. Results are represented as the mean plus or minus the standard error of the mean.

All animals were also examined to ensure that none were affected by pituitary pars intermedia dysfunction (PPID) by conducting thyrotropin-releasing hormone (TRH) stimulation and low-dose dexamethasone suppression testing. Thyrotropin-releasing hormone stimulation testing was accomplished similar to previously described [191, 192]. In brief, a morning baseline blood sample was taken via jugular venipuncture after which

1-mL IV dose of TRH dissolved to 1 mg/mL in 0.9% saline (Sigma-Aldrich, St. Louis, MO) was administered. Ten minutes after TRH injection, a second blood sample was collected. Blood samples were collected in EDTA-containing tubes, placed on ice, and transported to the laboratory for immediate centrifugation for plasma isolation [193]. Plasma was stored at -20^{o}C and shipped on dry ice to Cornell University for analysis of adrenocorticotropic hormone (ACTH) concentrations. Low-dose dexamethasone suppression testing was performed as previously described [194] by taking an afternoon (3:50 PM–6:00 PM) baseline jugular blood sample, administering an intramuscular dexamethasone bolus (0.04 mg/kg BW), and taking a second blood sample 19 hours later. Samples were collected in a gel serum separator tube and serum isolated and frozen at -20^{o}C until cortisol analysis. Adrenocorticotropic hormone concentrations 10 minutes after TRH injection >100 pg/mL or cortisol concentrations in excess of 1.0 μg/dL 19 hours after dexamethasone injection were considered indicative of PPID and any positive animals excluded. All blood samples were sent to Cornell University's Animal Health Diagnostic Center Endocrinology Laboratory for determination of insulin, ACTH, and cortisol concentrations. Insulin was measured using the Millipore Porcine Insulin RIA Kit (EMD Millipore Corporation, Darmstadt, Germany), ACTH using an automated chemiluminescent enzyme immunoassay system (Immulite, Erlangen, Germany) [195], and cortisol using Siemens Immulite Cortisol kit (Siemens, Erlangen, Germany). All blood samples and phenotypic measures for all horses were collected within a period of 2 weeks in the spring of 2014.

3.2.2. DNA extraction and 16s rRNA gene PCR

DNA was extracted from fecal samples using a commercial kit (E.Z.N.A. Stool DNA Kit, Omega Bio-tek Inc, Doraville, GA), according to manufacturer's instructions. Quantity and quality of DNA were assessed by spectrophotometry (NanoDrop; Roche, Mississauga, Canada), the V4 region of the 16S rRNA gene was amplified [211], and amplicons were sequenced by Illumina MiSeq (San Diego, CA). Sequence files are available at the University of Guelph Research Data Repository, DOI: http://dx.doi.org/10.5887/ UGRDR/10864/XER6B.

3.2.3. Data Analysis

Phenotypic data (insulin, BCS, and so forth) were analyzed by SIGMAPLOT version 12.3 (Systat Software Inc). A mixed model one-way analysis of variance was used for each quantitative variable and ordinal data analyzed via the Wilcoxon Mann-Whitney test. One EMS horse's CNS was not collected and thus not included in the analysis. Fixed effects were set as EMS versus control. Data were normally distributed and of equal variance. Data were considered statistically significant when $P \leq .05$.

Microbiota data were analyzed using the open-source platform mothur (v1.35) [212]. Initial sequence processing involved aligning paired-end reads, with a series of quality control steps to remove sequences that contained any ambiguous base calls, were not consistent with the target amplicon size (240 bp), contained holopolymers > 8 bp in length, or did not align with the correct 16S rRNA gene region. Chimeras were identified using UCHIME [213] and removed. Taxonomy was assigned using the Ribosomal Database Project taxonomy database (http://rdp.cme.msu.edu/index.jsp).

Subsampling was performed to normalize sequence numbers by random selection of number of sequences from each sample that corresponded to the sample with the smallest number of sequences. Comparison of the relative abundances of taxa between groups was done by linear modeling using robust (Huber) estimation to down-weight outliers, with *P* values that were adjusted for false discovery rate using the Benjamini–Hochberg technique. Alpha diversity was calculated using Chao1 richness, inverse Simpson's diversity, and Shannon's evenness tests and compared using Wilcoxon test or Steel–Dwass test. A P value of $\leq$.05 was considered significant.

Sequences were binned into phylotypes, and subsampling was performed to normalize sequence number for subsequent analyses [214]. Dendrograms for community membership (classical Jaccard index, a measure of shared OTUs) and structure (Yue and Clayton index of dissimilarity, which considers shared OTUs and their relative abundances) were created and compared using unifrac and analysis of similarity (ANOSIM). Community membership and structure were also visualized using principal coordinate analysis. Linear discriminate analysis effect size (LEfSe) [215] was used to identify overrepresented OTUs between horses with metabolic disease and controls. Samples were also evaluated using the Dirichlet multinomial mixtures method for

probabilistic modeling [216] to determine the number of different metacommunities (enterotypes) that they could be assigned to, with the K value that derived the minimum Laplace approximation indicating the number of different metacommunities. The observed learning technique, random forests [217], was used to determine whether a set of predictive features could be used to accurately identify samples from EMS versus control groups.

Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) [218] was used to predict the relative abundance of predicted Kyoto Encyclopedia of Genes and Genomes biochemical pathways and ortholog groups, and those were compared between horses with metabolic disease and controls using LEfSe.

3.3. Results

At the time of sampling, EMS horses were aged 13 ± 5 years (range 8–20 years), and non-EMS controls were aged 13 ± 3 years (range 10–19 years). Equine metabolic syndrome horses had greater values for fasting insulin, insulin 60 minutes after oral sugar administration, BCS, and CNS compared with controls (Table 3.2).

From the fecal samples analyzed for fecal microbiota differences, sequence processing and filtering yielded a total of 1,446,359 sequences (mean 72,318/sample, median 73,371.5, standard deviation 30,815.5, and range 11,491- 137,462). Subsampling of 11,491 sequences per sample was performed to normalize data. There were no differences in estimated richness (P = 97), observed richness (P = 79), or evenness (P = 12) between groups; however, diversity was lower in EMS horses (median 6.3 vs. 7.1, P = 026, Figure 3.1).

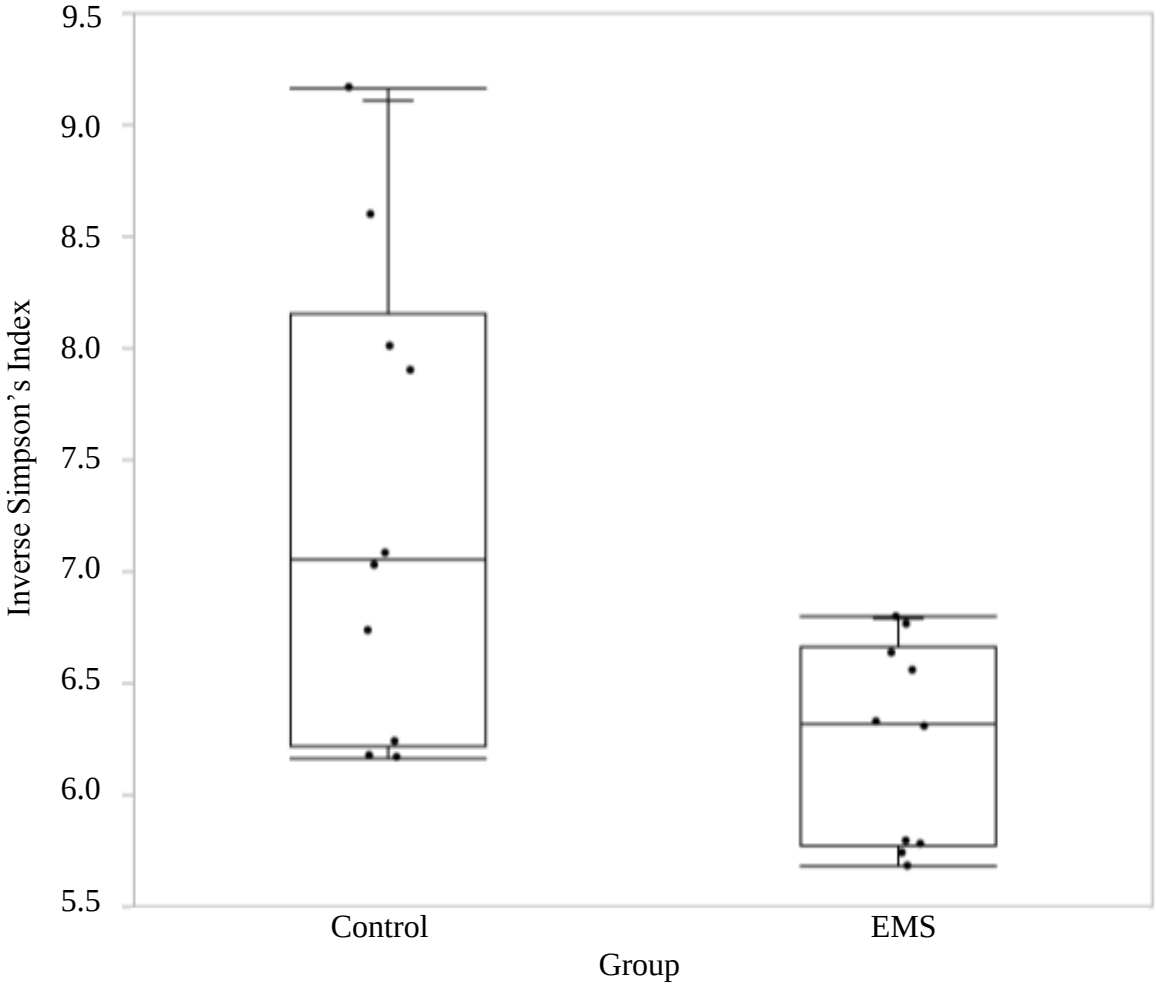

Figure 3.1. Intestinal microbiota diversity in equine metabolic syndrome (EMS) versus non-EMS control horses. Intestinal microbiota diversity as represented by an inverse Simpson's index in n = 10 EMS and n = 10 non-EMS control horses.

Based on unifrac analysis, there was a significant difference in community structure between groups (*P* = 011, Figure 3.2), with no difference in membership (*P* = 33, Figure 3.3). A significant difference in community structure was also identified with ANOSIM (*P* = 012), whereas the difference in membership approached significance (*P* = 056). However, the differences that were present were not to the degree that samples would be assigned to two different metacommunities. Similarly, random forest modeling was poorly predictive of group membership, with an error rate of 55%.

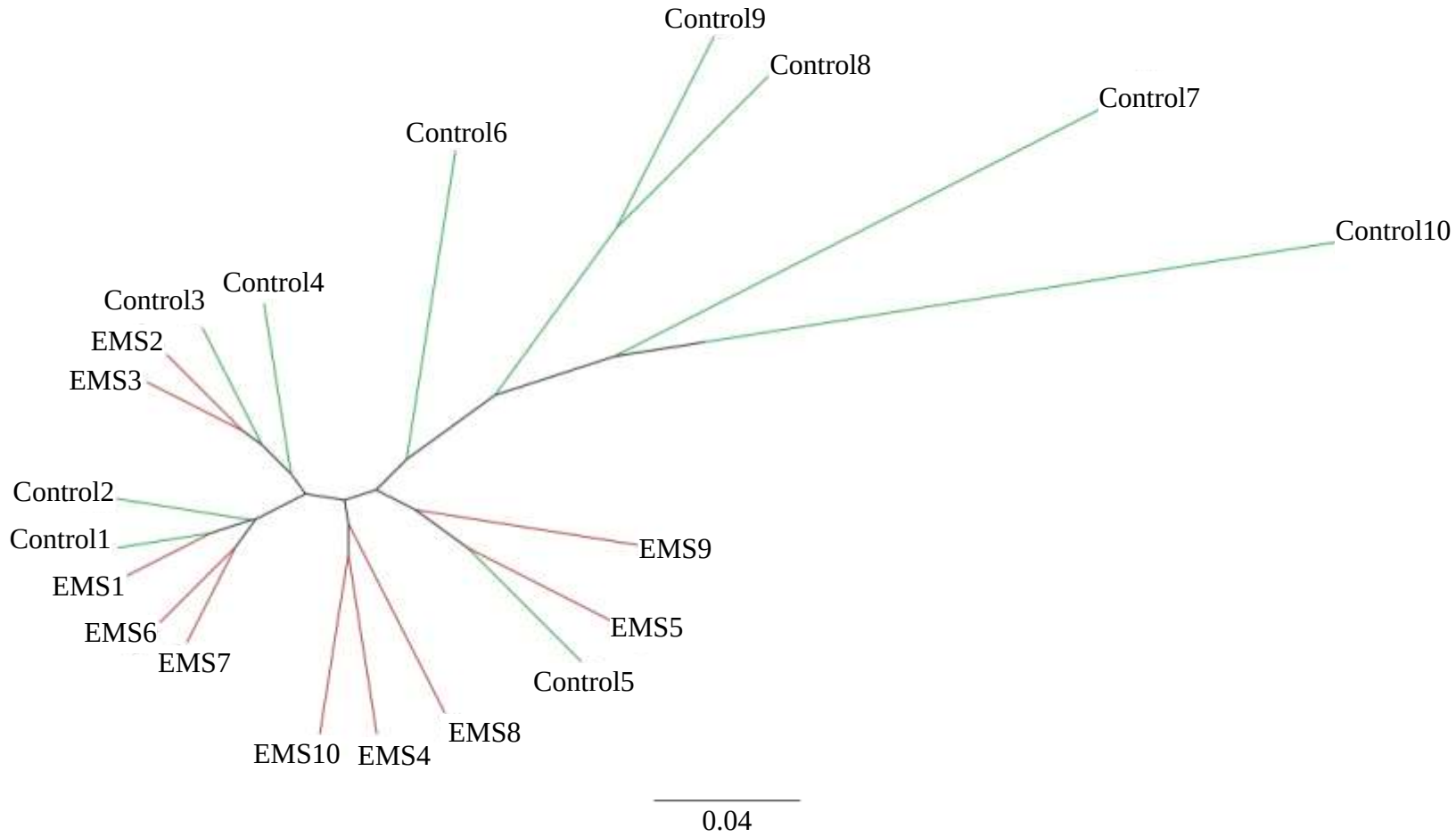

Figure 3.2. Bacterial population structure in equine metabolic syndrome (EMS) versus non-EMS control horses. Representation of bacterial population structure as measured by Yue & Clayton similarity coefficient in n = 10 EMS and n = 10 non-EMS control horses.

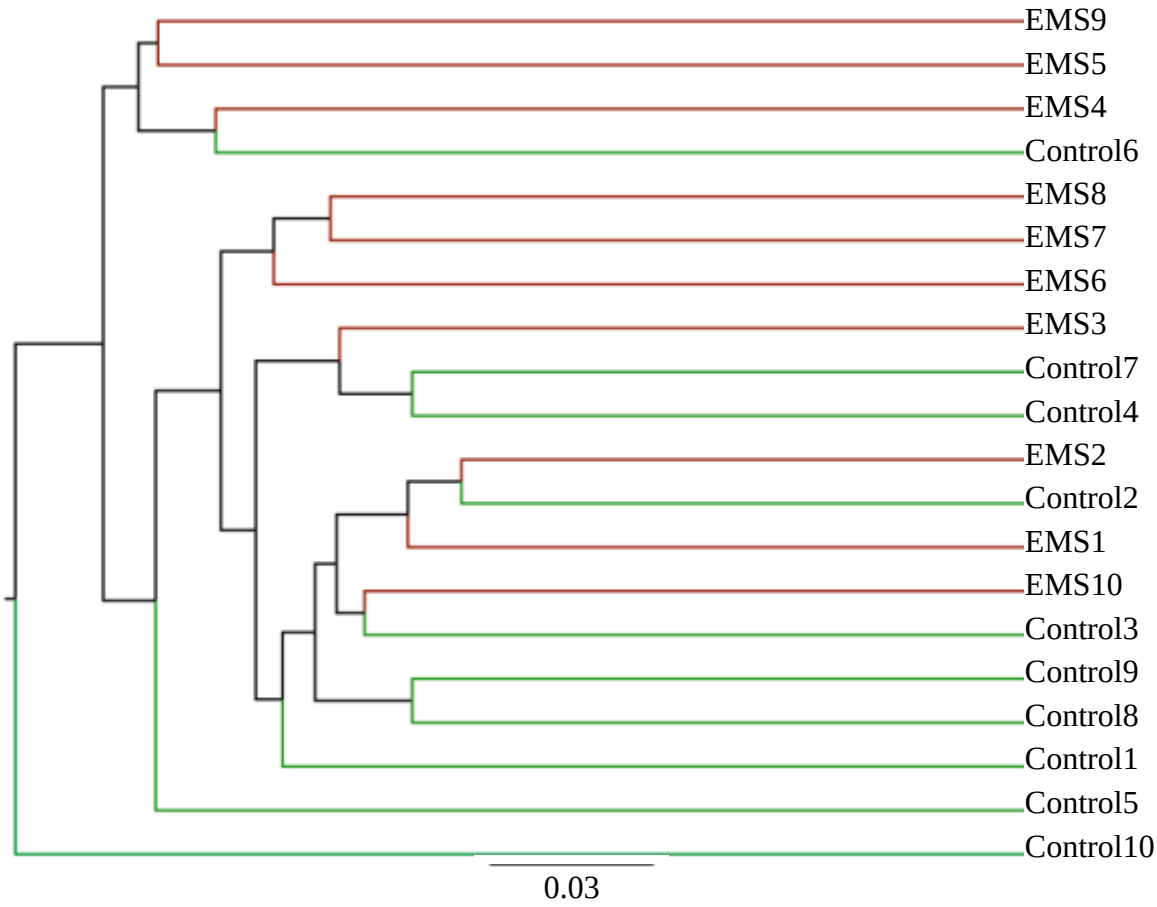

Figure 3.3. Bacterial membership for equine metabolic syndrome (EMS) versus non-EMS control horses. Intestinal microbiota membership as measured by a Jaccard index in n = 10 EMS and n = 10 non-EMS control horses.

No significant differences in relative abundances of different phyla were identified after correction of *P* values for false discovery rate. Similarly, no significant differences remained in lower taxonomic orders after correction of *P* values. Predominant genera are displayed in Figure 3.4. However, 12 OTUs were significantly different based on LEfSe (Figure 3.5). No significant differences in predicted functional capacity of the microbiota were identified by LEfSe analysis of PICRUSt data.

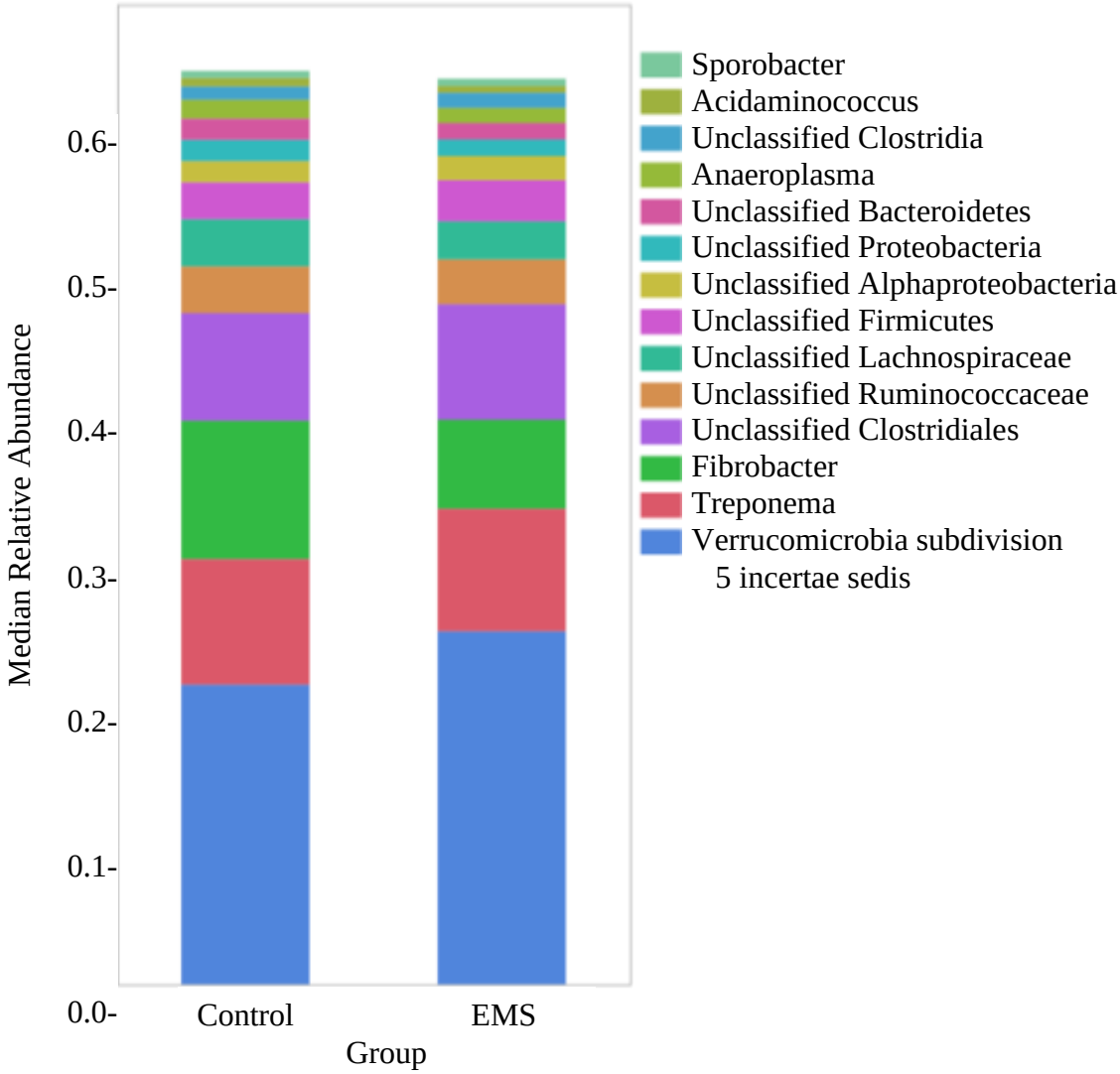

Figure 3.4. Microbiota genera in equine metabolic syndrome (EMS) versus non-EMS control horses. Comparison of the most common genera in the fecal microbiota and their relative abundance in n = 10 EMS and n = 10 non-EMS control horses.

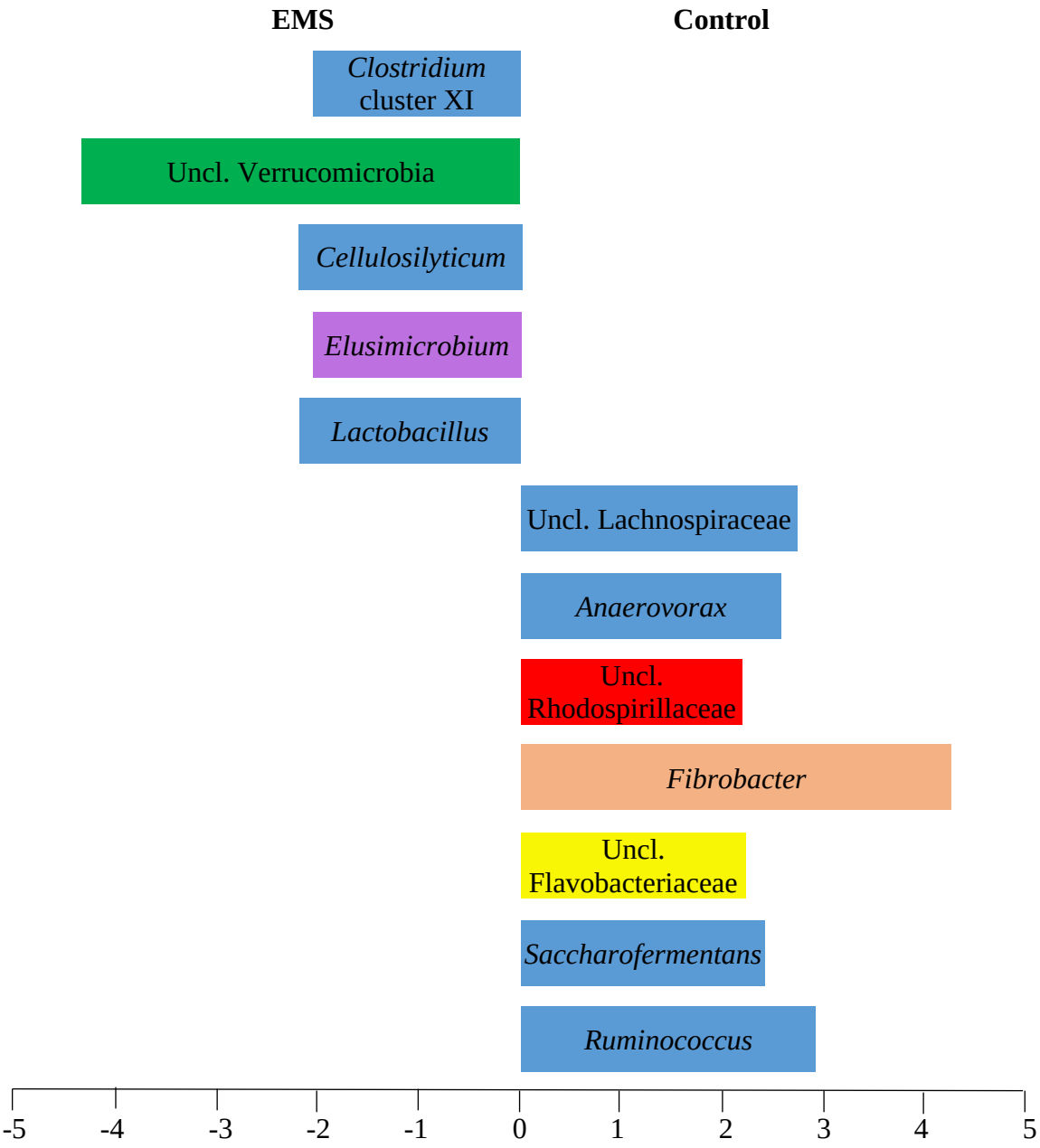

Figure 3.5. Linear discriminate analysis effect size (LEfSe) analysis in EMS versus non-EMS control horses. Over-represented OTU's as identified by LEfSe in n = 10 EMS and n = 10 non-EMS control horses.

3.4. Discussion

As expected, horses with a naturally occurring form of EMS had significantly greater measures of insulin resistance and obesity compared to a matched population of controls, considering these were the criteria used to establish the presence of EMS versus

control. The fecal microbiota of horses with EMS also differed from those of the controls. Although differences in individual taxa were limited, there were changes in community structure, an overall measure of the components of the microbiota, and their relative abundances. There was not a corresponding difference in community membership, although some results did approach significance. A difference in community structure but not membership implies alterations in relative abundances of a relatively conserved microbiota composition, such as might be present when some members are overgrown or depleted in the community but are still present. This suggests that EMS does not result in significant differences in bacteria colonization in the intestinal tract but can have an impact on their relative abundances, resulting in overgrowth or decreased growth of different members.

Despite the differences in community structure, direct comparison of relative abundances did not identify specific taxa that were differentially abundant. Yet numerous differences were identified by LEfSe. Closer evaluation of these groups raises some new questions and hypothesis. The OTU with the highest LDA score in association with EMS horses was an incertae sedis (uncertain classification) member of subdivision 5 of Verrucomicrobia. Verrucomicrobia is an interesting phylum that is getting increasing attention in obesity and metabolic disease. *Akkermansia* is a genus of mucin-degrading bacteria belonging to this phylum that helps maintain the integrity of the mucin layer and decrease inflammation [219]. Increases in this genus have been shown to improve glucose homeostasis in obese mice [220]. Similarly, normalization of *Akkermansia* in obese mice improved metabolic profiles, potentially through crosstalk between microbiota and host [219]. In minipigs with diet-induced obesity, Verrucomicrobia was increased compared with lean counterparts [78], and in humans, *Akkermansia muciniphila* abundance has been inversely associated with fasting glucose, waist-to-hip ratio, and subcutaneous adipose measures [221]. Verrucomicrobia abundance has been suggested as a microbial biomarker for the progression of glucose intolerance based on higher levels in newly diagnosed individuals, versus those with normal glucose control or prediabetes [222]. Therefore, it is possible that the increase in Verrucomicrobia noted here could be an adapted, protective effect of the microbiota in response to MetS- or EMS-associated obesity.

Fibrobacter was overrepresented in the control group. This genus of cellulolytic bacteria is most commonly identified in ruminants and hindgut fermenters, and it presumably plays an important role in digestion in horses. This genus has been identified as a component of the core microbiota in horses [223], with numbers increasing as foals age [224]. Decreased representation of this genus in horses with EMS that were on the same hay-based diet as control horses was interesting. *Fibrobacter* is a relatively acid-intolerant genus that has been reported (along with Ruminococcaceae, which were also associated with controls) to decrease concurrent with increases in lactic acid bacteria after dietary change and intestinal disease [225]. Thus, the association of *Lactobacillus* with EMS horses could be a possible explanation for the underrepresentation of *Fibrobacter* in that group. Although the individual roles of this, and other, members of the microbiota are difficult to assess given the relatively limited research, certain groups tend to be recurrently associated with "gut health." Particular attention is now being paid to Ruminococcaceae and Lachnospiraceae, members of the Clostridiales order [68, 69]. These butyrate-producing bacteria are often associated with intestinal health, with decreases associated with various disease states. For example, recent studies in horses have identified decreases in Clostridiales in horses with colitis [75], and in mares shortly before the onset of colic [76]. In the present study, two Ruminococcaceae (*Ruminococcus*, *Saccharofermentans*) and one Lachnospiraceae were associated with the control group, whereas only one (*Cellulosilyticum*) was associated with EMS horses. These results may provide potential links between alterations in gut microbiota, inflammation, and metabolic dysfunction. However, further work is warranted to explore these connections and to establish if changes in the gut microbiota in EMS horses contribute to inflammation, which in turn leads to insulin and glucose dysregulation or if the reverse is more likely.

A decrease in microbial diversity was also identified in EMS horses. Optimal values for diversity are poorly understood. Increasing diversity probably provides some benefits because the community may have a broader functional potential and greater ability to respond to alterations. However, ultimate diversity (all members of the community are different) is not desirable, and some members of the community should be present in higher levels because they perform important functions (e.g., cellulolytic bacteria). Lower diversity in EMS horses identified here could be of concern as decreased diversity has been

reported in association with obesity in humans [79], dogs [80], and in horses in response to antimicrobial administration [81] and diet change [69].

No differences in predicted functional capacity of the microbiota were identified by PICRUSt. This is perhaps not surprising given the relatively limited taxonomic differences that were noted (as PICRUSt is a predictive model based on taxa) and the small sample size. Further study of microbiota function through larger studies, either through inferred function or more specific identification of functional capacity through shotgun sequencing approaches is needed.

Small sample size is one limitation of this study, and the presence of identifiable differences indicates that broader studies should be undertaken. This study (similar to most microbiota studies) involved feces, not contents from different intestinal locations. Feces have been shown to be a good proxy for colon and to a lesser degree cecum [68], but these data may not accurately reflect the composition of more proximal regions. There are also potential impacts of various factors such as geography, management, diet, age, and a range of other exogenous influences. Although the use of farm-matched controls limits the impact of these on the analysis, it must be remembered that these data might only reflect horses under similar management.

3.5. Conclusion

This preliminary study identified differences in the microbiota of horses with EMS compared with non-EMS control horses. Although the differences were limited, changes that were present could influence health and be associated with some of the clinical characteristics of EMS. Understanding the impact of microbiota alterations and factors that influence these changes may be important for optimizing management of this common syndrome in horses.

CHAPTER 4 PLASMA LIPIDOMIC AND INFLAMMATORY CYTOKINE PROFILES OF HORSES F HORSES WITH EQUINE METABOLIC SYNDROME
Journal of Equine Veterinary Science, 2016: 40, 49-55

Equine metabolic syndrome (EMS) is a growing problem in the equine industry, particularly considering it is a risk factor for the development of laminitis. Equine metabolic syndrome is similar to metabolic syndrome in humans, which is associated with abnormal circulating plasma lipid concentrations. Thus, our objectives were to characterize the plasma lipid profiles, or lipidome, of horses with EMS compared to non-EMS controls and to further characterize the inflammatory state of these horses. Twenty-three horses of mixed breed and sex were selected. Of these, 14 were classified as EMS and 9 as non-EMS controls. Equine metabolic syndrome was determined by insulin resistance, general or regional adiposity, and a history of or predisposition to laminitis. Fasting serum and plasma samples were collected via jugular venipuncture. Serum samples were used to determine insulin, leptin, triglyceride, cholesterol, and nonesterified fatty acid concentrations. Heparinized plasma samples were used to isolate peripheral blood mononuclear cells for inflammatory cytokine determination and ethylenediaminetetraacetic acid plasma to analyze lipidomes. Equine metabolic syndrome horses had increased serum leptin and triglycerides. Plasma lipidomic analysis indicated that EMS horses had elevated triacylglycerides, diacylglycerides, monoacylglycerides, and ceramide compared to control horses. They had lower plasma sphingomyelins, suflatide, and choline plasmalogens. Peripheral blood mononuclear cell analysis for cytokine protein concentration via flow cytometry and gene transcription via real-time polymerase chain reaction showed no differences between the two groups; however, high variability may have influenced results. These data demonstrate that EMS horses have differences in their plasma lipidome compared to controls, similar to what has been observed in humans with metabolic syndrome.

4.1. Introduction

Obesity rates are rising not only in the human population, but in equids as well [2, 3, 7, 49]. Increased adiposity is one of the defining characteristics of equine metabolic syndrome (EMS). Equine metabolic syndrome was classified in 2010 in an ACVIM

consensus statement as insulin resistance, general, or regional adiposity and a history of or predisposition to laminitis [13, 23]. Equine metabolic syndrome is similar to metabolic syndrome (MetS) in humans, which is categorized by three or more of the following: visceral obesity, hypertriglyceridemia, glucose intolerance, low high-density lipoprotein cholesterol, or hypertension [207, 208].

Increased inflammation as well as altered lipid profiles have been associated with obesity in humans and mice [139, 226, 227]. Considering that abnormal lipid profiles may be a contributing factor to altered cell signaling or increased inflammation [139], as well as the influence of certain lipids on insulin resistance [140, 228], it is becoming increasingly important to examine possible connections between plasma lipid profiles, or lipidomes, and metabolic dysfunction. Lipidomic analysis is a relatively new field, but is providing promising new avenues for exploring questions related to metabolic dysfunction [108].

In humans, over 600 diverse molecular species make up the plasma lipidome [229]. It can be categorized into six primary mammalian lipid categories: fatty acyls, prenols, sterols, sphingolipids, glycerophospholipids, and glycerolipids. Changes in lipidome composition may affect human health [229, 230]. Differences in plasma lipidomes have been observed in humans and mice that experience obesity or hypertension. For example, changes in ether lipid or plasmalogen concentrations have been shown to be associated with atherogenic status and hypertension [231-233]. In particular, decreases in plasma ether phosphatidylethanolamines and phosphatidylcholines are indicative of hypertension in middle aged men [230] and patients with coronary artery disease with significant stenosis had a decrease in serum choline plasmalogens compared to patients without significant stenosis [232]. Additionally, differences in lipid composition between individuals who exercise regularly and those who consume a high fat diet have been demonstrated [125].

Research is also indicating a role of the immune response in lipid metabolism. The immune system has been shown to have the ability to recognize lipids as antigens [127]. One notable instance of this is the stimulation of T lymphocytes by CD1 antigen-presenting cells. CD1 cells recognize both foreign and self-lipids, such as phosphoglycerolipids and glycosphingolipids as stimulatory antigens [127-129]. Likewise, elevated levels of certain

lipids have been shown to impact cell signaling, including activation of NF-κB and programmed cell death [234-236].

Work in humans and mice has allowed for analysis of the lipidome in obese, hypertensive, or normal individuals, as well as those with metabolic dysfunction [112, 115]. However, no published work has been done to classify the lipidome of horses with EMS. Existing data in the horse have allowed for characterization of EMS and explored connections to laminitis, but have yielded conflicting results, particularly in regards to inflammation [31]. Some studies have shown differences in inflammatory cytokines such as gene expression of interleukin 1β (IL-1β) and IL-6 in equine nuchal adipose tissue in EMS horses versus controls [41], and an increase in circulating plasma and serum concentrations of tumor necrosis factor alpha (TNF-α) in horses with EMS [28] as well as in ponies with a history of pasture associated laminitis [237]. However, others have shown a trend for circulating plasma TNF-α concentrations to be lower in obese horses compared to controls as well as a decrease in gene expression of IL-1β and IL-6 in the peripheral blood mononuclear cells (PBMCs) of obese horses [31]. Conflicting results may in part stem from the fact that many studies were carried out on ponies or on horses with an induced form of hyperinsulinemia [28, 237]. Not only that, but it is unclear if many were tested for confounding factors, such as pituitary pars intermedia dysfunction (PPID), which can influence insulin/glucose dynamics.

Considering the negative effects of obesity and insulin dysregulation, in particular the increased risk of laminitis in horses, it is important to examine contributing mechanisms to the disorder. Thus, the objective of this study was to investigate and characterize the plasma lipidome as well as the inflammatory status of horses with naturally occurring EMS compared to non-EMS controls.

4.2. Materials and Methods

4.2.1 Horse Selection and Sample Collection

Twenty-three horses of mixed sex and breed were selected from the University of Kentucky's Department of Veterinary Science herd. Of these horses, n = 14 were classified as EMS (mean 13 ± 4 years) and n = 9 were non- EMS controls (mean 13 ± 3 years). There was no significant difference in age between EMS and control horses. Of the horses

classified as EMS, three were Standardbred or Standardbred crosses, three of mixed breed, and three were paints. In addition, one EMS horse was a Morgan, one a draft, one a warmblood cross, one a Walking horse, and one a Thoroughbred. Of the control horses, two were Quarter horses, three were Thoroughbreds, and four were of mixed breed. Ten of the 14 EMS horses were mares and four geldings. Seven of the controls were mares, and the remaining two were geldings. All horses were housed at the University of Kentucky's Main Chance or Woodford farm facilities, maintained on a similar all forage diet of free choice mixed grass hay and minimal pasture, and had access to water and a mineral block ad libitum. Horses were acclimated to their respective pastures for at least 2 months before sampling. All materials and methods were approved by the Institutional Care and Usage Committee of the University of Kentucky.

Equine metabolic syndrome was determined by the following criteria from the 2010 ACVIM consensus statement, insulin dysregulation, general or regional adiposity, and a history of or predisposition to laminitis. To ascertain the presence of insulin dysregulation, an oral sugar test was performed following overnight fasting. In brief, fasting serum sample collection was followed by PO administration of 0.15 mL/kg of Light Corn syrup (Karo; ACH Food Companies, Cordova, TN) and a second serum sample collected 1 hour later [13, 23]. All blood collection was carried out via jugular venipuncture. After centrifugation, aliquots of serum samples were kept at -20^{o}C until analysis. A fasting insulin level of >20 µIU/mL was considered indicative of hyperinsulinemia, and an increased insulin (>60µU/mL) 60 minutes post administration of oral sugar was defined as insulin resistance [13, 23]. The presence of either hyperinsulinemia or insulin resistance was considered positive for insulin dysregulation. Obesity and adiposity were determined by body condition score (BCS) and cresty neck score (CNS). Body condition score was established and averaged between three trained investigators using the 1 to 9 Henneke scoring system [186] with a 1 representing an extremely emaciated animal and a 9 representing an extremely obese animal. Investigators were blinded to which horses were positive for insulin dysregulation. The same trained individual's scores were averaged for CNS via the 0 to 5 system established by Carter et al [187], with a 0 representing no neck crest and a 5 representing a crest so large it permanently droops to one side of the neck. Equine metabolic syndrome was defined as having increased adiposity with a BCS of 6.5

or above or a CNS greater than 2.5. Equine metabolic syndrome horses also had a known history of or predisposition to laminitis. Body condition score and CNS scores, as well as insulin values from the oral sugar challenge are presented in Table 4.1.

Table 4.1. BCS, CNS, basal insulin, and insulin post glycemic challenge in horses with EMS and controls.

Phenotypic measure	Control	EMS	*P* value
BCS	5.9 ± 0.3	6.9 ± 0.2	.025
CNS	2.0 ± 0.3	3.2 ± 0.3	.015
Basal Insulin (uIU/mL)	13.9 ± 2.5	35.5 ± 1.9	<.001
Post Sugar Insulin (uIU/mL)	24.3 ± 6.1	72.2 ± 4.6	<.001

Abbreviation: EMS, equine metabolic syndrome.
Data are presented as least square means, mean ± standard error of the mean. n=9 controls and n=14 EMS. BCS (body condition score) and CNS (cresty neck score) are presented as an average condition score between 3 trained individuals.

All animals were also examined to ensure that none were effected by PPID by conducting thyrotropin-releasing hormone (TRH) stimulation and low-dose dexamethasone suppression testing. TRH stimulation testing was accomplished similar to previously described [191, 192] by taking an aseptic baseline blood sample, administering a 1 mg IV dose of TRH dissolved to 1 mg/mL in 0.9% saline (Sigma-Aldrich, St. Louis, MO) and taking a second sample 10 minutes following TRH injection. Samples were collected in ethylenediaminetetraacetic acid tubes, stored immediately on ice for transport, and centrifugation [193]. Plasma aliquots were removed and then stored at _20^{o} C until shipped on dry ice to Cornell University Animal Health Diagnostic Center's Endocrinology Laboratory. Low-dose dexamethasone suppression testing was performed similar to previously described [194] by taking an afternoon (15:50–18:00 hours) baseline sample, administering 0.04 mg/kg BW dexamethasone bolus IM and taking a second sample 19 hours later. Samples were collected in a gel serum separator tube and frozen at_2^{o} C until cortisol analysis. ACTH concentrations 10 minutes post-TRH injection >100 pg/mL and cortisol concentrations in excess of 1.0 µg/dL 19 hours following dexamethasone injection were considered indicative of PPID and any positive animals excluded. Samples were sent to Cornell University's endocrinology laboratory for determination of insulin, ACTH, and cortisol concentrations. Insulin was measured using the Millipore porcine insulin RIA kit

(EMD Millipore Corporation, Darmstadt, Germany), ACTH using an automated chemiluminescent enzyme immunoassay system (Immulite, Diagnostic Products Corporation, Los Angeles, CA) [195], and cortisol using Siemens Immulite Cortisol kit (Siemens, Washington, D.C.). All samples and phenotypic measures were collected within a period of 2 weeks in the spring of 2014.

4.2.2. Lipid and plasma lipidome analysis

Determination of fasting serum triglyceride, leptin, cholesterol, and nonesterified fatty acid (NEFA) concentrations were accomplished by Cornell University's endocrinology laboratory. Leptin analysis was carried out using the Millipore Multispecies Leptin RIA (EMD Millipore Corporation) and triglycerides, cholesterol, and NEFA determined using colorimetric assays with a Roche ModP chemical analyzer (Roche Diagnostics, Indianapolis, IN). Plasma samples were sent to Dr. Paul Wood at Lincoln Memorial University's metabolomics laboratory for lipidomics analysis by direct infusion electrospray ionization with high resolution (140,000; 0.3–3 ppm mass error) mass spectrometry of a large array of targeted lipid metabolites, followed by tandem mass spectrometry for further validation [238].

4.2.3. Inflammatory Cytokine analysis by Flow Cytometry

Heparinized blood was used to isolate PBMCs by Ficoll–Paque PlusTM (Amersham Biosciences, Piscataway, NJ) gradient centrifugation [197]. For in vitro stimulation, 10^7 PBMC in 1 mL c-RPMI (RPMI-1640; Gibco, Grand Island, NY), with 2.5% fetal equine serum (Sigma–Aldrich), 100 U/ml penicillin/streptomycin (Sigma), and 55 mM 2- mercaptoethanol (Gibco) media was incubated at 37°C, 5% CO_2 with Brefeldin A (10 mg/mL; Sigma) and select wells with the positive control C-RPMI media with phorbol 12- myristate 13-acetate (PMA; 25 ng/mL; Sigma) and ionomycin (1 mM; Sigma) for 4 hours. Following this, determination of cytokine gene expression was carried out by separating aliquots of the cells and placing them in Trizol (Ambion, Austin, TX) to isolate RNA (see below). Remaining cells were isolated for flow cytometry assay for interferon gamma (IFN-γ) and TNF-α production. Cells were fixed in 2% paraformaldehyde (Sigma) and stored overnight at 4°C. After overnight storage, IFN-γ and TNF-α intracellular staining was carried out as previously described [198, 239]. After the cells were stained, aliquots of cells were resuspended in fluorescence-activated cell sorting flow and flow

cytometric acquisition was performed on a FACSCALIBUR (Becton Dickinson, San Jose, CA). Determination of the percent of IFN-γ and TNF-α lymphocytes was accomplished with the use of CELL QUEST (Becton Dickinson).

4.2.4. Inflammatory Cytokine analysis by Real Time-PCR

A modified Trizol method was used to extract total RNA. RNA purity was assessed with a NanoDrop Spectrophotometer using 260/280 and 260/230 ratios of ~2.0 and between 2.0 and 2.2, respectively. One microgram of RNA was reverse transcribed into cDNA, as previously described [198]. In brief, 0.5 µg of each RNA sample and a reverse transcription master mix (Promega, Madison, WI) was incubated for 15 minutes at 42ºC and for 5 minutes at 95ºC. Reactions included 4.5 µl cDNA and 5 µl of SensiMix (Bio-Line, Taunton, MA). Equine-specific, inventoried intron-spanning primers and probes (Life Technologies, Carlsbad, CA) were used with Applied Biosystems (Foster City, CA) real-time polymerase chain reaction (RT-PCR) (ABI 7900HT) against the following genes: housekeeping gene beta-glucuronidase [198, 239] and the following genes: IFN-γ (catalog number 4331182), IL-6 (catalog number 4351372), IL-10 (catalogue number 4331182), TNF-α (catalog number 4331182), and transferrin (catalog number 4351372). Samples were processed in duplicate and incubated for 95ºC for 10 minutes. They then underwent 10 cycles at 95ºC for 15seconds and 60ºC for 60 seconds. Relative changes in gene expression were determined by the $\Delta\Delta C_T$ method [200], with mean ΔC_T of nonpositive controls set as the calibrator for all samples. Relative quantity, or RQ, was calculated as $2^{-\Delta\Delta CT}$ and used to express results.

4.2.5. Data Analysis

Data were analyzed by SIGMAPLOT version 12.3 (Systat Software Inc., San Jose, CA). A mixed model two-way ANOVA was used for each quantitative variable (insulin, triglyceride concentrations, and so forth). Fixed effects were set as EMS versus control and age. Data that were not normally distributed as determined by a Shapiro–Wilk test were log-transformed and resulted in normality. Data were considered statistically significant when $P \leq .05$. Any outliers identified by a modified Thompson tau technique was removed before statistical analysis.

4.3. Results

4.3.1. Lipid and lipidomics analysis

Triglyceride and leptin concentrations were significantly higher in EMS horses compared to controls (Table 4.2). Although there were differences between the two groups in triglycerides, only two of the horses (both EMS) fell outside the 0.16 to 0.87 mmol/L normal reference range. Cholesterol and NEFA concentrations did not differ between the two groups (Table 4.2).

Table 4.2. Serum triglycerides, leptin, cholesterol, and NEFA in horses with EMS and controls.

Biomarker	Control	EMS	*P* value
Triglycerides (mmol/L)	0.32 ± 0.06	0.62 ± 0.04	.002
Leptin (ng/mL)	3.4 ± 1.1	7.5 ± 0.8	.012
Cholesterol (mg/dL)	91.5 ± 3.8	91.6 ± 2.9	.975
NEFA (mEq/L)	0.15 ± 0.02	0.15 ± 0.01	.891

Abbreviation: EMS, equine metabolic syndrome; NEFA, nonesterified fatty acid.
Data are presented as least square means, mean ± standard error of the mean, n=9 controls and n=14 EMS.

Lipidomics analysis revealed that EMS horses had significantly increased plasma triacylglycerides, diacylglycerides, monoacylglycerides, and ceramide compared to controls. They also had significantly lower plasma sphingomyelins, suflatide, and choline ether lipids/plasmalogens (Figure 4.1 and Figure 4.2). No differences were seen between the two groups for ethanolamine plasmalogens (data not shown).

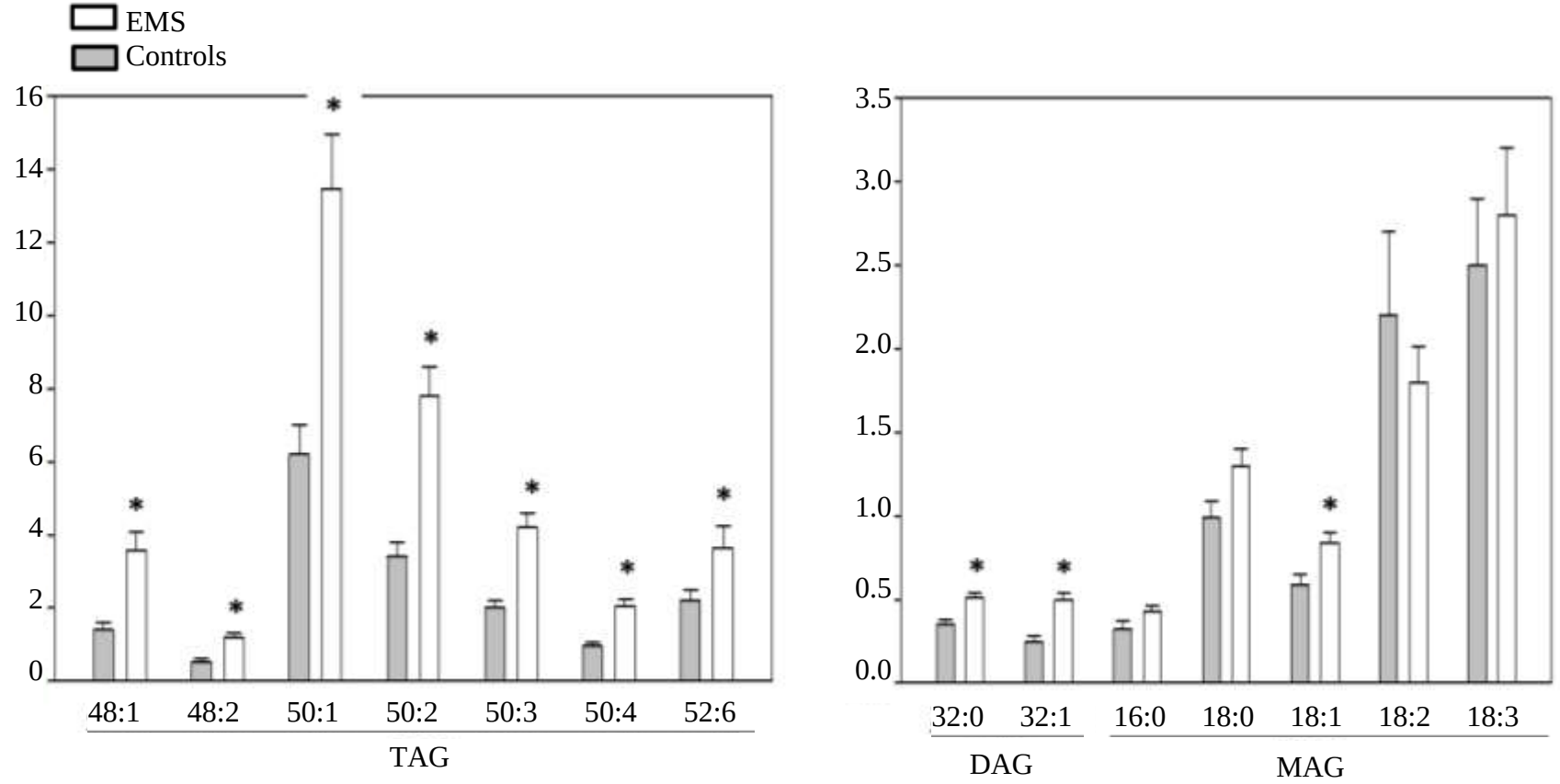

Figure 4.1. Plasma triacylglyceride (TAG), diacylglyceride (DAG), and monoacylglyceride (MAG) levels. Values are presented as the lipid ratio to an internal standard ± SEM * indicates significant ($p<0.05$) differences between EMS and controls. EMS, equine metabolic syndrome; SEM, standard error of the mean.

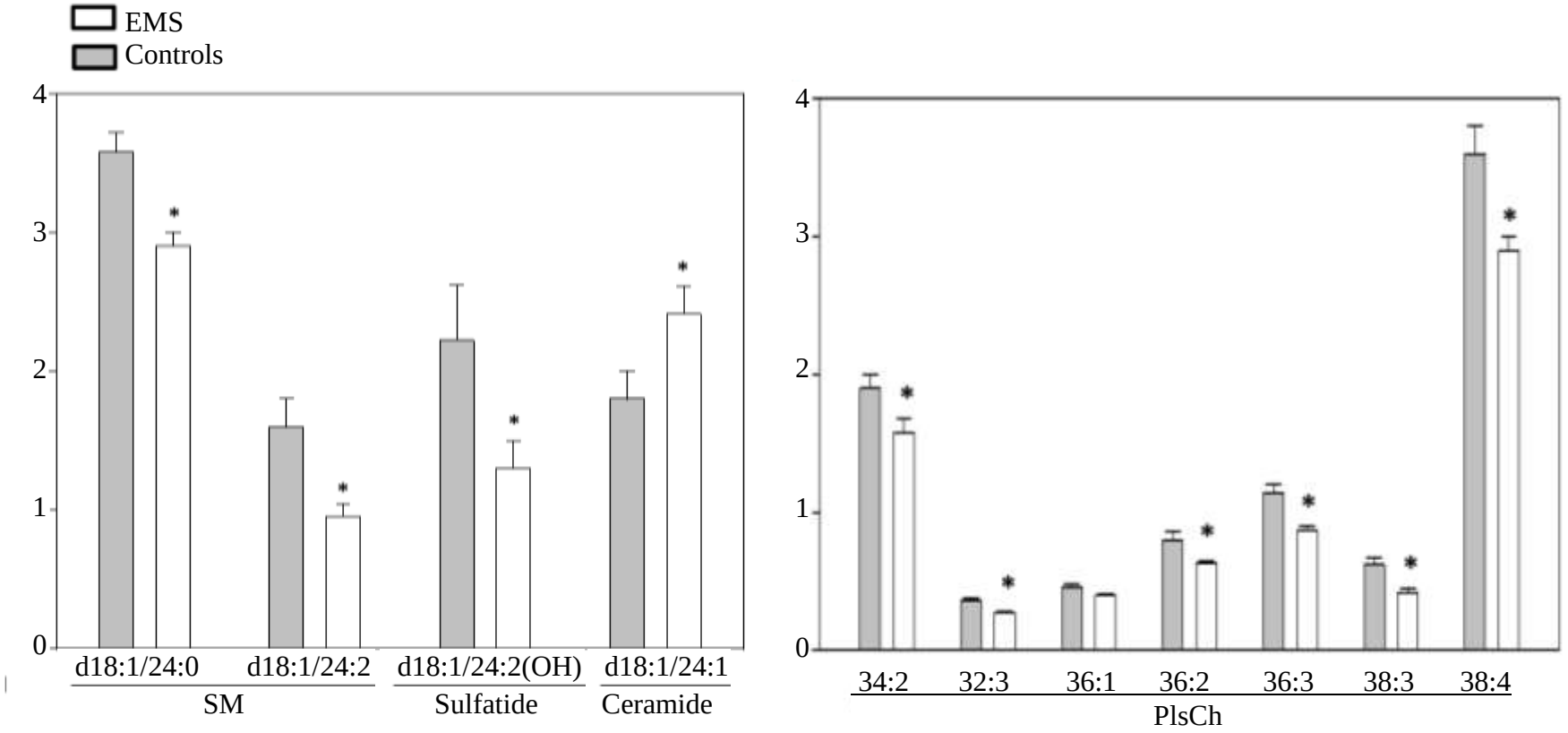

Figure 4.2. Plasma sphingomyelin (SM), sulfatide, ceramide, and choline plasmalogen (PlsCh) levels. Values are presented as the lipid ratio to an internal standard ± SEM * indicates significant ($p<0.05$) differences between EMS and controls. EMS, equine metabolic syndrome; SEM, standard error of the mean.

4.3.2. Inflammatory Cytokine analysis

No differences were observed for any of the PBMC values, either for intracellular protein production analyzed via flow cytometry or for gene expression analyzed via RTPCR (Table 4.3). However, high variability and low power may have influenced results. In addition, there was an observed trend for IL-6 and TNF-α RQs, with the EMS horses lower compared to controls.

Table 4.3. Flow cytometry and RT-PCR data in horses with EMS and controls.

Inflammatory marker	EMS	Control	*P* value
IFN % gated	8.7 + 2.3	12.8 + 1.8	.208
IFN geo mean	23.2 + 4.0	24.0 + 5.3	.911
TNF % gated	20.7 + 4.2	20.9 + 4.9	.978
TNF geo mean	19.1 + 2.2	17.3 + 3.0	.657
IFN RQ [a]	2.8 + 0.1	3.1 + 0.2	.251
IL10 RQ	10.7 + 4.1	16.2 + 5.2	.441
IL6 RQ	7.0 + 0.9	9.8 + 1.1	.076
TNF RQ	19.1 + 3.8	31.0 + 5.1	.098
Transferrin RQ	2.2 + 0.8	4.6 + 1.0	.101

Abbreviations: EMS, equine metabolic syndrome; IFN, interferon; IL, interleukin; RT-PCR, real-time polymerase chain reaction; RQ, relative quantity; TNF, tumor necrosis factor.

Data are presented as least square means, mean ± standard error of the mean, n=9 controls and n=14 EMS.

[a] Indicates log transformed data.

4.4. Discussion

This study used a group of horses with a naturally occurring form of hyperinsulinemia. As expected and similar to humans with MetS, EMS horses had an increase in serum triglycerides and leptin, as well as differences in lipid profiles compared to controls. These differences, in the plasma lipidome in particular, may have implications on cell signaling, membrane fluidity, and inflammation.

Cell signaling, including activation of inflammatory pathways in multiple cell types (such as macrophages, adipocytes, and hepatocytes), and gene expression of proteins involved in metabolism and inflammation (such as SOCS-3 and MCP-1) have been shown in humans and mice to be influenced by many different factors, including changes in circulating lipids [112-114]. Increases in these lipids appear to affect not only inflammation, but insulin

resistance as well [114, 228, 240]. In addition to influencing inflammation and insulin signaling, bioactive lipids such as ceramide and other sphingolipids can act as intracellular messengers and chemoattractants [118, 119], which may contribute to or exacerbate alterations in insulin dysregulation or inflammation. Further evidence of the role of the lipidome in metabolic dysfunction is demonstrated considering that nutritional modification can cause changes in the plasma lipidome, which can result in a decrease in markers of inflammation and increase in insulin sensitivity [121, 122].

Research has also compared circulating plasma lipidomes with tissue lipid profiles, finding that serum lipids correlate best with those lipids in the liver [208]. This same study also observed an increase in ceramide in subcutaneous adipose tissue of humans with fatty liver disease as well as in skeletal muscle of obese insulin insensitive individuals. Indeed, changes in ceramide concentrations in particular seem to play a major role in cell signaling and metabolism [113, 115, 116]. Therefore, increased levels of triacylglycerides, diacylglycerides, monoacylglycerides, and ceramide in EMS horses point to possible alterations in normal cell signaling and contribute to a viscous cycle of insulin resistance and lipid dysregulation in these animals. However, further research is warranted to understand the relationship between the lipidome and cell signaling in the horse.

Lipids play a vital role in almost all cell types' membrane health and function [109], particularly sphingomyelin and plasmalogens. Cell membrane health and function has direct implications on cell signaling, including influencing voltage-gated ion channels [110, 111]. Equine metabolic syndrome horses had decreased levels of these critical membrane lipids, which may point to changes in membrane fluidity and possibly impact membrane permeability [109-111]. However, results from this work are limited to circulating plasma, and more work is needed to understand the potential sources and influences of and on these lipids.

Not only do lipids impact cell signaling and cellular membrane health and function, there is considerable data linking lipids to immune function in humans and mice. For example, both foreign lipids such as KOD2-lipid A from Escherichia coli [126], and self-lipids such as gangliosides and glycosphingolipids [127-129] can be recognized as antigens. Not only that, but lipids can act a chemoattractants for lymphocytes [118]

Although there were differences in plasma lipidomes between EMS and control horses, there were no significant differences in markers of inflammation measured in PBMCs. However, high levels of variability, in particular with regard to flow cytometry data, may have masked differences. It is possible that a larger sample size is required to detect these differences. It is also important to note that these results are based on PBMCs stimulated with the mitogen PMA, and a different mitogen may yield different results. Regardless, EMS animals had numerically lower values for all RT-PCR results and a trend for lower IL-6 and TNF-α RQs which could point to changes in cell-mediated immunity. These trends may suggest that EMS horses have reduced systemic inflammation compared to controls. However, obese humans and mice have been shown to have decreased cell-mediated immunity compared to lean individuals [134-137], and some studies in obese insulin resistant horses have shown increases in inflammatory cytokines compared to controls [28, 41]. Considering this, as well as the fact that inflammatory data are from stimulated PBMC cultures which are typically more representative of cellular versus systemic responses, it is more likely that the trend for reduction in IL-6 and TNF-α RQs is due to reduced cell-mediated immunity as opposed to a reduction in systemic inflammation.

It is important to note that work in categorizing the inflammatory state of the obese insulin resistant horse has been mixed. Some report an increase in gene expression of IL-1β and IL-6 in equine nuchal adipose tissue in EMS horses versus controls [41] and others an increase in circulating plasma or serum concentrations of TNF-α in hoses with EMS [28] as well as in ponies with a history of pasture-associated laminitis [237]. Correlations in the horse have likewise been made between insulin sensitivity, obesity, and inflammatory cytokines where insulin sensitivity decreases with increasing obesity [8]. Also associated with increasing obesity and decreased insulin sensitivity has been elevated gene expression of TNF-α, IL- 1, and TNF-p, but no change in or decreasing IL-6 [8, 28]. However, these changes in inflammation, such as increasing TNF-α and decreasing IL-6, were in some instances age dependent [8]. Changes in inflammation have been more frequently observed in adipose tissue compared to circulation and often are only observed at the gene expression level [8, 39, 41]. Equine metabolic syndrome horses have also been observed to have an increase in macrophage and lymphocyte infiltration in adipocytes, as

well as increased adipocyte diameter [28]. In whole blood when increases in inflammation were observed in response to endotoxin administration, EMS and control horses both had an equivalent increase in gene expression of certain cytokines, however, the elevated expression of some, namely IL-6, IL-8, IL-10, and TNF-α persisted for a longer period of time in the EMS horses compared to controls [30].

Still, more research is needed in this area to determine true differences in inflammation in horses with naturally occurring EMS. Future work is also warranted to examine different cell types and tissues to further determine the presence inflammation as well as the presence of and effect from changes in lipid profiles. In particular, serological markers of inflammation should be examined to detect possible levels of low-grade systemic inflammation, similar to what is observed in humans with MetS.

4.5. Conclusion

These data indicate that horses with EMS have abnormal lipidomes compared to non-EMS control horses. This may have implications on cell membrane fluidity and cell signaling. Data support the idea that horses with metabolic dysfunction and obesity have altered lipidomes, similar to humans with MetS. These results allow for a more targeted exploration of mechanisms behind EMS, including changes in lipidomes as contributing factors to the disorder.

CHAPTER 5 DO HORSES WITH EQUINE METABOLIC SYNDROME (EMS) HAVE REDUCED IMMUNE RESPONSES TO VACCINATION?

Obesity is an increasing problem in the equine population with recent reports indicating that the percentage of overweight horses may range anywhere from 20.6-51%. Obesity in horses has been connected to more serious health concerns such as equine metabolic syndrome (EMS). The potentially devastating condition laminitis, insulin dysregulation (ID), and obesity are the three main characteristics of EMS, making this syndrome a serious problem in the equine industry. Ongoing and past research has been focused on better characterizing and understanding mechanisms responsible for EMS. However, little research has been conducted to determine the effects of EMS on routine healthcare of these horses, in particular how they respond to vaccination. It has been shown that obese humans and mice have decreased immune responses to vaccination. EMS may have similar effects on vaccine responses in horses. If this is the case, these animals may be more susceptible to disease, acting as unknown disease reservoirs. Therefore, we investigated the effects of EMS on immune responses to routine influenza vaccination. Twenty-five adult horses of mixed-sex and mixed-breed (8-21 years old) horses; 13 EMS and 12 non-EMS were selected. Within each group, 4 horses served as non-vaccinate saline controls and the remaining horses were vaccinated with a commercially available equine influenza vaccine. Vaccination (influenza or saline) was administered on weeks 0 and 3, and peripheral blood samples taken on week 0 prior to vaccination and on weeks 1, 2, 3, 4, and 5 post vaccination. Blood samples were used to measure hemagglutination inhibition (HI) titers and equine influenza specific IgGa, IgGb, and IgGT levels. Blood samples were also used to isolate peripheral blood mononuclear cells (PBMCs) for analysis of cell mediated immune (CMI) responses via real-time polymerase chain reaction (RT-PCR). All horses receiving influenza vaccination responded with significant increases ($P < 0.05$) in HI titers, and IgGa and IgGb EIV specific antibodies following vaccination compared to saline controls. EMS did not significantly affect ($P > 0.05$) humoral immune responses as measured by HI titers or IgG antibody isotypes to influenza vaccination. There was an effect of metabolic status on CMI responses, with influenza vaccinated EMS horses having lower gene expression of IFN-γ ($P = 0.02$) and IL-2 ($P = 0.01$) compared to vaccinated non-EMS control horses. Given these results, it appears that while metabolic status does

not influence humoral responses to an inactivated influenza vaccine in horses, horses with EMS appear to have a reduced CMI response to vaccination compared to metabolically normal, non-EMS control horses.

5.1. Introduction

An obesity epidemic is emerging in the horse population, with reports of 45% of 319 randomly selected horses classified as "fat" or "very fat" [7]. Still others have reported that of 300 horses, 54% were overweight or obese [3, 49]. Of these overweight or obese horses, 18% were hyperinsulinemic compared to only 1.4% of lean horses. While there is information regarding the number of horses affected by obesity, the prevalence of metabolic dysfunction in the horse is unknown. Both obesity and metabolic dysfunction are considered risk factors for the development of laminitis in the horse [14, 15]. All three of which (obesity, metabolic dysfunction, and laminitis) are components of equine metabolic syndrome (EMS).

EMS is closely related to MetS (metabolic syndrome) in humans. Associated characteristics of MetS include: adipose tissue dysfunction, insulin resistance, inflammation, and hypertension [207, 208]. Equine metabolic syndrome was defined in a 2010 ACVIM consensus statement [23]. The statement ascribes three major characteristics to horses with EMS; general or regional (fatty deposits around the neck, tail head, behind the shoulder, or in the mammary gland region) adiposity, an abnormal glycemic or insulinemic response upon challenge, and a history of or predisposition to laminitis. Hypertriglyceridemia, dyslipidemia, hyperleptinemia, arterial hypertension, abnormal reproductive cycling in mares, and an increase in systemic makers of inflammation associated with obesity are also considered possible hallmarks of EMS [23].

Considering correlations between obesity, insulin resistance, systemic inflammation, and the immune response [31, 130, 131], it begs the question; is there an effect of obesity and metabolic dysfunction on the immune response to vaccination in horses? Work in naïve mice has indicated that obesity significantly reduces antibody responses and antibody neutralizing capability to influenza vaccination [134]. In response to a hepatitis B vaccine, diet-induced obese mice exhibited an increased proliferation of T and B cells, increased levels of IFN-γ and TNF-α, but decreased levels of hepatitis specific

antibody and hepatitis specific T cell activation [135]. Type 1 diabetes in adult humans has also been associated with a risk for non-responsiveness to vaccination [136], and obesity is considered a risk factor for low levels of circulating antibody >3ys post hepatitis vaccination [137]. A human influenza vaccination study [138] positively correlated body mass index with higher initial IgG antibody responses, however 12 months following vaccination a greater body mass index was correlated with a greater decline in influenza antibody titers. In vivo, investigators also found that obese individuals had lower expression of functional proteins and decreased activation of their $CD8^+$ T-cells. Additionally, both adult and elderly obese individuals had impaired B cell function in response to influenza vaccination [241] and obese adult human PBMCs upon viral challenge with influenza, had a lower production of IFN-γ by their γδ T cells compared to lean controls [242]. In spite the evidence in humans and mice linking increased adiposity to an impaired immune response to vaccination, there has been no work to date regarding response to vaccination in the EMS horse.

Therefore, this study was designed to compare humoral and cell-mediated immune (CMI) responses to influenza vaccination of EMS and non-EMS horses, with the hypothesis that EMS horses would have a decreased immune response to vaccination compared to metabolically normal controls.

5.2. Materials and Methods

Horses of mixed-breed and mixed-sex (ranging in age from 8-21 years old) were utilized from the University of Kentucky's existing Department of Veterinary Science Main Chance herd. Criteria to determine EMS was as previously described; insulin resistance, regional or general adiposity, and a predisposition to or history of laminitis [13, 23]. Two weeks prior to the start of the study, EMS and non-EMS horses were screened for insulin resistance, existing HI influenza antibody titers, and to insure that they did not have Pituitary Pars Intermedia Dysfunction (PPID). PPID determination was established using both dexamethasone suppression and Thyroid Releasing Hormone (TRH) testing as previously described [191-194]. This was deemed especially important by investigators, as horses with PPID may have similar metabolic dysfunction as those with EMS [11]. Horses

were housed in a dry lot with ad libitum access to water, trace mineralized salt blocks, and a mixed-grass hay.

5.2.1. Phenotype determination

A portable agriculture scale (model 700, Tru Test Inc., Mineral Wells, TX) was used to determine body weight. General adiposity was measured by body condition scoring (BCS). BCS was established by three trained investigators using the 1Henneke scoring system [186]. The same trained individuals also performed cresty neck scoring (CNS) on horses with the 0-5 scale system established by Carter et al. [187] to measure regional adiposity. To ascertain the presence of insulin resistance, an oral sugar test was performed; following overnight fasting and 0800-1100 EST serum sample collection, 0.15 ml/kg of Karo Light Corn syrup was administered orally, and a second serum sample collected 60 min later [13, 23]. Serum samples were kept at -20º C until sent on dry ice to Cornell University's endocrinology lab for insulin determination. A fasting insulin level of >20 μIU/mL was considered indicative of hyperinsulinemia, and an increased insulin (>60μU/mL) 60 minutes post administration of oral sugar classified as insulin resistance [13, 23]. Horses were pre-screened and tested via HI as described below to establish IgG antibody levels specific to influenza. All animal methods were approved by the University of Kentucky's IACUC (institutional animal care and use committee).

5.2.2. Vaccination and Sample Collection

Thirteen EMS and 12 non-EMS horses were used in this study. Within each category (EMS vs non-EMS controls) horses were blocked by pre-existing antibody titers (HI titers) and systematically allocated to one of the following treatment groups: 1) vaccinate (Fluvac Innovator^{TM}, Zoetis, vaccine containing KY/97) (n=8 non-EMS control horses or n=9 EMS horses) or 2) saline controls (n=8, 4 horses within each metabolic group). There was no significant difference in median age between vaccine group vs saline (P = 0.55, vaccine group median age 11 years; IQR 9.5 – 16 years, and saline median 13 years; IQR 11.3 – 16.3 years), Nor was there a significant difference in median age between EMS group vs non-EMS control horses (P = 0.4, EMS group median age 13 years; IQR 10 – 18 years vs non-EMS control group median 12.5 years; IQR 9.3 – 14.8 years).

Blood collection was carried out via jugular venipuncture for all time points. For baseline and phenotypic determination, serum and plasma sample collection was

performed following fasting on day -14. As some horses had an unknown vaccine history as well as to determine if a second vaccination would result in additional effect on immune responses measured, horses received an inactivated influenza vaccination or saline at both weeks 0 and 3. Peripheral serum and heparinized blood samples were taken on week 0 prior to vaccination and on weeks 1, 2, 3, 4, and 5 post vaccination. Throughout the study horses were monitored for vaccine site reactions.

5.2.3. Equine Influenza Virus (EIV)-Specific Antibody Assays

5.2.3.1. HI assay

Humoral immune total IgG responses were determined using serum samples from all sampling time points by HI (hemagglutination inhibition) assay with ether treated KY/97 equine influenza virus, as previously described [243].

5.2.3.2. ELISA assay

Serum samples were also used as previously described [197] in a modified ELISA procedure to determine influenza specific antibody isotype responses to vaccination. The assay was carried out as follows: purified influenza virus (Eq/KY/97; 10 HA units per well) was used to coat a 96-well polystyrene plate (IMMULON®, Dynatech Laboratories Inc., Chantilly, VA) and incubated at 4°C overnight. The next morning, plates were washed (PBS/0.05% TWEEN®-20 (PBS-T; Sigma, St. Louis, MO)) and blocked (2% non-fat dried milk powder in PBS-T (Sigma)) at 37°C for 1 hr. Serum samples from an influenza virus hyperimmune horse were serial diluted and used to create a standard curve. Experimental serum samples were diluted in a volume of PBS-T to ensure that OD values were within the standard curve and plates were incubated at 37°C for 90 min. Plated were washed and incubated at 37°C for 1 hr with IgGa (CVS 40), IgGb (CVS 39), and IgG(T) (CVS 48) specific monoclonal antibodies. After being washed again, an incubation at 37°C for 1 hr took place with horseradish peroxidase-conjugated goat-anti-mouse IgG antibody (Jackson Laboratories Inc., West Grove, PA). Following the incubation, plates were washed and a substrate (KPL, Gaithersburg, MD) used for development. Development was stopped by the addition of a stop solution (KPL). A 450 nm optical density was determined with the use of an ELISA reader (BioRAD, Hercules, CA). Serum antibody concentrations relative to the standard curve were calculated and are represented as ELISA units.

5.2.4. EIV-specific CMI Assays

5.2.4.1. Peripheral blood processing

Heparinized blood was used to isolate peripheral mononuclear cells (PBMCs) by Ficoll-Paque PlusTM (Amersham Biosciences, Piscataway, NJ) gradient centrifugation [197]. For EIV *in vitro* stimulation, aliquots of 10^7 PBMC were plated in 1 ml c-RPMI [RPMI-1640 (Gibco, Grand Island, NY); supplemented with 2.5% fetal equine serum (FES; Sigma–Aldrich, St. Louis, MO), 100 U/ml penicillin/streptomycin (Sigma), and 55 mM 2-mercaptoethanol (Gibco) media. Select aliquots were incubated at 37 °C, 5% CO_2 for 45 min with influenza virus homologous strain used in the vaccine (KY/Eq/97). After incubation all flu simulated cells were pelleted by centrifugation at 500 × *g* for 5 min to remove the virus inoculum. All virus-stimulated and unstimulated PBMC were resuspended with c-RPMI. Brefeldin A (10 mg/ml; Sigma) was added to all wells. For all time points and horses an addition well was (non-flu stimulated) was stimulated with phorbol 12-myristate 13-acetate (PMA; 25 ng/ml; Sigma) and ionomycin (1 mM; Sigma) to serve as a positive control. Cells were incubated for an additional 4 hrs. Following this, determination of cytokine gene expression was carried out by separating an aliquot of the cells and placing them into Trizol (Ambion) to isolate RNA (see below).

5.2.4.2. Real Time-PCR

A modified Trizol method was used to extract total RNA. One µg of RNA was reverse transcribed into cDNA, as previously described [197]. In brief; 1 µg of each RNA sample and a reverse transcription master mix (Promega, Madison, WI) was incubated for 15 min at 42ºC and for 5 min at 95ºC. Reactions included 5µl cDNA and 20µl of master mix. Master mix included; 6.25µl nuclease-free water (Qiagen), 1.25µl primer/probe 20X assay mix (Applied Biosystems, Foster City, CA), and 12.5µl TaqMan™ (Applied Biosystems). Equine-specific, intron-spanning primers and probes were used with Applied Biosystems Real-Time PCR (ABI 7900HT) against the following genes: Housekeeping gene *beta*-glucuronidase (β-gus) and CMI induced cytokine genes; interferon gamma (IFN-γ), GrzB, and Perforin [244]. Samples were processed in duplicate and incubated for 95ºC for 10 min. They then underwent 10 cycles of 95ºC for 15s and 60ºC for 60s. Relative changes in gene expression were determined by the $\Delta\Delta C_T$ method [200], with mean ΔC_T

for week 0 from all horses set as the calibrator for all samples. Relative quantity, or RQ, was calculated as $2^{-\Delta\Delta CT}$ and was used to express results.

5.2.5. Statistical analysis

Data were analyzed utilizing SAS, version 9.4 (SAS Institute Inc., Cary, NC, USA). Data were analyzed with proc mixed with the repeated measure set at time and subject as horse ID. Horse ID, metabolic status, vaccination vs saline, and time point (week) were set as fixed effects and all possible interactions analyzed. A first-order autoregressive variance/covariance structure was used the statistical model. Results are expressed as least square means ± standard error of the mean (SEM). Normality was assessed using studentized residuals with a visual analysis as well as skew (>2) and kurtosis (>7). Non-normal data were natural log transformed and resulted in normality.

5.3. Results

Phenotypic data, including OST results used for EMS determination are presented in Table 5.1. As expected, EMS horses had a greater CNS ($P = 0.002$), and insulin (both pre ($P = 0.003$) and post ($P < 0.001$) oral sugar administration) compared to non-EMS control horses. However, EMS and non-EMS control horses did not different in BCS ($P = 0.227$) or weight ($P = 0.514$).

Table 5.1. Phenotypic and endocrine data for EMS and non-EMS horses.

Phenotypic measure	EMS horses	Non-EMS horses
Basal Insulin (µIU/ml)	37.4 ± 4.7 [b]	13.3 ± 5.4 [a]
Insulin 60 min post oral sugar administration (µIU/ml)	81.6 ± 5.8 [b]	23.8 ± 6.5 [a]
BCS	6.9 ± 0.3	6.6 ± 0.2
CNS	3.0 ± 0.3 [b]	1.6 ± 0.2 [a]
Weight (kg)	588.1 ± 22.1	604.4 ± 18.6

Phenotypic measures and endocrine data in EMS (n=13) versus non-EMS (n=12) horses. Results are presented as least square means (± SEM). Within a row, differences ($P < 0.05$) are represented by differing superscripts.

5.3.1. Antibody responses

All horses that received influenza vaccination had a significant antibody response to vaccination as compared to those given saline ($P < 0.05$) measured by HI titers against KY/97 (Figure 5.1). Horses given saline had lower HI titers compared to vaccinated horses

for all weeks post vaccination. Vaccinated horses at week 0 had lower HI titers compare to vaccinated horses at all other time points, whereas saline horses did not differ over time. Vaccinated horses at week 4 had higher HI titers compared weeks 2, 3, and 5. There was not effect of metabolic status on HI titers in response to vaccination.

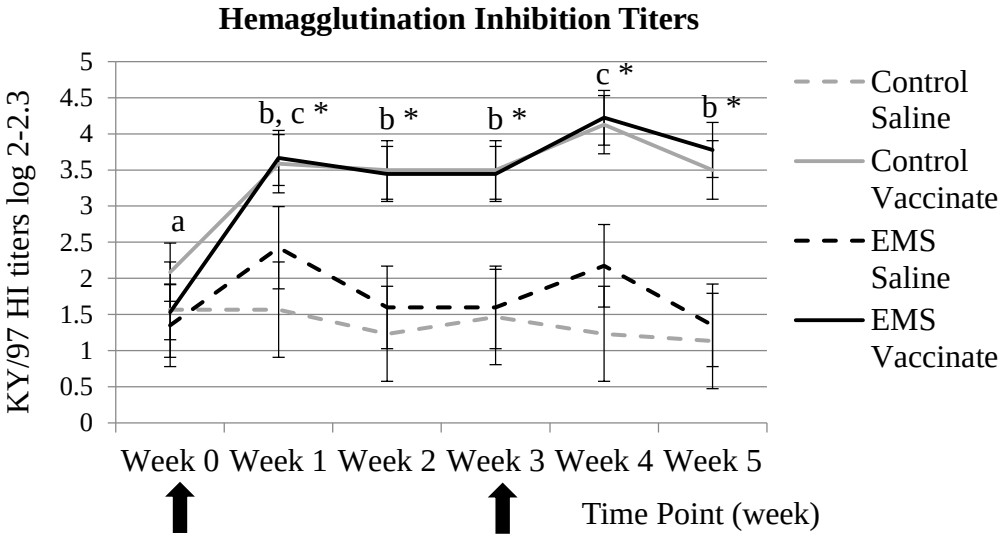

Figure 5.1. HI titers over time in vaccinated EMS horses (n = 9), vaccinated control horses (n = 8), EMS horses given saline (n = 4), and control horses given saline (n = 4). Results are presented as least square means (± SEM). Differences ($P < 0.05$) within time point between all vaccinated horses and all given saline are represented by *. Within all vaccinated horses differences between time points are represented by differing alphabetic superscripts. There were no significant differences over time point within all horses given saline. Arrows represent time points of vaccination or saline administration.

IgGa isotype flu specific antibody responses are represented in Figure 5.2. All vaccinated horses responded to vaccination, with higher levels of IgGa compared to horses given saline at all weeks post vaccination (Figure 5.2). Vaccinated horses were lower at week 0 compared to all other weeks. In addition, vaccinated horses were lower at week 3 compared to weeks 1, 2, 4, and 5. Again, there was no effect of metabolic status on flu specific IgGa isotype antibody responses.

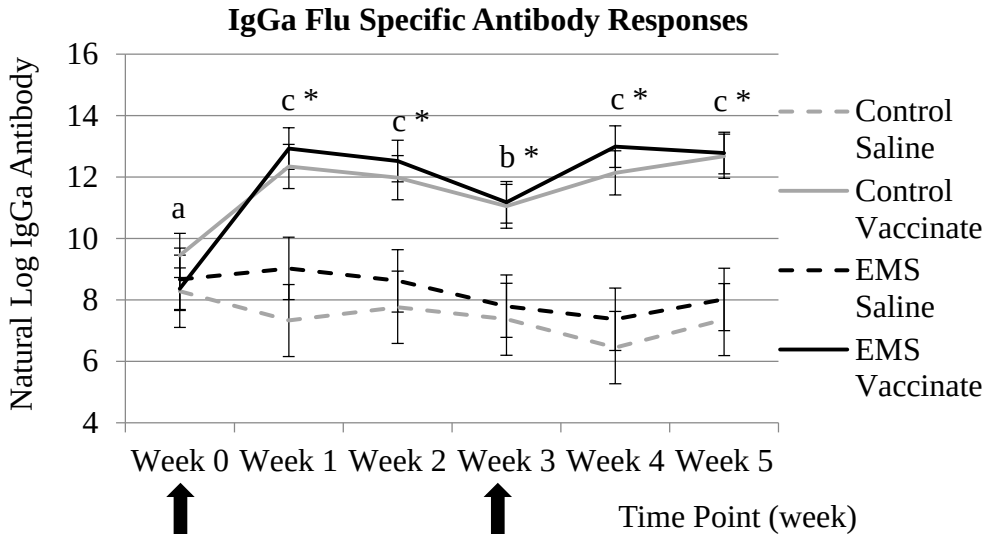

Figure 5.2. IgGa isotype flu specific antibody responses over time in vaccinated EMS horses (n = 9), vaccinated control horses (n = 8), EMS horses given saline (n = 4), and control horses given saline (n = 4). Results are presented as least square means (± SEM). Differences ($P < 0.05$) within time point between all vaccinated horses and all given saline are represented by *. Within all vaccinated horses differences between time points are represented by differing alphabetic superscripts. There were no significant differences over time point within all horses given saline. Arrows represent time points of vaccination or saline administration.

Similar to HI and IgGa antibody results and regardless of metabolic status, all vaccinated horses responded to vaccination in respect to their flu specific IgGb isotype antibody responses as compared to horses given saline (Figure 5.3). Horses given saline were lower in IgGb antibody compared to vaccinated horses for all weeks following vaccination, with the exception of week 1. Vaccinated horses were lower at week 0 compared to all other weeks.

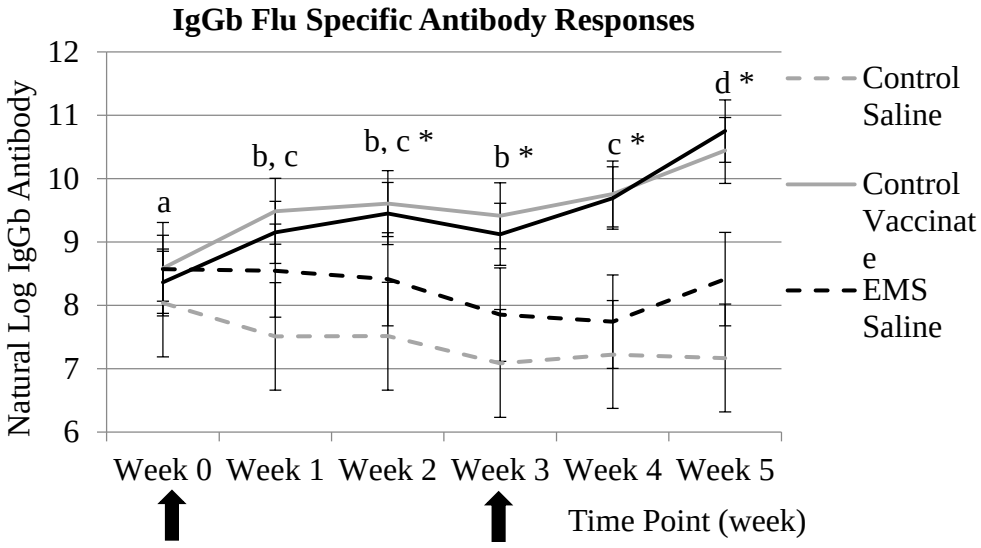

Figure 5.3. IgGb isotype flu specific antibody responses over time in vaccinated EMS horses (n = 9), vaccinated control horses (n = 8), EMS horses given saline (n = 4), and control horses given saline (n = 4). Results are presented as least square means (± SEM). Differences ($P < 0.05$) between all vaccinated horses and all given saline are represented by *. Within all vaccinated horses differences between time points are represented by differing alphabetic superscripts. There were no significant differences over time point within all horses given saline. Arrows represent time points of vaccination or saline administration.

There were no significant differences for time point, vaccine versus saline, or metabolic status, nor were there any significant interactions for IgGT isotype flu specific antibodies (data not shown). For all measures of humoral responses to vaccination, with the exception of IgGT isotype responses, vaccinated horses responded to vaccination compared to saline controls. There was, however, no effect of metabolic status on antibody responses to vaccination.

5.3.2. Flu specific cell mediated (CMI) responses

For granzyme B flu stimulated gene expression (Figure 5.4), there was a significant overall effect of vaccination ($P = 0.023$), with horses given saline lower in granzyme B compared to vaccinated horses. There was also an overall effect of time ($P = 0.018$); with horses having higher gene expression at weeks 2 and 3 compared to week 0. In addition, horses were higher at weeks 2, 3, and 5 compared to week 4. There were no differences or interactions for any variable for PMA stimulated granzyme B gene expression, data not shown.

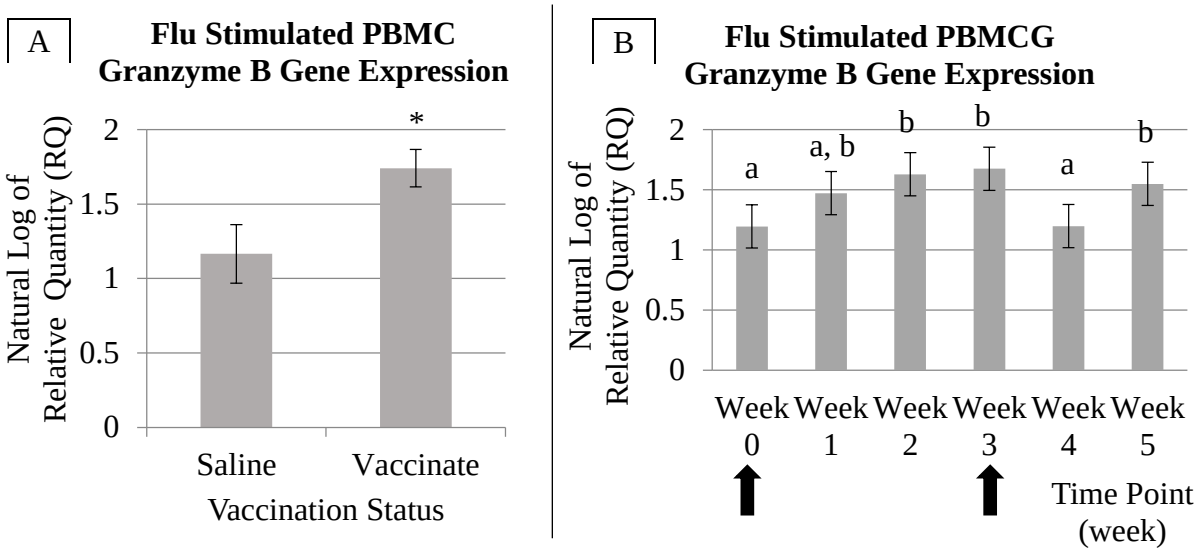

Figure 5.4. Relative quantity (RQ) of granzyme b gene expression for flu stimulated PBMCs (A) in vaccinated horses (n=17) and in horses given saline (n=8) and (B) over time for all horses (n = 25). Results are presented as least square means (± SEM). Differences ($P < 0.05$) between vaccinated horses and those given saline are represented by * and differences between time points are represented by differing alphabetic superscripts. Arrows represent time points of vaccination or saline administration.

Flu stimulated IFN-γ gene expression (Figure 5.5) was different between vaccinated horses and horses given saline, with horses given saline having lower expression compared to vaccinated horses ($P = 0.032$). In addition, there was an overall time point difference ($P = 0.002$). Horses were higher in flu stimulated IFN-γ gene expression at weeks 1, 2, and 3 compared to weeks 0 and 4. For PMA stimulated IFN-γ gene expression there were observed interactions (Figure 5.6). There was a metabolic status by vaccine vs saline ($P = 0.019$) interaction. Non-EMS control horses administered saline were lower in IFN-γ gene expression compared to non-EMS control horses administered vaccine. Non-EMS control vaccinated horses were higher compared to EMS horses regardless of vaccination status. In addition, there was a significant metabolic status by time point ($P = 0.015$) interaction, with non-EMS control horses lower in gene expression at weeks 0, 1, 2, and 3 compared to weeks 4 and 5. Non-EMS control horses at weeks 1 and 3 were higher compared EMS horses at those time points in IFN-γ gene expression. EMS horses were higher in gene expression at week 0 compared to weeks 1, 3, 4, and 5,

and higher at week 2 compared to weeks 1 and 5. Additionally, EMS horses at week 4 were higher compared to week 5.

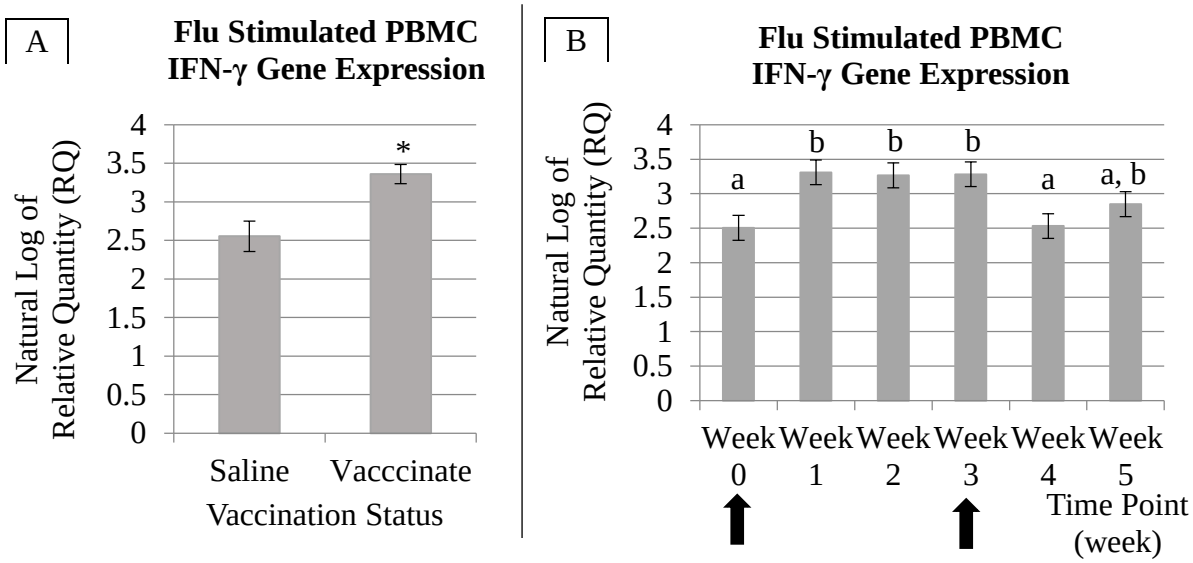

Figure 5.5. Relative quantity (RQ) of IFN-γ gene expression for flu stimulated PBMCs (A) in vaccinated horses (n = 17) and in horses given saline (n = 8) and (B) over time for all horses (n = 25). Results are presented as least square means (± SEM). Differences ($P < 0.05$) between vaccinated horses and those given saline are represented by * and differences between time points are represented by differing alphabetic superscripts. Arrows represent time points of vaccination or saline administration.

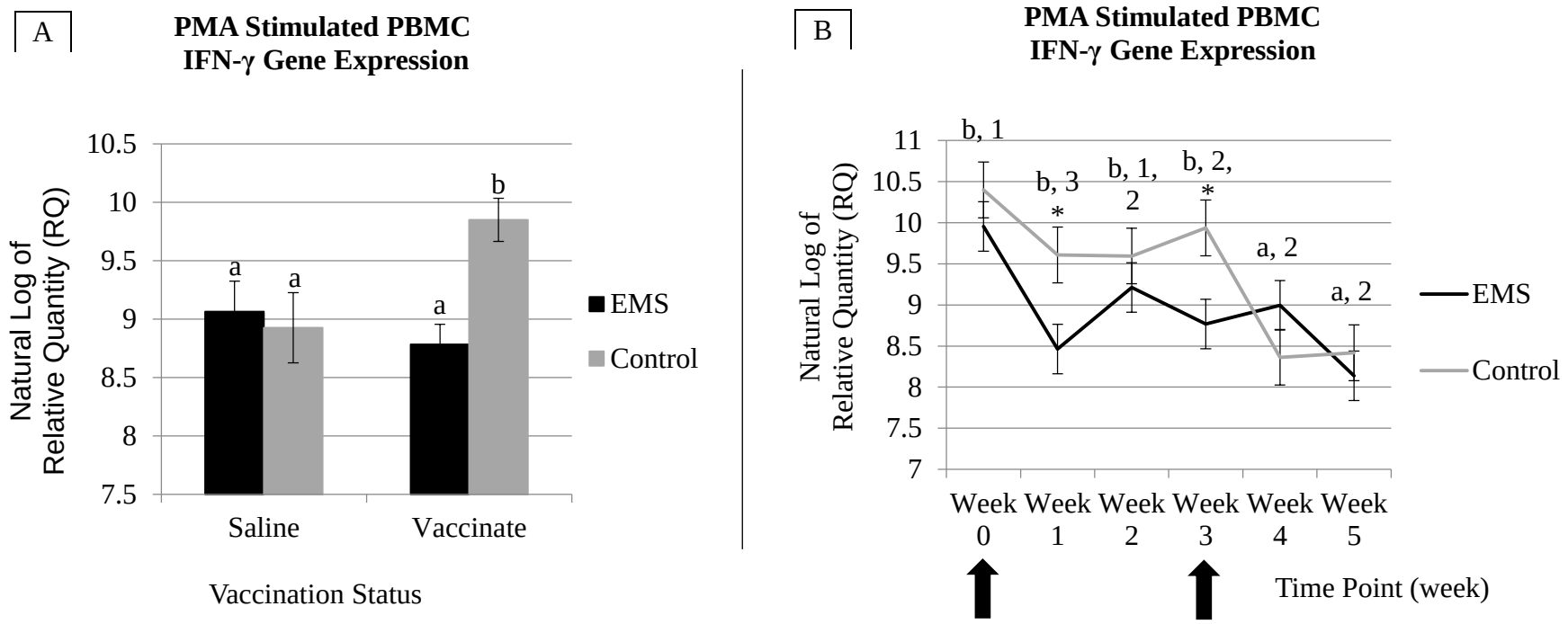

Figure 5.6. Relative quantity (RQ) of IFN-γ gene expression for PMA stimulated PBMCs (A) in vaccinated horses (EMS n = 9; non-EMS control n = 8) and in horses given saline (EMS n = 4; non-EMS control n = 4) and (B) over time in EMS (n = 13) vs non-EMS control horses (n = 12). Results are presented as least square means (± SEM). Differences ($P < 0.05$) between EMS vs non-EMS control horses within time point are represented by * and differences between time points within non-EMS control horses and for (A) are represented by differing alphabetic superscripts. Differences between time points within EMS horses are represented by differing numeric superscripts. Arrows represent time points of vaccination or saline administration.

Vaccinated horses were not significantly different from horses given saline for flu stimulated IL-2 gene expression (P = 0.461; data not shown). However, there was an overall effect of metabolic status, with non-EMS control horses higher compared to EMS horses (non-EMS control horses ln RQ = 1.28 ± 0.13, EMS horses ln RQ = 0.83 ± 0.11; P = 0.016). There was also an effect of time, with horses at week 0 lower in IL-2 gene expression compared to horses at week 3. Horses at week 1 were also lower compared to weeks 2 and 3, and horses at week 3 were higher compared to weeks 4 and 5. Similar to PMA stimulated IFN-γ gene expression, there were significant interactions for PMA stimulated IL-2 gene expression (Figure 5.7). There were significant interactions for metabolic status by vaccine vs saline (P = 0.013) and metabolic status by time point (P = 0.029). The only significant pairwise comparison for the metabolic status by vaccination status interaction involved vaccinated horses, where non-EMS controls were higher in PMA stimulated IL-2 gene expression than EMS horses. Metabolic status by time point differences showed non-EMS control horses to be higher at week 0 compared weeks 1, 4, and 5. Non-EMS control horses at week 1 were also higher compared to weeks 4 and 5. Additionally, at week 1 non-EMS control horses were higher in IL-2 gene expression compared to EMS horses. EMS horses were lower in gene expression at week 0 compared to all other time points, and EMS horses at week 5 were lower than week 2.

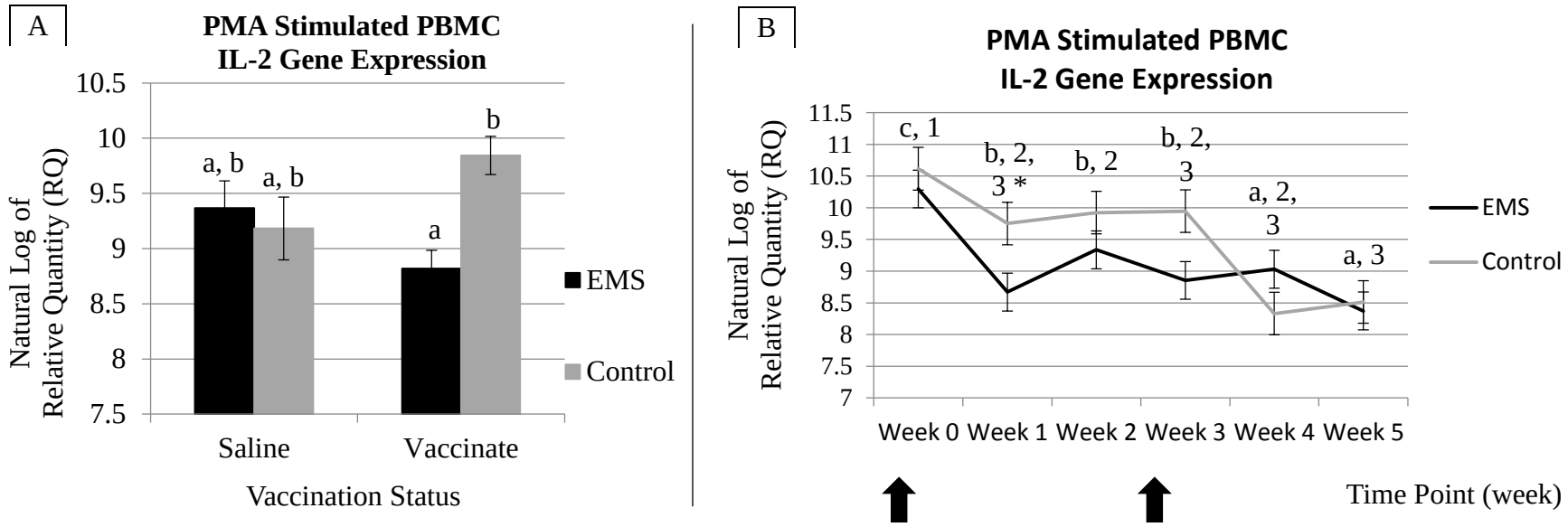

Figure 5.7. Relative quantity (RQ) of IL-2 gene expression for PMA stimulated PBMCs (A) in vaccinated horses (EMS n = 9; non-EMS control n = 8) and in horses given saline (EMS n = 4; non-EMS control n = 4) and (B) over time in EMS (n = 13) vs non-EMS control horses (n = 12). Results are presented as least square means (± SEM). Differences ($P < 0.05$) between EMS vs non-EMS control horses within time point are represented by * and differences between time points within non-EMS control horses and for (A) are represented by differing alphabetic superscripts. Differences between time points within EMS horses are represented by differing numeric superscripts. Arrows represent time points of vaccination or saline administration.

Perforin gene expression (data not shown) for flu stimulated lymphocytes had a significant vaccine vs saline by time point interaction ($P = 0.026$). Horses given saline had a lower gene expression at week 5 compared to all other weeks. Vaccinated horses were highest in perforin gene expression at weeks 1 and 4 compared to week 2. For gene expression of perforin in PMA stimulated lymphocytes, there was a significant metabolic status by time point interaction ($P = 0.007$; data not shown). Non-EMS control horses at weeks 0, 1, and 3 were higher compared to week 2, and higher at week 3 compared to week 5. EMS horses at weeks 0, 1, and 4 were higher in perforin gene expression compared to week 5. Within time point there were no differences between EMS and non-EMS control horses.

There was an observed metabolic status by time point interaction for flu stimulated lymphocyte TNF-α gene expression ($P = 0.009$; data not shown). Non-EMS control horses were lower at week 0 compared to weeks 1, 3, 4, and 5. Non-EMS control horses at weeks 1 and 3 were higher compared to week 2. Similar to perforin gene expression, within time point there were no differences between EMS and non-EMS control horses. There was likewise a metabolic status by time point interaction for PMA stimulated lymphocyte TNF-α gene expression ($P = 0.032$; Figure 5.8). Non-EMS control horses were highest at week 0 compared to weeks 2, 4, and 5, and at week 1 were higher compared to weeks 2 and 5. Non-EMS control horses were higher at week 3 compared to weeks 2, 4, and 5. Non-EMS control horses at week 4 were also higher compared week 5. At week 3, non-EMS control horses were higher compared to EMS horses at that same time point. EMS horses were highest in TNF-α gene expression at week 0 compared to weeks 2 and 5. EMS horses were also higher weeks 1 and 4 compared to week 5.

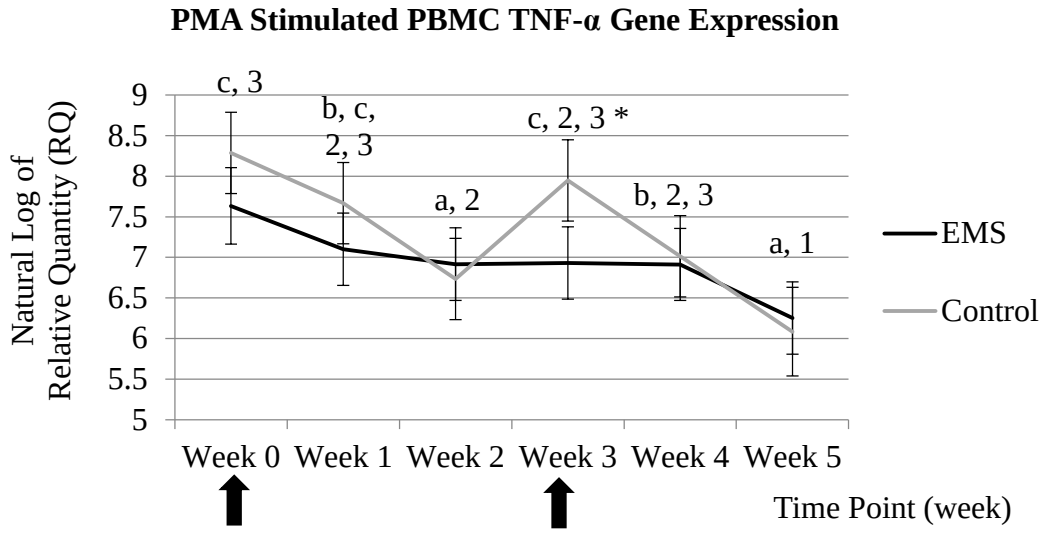

Figure 5.8. Relative quantity (RQ) of TNF-α gene expression for PMA stimulated PBMCs over time in EMS (n = 13) vs non-EMS control horses (n = 12). Results are presented as least square means (± SEM). Differences ($P < 0.05$) between EMS vs non-EMS control horses within time point are represented by * and differences between time points within non-EMS control horses are represented by differing alphabetic superscripts whereas differences between time points within EMS horses are represented by differing numeric superscripts. Arrows represent time points of vaccination or saline administration.

Overall and time point dependent effects of vaccination were seen for lymphocyte gene expression of flu stimulated granzyme B, flu and PMA stimulated IFN-γ, PMA stimulated IL-2, and flu stimulated perforin with vaccinated horses higher than controls. There were also both overall and time point dependent effects of metabolic status for lymphocyte gene expression of PMA stimulated IFN-γ, flu and PMA stimulated IL-2, PMA stimulated perforin, and flu and PMA stimulated TNF-α with EMS horses lower compared to non-EMS control horses.

5.4. Discussion

All horses responded to vaccination regardless of metabolic status as determined by influenza specific antibody responses. Vaccinated horses had an increase in HI titers, and IgGa and IgGb antibody isotypes following both the initial (week 0) and the second administration (week 3) of Fluvac Innovator™, whereas horses given saline did not change over time. There was, however, no effect of metabolic status on humoral immune measures. It is possible that obesity plays a larger role in the immune process as compared to

metabolic status. EMS horses all met the three criteria necessary for classification as EMS. However, some had increased regional but little to moderate general obesity. Indeed, although EMS horses had a significantly higher CNS (a measure of regional adiposity) compared to non-EMS control horses, they did not differ in BCS (a measure of general adiposity). In naïve, diet induced obese mice, differences in primary humoral immune responses to influenza vaccination [134, 135] have been observed. This has similarly been observed in humans where type 1 diabetes was associated with a risk for non-responsiveness to influenza vaccination [136], however investigators found no differences in humoral immune responses due to metabolic status. Obesity in humans was a risk factor >3ys post hepatitis vaccination for lower circulating levels of antibody [137] and body mass index, but not diabetes status, was positively correlated with a greater decline in influenza antibody titers 12 months following influenza vaccination [138]. A greater body mass index in a group of non-diabetic women given a HIV vaccine was associated with decreased HIV specific IgG titers and obese women had a greater likelihood to be non-responders [245]. Further, independent from age and in response to influenza vaccination obese humans had lower HI titers, decreased percentage of memory B cells, increased B cell production of the pro-inflammatory IL-6, and decreased B cell production of the anti-inflammatory IL-10 [241]. Also, the number of human γδ T cells is negatively correlated with obesity and obese individuals γδ T cells produce less IFN-γ upon *in vitro* influenza challenge compared to lean controls [242]. It is important to note that for human studies with influenza vaccine, similar to this work, participants presumably have been previously exposed to influenza and responses to vaccination are primarily recall responses.

Inactivated or killed vaccines, similar to the inactivated vaccine used in this study, tend to produce a robust humoral immune response, but a weaker CMI response [246]. This may have contributed to the results seen here considering that differences between EMS and non-EMS controls were observed not for humoral but for CMI responses. For this study, results for CMI responses when comparing vaccinated horses to those given saline were few. An overall effect of vaccination was seen for flu stimulated PBMC granzyme B and IFN-γ gene expression, and a vaccine vs saline by time point interaction for perforin. Vaccinated horses were higher overall for granzyme B and IFN-γ compared to horses given saline. Within time point, there were no differences between vaccinated

horses and those given saline for flu stimulated perforin gene expression, however vaccinated horses did have increases in gene expression at weeks 1 and 4 (the weeks following vaccine administration).

There was an overall effect of metabolic status for flu stimulated PBMC IL-2 gene expression with EMS horses lower compared to non-EMS controls. There was also an interaction between metabolic status and time point for flu stimulated PBMC TNF-α gene expression. Although within time point there were no differences due to metabolic status, the non-EMS control horses had significant changes over time in TNF-α whereas the EMS horses did not. There were several interactions between metabolic status and vaccination status and/or time point for PMA stimulated PBMC gene expression of IFN-γ, IL-2, perforin, and TNF-α. EMS horses were lower in gene expression of IFN-γ at weeks 1 and 3 compared to non-EMS controls. Also, EMS horses were lower in IL-2 gene expression at week 1 compared to non-EMS controls. Vaccinated EMS horses were overall lower in IFN-γ and IL-2 compared to vaccinated non-EMS control horses.

Lower gene expression of IL-2 in flu and PMA stimulated PBMCs indicate that EMS horses may have a decrease in lymphocyte proliferation [247] compared to metabolically normal controls. Pro-inflammatory cytokines TNF-α and IFN-γ play an important role in antigen-specific CMI responses [248, 249]. A lack of change in TNF-α gene expression in flu stimulated PBMCs and over time in EMS horses and lower gene expression of IFN-γ in PMA stimulated PBMCs at weeks 1 and 3 compared to non-EMS control horses, further points to a reduced CMI response to vaccination in these animals. Further supporting a reduced CMI in EMS horses were the differences in PMA stimulated PMBCs between vaccinated EMS and vaccinated non-EMS control horses for IFN-γ and IL-2, with vaccinated non-EMS controls significantly higher compared to vaccinated EMS horses.

These data indicate that the EMS horse is able to produce a robust humoral immune response to vaccination. However, their ability to mount a CMI response may be reduced in comparison to horses without metabolic dysfunction, and requires further study. In particular, the impact of obesity as opposed to metabolic status, as well as a better characterization of CMI responses to vaccination in horses with metabolic dysfunction utilizing an attenuated live or other vaccine able to induce stronger CMI responses should

be investigated. Furthermore, given data in other species and indications here of potential differences in CMI responses, vaccine efficacy and duration of immunity in the obese or EMS horse should be evaluated to further understand the effects of theses parameters on immune responses.

CHAPTER 6 EFFECTS OF SUPPLEMENTATION WITH YEAST CELL WALL ON METABOLIC AND INFLAMMATORY MEASURES IN INSULIN DYSREGULATED HORSES

Insulin dysregulation (ID) is a common problem associated with serious health concerns in the equine, however its pathogenesis and underlying mechanisms are not well understood. There has been increasing attention on the role of the gut in metabolic dysfunction, including the influence of an oral glycemic or meal challenge on both metabolic and inflammatory processes. Therefore, the objective of this study was to determine the effects of supplementation with a mannan-rich fraction of the yeast cell wall (Actigen^{TM}) on metabolic and inflammatory measures in ID horses compared to non-ID controls. To accomplish this, we supplemented 24 ID and non-ID control horses (13 ID and 11 non-ID, 4 non-supplemented horses within each metabolic group) with supplemented horses receiving 6 g/horse/day of Actigen^{TM} for 45 days, with blood samples collected via jugular venipuncture both pre and post oral glycemic challenge (time point) as well as pre and post the 45-day supplementation period (period). Samples were analyzed for serum glucose and insulin concentrations, circulating and peripheral blood mononuclear cells (PBMCs) inflammatory markers at both the protein and gene expression levels, and circulating plasma endotoxin concentrations were established. Metabolic results indicated that serum glucose and insulin increased following the supplementation period and in response to oral glycemic challenge, with the ID horses having a greater increase compared to non-ID controls ($P < 0.04$). There was no effect of treatment on either serum glucose or insulin concentrations. Few effects of metabolic status or treatment on inflammatory markers were observed, however non-supplemented horses or non-ID controls often had a decrease in markers of inflammation at both the protein and gene expression levels in response to the oral sugar challenge, as opposed to ID horses. There were, however, multiple inflammatory parameters affected by period, including LPS stimulated lymphocyte or whole blood gene expression and lymphocyte inflammatory protein as well as circulating LPS plasma concentrations. Overall these results indicate that supplementation had minimal effects on metabolic and inflammatory responses, however these variables were affected by both oral sugar challenge and period. In addition, it appears

that metabolic and inflammatory responses of horses with ID may be especially sensitive to changes in either metabolic stimuli or season, however further investigation regarding the influence of these factors is needed.

6.1. Introduction

Insulin dysregulation (ID) is a major problem in the equine industry, particularly as it predisposes the horse to the development of laminitis, which can be a career or even life ending condition [12, 14, 15]. However, underlying mechanisms of ID remain unclear. Some have proposed a role of inflammation in ID, but results concerning this have been mixed with reports of both increases in circulating or tissue markers of inflammation [28, 40, 41] as well as no differences or a trend for lower circulating markers of inflammation [31, 42]. This may in part be explained as in some cases ponies were utilized instead of horses, or ID was induced rather that naturally occurring. In addition, many studies were carried out in a fasted state. Establishing fed versus fasted state becomes important considering the role of the gut in metabolic regulation and hormonal control [250, 251]. Further, it has been demonstrated in humans and mice that inflammatory markers increase postprandially following a meal high in fat or high in both fat and sugar/starch [43-45]. This has similarly been observed in the horse in response sugar/starch given either as a meal or orally administered [46, 47].

Yeast cell wall supplementation has been shown to reduce inflammation in dairy cows [95], poultry [96], humans [97-99], and horses [100, 101]. Supplementation with yeast cell wall has also influenced metabolic parameters in cattle [102], and reduced circulating triglyceride and cholesterol levels in poultry [103, 104]. The mannan oligosaccharides (MOS) found in the yeast cell wall modulate the immune response by binding to mannose-specific lectin on gram negative bacteria, thus preventing it from binding to intestinal epithelial cells [252, 253]. Concentrations of lipopolysaccharide (LPS), a component of the outer membranes of gram negative bacteria, have been shown to be reduced following MOS supplementation in pigs [105], and modulated the inflammatory response to LPS challenge in chickens [254]. Given the fact that there have been multiple studies showing probable links between inflammation and metabolic dysfunction [127, 139, 140], there is ample evidence to further investigate not only these

relationships in the horse with ID, but also possible ways to improve these parameters. Therefore, the aim of this study was to further establish the role of inflammation in the ID horse in response to an oral sugar challenge as well as the ability of the gut modulating product Actigen^{TM} to modify circulating LPS concentrations, and improve metabolic and inflammatory parameters in these animals.

6.2. Materials and methods

6.2.1. Horse selection and study design

Twenty-four horses of mixed-breed and mixed-sex, both ID (n=13) and non-ID controls (n=11) [12], were selected from the University's Department of Veterinary Science Woodford County herd for use in this study. ID was established three months prior to the start of the study with an oral sugar test (OST) as previously reported [13, 47], with hyperinsulinemia diagnosed as a fasting insulin level of > 20 μIU/mL and a concentration of insulin > 60μU/mL 60 minutes post OST considered further indicative of ID. General and regional adiposity were assessed for all horses by body condition scoring (BCS; based on the 1-9 Henneke scoring system [186]) and cresty neck scoring (CNS; based on the 0-5 Carter et. al. scoring system [187], respectively. Screening results presented in Table 6.1. Of the 13 ID horses (7 geldings and 6 mares), 4 were of mixed breed, 2 were Warmbloods, 2 were Standardbred or Standardbred cross, 2 were walking horses, 1 was a Thoroughbred cross, 1 a Morgan, and 1 a Quarter Horse. Of the 11 non-ID control horses (6 geldings and 5 mares), 5 were of mixed breed, 4 were Thoroughbreds or Thoroughbred cross, 1 was a Quarter Horse, and 1 a walking horse cross.

Table 6.1. Endocrine and measures of adiposity in ID and control horses.

	Baseline Insulin	Insulin 60 min post oral sugar administration	BCS	CNS
ID	36.8 ± 6.3	76.5 ± 12.1	7.0 ± 0.2	2.7 ± 0.3
Non-ID control	12.9 ± 0.9	24.2 ± 3.7	6.1 ± 0.1	1.4 ± 0.2
p value	*0.0026*	*0.00095*	*0.0019*	*0.00131*

Data are presented as least square means ± standard error of the mean for n = 13 ID and n = 11 controls. Body condition score (BCS) and cresty neck score (CNS) are presented as an average score between 3 trained individuals.

To ensure that none of the horses were affected with PPID (Pituitary Pars Intermedia Dysfunction), all horses were screened at least 3 mo prior to the start of the study via thyrotropin releasing hormone (TRH) stimulation testing as previously described [47, 191, 192]. Briefly, a.m. (between 0800 and 1000, EST) blood samples from jugular venipuncture were collected and a subsequent 1 mg/mL of TRH (Sigma-Aldrich, St. Louis, MO) given intravenously (IV). Ten min following TRH administration, a second blood sample was collected. All samples were collected in ethylenediaminetetraacetic acid (EDTA) containing tubes which were kept on ice for transport to the lab and immediate centrifugation and isolation of plasma [193]. ETDA plasma was stored -20 º C until sent on dry ice for ACTH analysis by Cornell University's Animal Health Diagnostic Center Endocrinology Laboratory (Ithaca, NY) by chemiluminescence (Immulite, Erlangen, Germany) as previously described [195]. A 10 min post TRH administration ACTH concentration of > 110 pg/mL was considered positive for PPID and any horse exceeding this concentration excluded.

To determine the response of metabolic and inflammatory parameters following supplementation with a mannan-rich fraction of the yeast cell wall (YCW) (Actigen^{TM}; Alltech Inc., Nicholasville, KY) in the equine, 24 non-PPID mixed-breed and mixed-sex animals (13 ID and 11 non-ID controls; 4 non-supplemented horses in each metabolic group) were selected as described above. Horses were blocked by insulin responses to the OST as well as age and randomly assigned to either the YCW supplemented or non-supplemented groups. All horses were acclimated to their respective paddocks at the University of Kentucky's Department of Veterinary Science Woodford County facility for > 9 months with *ad libitum* access to mixed-grass hay, a trace mineralized salt block, and water. None of the horses received any form of concentrate prior to the study. Two weeks prior to the start of supplementation, all horses were acclimated to study feeding protocols, with a 0800-0900 EST individual pen feeding with 0.45 kg balancer pellet (M30; Mc Cauley Bros Inc., Versailles, KY) which was used for the duration of the study. The OST was performed as described above prior to supplementation (July of 2016) as well as following 45 days of supplementation (September of 2016) with 6 g/horse/day of Actigen^{TM} top-dressed on balancer pellet or balancer pellet only for the non-supplemented horses. For both the OST 0 and 60 min time points, blood samples were collected for

determination of metabolic (serum glucose and insulin), inflammatory (cytokine or TLR protein and gene expression in lymphocytes as well as circulating), and plasma LPS concentrations.

6.2.2. Metabolic parameters

All serum samples for insulin determination from the OST were sent to Cornell University's Animal Health Diagnostic Centers Endocrinology Laboratory using a commercially available radioimmunoassay for human insulin (RIA) (EMD Millipore Corp, Billerica, MA). This RIA assay was validated for use on equine serum samples as follows; serial dilutions of 4 equine samples with assay buffer were parallel to the standard curve, and samples that were spiked with four different quantities of porcine insulin (Sigma-Aldrich, St. Louis, MO) had observed concentrations that averaged 96% of expected. The manufacturer did not report the cross-reactivity of the RIA antibody for equine insulin. However, the cross-reactivity for porcine insulin was reported as 100%, and the homology between equine and porcine insulin is 98%. The sensitivity of the assay, as reported by the manufacturer, is 2.72 μIU/mL. The mean intra- and inter-assay coefficients of variation were 7.4 and 6.3%, respectively. In addition to serum insulin analysis, serum was analyzed for glucose concentrations. Glucose analysis was performed by Cornell University's Animal Health Diagnostic Center Clinical Pathology Laboratory (Ithaca, NY) with a hexokinase kinetic method as previously described [47].

6.2.3. Inflammatory parameters

6.2.3.1. Lymphocyte inflammatory cytokine production as measured by flow cytometry

Heparinized blood samples were taken both pre and post OST as well as pre and post supplementation. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood by Ficoll-Paque PlusTM (Amersham Biosciences, Piscataway, NJ) gradient centrifugation [197]. PBMCs at a concentration of 2×10^8 were frozen suspended in media (10% dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO), 40% fetal bovine serum (FBS; Sigma), and 50% RPMI-1640 (Gibco, Grand Island, NY)) in liquid nitrogen until the end of the study, at which point they were thawed by horse for stimulation and analysis. For *in vitro* stimulation, cells were thawed in c-RPMI media [RPMI-1640 supplemented with 2.5% fetal equine serum (FES; Sigma–Aldrich, St. Louis, MO), 100

U/mL penicillin/streptomycin (Sigma), and were plated and incubated for 4H at 37 ° C, 5% CO_2. Select wells were incubated with 10 mg/mL Brefeldin A (Sigma) and select wells additionally incubated with 25 ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma) and ionomycin (1 mM; Sigma) as a positive control. Separate select wells were also incubated with 10 ng LPS (catalogue #L5293; Sigma) for 2 H. Following incubation, aliquots of the cells were isolated for determination of gene expression by a Trizol (Ambion) method to isolate RNA (see below). Remaining cells incubated with BFA alone, or the combination of BFA and PMA, were used to determine IFN-γ and TNF-α protein production by flow cytometry. In brief, using 2% paraformaldehyde (Sigma), cells were fixed and allowed to incubate at 4º C overnight. Following this, intracellular staining was performed as previously described [198]. Stained cells were re-suspended in FACS flow (Becton Dickinson (BD), San Jose, CA) and a BD Accuri™ (BD) used for flow cytometric acquisition and determination of IFN-γ and TNF-α protein production.

6.2.3.2. Lymphocyte gene expression measured by real-time polymerase chain reaction (RT-PCR)

RNA was extracted using a modified Trizol method. cDNA was generated by reverse transcription using 1 µg of RNA as previously described [197]. Briefly, RNA sample and a master mix (Promega, Madison, WI) were combined and incubated at 42º C for 15 min and at 95º C for 5 min. cDNA (4.5 µL) was then combined with a Master Mix (5 µL; SensiMix, HI-ROX 2x, Bioline) and the equine-specific, intron-spanning, primer-probe of interest (0.5 µL; TaqManTM, Applied Biosystems). An Applied Biosystems RT-PCR ABI 7900HT was used to run samples for the following genes of interest: *beta*-glucuronidase (house-keeping gene; β-gus; Ec03470630_m1), interferon gamma (IFN-γ; Ec03468606_m1), IL-6 (Ec03468678_m1), IL-1β (Ec04260298_s1), TNF-α (Ec03467871_m1), and TLR4 (Ec03468994_m1) [199]. All samples were run in duplicate. Following a 10 min incubation at 95º C, they underwent 40 cycles of 15 sec at 95º C and 60 sec at 60º C. The $\Delta\Delta C_T$ method [200] was used to determine relative changes in gene expression, with the average pre supplementation time 0 samples mean ΔC_T for all horses set at the calibrator for all samples. Results are expressed as relative quantities (RQ), calculated as $2^{-\Delta\Delta CT}$.

6.2.3.3. Whole blood gene expression measured by real-time polymerase chain reaction (RT-PCR)

Samples (3 ml whole blood) were collected pre and 60 minutes post oral sugar test as well as pre and post supplementation into Tempus™ Blood RNA Tubes (Life Technologies, Grand Island, NY). Tubes were shaken vigorously following blood collection and incubated at room temperature for 24 hrs. Following incubation, they were stored at -20 ºC for later analysis. Prior to analysis, tubes were thawed and total RNA extracted using iPrep RNA isolation kit (Thermo Fisher Scientific, Waltham, MA) following the manufacturer's instructions. Following this, reverse transcription reactions and RT-PCR took place as described above.

6.2.3.4. Serum inflammatory parameters as measured by enzyme-linked immunosorbent assay (ELISA) and Luminex bead-based multiplex assay

The commercially available equine TNF-α ELISA (R&D Systems, Inc., Minneapolis, MN) was used for determination of serum concentrations of TNF-alpha protein both pre and post OST as well as pre and post supplementation. Assay was carried out per the manufacturer's instructions, with the exception the following: dilutions were performed with buffer (2.5 mmol NaH_2PO_4, 7.5 mmol Na_2HPO_4, 145 mmol NaCl, 0.1% (v/v) Tween 20, pH 7.2) and samples plated in duplicate in a 96-well plate. The sensitivity of the ELISA was 15.6 pg/ml with a mean intra- and inter-assay coefficients of variation of 2.0% and 5.4%, respectively.

Serum C-reactive Protein (CRP) concentrations were also measured pre and post OST as well as pre and post supplementation. CRP concentrations were determined by the equine specific commercially available kit (Kamiya Biomedical Company, Tukwila, WA) and was used according to the manufacturer's instructions.

Pre and post OST as well as pre and post supplementation, a multiplex assay using a Luminex x200 (Luminex Corporation, Austin, TX) was used to further measure serum markers of inflammation. The equine specific assay (EMD Millipore Corporation Billerica, MA; EQCYTMAG-93K) contained antibodies against IL-6, IFN-γ, and MCP-1 (Monocyte chemotactic protein-1). The protocol was performed per manufacturer's instructions.

6.2.4. LPS as measured by LAL chromogenic endotoxin assay

Heparinized plasma collected from all time points and immediately isolated using sterile techniques following collection upon arrival to the lab. Plasma was transferred to LPS free glass 15 ml tubes and frozen at -20 º C until further analysis. Samples were analyzed using a Pierce™ LAL Chromogenic Endotoxin Quantitation Kit (Thermo Fisher Scientific, Waltham, MA) per the manufactures' instructions, with the following exceptions; plasma samples were diluted 1:3 in endotoxin free water and reactions were mixed by gently swirling plates.

6.2.5. Data analysis

Prior to analysis, data points within variables determined to be outliers were removed from the analysis. Outliers were established as being 1.5 times the interquartile range below or above the 1st or 3rd quartiles, respectively. Analysis of data were completed with a repeated measures ANOVA using PROC MIXED, SAS 9.4 (SAS Institute Inc., Cary, NC, USA). Horse ID, metabolic group (ID vs non-ID controls), period (pre vs post supplementation), time (0 vs 60 min OST time points), and treatment (supplemented vs non-supplemented horses) were set at fixed effects and all potential interactions between these effects analyzed. Horse ID was set as a random effect and time and period with the subject horse set as the repeated measures. Pairwise comparisons were made using protected LSD. Significance was set at $P < 0.05$ and trends considered at $P < 0.10$. Pairwise comparisons were examined for both trends and significant differences as determined by ANOVA. Normality was determined by analysis of residuals. Data with residuals not visually normal or with a skew of < 2 and/or kurtosis of < 7 were natural log transformed and resulted in normality.

6.3. Results

6.3.1. Metabolic parameters

Metabolic results are presented in Figures 6.1 and 6.2. There was no effect of treatment on serum insulin concentrations (Figure 6.1). All horses responded to OST (P = 0.0005) with an increase in insulin concentrations from time point 0 to time point 60. Overall (P = 0.0003), ID horses had higher insulin responses compared to non-ID controls and there was a significant metabolic group (ID vs non-ID controls) by period (pre vs post

supplementation) interaction ($P = 0.0067$). Prior to supplementation, non-ID control horses were lower compared to ID horses ($P = 0.0111$) in serum insulin. This did not change following supplementation, with non-ID control horses still lower in serum insulin compared to ID horses ($P < 0.0001$) however, ID horses had a significant increase in insulin following the supplementation period ($P = 0.0023$) whereas non-ID control horses did not ($P = 0.4116$).

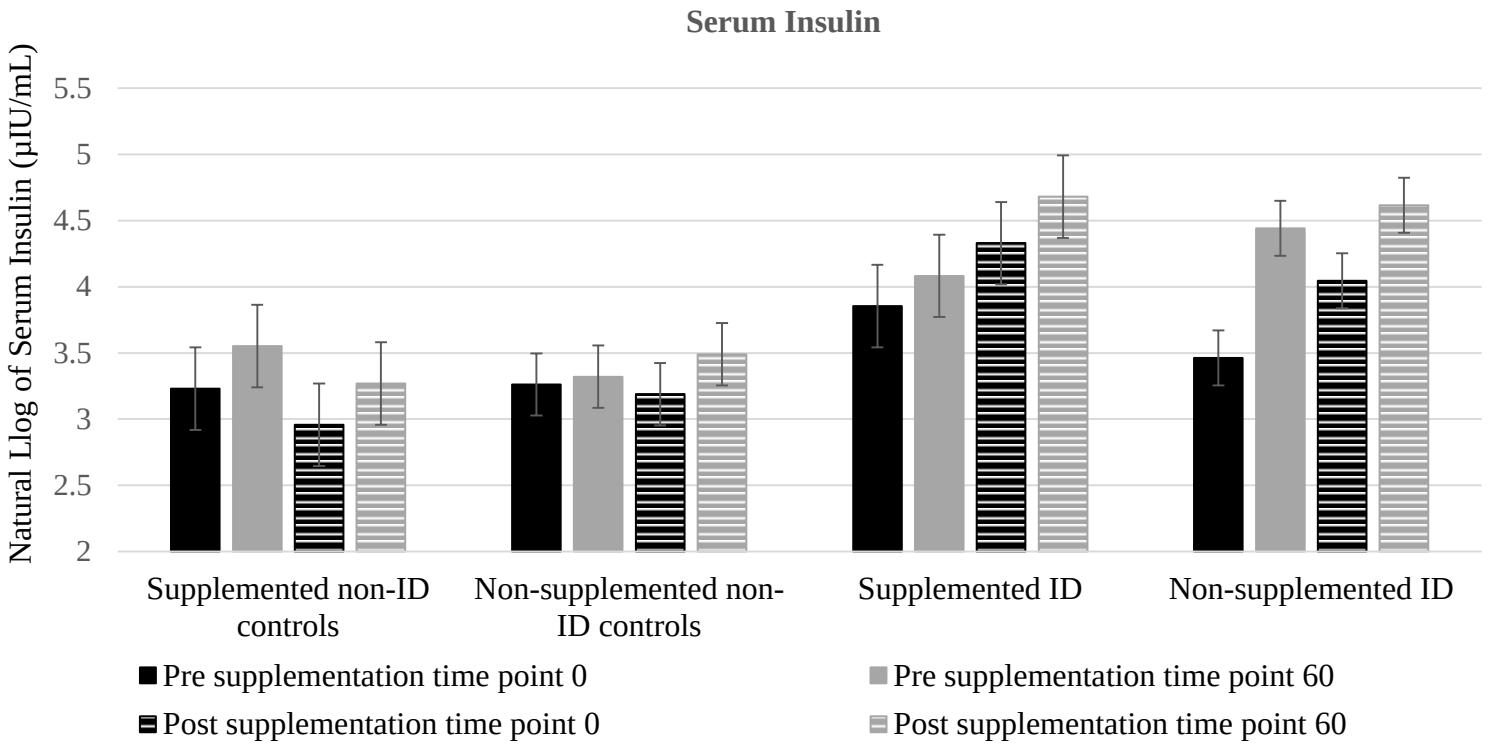

Figure 6.1. Mean (± SEM) natural log of serum insulin concentrations (µIU/mL) in supplemented non-ID control horses (n = 7), non-supplemented non-ID control horses (n = 4), supplemented ID horses (n = 9) and non-supplemented ID horses (n = 4) at two different time points (baseline prior to oral sugar administration (time point 0; represented in black) and 60 min following (time point 60; represented in gray) as well as at the pre (represented by solid bars) and post (represented by corregated bars) supplementation periods. Significant ($P < 0.05$) group by period interaction with non-ID control horses having lower insulin than ID horses regardless of period, and ID horses higher at the post supplementation period compared to pre. Significant overall effect of time with time point 0 lower compared to time point 60. Significant overall effect of group with ID horses higher compared to non-ID controls horses.

Serum glucose concentrations (Figure 6.2) were likewise not affected by YCW supplementation. However, there was a trend for a metabolic group by treatment by period interaction ($P = 0.0737$) where YCW supplemented ID horses increased in serum glucose from pre to post supplementation ($P = 0.0084$) and were higher post supplementation compared to supplemented non-ID control horses ($P = 0.0295$). There was an effect of OST on serum glucose, with a significant time (time point 0 vs time point 60) by period (pre vs post supplementation) interaction ($P = 0.0137$) where time point 0 was consistently lower compared to time point 60, regardless of period ($P < 0.0001$), and glucose concentrations increased pre vs post supplementation within time point 0 ($P = 0.002$). Further, there was also a significant metabolic group by time interaction ($P < 0.0001$) for serum glucose, with ID and non-ID control horses both having a significant increase in glucose in response to the OST ($P < 0.0001$). ID horses had no differences in glucose compared to non-ID controls at time point 0 ($P = 0.4415$), but were higher at time point 60 ($P = 0.0233$). In addition, there was an overall effect of period ($P = 0.0401$), with horses higher in serum glucose following the supplementation period.

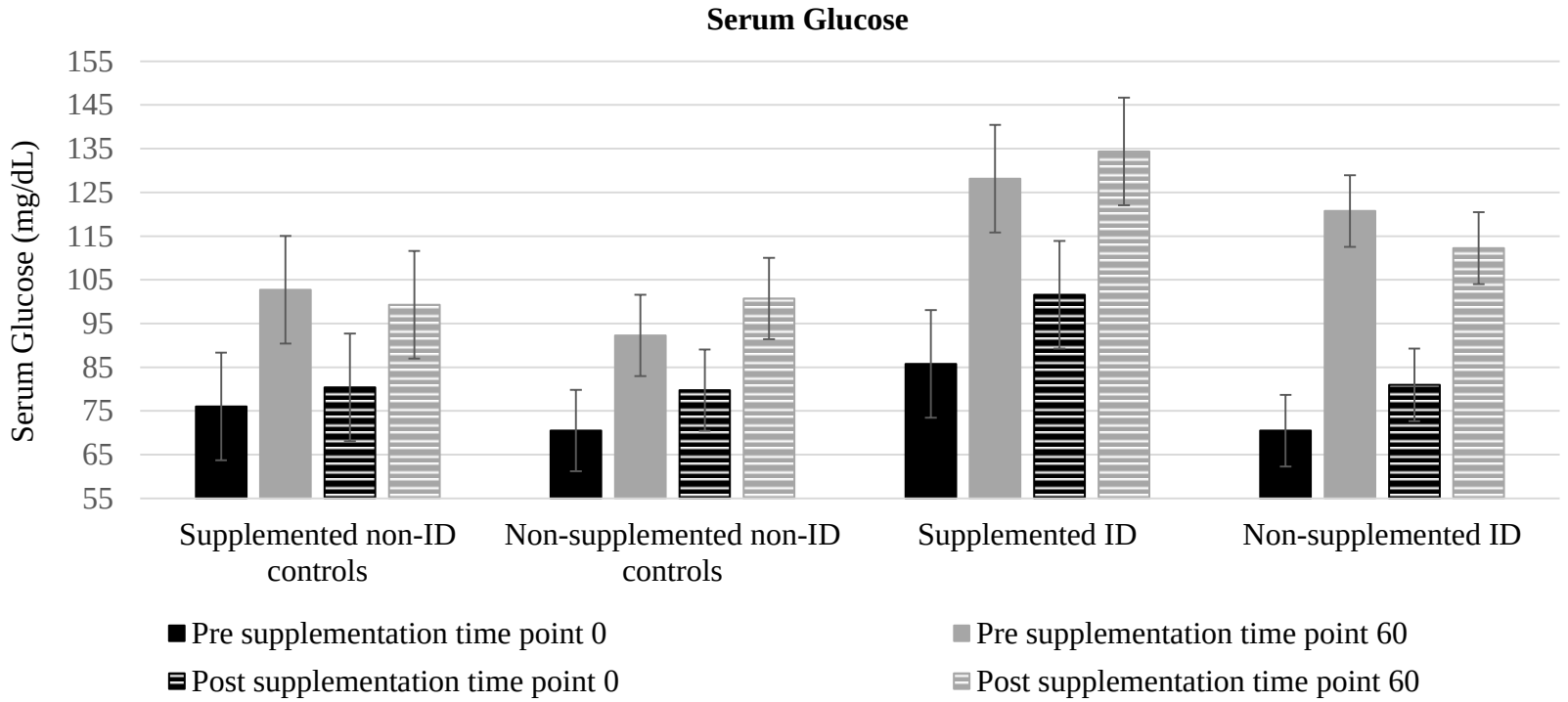

Figure 6.2. Mean (± SEM) serum glucose concentrations (mg/dL) in supplemented non-ID control horses (n = 7), non-supplemented non-ID control horses (n = 4), supplemented ID horses (n = 9) and non-supplemented ID horses (n = 4) at two different time points (baseline prior to oral sugar administration (time point 0; represented in black) and 60 min following (time point 60; represented in gray) as well as at the pre (represented by solid bars) and post (represented by corregated bars) supplementation periods. Significant ($P < 0.05$) time by period interaction with horses lower in glucose at time point 0 compared to time point 60 regardless of period, and horses within time point 0 at the pre supplementation period lower compared to post. Significant group by time interaction with both groups increasing in glucose from time point 0 to time point 60 and ID horses higher compared to non-ID control horses at time point 60. Significant overall effects of time and period with horses higher at time point 60 compared to time point 0 and higher at the post supplementation period compared to pre, respectively.

While there was no effect of treatment, horses responded as expected to the OST for both serum insulin and glucose, and had an increase in both of these metabolic parameters from the pre to post supplementation period.

6.3.2. Inflammatory parameters

6.3.2.1. Lymphocyte inflammatory cytokine production

Lymphocytes stimulated with PMA and intracellularly stained with TNF-α for flow cytometric cytokine protein analysis (Figure 6.3) were not significantly affected by YCW supplementation. However, there was a trend for a four-way metabolic group by treatment by time by period interaction ($P = 0.0828$). When examining pairwise comparisons, for most horses there was a decline in TNF-α % gated (percentage of the lymphocyte population stained positive for TNF-α %) in response to OST and from pre to post supplementation. Prior to supplementation, non-supplemented non-ID control horses at time point 0 had the highest TNF-α % gated, and were higher compared to all other periods and time points ($P \leq 0.0233$). They were also higher compared to supplemented non-ID control horses for all time points and periods ($P \leq 0.0247$), with the exception time point 60 prior to supplementation. Supplemented non-ID control horses did not change in TNF-α % gated in response to OST ether pre ($P = 0.247$) or post supplementation ($P = 0.383$). Prior to supplementation, supplemented non-ID control horses at time point 0 were lower compared to supplemented ID horses ($P = 0.0355$). This was similarly true for the post supplementation period ($P = 0.0434$). Supplemented non-ID control horses decreased significantly from pre supplementation at time point 60 compared to that same time point post supplementation ($P = 0.0024$). They were also lower compared to ID horses, regardless of treatment, for all periods and time points ($P \leq 0.0302$), with the exception of non-supplemented ID horses post supplementation at time point 60 ($P = 0.9203$). Finally, supplemented ID horses at time point 0 decreased from pre to post supplementation in TNF-α % gated ($P = 0.022$). There was an overall trend ($P = 0.0773$) for an effect of time, with horses decreasing in TNF-α in response to OST. There was a significant overall effect of period ($P < 0.0001$) for TNF-α % gated where, regardless of metabolic status or supplementation, horses were overall lower following the supplementation period.

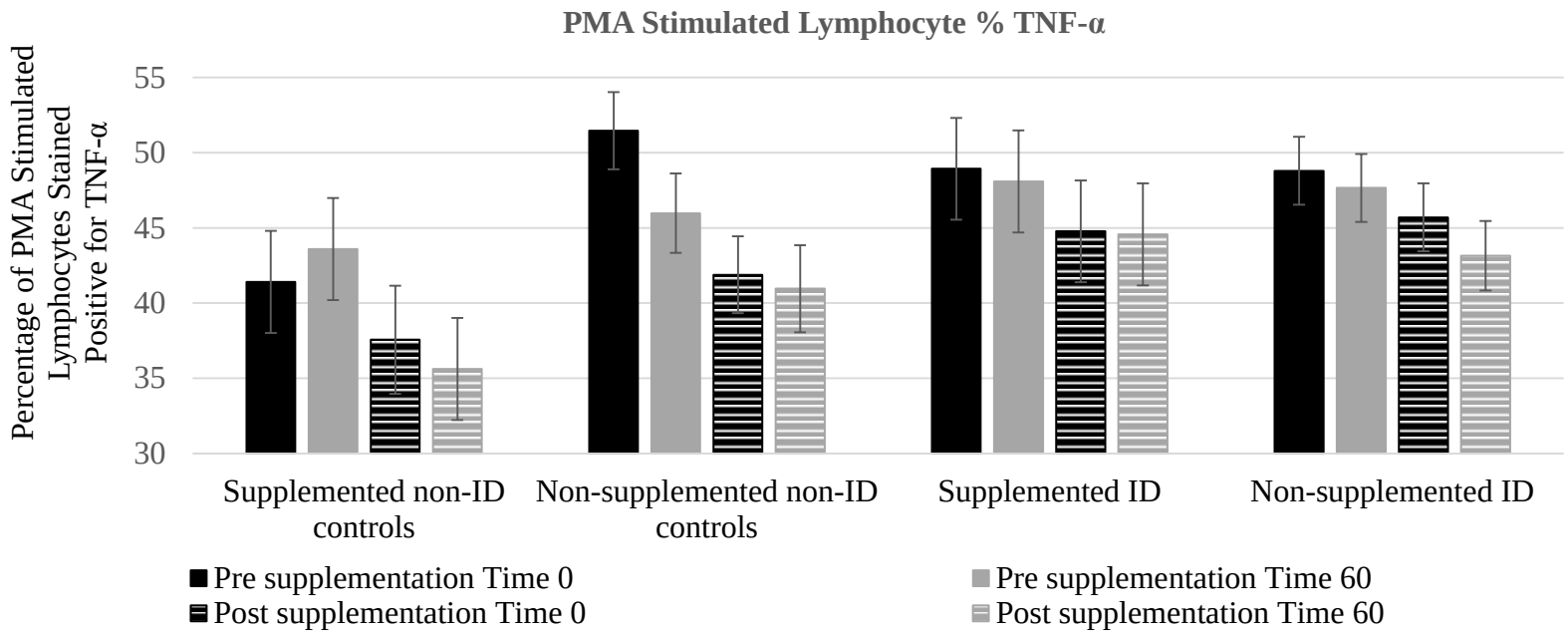

Figure 6.3. Mean (± SEM) percentage of PMA stimulated lymphocytes intracellularly stained positive for TNF-α in supplemented non-ID control horses (n = 7), non-supplemented non-ID control horses (n = 4), supplemented ID horses (n = 9) and non-supplemented ID horses (n = 4) at two different time points (baseline prior to oral sugar administration (time point 0; represented in black) and 60 min following (time point 60; represented in gray) as well as at the pre (represented by solid bars) and post (represented by corregated bars) supplementation periods. Significant ($P < 0.05$) overall effect of period, with horses higher pre vs post supplementation.

There was no effect of treatment on PMA stimulated lymphocytes intracellularly stained with IFN-γ (data not shown). While there was a trend for a metabolic group by treatment interaction (P = 0.0544), there were no significant pairwise comparisons. There was also a treatment by period interaction (P = 0.0628) with non-supplemented horses lower in IFN-γ post supplementation compared to pre (P = 0.0029). There was no effect of OST on IFN-γ in PMA stimulated lymphocytes. However, there was a significant overall effect of period (P = 0.0021) with horses lower post supplementation compared to pre supplementation, and a metabolic group by period interaction (P = 0.0822) with non-ID control horses lower post supplementation compared to pre supplementation (P = 0.0015).

6.3.2.2. Lymphocyte gene expression

For lymphocytes stimulated with PMA (data not shown) cytokine and TLR gene expression did not differ significantly for any of the variables measured, with the exception of IFN-γ. There was a significant effect of time (P = 0.0319) on IFN-γ PMA stimulated gene expression with all horses decreasing in response to OST. There were a number of trends observed. There was a trend (P = 0.0883) for a metabolic group by time by period interaction for TNF-α gene expression where post supplementation, non-ID control horses were lower compared to ID horses at time point 60 (P = 0.012). TLR-4 gene expression had trends for overall difference of time (P = 0.0554; horses increasing in response to OST) and period (P = 0.066; horses increasing from pre to post supplementation). There was also a trend for metabolic group by time interaction (P = 0.0886) for IL-6 gene expression, however none of the pairwise comparisons were significant.

There were differences or trends for cytokine and TLR gene expression of lymphocytes stimulated with LPS (Figures 6.4 - 6.7). There was no effect of treatment on LPS stimulated TNF-α gene expression, however there was a trend for a metabolic group by treatment by period interaction (P = 0.0676). Non-supplemented ID horses prior to supplementation were higher compared to supplemented ID horses both pre (P = 0.0411). There was also a trend for an overall effect of treatment (P = 0.0966), with non-supplemented horses having a trend to be higher compared to supplemented horses. The only significant effect on LPS stimulated TNF-α gene expression (P = 0.0379) was an effect of time, with all horses increasing in response to the OST (Figure 6.4).

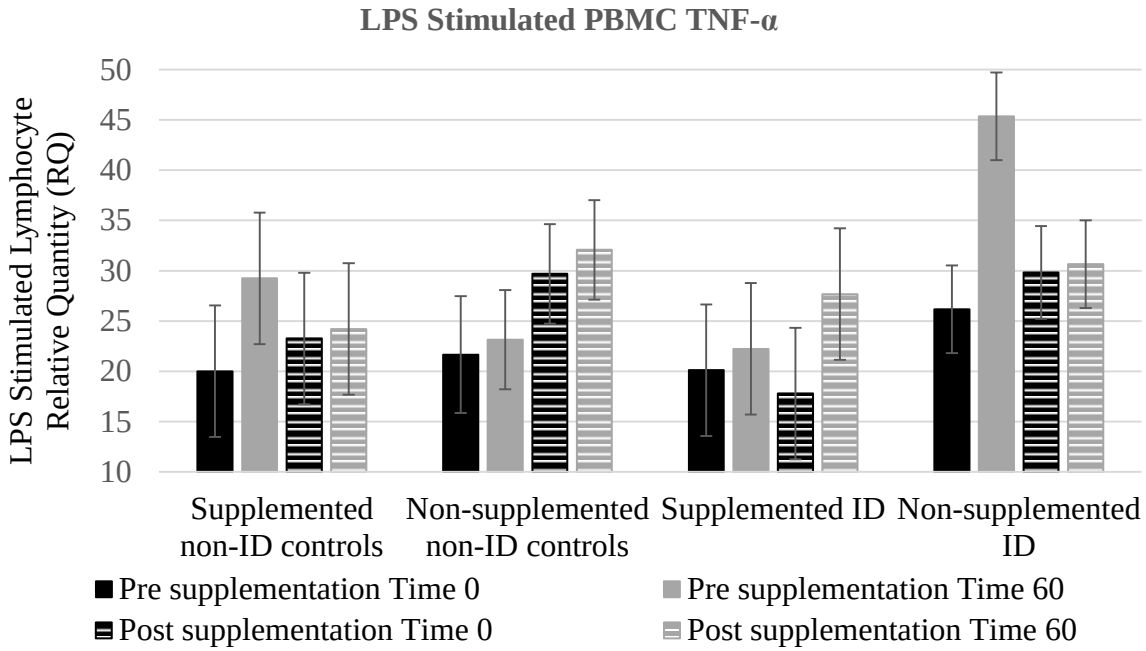

Figure 6.4. Mean (± SEM) relative quantity (RQ) of LPS stimulated PBMC TNF-α gene expression in supplemented non-ID control horses (n = 7), non-supplemented non-ID control horses (n = 4), supplemented ID horses (n = 9) and non-supplemented ID horses (n = 4) at two different time points (baseline prior to oral sugar administration (time point 0; represented in black) and 60 min following (time point 60; represented in gray)) as well as at the pre (represented by solid bars) and post (represented by corregated bars) supplementation periods. Significant ($P < 0.05$) overall effect of time point with horses lower at time point 0 compared to time point 60.

There was no significant effect of treatment on TLR-4 LPS stimulated gene expression (Figure 6.5), however there was a trend for a metabolic group by treatment by period interaction ($P = 0.0934$), with non-supplemented ID horses post supplementation lower in TLR-4 gene expression compared to pre supplementation ($P = 0.0395$). There were significant effects of OST on TLR-4, with an overall increase in response to OST for all horses ($P = 0.007$). Further, there was a significant metabolic group by time by period interaction ($P = 0.0334$) where ID horses had a decrease in TLR-4 gene expression from the pre to post supplementation periods within time point 60 ($P = 0.0042$). Prior to supplementation, ID horses also had a significant increase in gene expression in response to OST ($P = 0.0005$). In addition, there was a significant metabolic group by period interaction ($P = 0.039$) for TLR-4 LPS stimulated gene expression, but no pairwise comparisons were significant.

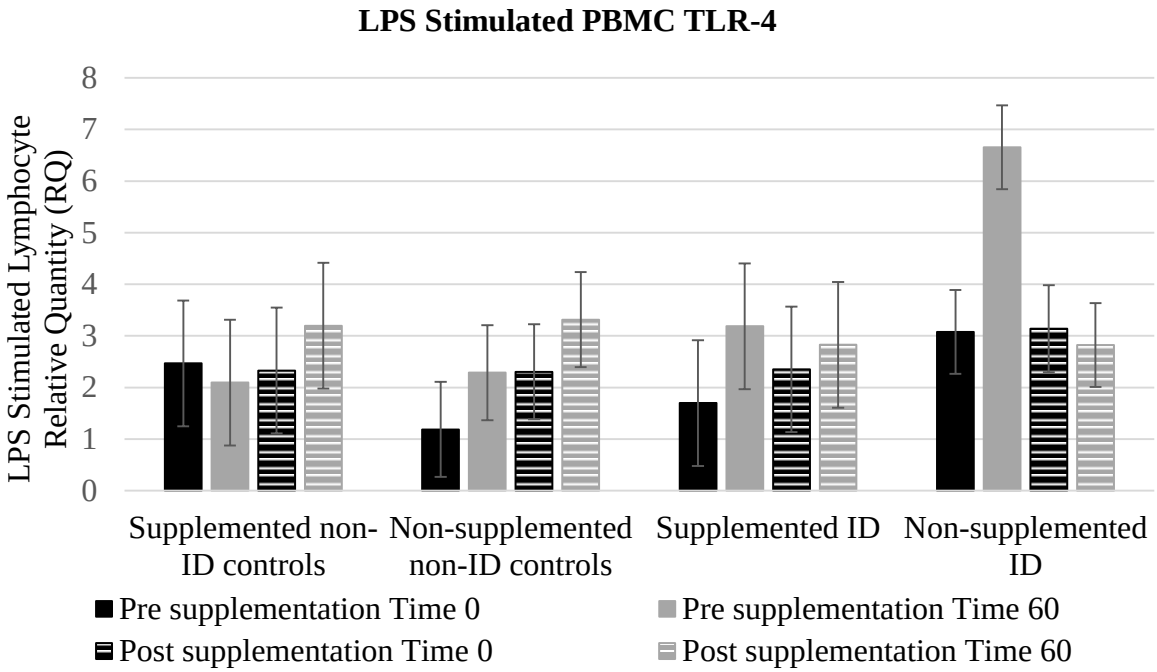

Figure 6.5. Mean (± SEM) relative quantity (RQ) of LPS stimulated PBMC TLR-4 gene expression in supplemented non-ID control horses (n = 7), non-supplemented non-ID control horses (n = 4), supplemented ID horses (n = 9) and non-supplemented ID horses (n = 4) at two different time points (baseline prior to oral sugar administration (time point 0; represented in black) and 60 min following (time point 60; represented in gray) as well as at the pre (represented by solid bars) and post (represented by corregated bars) supplementation periods. Significant ($P < 0.05$) group by time by period interaction with ID horses increasing in TLR-4 between time points where they were higher at time point 60 compared to non-ID controls at the pre supplementation period. Significant group by period interaction with no significant pairwise comparisons and an overall significant effect of time point with horses lower at time point 0 compared to time point 60.

LPS stimulated IL-6 lymphocyte gene expression (data not shown) had no significant differences for any of the variables measured. There was however a trend for a metabolic group by treatment by time interaction ($P = 0.0585$), with non-supplemented non-ID control horses increasing in IL-6 gene expression in response to OST ($P = 0.0479$). Non-supplemented non-ID control horses at time point 60 were also higher compared to supplemented non-ID control horses at that same time point ($P = 0.0275$).

There was an overall effect of treatment ($P = 0.0041$) for IL-10 (Figure 6.6), with treated horses lower compared to non-supplemented horses. There were no effects or trends of metabolic status, time, or period on IL-10 LPS stimulated gene expression. For IFN-γ (Figure 6.7), there was a significant metabolic group by treatment by time by period

interaction (P = 0.036). Post supplementation, non-supplemented horses had a significant increase in IFN-γ in response to OST (P = 0.042). Time points 0 and 60 pre supplementation were lower compared to time point 0 post supplementation for non-supplemented horses (P < 0.0265). Supplemented horses at the post supplementation period had a significant increase in IFN-γ LPS stimulated gene expression (P = 0.0183). Supplemented horses post supplementation at time point 60 were also higher than compared to pre supplementation at that same time point (P = 0.0182). Further, there was a treatment by time interaction (P = 0.0259), with supplemented horses increasing in IFN-γ in response to OST (P = 0.0328). Similarly, there was a significant metabolic group by time interaction (P = 0.0239) for IFN-γ LPS stimulated gene expression, with a trend for ID horses to have an increase in gene expression in response to OST (P = 0.0631). Finally, there was a significant effect of period (P = 0.0107) with horses increasing from the pre to the post supplementation period.

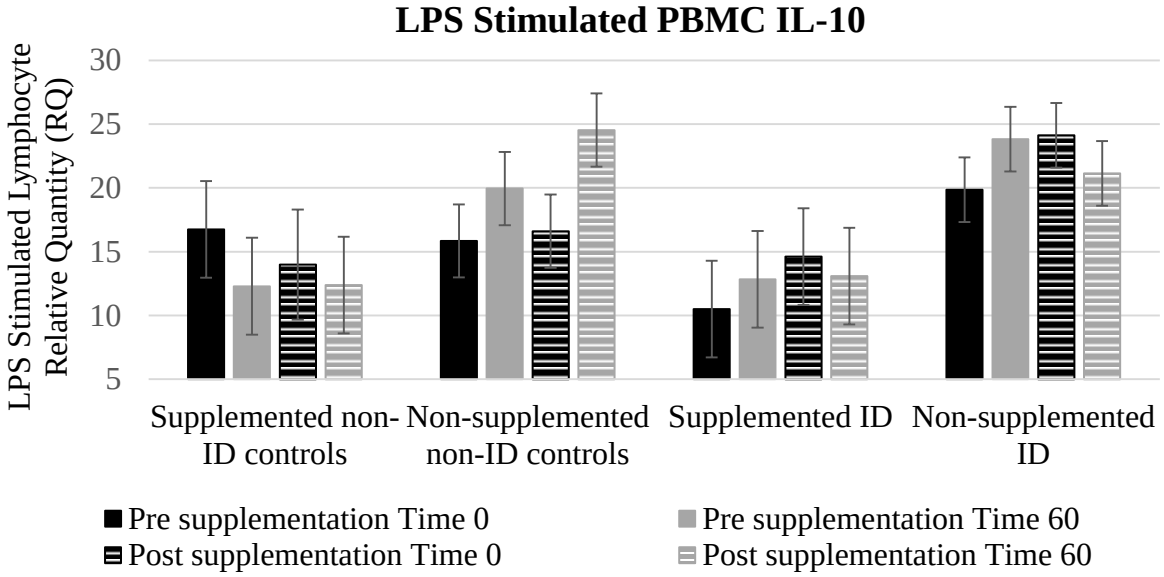

Figure 6.6. Mean (± SEM) relative quantity (RQ) of LPS stimulated PBMC IL-10 gene expression in supplemented non-ID control horses (n = 7), non-supplemented non-ID control horses (n = 4), supplemented ID horses (n = 9) and non-supplemented ID horses (n = 4) at two different time points (baseline prior to oral sugar administration (time point 0; represented in black) and 60 min following (time point 60; represented in gray) as well as at the pre (represented by solid bars) and post (represented by corregated bars) supplementation periods. Significant (P < 0.05) overall effect of treatment with non-supplemented horses lower compared to supplemented horses.

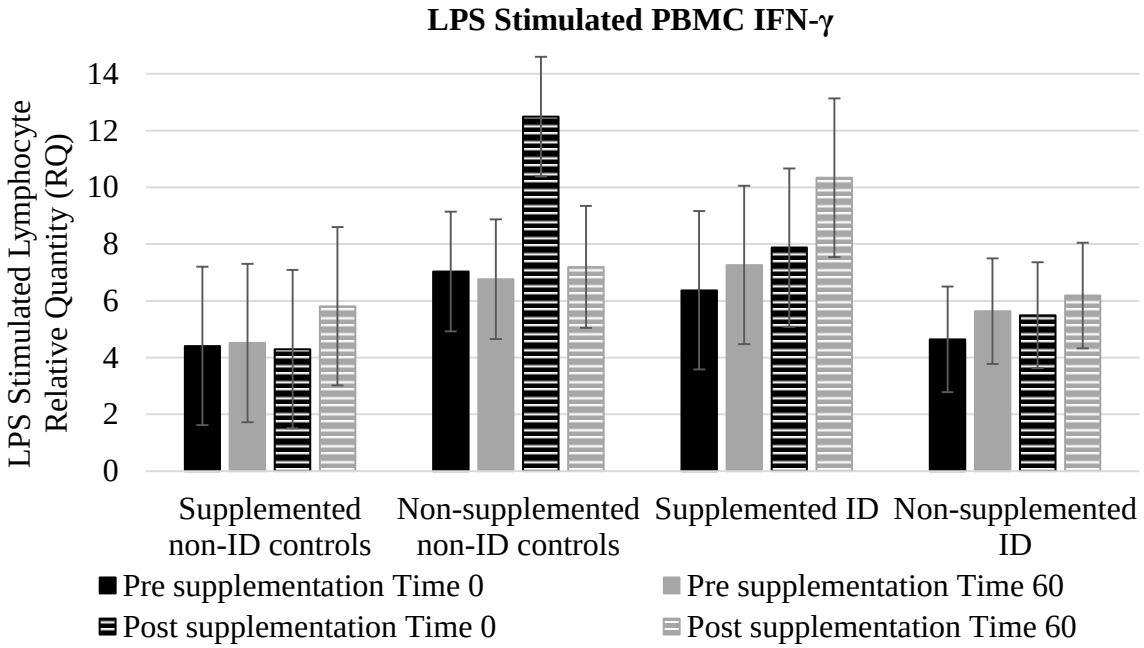

Figure 6.7. Mean (± SEM) relative quantity (RQ) of LPS stimulated PBMC IFN-γ gene expression in supplemented non-ID control horses (n = 7), non-supplemented non-ID control horses (n = 4), supplemented ID horses (n = 9) and non-supplemented ID horses (n = 4) at two different time points (baseline prior to oral sugar administration (time point 0; represented in black) and 60 min following (time point 60; represented in gray) as well as at the pre (represented by solid bars) and post (represented by corregated bars) supplementation periods. Significant ($P < 0.05$) treatment by time by period interaction with non-supplemented horses higher in IFN-γ at the post supplementation period for time point 0 compared to time point 60 as well as pre supplementation for time point 0, and supplemented horses were higher in IFN-γ at the post supplementation period for time point 60 compared to time point 0 as well as pre supplementation for time point 60. Significant treatment by time interaction with supplemented horses lower in IFN-γ at time point 0 compared to time point 60. Significant group by time interaction, no significant pairwise comparisons. Significant overall effect of period with horses higher at the post supplementation period compared to pre.

6.3.2.3. Whole blood inflammatory gene expression

There were no differences in whole blood gene expression of TLR-4 (data not shown), regardless of time point, metabolic group, period, or treatment. There was an effect of period on IL-6 ($P = 0.0009$) and IL-10 ($P = 0.0009$) gene expression (data not shown). Horses had higher IL-6 but lower IL-10 at the pre supplementation period compared to post. There were no other effects on IL-6 or IL-10 whole blood gene expression. There were no significant effects for any variable measured on TNF-α, but there was a trend for

an effect of period (P = 0.0539) (Figure 6.8), where horses increased in TNF-α from the pre supplementation to post supplementation period. Additionally, there was a trend for a metabolic group by period interaction (P = 0.0766), however while results reflected an increase in gene expression of TNF-α in non-ID control horses from the pre to post supplementation period, pairwise comparisons were only trending.

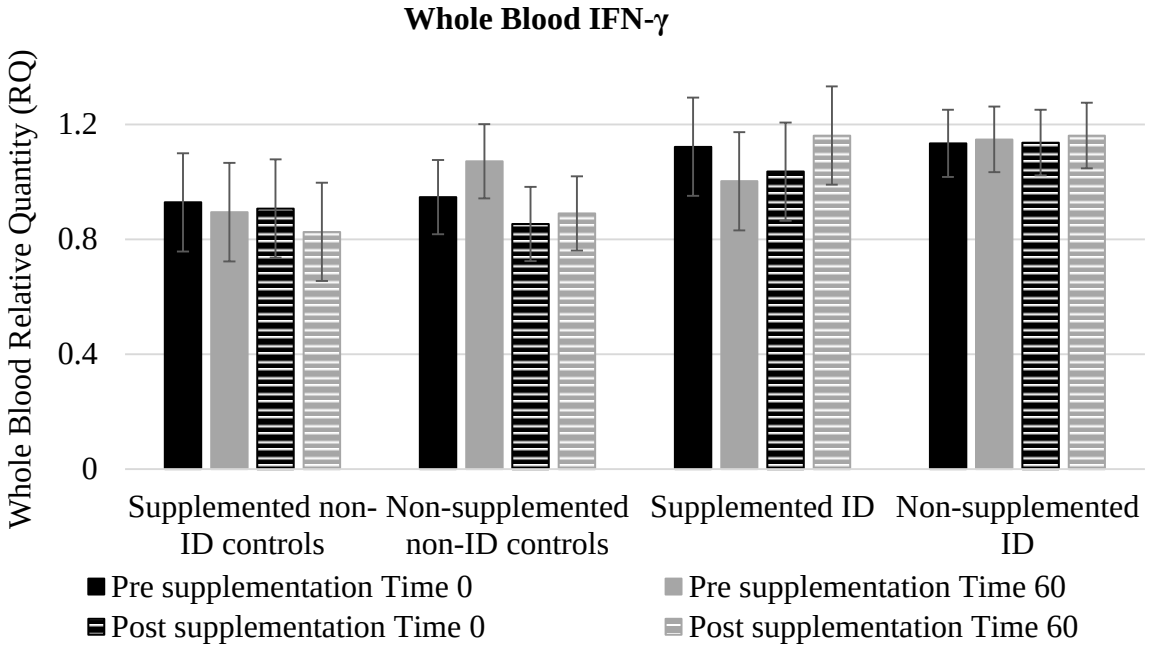

Figure 6.8. Mean (± SEM) relative quantity (RQ) of whole blood IFN-γ gene expression in supplemented non-ID control horses (n = 7), non-supplemented non-ID control horses (n = 4), supplemented ID horses (n = 9) and non-supplemented ID horses (n = 4) at two different time points (baseline prior to oral sugar administration (time point 0; represented in black) and 60 min following (time point 60; represented in gray) as well as at the pre (represented by solid bars) and post (represented by corregated bars) supplementation periods.

6.3.3. Serum inflammatory protein markers

There were no differences or trends for serum inflammatory markers as measured by CPR ELISA, or Luminex multiplex assay for IL-6, MCP-1, or IFN-γ concentrations for any variable measured (data not shown). There was an effect of time on TNF-α serum protein concentrations (P = 0.0302) (Figure 6.9) as measured by ELISA, with horses decreasing in TNF-α in response to OST.

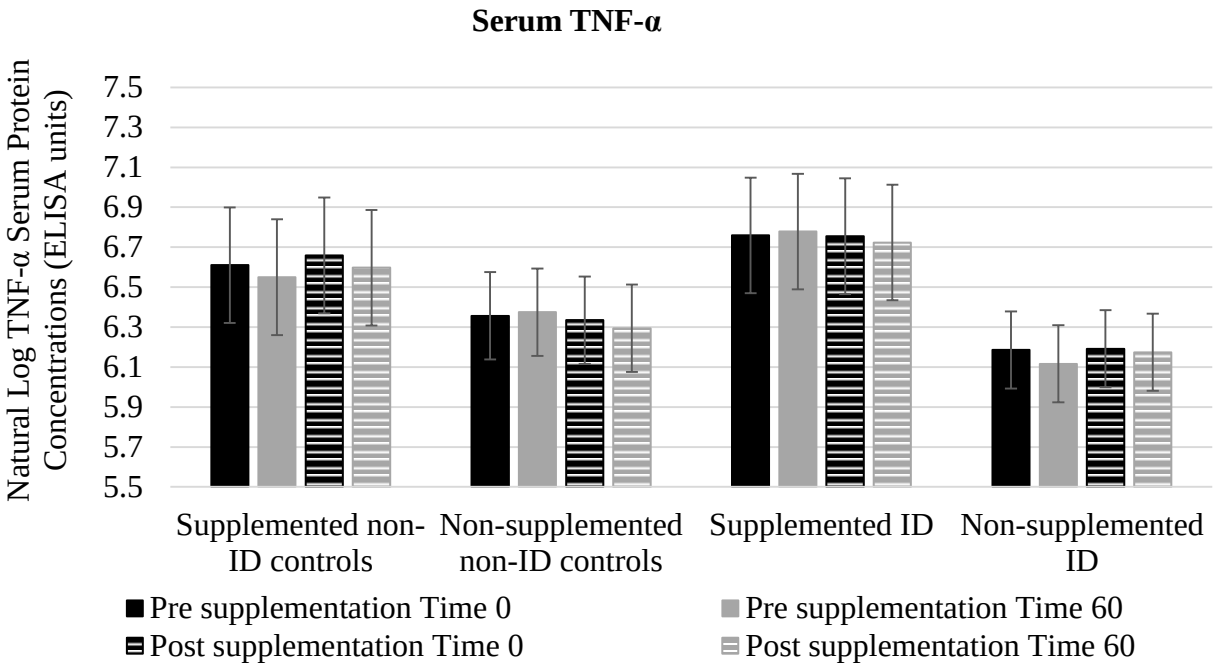

Figure 6.9. Mean (± SEM) natural log of serum TNF-α concentrations (ELISA) in supplemented non-ID control horses (n = 7), non-supplemented non-ID control horses (n = 4), supplemented ID horses (n = 9) and non-supplemented ID horses (n = 4) at two different time points (baseline prior to oral sugar administration (time point 0; represented in black) and 60 min following (time point 60; represented in gray) as well as at the pre (represented by solid bars) and post (represented by corregated bars) supplementation periods. Significant ($P < 0.05$) overall effect of time point with horses higher at time point 0 compared to time point 60.

6.3.4. Plasma LPS concentrations

LAL assay was able to detect differences in LPS plasma concentrations (Figure 6.10). There was a metabolic group by treatment by period interaction ($P = 0.0261$). Non-supplemented non-ID control horses at prior to supplementation were higher compared to post ($P = 0.009$), as well as compared to supplemented ID horses post supplementation ($P = 0.042$). They also had trends to be higher compared to non-supplemented ID horses at either periods ($P = 0.0761$ pre, $P = 0.0562$ post). Additionally, there was a treatment by period interaction ($P = 0.0338$) with non-supplemented horses decreasing in LPS concentrations from the pre to post supplementation period ($P = 0.0159$), and an overall effect of period ($P = 0.0338$) with horses having lower LPS concentrations following the supplementation period.

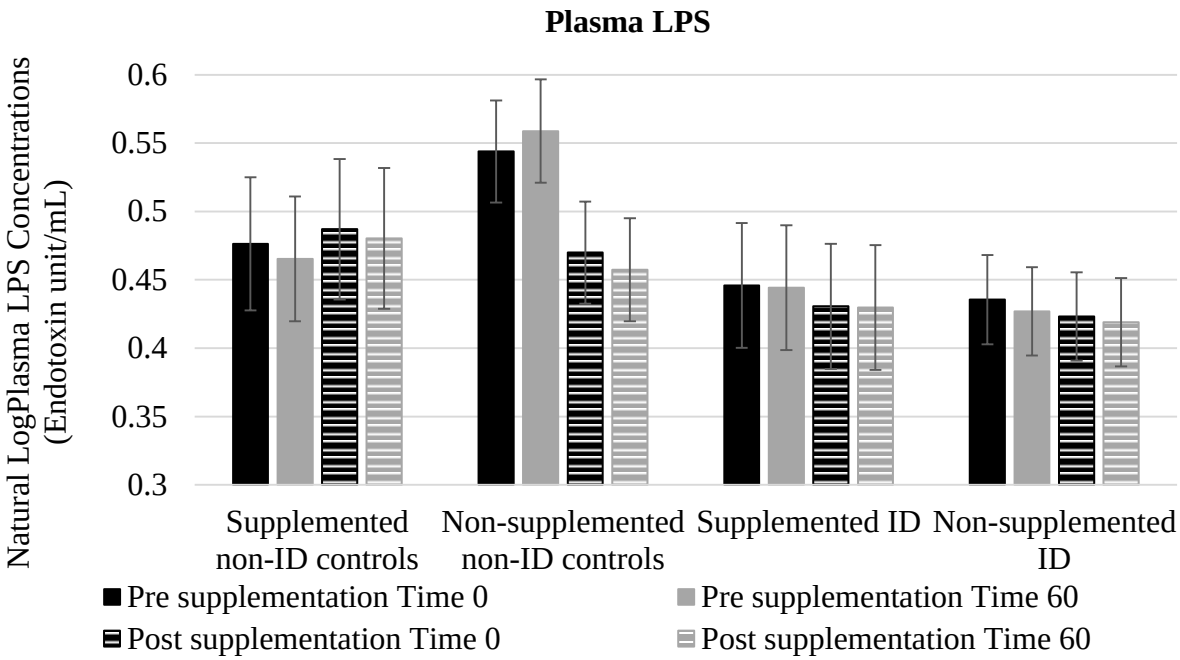

Figure 6.10. Mean (± SEM) natural log of plasma LPS concentrations in supplemented non-ID control horses (n = 7), non-supplemented non-ID control horses (n = 4), supplemented ID horses (n = 9) and non-supplemented ID horses (n = 4) at two different time points (baseline prior to oral sugar administration (time point 0; represented in black) and 60 min following (time point 60; represented in gray) as well as at the pre (represented by solid bars) and post (represented by corregated bars) supplementation periods. Significant group by treatment by date interaction, with non-supplemented non-ID controls higher in LPS pre supplementation period compared to post. Significant ($P < 0.05$) treatment by period interaction, with non-supplemented horses higher pre supplementation period in LPS compared to post, and an overall effect of date with horses higher pre compared to post supplementation period.

6.4. Discussion

There was no effect of supplementation with a manna-rich fraction of the YCW on either serum insulin or glucose concentrations in either ID or non-ID control horses. However, there was a trend for a metabolic group by treatment by period interaction for serum glucose. This trend indicated that supplemented ID horses increased in serum glucose from pre to post supplementation, and at the post supplementation period were higher compared to supplemented non-ID controls. In a bovine study, the opposite effect was seen in metabolically normal heifers supplemented for 52 days with Saccharomyces cerevisiae yeast cell wall [102], where heifers had a decrease in serum glucose and an increase in insulin. However, while the increase in insulin was seen following

supplementation, the decrease in glucose was observed following supplementation but only in response to an endotoxin challenge in supplemented heifers. In addition, pigs fed a high chromium yeast supplement had an increased glucose tolerance and clearance following 23-30 days of supplementation [255]. The differences in these results may indicate that yeast cell wall supplementation contributes to an increase in serum glucose concentrations in horses with ID. Conversely, this increase in glucose may be due to an increased susceptibility to dietary changes in ID horses, be it caloric or other nutrient intake, or treatment period or dosing may have been inadequate to positively impact the metabolic parameters measured. Metabolically, horses responded as expected to glycemic challenge in the form of an OST, increasing in glucose and insulin concentrations between the 0 and 60 time points, with ID horse having greater serum concentrations compared to non-ID control horses. There was an effect of period on concentrations of both glucose and insulin, with horses increasing from the pre supplementation (July) to post supplementation (September) period. This may indicate an effect of season on metabolic parameters, which have been proposed to be higher in the fall months in horses with PPID [256] and higher in February compared to August and November and June compared to November in healthy horses [257]. However, others have not observed the same in a group of healthy or ID horses [258]. Regardless, these data support an effect of season on serum concentrations of both glucose and insulin, in particular in horses with ID.

Effects of YCW supplementation on inflammatory markers were minimal. Non-supplemented horses had a decrease in LPS concentrations following the 45 day supplementation period whereas supplemented horses did not. Further, when examining pairwise comparisons for the metabolic group by treatment by period interaction, it was the non-ID control horses who were non-supplemented that had this decrease in LPS concentrations between periods. For LPS stimulated lymphocyte IFN-γ gene expression, there was a significant treatment by time by period interaction, indicating that both supplemented and non-supplemented horses had an increase following the 45 day supplementation period (within time point 60 for supplemented horses and within time point 0 for non-supplemented horses). Interesting, post supplementation, supplemented and non-supplemented horses had opposing responses to the OST with supplemented horses increasing from time point 0 to time point 60 in LPS stimulated lymphocyte IFN-γ gene

expression whereas non-supplemented horses decreased. There was also a significant overall effect of treatment on LPS stimulated lymphocyte IL-10 gene expression, with non-supplemented horses having higher gene expression compared to those who received YWC. There were no other significant effects of treatment, although several trends were observed. Of interest was the trend for the 4 way interaction between metabolic group, treatment, time, and period for the percentage of lymphocytes stained positive for TNF-α. Within time point 0, ID supplemented horses had a decrease in TNF-α following supplementation. Non-ID control horses, regardless of supplementation status, had a similar decrease between periods. However, non-supplemented ID horses had no difference in their percentage of lymphocytes stained positive for TNF-α between the pre and post supplementation period. These data suggest that supplementation with yeast cell wall in horses may have some impact on lymphocyte mediated inflammation.

Differences due to metabolic status, in particular in response to the OST, in respect to inflammatory parameters were varied. We and others have previously shown that in response to a meal or an oral sugar challenge, ID and non-ID control horses have an inflammatory response [46, 47], and that this response can differ between the two metabolic groups [47]. There was a significant metabolic group by time by period interaction for LPS stimulated lymphocyte TLR-4 gene expression. ID horses had a large increase in TLR-4 gene expression in response to oral sugar prior to supplementation that was not seen post supplementation or in the non-ID control horses. There was also a significant metabolic group by time interaction for LPS stimulated lymphocyte IFN-γ gene expression with ID horses having a trend to increase in IFN –γ in response oral sugar administration whereas non-ID horses had a (non-significant) decrease. For TNF-α gene expression in PMA stimulated lymphocytes, post supplementation ID horses were higher in TNF-α gene expression compared to non-ID control horses at time point 60. In addition, there was a trend for a metabolic group by treatment by time interaction for LPS stimulated lymphocyte IL-6 gene expression. This trend was due to the higher IL-6 gene expression in non-supplemented non-ID control horses at time point 60. There were also trends for an effect of metabolic group on the percentage of lymphocytes stained positive for both TNF-α and IFN-γ. ID and non-ID control horses supplemented with yeast cell wall decreased in TNF-α following supplementation, as discussed above, whereas non-supplemented ID

horses did not. For IFN-γ, non-ID control horses decreased in IFN-γ between the two periods, whereas the ID horses did not change. Finally there was a trend for a metabolic group by period interaction for whole blood IFN-γ gene expression, and while pairwise comparisons were only trends, they indicated that post supplementation non-ID controls had a tendency to be lower compared to ID horses regardless of period. These data may further point to an increased sensitivity to changes in season in horses with ID.

In respect to overall differences between the 0 and 60 time points, PMA stimulated lymphocyte IFN –γ gene expression and TNF-α serum protein concentrations (ELISA) in horses were overall higher at time point 0 vs time point 60. However, for LPS stimulated lymphocyte TNF-α and TLR-4 horses were higher at time point 60 compared to time point 0. Further indications of an effect of time were observed in significant interactions for LPS stimulated lymphocyte TLR-4 gene expression (discussed above) and LPS stimulated lymphocyte IFN –γ gene expression. Interactions involving time point seen with IFN –γ show that following the supplementation period, supplemented horses had an increase in gene expression in response to the OST whereas non-supplemented horses had a decrease. Further, regardless of period and treatment, this pattern was observed in respect to metabolic status (discussed above). Trends were observed for an overall effect of time as well. They indicated that gene expression of TLR-4 in PMA stimulated lymphocytes was increased in response to OST, likely due to a large increase prior to supplementation in the ID horses. Gene expression of IL-6 in PMA and LPS stimulated lymphocytes was also increased in response to OST, but this increase was seen in non-ID controls or non-supplemented non-ID controls, not in ID horses or horses receiving YCW supplementation. Additionally, the percentage of lymphocytes producing TNF-α (% gated) had a trend to decrease from the 0 to 60 time points and a trend for a 4 way interaction indicating that non-supplemented non-ID control horses pre supplementation were primarily responsible for this decrease in TNF-α % gated in response to the OST.

Similar to metabolic parameters, there was an apparent effect of period on inflammatory markers. There was an overall increase following the 45 day supplementation period in IFN-γ gene expression of LPS simulated lymphocytes, and horses had trends to be higher in TLR-4 gene expression of PMA simulated lymphocytes and TNF-α gene expression in whole blood. On the other hand, they had a decrease

following the supplementation period in plasma LPS concentrations, the percentage of lymphocytes stained positive for both TNF-α and IFN-γ, whole blood gene expression of IL-10, and whole blood gene expression of IL-6. There have been indications in other species, such as baboons, fish, and rodents that season is able to impact the immune response [259-261]. Specifically, serum concentrations of CRP were greater in June compared to December, and a seasonal influence was also seen for serum IL-6 levels in baboons [259]. In fact, a decrease in IL-6 and IL-1β in Siberian hamsters in response to LPS and shorter day lengths indicate a that the immune response may be reduced in the winter months [260]. There has been some work related to the immune response and season in the horse [262-264]. Seasonal allergy responses in PBMCs in particular have been utilized [265, 266] and show increased PBMC production of IFN-γ and a decreased production of IL-4 in the winter compared to summer months [265]. However, another study in horses with summer pasture-associated obstructive pulmonary disease (SPAOPD) as well as controls, showed increases in PBMC IFN-γ gene expression in the summer as compared to the winter [266]. For this study horses had an increase from July to September in pro-inflammatory cytokine or TLR gene expression of IFN-γ, TNF-α, and TLR-4. However, horses had a decrease in gene expression of the anti-inflammatory IL-10 as well as IL-6, lymphocyte protein concentrations of IFN-γ and TNF-α, and plasma LPS concentrations. This may point to seasonal variations in post transcriptional factors, but more research is needed to better understand possible seasonal influences on inflammatory responses in both the ID and metabolically normal horse.

Metabolic parameters were increased from pre to post supplementation and in response to oral sugar administration, in particular in ID horses. However there was not a significant effect of YCW supplementation on either basal or OST responses of serum insulin or glucose. There were limited effects of treatment and metabolic status on inflammatory markers, however non-supplemented horses or non-ID controls frequently had a decrease in pro-inflammatory markers of inflammation at both the protein and gene expression levels in response to OST whereas ID horses increased. Overall, these data show a strong effect of period and OST on the variables measured and suggest that ID horses may be particularly sensitive to changes in metabolic stimuli or season.

CHAPTER 7 EQUINE WHOLE BLOOD INFLAMMATORY GENE EXPRESSION IN INSULIN DYSREGULATED (ID) AND CONTROL HORSES IN RESPONSE TO A TLR-4 AGONIST, LPS

Insulin dysregulation (ID) in the horse is considered to be an increasing problem, due in large part to its association with the often career or life ending condition, laminitis. However, underlying mechanisms of ID and the development of laminitis, in particular as they relate to inflammation, remain unclear. There have been reports that horses with ID have increased levels of inflammatory cytokines, especially in response to oral glycemic challenge. It has also been shown that both levels of inflammation as well as bacterial endotoxin, or lipopolysaccharide (LPS), are increased in humans in response to a meal. The primary pattern recognition LPS receptor in humans and horses is a member of the toll like receptor family, TLR-4 (toll like receptor 4). It can function in response to LPS through the MyD88 (Myeloid differentiation primary response gene 88) dependent or independent pathways. However, it remains to be seen if differences in postprandial inflammatory responses in ID horses are driven in part by differential regulation of LPS TLR-4 inflammatory signaling. We therefore hypothesized that horses with ID would have differences in their TLR4 mediated inflammatory response to an oral glycemic challenge in response to LPS stimulation. To accomplish this, a total of 14 adult, non-PPID, mixed-breed and mixed-sex horses (7 ID and 7 non-ID controls, not different in age) were used in this study. Horses were administered an oral sugar test (OST), and blood collected via jugular venipuncture prior to (time point 0) and 60 min post oral sugar administration (time point 60). Blood was analyzed at both time points for serum insulin concentrations as well as whole blood inflammatory markers in both LPS stimulated and unstimulated samples. As expected, ID horses had significantly greater serum insulin at time point 60 in comparison to controls ($P < 0.0001$). LPS was able to induce gene expression in relation to unstimulated samples in all horses for all inflammatory markers, with the exception of the chemokine CCL2 (chemokine (C-C motif) ligand 2; $P < 0.005$). Inflammatory markers associated with the MyD88 independent pathway were however only minimally increased or did not change in response to LPS stimulation. There was an effect of oral sugar administration, with decreases in CCL2 and TLR-4 in LPS stimulated samples. Further, whole blood gene expression of the chemokines IL-8 (interleukin 8) and CCL2 were lower

in ID compared to control horses. This indicates that in whole blood, LPS stimulates inflammatory responses primarily of MyD88 dependent associated genes, regardless of metabolic status, and that horses with ID may have an impaired innate immune response. However, more work is needed to understand possible differences in immune responses of ID compared to metabolically normal horses.

7.1. Introduction

Metabolic dysfunction, in particular insulin dysregulation, is considered to be of concern in the equine due to its association with numerous health concerns including the potentially fatal condition, laminitis [12, 15]. In humans, low grade systemic inflammation is often observed in individuals with metabolic dysfunction [267], but although this is suggested to be the case in the horse as well [127, 139, 140], the link between inflammation and ID, as well as underlying mechanisms of ID and relationship to laminitis in the horse remains unclear. Environmental factors, in particular over-nutrition and a lack of exercise leading to an increased fat mass are thought to be influential in the development of metabolic dysfunction in the horse [142]. Adipose tissue is itself categorized as an endocrine organ, and as such is capable of producing inflammatory cytokines [268]. There has been evidence in obese or insulin resistant humans and mice however, that adipose tissue macrophages, not adipose tissue, have a greater role in the production of inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α) [145, 146]. Adipose tissue dysfunction is often a prominent aspect of obesity and ID. This dysfunction involves macrophage accumulation, wherein these cells accumulate in greater numbers and size in the tissue, and favor M1 (largely proinflammatory) over M2 (largely anti-inflammatory) macrophages [131, 147].

Recognition of pathogens by macrophages principally occurs via pattern recognition receptors (PRR). In particular, the PRR toll-like receptor 4 (TLR4), is primarily responsible for recognition of bacterial lipopolysaccharide (LPS) [148]. Once triggered by LPS, TLR4 (along with its co-receptors cluster of differentiation 14 (CD14) and lymphocyte antigen 96 (MD-2)) activate a signaling cascade which ultimately allows the release of NF- κB (nuclear factor kappa-light-chain-enhancer of activated B cells) and its translocation into the cell's nucleus for induction of proinflammatory gene expression

[149]. TLR-4 predominantly functions in response to LPS through one of two pathways; MyD88 (Myeloid differentiation primary response gene 88) dependent TIRAP (TIR Domain Containing Adaptor Protein) pathway, resulting in proinflammatory cytokine production (such as TNF-α and IL-6 (interleukin 6)), or the MyD88 independent TRIF (TIR-domain-containing adapter-inducing interferon-β) pathway, resulting in the production of type I interferons (such as interferon beta, or IFN-β), and chemokines such as CCL5 (Chemokine (C-C motif) ligand 5, or RANTES) and CCL2 (Chemokine (C-C motif) ligand 2, or MCP-1) [269, 270]. Not only is this receptor complex able to recognize LPS, but it is also able to recognize free fatty acids (FFA), resulting in a similar activation of NF- κB and subsequent production of proinflammatory cytokines, including TNF-α [148, 150, 270].

There have been reports that TLR4 concentrations and gene expression are elevated in both animals and humans with metabolic dysfunction [271, 272]. Indeed, high fat fed mice have been used to show that endotoxemia can induce both inflammation and insulin resistance [61]. Further, increases in inflammatory markers and endotoxin concentrations [43, 46, 47, 59, 60, 273] have been observed in multiple species, including the horse, following a meal. Considering the proposed involvement of the gut both in metabolism and the inflammatory process, these postprandial increases in concentrations of bacterial endotoxin are not surprising [62, 63]. Therefore, the goal of this work was to examine mechanisms relating to differences in inflammatory responses in ID horses, possibly due to alterations in TLR-4 inflammatory signaling in response to glycemic challenge as well as endotoxin stimulation.

7.2. Materials and methods

7.2.1. Horse selection and study design

A total of 14 mixed-breed and mixed-sex horses were selected from the University's Department of Veterinary Science Woodford County herd; 7 ID and 7 non-ID controls. ID was established using an oral sugar test (OST) [13, 47], with hyperinsulinemia diagnosed as a fasting serum insulin level of > 20 μIU/mL and a concentration of insulin > 60μU/mL 60 minutes post OST was considered further indicative of ID. Horses were also scored for body condition (BCS) based on the 1-9

Henneke scoring system [186] and cresty neck score (CNS) based on the 0-5 Carter et. al. scoring system [187] to determine overall and regional adiposity, respectively. Phenotypic data is presented in Table 7.1. Breed distribution of the ID horses (3 geldings and 4 mares; average age 13.6 yrs) was as follows; 1 was of mixed breed, 2 were Warmbloods, 2 were Standardbred or Standardbred cross, 1 a Morgan, and 1 a Quarter Horse. Breed distribution of the 7 non-ID controls horses (3 geldings and 4 mares; average age 13.1 yrs) was as follows; 3 were of mixed breed, 3 were Thoroughbreds, and 1 was a Quarter Horse. Horses were housed at the University of Kentucky's Department of Veterinary Sciences Woodford County facility and allowed to acclimate for > 9 mo to their respective pastures prior to the start of the study. They were provided ad libitum access water and a trace mineralized salt block. While none of the horses were fed any form of concentrate and pasture was minimal and composed of mixed-grass, they were given unrestricted access to a mixed-grass hay.

Table 7.1. Phenotypic measures of ID and control horses.

Group	Baseline Insulin	Insulin 60 min post oral sugar administration	BCS	CNS
Control	13.9 ± 11.4	21.8 ± 11.4	6 ± 0.3	1.8 ± 0.3
ID	40.7 ± 11.4	113.8 ± 11.4	7 ± 0.4	2.8 ± 0.4
p value	0.1108	< 0.0001	0.1061	0.0798

Data are presented as least square means ± standard error of the mean for insulin, body condition score (BCS), and cresty neck score (CNS). n = 7 ID and n = 7 controls. BCS and CNS are presented as an average score between 4 trained individuals.

This was an observational study with all horses sampled during the winter season of 2017. Fourteen non-PPID (see below) mixed-breed and mixed-sex animals (7 ID and 7 non-ID controls) were selected. An OST (described above) was administered, and blood samples from time points 0 and 60 were collected for determination of serum insulin levels as well as whole blood gene expression of markers of inflammation.

Thyrotropin resealing hormone (TRH) stimulation was used to rule out any horses affected by Pituitary Pars Intermedia Dysfunction (PPID). TRH stimulation testing was performed as previously described [47, 191, 192], with a blood sample collected via jugular venipuncture in the a.m. (between 0800 and 1000, EST). Following this, TRH (1 mg/mL; Sigma-Aldrich, St. Louis, MO) was administered intravenously and 10 min later a second

blood sample collected. Blood tubes (containing ethylenediaminetetraacetic acid, or EDTA) were placed on ice, transported to the lab, and upon arrival were centrifuged to isolate plasma [193] which was stored at -20 º C. Samples were sent on dry ice to Cornell University's Animal Health Diagnostic Center Endocrinology Laboratory (Ithaca, NY) for ACTH analysis. Analysis of ACTH was accomplished with a chemiluminescent assay (Immulite, Elangen, Germany) as previously described [195]. ACTH concentrations exceeding either > 35 pg/mL for the baseline sample or > 180 pg/mL for the 10 min sample were considered positive for PPID and were the basis for exclusion from the study.

7.2.2. Insulin determination

Serum insulin concentrations were analyzed by Cornell University's Animal Health Diagnostic Center Endocrinology Laboratory using a commercially available human insulin radioimmunoassay (RIA; EMD Millipore Corp, Billerica, MA). This assay was validated for use in equine serum as follows; 4 equine samples were serial dilutions with assay buffer parallel to the standard curve. Four different quantities of porcine insulin (Sigma-Aldrich, St. Louis, MO) were used to spike samples and concentrations averaged 96% of expected. Cross-reactivity of the RIA antibody with equine insulin was not reported by the manufacturer however, cross-reactivity was reported as 100% with porcine insulin. Homology between equine and porcine insulin is 98%. Assay sensitivity is reported as 2.72 μIU/mL by the manufacturer. Mean intra-assay coefficient of variation was 7.4 and inter-assay coefficient of variation was 6.3%.

7.2.3. Whole blood gene expression

Blood was collected via jugular venipuncture into 2 (15 mL) glass heparinized vacutainer tubes at each time point (0 and 60) and kept warm and consistently inverted to prevent separation until arrival at the lab. Following arrival, one tube per horse per time point was stimulated with 1 μg LPS (catalogue #L5293; Sigma). This dose was based on an earlier study in equine monocytes [274] and modified based on data from a pilot study that determined responses to LPS in equine whole blood (data not shown). The second tube served as an unstimulated control. All tubes were incubated at 37º Celsius on an automated shaker (Max Q4450, Thermo Fisher Scientific, Waltham, MA) for 2 hours. Following this 2 H incubation, 3 mL of blood was transferred into Tempus™ Blood RNA Tubes (Life Technologies, Grand Island, NY). Tubes were vigorously shaken following addition of

blood and incubated at room temperature for 24 hrs. Tubes were then stored at -20 ºC for later analysis. Tubes were thawed prior to analysis and Tempus Spin RNA isolation kit (Thermo Fisher Scientific, Waltham, MA) was used following the manufacturer's instructions to isolate total RNA. Once isolated, cDNA was generated by transcription of 1 µg of RNA as previously described [197]. Briefly, RNA was combined with a master mix (Promega, Madison, WI) and incubated at 42º C for 15 min and at 95º C for 5 min. The resulting cDNA (4.5 µL) was combined with 5 µL of Master Mix (SensiMix HI-ROX 2x, Bioline) and the primer-probe of interest (0.5 µL; TaqMan™). Primer-probes were equine-specific (Applied Biosystems, Foster City, CA). An Applied Biosystems Real-Time PCR system (ABI Viia7) was used to run samples against: Housekeeping gene *beta*-glucuronidase (β-gus; Ec03470630_m1), IL-6 (Ec03468678_m1), IL-1β (Ec04260298_s1), TNF-α (Ec03467871_m1), TLR4 (Ec03468994_m1), IFN-γ (Ec03468606_m1), IL-8 (Ec0346880_m1), IL-10 (Ec03468647_m1), CCL5 (Ec03468106_m1), and CCL2 (Ec03468496_m1) [199]. All samples analyzed in duplicate and after an initial 10 min incubation at 95º C underwent 10 cycles of 15s at 95º C followed by 60s at 60º C. Changes in gene expression, relative to both β-gus and mean ΔC_T for non-ID horses' time 0 media samples set as a calibrator, were calculated using the $\Delta\Delta C_T$ method [200]. Results are expressed as relative quantity (RQ), calculated as $2^{-\Delta\Delta CT}$.

7.2.6. Statistical analysis

Prior to analysis, data points determined to be outliers as established as being 1.5 times the interquartile range below or above the 1st or 3rd quartiles, respectively were removed from the data set. Analysis of data were completed with repeated measures ANOVA using PROC MIXED, SAS 9.4 (SAS Institute Inc., Cary, NC). Horse ID, group (ID vs non-ID controls), time (0 vs 60 min OST time points), and treatment (non-stimulated tubes vs those stimulated with LPS) were set at fixed effects and all possible interactions were considered. The repeated measure was set as time, with the subject of horse ID. Pairwise comparisons were made using protected LSD. All data were normally distributed as determined by analysis of residuals. Residuals were visually normally distributed and had a skew of < 2 and kurtosis of < 7. Differences were considered statistically significant when $P < 0.05$ and trends were considered at $P < 0.10$.

7.3. Results

While ID horses met the criteria for hyperinsulinemia, having resting insulin concentrations > 20 μIU/mL, they were not significantly in higher serum insulin concentrations (Table 7.1) compared to controls at time point 0 prior to oral sugar administration ($P = 0.1108$). However, they significantly increased from time point 0 to time point 60 ($P = 0.0002$) and were higher at that time point compared to controls ($P < 0.0001$).

Results for gene expression of inflammatory markers in LPS treated and unstimulated whole blood samples from ID and control horses both prior to (time point 0) and 60 min following oral sugar administration (time point 60) are presented in Figures 7.1 and 7.2. Regardless of metabolic status or time point, LPS stimulation was able to significantly increase whole blood gene expression for all variables measured ($P < 0.005$), with the exception of CCL2 ($P = 0.3013$, Figure 7.2). It especially effected interleukin gene expression, inducing the highest expression in IL-1β and IL-6 (Figure 7.1).

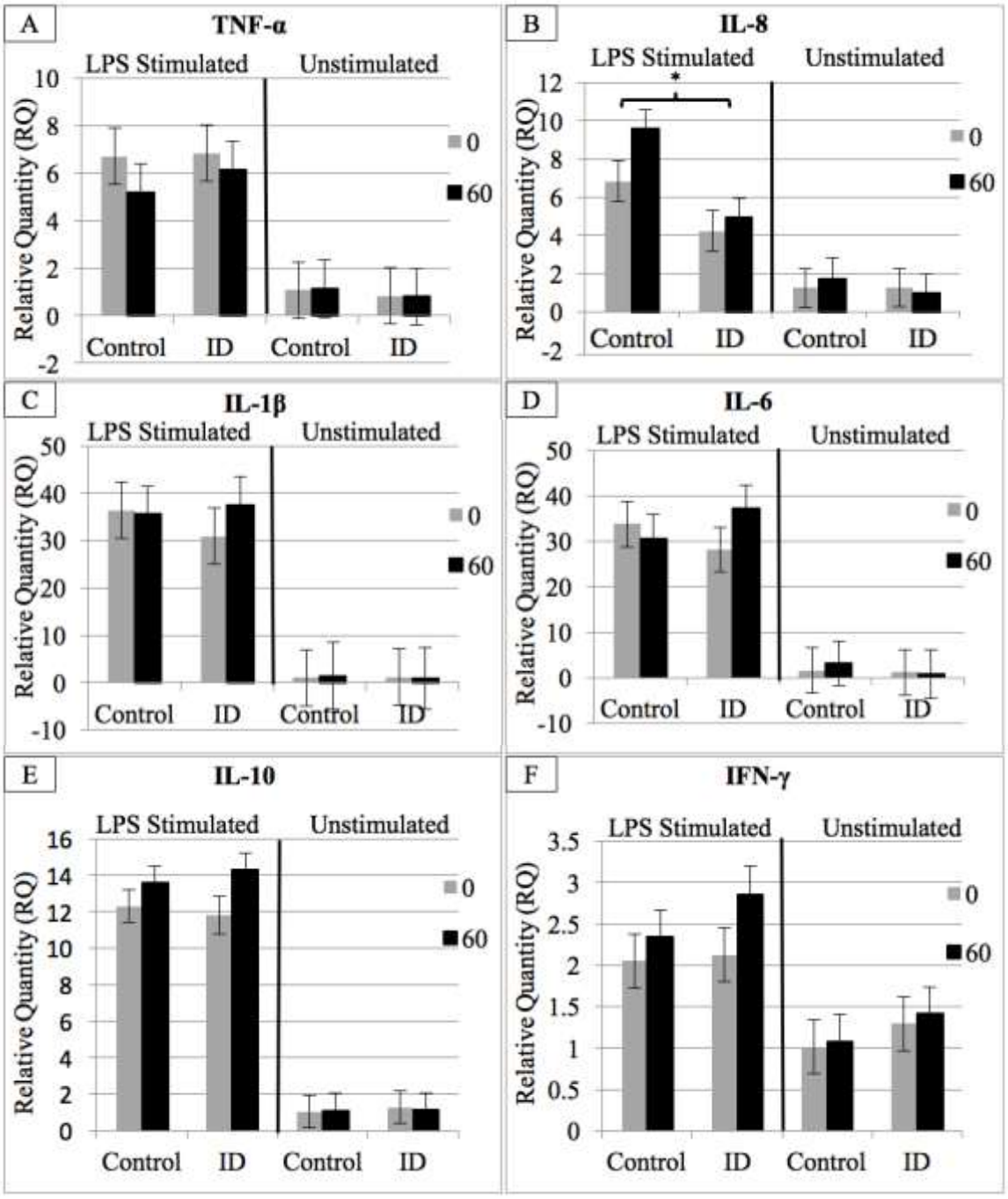

Figure 7.1. Mean (± SEM) mRNA gene expression represented as relative quantities (RQ) of (A) TNF-α, (B) IL-8, (C) IL-1β, (D) IL-6, (E) IL-10, and (F) IFN-γ in equine whole blood both incubated for 2 hrs with 1 µg LPS (LPS stimulated; left panel of the graphs) and incubated without LPS (Unstimulated; right panel of the graphs) at two different time points (baseline prior to oral sugar administration (time point 0) and 60 min following (time point 60)). Differences between ID and controls ($P < 0.05$) are represented by *.

There were some overall effects of time on gene expression. For TLR-4 (Figure 7.2), time point 0 was lower compared to time point 60 (P = 0.0126). However, time point 0 was higher compared to time point 60 for CCL2 (MCP-1) gene expression (P = 0.018). Additionally, there was a trend for an effect of time (P = 0.0972) for IFN-γ (Figure 7.1), with horses at time point 0 lower compared to time point 60.

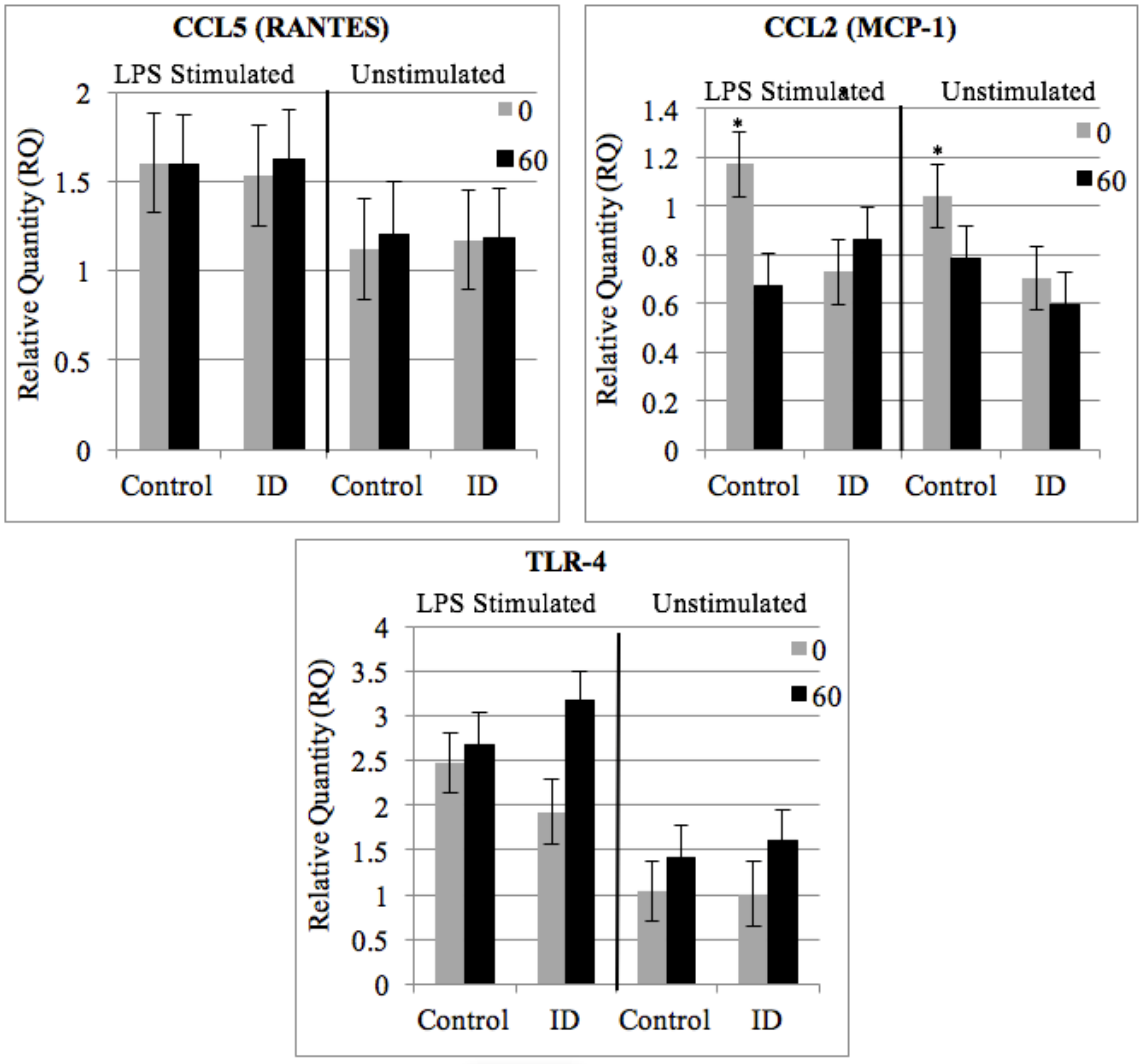

Figure 7.2. Mean (± SEM) mRNA gene expression represented as relative quantities (RQ) of (A) CCL5 (RANTES), (B) CCL2 (MCP-1), and (C) TLR-4 in equine whole blood both incubated for 2 hrs with 1 µg LPS (LPS stimulated; left panel of the graphs) and incubated without LPS (Unstimulated; right panel of the graphs) at two different time points (baseline prior to oral sugar administration (time point 0) and 60 min following (time point 60)). Differences between ID and controls within time point ($P < 0.05$) are represented by *.

IL-8 gene expression had a significant overall effect of group (P = 0.0189; Figure 7.1), with control horses higher compared to ID horses. There was also a significant group by treatment interaction (P = 0.0329). All groups and treatments were significantly different from each other for IL-8 gene expression (P < 0.0018), with the exception of unstimulated samples where control and ID horses were not different (P = 0.726). There was also a significant group by time point interaction (P = 0.0125) for CCL2 gene expression, with control horses higher compared to ID horses within time point 0 (P = 0.0217) and control horses having a significant decrease in gene expression between time points 0 and 60 (P = 0.0009).

While there were no significant three-way group by time by treatment interactions, there were some significant pairwise comparisons. For TLR-4 gene expression (Figure 7.2), ID horses had an increase from time point 0 to time point 60 in their LPS stimulated samples (P = 0.0098). ID horses were lower compared to controls for gene expression of IL-8 in LPS stimulated samples at time point 60 (P = 0.0021) and there was a trend for a difference for ID to be lower compared to control horses for LPS stimulated samples at time point 0 (0.0976), as well as a trend for an increase in control horses' LPS simulated samples between time point 0 and 60 (P = 0.0722). ID horses had a trend for an increase in IL-10 (Figure 7.2) from time point 0 to time point 60 in LPS stimulated samples (P = 0.0663). IFN-γ gene expression in ID horses increased from time point 0 to time point 60 in LPS stimulated samples (P = 0.055). Finally, for CCL2 gene expression, control horses LPS treated samples decreased in CCL2 gene expression between time points 0 and 60 (P = 0.0017). Control horses had a higher gene expression in LPS treated samples at time point 0 compared to ID horses (P = 0.0248). There was also a trend for control horses unstimulated samples to be higher at time point 0 compared to 60 (P = 0.0938), and for time point 0 unstimulated samples the control horses had a trend to be higher compared to the ID horses (P = 0.0805).

7.4. Discussion

While gene expression was increased in response to incubation with LPS for all markers of inflammation (with the exception of CCL2), expression was increased minimally for most. It was particularly low for CCL5 (RANTES) and CCL2 (MCP-1),

genes associated with the MyD88-independent TRIF pathway. Similar results have previously been seen by others in monocytes of metabolically normal horses stimulated with 100 pg/mL LPS [274]. Surprisingly, TLR-4 gene expression was also low. This may be due to potential crosstalk between TLR-4 and TLR-2 [275]. Interestingly, the cytokine and chemokine with significant differences between ID and controls, IL-8 and CCL2, have been shown to be released in human keratinocytes in response to a TLR2/TLR1 heterodimer specific mitogen [276]. In other species, MyD88 dependent TLR-4 signaling has been demonstrated to be activated prior to MyD88 independent signaling, and TLR-4 internalized within 60-120 min following LPS binding [277, 278]. Therefore, lower gene expression of TLR-4 and other proinflammatory cytokines typically associated with MyD88 dependent signaling (such as TNF-α) is likely due to LPS incubation length. After 2 h, MyD88 dependent signaling has presumably passed its peak activity, TLR-4 internalized and in the process of being degraded, and later stage activation of MyD88 independent signaling in process. However, more work would be needed to see if this is the case in the horse. In particular, further investigations of these pathways in adipose tissue macrophages would be of interest.

Small sample size and subsequent lack of power likely contributed to the lack of differences between ID and control horses. Interestingly, though not statically significant, horses with ID often had an opposing inflammatory responses to oral sugar administration in stimulated samples compared to controls. This is similar to previous, where we demonstrated that in response to the OST, PMA (phorbol myristate acetate) stimulated lymphocytes of horses with ID had either an increase or no change in markers of inflammation as opposed to metabolically normal controls, who had a decrease in these same markers of inflammation [47]. For this work, CCL2, CCL5, IL-1β, and IL-6 gene expression in ID horses LPS stimulated samples numerically increased in response to oral sugar whereas control horses decreased. In addition, while the three-way group by time by treatment interaction was not significant for any variable measured, some pairwise comparisons were significant. These differences showed that ID horses LPS stimulated samples had an increased TLR-4 and IFN-γ gene expression in response to oral sugar for with ID horses higher at time point 60 compared to controls. Additionally, control horses

LPS treated samples decreased in CCL2 gene expression between time points 0 and 60, whereas ID horses did not.

There were significant effects of metabolic status on IL-8 and CCL2 gene expression. Obese humans or those with metabolic dysfunction have been shown to have increased circulating or levels of TNF-α, IL-6, CCL2, and IL-8 [279-282]. Healthy elderly humans have been shown to have an increased whole blood inflammatory cytokine protein production (IL-1, IL-6, and IL-8) in response to LPS stimulation compared with young controls [283]. This has similarly been observed in elderly horses and those with PPID, with reported increases in IL-8 LPS stimulated gene expression in equine leukocytes [284]. Further, McFarlane and colleagues described increased chemotactic ability in elderly horses and those with PPID [285]. However, IL-8 lymphocyte gene expression was not different between obese hyperinsulinemic and non-obese normoinsulinemic controls in another study [31], although investigators did see an increase in neutrophil oxidative activity in the obese hyperinsulinemic group. For the horses in this work, all were classified as adults with no difference in age between the control and ID groups. Therefore, lower IL-8 LPS stimulated whole blood gene expression in ID horses, in particular in response to oral sugar administration, indicates a reduced chemotactic and therefore reduced neutrophil function in metabolically dysfunctional animals. Interestingly, the other inflammatory marker with significant differences related to group was the chemokine CCL2, or MCP-1. At time point 0 control horses were higher compared to ID horses for this chemokine, further supporting the hypothesis that horses with metabolic dysfunction may have a decreased innate immune response.

7.5. Conclusions

These data suggest that equine whole blood gene expression of inflammatory markers, particularly interferons, are induced by LPS. Increases in gene expression in response to LPS appear to occur primarily in MyD88 dependent associated genes, with little or no increase in gene expression MyD88 independent TRIF associated genes, as evidenced by weak induction of TRIF associated inflammatory markers, CCL5 (RANTES) and CCL2 (MCP-1). Oral sugar administration appears to affect LPS induced gene expression of inflammatory markers, with a significant effect on CCL2 (MCP-1) and TLR-

4, and a trend for changes in IFN-γ and IL-10 gene expression. Further, reduced whole blood gene expression of chemokines IL-8 and CCL2 in ID compared to control horses indicate that horses with metabolic dysfunction may have an impaired innate immune response, however more work is need to further understand possible differences and their implications in the horse.

CHAPTER 8 THE EFFECT OF ALGAL DOCOSAHEXAENOIC ACID (DHA) CONTAINING SUPPLEMENTATION ON METABOLIC AND INFLAMMATORY PARAMETERS OF HORSES WITH EQUINE METABOLIC SYNDROME (EMS)

There is a high incidence of obesity in the equine population, with some reporting up to 51% of the population classified as overweight or obese. Obesity in horses is associated with more serious health concerns such as insulin dysregulation (ID) and equine metabolic syndrome (EMS). Accepted treatments for EMS are limited and not always effective. Omega-3 fatty acid supplementation has been suggested as a therapeutic for humans with metabolic dysfunction, as it has been purported to improve insulin sensitivity and reduce inflammation in these individuals. However, no work has been conducted to investigate the effects of the omega-3 fatty acid docosahexaenoic acid (DHA) provided from an algal source on metabolic or inflammatory parameters in the metabolic syndrome horse. To investigate its possible effects on insulin and glucose dynamics as well as inflammation in horses with EMS, 10 mixed-sex and mixed-breed adult (8-21 years old) EMS horses were selected. Horses were supplemented with a DHA-rich microalgae containing 16 g DHA/horse/day top dressed on a vehicle or vehicle only for 46 days. Pre and post supplementation blood samples were collected via jugular venipuncture. Blood samples were used to isolate serum and plasma. Further, plasma was utilized for isolation of peripheral blood mononuclear cells (PBMCs). Classification of the animals' inflammatory state was carried out by serologic enzyme-linked immunosorbent assay (ELISA), and analysis of PBMCs via flow cytometry and reverse transcription polymerase chain reaction (RT-PCR). Circulating fatty acids and triglyceride, leptin, and adiponectin concentrations were also determined. Insulin and glucose dynamics were assessed pre and post supplementation with an oral sugar test (OST) and a frequently sampled intravenous glucose tolerance test (FSIGTT). Post supplementation, horses receiving DHA rich microalgae had a significant increase in many circulating fatty acids, in particular an increase in DHA ($P < 0.001$), compared to controls. Treated horses also had lower serum triglycerides post supplementation ($P = 0.023$) and there was a trend ($P = 0.066$) for a reduction in the amount of TNF-α produced per lymphocyte post supplementation in the treated horses. Interestingly, post supplementation horses given DHA rich microalgae did not have the same rise in insulin concentrations 60 minutes post oral sugar administration

($P = 0.689$) as seen in controls ($P = 0.012$). These results indicate that DHA rich microalgae supplementation is able to affect circulating fatty acids, reduce inflammation, and modulate metabolic parameters in EMS horses. Considering the negative impact of obesity on the health of the horse, continued research on the underlying mechanisms and treatment of associated diseases is imperative.

8.1. Introduction

More than 30% of the human population is considered to be obese [2]. Obesity is an increasing problem not only in humans, but in the horse population as well. Similar to statistics in people, reports indicate that the percentage of overweight or obese horses may range anywhere from 20.6-51% [3-6]. Obesity in horses, as in humans, has been connected to more serious health concerns such as insulin dysregulation (ID) [12] and equine metabolic syndrome (EMS). EMS was defined in a 2010 ACVIM consensus statement as the following: regional (neck crest, rump, etc.) or general adiposity, hyperinsulinemia or abnormal glycemic or insulinemic response to challenge, and a history of or predisposition towards laminitis [23]. Traditionally EMS is treated with dietary restriction and exercise. While this treatment is effective in most instances by increasing insulin sensitivity and decreasing inflammation [29], it is a process that can take months. In addition, due to complications from underlying or other conditions (such as laminitis), an increased exercise regimen is not always possible. There are two pharmaceutical options available at this time. They act to decrease hepatic glucose production (Metformin) and increase metabolism via synthetic thyroid hormone (levothyroxine sodium). However, metformin and levothyroxine sodium are used off label in the EMS animal and the mechanism of action of either is not well known. In addition, published findings have yielded conflicting results as to the long term safety and efficacy of these drugs in the horse [9, 154-156]. Therefore investigation into alternative methods of treatment for inflammation and insulin resistance associated with EMS is warranted.

One method that has proven effective in humans with Metabolic Syndrome (MetS) [24], diabetes, obesity, or those with metabolic dysfunction [157] is supplementation with omega-3 fatty acids. Supplementation with the long chain omega-3 fatty acids docosahexaenoic acid (DHA) or eicosapentaenoic acid (EPA), is shown to improve insulin

sensitivity [162, 163], promote oxidation of body fat, decrease lipogenesis, and reduce inflammation [167, 168, 286]. Omega-3 fatty acid supplementation in horses has been shown to positively influence glucose clearance [171] and reduce inflammatory mediators [174-176]. It has also been shown in horses to change circulating fatty acid concentrations, including DHA, triglycerides, and phospholipids [159, 177, 178]. In addition, increases in DHA concentrations following supplementation have been noted not only in circulation, but are incorporated into skeletal muscle [160]. Therefore, the objective of this study was to determine the effects of omega-3 fatty acid supplementation in the form of a DHA-rich microalgae on metabolic and inflammatory responses in horses with EMS.

8.2. Materials and Methods

Ten EMS horses of mixed-sex and mixed-breed who were residing on the University of Kentucky's Maine Chance Farm were selected for this study. All horses were acclimated at least 2 months prior to the start of study to dry lot paddocks, with 2 horses housed per paddock, and to feed and feeding protocols. Horses were provided a low fat diet of mixed grass hay fed at 2% body wt (BW)/day split between a morning (0800-1000, EST) and afternoon (1500-1600, EST) feeding. Nutrient analysis of the hay is presented in Table 8.1. Horses were also provided with the morning feeding; 1 lb/day balancer pellet (Essential K®, Tribute Equine Nutrition, OH) and 1 lb/day alfalfa pellet (Hallway feeds, Farmers Feed Mill, KY), and had access to water and a mineral block ad libitum. EMS horses were randomly assigned to one of two groups, treatment group (n=6; mean age 13.2 ± 4.4 years) or control (n=4; mean age 11.5 ± 2.6 years). Horses in were not different in age between treated groups (P = 0.48). At the start of the 46 day supplementation period the treatment group received a DHA-rich microalgae supplement (FOR$^{PLUS™}$, Alltech, Inc, Nicholasville KY) containing 16 g DHA mixed with 25 ml water, 15 ml molasses (Double S Liquid Feed Services, Inc., Danville, IL), and 4 ml anise extract (A1 Spice World, Glen Head, NY) top dressed onto their balancer and alfalfa pellets to make the supplement palatable and to ensure horses received their entire daily portion of supplement. Control horses received the vehicle of 25 ml water, 15 ml molasses, and 4 ml anise extract without the algal supplement top dressed onto their balancer and alfalfa pellets only. Analysis of

algal supplements nutritional and fatty acid content was performed by Eurofins Analytical Laboratories (New Orleans, LA, USA) and is presented in Table 8.2.

Table 8.1. Mixed grass hay dietary analysis.

Component	DM
DE (Mcal/kg) 1X	2.68
ME (Mcal/kg) 1X	2.26
% Moisture (as fed)	8.5
% Dry Matter (as fed)	91.5
% Crude Protein	16.3
% Adjusted Crude Protein	16.3
% Acid Detergent Fiber	41.0
% Neutral Detergent Fiber	55.3
% NFC	22.5
% Starch	1.6
% WSC (water soluble carbohydrates)	6.0
% NSC	7.6
% ESC (simple sugars)	4.5
% Crude Fat	2.8
% TDN	59
NEL, Mcal/Lb	0.56
NEM, Mcal/Lb	0.54
NEG, Mcal/Lb	0.29
% Calcium	1.01
% Phosphorus	0.27
% Magnesium	0.18
% Potassium	2.18
% Sodium	0.043
PPM Iron	137
PPM Zinc	23
PPM Copper	9
PPM Manganese	26
PPM Molybdenum	3.5

Table 8.2. Algal supplement dietary analysis and fatty acid content.

Component	As fed basis
% Moisture	2.4
% Acid Hydrolysis Fat	58
% Crude Fiber	0.9
Calculated % Carbohydrate	17.3
Combustion % Protein	13.5
% Total Ash	3.1
% Calcium	0.34
% Phosphorus	0.47
% Sulfur	0.74
% Potassium	0.55
% Sodium	0.10
PPM Iron	13
PPM Zinc	36
PPM Copper	2
PPM Selenium	0.13
Fatty Acid	**% of fat content**
C6:0 Caproic acid	< 0.10%
C7:0 Heptanoic acid	< 0.10%
C8:0 Caprylic acid	< 0.10%
C9:0 Nonanoic acid	< 0.10%
C10:0 Capric acid	< 0.10%
C11:0 Undecaniod acid	< 0.10%
C12:0 Lauric acid	0.13 %
C13:0 Tridecanoic acid	< 0.10%
C14:0 Myristic acid	4.16%
C14:1 Myristoleic acid	< 0.10%
C15:0 Pentadecanoic acid	1.98%
C15:1 Pentadecenoic acid	< 0.10%
C16:0 Palmitic acid	52.55%
C16:1 Palmitoleic acid	0.11%
C17:0 Margaric acid	0.82%
C17:1 Margaroleic acid	< 0.10%
C18:0 Stearic acid	1.65%
C18:1n7c Vaccenic acid	< 0.10%
C18:1n9c Oleic acid	< 0.10%
C18:1n9t Elaidic acid	< 0.10%
C18:2n6c Linoleic acid	< 0.10%
C18:2n6t Linolelaidic acid	< 0.10%
C18:3n3c alpha-linolenic acid	< 0.10%
C18:3n6c gamma-linolenic acid	< 0.10%
C19:0 Nonadecanoic acid	< 0.10%
C20:0 Aracidic acid	0.21%
C20:1 Eicosenoic acid	< 0.10%

Table 8.2 Algal supplement dietary analysis and fatty acid content. (continued)

Fatty Acid	% of fat content
C20:2n6c Eicosadienoic acid	< 0.10%
C20:3n3c Eicosatrienoic acid	0.74%
C20:3n6c homo-gamma-Linolenic acid	0.13%
C20:4n6c Arachidonic acid	0.14%
C20:5n3c Eicosapentaenoic acid	0.56%
C21:0 Heneicosanoic acid	< 0.10%
C22:0 Behenic acid	0.11%
C22:1 Erucic acid	< 0.10%
C22:2n6c Docosadienoic acid	0.41%
C22:5n3c Docosahexaenoic acid	0.17%
C22:6n3c Docosahexaenoic acid	27.74%
C23:0 Tricosanoic acid	< 0.10%
C24:0 Lingoceric acid	0.11%
C24:1 Nervonic acid	0.32%

Analysis of algal supplement fatty acid content by high-performance liquid chromatography (HPLC)

Originally, the study was intended to be carried out with 12 horses and as a crossover-design, with a 60 day washout period. However, 2 horses were dropped from the study for health reasons. In addition, the second half of the crossover was not completed due to significantly elevated concentrations of circulating DHA in treated horses compared to controls as far as 263 days (P = 0.01, DHA-rich microalgae treated horses median 0.30; IQR 0.24 – 0.40 versus control horses median 0.06; IQR 0.06 – 0.172) following cessation of supplementation. All experimental procedures were approved by the University of Kentucky's IACUC (institutional animal care and use committee).

8.2.1. EMS determination

EMS was determined by the criteria established in a 2010 ACVIM consensus statement [23]. In brief, hyperinsulinemia, general or regional obesity, and a history of or predisposition to laminitis. A portable agriculture scale (model 700, Tru Test Inc., Mineral Wells, TX) was used to establish body weight, which was monitored weekly. Body condition score (BCS) was determined and averaged between three trained investigators using the 1-9 Henneke scoring system [186], with a 1 representing an extremely emaciated animal and a 9 representing an extremely obese animal. The same trained individuals scored and regional adiposity via the 0-5 cresty neck score (CNS) system established by Carter et al. [187], with a 0 representing no neck crest and a 5 representing a crest so large

it permanently droops to one side of the neck. Blood collection for serum and plasma samples was carried out via jugular venipuncture. To ascertain the presence of insulin dysregulation, an oral sugar test (OST) was performed; following overnight fasting serum samples were collected and 0.15 ml/kg of Karo Light Corn syrup (ACH food companies, Cordova, TN) orally administered and a second serum sample collected 60 minutes later [13, 23]. Serum samples were kept at -20º C until analysis. A fasting insulin level of >20 µIU/mL was considered indicative of hyperinsulinemia, and an increased insulin (>60µU/mL) 60 minutes post administration of oral sugar was classified as insulin dysregulation [13, 23]. These cut-off values were also utilized as they are recommended by the Tufts University Equine Endocrinology Group (EEG).

Horses were screened using the TRH (thyrotropin releasing hormone) stimulation test and low-dose dexamethasone suppression testing to ensure that none were affected by pituitary pars intermedia dysfunction (PPID). TRH stimulation testing was carried out as previously described [191, 192]. Briefly; an A.M. (between 0800 and 1200, EST) baseline blood sample was taken via jugular venipuncture. Following this, a 1ml dose of TRH dissolved to 1 mg/ml in 0.9% saline (Sigma-Aldrich, St. Louis, MO) was administered intravenously (IV) and 10 minutes post TRH injection a second blood sample was taken. Ethylenediaminetetraacetic acid (EDTA) containing tubes were used to collect blood samples. Tubes were placed on ice and transported to the lab for immediate centrifugation and plasma isolation [193]. Plasma was stored at -20 º C until shipped on dry ice to Cornell University for determination of ACTH concentrations. Low-dose dexamethasone suppression testing was performed as previously described [194]. In brief, an afternoon (1550-1800 hours) baseline jugular blood sample was taken. Following this, an intramuscular (IM) dexamethasone bolus (0.04 mg/kg BW) was administered and a second blood sample taken 19 hours later. A gel serum separator tube was used to collect samples. Tubes were centrifuged, and serum isolated and frozen at -20 º C until cortisol analysis. As recommended by the EEG, concentrations of ACTH 10 minutes post TRH injection in excess of 100 pg/mL or concentrations of cortisol exceeding 1.0 µg/dL 19 hours following dexamethasone injection were considered positive for PPID. Any animals meeting these criteria were excluded.

Analysis of ACTH, cortisol, and insulin were performed by Cornell University's Animal Health Diagnostic Center Endocrinology Laboratory. The Millipore porcine insulin RIA kit (EMD Millipore Corporation, Darmstadt, Germany) was used to measure insulin concentrations [47], ACTH was measured via an automated chemiluminescent enzyme immunoassay system (Immulite, Erlangen, Germany) [195], and cortisol concentrations were determined using the Siemens Immulite Cortisol kit (Siemens, Erlangen, Germany).

8.2.2. Sample collection and timeline

Serum and EDTA plasma samples were taken after an overnight fast at baseline and following the OST both prior to (day 0; time point 1) and following supplementation (day 46; time point 2). Horses were administered a frequently sampled intravenous glucose tolerance test (FSIGTT; see below) one day following blood collection and OST. Serum samples were utilized for determination of insulin, leptin, and triglyceride analysis. EDTA plasma samples were used to determine fatty acid concentrations.

8.2.3 Frequently sampled intravenous glucose tolerance test (FSIGTT)-Optimized

At time points 1 and 2 a FSIGTT was performed as previously described [287]. In brief; an indwelling, long-term intravenous catheter was placed to administer treatments and collect blood samples. Following baseline blood sample collection, a 50% glucose (100 mg/kg, IV) was administered, followed 20 minutes later by administration of insulin (20 mU/kg, IV). Blood samples were obtained at -10, -5, 0, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, 19, 22, 23, 24, 25, 27, 30, 35, 40, 50, 60, 70, 80, 90, 100, 120, 150, and 180 minutes after glucose administration. Blood samples from the FSIGTT were analyzed for glucose concentrations on an YSI analyzer (YSI Incorporated, Xylem Inc., Yellow Springs, OH).

8.2.4 Endocrine and lipid measures

Serum samples from the OST for time points 1 and 2, as well as EDTA plasma from the FSIGTT were sent to Cornell University's endocrinology lab for determination of insulin using RIA (see above). In addition, fasting serum samples were sent to Cornell University's Clinical Pathology lab for leptin and triglyceride analysis via colometric assay using a Roche ModP analyzer (Roche Diagnostics, Indianapolis, IN) [49]. Fasting EDTA plasma samples from time points 1 and 2 were sent to Michigan State University's

Diagnostic Center for Population and Animal Health for plasma fatty acid analysis using high-performance liquid chromatography (HPLC) [288].

8.2.4. Peripheral blood mononuclear cell (PBMC) inflammatory cytokine production

Heparinized blood was used to isolate peripheral mononuclear cells (PBMCs) by Ficoll-Paque PlusTM (Amersham Biosciences, Piscataway, NJ) gradient centrifugation [197]. Cells were frozen in 10% fetal bovine serum (FBS; Sigma–Aldrich, St. Louis, MO), 40% dimethyl sulfoxide (DMSO), and 50% Roswell Park Memorial Institute medium (RPMI-1640, Gibco, Grand Island, NY) until thawed for *in vitro* stimulation. For stimulation, 10^7 PBMC were incubated in 1 ml c-RPMI (RPMI-1640 with 2.5% fetal equine serum (FES; Sigma–Aldrich, St. Louis, MO), 100 U/ml penicillin/streptomycin (Sigma), and 55 mM 2-mercaptoethanol (Gibco) media. Cells were incubated at 37 °C, 5% CO_2 with Brefeldin A (10 mg/ml; Sigma) and select wells with the positive control phorbol 12-myristate 13-acetate (PMA; 25 ng/ml; Sigma) and ionomycin (1 mM; Sigma) for 4 hrs. Following this, determination of cytokine gene expression was carried out by separating aliquots of the cells and placing them into Trizol (Ambion) to isolate RNA (see below). Remaining cells were assayed by flow cytometry for interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) production. Cells were fixed in 2% paraformaldehyde (Sigma) and stored overnight at 4°C. After overnight storage, IFN-γ and TNFα intracellular staining was carried out as previously described [198]. After the cells were stained, aliquots of cells were resuspended in FACS flow and flow cytometric acquisition was performed on a FACSCALIBUR™ (Becton Dickinson, San Jose, CA). Determination of the percent of IFN-γ and TNFα lymphocytes was accomplished with the use of CELL QUEST™ (Becton Dickinson) [42].

A modified Trizol method was used to extract total RNA from PBMC cultures (see above). One µg of RNA was reverse transcribed into cDNA, as previously described [42, 197]. In brief; 1 µg of each RNA sample and a reverse transcription master mix (Promega, Madison, WI) was incubated for 15 min at 42°C and for 5 min at 95°C. Reactions included 5µl cDNA and 20µl of master mix. Master mix included; 6.25µl nuclease-free water (Qiagen), 1.25µl primer/probe 20X assay mix (Applied Biosystems, Foster City, CA), and 12.5µl TaqMan™ (Applied Biosystems). Equine-specific, inventoried, intron-spanning primers and probes were used with Applied Biosystems Real-Time PCR (ABI 7900HT)

against the following genes: Housekeeping gene *beta*-glucuronidase (β-gus) and the following genes; IFN-γ (catalog number 4331182), interleukin 6 (IL-6; catalog number 4351372), interleukin 10 (IL-10; catalogue number 4331182), TNF-α (catalog number 4331182), and transferrin (catalog number 4351372). Samples were processed in duplicate and incubated for 95^{o}C for 10 min. They then underwent 40 cycles at 95^{o}C for 15s and 60^{o}C for 60s. Relative changes in gene expression were determined by the $\Delta\Delta C_T$ method [200], with mean ΔC_T for week 0 set as the calibrator for all samples. Relative quantity, or RQ, was calculated as $2^{-\Delta\Delta CT}$ and used to express results.

8.2.5. Serum Cytokine Analysis

8.2.5.1. TNF-α ELISA

Serum concentrations of TNF-α protein were determined using a commercially available equine TNF-α ELISA kit (R&D, Minneapolis, MN). This assay is optimized for equine serum samples diluted at a minimum of 1:2 [289]. All steps of the ELISA were performed according to the manufacturer's instructions, with the exception of the following; coating antibody provided was prepared in carbonate buffer (15 mmol Na_2CO_3, 35 mmol $NaHCO_3$, pH 9.6) and the final 2 incubation times were increased from 20 min to 30 min. In addition, single absorbance was measured at A_{450}.

8.2.5.2. IL-6 ELISA

Serum concentrations of IL-6 were determined using an IL-6 ELISA validated for use in the horse with an analytical sensitivity of 780 pg/mL [290]. The ELISA utilizes a polyclonal goat anti-horse IL-6 antibody (AF1886, R&D Systems, Inc., Minneapolis, MN) to coating ELISA plates (Immunoplate Maxisorp, Nalge Nunc Int., Rochester, NY). The antibody was diluted to a final concentration of 1 μg/mL in carbonate buffer (15 mmol Na_2CO_3, 35 mmol $NaHCO_3$, pH 9.6) and incubated overnight at 4 °C. Afterwards, the coating solution was discarded and the plates blocked for 30 min at room temperature with the addition of phosphate buffered saline (PBS, pH 7.2) containing 0.5% (w/v) bovine serum albumin. Plates were washed five times with phosphate buffer (2.5 mmol NaH_2PO_4, 7.5 mmol Na_2HPO_4, 145 mmol NaCl, 0.1% (v/v) Tween 20, pH 7.2). A recombinant equine IL-6 (1886-EL, R&D Systems, Inc., Minneapolis, MN) diluted in 2-fold serial dilutions ranging from 50 to 0.78 ng/mL was used as standard to determine IL-6 concentrations in the samples. The serum was diluted in phosphate buffer, added to the

plates in triplicate wells and incubated for 90 min at room temperature. After five washes, biotinylated goat anti-horse IL-6 (AF1886, R&D Systems, Inc., Minneapolis, MN) diluted 1:100 in phosphate buffer was added, incubated for 60 min, and washed again. A streptavidin–horseradish peroxidase solution (Jackson ImmunoResearch Lab., West Grove, PA) was added to the plates for another 30 min. After a final wash, substrate buffer (33.3 mmol citric acid, 66.7 mmol NaH_2PO_4, pH 5.0) was added and incubated for 20 min in the dark. The reaction was stopped by adding one volume of 0.5 mol H_2SO_4. Plates were read in an ELISA reader (Bio-Tek, Winooski, VT) at 450 nm absorbance.

8.2.5.3. CRP ELISA

A commercially available equine specific kit (Kamiya Biomedical Company, Tukwila, WA) was used to measure C - reactive protein (CRP) in the serum according to manufacturer's instructions and as previously described [291].

8.2.6. Data Analysis

Data were analyzed via Sigma Plot 13.0 (Systat Software, San Jose, CA). A two-way repeated measured ANOVA was used with fixed effects set as treated vs control and time point and all possible interactions analyzed. Data not normal were log transformed and resulted in normality. Results were considered statistically significant when $P \leq 0.05$ and trends considered at $P \leq 0.10$.

8.3. Results

8.3.1. Lipid, phenotypic, and endocrine measures

Plasma fatty acid concentrations as a percentage of total circulating fatty acids are presented in Table 8.3. DHA-rich microalgae treated horses were higher at time point 2 compared to all horses at time point 1 and control horses at time point 2 in DHA ($P < 0.001$) and C:22 2n6c ($P < 0.001$) concentrations. However, DHA-rich microalgae treated horses at time point 2 were lower compared to all others for C18:1n9c ($P = 0.02$) and C18:3n6c ($P = 0.035$) plasma concentrations. Control horses at time point 2 had higher concentrations of C16:1n7c compared to control horses at time point 1 ($P = 0.009$), but were not different compared to treated horses regardless of time point. In addition, there was an overall effect of time point for C20:3n3c ($P = 0.011$) and C24:1n9c ($P = 0.025$) concentrations, with horses at time point 2 higher compared to horses at time point 1.

Table 8.3. Plasma fatty acid results for DHA-rich microalgae treated and control EMS horses both pre (time point 1) and post (time point 2) a 46-day supplementation period.

Plasma Fatty Acid Concentrations (% of total fatty acids)	Control horses time point 1	Control horses time point 2	Treated horses time point 1	Treated horses time point 2
C22:6n3c Docosahexaenoic acid	0.05 ± 0.1 [a]	0.05 ± 0.1 [a]	0.01 ± 0.1 [a]	1.94 ± 0.1 [b]
C14:0 Myristic Acid	0.86 ± 0.1	0.97 ± 0.1	0.88 ± 0.1	0.89 ± 0.1
C16:0 Palmitic Acid	15.87 ± 0.7	16.69 ± 0.7	16.31 ± 0.6	17.22 ± 0.6
C16:1n7c Palmitoleic Acid	1.10 ± 0.2 [a]	1.49 ± 0.2 [b]	1.13 ± 0.1 [a, b]	1.15 ± 0.1 [a, b]
C17:0 Margaric Acid	0.36 ± 0.03	0.35 ± 0.03	0.44 ± 0.02	0.39 ± 0.02
C18:0 Steric Acid	15.38 ± 0.7	15.40 ± 0.7	15.97 ± 0.6	15.35 ± 0.6
C18:1n7c Cis-Vaccenic Acid	1.50 ± 0.2	1.43 ± 0.2	1.55 ± 0.1	1.53 ± 0.1
C18:1n9c Oleic Acid	16.10 ± 1.5 [b]	17.67 ± 1.5 [b]	15.74 ± 1.2 [b]	12.64 ± 1.2 [a]
C18:2n6c Linoleic Acid Methyl Ester	43.76 ± 2.0	41.13 ± 2.0	43.49 ± 1.6	44.89 ± 1.6
C18:3n3c Alpha linolenic Acid	0.23 ± 0.04	0.23 ± 0.04	0.25 ± 0.03	0.18 ± 0.03
C18:3n6c y-Linolenic Acid	3.64 ± 0.3 [b]	3.15 ± 0.3 [b]	3.24 ± 0.3 [b]	1.81 ± 0.3 [a]
C20:2n6c Eicosadienoic Acid	0.15 ± 0.02	0.21 ± 0.02	0.14 ± 0.01	0.16 ± 0.01
C20:3n3c Eicosatrienoic Acid	0.59 ± 0.1	0.75 ± 0.1	0.56 ± 0.1	0.89 ± 0.1
C20:3n6c Homo-y Linolenic Acid	0.19 ± 0.04	0.17 ± 0.04	0.12 ± 0.03	0.24 ± 0.03
C:22 2n6c Docosadienoic Acid	0.07 ± 0.02 [a]	0.07 ± 0.02 [a]	0.04 ± 0.02 [a]	0.22 ± 0.02 [b]
C22:4n6c	0.004 ± 0.01 [a]	0.009 ± 0.01 [a]	-0.002 ± 0.01 [a]	0.316 ± 01 [b]
C22:5n3c Docosapentaenoic Acid	0.12 ± 0.02	0.15 ± 0.02	0.09 ± 0.02	0.13 ± 0.02
C24:1n9c Nervonic Acid	0.07 ± 0.02	0.10 ± 0.02	0.04 ± 0.02	0.09 ± 0.02

Plasma fatty acid concentrations as measured by high performance liquid chromatography (HPLC) in EMS control (n=4) versus DHA-rich microalgae treated (n=6) horses. Data are expressed as the percentage of total fatty acid concentrations. Results are presented as least square mean plus or minus standard error of the mean. Within a row, differences ($P < 0.05$) are represented by differing superscripts.

OST results and phenotypic data, along with serum leptin and triglycerides are presented in Table 8.4. There were no differences in fasting insulin between control and treated horses' both pre and post supplementation ($P = 0.568$). However, control horses had higher insulin concentrations 60 minutes post oral sugar administration at time point 2 compared to time point 1 ($P = 0.012$). Similarly, when considering the change in insulin concentrations 60 minutes post oral sugar administration compared to baseline insulin concentrations (delta change), control horses had an increase ($P = 0.009$) from time point 1 to time point 2 whereas DHA-rich microalgae treated horses did not ($P = 0.419$). In addition, control horses did not have greater delta insulin concentrations at time point 1 ($P = 0.143$) compared to treated horses, but they did have a greater delta insulin at time point 2 ($P = 0.027$). DHA-rich microalgae treated horses had lower serum triglycerides at time point 2 compared control horses ($P = 0.017$) and compared to treated horses at time point 1 ($P = 0.023$). Control horses at time point 1 were not different from control horses at time point 2 or from treated horses at any time point. There were no differences between treated or control horses for serum leptin, regardless of time point. While there was an overall difference in time point for BCS ($P < 0.001$), with all horses being higher at time point 2 versus time point 1, there were no differences either pre or post supplementation between treated and control horses. Nor were there any differences between treated or control horses for CNS or weight.

Table 8.4. Endocrine data, phenotypic measures, and serum leptin and triglyceride concentrations for DHA-rich microalgae treated and control EMS horses both pre (time point 1) and post (time point 2) a 46-day supplementation period.

Endocrine and phenotypic measures	Control horses time point 1	Control horses time point 2	Treated horses time point 1	Treated horses time point 2
Basal Insulin (µIU/ml)	21.58 ± 6.89	22.85 ± 6.89	32.61 ± 5.62	29.76 ± 5.62
Insulin 60 min post oral sugar administration (µIU/ml)	68.90 ± 12.29 [a]	106.25 ± 12.29 [b]	78.13 ± 10.04 [a, b]	82.02 ± 10.04 [a, b]
Change in insulin concentrations (OST 60 min-basal insulin; µIU/ml)	47.31 ± 9.69	83.40 ± 9.69	45.53 ± 7.91	52.26 ± 7.91
Log BCS	0.79 ± 0.01 [a]	0.84 ± 0.01 [b]	0.82 ± 0.01 [a]	0.86 ± 0.01 [b]
CNS	2.29 ± 0.44	2.25 ± 0.44	2.97 ± 0.36	3.08 ± 0.36
Weight (kg)	515.60 ± 34.54	517.19 ± 34.54	562.63 ± 28.03	568.16 ± 28.03
Leptin (ng/mL)	7.23 ± 1.40	6.72 ± 1.40	7.18 ± 1.14	7.94 ± 1.14
Triglycerides (mg/dL)	47.75 ± 6.62 [a, b]	59.00 ± 6.62 [b]	49.17 ± 5.40 [b]	35.33 ± 5.40 [a]

Phenotypic measures, endocrine data, and serum leptin and triglyceride concentrations in EMS control (n=4) versus DHA-rich microalgae treated (n=6) horses. Results are presented as least square mean plus or minus standard error of the mean. Within a row, differences ($P < 0.05$) are represented by differing superscripts.

8.3.2. FSIGTT

As shown in Table 8.5, there were no treatment differences in insulin sensitivity (SI), acute insulin response to glucose (AIRg), disposition index (DI), or glucose effectiveness (Sg). Nor were there any differences over time in SI, DI, or Sg. While there was an increase in acute insulin response to glucose for time point 2 compared to time point 1 ($P = 0.008$), there was not a treatment by time point interaction.

Table 8.5. MinMod analysis of FSIGTT data for DHA-rich microalgae treated and control EMS horses both pre (time point 1) and post (time point 2) a 46-day supplementation period.

Insulin sensitivity measures	Control horses time point 1	Control horses time point 2	Treated horses time point 1	Treated horses time point 2
AIRg (acute insulin response to glucose)	158.9 ± 73.8 [a]	394.4 ± 73.8 [b]	153.5 ± 60.2 [a]	335.8 ± 60.2 [b]
SI (insulin sensitivity)	0.442 ± 0.215	0.352 ± 0.215	0.324 ± 0.176	0.449 ± 0.176
DI (disposition index)	73.4 ± 77.8	106.0 ± 77.8	73.5 ± 63.5	185.8 ± 63.5
Sg (glucose effectiveness)	0.022 ± 0.009	0.025 ± 0.009	0.034 ± 0.007	0.018 ± 0.007

Measures of insulin resistance in EMS control (n=4) versus DHA-rich microalgae treated (n=6) horses from MinMod analysis of FSIGTT data obtained from insulin and glucose EDTA plasma concentrations. Results are presented as least square mean plus or minus standard error of the mean. Within a row, differences ($P < 0.05$) are represented by differing superscripts.

8.3.3. Peripheral blood mononuclear cell (PBMC) inflammatory cytokine production

Flow cytometry analysis of TNF-α and IFN-γ intracellular protein (Table 8.6) showed no significant differences between DHA-rich microalgae treated and control horses for the percent of lymphocytes positive (% gated) for IFN-γ or TNF-α protein, nor for IFN-γ intensity of fluorescence per lymphocyte (mean fluorescence activity-MFI). There was a trend ($P = 0.066$) for an interaction between treatment and time point for TNF-α MFI, with DHA-rich microalgae treated horses having a lower MFI at time point 2 compared to time point 1. Control horses were not different between time points or from treated horses, regardless of time point. There was an overall time point difference for IFN-γ MFI ($P =$

0.006), with horses at time point 1 higher compared to time point 2, and an overall treatment versus control difference with TNF-α % gated higher in treated horses compared to controls ($P < 0.001$).

Table 8.6. PBMC inflammatory cytokine production for DHA-rich microalgae treated and control EMS horses both pre (time point 1) and post (time point 2) a 46-day supplementation period.

Inflammatory cytokine measures	Control horses time point 1	Control horses time point 2	Treated horses time point 1	Treated horses time point 2
IFN-γ % gated	20.13 ± 2.55	20.22 ± 2.55	24.51 ± 2.08	23.56 ± 2.08
IFN-γ MFI	126.43 ± 7.98	101.86 ± 7.98	108.45 ± 6.52	94.43 ± 6.52
TNF-α % gated	39.56 ± 2.76	40.96 ± 2.76	53.10 ± 2.25	58.79 ± 2.25
TNF-α MFI	57.39 ± 6.12	58.52 ± 6.12	64.11 ± 5.00	54.98 ± 5.00
IFN-γ RQ	13296 ± 2192	9383 ± 2192	11395 ± 1790	9642 ± 1790
TNF-α RQ	330.5 ± 136.6	315.4 ± 136.6	619.0 ± 111.6	605.3 ± 111.6
IL-6 RQ	48.41 ± 8.74	40.75 ± 8.74	42.91 ± 7.14	39.28 ± 7.14
IL-1β RQ	3.31 ± 0.91	3.36 ± 0.91	1.86 ± 0.74	1.12 ± 0.74
IL-10 RQ	142.56 ± 34.56	144.06 ± 34.56	221.66 ± 28.22	173.84 ± 28.22

PBMC inflammatory data in EMS control (n=4) versus DHA-rich microalgae treated (n=6) horses. Flow cytometry data is represented by % gated and MFI, which characterize the percent of lymphocytes positive for IFN-γ or TNF-α protein (% gated) and the intensity of florescence, or mean cytokine activity, per lymphocyte. RT-PCR data is represented by relative quantity (RQ) of gene expression. Results are presented as least square mean plus or minus standard error of the mean. Within a row, differences ($P < 0.05$) are represented by differing superscripts.

Lymphocyte gene expression of IFN-γ, TNF-α, IL-6, IL-1β, and IL-10 was not different between time points, regardless of treatment. There was an overall treatment versus control difference, with IL-1β expression higher in controls compared to treated horses ($P = 0.009$).

8.3.4. Serum cytokine analysis

Circulating concentrations of IL-6, TNF-α, and CRP protein as measured by ELISA were not different between DHA-rich microalgae treated or control horses (Table 8.7). There were no overall differences between time points 1 or 2, or any overall differences between treated or control horses.

Table 8.7. Serum inflammatory cytokine and CRP results for DHA-rich microalgae treated and control EMS horses both pre (time point 1) and post (time point 2) a 46-day supplementation period.

Serum inflammatory cytokines and CRP	Control horses time point 1	Control horses time point 2	Treated horses time point 1	Treated horses time point 2
TNF-α	18.5 ± 596.6	19.1 ± 596.6	57.0 ± 487.1	1375.1 ± 487.1
Log IL-6	0.32 ± 0.32	0.34 ± 0.32	1.05 ± 0.26	1.25 ± 0.26
C-reactive Protein	272.4 ± 837.1	566.9 ± 837.1	278.5 ± 683.5	1636.5 ± 638.5

Circulating inflammatory cytokines and CRP in EMS control (n=4) versus DHA-rich microalgae treated (n=6) horses. Data are expressed as ELISA units. Results are presented as least square mean plus or minus standard error of the mean. Within a row, differences ($P < 0.05$) are represented by differing superscripts.

8.4. Discussion

As expected, treated horses had increases in circulating DHA as well as other fatty acid concentrations post supplementation, similar to what has been observed in other work [159, 288, 292]. Also similar to previous studies [177], DHA-rich microalgae treated horses in this study had a reduction in serum triglycerides following supplementation. Human studies have likewise indicated a positive effect of omega 3 fatty acid supplementation on lipids and lipid metabolism [169, 170], including lipids which are implicated in insulin resistance and inflammation, such as ceramide [140]. Indeed, linoleic and DHA containing phosphatidylcholine and sphingomyelin were found to be increased in rats following an exercise regimen, whereas arachidonic acid containing phosphatidylcholines, phosphatidylethanolamine, and phosphatidylinositol were increased in rats following high fat feeding [125]. In addition, exercised rats were more insulin sensitive compared to rats fed a high fat diet. Considering that dietary supplementation with omega-3 fatty acids can improve concentrations of these lipids [169, 293], this supports their usage in individuals with metabolic dysfunction. Further, these studies further indicate a link between circulating fatty acids, insulin sensitivity, and inflammation.

Supplementation with omega 3 fatty acids in equines has been proposed as a way to improve glucose and insulin dynamics [172], similar to the improved insulin sensitivity seen in rats, pigs, and humans following supplementation [164-166]. DHA rich algal supplementation has also been shown in horses following dexamethasone administration to improve baseline glucose and insulin concentrations, as well as the modified insulin to glucose ratio [173]. In this study, there was a positive effect on metabolic parameters in

horses fed 16 g/day DHA from an algal source for 46 days. Specifically, treated horses did not have the same increase in insulin responses to an OST. These improved insulin responses to the OST indicate that supplementation provided a protective effect from increases in insulin responses seen in the control group. This increase in insulin measured in the control horses may be in part due to seasonal changes, however more work is needed to explore possible seasonal effects on insulin and insulin responses to the OST in the EMS animal.

In humans and in mice, metabolic dysfunction has long been associated with increases in inflammation [267, 294]. These increases in inflammation have been proposed for the EMS horse or pony as well [28, 295, 296]. For this study, treated horses had a trend for a decrease in TNF-α MFI post supplementation, indicating immune modulating anti-inflammatory effect of DHA-rich microalgae supplementation in EMS horses. There were no differences in gene expression of PBMC inflammatory markers as measured by RT-PCR, suggesting modification likely occurs post transcriptionally. Indeed, no differences were seen in circulating inflammatory markers as measured by ELISA either pre or post supplementation. This is likely due in part to small sample sizes and large variability. Results from other work regarding the effect of omega 3 fatty acid supplementation on inflammatory parameters in the horse has been mixed. While fish oil supplementation has not been shown to lower prostaglandin E2 (PGE2) production in lipopolysaccharide (LPS) stimulated PBMCs from yearling horses [292], there has been an effect following 14 weeks of fish oil supplementation on PGE2 production in healthy equine LPS stimulated bronchoalveolar lavage fluid (BAL) cells compared to those supplemented with corn oil [297]. Feeding seal blubber oil as a source of omega 3 fatty acids has also been shown to have anti-inflammatory effects in the BAL of horses with recurrent airway obstruction [298]. The algal source used for this work largely contained DHA with only minor amounts of EPA, and DHA has been shown to be more effective in reducing inflammation compared to EPA [299, 300]. This may have been one of the reasons for the observed the trend in reduction of TNF-α MFI following supplementation.

The major limitation of this study was small sample size. Interestingly, treated horses had significantly elevated circulating DHA concentrations 263 days following cessation of supplementation. Others have reported that 42 days [159] and 56 days [301]

post supplementation DHA and EPA concentrations were not different from controls. This discrepancy may be explained by the fact that in the current study, horses had increased adiposity compared to presumably metabolically normal horses used in other studies. In people and in horses, DHA and EPA supplementation appears to be dose responsive [158, 159]. In addition, it has been shown in humans that DHA can be stored in adipose tissue for years [161]. Therefore, treated horses may have stored DHA in large amounts in adipose tissue and slowly released it over an extended period of time. An addition explanation may be that most other equine studies have utilized a fish oil source of DHA and EPA, whereas the current study which utilized an algal source of DHA. Considering that DHA has been shown to be more readily incorporated into tissue than EPA [302], DHA vs EPA concentrations or the ratio of DHA to EPA may influence DHA incorporation into and release from tissue.

8.5. Conclusions

DHA-rich microalgae supplementation positively affected metabolic, inflammatory, and lipid parameters following 46 days of 16 g/day algal DHA supplementation in a group of EMS horses. While supplementation did not normalize insulin levels, given long-term it may prove useful as a nutritional therapy in addition to diet and exercise for horse with this syndrome. However, more work is needed to investigate this further, and to better understand mechanisms responsible for the capacity of DHA-rich microalgae to modulate these parameters.

CHAPTER 9 DISCUSSION

Given the health concerns associated with insulin dysregulation (ID) in the horse, little is known about its underlying mechanisms or the involvement of the immune system and inflammation in this endocrine and metabolic dysregulation. Our overall hypothesis for this work was that due in part to changes in their gut microbiome and plasma lipidome, horses with ID will have changes in circulating proinflammatory markers, in particular in response to glycemic challenge, that further drive metabolic dysfunction.

It has been well established in human and mouse models that metabolic dysfunction has an inflammatory component [267, 294]. While this is proposed to be the case in the ID horse or pony [28, 295, 296], results have been mixed. There have been reports of increased IL-1β and IL-6 gene expression in equine nuchal adipose tissue [41] and increased TNF-α plasma or serum concentrations in ID horses [28]. Increases in plasma TNF-α concentrations have also been observed in ponies with a history of pasture-associated laminitis [237]. Correlative analysis has likewise linked insulin insensitivity, obesity, and inflammation in horses with ID [8]. Often, differences in inflammatory markers in horses with ID are seen at the gene expression level, are limited to adipose tissue, and are not observed in circulation [8, 39, 41]. Indeed, others have shown lower peripheral blood mononuclear cell (PBMC) cytokine gene expression of IL-1, IL-6, and a trend for lower TNF- α in horses with ID compared to normal controls [31]. Similarly, we observed no differences in baseline PBMC PMA stimulated cytokine protein concentrations or gene expression, although there was a trend for lower IL-6 and TNF- α gene expression in ID horses [42]. In fact, in a subsequent study, we further observed that control horses were higher at baseline in PBMC PMA stimulated cytokine protein concentration or gene expression [47]. However, ID horses then had an opposing inflammatory response to oral glycemic challenge wherein they were higher in IFN-γ and TNF-α protein concentrations as well as IFN-γ gene expression 60 min following oral administration of a glycemic challenge compared to controls.

There has been evidence in other species that there is an inflammatory response to feeding, so called postprandial inflammation, similar to what we have observed. There have been reported increases following a high fat or high fat meal combined with glucose in

circulating concentrations of intercellular Adhesion Molecule-1 (ICAM-1), a systemic soluble adhesion molecule [43, 44]. Soluble adhesion molecules have a role in both the inflammatory process and immune response [202] and have been proposed as a biomarker of cardiovascular disease in humans [203, 204]. There have also been reports in both normal and diabetic individuals of postprandial increases in adhesion molecules or pro-inflammatory cytokines where diabetic subjects have a greater postprandial response compared to normal controls [43, 45]. While others did not see differences in postprandial inflammatory markers between diabetic and non-diabetic individuals [205], this may be due to differences in meal type or carbohydrate complexity [206]. In horses, the majority of data assessing the inflammatory status of ID horses has been in a basal or fasting state and examined circulating or tissue levels of inflammation. Postprandial inflammatory responses in the horse are not well studied. In metabolically normal horses, IL-1β was shown to increase in response to a high sugar high starch meal [46]. Differences in postprandial inflammation in individuals with metabolic dysfunction has several proposed explanations. One of which may be difference in circulating endotoxin concentrations similar to what is seen in humans with type 2 diabetes [303]. Endotoxin concentrations have also increased in response to a meal in humans, particularly one high in fat [59, 60]. These increases in endotoxin may stem from differences in the gut microbiota, especially considering the proposed interactions between the gut microbiota, metabolic, and inflammatory processes [62, 63]. We were able to show that horses with ID have differences in their gut microbiota compared to metabolically normal controls. Specifically changes in community structure, suggesting overgrowth or undergrowth of different community members. A member of the Verrucomicrobia phylum was the most abundant in ID horses. This phyla has been shown to be increased in diet-induced obese minipigs [78]. One of the genus in this phyla, *Akkermansia*, is inversely associated in humans with fasting glucose, waist-to-hip ratio, and subcutaneous adipose measures [221]. Further, the abundance of Verrucomicrobia is proposed as a method to monitor the progression of glucose intolerance in people [222]. ID horses also had a decreased representation of *Fibrobacter* as compared to controls. *Fibrobacter* has been shown to decrease, along with increases in lactic acid producing bacteria, in response to both intestinal disease and changes in diet [225]. Increases in Verrucomicrobia and under representation of

Fibrobacter in ID horses may indicate that horses with metabolic dysfunction have adapted protective mechanisms, perhaps due in part to an acidic environment, however this has yet to be established in the ID horse.

While alterations in the gut microbiota of horses with ID possibly contributed to the differences in postprandial inflammatory responses we observed, we did not see any differences between ID and control horses in circulating lipopolysaccharide (LPS) concentrations either prior to or following supplementation with a mannan rich fraction of the yeast cell wall or in response to glycemic challenge. Nor did supplementation have an appreciable effect on metabolic or inflammatory parameters. However, there was an effect of date (pre vs post supplementation period) and glycemic challenge on serum insulin and glucose, as well as many inflammatory markers. In horses there have been some indications that metabolic parameters are influenced by season. Horses with PPID are reportedly higher in serum insulin in the fall months [256] and healthy horses had a higher area under the curve for glucose concentrations in response to the combined glucose-insulin tolerance test in February compared to August and November and June compared to November [257]. This was not observed by others who saw no differences in serum glucose or insulin concentrations in respect to season in a group of healthy or ID horses [258]. For our work, serum glucose and insulin had an apparent effect of season wherein the ID horses had an even greater increase from July to September as compared to controls.

Inflammation and the immune response have in other species been shown to be affected by season [259-261]. In baboons and Siberian hamsters the immune response appears to be suppressed in the fall and into the winter seasons [259, 260]. This may be the case in the horse as well [262-264], with a reported increase in IFN-γ and decrease in IL-4 in the winter as opposed to the summer months [265]. However, PBMC IFN-γ gene expression was conversely shown to be increased in the summer as opposed to winter months in another group of horses [266]. We observed an increase in gene expression of the proinflammatory cytokines IFN-γ and TNF-α as well as TLR-4 from the summer (July) to the fall (September) and a decrease in anti-inflammatory gene expression of IL-10 as well as IL-6, and protein concentrations of IFN-γ and TNF-α from lymphocytes. This supports the hypothesis that seasonal variations in inflammatory cytokines are present in both ID and metabolically normal horses. In fact, our results indicate that ID horses may

be particularly sensitive to seasonal changes in inflammation, as they had differences or trends to differ from controls dependent upon date for gene expression of TLR-4, IL-6, and TNF-α as well as lymphocyte protein concentrations of TNF-α and IFN-γ.

Not only have we observed differences between ID and metabolically normal horses' inflammatory responses and gut microbiota with this work, but we have also seen changes in circulating plasma lipid concentrations. ID horses had increases in serum triglycerides and leptin, as well as plasma diacylglycerides, triacylglycerides, monoacylglyceride, and ceramide. They also had decreases in plasma sphingomyelins, sulfatide, and choline plasmalogens as compared to controls. Lipids have been shown in humans and mice to be able to influence multiple biological functions, including; cell signaling, gene expression, and activation of inflammatory pathways in multiple cell types [112-114]. Changing lipid concentrations has also been shown to impact insulin resistance [114, 228, 240]. Bioactive lipids (including ceramide and sulfatides) are also able to serve as chemoattractant and intracellular messengers [118, 119]. One of the lipid moieties we observed to be increased in ID horses, ceramide, has received attention in human research as it is increased in skeletal muscle of obese individuals with metabolic dysfunction [208]. Further, it appears to have a prominent role in both cell signaling and metabolism [113, 115, 116]. ID horses also had decreased levels of critical membrane lipids, sphingomyelins and plasmalogens, indicating possible changes in membrane fluidity and permeability [109-111]. These differences, along with increased levels of tri-, di-, and monoacylglycerides, may point to changes in cell signaling and add to existing metabolic dysfunction in ID horses. However, the full impact of changes in plasma lipid concentrations in ID horses as it relates to cell signaling and metabolism is unclear.

Considering that lipids can influence inflammatory signaling, including acting as chemoattractants for lymphocytes [118], and the fact that inflammation has a role in the immune response, it is perhaps not surprising that the immune system can recognize both foreign [126] and self-lipids [127-129] as antigens. In addition, a reduced cell mediated immune response (CMI) has been observed in obese humans and mice [134-137]. Data generated from stimulated PBMCs, as in our results, more strongly reflects cellular responses opposed to systemic responses. Indeed, we show that at fasting in PBMC stimulated lymphocytes, ID horses often are lower in proinflammatory cytokine protein or

gene expression compared to controls. This indicates a reduced CMI response in these animals. In response to a Hepatitis B vaccine, a reduced hepatitis specific antibody and T cell activation but increased levels of IFN-γ and TNF-α as well as T and B cell proliferation have been observed in diet-induced obese mice [135]. In influenza vaccinated obese humans, PBMCs had a lower functional protein expression in response to viral challenge [138]. When we vaccinated a group of ID and non-ID controls, we observed a normal humoral response to vaccination, regardless of metabolic status, compared to non-vaccinated saline controls. This is similar to what is seen in humans where body mass index, but not diabetes status, was positively correlated with a greater decline in influenza antibody titers 12 months following influenza vaccination [138]. While we did not observe an effect of metabolic status on humoral immune responses to vaccination, there was some evidence that CMI responses were different in ID compared to control horses. Flu stimulated PBMC IL-2 gene expression was lower in ID horses and TNF-α gene expression did not change over time in ID horses whereas control horses had differences between pre and post vaccination time points. ID horses also had lower PMA stimulated PBMC gene expression of IFN-γ and IL-2 compared to controls, dependent upon time point. As TNF-α and IFN-γ play important roles in CMI immune responses [248, 249] and IL-2 is associated with lymphocyte proliferation [247], it lends further evidence for a reduced CMI response in horses with ID. However, it is important to note that while this work observed differences in immune responses of horses with ID, the physiological impact of these differences, in particular in the face of challenge, remains to be seen.

To better understand mechanisms behind alterations is inflammatory signaling pathways, we also examined gene expression of inflammatory cytokines and markers involved in TLR-4 inflammatory signaling pathways in equine whole blood both unstimulated and stimulated with endotoxin. For all horses and all inflammatory markers (with the exception of CCL2; chemokine (C-C motif) ligand 2), LPS was able to stimulate gene expression. However, stimulation was minimal or nonexistent for chemokine (C-C motif) ligand 5 (CCL5, or RANTES) and CCL2 (or MCP-1, monocyte chemoattractant protein-1), which are associated with the MyD88 independent signaling pathway. Others have similarly demonstrated this in LPS stimulated monocytes from metabolically normal horses [274]. Interesting, there was an effect of metabolic status on two chemokines,

interleukin 8 (IL-8) and CCL2, with ID horses having lower gene expression compared to controls. Conversely, healthy elderly humans LPS stimulated whole blood gene expression [283], obese humans or those with metabolic dysfunction circulating levels [279-282] and LPS stimulated leukocytes in elderly horses or those with PPID [284] have all shown an increased IL-8 gene expression as compared to controls. However, others have shown no difference in lymphocyte gene expression of IL-8 in obese hyperinsulinemic horses compared to non-obese normoinsulinemic controls [31], although neutrophil oxidative activity was increased in the obese hyperinsulinemic group. Our horses did not differ in age and were all classified as adults. Lower IL-8 and CCL2 concentrations therefore indicate that ID horses may have decreased innate immune function.

Treatment strategies for both humans and animals with metabolic dysfunction have been varied. One of the proposed dietary strategies in humans and horses has been supplementation with omega-3 fatty acids, specifically docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) [157]. We observed, similar to others [159, 288, 292], that following supplementation with a DHA rich algae, ID horses had significant increases in serum DHA concentrations, among other fatty acids. Also similar to others [177], we observed a significant decrease in serum triglycerides in response to supplementation. There was also a trend for a decrease in lymphocyte TNF-α protein production. Others have not seen an effect of omega 3 fatty acid supplementation on prostaglandin E2 (PGE2) as an inflammatory marker in PBMCs of yearling horses [292], although it did impact PGE2 production and other markers of inflammation in adult horses bronchoalveolar lavage fluid (BAL) in other studies [297, 298]. These differences in results are possibly due to different sources of omega 3 fatty acids as well as the resulting differences in DHA to EPA ratio, especially considering that DHA appears to have a more potent anti-inflammatory effect compared to EPA [299, 300]. Importantly for this work, supplementation with a DHA rich algae in ID horses also resulted in an improved insulinemic response to an oral sugar challenge. Others have shown that following dexamethasone administration in horses, DHA rich algae reduced both glucose and insulin concentrations and improved their modified insulin to glucose ratio [173]. However, we are the first to demonstrate a positive metabolic effect of DHA rich microalgae supplementation in the ID horse.

With these results we have been able to show that compared to metabolically normal controls, horses with ID have differences in their gut microbiota, specifically a decrease in microbial diversity and overall community structure. Horses with ID also have differences in their plasma lipidome, with increased tri-, di- and mono-acylglycerides as well as ceramide but lower levels of sphingomyelins, sulfatide, and choline plasmalogens. In addition, ID horses demonstrated an abnormal inflammatory response to glycemic challenge, having an inverse, often proinflammatory response, compared to controls. ID horses were able to mount a normal humoral response to routine vaccination, however lower IFN-γ and IL-2 gene expression indicated a possible reduced cell mediated immune (CMI) responses to vaccination. Circulating endotoxin concentrations did not appear to be influenced by metabolic status or glycemic challenge. While there was a minor effect of yeast cell wall supplementation on inflammatory markers, it did not influence serum glucose of insulin concentrations. However, there appeared to be a seasonal effect as well as an effect of glycemic challenge on both metabolic and inflammatory markers, indicating that ID horses may be particularly sensitive to changes in either diet or season. Endotoxin stimulation of whole blood was able to induce inflammatory gene expression primarily in MyD88 dependent associated genes for all horses. In ID horses, a reduced whole blood gene expression of the chemokines IL-8 and CCL2 may further point to a suppressed innate immune response in these horses. Finally, we were able to show that supplementation with DHA rich algae can improve insulin responses and may potentially improve inflammatory parameters in ID horses. Overall this work has indicated differences in inflammation in the ID horse, primarily in a TLR-4 MyD88 dependent associated gene and particularly in response to oral glycemic challenge. It also demonstrated changes in their circulating lipid concentrations and gut microbiota as well as a possible reduced innate immune or cell mediated immune responses in these animals.

REFERENCES

[1] Flegal KM, Carroll MD, Kit BK, Ogden CL. Prevalence of obesity and trends in the distribution of body mass index among US adults, 1999-2010. Jama. 2012;307:491-7.

[2] Ogden CL, Carroll MD, Kit BK, Flegal KM. Prevalence of childhood and adult obesity in the United States, 2011-2012. Jama. 2014;311:806-14.

[3] Geor R, Thatcher C, Pleasant R, Elvinger F, Gay L, Werre S. Prevalence of hyperinsulinemia in mature horses: Relationship to adiposity. Journal of Veterinary Internal Medicine: AMER COLL VETERINARY INTERNAL MEDICINE 1997 WADSWORTH BOULEVARD, STE A, LAKEWOOD, CO 80214-5293 USA; 2007. p. 601-.

[4] Thatcher C, Pleasant R, Geor R, Elvinger F, Negrin K, Franklin J, et al. Prevalence of obesity in mature horses: an equine body condition study. Journal of Animal Physiology and Animal Nutrition. 2008;92:222-.

[5] Robin C, Ireland J, Wylie C, Collins S, Verheyen K, Newton J. Prevalence of and risk factors for equine obesity in Great Britain based on owner-reported body condition scores. Equine veterinary journal. 2015;47:196-201.

[6] Potter S, Bamford N, Harris P, Bailey S. Prevalence of obesity and owners' perceptions of body condition in pleasure horses and ponies in south-eastern Australia. Australian Veterinary Journal. 2016;94:427-32.

[7] Wyse C, McNie K, Tannahil V, Murray J, Love S. Prevalence of obesity in riding horses in Scotland. Veterinary Record. 2008;162:590.

[8] Vick M, Adams A, Murphy B, Sessions D, Horohov D, Cook R, et al. Relationships among inflammatory cytokines, obesity, and insulin sensitivity in the horse. Journal of animal science. 2007;85:1144-55.

[9] Vick M, Sessions D, Murphy B, Kennedy E, Reedy S, Fitzgerald B. Obesity is associated with altered metabolic and reproductive activity in the mare: effects of metformin on insulin sensitivity and reproductive cyclicity. Reproduction, Fertility and Development. 2006;18:609-17.

[10] McGowan T, Pinchbeck G, McGowan C. Prevalence, risk factors and clinical signs predictive for equine pituitary pars intermedia dysfunction in aged horses. Equine veterinary journal. 2013;45:74-9.

[11] Klinkhamer K, Menheere PP, van der Kolk JH. Basal glucose metabolism and peripheral insulin sensitivity in equine pituitary pars intermedia dysfunction. Veterinary Quarterly. 2011;31:19-28.

[12] Frank N, Tadros E. Insulin dysregulation. Equine veterinary journal. 2014;46:103-12.

[13] Schuver A, Frank N, Chameroy KA, Elliott SB. Assessment of insulin and glucose dynamics by using an oral sugar test in horses. Journal of Equine Veterinary Science. 2014;34:465-70.

[14] de Laat MA. Metabolic dysfunction and laminitis. Australian Equine Veterinarian. 2014;33:44-52.

[15] Menzies-Gow N, Harris P, Elliott J. Prospective cohort study evaluating risk factors for the development of pasture-associated laminitis in the United Kingdom. Equine veterinary journal. 2016.

[16] Lane H, Burns T, Hegedus O, Watts M, Weber P, Woltman K, et al. Lamellar events related to insulin-like growth factor-1 receptor signaling in two models relevant to endocrinopathic laminitis. Equine Veterinary Journal. 2017.
[17] Geor RJ, Belknap JK. Equine Laminitis: John Wiley & Sons; 2016.
[18] Belknap J, Giguere S, Pettigrew A, Cochran A, Eps A, Pollitt C. Lamellar pro-inflammatory cytokine expression patterns in laminitis at the developmental stage and at the onset of lameness: innate vs. adaptive immune response. Equine veterinary journal. 2007;39:42-7.
[19] Asplin KE, Sillence MN, Pollitt CC, McGowan CM. Induction of laminitis by prolonged hyperinsulinaemia in clinically normal ponies. The Veterinary Journal. 2007;174:530-5.
[20] Karikoski NP, Patterson-Kane JC, Asplin KE, McGowan TW, McNutt M, Singer ER, et al. Morphological and cellular changes in secondary epidermal laminae of horses with insulin-induced laminitis. American journal of veterinary research. 2014;75:161-8.
[21] Wylie CE, Collins SN, Verheyen KL, Newton JR. Frequency of equine laminitis: a systematic review with quality appraisal of published evidence. The Veterinary Journal. 2011;189:248-56.
[22] Karikoski N, Horn I, McGowan T, McGowan C. The prevalence of endocrinopathic laminitis among horses presented for laminitis at a first-opinion/referral equine hospital. Domestic animal endocrinology. 2011;41:111-7.
[23] Frank N, Geor R, Bailey S, Durham A, Johnson P. Equine metabolic syndrome. Journal of Veterinary Internal Medicine. 2010;24:467-75.
[24] Kaur J. A comprehensive review on metabolic syndrome. Cardiology research and practice. 2014;2014.
[25] Alberti K, Eckel RH, Grundy SM, Zimmet PZ, Cleeman JI, Donato KA, et al. Harmonizing the metabolic syndrome. Circulation. 2009;120:1640-5.
[26] Ertelt A, Barton A-K, Schmitz RR, Gehlen H. Metabolic syndrome: is equine disease comparable to what we know in humans? Endocrine connections. 2014;3:R81-R93.
[27] Bremer AA, Devaraj S, Afify A, Jialal I. Adipose tissue dysregulation in patients with metabolic syndrome. The Journal of Clinical Endocrinology & Metabolism. 2011;96:E1782-E8.
[28] Marycz K, Basinska K, TOKER NY. The Activity of IL-6 and TNF-α in Adipose Tissue and Peripheral Blood in horses Suffering from Equine Metabolic Syndrome (EMS). Kafkas Üniversitesi Veteriner Fakültesi Dergisi. 2014;20:493-9.
[29] McGowan C, Dugdale A, Pinchbeck G, Argo CM. Dietary restriction in combination with a nutraceutical supplement for the management of equine metabolic syndrome in horses. The Veterinary Journal. 2013;196:153-9.
[30] Tadros EM, Frank N, Donnell RL. Effects of equine metabolic syndrome on inflammatory responses of horses to intravenous lipopolysaccharide infusion. American journal of veterinary research. 2013;74:1010-9.
[31] Holbrook TC, Tipton T, McFarlane D. Neutrophil and cytokine dysregulation in hyperinsulinemic obese horses. Veterinary immunology and immunopathology. 2012;145:283-9.

[32] Tiley HA, Geor RJ, McCutcheon LJ. Effects of dexamethasone administration on insulin resistance and components of insulin signaling and glucose metabolism in equine skeletal muscle. American journal of veterinary research. 2008;69:51-8.
[33] Carter RA, McCutcheon LJ, George LA, Smith TL, Frank N, Geor RJ. Effects of diet-induced weight gain on insulin sensitivity and plasma hormone and lipid concentrations in horses. American journal of veterinary research. 2009;70:1250-8.
[34] Banse HE, McCann J, Yang F, Wagg C, McFarlane D. Comparison of two methods for measurement of equine insulin. Journal of Veterinary Diagnostic Investigation. 2014;26:527-30.
[35] Carslake H, Pinchbeck G, McGowan C. Evaluation of a Chemiluminescent Immunoassay for Measurement of Equine Insulin. Journal of Veterinary Internal Medicine. 2017.
[36] Warnken T, Huber K, Feige K. Comparison of three different methods for the quantification of equine insulin. BMC Veterinary Research. 2016;12:196.
[37] Bertin F, Taylor S, Bianco A, Sojka-Kritchevsky J. The Effect of Fasting Duration on Baseline Blood Glucose Concentration, Blood Insulin Concentration, Glucose/Insulin Ratio, Oral Sugar Test, and Insulin Response Test Results in Horses. Journal of veterinary internal medicine. 2016;30:1726-31.
[38] Knowles E, Harris P, Elliott J, Menzies-Gow N. Use of the oral sugar test in ponies when performed with or without prior fasting. Equine veterinary journal. 2016.
[39] Vick MM, Murphy BA, Sessions DR, Reedy SE, Kennedy EL, Horohov DW, et al. Effects of systemic inflammation on insulin sensitivity in horses and inflammatory cytokine expression in adipose tissue. American journal of veterinary research. 2008;69:130-9.
[40] Suagee J, Corl B, Crisman M, Pleasant R, Thatcher C, Geor R. Relationships between Body Condition Score and Plasma Inflammatory Cytokines, Insulin, and Lipids in a Mixed Population of Light-Breed Horses. Journal of Veterinary Internal Medicine. 2013;27:157-63.
[41] Burns T, Geor R, Mudge M, McCutcheon L, Hinchcliff K, Belknap J. Proinflammatory Cytokine and Chemokine Gene Expression Profiles in Subcutaneous and Visceral Adipose Tissue Depots of Insulin-Resistant and Insulin-Sensitive Light Breed Horses. Journal of Veterinary Internal Medicine. 2010;24:932-9.
[42] Elzinga S, Wood P, Adams AA. Plasma Lipidomic and Inflammatory Cytokine Profiles of Horses With Equine Metabolic Syndrome. Journal of Equine Veterinary Science. 2016;40:49-55.
[43] Nappo F, Esposito K, Cioffi M, Giugliano G, Molinari AM, Paolisso G, et al. Postprandial endothelial activation in healthy subjects and in type 2 diabetic patients: role of fat and carbohydrate meals. Journal of the American College of Cardiology. 2002;39:1145-50.
[44] Ceriello A, Quagliaro L, Piconi L, Assaloni R, Da Ros R, Maier A, et al. Effect of postprandial hypertriglyceridemia and hyperglycemia on circulating adhesion molecules and oxidative stress generation and the possible role of simvastatin treatment. Diabetes. 2004;53:701-10.

[45] Esposito K, Nappo F, Giugliano F, Giugliano G, Marfella R, Giugliano D. Effect of dietary antioxidants on postprandial endothelial dysfunction induced by a high-fat meal in healthy subjects. The American journal of clinical nutrition. 2003;77:139-43.
[46] Suagee JK, Splan RK, Swyers KL, Geor RJ, Corl BA. Effects of High-Sugar and High-Starch Diets on Postprandial Inflammatory Protein Concentrations in Horses. Journal of Equine Veterinary Science. 2015;35:191-7.
[47] Elzinga S, Rohleder B, Schanbacher B, McQuerry K, Barker V, Adams A. Metabolic and inflammatory responses to the common sweetener stevioside and a glycemic challenge in horses with equine metabolic syndrome. Domestic Animal Endocrinology. 2017.
[48] Council NR. Nutrient Requirements of Horses: Sixth Revised Edition. Washington D.C.: The National Academies Press; 2007.
[49] Shepherd M, Pleasant R, Crisman M, Werre S, Milton S, Swecker Jr W. Effects of high and moderate non-structural carbohydrate hay on insulin, glucose, triglyceride, and leptin concentrations in overweight Arabian geldings. Journal of animal physiology and animal nutrition. 2012;96:428-35.
[50] Eps Av, Pollitt C. Equine laminitis induced with oligofructose. Equine veterinary journal. 2006;38:203-8.
[51] Tadros EM, Frank N, Horohov DW. Inflammatory cytokine gene expression in blood during the development of oligofructose-induced laminitis in horses. Journal of Equine Veterinary Science. 2013;33:802-8.
[52] Bonnard C, Durand A, Peyrol S, Chanseaume E, Chauvin M-A, Morio B, et al. Mitochondrial dysfunction results from oxidative stress in the skeletal muscle of diet-induced insulin-resistant mice. The Journal of clinical investigation. 2008;118:789-800.
[53] Marycz K, Kornicka K, Basinska K, Czyrek A. Equine metabolic syndrome affects viability, senescence, and stress factors of equine adipose-derived mesenchymal stromal stem cells: new insight into EqASCs isolated from ems horses in the context of their aging. Oxidative medicine and cellular longevity. 2015;2016.
[54] Meigs JB, Larson MG, Fox CS, Keaney JF, Vasan RS, Benjamin EJ. Association of oxidative stress, insulin resistance, and diabetes risk phenotypes. Diabetes care. 2007;30:2529-35.
[55] Urakawa H, Katsuki A, Sumida Y, Gabazza EC, Murashima S, Morioka K, et al. Oxidative stress is associated with adiposity and insulin resistance in men. The Journal of Clinical Endocrinology & Metabolism. 2003;88:4673-6.
[56] Ceriello A, Taboga C, Tonutti L, Quagliaro L, Piconi L, Bais B, et al. Evidence for an independent and cumulative effect of postprandial hypertriglyceridemia and hyperglycemia on endothelial dysfunction and oxidative stress generation. Circulation. 2002;106:1211-8.
[57] Annuzzi G, Bozzetto L, Costabile G, Giacco R, Mangione A, Anniballi G, et al. Diets naturally rich in polyphenols improve fasting and postprandial dyslipidemia and reduce oxidative stress: a randomized controlled trial. The American journal of clinical nutrition. 2014;99:463-71.

[58] Sottero B, Gargiulo S, Russo I, Barale C, Poli G, Cavalot F. Postprandial dysmetabolism and oxidative stress in type 2 diabetes: pathogenetic mechanisms and therapeutic strategies. Medicinal research reviews. 2015;35:968-1031.
[59] Erridge C, Attina T, Spickett CM, Webb DJ. A high-fat meal induces low-grade endotoxemia: evidence of a novel mechanism of postprandial inflammation. The American journal of clinical nutrition. 2007;86:1286-92.
[60] Lyte JM, Gabler NK, Hollis JH. Postprandial serum endotoxin in healthy humans is modulated by dietary fat in a randomized, controlled, cross-over study. Lipids in Health and Disease. 2016;15:186.
[61] Cani PD, Amar J, Iglesias MA, Poggi M, Knauf C, Bastelica D, et al. Metabolic endotoxemia initiates obesity and insulin resistance. Diabetes. 2007;56:1761-72.
[62] Laugerette F, Vors C, Peretti N, Michalski M-C. Complex links between dietary lipids, endogenous endotoxins and metabolic inflammation. Biochimie. 2011;93:39-45.
[63] Cani PD, Bibiloni R, Knauf C, Waget A, Neyrinck AM, Delzenne NM, et al. Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet–induced obesity and diabetes in mice. Diabetes. 2008;57:1470-81.
[64] Costa MC, Weese JS. The equine intestinal microbiome. Animal Health Research Reviews. 2012;13:121-8.
[65] Dougal K, de la Fuente G, Harris PA, Girdwood SE, Pinloche E, Geor RJ, et al. Characterisation of the faecal bacterial community in adult and elderly horses fed a high fibre, high oil or high starch diet using 454 pyrosequencing. PloS one. 2014;9:e87424.
[66] Fernandes KA, Kittelmann S, Rogers CW, Gee EK, Bolwell CF, Bermingham EN, et al. Faecal microbiota of forage-fed horses in New Zealand and the population dynamics of microbial communities following dietary change. PloS one. 2014;9:e112846.
[67] Shepherd ML, Swecker Jr WS, Jensen RV, Ponder MA. Characterization of the fecal bacteria communities of forage-fed horses by pyrosequencing of 16S rRNA V4 gene amplicons. FEMS microbiology letters. 2012;326:62-8.
[68] Costa M, Silva G, Ramos R, Staempfli H, Arroyo L, Kim P, et al. Characterization and comparison of the bacterial microbiota in different gastrointestinal tract compartments in horses. The Veterinary Journal. 2015.
[69] Fernandes KA, Kittelmann S, Rogers CW, Gee EK, Bolwell CF, Bermingham EN, et al. Faecal Microbiota of Forage-Fed Horses in New Zealand and the Population Dynamics of Microbial Communities following Dietary Change. 2014.
[70] Biddle AS, Black SJ, Blanchard JL. An in vitro model of the horse gut microbiome enables identification of lactate-utilizing bacteria that differentially respond to starch induction. PloS one. 2013;8:e77599.
[71] Lecomte V, Kaakoush NO, Maloney CA, Raipuria M, Huinao KD, Mitchell HM, et al. Changes in gut microbiota in rats fed a high fat diet correlate with obesity-associated metabolic parameters. PloS one. 2015;10:e0126931.
[72] Steelman SM, Chowdhary BP, Dowd S, Suchodolski J, Janečka JE. Pyrosequencing of 16S rRNA genes in fecal samples reveals high diversity of hindgut microflora in horses and potential links to chronic laminitis. BMC veterinary research. 2012;8:231.

[73] Milinovich GJ, Trott DJ, Burrell PC, Croser EL, Al Jassim RA, Morton JM, et al. Fluorescence in situ hybridization analysis of hindgut bacteria associated with the development of equine laminitis. Environmental microbiology. 2007;9:2090-100.
[74] Milinovich G, Trott D, Burrell P, Van Eps A, Thoefner M, Blackall L, et al. Changes in equine hindgut bacterial populations during oligofructose-induced laminitis. Environmental Microbiology. 2006;8:885-98.
[75] Costa MC, Arroyo LG, Allen-Vercoe E, Stampfli HR, Kim PT, Sturgeon A, et al. Comparison of the fecal microbiota of healthy horses and horses with colitis by high throughput sequencing of the V3-V5 region of the 16S rRNA gene. PLoS One. 2012;7:e41484.
[76] Weese J, Holcombe S, Embertson R, Kurtz K, Roessner H, Jalali M, et al. Changes in the faecal microbiota of mares precede the development of post partum colic. Equine veterinary journal. 2015.
[77] Fava F, Gitau R, Griffin B, Gibson G, Tuohy K, Lovegrove J. The type and quantity of dietary fat and carbohydrate alter faecal microbiome and short-chain fatty acid excretion in a metabolic syndrome 'at-risk'population. International Journal of Obesity. 2013;37:216-23.
[78] Pedersen R, Ingerslev H-C, Sturek M, Alloosh M, Cirera S, Christoffersen BØ, et al. Characterisation of gut microbiota in Ossabaw and Göttingen minipigs as models of obesity and metabolic syndrome. PloS one. 2013;8:e56612.
[79] Turnbaugh PJ, Hamady M, Yatsunenko T, Cantarel BL, Duncan A, Ley RE, et al. A core gut microbiome in obese and lean twins. nature. 2009;457:480-4.
[80] Park HJ, Lee SE, Kim HB, Isaacson R, Seo KW, Song KH. Association of Obesity with Serum Leptin, Adiponectin, and Serotonin and Gut Microflora in Beagle Dogs. Journal of Veterinary Internal Medicine. 2015;29:43-50.
[81] Costa MC, Stämpfli HR, Arroyo LG, Allen-Vercoe E, Gomes RG, Weese JS. Changes in the equine fecal microbiota associated with the use of systemic antimicrobial drugs. BMC veterinary research. 2015;11:19.
[82] Elzinga SE, Weese JS, Adams AA. Comparison of the Fecal Microbiota in Horses With Equine Metabolic Syndrome and Metabolically Normal Controls Fed a Similar All-Forage Diet. Journal of Equine Veterinary Science. 2016;44:9-16.
[83] Kim K-A, Jeong J-J, Yoo S-Y, Kim D-H. Gut microbiota lipopolysaccharide accelerates inflamm-aging in mice. BMC Microbiology. 2016;16:1.
[84] Haro C, Garcia-Carpintero S, Alcala-Diaz JF, Gomez-Delgado F, Delgado-Lista J, Perez-Martinez P, et al. The gut microbial community in metabolic syndrome patients is modified by diet. The Journal of nutritional biochemistry. 2016;27:27-31.
[85] Isabel M-I, Lidia S-A, Pablo P-M, Cristina A-L, Fernando C, Francisco T, et al. Red wine polyphenols modulate fecal microbiota and reduce markers of the metabolic syndrome in obese patients. Food & function. 2016.
[86] Han SH, Suk KT, Kim DJ, Kim MY, Baik SK, Kim YD, et al. Effects of probiotics (cultured Lactobacillus subtilis/Streptococcus faecium) in the treatment of alcoholic hepatitis: randomized-controlled multicenter study. European journal of gastroenterology & hepatology. 2015;27:1300-6.

[87] Collins MD, Gibson GR. Probiotics, prebiotics, and synbiotics: approaches for modulating the microbial ecology of the gut. The American Journal of Clinical Nutrition. 1999;69:1052s-7s.
[88] Berg E, Fu C, Porter J, Kerley M. Fructooligosaccharide supplementation in the yearling horse: effects on fecal pH, microbial content, and volatile fatty acid concentrations. Journal of animal science. 2005;83:1549-53.
[89] Respondek F, Goachet A, Julliand V. Effects of dietary short-chain fructooligosaccharides on the intestinal microflora of horses subjected to a sudden change in diet. Journal of animal science. 2008;86:316-23.
[90] Bornet F, Brouns F, Tashiro Y, Duvillier V. Nutritional aspects of short-chain fructooligosaccharides: natural occurrence, chemistry, physiology and health implications. Digestive and Liver Disease. 2002;34:S111-S20.
[91] Canfora EE, Jocken JW, Blaak EE. Short-chain fatty acids in control of body weight and insulin sensitivity. Nature reviews Endocrinology. 2015;11:577.
[92] Puertollano E, Kolida S, Yaqoob P. Biological significance of short-chain fatty acid metabolism by the intestinal microbiome. Current Opinion in Clinical Nutrition & Metabolic Care. 2014;17:139-44.
[93] Respondek F, Myers K, Smith T, Wagner A, Geor R. Dietary supplementation with short-chain fructo-oligosaccharides improves insulin sensitivity in obese horses. Journal of animal science. 2011;89:77-83.
[94] Respondek F, Swanson KS, Belsito KR, Vester BM, Wagner A, Istasse L, et al. Short-chain fructooligosaccharides influence insulin sensitivity and gene expression of fat tissue in obese dogs. The Journal of nutrition. 2008;138:1712-8.
[95] Yuan K, Mendonça L, Hulbert L, Mamedova L, Muckey M, Shen Y, et al. Yeast product supplementation modulated humoral and mucosal immunity and uterine inflammatory signals in transition dairy cows. Journal of dairy science. 2015;98:3236-46.
[96] Świątkiewicz S, Arczewska-Włosek A, Jozefiak D. Immunomodulatory efficacy of yeast cell products in poultry: a current review. World's Poultry Science Journal. 2014;70:57-68.
[97] Smith IM, Christensen JE, Arneborg N, Jespersen L. Yeast modulation of human dendritic cell cytokine secretion: an in vitro study. PloS one. 2014;9:e96595.
[98] Stier H, Ebbeskotte V, Gruenwald J. Immune-modulatory effects of dietary Yeast Beta-1, 3/1, 6-D-glucan. Nutrition journal. 2014;13:38.
[99] Spatuzza C, Postiglione L, Covelli B, Ricciardone M, Benvenuti C, Mondola P, et al. Effects of berberine and red yeast on proinflammatory cytokines IL-6 and TNF-α in peripheral blood mononuclear cells (PBMCs) of human subjects. Frontiers in pharmacology. 2014;5:230.
[100] Koke K. Effects of dietary yeast supplementation on serum immunoglobulin concentrations in quarter horse mares: The Ohio State University; 2014.
[101] Schoster A, Weese J, Guardabassi L. Probiotic Use in Horses–What is the Evidence for Their Clinical Efficacy? Journal of veterinary internal medicine. 2014;28:1640-52.
[102] Sanchez NCB, Young TR, Carroll JA, Corley JR, Rathmann RJ, Johnson BJ. Yeast cell wall supplementation alters the metabolic responses of crossbred heifers to an endotoxin challenge. Innate immunity. 2014;20:104-12.

[103] Kannan M, Karunakaran R, Balakrishnan V, Prabhakar T. Influence of prebiotics supplementation on lipid profile of broilers. Int J Poult Sci. 2005;4:994-7.
[104] Huff G, Huff W, Farnell M, Rath N, de Los Santos FS, Donoghue A. Bacterial clearance, heterophil function, and hematological parameters of transport-stressed turkey poults supplemented with dietary yeast extract. Poultry science. 2010;89:447-56.
[105] Jin L, Dong G, Lei C, Zhou J, Zhang S. Effects of dietary supplementation of glutamine and mannan oligosaccharides on plasma endotoxin and acute phase protein concentrations and nutrient digestibility in finishing steers. Journal of Applied Animal Research. 2014;42:160-5.
[106] Hansen ME, Simmons KJ, Tippetts TS, Thatcher MO, Saito RR, Hubbard ST, et al. Lipopolysaccharide disrupts mitochondrial physiology in skeletal muscle via disparate effects on sphingolipid metabolism. Shock (Augusta, Ga). 2015;44:585.
[107] Frank N, Elliott SB, Brandt LE, Keisler DH. Physical characteristics, blood hormone concentrations, and plasma lipid concentrations in obese horses with insulin resistance. Journal of the American Veterinary Medical Association. 2006;228:1383-90.
[108] Gross RW, Han X. Lipidomics in diabetes and the metabolic syndrome. Methods in enzymology. 2007;433:73-90.
[109] Pietiläinen KH, Sysi-Aho M, Rissanen A, Seppänen-Laakso T, Yki-Järvinen H, Kaprio J, et al. Acquired obesity is associated with changes in the serum lipidomic profile independent of genetic effects–a monozygotic twin study. 2007.
[110] Hunte C, Richers S. Lipids and membrane protein structures. Current opinion in structural biology. 2008;18:406-11.
[111] Kasimova MA, Tarek M, Shaytan AK, Shaitan KV, Delemotte L. Voltage-gated ion channel modulation by lipids: Insights from molecular dynamics simulations. Biochimica et Biophysica Acta (BBA)-Biomembranes. 2014;1838:1322-31.
[112] Dennis EA, Deems RA, Harkewicz R, Quehenberger O, Brown HA, Milne SB, et al. A mouse macrophage lipidome. Journal of Biological Chemistry. 2010;285:39976-85.
[113] Yang G, Badeanlou L, Bielawski J, Roberts AJ, Hannun YA, Samad F. Central role of ceramide biosynthesis in body weight regulation, energy metabolism, and the metabolic syndrome. American Journal of Physiology-Endocrinology and Metabolism. 2009;297:E211-E24.
[114] Glass CK, Olefsky JM. Inflammation and lipid signaling in the etiology of insulin resistance. Cell metabolism. 2012;15:635-45.
[115] Meikle PJ, Christopher MJ. Lipidomics is providing new insight into the metabolic syndrome and its sequelae. Current opinion in lipidology. 2011;22:210-5.
[116] Turpin SM, Lancaster GI, Darby I, Febbraio MA, Watt MJ. Apoptosis in skeletal muscle myotubes is induced by ceramides and is positively related to insulin resistance. American Journal of Physiology-Endocrinology and Metabolism. 2006;291:E1341-E50.
[117] Chavez JA, Siddique MM, Wang ST, Ching J, Shayman JA, Summers SA. Ceramides and glucosylceramides are independent antagonists of insulin signaling. Journal of Biological Chemistry. 2014;289:723-34.

[118] Kihara A, Mitsutake S, Mizutani Y, Igarashi Y. Metabolism and biological functions of two phosphorylated sphingolipids, sphingosine 1-phosphate and ceramide 1-phosphate. Progress in lipid research. 2007;46:126-44.
[119] Samad F, Hester KD, Yang G, Hannun YA, Bielawski J. Altered Adipose and Plasma Sphingolipid Metabolism in Obesity A Potential Mechanism for Cardiovascular and Metabolic Risk. Diabetes. 2006;55:2579-87.
[120] Katz S, Ernst O, Avni D, Athamna M, Philosoph A, Arana L, et al. Exogenous ceramide-1-phosphate (C1P) and phospho-ceramide analogue-1 (PCERA-1) regulate key macrophage activities via distinct receptors. Immunology letters. 2016;169:73-81.
[121] Lankinen M, Schwab U, Erkkila A, Seppanen-Laakso T, Hannila M-L, Mussalo H, et al. Fatty fish intake decreases lipids related to inflammation and insulin signaling–a lipidomics approach. PLoS one. 2009;4:e5258.
[122] Browning L, Krebs J, Moore C, Mishra G, O'Connell M, Jebb S. The impact of long chain n-3 polyunsaturated fatty acid supplementation on inflammation, insulin sensitivity and CVD risk in a group of overweight women with an inflammatory phenotype. Diabetes, Obesity and Metabolism. 2007;9:70-80.
[123] Wall R, Ross RP, Fitzgerald GF, Stanton C. Fatty acids from fish: the anti-inflammatory potential of long-chain omega-3 fatty acids. Nutrition reviews. 2010;68:280-9.
[124] Davies P, Bailey PJ, Goldenberg MM, Ford-Hutchinson AW. The role of arachidonic acid oxygenation products in pain and inflammation. Annual review of immunology. 1984;2:335-57.
[125] Goto-Inoue N, Yamada K, Inagaki A, Furuichi Y, Ogino S, Manabe Y, et al. Lipidomics analysis revealed the phospholipid compositional changes in muscle by chronic exercise and high-fat diet. Scientific reports. 2013;3.
[126] Raetz CR, Reynolds CM, Trent MS, Bishop RE. Lipid A modification systems in gram-negative bacteria. Annual review of biochemistry. 2007;76:295.
[127] De Libero G, Mori L. How the immune system detects lipid antigens. Progress in lipid research. 2010;49:120-7.
[128] Kain L, Webb B, Anderson BL, Deng S, Holt M, Costanzo A, et al. The identification of the endogenous ligands of natural killer t cells reveals the presence of mammalian α-linked glycosylceramides. Immunity. 2014;41:543-54.
[129] Kim J, Kim JH, Winau F. Thinking inside the box: endogenous α-anomeric lipid antigens. Immunity. 2014;41:505-6.
[130] Shi H, Kokoeva MV, Inouye K, Tzameli I, Yin H, Flier JS. TLR4 links innate immunity and fatty acid–induced insulin resistance. Journal of Clinical Investigation. 2006;116:3015.
[131] Balasubramanyam A. The role of the immune system in obesity and insulin resistance. Journal of obesity. 2013;2013.
[132] Pashine A, Valiante NM, Ulmer JB. Targeting the innate immune response with improved vaccine adjuvants. Nature medicine. 2005;11:S63-S8.
[133] Iwasaki A, Medzhitov R. Regulation of adaptive immunity by the innate immune system. science. 2010;327:291-5.

[134] Kim Y-H, Kim J-K, Kim D-J, Nam J-H, Shim S-M, Choi Y-K, et al. Diet-induced obesity dramatically reduces the efficacy of a 2009 pandemic H1N1 vaccine in a mouse model. Journal of Infectious Diseases. 2011:jir731.
[135] Chen S, Akbar SMF, Miyake T, Abe M, Al-Mahtab M, Furukawa S, et al. Diminished immune response to vaccinations in obesity: Role of myeloid-derived suppressor and other myeloid cells. Obesity research & clinical practice. 2015;9:35-44.
[136] Diepersloot R, Bouter K, Beyer W, Hoekstra J, Masurel N. Humoral immune response and delayed type hypersensitivity to influenza vaccine in patients with diabetes mellitus. Diabetologia. 1987;30:397-401.
[137] Horowitz MM, ERSHLER WB, MCKINNEY WP, BATTIOLA RJ. Duration of immunity after hepatitis B vaccination: efficacy of low-dose booster vaccine. Annals of internal medicine. 1988;108:185-9.
[138] Sheridan PA, Paich HA, Handy J, Karlsson EA, Hudgens MG, Sammon AB, et al. Obesity is associated with impaired immune response to influenza vaccination in humans. International journal of obesity. 2012;36:1072-7.
[139] Kotronen A, Seppänen-Laakso T, Westerbacka J, Kiviluoto T, Arola J, Ruskeepää AL, et al. Comparison of Lipid and Fatty Acid Composition of the Liver, Subcutaneous and Intra-abdominal Adipose Tissue, and Serum. Obesity. 2010;18:937-44.
[140] Hla T, Kolesnick R. C16: 0-Ceramide Signals Insulin Resistance. Cell metabolism. 2014;20:703-5.
[141] Bamford N, Potter S, Baskerville C, Harris P, Bailey S. Effect of increased adiposity on insulin sensitivity and adipokine concentrations in different equine breeds adapted to cereal-rich or fat-rich meals. The Veterinary Journal. 2016;214:14-20.
[142] McCue ME, Geor RJ, Schultz N. Equine metabolic syndrome: a complex disease influenced by genetics and the environment. Journal of Equine Veterinary Science. 2015;35:367-75.
[143] McGowan C, Cooper D, Ireland J. No evidence that therapeutic systemic corticosteroid administration is associated with laminitis in adult horses without underlying endocrine or severe systemic disease. Veterinary Evidence. 2016;1.
[144] Welsh CE, Duz M, Parkin TD, Marshall JF. Disease and pharmacologic risk factors for first and subsequent episodes of equine laminitis: A cohort study of free-text electronic medical records. Preventive Veterinary Medicine. 2017;136:11-8.
[145] Spencer M, Yang L, Adu A, Finlin BS, Zhu B, Shipp LR, et al. Pioglitazone treatment reduces adipose tissue inflammation through reduction of mast cell and macrophage number and by improving vascularity. 2014.
[146] Ebke LA, Nestor-Kalinoski AL, Slotterbeck BD, Al-Dieri AG, Ghosh-Lester S, Russo L, et al. Tight association between macrophages and adipocytes in obesity: Implications for adipocyte preparation. Obesity. 2014;22:1246-55.
[147] Mauer J, Chaurasia B, Goldau J, Vogt MC, Ruud J, Nguyen KD, et al. Signaling by IL-6 promotes alternative activation of macrophages to limit endotoxemia and obesity-associated resistance to insulin. Nature immunology. 2014;15:423-30.

[148] Shi H, Kokoeva MV, Inouye K, Tzameli I, Yin H, Flier JS. TLR4 links innate immunity and fatty acid–induced insulin resistance. The Journal of clinical investigation. 2006;116:3015-25.
[149] Vaure C, Liu Y. A comparative review of toll-like receptor 4 expression and functionality in different animal species. Frontiers in immunology. 2014;5:316.
[150] Schaeffler A, Gross P, Buettner R, Bollheimer C, Buechler C, Neumeier M, et al. Fatty acid-induced induction of Toll-like receptor-4/nuclear factor-κB pathway in adipocytes links nutritional signalling with innate immunity. Immunology. 2009;126:233-45.
[151] Zhang Z, Amorosa LF, Coyle SM, Macor MA, Birnbaum MJ, Lee LY, et al. Insulin-dependent regulation of mTORC2-Akt-FoxO suppresses TLR4 signaling in human leukocytes: Relevance to type 2 diabetes. Diabetes. 2016;65:2224-34.
[152] Hundal RS, Krssak M, Dufour S, Laurent D, Lebon V, Chandramouli V, et al. Mechanism by which metformin reduces glucose production in type 2 diabetes. Diabetes. 2000;49:2063-9.
[153] Visser T. Pathways of thyroid hormone metabolism. Acta Medica Austriaca. 1995;23:10-6.
[154] Durham A, Rendle D, Newton J. The effect of metformin on measurements of insulin sensitivity and β cell response in 18 horses and ponies with insulin resistance. Equine veterinary journal. 2008;40:493-500.
[155] Frank N, Elliott SB, Boston RC. Effects of long-term oral administration of levothyroxine sodium on glucose dynamics in healthy adult horses. American journal of veterinary research. 2008;69:76-81.
[156] Frank N, Sommardahl CS, Eiler H, Webb LL, Denhart JW, Boston RC. Effects of oral administration of levothyroxine sodium on concentrations of plasma lipids, concentration and composition of very-low-density lipoproteins, and glucose dynamics in healthy adult mares. American journal of veterinary research. 2005;66:1032-8.
[157] Spencer M, Finlin BS, Unal R, Zhu B, Morris AJ, Shipp LR, et al. Omega-3 fatty acids reduce adipose tissue macrophages in human subjects with insulin resistance. Diabetes. 2013;62:1709-17.
[158] Skulas-Ray AC, Kris-Etherton PM, Harris WS, Heuvel JPV, Wagner PR, West SG. Dose-response effects of omega-3 fatty acids on triglycerides, inflammation, and endothelial function in healthy persons with moderate hypertriglyceridemia. The American journal of clinical nutrition. 2011;93:243-52.
[159] King S, AbuGhazaleh A, Webel S, Jones K. Circulating fatty acid profiles in response to three levels of dietary omega-3 fatty acid supplementation in horses. Journal of animal science. 2008;86:1114-23.
[160] Hess T, Rexford J, Hansen D, Harris M, Schauermann N, Ross T, et al. Effects of two different dietary sources of long chain omega-3, highly unsaturated fatty acids on incorporation into the plasma, red blood cell, and skeletal muscle in horses. Journal of animal science. 2012;90:3023-31.
[161] Katan MB, Deslypere J, Van Birgelen A, Penders M, Zegwaard M. Kinetics of the incorporation of dietary fatty acids into serum cholesteryl esters, erythrocyte membranes, and adipose tissue: an 18-month controlled study. Journal of lipid research. 1997;38:2012-22.

[162] Tsitouras P, Gucciardo F, Salbe A, Heward C, Harman S. High omega-3 fat intake improves insulin sensitivity and reduces CRP and IL6, but does not affect other endocrine axes in healthy older adults. Hormone and metabolic research. 2008;40:199-205.
[163] Lombardo YB, Hein G, Chicco A. Metabolic syndrome: effects of n-3 PUFAs on a model of dyslipidemia, insulin resistance and adiposity. Lipids. 2007;42:427-37.
[164] Behme MT. Dietary fish oil enhances insulin sensitivity in miniature pigs. The Journal of nutrition. 1996;126:1549.
[165] Lou J, Rizkalla S, Boillot J, Alamowitch C. Dietary (n-3) polyunsaturated fatty acids improve adipocyte insulin action and glucose metabolism in insulin-resistance rats: relation to membrane fatty acids. J Nutr. 1996;126:1951-8.
[166] Rasic-Milutinovic Z, Perunicic G, Pljesa S, Gluvic Z, Sobajic S, Djuric I, et al. Effects of N-3 PUFAs supplementation on insulin resistance and inflammatory biomarkers in hemodialysis patients. Renal failure. 2007;29:321-9.
[167] Baillie R, Takada R, Nakamura M, Clarke S. Coordinate induction of peroxisomal acyl-CoA oxidase and UCP-3 by dietary fish oil: a mechanism for decreased body fat deposition. Prostaglandins, leukotrienes and essential fatty acids. 1999;60:351-6.
[168] Price PT, Nelson CM, Clarke SD. Omega-3 polyunsaturated fatty acid regulation of gene expression. Current opinion in lipidology. 2000;11:3-7.
[169] Lankinen M, Schwab U, Erkkilä A, Seppänen-Laakso T, Hannila M-L, Mussalo H, et al. Fatty fish intake decreases lipids related to inflammation and insulin signaling—a lipidomics approach. PLoS one. 2009;4:e5258.
[170] Ottestad I, Hassani S, Borge GI, Kohler A, Vogt G, Hyötyläinen T, et al. Fish oil supplementation alters the plasma lipidomic profile and increases long-chain PUFAs of phospholipids and triglycerides in healthy subjects. PLoS One. 2012;7:e42550.
[171] O'Connor C, Lawrence L, St Lawrence A, Janicki K, Warren L, Hayes S. The effect of dietary fish oil supplementation on exercising horses. Journal of animal science. 2004;82:2978-84.
[172] Hess TM, Rexford J, Hansen DK, Ahrens NS, Harris M, Engle T, et al. Effects of Ω-3 (n-3) fatty acid supplementation on insulin sensitivity in horses. Journal of Equine Veterinary Science. 2013;33:446-53.
[173] Brennan KM, Graugnard DE, Spry ML, Brewster-Barnes T, Smith AC, Schaeffer RE, et al. Effects of a docosahexaenoic acid–rich microalgae nutritional product on insulin sensitivity after prolonged dexamethasone treatment in healthy mature horses. American journal of veterinary research. 2015;76:889-96.
[174] McCann ME, Moore JN, Carrick JB, Barton MH. Effect of intravenous infusion of omega-3 and omega-6 lipid emulsions on equine monocyte fatty acid composition and inflammatory mediator production in vitro. Shock. 2000;14:222-8.
[175] Dinnetz JM, Furtney SR, Pendergraft JS, Davis EG, Epp TS, Minton JE. Omega-3 fatty acid supplementation reduces basal TNFα but not toll-like receptor-stimulated TNFα in full-sized and miniature mares. Journal of Equine Veterinary Science. 2013;33:523-9.

[176] Morris DD, Henry M, Moore J, Fischer J. Effect of dietary alpha-linolenic acid on endotoxin-induced production of tumor necrosis factor by peritoneal macrophages in horses. American journal of veterinary research. 1991;52:528-32.
[177] O'Connor C, Lawrence L, Hayes S. Dietary fish oil supplementation affects serum fatty acid concentrations in horses. Journal of animal science. 2007;85:2183-9.
[178] McHaney AM, Welti R, Roth MR, Dinnetz JM, Furtney SR, Pendergraft JS, et al. Omega-3 Fatty Acid Supplementation Affects Selected Phospholipids in Peripheral White Blood Cells and in Plasma of Full-Sized and Miniature Mares. Journal of Equine Veterinary Science. 2013;33:779-86.
[179] Goyal S, Goyal R. Stevia (Stevia rebaudiana) a bio-sweetener: a review. International Journal of Food Sciences and Nutrition. 2010.
[180] Barriocanal LA, Palacios M, Benitez G, Benitez S, Jimenez JT, Jimenez N, et al. Apparent lack of pharmacological effect of steviol glycosides used as sweeteners in humans. A pilot study of repeated exposures in some normotensive and hypotensive individuals and in Type 1 and Type 2 diabetics. Regulatory toxicology and pharmacology. 2008;51:37-41.
[181] Chang J, Wu M, Liu I, Cheng J. Increase of insulin sensitivity by stevioside in fructose-rich chow-fed rats. Hormone and metabolic research= Hormon-und Stoffwechselforschung= Hormones et metabolisme. 2005;37:610-6.
[182] Chen T-H, Chen S-C, Chan P, Chu Y-L, Yang H-Y, Cheng J-T. Mechanism of the hypoglycemic effect of stevioside, a glycoside of Stevia rebaudiana. Planta medica. 2005;71:108-13.
[183] Anton SD, Martin CK, Han H, Coulon S, Cefalu WT, Geiselman P, et al. Effects of stevia, aspartame, and sucrose on food intake, satiety, and postprandial glucose and insulin levels. Appetite. 2010;55:37-43.
[184] Jeppesen PB, Gregersen S, Poulsen C, Hermansen K. Stevioside acts directly on pancreatic β cells to secrete insulin: Actions independent of cyclic adenosine monophosphate and adenosine triphosphate—sensitivie K+-channel activity. Metabolism. 2000;49:208-14.
[185] Williams O, Pharm B. Botanicals in diabetes treatment: A look at stevia. 2014.
[186] Henneke D, Potter G, Kreider J, Yeates B. Relationship between condition score, physical measurements and body fat percentage in mares. Equine veterinary journal. 1983;15:371-2.
[187] Carter RA, Geor RJ, Staniar WB, Cubitt TA, Harris PA. Apparent adiposity assessed by standardised scoring systems and morphometric measurements in horses and ponies. The Veterinary Journal. 2009;179:204-10.
[188] Herieka M, Erridge C. High-fat meal induced postprandial inflammation. Molecular nutrition & food research. 2014;58:136-46.
[189] Abraham G, Allersmeier M, Schusser GF, Ungemach FR. Serum thyroid hormone, insulin, glucose, triglycerides and protein concentrations in normal horses: Association with topical dexamethasone usage. The Veterinary Journal. 2011;188:307-12.
[190] Brennan K, Urschel K. Recovery of insulin sensitivity in mature horses after a 3 week course of dexamethasone therapy. Equine veterinary journal. 2014;46:718-21.

[191] Beech J, Boston R, Lindborg S, Russell GE. Adrenocorticotropin concentration following administration of thyrotropin-releasing hormone in healthy horses and those with pituitary pars intermedia dysfunction and pituitary gland hyperplasia. Journal of the American Veterinary Medical Association. 2007;231:417-26.
[192] McFarlane D, Beech J, Cribb A. Alpha-melanocyte stimulating hormone release in response to thyrotropin releasing hormone in healthy horses, horses with pituitary pars intermedia dysfunction and equine pars intermedia explants. Domestic animal endocrinology. 2006;30:276-88.
[193] de Castro ED, Lopez I, Cortes B, Pineda C, Garfia B, Aguilera-Tejero E. Influence of feeding status, time of the day, and season on baseline adrenocorticotropic hormone and the response to thyrotropin releasing hormone-stimulation test in healthy horses. Domestic animal endocrinology. 2014;48:77-83.
[194] Donaldson MT, McDonnell SM, Schanbacher BJ, Lamb SV, McFarlane D, Beech J. Variation in plasma adrenocorticotropic hormone concentration and dexamethasone suppression test results with season, age, and sex in healthy ponies and horses. Journal of veterinary internal medicine. 2005;19:217-22.
[195] Perkins G, Lamb S, Erb H, Schanbacher B, Nydam D, Divers T. Plasma adrenocorticotropin (ACTH) concentrations and clinical response in horses treated for equine Cushing's disease with cyproheptadine or pergolide. Equine veterinary journal. 2002;34:679-85.
[196] Reimers T, Salerno V, Lamb S. Validation and application of solid-phase chemiluminescent immunoassays for diagnosis of endocrine diseases in animals. Comparative Haematology International. 1996;6:170-5.
[197] Adams AA, Sturgill TL, Breathnach CC, Chambers TM, Siger L, Minke JM, et al. Humoral and cell-mediated immune responses of old horses following recombinant canarypox virus vaccination and subsequent challenge infection. Veterinary immunology and immunopathology. 2011;139:128-40.
[198] Adams AA, Katepalli MP, Kohler K, Reedy SE, Stilz J, Vick MM, et al. Effect of body condition, body weight and adiposity on inflammatory cytokine responses in old horses. Veterinary immunology and immunopathology. 2009;127:286-94.
[199] Adams AA, Betancourt A, Barker VD, Siard MH, Elzinga S, Bellaw JL, et al. Comparison of the Immunologic Response to Anthelmintic Treatment in Old Versus Middle-Aged Horses. Journal of Equine Veterinary Science. 2015;35:873-81. e3.
[200] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. methods. 2001;25:402-8.
[201] FDA U. Agency Response Letter GRAS Notice No. GRN 000348 [Stevioside purified from the leaves of Stevia rebaudiana (Bertoni) Bertoni (stevioside)]. Center for Food Safety and Applied Nutrition (CFSAN). 2011.
[202] Albelda SM, Buck CA. Integrins and other cell adhesion molecules. The FASEB Journal. 1990;4:2868-80.
[203] Blankenberg S, Rupprecht HJ, Bickel C, Peetz D, Hafner G, Tiret L, et al. Circulating cell adhesion molecules and death in patients with coronary artery disease. Circulation. 2001;104:1336-42.

[204] Park M, Vittinghoff E, Ganz P, Peralta CA, Whooley M, Shlipak MG. Role of Soluble Endothelial Cell–Selective Adhesion Molecule Biomarker in Albuminuria and Kidney Function Changes in Patients With Coronary Artery Disease The Heart and Soul Study. Arteriosclerosis, thrombosis, and vascular biology. 2014;34:231-6.
[205] Esposito K, Nappo F, Giugliano F, Di Palo C, Ciotola M, Barbieri M, et al. Meal modulation of circulating interleukin 18 and adiponectin concentrations in healthy subjects and in patients with type 2 diabetes mellitus. The American journal of clinical nutrition. 2003;78:1135-40.
[206] Burdge GC, Calder PC. Plasma cytokine response during the postprandial period: a potential causal process in vascular disease? British journal of nutrition. 2005;93:3-9.
[207] Consultation W. Definition, diagnosis and classification of diabetes mellitus and its complications. Part; 1999.
[208] Wilson PW, D'Agostino RB, Parise H, Sullivan L, Meigs JB. Metabolic syndrome as a precursor of cardiovascular disease and type 2 diabetes mellitus. Circulation. 2005;112:3066-72.
[209] Geor R, Frank N. Metabolic syndrome—from human organ disease to laminar failure in equids. Veterinary Immunology and immunopathology. 2009;129:151-4.
[210] Xiao S, Zhao L. Gut microbiota-based translational biomarkers to prevent metabolic syndrome via nutritional modulation. FEMS microbiology ecology. 2014;87:303-14.
[211] Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. Nature methods. 2010;7:335-6.
[212] Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, et al. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Applied and environmental microbiology. 2009;75:7537-41.
[213] Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics. 2011;27:2194-200.
[214] Gihring TM, Green SJ, Schadt CW. Massively parallel rRNA gene sequencing exacerbates the potential for biased community diversity comparisons due to variable library sizes. Environmental microbiology. 2012;14:285-90.
[215] Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, et al. Metagenomic biomarker discovery and explanation. Genome Biol. 2011;12:R60.
[216] Holmes I, Harris K, Quince C. Dirichlet multinomial mixtures: generative models for microbial metagenomics. PloS one. 2012;7:e30126.
[217] Knights D, Costello EK, Knight R. Supervised classification of human microbiota. FEMS microbiology reviews. 2011;35:343-59.
[218] Langille MG, Zaneveld J, Caporaso JG, McDonald D, Knights D, Reyes JA, et al. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nature biotechnology. 2013;31:814-21.
[219] Everard A, Belzer C, Geurts L, Ouwerkerk JP, Druart C, Bindels LB, et al. Cross-talk between Akkermansia muciniphila and intestinal epithelium controls diet-

induced obesity. Proceedings of the National Academy of Sciences. 2013;110:9066-71.
[220] Shin N-R, Lee J-C, Lee H-Y, Kim M-S, Whon TW, Lee M-S, et al. An increase in the Akkermansia spp. population induced by metformin treatment improves glucose homeostasis in diet-induced obese mice. Gut. 2013:gutjnl-2012-303839.
[221] Dao MC, Everard A, Aron-Wisnewsky J, Sokolovska N, Prifti E, Verger EO, et al. Akkermansia muciniphila and improved metabolic health during a dietary intervention in obesity: relationship with gut microbiome richness and ecology. Gut. 2015.
[222] Zhang X, Shen D, Fang Z, Jie Z, Qiu X, Zhang C, et al. Human gut microbiota changes reveal the progression of glucose intolerance. PLoS One. 2013;8:e71108.
[223] O'Donnell M, Harris H, Jeffery I, Claesson M, Younge B, O'Toole P, et al. The core faecal bacterial microbiome of Irish Thoroughbred racehorses. Letters in applied microbiology. 2013;57:492-501.
[224] Faubladier C, Julliand V, Danel J, Philippeau C. Bacterial carbohydrate-degrading capacity in foal faeces: changes from birth to pre-weaning and the impact of maternal supplementation with fermented feed products. British Journal of Nutrition. 2013;110:1040-52.
[225] Daly K, Proudman CJ, Duncan SH, Flint HJ, Dyer J, Shirazi-Beechey SP. Alterations in microbiota and fermentation products in equine large intestine in response to dietary variation and intestinal disease. British Journal of Nutrition. 2012;107:989-95.
[226] Bagi Z, Broskova Z, Feher A. Obesity and Coronary Microvascular Disease–Implications for Adipose Tissue-Mediated Remote Inflammatory Response. Current vascular pharmacology. 2014;12:453.
[227] Yao L, Herlea-Pana O, Heuser-Baker J, Chen Y, Barlic-Dicen J. Roles of the chemokine system in development of obesity, insulin resistance, and cardiovascular disease. Journal of immunology research. 2014;2014.
[228] Borkman M, Storlien LH, Pan DA, Jenkins AB, Chisholm DJ, Campbell LV. The relation between insulin sensitivity and the fatty-acid composition of skeletal-muscle phospholipids. New England Journal of Medicine. 1993;328:238-44.
[229] Quehenberger O, Armando AM, Brown AH, Milne SB, Myers DS, Merrill AH, et al. Lipidomics reveals a remarkable diversity of lipids in human plasma. Journal of lipid research. 2010;51:3299-305.
[230] Jové M, Naudí A, Portero-Otin M, Cabré R, Rovira-Llopis S, Bañuls C, et al. Plasma lipidomics discloses metabolic syndrome with a specific HDL phenotype. The FASEB Journal. 2014;28:5163-71.
[231] Graessler J, Schwudke D, Schwarz P, Herzog R, Shevchenko A, Bornstein SR. Top-down lipidomics reveals ether lipid deficiency in blood plasma of hypertensive patients. PloS one. 2009;4:e6261.
[232] Maeba R, Hara H. Serum choline plasmalogen is a reliable biomarker for atherogenic status. Coronary artery disease–new insights and novel approaches. 2012:243.
[233] Hellmann J, Zhang MJ, Tang Y, Rane M, Bhatnagar A, Spite M. Increased saturated fatty acids in obesity alter resolution of inflammation in part by

stimulating prostaglandin production. The Journal of Immunology. 2013;191:1383-92.
[234] Wu D, Ren Z, Pae M, Guo W, Cui X, Merrill AH, et al. Aging up-regulates expression of inflammatory mediators in mouse adipose tissue. The Journal of Immunology. 2007;179:4829-39.
[235] Ren H, Zhang C, Su L, Bi X, Wang C, Wang L, et al. Type II anti-CD20 mAb-induced lysosome mediated cell death is mediated through a ceramide-dependent pathway. Biochemical and biophysical research communications. 2015;457:572-7.
[236] Huang C, Freter C. Lipid metabolism, apoptosis and cancer therapy. International journal of molecular sciences. 2015;16:924-49.
[237] Treiber K, Carter R, Gay L, Williams C, Geor R. Inflammatory and redox status of ponies with a history of pasture-associated laminitis. Veterinary immunology and immunopathology. 2009;129:216-20.
[238] Wood PL. Mass spectrometry strategies for clinical metabolomics and lipidomics in psychiatry, neurology, and neuro-oncology. Neuropsychopharmacology. 2014;39:24-33.
[239] Breathnach C, Sturgill-Wright T, Stiltner J, Adams A, Lunn D, Horohov D. Foals are interferon gamma-deficient at birth. Veterinary Immunology and immunopathology. 2006;112:199-209.
[240] Park E, Wong V, Guan X, Oprescu AI, Giacca A. Salicylate prevents hepatic insulin resistance caused by short-term elevation of free fatty acids in vivo. Journal of Endocrinology. 2007;195:323-31.
[241] Frasca D, Ferracci F, Diaz A, Romero M, Lechner S, Blomberg BB. Obesity decreases B cell responses in young and elderly individuals. Obesity. 2016;24:615-25.
[242] Costanzo AE, Taylor KR, Dutt S, Han PP, Fujioka K, Jameson JM. Obesity impairs γδ T cell homeostasis and antiviral function in humans. PloS one. 2015;10:e0120918.
[243] Chambers TM, Reedy SE. Equine Influenza Serological Methods. Animal Influenza Virus: Springer; 2014. p. 411-22.
[244] Liu C, Betancourt A, Cohen D, Adams A, Sun L, Horohov D. Granzyme B-mRNA expression by equine lymphokine activated killer (LAK) cells is associated with the induction of apoptosis in target cells. Veterinary immunology and immunopathology. 2011;143:108-15.
[245] Young KM, Gray CM, Bekker L-G. Is obesity a risk factor for vaccine non-responsiveness? PloS one. 2013;8:e82779.
[246] He X-S, Holmes TH, Zhang C, Mahmood K, Kemble GW, Lewis DB, et al. Cellular immune responses in children and adults receiving inactivated or live attenuated influenza vaccines. Journal of virology. 2006;80:11756-66.
[247] Lord JD, McIntosh BC, Greenberg PD, Nelson BH. The IL-2 receptor promotes lymphocyte proliferation and induction of the c-myc, bcl-2, and bcl-x genes through the trans-activation domain of Stat5. The Journal of Immunology. 2000;164:2533-41.
[248] O'Garra A. Cytokines induce the development of functionally heterogeneous T helper cell subsets. Immunity. 1998;8:275-83.

[249] Li Q, Verma IM. NF-κB regulation in the immune system. Nature Reviews Immunology. 2002;2:725-34.
[250] de Laat MA, McGree JM, Sillence MN. Equine hyperinsulinemia: investigation of the enteroinsular axis during insulin dysregulation. American Journal of Physiology-Endocrinology and Metabolism. 2016;310:E61-E72.
[251] Bamford N, Baskerville C, Harris P, Bailey S. Postprandial glucose, insulin, and glucagon-like peptide-1 responses of different equine breeds adapted to meals containing micronized maize. Journal of animal science. 2015;93:3377-83.
[252] Ofek I, Beachey EH. Mannose binding and epithelial cell adherence of Escherichia coli. Infection and Immunity. 1978;22:247-54.
[253] Baurhoo B, Letellier A, Zhao X, Ruiz-Feria C. Cecal populations of lactobacilli and bifidobacteria and Escherichia coli populations after in vivo Escherichia coli challenge in birds fed diets with purified lignin or mannanoligosaccharides. Poultry Science. 2007;86:2509-16.
[254] Baurhoo B, Ferket P, Ashwell CM, de Oliviera J, Zhao X. Cell walls of Saccharomyces cerevisiae differentially modulated innate immunity and glucose metabolism during late systemic inflammation. PloS one. 2012;7:e30323.
[255] Guan X, Matte JJ, Ku PK, Snow JL, Burton JL, Trottier NL. High chromium yeast supplementation improves glucose tolerance in pigs by decreasing hepatic extraction of insulin. The Journal of nutrition. 2000;130:1274-9.
[256] Frank N, Elliott SB, Chameroy K, Toth F, Chumbler N, McClamroch R. Association of season and pasture grazing with blood hormone and metabolite concentrations in horses with presumed pituitary pars intermedia dysfunction. Journal of veterinary internal medicine. 2010;24:1167-75.
[257] Funk RA, Wooldridge A, Stewart A, Behrend E, Kemppainen R, Zhong Q, et al. Seasonal changes in the combined glucose-insulin tolerance test in normal aged horses. Journal of veterinary internal medicine. 2012;26:1035-41.
[258] Place N, McGowan C, Lamb S, Schanbacher B, McGowan T, Walsh D. Seasonal variation in serum concentrations of selected metabolic hormones in horses. Journal of veterinary internal medicine. 2010;24:650-4.
[259] McFarlane D, Wolf RF, McDaniel KA, White GL. The effect of season on inflammatory response in captive baboons. J Med Primatol. 2012;41:341-8.
[260] Bilbo SD, Drazen DL, Quan N, He L, Nelson RJ. Short day lengths attenuate the symptoms of infection in Siberian hamsters. Proc Biol Sci. 2002;269:447-54.
[261] Bowden TJ, Thompson KD, Morgan AL, Gratacap RM, Nikoskelainen S. Seasonal variation and the immune response: a fish perspective. Fish Shellfish Immunol. 2007;22:695-706.
[262] Riihimaki M, Raine A, Elfman L, Pringle J. Markers of respiratory inflammation in horses in relation to seasonal changes in air quality in a conventional racing stable. Can J Vet Res. 2008;72:432-9.
[263] Secombe CJ, Lester GD, Robertson ID, Cullimore AM. Retrospective survey of bronchoalveolar lavage fluid cytology in Western Australian horses presented for evaluation of the respiratory tract: effect of season on relative cell percentages. Aust Vet J. 2015;93:152-6.

[264] Wray H, Elliott J, Bailey SR, Harris PA, Menzies-Gow NJ. Plasma concentrations of inflammatory markers in previously laminitic ponies. Equine Vet J. 2013;45:546-51.
[265] Hamza E, Doherr MG, Bertoni G, Jungi TW, Marti E. Modulation of allergy incidence in icelandic horses is associated with a change in IL-4-producing T cells. Int Arch Allergy Immunol. 2007;144:325-37.
[266] Beadle RE, Horohov DW, Gaunt SD. Interleukin-4 and interferon-gamma gene expression in summer pasture-associated obstructive pulmonary disease affected horses. Equine Vet J. 2002;34:389-94.
[267] Jung UJ, Choi M-S. Obesity and its metabolic complications: the role of adipokines and the relationship between obesity, inflammation, insulin resistance, dyslipidemia and nonalcoholic fatty liver disease. International journal of molecular sciences. 2014;15:6184-223.
[268] Kershaw EE, Flier JS. Adipose tissue as an endocrine organ. The Journal of Clinical Endocrinology & Metabolism. 2004;89:2548-56.
[269] Zughaier SM, Zimmer SM, Datta A, Carlson RW, Stephens DS. Differential induction of the toll-like receptor 4-MyD88-dependent and-independent signaling pathways by endotoxins. Infection and immunity. 2005;73:2940-50.
[270] Yamamoto M, Sato S, Hemmi H, Hoshino K, Kaisho T, Sanjo H, et al. Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. Science. 2003;301:640-3.
[271] Song MJ, Kim KH, Yoon JM, Kim JB. Activation of Toll-like receptor 4 is associated with insulin resistance in adipocytes. Biochemical and biophysical research communications. 2006;346:739-45.
[272] Reyna SM, Ghosh S, Tantiwong P, Meka CR, Eagan P, Jenkinson CP, et al. Elevated toll-like receptor 4 expression and signaling in muscle from insulin-resistant subjects. Diabetes. 2008;57:2595-602.
[273] Kuhel DG, Konaniah ES, Basford JE, McVey C, Goodin CT, Chatterjee TK, et al. Apolipoprotein E2 accentuates postprandial inflammation and diet-induced obesity to promote hyperinsulinemia in mice. Diabetes. 2013;62:382-91.
[274] Figueiredo MD, Vandenplas ML, Hurley DJ, Moore JN. Differential induction of MyD88-and TRIF-dependent pathways in equine monocytes by Toll-like receptor agonists. Veterinary immunology and immunopathology. 2009;127:125-34.
[275] Jagannathan M, Hasturk H, Liang Y, Shin H, Hetzel JT, Kantarci A, et al. TLR cross-talk specifically regulates cytokine production by B cells from chronic inflammatory disease patients. The Journal of Immunology. 2009;183:7461-70.
[276] Niebuhr M, Baumert K, Werfel T. TLR-2-mediated cytokine and chemokine secretion in human keratinocytes. Experimental dermatology. 2010;19:873-7.
[277] Kobayashi M, Saitoh S-i, Tanimura N, Takahashi K, Kawasaki K, Nishijima M, et al. Regulatory roles for MD-2 and TLR4 in ligand-induced receptor clustering. The Journal of Immunology. 2006;176:6211-8.
[278] Klein DC, Skjesol A, Kers-Rebel ED, Sherstova T, Sporsheim B, Egeberg KW, et al. CD14, TLR4 and TRAM show different trafficking dynamics during LPS stimulation. Traffic. 2015;16:677-90.
[279] Roytblat L, Rachinsky M, Fisher A, Greemberg L, Shapira Y, Douvdevani A, et al. Raised Interleukin-6 Levels in Obese Patients. Obesity research. 2000;8:673-5.

[280] Straczkowski M, Dzienis-Straczkowska S, Stêpieñ A, Kowalska I, Szelachowska M, Kinalska I. Plasma interleukin-8 concentrations are increased in obese subjects and related to fat mass and tumor necrosis factor-α system. The Journal of Clinical Endocrinology & Metabolism. 2002;87:4602-6.
[281] Hotamisligil GS, Spiegelman BM. Tumor necrosis factor α: a key component of the obesity-diabetes link. Diabetes. 1994;43:1271-8.
[282] Sartipy P, Loskutoff DJ. Monocyte chemoattractant protein 1 in obesity and insulin resistance. Proceedings of the National Academy of Sciences. 2003;100:7265-70.
[283] Gabriel P, Cakman I, Rink L. Overproduction of monokines by leukocytes after stimulation with lipopolysaccharide in the elderly. Experimental gerontology. 2002;37:235-47.
[284] McFarlane D, Holbrook T. Cytokine dysregulation in aged horses and horses with pituitary pars intermedia dysfunction. Journal of veterinary internal medicine. 2008;22:436-42.
[285] McFarlane D, Hill K, Anton J. Neutrophil function in healthy aged horses and horses with pituitary dysfunction. Veterinary immunology and immunopathology. 2015;165:99-106.
[286] Haghiac M, Yang X-h, Presley L, Smith S, Dettelback S, Minium J, et al. Dietary omega-3 fatty acid supplementation reduces inflammation in obese pregnant women: a randomized double-blind controlled clinical trial. PloS one. 2015;10:e0137309.
[287] Toth F, Frank N, Elliott SB, Perdue K, Geor R, Boston R. Optimisation of the frequently sampled intravenous glucose tolerance test to reduce urinary glucose spilling in horses. Equine veterinary journal. 2009;41:844-51.
[288] Woodward A, Nielsen B, O'Connor C, Skelly C, Webel S, Orth M. Supplementation of dietary long-chain polyunsaturated omega-3 fatty acids high in docosahexaenoic acid (DHA) increases plasma DHA concentration and may increase trot stride lengths in horses. Equine and Comparative Exercise Physiology. 2007;4:71-8.
[289] Lavoie-Lamoureux A, Maghni K, Lavoie J-P. Optimization of a procedure to accurately detect equine TNFα in serum samples. Veterinary immunology and immunopathology. 2010;138:118-23.
[290] Burton A, Wagner B, Erb H, Ainsworth D. Serum interleukin-6 (IL-6) and IL-10 concentrations in normal and septic neonatal foals. Veterinary immunology and immunopathology. 2009;132:122-8.
[291] Leclere M, Lavoie-Lamoureux A, Lavoie JP. Acute phase proteins in racehorses with inflammatory airway disease. Journal of Veterinary Internal Medicine. 2015;29:940-5.
[292] Vineyard K, Warren L, Kivipelto J. Effect of dietary omega-3 fatty acid source on plasma and red blood cell membrane composition and immune function in yearling horses. Journal of animal science. 2010;88:248-57.
[293] Blank ML, Smith ZL, Cress EA, Snyder F. Molecular species of ethanolamine plasmalogens and transacylase activity in rat tissues are altered by fish oil diets. Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism. 1994;1214:295-302.

[294] Jia L, Vianna CR, Fukuda M, Berglund ED, Liu C, Tao C, et al. Hepatocyte Toll-like receptor 4 regulates obesity-induced inflammation and insulin resistance. Nature communications. 2014;5.
[295] de Laat M, Clement C, McGowan C, Sillence M, Pollitt C, Lacombe V. Toll-like receptor and pro-inflammatory cytokine expression during prolonged hyperinsulinaemia in horses: implications for laminitis. Veterinary immunology and immunopathology. 2014;157:78-86.
[296] Basinska K, Marycz K, Smieszek A, Nicpoń J. The production and distribution of IL-6 and TNF-α in subcutaneous adipose tissue and their correlation with serum concentration in Welsh Ponies with EMS. Journal of veterinary science. 2014.
[297] Hall JA, Saun RJ, Tornquist SJ, Gradin JL, Pearson EG, Wander RC. Effect of type of dietary polyunsaturated fatty acid supplement (corn oil or fish oil) on immune responses in healthy horses. Journal of veterinary internal medicine. 2004;18:880-6.
[298] Khol-Parisini A, van den Hoven R, Leinker S, Hulan HW, Zentek J. Effects of feeding sunflower oil or seal blubber oil to horses with recurrent airway obstruction. Canadian journal of veterinary research. 2007;71:59.
[299] Weldon SM, Mullen AC, Loscher CE, Hurley LA, Roche HM. Docosahexaenoic acid induces an anti-inflammatory profile in lipopolysaccharide-stimulated human THP-1 macrophages more effectively than eicosapentaenoic acid. The Journal of nutritional biochemistry. 2007;18:250-8.
[300] Mullen A, Loscher CE, Roche HM. Anti-inflammatory effects of EPA and DHA are dependent upon time and dose-response elements associated with LPS stimulation in THP-1-derived macrophages. The Journal of nutritional biochemistry. 2010;21:444-50.
[301] Vineyard K, Warren L, Webel S, Kivipelto J. Effect of fish oil supplementation on neutrophil function and antibody production in yearling horses. Proceedings of the 20th Equine Science Society Hunt Valley, MD: Equine Science Society, Champaign, Illinois. 2007:13-4.
[302] Kaur G, Begg DP, Barr D, Garg M, Cameron-Smith D, Sinclair AJ. Short-term docosapentaenoic acid (22: 5 n-3) supplementation increases tissue docosapentaenoic acid, DHA and EPA concentrations in rats. British journal of nutrition. 2010;103:32-7.
[303] Creely SJ, McTernan PG, Kusminski CM, Da Silva N, Khanolkar M, Evans M, et al. Lipopolysaccharide activates an innate immune system response in human adipose tissue in obesity and type 2 diabetes. American Journal of Physiology-Endocrinology And Metabolism. 2007;292:E740-E7.

VITA

Sarah Elzinga

EDUCATION

2009-2011	**Michigan State University** *MS Degree in Animal Science*	East Lansing, MI
2002-2005	**Saint Mary-of-the-Woods College** *BS Degree in Equine Science*	Saint Mary-of-the-Woods, IN

WORK EXPERIENCE

2013-present	**University of Kentucky** *Graduate Research Assistant, Veterinary Science Department, Gluck Equine Research Center*	Lexington, KY
2011-2013	**Chemical Bank** *Teller*	Grand Rapids, MI
2009-2011	**Michigan State University** *Research Assistant, Equine Nutrition Lab*	East Lansing, MI
2008-2009	**Schmitt's Animal Hospital** *Veterinary Assistant*	Grand Rapids, MI
2007	**Melissa Sexton Performance Horses** *Assistant Trainer*	Okanogan, WA
2006-2007	**Rivendell Stables** *Assistant Manager, Riding Instructor/trainer*	Lowell, MI

HONORS AND AWARDS

- Veterinary Science Annual 3MT Graduate Student Competition Winner (2017)
- AQHF Young Investigator Award for Equine Research (2015)
- Deans Scholarship Recipient (2002-2005)
- Equestrian Scholarship Recipient (2002-2005)
- Saint Mary-of-the-Woods college Outstanding Academic Achievement (2003-2005)
- Saint Mary-of-the-Woods college Honors List and National Dean's List (2003)
- Saint Mary-of-the-Woods college Knoerle Honors Forum for Academic Scholarship Award (2002 & 2003)
- Saint Mary-of-the-Woods College Graduate with Honors (2005)
- Michigan Merit Award Recipient (May 2002)
- Michigan Competitive Scholarship Recognition (May 2002)

GRANT PARTICIPATION

- **Neogen Pony:** Characterization of cell-mediated and humoral immune responses and stress to an immunostimulant in ponies, utilizing weaning as a model of stress.
- **BI PPID:** Compare immune response to multi-path vaccination of PPID (Pituitary Pars Intermedia Dysfunction) vs non-PPID controls
- **Seasonal Cytokines:** Comparison of adult and old horse inflammatory markers and assessment of lymphocyte proliferation monthly over the course of a year
- **Buckeye:** Relationship between circulating vitamin and fatty acid levels as well as systemic inflammation and muscle mass in aged horses to determine if a rating balancer pellet can modulate these factors
- **Zoetis Dewormer:** Comparison of immunologic responses to anthelmintic treatment in old versus middle-aged adult horses
- **Alltech Omega 3 Fatty Acids:** Metabolic and inflammatory responses of EMS horses to algal DHA supplementation
- **Lipidomic and inflammatory profiling:** Characterization of the lipidomic and inflammatory status of a group of Equine Metabolic Syndrome (EMS) and non-EMS controls
- **Microbiota profiling:** Characterization of the fecal microbiota of a group of EMS and non-EMS control horses
- **AHQA vaccine:** Humoral and cell-mediated immune responses to an influenza vaccine in EMS and non-EMS controls
- **Lincoln Memorial**: Inflammatory responses to an oral sugar challenge utilizing a common sweetener as well as a glucose and maltose syrup in a group of EMS and non-EMS controls
- **Alltech yeast cell wall:** Metabolic and inflammatory responses of EMS and non-EMS control horses to yeast cell wall supplementation

PEER REVIEWED PUBLICATIONS

Elzinga, S. E., et al. "Metabolic and inflammatory responses to the common sweetener stevioside and a glycemic challenge in horses with equine metabolic syndrome." *Domestic Animal Endocrinology* 60 (2017): 1-8.

Elzinga, Sarah E., J. Scott Weese, and Amanda A. Adams. "Comparison of the Fecal Microbiota in Horses With Equine Metabolic Syndrome and Metabolically Normal Controls Fed a Similar All-Forage Diet." *Journal of Equine Veterinary Science* 44 (2016): 9-16.

Elzinga, Sarah, Paul Wood, and Amanda A. Adams. "Plasma Lipidomic and Inflammatory Cytokine Profiles of Horses With Equine Metabolic Syndrome." *Journal of Equine Veterinary Science* 40 (2016): 49-55.

Adams, Amanda A., et al. "Effects of an immunostimulant containing Propionibacterium acnes (EqStim TM) on cell-mediated immunity and nasal shedding of respiratory pathogens using a model of ‘weaning’ stress in foals." *Journal of Equine Veterinary Science* (2016).

Adams, Amanda A., et al. "Comparison of the Immunologic Response to Anthelmintic Treatment in Old Versus Middle-Aged Horses." *Journal of Equine Veterinary Science* 35.11 (2015): 873-881.

Elzinga, Sarah, et al. "Comparison of Nutrient Digestibility Between Adult and Aged Horses." *Journal of Equine Veterinary Science* 34.10 (2014): 1164-1169.

Dougal, Kirsty, et al. "Characterisation of the faecal bacterial community in adult and elderly horses fed a high fibre, high oil or high starch diet using 454 pyrosequencing." *PloS one* 9.2 (2014): e87424.