THESIS

EFFECT OF N-3 POLYUNSATURATED FATTY ACID SUPPLEMENTATION ON CIRCULATING CONCENTRATIONS AND mRNA EXPRESSION OF INFLAMMATORY CYTOKINES IN HORSES

Submitted by

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WE HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER OUR SUPERVISION BY NICOLETTE SCHAUERMANN ENTITLED EFFECT OF N-3 POLYUNSATURATED FATTY ACID SUPPLEMENTATION ON CIRCULATING CONCENTRATIONS AND mRNA EXPRESSION OF INFLAMMATORY CYTOKINES IN HORSES BE ACCEPTED AS FULLFILLING IN PART REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE.

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ABSTRACT OF THESIS

EFFECT OF N-3 POLYUNSATURATED FATTY ACID SUPPLEMENTATION ON CIRCULATING CONCENTRATIONS AND mRNA EXPRESSION OF INFLAMMATORY CYTOKINES IN HORSES

Introduction: Studies in horses have shown an association between increased levels of the inflammatory cytokines and inflammatory conditions. Ingestion of n-3 polyunsaturated fatty acids (PUFA) can produce inflammatory mediators with the potential to down regulate the production of cytokines.

Methods: Twenty one mares were blocked by age, body weight and body condition score and randomly assigned to one of three dietary treatment supplements. Treatments consisted of: 1) fish oil supplement and diet (FISH; Magnitude^{TM}); 2) flaxseed supplement and diet (FLAX; Nutra-Flax^{TM}); and 3) control diet (CON). Horses were supplemented for 12 weeks. Serum and white blood cells were collected on weeks 0, 4, 8 and 12 and assayed for TNFα protein concentrations and IL-1, IL-6, IL-10, and TNFα mRNA expression. Data was log transformed for normalization and analyzed by ANOVA. Significant differences ($P<0.05$) were compared by least square means analysis.

Results: Statistical analysis of mRNA expression was not completed because of failure to produce consistent, reliable results from white blood cell samples. The serum

analysis revealed no treatment effects, however FISH treated horses had a trend for lower TNFα than FLAX during week 4 ($P=0.1202$). Both the FISH and CON groups showed an increase between week 4 and 8 and a decrease between week 8 and 12 ($P<0.035$). The Flax group only showed a significant change between week 8 and 12 which was similar to the other treatments ($P<0.040$).

Discussion: For more consistent results, a standard amount of whole blood should be used for mRNA expression analysis and include a RNA quality check procedure after RNA isolation. Results indicate that supplementation is not effective in altering TNFα concentrations in adult, healthy mares. The effect of n-3 PUFA may only be evident when inflammatory challenges are present as shown in previous studies of horses prone to laminitis, suffering from arthritis or stimulated with lipopolysaccharides.

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The twenty-one stock horse mares in this study

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CHAPTER I: INTRODUCTION

The equine industry continually struggles to keep horses healthy and free of lameness for the duration of a horse's natural life. Contributing to this struggle are many inflammatory conditions that can lead to significant treatment expenses, reduced performance, rehabilitation time, retirement or possibly even euthanasia of the horse. Common inflammatory conditions in horses include laminitis (Treiber et al., 2006), osteoarthritis (Manhart et al., 2009), Cushing's disease (McFarlane and Holbrook, 2008), metabolic syndrome (Geor and Frank, 2009) and obesity (Vick et al., 2007). The benefit of including n-3 polyunsaturated fatty acids (PUFA) in the diet is evident in humans. Eating fish or taking a daily dose of fish oil has been shown to protect against heart disease (Hirafuji et al., 2003; Leaf et al., 2003), reduce the risk of certain cancers (Gonzalez et al., 1993) and help treat arthritis and other inflammatory conditions (Cleland et al., 2003; Geusens et al., 1994; MacLean et al., 2005). With such a positive effect, it may prove beneficial to apply this treatment to horses suffering from inflammatory conditions. The purpose of this research was to determine if altering the fatty acid content of a horse's diet would produce a change in circulating concentrations of inflammatory markers linked to common inflammatory diseases.

CHAPTER II: REVIEW OF LITERATURE

Inflammation and Inflammatory Markers

The immune system responds to infections and injury by inducing acute inflammation (Calder, 2001; 2006). The inflammatory response is a natural and immediate response noted by the presence of redness, swelling, heat and pain due to increased blood flow and vessel wall permeability of the surrounding vasculature (Calder, 2006). The inflammatory process allows for immune cells and large molecules involved in the immune response (ie. chemokines, cytokines) to enter the site of infection or injury from the blood stream (Calder, 2006). These immune cells include granulocytes (neutrophils, eosinophils and basophils) and monocytes and macrophages needed for killing pathogens and clearing up damaged tissue (Calder, 2006). Other immune cells apart of the memory (acquired) immune response known as lymphocytes (B and T cells) will arrive later at the site of inflammation if reinforcement is needed (Calder, 2006). Communication between immune cells can be either direct or by the production of protein chemical messengers such as cytokines (Calder, 2001). Cytokines influence the immune response by binding to specific receptors on cells that signal changes in the cells' behavior (Calder, 2001). The cytokines TNFα, IL-1, and IL-6 are produced by monocytes and macrophages in order to activate neutrophils and other monocytes and macrophages, stimulate the proliferation of T and B cell lymphocytes and initiate the production of

other cytokines (Calder, 2001). They are also released by adipose tissue because of residing macrophages that clean up dead adipocytes (D. C. Lau et al., 2005). These cytokines influence both the innate (natural) and acquired immune response and are an important link between the two (Calder, 2001; 2006). Cytokines influence systemic effects of inflammation by mediating fever and weight loss (Calder, 2001). The effect of these cytokines is opposed by the production of cytokines such as IL-10 by regulatory cells (Calder, 2006). The role of these cytokines in the immune response is very important and beneficial for combating invading pathogens along with regulating and resolving inflammation but can be dangerous when present in inappropriate and uncontrolled concentrations (Calder, 2001; 2003b; 2006). When the immune system lacks regulation and resolution of the inflammatory response, the system enters a chronic state of inflammation leading to damage of host tissue and disease (Calder, 2006). High levels of TNFα, IL-1 and IL-6 are particularly destructive and implicated in inflammatory diseases such as rheumatoid arthritis, neurogenerative diseases of aging in humans and septic/systemic inflammatory response syndrome (Calder, 2003a; b; 2006; Feldmann et al., 1996; Fetterman and Zdanowicz, 2009; Stulnig, 2003). In addition, TNFα is believed to be associated with the development of inflammatory bronchial conditions and inflammatory bowel disease both in animals and humans (Fetterman and Zdanowicz, 2009; Fritsche, 2006). Chronic inflammation in humans is similar in horses and is characterized by increased concentrations of eicosanoids and cytokines as the following sections will present.

Exercised Induced Inflammation

The effect of exercise stress on inflammation in horses is unclear and seems to be dependent on the type of exercise preformed but not the duration. Single bouts of intense exercise in horses produced increased total neutrophil and leukocyte counts at 6 hours post exercise and increased lymphocyte counts at 24 and 120 hours post exercise ($P < 0.05$) (Wong et al., 1992). Also the ratio of neutrophils to lymphocytes was increased at 6 hours post exercise (Wong et al., 1992). This study indicates that the non-specific (innate) immune response may be impaired by strenuous exercise (Wong et al., 1992). Healthy thoroughbred fillies exposed to four trials of varying exercise intensity (low: 4 m/s for 10 min, medium: 7 m/s for 5 min, high: 9 m/s for 2 1/5 min and none: no exercise) had increased plasma concentrations of the inflammatory mediator thromboxane (TX) B_2 during and after exercise at all three intensities but with no difference between exercise intensities (Mitten et al., 1995). A lack of difference between exercise intensities is uncertain but may be due to the decreased length of higher speed trails that equalized the overall intensity of the exercise and immune response. Exercise type influenced the immune response in four healthy standard bred mares exposed to three different treadmill exercises (Lamprecht et al., 2005). Four bouts of near maximal sprints produced the greatest up-regulation of the mRNA expression of cytokines TNFα and IL-10 compared to two other less intense treadmill exercises (Lamprecht et al., 2005). Another study on yearlings exercising daily showed a decrease in PGE_2 production over time speculated to be due to adaptation to the exercise protocol (Ross et al., 2005). It is clear that exercise has an effect on immune function that may or may not be dependent on the duration, type and overall intensity of the exercise.

Inflamm-aging

Aged horses show an increase in inflammation referred to as inflamm-aging and made evident by an increase in inflammatory markers such as cytokines. Serum levels of TNFα were positively related to age in sixty mares sampled during the autumn season (Vick et al., 2007). Aged horses over 20 years produce a higher percentage of TNFα positive lymphocytes and monocytes compared to young horses of 4 or 5 years old (Adams et al., 2009). This pro-inflammatory state of the aged horse may contribute to the development of age-associated diseases such as osteoarthritis and pituitary pars intermedia dysfunction (PPID) also known as Cushing's disease (McFarlane and Holbrook, 2008).

Cushing's disease is an endocrine disorder with symptoms including a long, wavy coat, weight loss, increased sweating, infertility, various infections, laminitis and insulin resistance (Dybdal, 2000). A study by McFarlane and Holbrook compared inflammation levels between PPID horses (2 or more clinical signs), aged horses (over 16 years) or adult healthy horses (6 to 14 years) (McFarlane and Holbrook, 2008). Cytokine levels were evaluated from circulating white blood cells (WBC), plasma and peripheral blood mononuclear cells (PBMC) (McFarlane and Holbrook, 2008). Although the PPID horses were similar in age to the aged horses, cytokine expression differed between these groups (McFarlane and Holbrook, 2008). Aged horses had greater expression of IL-6, and ratios of IL-6/IL-10 and TNFα/IL-10 than both PPID or adult horses (McFarlane and Holbrook, 2008). Only IL-8 expression was determined higher in both aged and PPID compared to adult (McFarlane and Holbrook, 2008). When extracted PBMC were stimulated with lipopolysaccharide (LPS), PPID horses had a significantly higher release of TNFα

compared to the adult horses but did not differ from the aged horses (McFarlane and Holbrook, 2008). It is evident an increase in inflammatory cytokines is associated with age (McFarlane and Holbrook, 2008). Differences between PPID and aged horses may be due to increased hormone levels from the pituitary gland. Extremely high plasma concentrations of α-melanocyte stimulating hormone (αMSH) were found in PPID horses compared to both aged and adult horses (McFarlane and Holbrook, 2008). This hormone has been shown to suppress the activation of nuclear factor (NF)-κB needed for DNA transcription which may have reduced the expression of cytokines in PPID horses compared to aged horses (D'Acquisto and Ianaro, 2006; McFarlane and Holbrook, 2008). Once cells were removed from all hormones and stimulated, PBMC from PPID horses released almost 40% more TNFα than the adult horses which was similar to the aged horses (McFarlane and Holbrook, 2008). This study indicates that aged horses have higher concentrations of inflammatory cytokines that may initially contribute to the development of PPID but are masked by the increased outputs of certain pituitary gland hormones after a horse show signs of PPID.

Studies of equine joint disease to our knowledge have not yet directly determined an association with increased age but have shown designated cytokines and eicosanoids as markers of inflammation in synovial fluid. The cytokines TNFα, IL-1 and IL-6 are involved in stimulating cartilage degradation and the development of arthritis (Shinmei et al., 1989). Evaluation of synovial fluid from horses with or without joint disease indicated that IL-6 concentrations were a great indicator of joint disease mainly due to a lack of detection in horses with normal, healthy joints. TNFα and IL-1 were also good indicators predicting over 80% of horses with acute severe joint disease. Eicosanoids also

provided good detection of joint disease in general at 57% with PGE_2 providing the most accurate assessment of acute joint disease (Bertone et al., 2001). Arthritis in horses is clearly associated with increased inflammation in the affected joints. The increased systemic state of inflammation in aged horses may contribute to increased cytokine levels involved in the destruction of joints.

Insulin Resistance, Obesity and Inflammation

Insulin resistance refers to the reduce ability of normal insulin concentrations to produce normal responses of cells due to insensitivity or ineffectiveness of insulin (Kronfeld et al., 2005; Treiber et al., 2006). Insulin resistance can be measured in several ways. The minimal model provides the most elaborate assessment by determining several parameters based on plasma concentrations of glucose and insulin over time when horses are infused first with glucose and later with insulin (Treiber et al., 2006). The change in the rate of glucose clearance to the insulin infusion indicates insulin sensitivity (SI) while the insulin response to the glucose infusion indicates the pancreatic β-cell response (AIRg) (Treiber et al., 2006). When research requires repeated testing or large sample sizes, basal proxies provide the best assessment of insulin resistance. In order to determine proxies, plasma concentrations of glucose and insulin are measured just from basal samples without any infusion of glucose or insulin. From these basal samples a similar measures of SI and AIRg are determined via the reciprocal of the insulin square-root index (RISQI) and modified insulin ratio to glucose (MIRG) (Treiber et al., 2006).

Obesity is recognized to be a mild but chronic inflammatory state and contributor of insulin resistance (Das, 2001; Hoffman et al., 2003; Ramos et al., 2003; Uysal et al.,

1997). Adipose tissue is a major expresser of inflammatory cytokines especially TNFα because of the increased death of adipocytes in obese individuals leading to increased accumulation of cytokine secreting macrophages (Zeyda and Stulnig, 2009). In horses, adiposity (as designated by BCS or % fat) shares a positive correlation with inflammatory markers IL-1 and TNFα (Vick et al., 2007). The relationship between obesity and insulin resistance is thought to be due to the many inflammatory mediators secreted by adipocytes that are also known to contribute to insulin resistance (Tilg and Moschen, 2006). The role of obesity induced inflammation in insulin resistance was made clear when genetically obese or diet induced obese mice lacking the ability to produce TNFα protein or receptors showed significant improvements in insulin sensitivity (Uysal et al., 1997). In addition, the neutralization of TNFα in obese mice has been shown to increase glucose uptake in response to insulin (Hotamisligil et al., 1993). Insulin resistance may be induced by TNFα because of its interaction with insulin receptors and influence on signaling pathways (Hotamisligil et al., 1996; Ye, 2008).

An initial relationship between insulin resistance and inflammation in vivo in horses has been demonstrated by inducing inflammation via LPS injections that resulted in increased glucose infusion rate (GIR) compared to horses administered saline (Vick et al., 2008). Additional observational studies found an inverse relationship between insulin sensitivity and serum levels of TNFα along with mRNA expression of IL-1 (Vick et al., 2007).

Metabolic Syndrome. Laminitis and Inflammation

The combination of obesity, insulin resistance and obesity-related inflammation may lead to what's known as pre-laminitic metabolic syndrome also known as equine metabolic syndrome (EMS) coined from metabolic syndrome in humans (Calder, 2006; Geor and Frank, 2009). The concern associated with this condition is the increased risk of laminitis in horses (Geor and Frank, 2009). Evaluations of laminitis-prone ponies showed increased serum levels of TNFα indicating increased inflammation of cytokines produced by adipose tissue that may be contributing to the development of laminitis (Treiber et al., 2005a). Metabolic syndrome (MS) in humans is recognized as a chronic state of acute inflammation due to abnormal levels of cytokines including TNFα, IL-1, IL-6 and IL-10 (D. C. Lau et al., 2005). There is a positive relationship between the concentration of circulating IL-6 and the number of signs of MS along with increased risk of type 2 diabetes and cardio vascular disease (Pickup et al., 1997).

By influencing the concentration of circulating inflammatory cytokines in the previously described conditions, the immune response may be altered to reduce or prevent the damage caused by these conditions. Current research is focusing on the role of fatty acids in the immune response and begun to show an influence of n-3 polyunsaturated fatty acids on inflammatory cytokines. (Cleland et al., 2003; Fritsche, 2006; Perez-Matute et al., 2007; Sierra et al., 2008; Todoric et al., 2006).

Fatty Acids

Fatty acids are a form of fat or lipid and are made up of chains of carbons that can be manipulated by enzymes to form other fatty acids. They are an energy source for cells

and make up cell membrane phospholipids providing an influence on the function and physical properties of the cell (Calder, 2008). Fatty acids are classified according to the number of double bonds they contain in their chain of hydrocarbons. Saturated fatty acids have no double bonds and are solid at room temperature (ie. butter). Unsaturated fatty acids are liquid at room temperature (ie. vegetable oils) and can be either mono, containing one double bond, or poly, containing two or more double bonds. Within each classification, fatty acids have different forms based on carbon chain length and arrangement of double bonds. Fatty acids can be converted to different forms by biological enzymes that add carbons (elongase) or form additional double bonds (desaturase).

Polyunsaturated fatty acids (PUFA) make up two major classes: n-3 and n-6 (Figure 1.1). These classes of fatty acids are essential because of the absence of the required enzymes for conversion de novo from other fatty acids (Arterburn et al., 2006; Goyens et al., 2006). The family of n-3 fatty acids includes the parent fatty acid α-linolenic acid (ALA) and the eicosanoid producing derivatives eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The family of n-6 fatty acids include the parent fatty acid linoleic acid (LA) and the eicosanoid producing derivative arachidonic acid (ARA). The process of conversion along each line of PUFA requires a set of enzymes shared between the two classes of PUFA with no conversion occurring between the two classes. The conversion of LA to ARA requires the same set of enzymes for the conversion of ALA to EPA. Additional enzymes are required in order to further convert EPA into DHA with evidence of the ability to reverse conversion of DHA to EPA (Arterburn et al., 2006).

Fatty acids compete for incorporation into the membranes of cells and it has been shown that the fatty acid make up of plasma, serum and cell membranes is dependent on the fatty acid content of the diet (Calder, 2008). The serum fatty acid profile of horses in a PUFA supplementation study was significantly dependent on treatment diet: corn oil (12.2 mg/kg BW of n-3, 179.5 mg/kg BW of n-6) or fish oil (73.4 mg/kg BW, 13.93 mg/kg BW of n-6) (O'Connor et al., 2007). Fish oil fed horses had increased concentrations of total n-3 fatty acids ($P = 0.014$) while corn oil fed horses had increased concentrations of total n-6 fatty acids ($P = 0.050$) both compared to baseline values (O'Connor et al., 2007). Therefore if more n-3 is included in the diet, more n-3 fatty acids will be incorporated into cell membranes including those of immune cells.

Influence of Fatty Acids on Inflammatory Response

Evidence of fatty acid influence on the immune system has been evident for over 30 years beginning back in 1978 (Calder, 2008). However, there are a lot of inconsistencies in the effect of PUFA supplementation (Calder, 2008). Fatty acids influence the immune response because of their role in the eicosanoid pathway. Eicosanoids are a set of lipid mediators that modulate the intensity and duration of the inflammatory response by influencing the actions of immune cells, production of cytokines and platelet aggregation (Calder, 2006; Fetterman and Zdanowicz, 2009). Fatty acids affect the immune system by acting as precursors for the production of inflammatory mediators such as prostaglandins (PG), thromboxanes (TX), leukotrienes (LT) (Calder, 2008; Fetterman and Zdanowicz, 2009). These eicosanoids affect the intensity along with the duration of the inflammatory response (Calder, 2006). In

response to inflammation, fatty acids incorporated into cell membranes are released from phospholipids and become a substrate for the generation of eicosanoids by one of two enzymes, cyclooxygenase (COX) or 5-lipooxygenase (5-LOX) (Fetterman and Zdanowicz, 2009; Stulnig, 2003). Different fatty acids produce certain eicosanoids with a specific influence on the immune system (Figure 1.2). The n-6 pathway produces the inflammatory mediators PGE_2, TXA_2 and LTB_4 as a product of ARA which provide a more potent influence on the immune system leading to a longer and more intense inflammatory and immune cell responses possibly contributing to the development of inflammatory conditions (Fetterman and Zdanowicz, 2009). The n-3 pathway produces EPA derived PGE_3, TXA_3 and LTB_5 which provide a less potent influence on the immune system leading to down regulation of immune cells and inflammatory cytokines possibly reducing the risk of developing an inflammatory condition (Fetterman and Zdanowicz, 2009).

When EPA is incorporated into the cell membrane, it out competes ARA as a substrate for COX potentially decreasing the amount of potent ARA derived eicosanoids. Both humans and horses supplemented with EPA and DHA had a reduced production of ARA derived PGE_2 (Caughey et al., 1996; Munsterman et al., 2005). In addition, EPA has shown to be a relatively poor substrate for COX (Obata et al., 1999). This difference in eicosanoid production was shown in horses supplemented 272 g of either fish oil (27.2% of total dietary fatty acids as n-3) or corn oil (0.88% of total dietary fatty acids as n-3) (Hall et al., 2004). Neutophils were isolated from ten mature horses receiving supplementation for 14 weeks and chemically stimulated for the production of LT (Hall et al., 2004). The fish oil supplemented horses had a significant increase (9.4 fold) in the

ratio of LTB_5 to LTB_4 compared to baseline that was highly correlated to the ratio of EPA to ARA in the plasma of these horses (Hall et al., 2004). Corn oil supplemented horses had no significant change in LTB_5:LTB_4 or EPA:ARA from baseline (Hall et al., 2004). Therefore, the horses receiving fish oil have higher levels of EPA derived inflammatory mediators that could lead to reduced inflammatory and immune cell responses.

The replacement of n-6 eicosanoids with n-3 eicosanoids may mediate a lower inflammatory immune response and act as therapy for inflammatory conditions by reducing the concentrations of inflammatory cytokines. Supplementation of n-3 in healthy humans resulted in reduced monocyte production of ARA eicosanoids PGE_2 and TXB_2 (from 30 to 55%) that translated into reduced production of inflammatory cytokines TNFα and IL-1β (from 30 to 80%) by monocytes after 8 weeks compared to those receiving a n-6 supplementation (Caughey et al., 1996). In addition, there was a significant inverse relationship between cellular EPA concentrations and production of TNFα and IL-1β (Caughey et al., 1996). There is expansive evidence in mice the n-3 PUFA supplementation has an influence on the production of inflammatory markers especially TNFα. Mice fed a diet high in EPA for 3 weeks had reduced macrophage production of TNFα and IL-12, and Th2 response production of IL-4 (Sierra et al., 2008). Additional studies have indicated the role of n-3 PUFA on the prevention of obesity related inflammation (ie. TNFα) induced by high fat diets in mice (Perez-Matute et al., 2007; Todoric et al., 2006).

There is growing evidence of the benefits of n-3 PUFA supplementation in horses. In a study of sixteen mares with osteoarthritis, eight mares were supplemented

with 15 g of EPA and 19.8 g of DHA each day with a total treatment diet (excluding hay) n-6:n-3 ratio of 5:1. These mare were compared with a control group comprised of the other eight mares with a dietary n-6:n-3 ratio of 11:1 (Manhart et al., 2009). Results showed an overall reduced amount of synovial fluid white blood cells (WBC) ($P = 0.027$) and PGE_2 concentrations ($P = 0.016$) in the treatment horses compared with the control horses (Manhart et al., 2009). This suggests a reduction in inflammation in the joints and circulation of these arthritic horses supplemented n-3 PUFA. In addition, reduced pain has shown to be positively correlated with PGE_2 in osteoarthritic canines (Manhart et al., 2009; Trumble et al., 2004). Evaluation of the mean BW placement on each mare's four limbs by force plate analysis showed a trend ($P = 0.12$) toward greater weight placement on affected limbs by treatment horses compared to control (Manhart et al., 2009). A significant effect may not have been evident because of the high variation in the degree of arthritis between horses but may indicate potential joint improvement from n-3 PUFA supplementation.

Further evidence of the potential for reduced pain when supplemented n-3 PUFA was reported in another study of horses with arthritis (Woodward et al., 2005). The horses receiving n-3 PUFA supplementation had increased concentrations of EPA and DHA in plasma that translated to increased stride length in these horses suggesting a reduction in pain (Woodward et al., 2005). More definite evidence is shown in a long term study of humans with stable arthritis comparing the effect of Maxepa, a pill containing both EPA (171 mg/capsule) and DHA 114 mg/capsule), with an air-filled placebo (C. S. Lau et al., 1993). At the start of the study, all patients required 100% NSAID therapy to combat the symptoms of arthritis (C. S. Lau et al., 1993). After 12

years of treatment, patients taking Maxepa daily reduced their NSAID usage to 40% which was significantly lower than the placebo group at 84.1% (C. S. Lau et al., 1993).

Fatty Acid Source: Plant vs. Animal

Major amounts of ALA are present in vegetable oils including flaxseed, linseed, canola and soy oil. Hay is also a significant source of ALA for horses (Davidson, 2009). The long chained n-3 fatty acids EPA and DHA are produced by algae a common diet of fish. Therefore, marine foods and fish oils provide a major source of EPA and DHA. The conversion of ALA to EPA and DHA is limited in humans and other mammals. This is due to ALA having the highest oxidation rate of all unsaturated fatty acids along with moderate activity by the enzymes needed for fatty acid conversion (Nettleton, 1991; Robinson et al., 2007; Sierra et al., 2008). Humans and nonhuman primates receiving high doses of ALA had a small conversion of ALA to EPA and sometimes DHA in plasma (Arterburn et al., 2006; Wu et al., 1996). However, investigations comparing different sources of n-3 PUFA are important because some studies suggest that sources containing ALA result in less adverse effects and are more palatable for horses than fish oil (Davidson, 2009; Simpoloulos and Salem, 1987).

It is hypothesized that the lack of conversion may also be due to the typical western diet of humans containing high levels of n-6 competing for the same conversion enzymes (Arterburn et al., 2006). This concept was tested in humans by providing three diets that either varied from each other in the level of ALA or level of LA or ratio of LA:ALA (Goyens et al., 2006). Consumption of low LA and high ALA resulted in highly significant increases in ALA ($P \leq 0.001$) and EPA ($P < 0.001$) in plasma phospholipids

compared to the high LA/low ALA control group (Goyens et al., 2006). There was no difference between groups in the level of DHA (Goyens et al., 2006). These results indicate that by exchanging LA for ALA in the diet there is an increase in synthesis of EPA but these levels are not as high as those provided by direct consumption of EPA (Goyens et al., 2006). In addition, the levels provided by ALA supplementation may not translate to the same reduced inflammation response seen with direct supplementation. A comparison of plant (5.3% of energy as ALA) and marine (3.3% of energy as EPA/DHA) derived n-3 PUFA in 20 nonhuman primates resulted in decreased PGE_2 and percentage of T cells which was greater in the marine fed primates (Wu et al., 1996). At the same time, an increase in T cell-mediated mitogenic response and IL-2 production was only evident in the marine fed primates (Wu et al., 1996). In horses, flaxseed oil supplementation (47.4% ALA) increased plasma ALA and EPA concentrations but did not alter platelet aggregation which is a part of the inflammatory response to tissue injury (Hansen et al., 2002). Horses may be similarly limited in their conversion of ALA therefore not able to influence the production of eicosanoids with plant sources of n-3 PUFA enough to provide a significant effect on the immune response.

The fatty acid content of a horse's natural diet is quite different from the western diet of humans, so there is uncertainty as to whether the same effects of PUFA in humans will translate to horses. The majority of a horse's diet tends to be forage. Forage is low in total fat but what fat it has is predominantly ALA. Green pasture can provide three times more n-3 than n-6 (Davidson, 2009) which is reverse to the typical human western diet (Goyens et al., 2006). Hay provides about half as much ALA as pasture but still dominates over the amount of n-6 even if grains high in n-6 are fed with hay (Davidson,

2009). The benefits of adding more ALA to a diet already high in ALA is unclear especially if conversion to EPA and DHA is limited.

Justification of Study

Inflammation related conditions affect a large number of horses around the world and create a need for effective therapies. Traditionally anti-inflammatory drugs and surgeries are used to treat the symptoms related to these conditions and are paired with adverse effects and often only mask the symptoms temporarily. The equine industry has a growing need for a non-invasive way of reducing inflammation and the damage these conditions can cause. There are many claims of the benefits of providing a source of n-3 for a horse from reduced seasonal skin irritation to reduced joint inflammation (Davidson, 2009). Actual evidence of these improvements is limited along with a comparison of different sources of n-3 PUFA. These claims are commonly based on the supplementation of flaxseed and fish oil, so this study provided an opportunity to directly compare these two sources while contrasting each with a more common equine diet as a control.

CHAPTER III: MATERIALS AND METHODS

Horses

Twenty one mares of stock horse mixed breed were obtained from Equine Reproductive Laboratory (ERL) at Colorado State University (CSU). Prior to arriving at CSU, these mares were kept on a ranch in Canadian, Texas where they were provided access to free choice pasture native to the area. Thirteen of the mares had been housed at the CSU equine reproductive laboratory (ERL) during the year prior to this study. The other eight mares were brought in from Texas to the ERL at least a month prior to the start of this study. Mares were kept on a regular deworming and vaccination program and provided only free choice alfalfa during their time at the ERL. Protocols for care and use of horses were approved by the Colorado State Institutional Animal Care and Use Committee. The mares in this study ranged from 5 to 14 years of age with an average of 9 within each treatment group. Weight (kg) and body condition score (BCS) ranged from 525 to 673 kg and 5 to 8 respectively. Age, weight and BCS means and ranges by treatment are given in Table 2.1. Body condition score was based on a scale of 1 to 9 with 1 being emaciated and 9 representing extreme obesity (Henneke et al., 1983). Final marks were based on the average scores from two individuals.

Treatment Diets

The twenty one mares were blocked by age, weight and BCS and randomly assigned to one of three dietary treatment supplements, fish oil (FISH; Magnitude^{TM}), ground flaxseed (FLAX; Nutra-Flax^{TM}), and control (CON). All three diets included rolled barley in variable amounts in order to equalize the amount of protein, digestible energy, starch and fiber between the treatment groups. Analysis of supplements, barley and hay were completed prior to supplementation (Table 2.2 and 2.3). The fish oil supplement provided 13 and 45.5 mg/kg body weight (BW) of EPA and DHA respectively which were not present in the other treatment diets. The flaxseed supplement provided an additional 68.8 and 71.63 mg/kg BW of ALA compared to the FISH and CON treatment diets. The FISH and FLAX diets shared a n-6:n-3 fatty acid ratio of 0.44:1. The CON diet contained a similar amount of total n-6 and almost half as much total n-3 with a fatty acid ratio of 0.79:1. A small amount of water was added to the barley of the FLAX diet so the ground flaxseed supplement would stick to the grain and ensure complete ingestion of the supplement by the subjects. The grain supplementation was provided to the horses once a day (1000) via individual feed bags. The horses were introduced to the supplement over two weeks after baseline samples. Refusals were rare among the majority of the horses after the adaptation period except the FISH group. Refusals were summed for each horse and compared to the total amount of that treatment diet offered during the study to develop a percentage of refusals. All treatment groups were fed an alfalfa and grass mixture daily (0700) in cement feed bunks at 1.5% BW for FISH and CON and 1.7% BW for FLAX. More hay was fed to the flax group to minimize caloric differences between diets. Horses were housed according to treatment

group in three uncovered, dirt pens containing a centrally located automatic water tank and salt block.

Blood Sample Collection

Blood samples were collected prior to daily supplementation on week 0 (baseline), 2, 4, 6, 8, 10 and 12 for a total of 6 treatment samplings. Treatment groups began supplementation and were sampled on different days of the week, FISH on Wednesday, FLAX on Thursday and CON on Friday, in order to minimize the time between blood sample collection and laboratory processing. Samples were collected according to treatment groups as opposed to random selection so each horse within a treatment pen could begin supplementation on the same day. Blood samples were collected from the jugular vein switching sides of the horse each sampling. A 25 G needle and vacutainer holder were used to collect 10 cc of whole blood into a serum separation tube containing a polymer gel and a sterile blood collection tube, containing dried sodium heparin each week of sampling. The serum separation tubes were allotted two hours to clot at room temperature and then spun for 15 min at 3,000 rpm. Aliquots of serum were stored at -20°C until assayed. The sodium heparin tubes were spun within one hour post sampling at 2,000 rpm for 10 min. The buffy coat was extracted, washed twice with phosphate buffered solution (PBS, pH=7.5) and combined with 1 mL of RNAlater® (Ambion, Austin, TX) in nuclease-free eppendorfs. These samples were kept for 24 hours at 4°C and then stored at -80°C until RNA extraction.

CHAPTER IV: EFFECT OF N-3 PUFA SUPPLEMENTATION ON MRNA EXPRESSION OF INFLAMMATORY CYTOKINES

RNA Extraction and Isolation

White blood cell samples were centrifuged for 5 min at 10,000 rpm in order to remove the supernatant of RNAlater® from the samples. Total RNA was extracted from 250 μL of sample using the RNeasy® Mini Kit (Qiagen, Valencia, CA) and following the RNeasy® Handbook protocol 'Purification of Total RNA from Animal Cells Using Spin Technology'. First, the cells were disrupted in order to release the RNA contained within them by adding 600 μL of a lysis buffer (Buffer RLT) provided in the RNeasy® Mini Kit. The product after addition of the lysis buffer did not require homogenization. One volume of 70% ethanol (850 μL) was added to the lysate and mixed well via pipetting. RNA was collected by centrifuging the samples (700 μL at a time) in RNeasy® spin columns for 15 sec at 13,000 rpm. The flow-through left over after each centrifuge was discarded. Two more washes were preformed with 500 μL of Buffer RPE first for 15 sec and next for 2 min at 13,000 rpm. The spin-columns were centrifuged an additional minute at 13,000 rpm in a new collection tube in order to eliminate any leftover ethanol from the Buffer RPE. RNA was eluted from the spin-columns in 30 μL of RNase-free water run through the spin-column twice.

RNase-Free DNase Treatment

Previously extracted RNA samples were re-filtered through a spin-column in order to eliminate any contaminating DNA. This was performed via the RNase-Free DNase Set (Qiagen, Valencia, CA). The membrane bound RNA was washed first with 350 µL of Buffer RW1 for 15 sec at 10,000 rpm and then incubated with 80 µL of a DNase I mix for 15 min at room temperature. The DNase I mix was a 1:8 dilution of reconstituted DNase I in Buffer RDD. Post incubation, the membranes were washed with another 350 µL of Buffer RW1. RNA was then washed twice with Buffer RPE and eluted as previously described.

Reverse Transcription

Total RNA was reverse transcribed into cDNA using a Mastercycler gradient (Eppendorf, Hamburg, Germany). Reverse transcription was carried out on 20 µL reactions comprising of 10 µL of RNA, 5 µL of RNase-free water, 4 µL of 5x iScript Reaction Mix and 1 µL of iScript Reverse Transcriptase (iScript cDNA Synthesis Kit, Bio-Rad, Hercules, CA). Reactions were incubated at 25°C for 5 min, 42°C for 30°min and 85°C for 5 min and then diluted in RNase-free water to a final volume of 200 µL and stored at -20°C. Eluted RNA not used for reverse transcription was stored at -80°C.

Primer Design

Equine specific primer sets were designed using the NCBI primer tools (www.ncbi.nlm.nih.gov) on published equine sequences for *Equus caballus* target genes TNFα (NM_001081819), IL-1 (NM_001082526), IL-6 (NM_001082496) and IL-10

(NM_001082490) along with housekeeping genes 18S ribosomal RNA (18S) (AJ311673) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (AF157626). Primers were chosen with a product length between 200 and 300 base pairs, a Tm of 58 to 62 and with the inclusion of introns. Primers were verified using NCBI Primer-BLAST on each forward and reverse primer. Standard primers were ordered from Invitrogen (Carlsbad, CA) as listed on Table 3.1. Upon arrival, primers were reconstituted, diluted to a 5μM solution and separated into 30 μL aliquots for use on individual plates.

Real-time PCR

Real-time PCR was preformed on cDNA using an iQ5 Real-Time PCR Detection System iCycler with iQ5 2.0 program (Bio-Rad, Hercules, CA). Samples of cDNA were run in duplicate in a 25 μL reaction containing 1 μg of cDNA in 10.5 μL and 14.5 μL of master mix (12.5 μL of SYBR Green super mix (iQ SYBR Green kit, Bio-Rad, Hercules, CA), 1 μL of forward and reverse primers and .5 μL of nuclease-free water (Qiagen, Valencia, CA). Concentrations of cDNA were determined by performing UV spectrometry on 2 μL of sample using a ND-1000 Spectrophotometer with respective V3.3.0 program (NanoDrop, Thermo Scientific, Wilmington, DE). Reactions were incubated for 2 min and 30 sec at 95°C, followed by 45 cycles of 30 sec at 95°C and 30 sec at 65°C. The real-time PCR procedure concluded with 1 min at 95°C followed by a melt curve determined by 10 sec incubations for each half a degree increase in temperature from 55°C to 95°C.

Primer Sequencing

Real-time PCR was run on two samples including each target and housekeeping gene and followed by electrophoresis on a 1 M agarose gel containing ethidium bromide for 50 min at 100 volts. This preliminary run revealed sharp bands for all target and housekeeping genes (Figure 3.1). Extraction of cDNA was performed via a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). Amplicons were extracted and weighted then QG Buffer was added to each amplicon at a volume of three times the weight of the band. The combination was incubated at 50°C for 10 min. After incubation, isopropanol was added at one time the weight of the band and then centrifuged for one min through a DNA column followed by 500 µL of QG Buffer and 750 µL of PE buffer. Amplicon was eluted out in 20 µL of RNase-free water run twice through the spin-column membrane. The eluted cDNA and each primer set were sent to the Microbiology Lab at Colorado State University for sequencing.

Primer Efficiency Testing

Housekeeping genes were tested on their consistency between samples by running real-time PCR on a set of different samples with GAPDH and 18S primers. Greater consistency was found between samples using GAPDH. 18S was diluted 16x during all future PCR in order to increase the number of cycles closer to target genes.

Primer efficiency was tested by running target and housekeeping genes separately on a set of sample half dilutions (1x, 2x, 4x, 8x and 16x). Real-time PCR was run as described previously. Primer efficiency for all genes is given in Figures 3.2-3.6. IL-6 did

not produce enough product under each dilution to calculate an efficiency graph or percent.

Results and Discussion

Real-Time PCR

Initial real-time PCR gave inconsistent and often unidentifiable results that may have been due to loading amounts of cDNA being to low for detection. Amplification that was detected produced a correct product for all genes except IL-10. The RNase-free DNase treatment explained previously was adopted at this point in order to remove any DNA that may be causing incorrect amplification. RNA was extracted again from all WBC samples and combined with the previously extracted RNA for DNase treatment. For the majority of samples, amplification of target genes was still undetectable using the treated cDNA. Adjustments in the real-time PCR procedure were made including temperature and cycle length in order to increase the chance of reaching detectable levels. Improvement was only evident for target gene IL-1. Electrophoresis revealed possible contamination of primers or nuclease-free water (Figures 3.7). Because IL-6 was consistently showing no amplification of most samples, it was concluded that circulating IL-6 concentrations in WBC are too low for our detection in most of the horses of this study. New aliquots of primers and nuclease-free water were made and tested resulting in the same issues as before. In order to test for contamination, each gene was run on both a mixed sample and just water without any added cDNA. Reactions were then run on a gel (Figure 3.8). Results provided evidence of cDNA amplification in water samples so further precautions to avoid water contamination were made.

In order to possibly eliminate the amplification of cDNA below 100 bp, PCR temperature was set back to 65°C. Results indicated that TNFα and IL-1 produced a correct product, IL-10 produced a false product and IL-6 produced no product (Figure 3.9). In hopes of eliminating false products, IL-6 and IL-10 primers were redesigned (Table 3.1). A nine point gradient temperature test from 56 to 70°C was preformed on all four target gene primers using a Mastercycler gradient (Eppendorf, Hamburg, Germany). The results for IL-1 are shown in Figure 3.10. Results for TNFα were not captured on film but were very similar to IL-1. No cDNA markers were visible for IL-6 or IL-10. The temperature gradient indicated that 65°C was the best temperature to run real-time PCR for these primers. A final run of all samples was completed with limited results. Only a few correct products were shown and these were limited to a few select samples (Figure 3.11). The false products may indicate that the quality of cDNA was poor and degraded.

RNA Quality Assessment

Inconsistent and low RNA quality may have been the cause for continued problems throughout this study. Initial primer products and sequences indicate that primer designs were sound. If contamination was the issue, improvements would have been made when new primer and nuclease-free water aliquots were used. A temperature gradient test revealed that the initial temperature utilized for PCR should have provided the cleanest product. By diluting samples for 18S primers and increasing the number of cycles, the chance for producing detectable amplification levels was optimized. When successful results could not be obtained, the concern became low RNA quality. Previous studies reviewing the effect of RNA integrity on the results of PCR analysis indicate the

importance of starting with high quality RNA (Fleige et al., 2006; Imbeaud et al., 2005). Quality RNA must not be degraded and must be free of protein, genomic DNA, salts (i.e. Mg^{2+} or Mn^{2+}) and nucleases for extended storage (Bustin and Nolan, 2004; Pfaffl, 2005). The RNA extraction and purification method combined with the Nuclease-free DNase treatment used in this study should have provided RNA free of proteins and salt. This leaves the possibility of degraded RNA or nuclease contamination.

Signs of degradation or contamination were not present initially in this study but seem to increase over time. This indicates that RNA may not only have been degraded during collection, extraction or purification procedures but also might have contained nuclease enzymes that contribute to long term and inconsistent degradation of RNA during storage. Lab equipment utilized for WBC extraction and washing were not treated prior to sample processing and may have contained nuclease enzymes that were transferred to RNA samples during the extraction process.

In order to avoid applying real-time PCR, a labor intensive, time consuming and highly expensive procedure (Imbeaud et al., 2005; Raeymaekers, 1993), on RNA with poor integrity, a RNA quality assessment should be applied (Fleige and Pfaffl, 2006). RNA purity can be measured via a spectrophotometer at 260 nm and 280 nm. The ratio of these two readings indicates the RNA is free of significant amounts of protein or phenol when the value is between 1.8 and 2.2. However, this method should not be the only assessment due to the high amount of protein that may still be present with a value of 1.8 and lack of quantification of the other contaminates that could be present (Bustin and Nolan, 2004). RNA integrity can be inspected by running RNA by electrophoresis on a denaturing agarose gel. Intact RNA will show two sharp bands that indicate 28S and 18S

ribosomal RNA. Degraded RNA will show a smear at a lower molecular weight. Only nucleic acid content will be measured using this technique. This technique is often undesirable because it requires significantly extra time when working with large sample sizes along with requiring large quantities of often limited amounts of RNA (Bustin and Nolan, 2004).

Alternatively, the lab-on-chip capillary gel-electrophoresis method only requires 200 pg of total RNA and is a more convenient way to assess RNA integrity especially when working with a large number of RNA preparations (Fleige and Pfaffl, 2006). This technology separates and detects RNA samples to create a electropherogram. Similar to the previous method, a ratio of the plot peak of 18S and 28S indicates the quality of RNA. Agilent Technologies developed a tool for standardization of the lab-on-chip capillary gel-electrophoresis. This tool is called the RNA Integrity Number (RIN) and is determined by the shape of the electropherogram curve (Mueller et al., 2004). RIN ranges from 1 (degraded) to 10 (intact) with a score for good quality RNA dependent on the tissue used for RNA extraction (Fleige et al., 2006). For example, quality RNA from WBC will yield a RIN value just above 9 (Fleige et al., 2006).

Inconsistent amplification of samples may have been due to degraded RNA. A RNA quality assessment would have determined poor RNA integrity early on and prevented the use of degraded samples. In order for an accurate representation of gene expression to be provided by real-time PCR, RNA integrity needs to be ensured (Fleige and Pfaffl, 2006).

Problem With Varying WBC Counts

Further investigation indicated that the failure of consistent PCR products was insignificant because the method of WBC collection does not provide a reliable conclusion of circulating levels of inflammatory markers. The total volume of WBC within the blood may vary from horse to horse and this variability can not be accounted for unless the individual cells are counted (Satue et al., 2010). This was visually noticeable during WBC extraction and processing. By using a standard amount of WBC from each horse without any idea of the total count, the circulating inflammatory variable that may be present due to the difference in the number of lymphocytes is lost. The results will be the variability and change within 250 μL of WBC instead of within the circulating blood. In order to provide evidence of change and differences within the circulating blood, a standard amount of whole blood should be used for RNA isolation therefore allowing for differences in the total count of WBC from horse to horse. There are several options for whole blood RNA isolation ranging in price, length of assay run and level of difficulty. The PAXgene® Blood RNA System, RNeasy® Mini Kit , TRI® Reagent BD assay and RiboPure-Blood RNA kit established processes used for RNA extraction from whole blood in humans and often other species including horses.

PAXgene® Blood RNA System

This system includes a specific set of materials for blood collection and RNA extraction. PAXgene® Blood RNA Tubes (PreAnalytiX, Qiagen, Valencia, CA) are a 2.5 mL vacutainer blood collection tube containing an additive for RNA stabilization. This additive not only reduces RNA degradation but also minimizes gene induction. Whole

blood RNA can be stored for several days at temperatures higher than 4°C or 6 months at temperatures below -20°C. Lysis of blood cells to release RNA occurs during incubation at room temperature for at least two hours after blood collection. RNA is extracted using the PAXgene® Blood RNA Kit (PreAnalytiX, Qiagen, Valencia, CA) via a manual procedure using PAXgene® Shredder and RNA spin columns or an automated procedure using a QIAcube. The manual procedure requires only an hour and a half for 12 samples with less than half the time as hands-on (40 min). By utilizing a QIAcube the total procedure is longer (125 min) but requires less hands-on time (20 min) than the manual procedure which is mostly required at the start of the process. The manual procedure requires a microcentrifuge and shaker-incubator to process samples. Assuming the shaker-incubator for the manual procedure or QIAcube for the automated procedure are already available, the estimated cost per sample is $20.12[1] contributed mostly by the cost of the PAXgene® Blood tubes ($10.10 per tube) and kit ($8.28 per sample). However, this procedure will provide 3 or more μg of RNA for reverse transcription.

RNeasy® Mini Kit

This kit for RNA extraction provided by Qiagen (Valencia, CA). This is the same system used for RNA extraction in this study, however, instead of using extracted WBC, 200 μL of whole blood would be processed and reverse transcribed to limit RNA degradation and gene induction. This procedure would require less than one hour of hands-on processing each day of sampling. This system was designed for animal cell and

[1] Estimated cost per sample based on pricing information collected in May 2010 for the following: PAXgene® Blood RNA Kit (50 samples, $414.00, Qiagen); PAXgene® Blood RNATubes (100 tubes, $1009.64, Optics Plant); RNase-free DNase Set (50 samples, $83, Qiagen); Ethanol, 99.5% (500 mL, $45.20, Acros Organics) for dilution of Buffer BR4 (44 mL).

tissue processing and is not verified for use on whole blood. My study has shown that RNA can be extracted from blood cells via the RNeasy system but requires further investigation to determine RNA quality and purity. Purification with RNeasy® Mini spin columns does not require the use of a DNase I treatment. The estimated cost per sample is $7.913[2]. This procedure has the potential to extract up to 100 μg of RNA but needs to be confirmed for blood samples.

TRI®Reagent BD

TRI®Reagent BD is a an adaptation of the original single-step TRI®Reagent method (Chomczynski and Sacchi, 1987) and works as a RNA stabilization and cell lysis reagent of 200 μL of whole blood during collection. The addition of chloroform separates the blood into three phases (Chomczynski, 1993). The organic phase is made up of proteins, the interphase contains DNA and the aqueous phase holds RNA. The aqueous phase with RNA is recovered by precipitation with isopropyl alcohol, two washes with ethanol (75%) and dissolved in nuclease-free water. The process takes approximately an hour for a dozen samples. The estimated total cost per sample is $2.02[3] with the majority of the cost due to the purchase of the TRI®Reagent BD and green top tubes ($1.58). This procedure provides 30 to 40 μg of RNA to be used directly in reverse transcription. Therefore, it is common to process several aliquots of whole blood from each sample. If

[2] Estimated cost per sample based on pricing information collected in May 2010 for the following: RNeasy® Mini Kit (50 samples, $242, Qiagen); Sodium Heparin Blood Collection Tubes (100 tubes, $55.85, BD); RNase-Free DNase Set (50 samples, $83, Qiagen); Ethanol, 99.5% (500 mL, $45.20, Acros Organics) for spin-columns and dilution of Buffer RPE (44 mL).

[3] Estimated cost per sample based on pricing information collected in May 2010 for the following: TRIzol® Reagent (200 mL, $256.00, Invitrogen); Sodium Heparin Blood Collection Tubes (100 tubes, $55.85, BD); Glacial Acetic Acid (250 mL, $17.20, Fisher Scientific), Chloroform (1 L, $47.15, Fisher Scientific); Isopropyl alcohol (500 mL, $174.30, Fisher Scientific); Ethanol, 99.5% (500 ml, $45.20, Acros Organics); RNase-free water (12 x 1.9 mL, $95, Qiagen).

three aliquots are collected the estimated total cost per sample becomes $6.06 with $4.74 covering the TRI®Reagent BD and green top tubes.

RiboPure™-Blood Kit

This system provided by Ambion (Austin, TX) includes steps for RNA isolation, purification and RNA quality assessment. Each kit purifies up to 40 samples of 500 µL using a similar method of lysis solution, chloroform and ethanol as the TRI®Reagent BD assay. Final RNA purification is accomplished via a Filter Cartridge system similar to the spin-columns of the PAXgene® Blood RNA Kit and RNeasy® Mini Kit. Expected RNA yields are between 2 and 4 µg. A DNase I treatment set is included in this kit for application post RNA elution. The assay requires less than half an hour to process a dozen samples. This kit also includes 100 mL of RNAlater® (Ambion, Austin, TX) for stabilization of RNA in whole blood samples during storage. The cost per sample for this procedure of RNA extraction is around $10.24[4] with the majority of the cost from the kit itself and additional cost from collection tubes and ethanol. This kit provides a procedure and formaldehyde loading dye for assessing RNA quality but requires additional materials to run electrophoresis on RNA.

RNA Extraction Method Comparison

Both the TRI®Reagent BD assay and PAXgene® Blood RNA System are commonly used and accepted by various veterinary and animal science journals. Studies

[4] Estimated cost per sample based on pricing information collected in May 2010 for the following: RiboPure™-Blood Kit (40 samples, $380, Applied BioSystems); Sodium Heparin Blood Collection Tubes (100 tubes, $55.85, BD); Ethanol, 99.5% (500 mL, $45.20, Acros Organics) for Filter Cartridge prep and dilution of Wash Solution 2/3 (80 mL).

evaluating cytokines involved in respiratory and endocrinology inflammation utilized the TRI®Reagent assay for RNA isolation in tissue and cells (McFarlane and Holbrook, 2008; Riihimaki et al., 2008a; Riihimaki et al., 2008b). The PAXgene® Blood RNA System is commonly used for whole blood RNA extraction in equine studies of the relationship of inflammatory cytokines with exercise, obesity and insulin sensitivity (Lamprecht et al., 2005; Vick et al., 2007; Vick et al., 2008). An alternative method has been developed and tested that allows for double the amount of whole blood (5 mL) to be collected into each PAXgene™ Blood RNA Tube (Vu, 2004). This requires a few modifications to the standard PAXgene™ protocol including addition of more proteinkinase - K and extended time for protein digestion (Vu, 2004). This study utilized this alternative method to analyze RNA quantity, purity and RT – PCR performance when the PAXgene™ System is used for RNA extraction. The study concluded that samples collected and processed within 72 hours at room temperature or 10 days at 4°C produced quality RNA with negligible degradation and easy amplification of β-actin from 0.6 μg total RNA (Vu, 2004). By utilizing this alternative method, the higher concentration of RNA collected would help off set the high cost of PAXgene™ Blood RNA Tubes. An additional supply of proteinkinase – K solution[5] above what is supplied in the kit would have to be purchased. Both the RNeasy®Mini Kit and TRI®Reagent BD assay still yield higher amounts of RNA but the RiboPure®-Blood Kit yields the lowest out of all four methods and is not often cited in equine literature.

The use of the RNeasy® Mini Kit for PCR-analysis of inflammatory markers in human and mice studies is also common but modified in order to allow for RNA to be stabilized and stored after sampling (Salway et al., 2008; Todoric et al., 2006). This is

[5] Proteinkinase-K Solution (2 mL, $74, Qiagen, May 2010)

accomplished by collecting whole blood samples and isolating RNA using the TRI®Reagent procedure and then purifying the aqueous phase containing RNA using the RNeasy® Mini Kit. By adding the cost of the TRI®Reagent BD and chloroform to the RNeasy® Mini Kit without the RNase-Free DNase Kit, this process totals around $888.20[6] or $7.29 per sample. A dozen samples can be processed at a time using this method still requiring only about 1 hour. By including a RNA stabilization method, samples do not have to be processed immediately allowing RNA to be isolated from more samples at a time to reduce the amount of time.

It is important to evaluate the length, level of difficulty, accuracy and reliability of each procedure. The RiboPure®-Blood Kit requires the least amount of time at about three quarters of an hour for a dozen samples when the DNase I treatment is included after the purification process. TRI®Reagent BD assay also requires a low amount of time at about 1 hour for a dozen samples. The PAXgene® Blood RNA System takes almost one and a half hours to do the same number of samples. The RNeasy® Mini Kit requires less than an hour to purify a dozen samples. However, because the RNeasy® Mini Kit does not provide an RNA stabilizing reagent, samples would need to be processed for RNA immediately after sampling each day. Dependent on the frequency and number of samples being collected, this could add considerable processing time when only a few samples are taken over several days.

In addition, when following the TRI®Reagent BD assay to isolate RNA, there is some uncertainty and more room for error compared to the spin column kits. The

[6] Estimated cost per sample based on pricing information collected in May 2010 for the following: TRIzol® Reagent (200 mL, $256.00, Invitrogen); Chloroform (1 L, $47.15, Fisher Scientific); Isopropyl alcohol (500 mL, $174.30, Fisher Scientific); RNeasy® Mini Kit (50 samples, $242, Qiagen); Sodium Heparin Blood Collection Tubes (100 tubes, $55.85, BD); Ethanol, 99.5% (500 mL, $45.20, Acros Organics) for spin-columns and dilution of Buffer RPE (44 mL).

combined TRI®Reagent BD and RNeasy® Mini Kit procedure would reduce some of the uncertainty when forming a RNA pellet. The repetitive procedure and easy to use columns of the PAXgene™ Blood RNA, RiboPure®-Blood Kit and RNeasy® Mini Kit allow for persons with little lab experience to extract RNA successfully. The PAXgene™ Blood RNA also requires very little handling and includes all components needed except ethanol which may be further useful for those with little laboratory experience and working in a laboratory with poor air quality.

Levels of accuracy and reliability are established by the companies that release these RNA purification procedures. However, there is a need to compare them side-by-side on equine blood before any conclusions on actual RNA yield, quality and purity levels can be established. Further research to determine the reliability and accuracy of these methods is required.

The four methods clearly differ in amount of cost per sample. The TRI®Reagent BD assay was by far the most cost effective even when multiple aliquots of each sample are processed. The other solutions needed for this procedure (chloroform, isopropyl alcohol, nuclease-free water and ethanol) are common laboratory items often kept on-hand in sufficient quantities. If this is the case, the cost for these items may be negligible leaving only the purchase of TRI®Reagent BD and heparin containing blood collection tubes at under $5 per sample for over 30 μg of RNA. The most costly method is by far the PAXgene® Blood RNA System because of its high priced specialized blood collection tubes and kit along with a DNase I treatment set totaling at just over $20 per sample for 3 or more μg of RNA. The RNeasy® Mini Kit was not much more than the TRI®Reagent BD assay and includes the same cost from blood collection tubes along with a specialized

kit and DNase I treatment. This procedure cost just under $8 per sample for isolation of up to100 μg of RNA. Costing just over $10 a sample, the RiboPure®-Blood Kit is half the price of the PAXgene® Blood RNA System but still more than the TRI®Reagent BD assay and RNeasy® Mini Kit and yields only 2 to 4 μg of RNA.

A per sample cost may not be relevant for developing labs and research programs. If a project has a limited budget without the availability small aliquots of chemicals sold in bulk, the total cost to purchase all products needed may be more significant. When evaluating the total start up cost for at least 3 μg of RNA isolation per sample for 100 samples, the order of the procedures cost wise stays the same but the gap in price is narrowed due to increased cost of bulk chemicals needed for the TRI®Reagent BD assay and reduced cost for kits that provide just enough of each chemical for a set number of samples. The PAXgene® Blood RNA System requires over $2000 to run 100 samples. The TRI®Reagent BD assay and RNeasy® Mini Kit require almost 75% less at $673.5 and $751.05. The RiboPure®-Blood Kit is also considerably less than the PAXgene® Blood RNA System at $861.05 but this only covers 80 samples. A third kit is required to purify at least 100 samples totaling $1241.50 almost twice as much as the other two procedures.

Conclusion

When determining which RNA isolation procedure to use, the acceptance of the assay in equine literature and its ability to produce quality RNA usable for PCR along with the skill level of the individual completing the assay, length of processing, cost per sample and total start up cost all need to be considered. Both TRI®Reagent BD and the

PAXgene® Blood RNA System are highly accepted and respected in equine literature. The RiboPure®-Blood Kit is simple and requires very little processing time but it provides low RNA yields and is not found in equine literature. TRI®Reagent BD is a very cost effective option. The PAXgene® Blood RNA System provides an expensive but all included assay with the option of an automated procedure requiring very little handling. RNeasy®Mini Kit is great for developing laboratories and students lacking laboratory experience but caution is needed to determine its reliability for isolating quality RNA from whole blood. When combined with TRI®Reagent BD for RNA stabilization it provides a simple and fairly low cost RNA isolation procedure requiring only a small amount of processing time. However further research is necessary to determine true RNA yields from whole blood and the effect each isolation technique has on the outcome of PCR.

CHAPTER V: EFFECT OF N-3 PUFA SUPPLEMENTATION ON CIRCULATING CONCENTRATIONS OF TUMOR NECROSIS FACTOR ALPHA

TNFα ELISA Assay

Serum samples were assayed using the ELISA Equine TNFα Screening Set by Endogen (Piercenet, Rockford, IL) for concentrations of TNFα protein (TNFα). Currently, an ELISA set for measuring the concentration of cytokines other than TNFα in horses has not been validated. Additional supplies included BupH Modified Dulbecco's PBS Packs (Piercenet, Rockford, IL), BupH Carbonate-Bicarbonate Buffer Packs (Piercenet, Rockford, IL), ELISA Blocker Blocking Buffer (Piercenet, Rockford, IL), ELISA Wash Buffer (Piercenet, Rockford, IL), ELISA Plates (Piercenet, Rockford, IL), pre-cut Sealing Tape for 96-well plates (Piercenet, Rockford, IL), Reagent Reservoirs (Piercenet, Rockford, IL) and Bovine Serum Albumin (EMD Chemicals, Gibbstown, NJ). Plates were prepped the afternoon prior with 100 ul of diluted coating antibody and incubated 16 hours. Every step of this ELISA requires a plate sealer during incubation and is incubated at room temperature. The provided concentrated TNFα Coating Antibody was diluted 1:100 into reconstituted Carbonate-Bicarbonate Buffer. Reagent Diluent (RD) was also prepared the afternoon before by dissolving 2 g of BSA into reconstituted PBS with a final volume of 50 ml multiplied times the number of plates. Samples were thawed over night at 4°C.

The coated plates were aspirated first thing the next day. Plates were then blocked with 300 ul per well of Blocking Buffer and incubated for 1 hour. During blocking, samples were diluted 1:50 into RD and the standards were made from 10,000 to 7.8125 pg/ml. The provided Equine TNFα Standard was reconstituted into 1.35 ml of RD to make 10,000 pg/ml. The second Standard was diluted 1:10 by adding 200 ul of the 10,000 standard to 1800 ul of RD. The rest of the standards were diluted 1:2 by adding 1000 ul of the higher dilution to 100 ul of RD (500, 250, 125, 62.5, 31.25, 15.63, 7.8125 pg/ml). A blank standard containing only RD was included on each plate. After aspiration of the Blocking Buffer and three washings, samples and standards were added at 100 ul per well to plates. All washings require 300 ul of Wash Buffer added to each well and were completed using a wash bottle. The rest of the ELISA procedure comprised of repeated incubations with 100 ul in each well of different reagents. The plates were aspirated and washed three times between incubations. The provided TNFα Detection Antibody was diluted 1:100 in RD and the provided horseradish peroxidase labeled Streptavidin (Streptavidin-HRP) was diluted (30 ul) in 12 ml of RD. Plates were incubated first with biotin labeled Detection Antibody for 1 hour that attached to the TNFp bound to the coating antibody. This formed a coating antibody - TNFp - detection antibody (CA-TNFp-DA) complex. Then plates were incubated with Streptavidin HRP for 30 min. The Streptavidin-HRP bound to the detection antibody end of the CA-TNFp-DA complex. Lastly, plates were incubated in the dark with Substrate Solution containing tetramethyl benzidine (TMB) and peroxide. TMB is a chromogen that yields blue in the presence of peroxide and in reaction with HRP. The amount of reaction occurs in accordance to the amount of bound TNFp in the CA-TNFp-DA complex. After precisely

20 min of incubation, 100 ul of Stop Solution containing 0.18 M sulfuric acid was added to stop the reaction while turning the solution yellow. Each plate was measured at 450 nm and 550 nm on a BioTek Synergy HT plate reader via Gen5 software.

Insulin Resistance Markers

Minimal model parameters (SI and AIRg) were obtained through a companion study via frequently sampled intravenous glucose tolerance test (FSIGT) on the same 21 horses during the same sampling weeks (Rexford, 2010). Horses were diagnosed as insulin resistant based on their level of SI as established in previous research (Treiber et al., 2005b). Horses with a SI value below 0.79 $L*min^{-1*}mU^{-1}$ were labeled insulin resistant while horses with an SI value at or above 0.79 $L*min^{-1*}mU^{-1}$ were labeled non-insulin resistant. These resulting labels of insulin resistance or non-insulin resistance were designated IR for statistical analysis. Ten of the twenty one horses had SI values below this level. Just prior to the FSIGT testing, basal blood samples were collected in order to determine plasma concentrations of glucose and insulin for the insulin resistance makers called proxies (MIRG and RISQI). Glucose concentrations were determined using a YSI 2700 SELECT biochemistry analyzer while insulin concentrations were determined by radioimmunoassay (Treiber et al., 2005b). Glucose and insulin concentrations were then used in established equations to estimate insulin sensitivity and insulin response (Treiber et al., 2006).

Statistical Analysis

Measures from the vet teaching hospital were compared to the blank standard measurement ($Sample_{450}$ - $Blank_{450}$). Any background in the plate readings from Animal Science was accounted for by taking the difference of the 550 nm reading from the 450 nm reading. The data obtained from Animal Science were used for statistical analysis because of the significant background determined by the 550 nm reading. Serum concentrations were determined based on the standard curve on each plate. This was accomplished by formulating a standard curve equation from the absorbancy readings of the standard curve wells at each known concentration of TNFα and applying the curve to the sample readings to calculate the actual amount (pg/ml) of TNFα in each sample. Following this, the calculated amounts of TNFα based on the standard were multiplied by 50 to account for sample dilution. Statistical analysis was completed using the SAS 9.2 program PROC MIXED with fixed variables treatment, week and treatment by week interaction, and the random variable horse nested in treatment. Week was analyzed as a repeated measurement. Age, weight, BCS and insulin resistance parameters (RISQI, MIRG, AIRg, SI and IR) were run as covariates in the model. In addition, Pearson correlations were calculated between TNFα, age, weight, BCS, RISQI, MIRG, AIRg, SI and IR.

Results and Discussion

Equine TNFα ELISA

Because data was not normally distributed due to the exponential variation between horses, log transformation was preformed similar to previous studies using the same equine TNFα screening set (Vick et al., 2008). An analysis of variance (ANOVA) was run on the transformed data and differences were compared by least square means analysis ($P<0.05$). Log transformed data is shown in Figure 4.1. Due to treatment variation at baseline, week 0 was included as a covariate (estimate = 0.918; $P < 0.001$). Age, weight and BCS were not significant in the model ($P > 0.127$) and were not included as covariates.

Results are shown in Table 4.1 and Figure 4.2. No treatment effects were seen ($P = 0.678$). There was a significant treatment by week interaction ($P = 0.009$). There were no treatment effects within weeks although FISH treated horses had a trend of lower TNFα than both CON and FLAX during week 4 ($P = 0.120$). From week to week, FISH and CON treated horses showed significant differences. CON and FISH showed an increase in TNFα from week 4 to week 8 ($P < 0.035$) and a decrease between week 8 and 12 ($P < 0.001$) to levels close to week 4 ($P > 0.091$). FLAX treated horses had a trend of increased TNFα concentrations between week 4 and week 8 ($P = 0.107$) and a significant decrease from week 8 to week 12 ($P = 0.040$) sill similar to week 4 concentrations ($P = 0.723$).

Correlations are shown in Table 4.2. Throughout the study, there was a positive correlation between TNFα and age ($r = 0.235$; $P = 0.031$) and a negative correlation between TNFα and weight ($r = -0.288$; $P = 0.008$). There was not a significant correlation

between TNFα and BCS only a slight trend of negative correlation (r = -0.19286; P = 0.0788). AIRg was the only significant insulin resistance marker as a covariate in the model (estimate = -0.00018; P = 0.031). No significant correlations were present between TNFα and any of the markers of insulin resistance, AIRg, SI, RISQI, MIRG or IR (P > 0.161).

Weight and BCS of these mares was not consistent during the 12 weeks of supplementation (Figure 4.3 and Table 4.3). There was a 1.2% decrease in weight across all treatments between week 0 and week 4 (7.42 kg, P = 0.020) and a 2.5% decrease between week 4 and week 8 (13.42 kg, P < 0.001). Then a 2.0% increase in weight across all treatments was determined between week 8 to 12 (11.04 kg, P <0.001) back to a similar mean weight of week 4 (P = 0.573). There was a trend effect of week on BCS (P = 0.056). No difference in BCS across all treatments was found between week 0, 4 and 8 (P > 0.077). However, BCS was as much as 5.3% lower in week 12 compared to the previous weeks (P < 0.044).

Temperature averages and precipitation totals during the four weeks prior to each sampling period indicate inconsistent weather during the 90 days of supplementation (Table 4.4). The temperature average of week 8 was similar to week 4 but higher in precipitation including a mix of snow and rain. Week 12 had a warmer average temperature and a lot less precipitation with no snow.

Obesity, Age and Inflammation

A positive correlation between TNFα levels in serum and age was determined despite small variation in age between horses (r = 0.235, P = 0.031). McFarlane and

Holbrook (2008) found significant differences in levels of cytokines including TNFα in healthy horses where aged horses (≥ 16 years) had higher concentrations of inflammatory markers than adult horses (6-14 years). Vick (2007) also utilized a wide range of horses from 3 to 29 years of age yet did not find a significant direct correlation between age and circulating levels of TNFα. This indicates that there may be less correlation between TNFα and age when horses reach a certain age out of the range of this study. Further research involving a wide range of older horses may require grouping old horses vs. young horses in order to determine relationships with inflammation.

Correlations between weight and TNFα have not been reported in studies because weight is not a good indicator of obesity. However in this study, BCS and weight were highly correlated ($P < 0.001$) and inversely related to TNFα concentrations. Previous research indicates that TNFα increases with increased levels of obesity but it was not seen in the current study (Vick et al., 2007; Vick et al., 2008). This may be due to the limited range of BCS of the horses in this study. Significant correlations between obesity and TNFα may be more detectible in a population including horses well above and below a BCS of five.

Lack of Translation from Fatty Acid Incorporation to Cytokine Production

A companion study reported incorporation of n-3 fatty acids into plasma, red blood cells (RBC) and muscle cells in the same twenty one mares of this study mirroring different treatments (Rexford, 2010). FISH treated horses showed an increase in the level of EPA and DHA over time within both the plasma and red blood cells and there was no trace of either long chain n-3 in the other treatment groups. Muscle cell EPA and DHA

incorporation was also shown to be higher in the FISH treated horses compared to FLAX and CON. The results from this companion study indicate a treatment effect on the type of fatty acids incorporated into the plasma and membranes of cells. In addition, plasma ARA was higher in the FISH treated horses. This indicates limited conversion of ARA due to EPA out competing as a substrate for COX and LOX enzymes in the eicosanoid pathway as consistent with human and horse studies (Hall et al., 2004). Incorporation of EPA and DHA into plasma and cells has been shown to decrease n-6 derived eicosanoids and TNFα production in humans, mice, apes and horses (Caughey et al., 1996; Hall et al., 2004; Sierra et al., 2008; Vineyard et al., 2005; Wu et al., 1996). Despite its incorporation in this study, dietary EPA and DHA did not produce a significant decrease in circulating levels TNFα compared to control.

Inflammatory Stimulation may be Required for a Treatment Effect

Studies investigating inflammation in horses differ from this study because they included a source of inflammation stimulation in vivo or in vitro prior to determining cytokine levels. There are limited examples of dietary n-3 PUFA influencing systemic inflammatory markers in horses via a natural inflammatory condition. Arthritic horses showed a decrease in plasma PGE_2 when provided a supplement containing EPA and DHA (Manhart et al., 2009). However, exercising yearlings receiving various forms PUFA failed to demonstrate a treatment effect on plasma PGE_2 (Ross et al., 2005). Studies have indicated that only certain forms of exercise produce an inflammatory response (Lamprecht et al., 2005). This indicates that providing a naturally occurring inflammatory response in vivo is complex. Further research will indicate which

conditions and stresses best provide a natural systemic inflammation response for studying treatment effects.

Lipopolysaccharide administration is a common artificial inflammation stimulant. When this stimulant is used in vivo, horses have shown a major up regulation in cytokine levels including TNFα compared to baseline and control horses receiving saline injections (Vick et al., 2008). In vitro studies have also shown the necessity to administer LPS with inflammatory cells in order to find detectable differences in the level of cytokines (Munsterman et al., 2005). This same study was then able to demonstrate the effect of PUFA on PGE_2 concentrations (Munsterman et al., 2005). When investigating the effect of PUFA, changes in the level of response of inflammatory cells may only be detectable when cells are stimulated. Therefore, an effect of dietary PUFA supplementation on the concentration of inflammatory markers in a healthy horse may be detectible without some form of inflammatory stimulation.

It should be noted that there was great variation in the level of TNFα between horses throughout this study. This high variance may have limited the power for detecting differences between treatment groups so only a slight reduction of TNFα concentrations in FISH treated horses compared to FLAX treated horses was shown. A larger sample size may improve statistical power of studies measuring inflammatory markers in mares of mixed breeds. When funding is limited, variance may be controlled by screening a group of horses prior to supplementation to reduce the range of pre-existing inflammatory conditions in the study population.

Week Effect due to Environment and Reproductive Factors

Other equine studies have reported significant difference over time in inflammatory markers because of initial acclimation to the environment or long term acclimation to an exercise protocol (Manhart et al., 2009; Ross et al., 2005). The continued weekly variation in this study indicates an unknown outside factor may have been contributing to changes in TNFα levels. The horses in this study were kept in uncovered pens and subject to Colorado's spring climate. Several cold storms with high winds produced a low temperature average and high precipitation during April of 2009 (Table 4.4). A study applying cold stress on mice provided evidence of altered immune cell function and cytokine production (Sesti-Costa et al., 2010). The cold and wet weather conditions in April may have provided enough stress to the animals to contribute to a systemic spike in TNFα levels at week 8 that was then resolved by week 12 when warmer temperatures and very little precipitation no longer caused high stress on these mares.

An important difference between this study and others previously investigating the effect of PUFA on inflammatory markers in horses is the exclusion of geldings or stallions. The stage of a mare's reproductive cycle may have an effect on the level of inflammatory markers. It is believed that luteal function is regulated by immune cells and their products, cytokines (Pate and Landis Keyes, 2001; Penny et al., 1999). Previous research in mares has demonstrated how changes in IL-1β take place during oocyte development and ovulation (Martoriati et al., 2003a; Martoriati et al., 2003b; Martoriati and Gerard, 2003). Similarly in cattle, research indicates that corpus luteum development and estrous cycle length is dependent on the level of TNFα (Korzekwa et al., 2008; Skarzynski et al., 2003). Reproductive parameters were not evaluated during this study.

However, research linking cytokine levels with the reproductive cycles of female mammals may also indicate an explanation for the week variation of circulating levels of TNFα in a group of mares.

Insulin Resistance and TNFα

The relationship between insulin resistance and circulating levels of TNFα was inconclusive and practically nonexistent in this study. This conflicts with other studies relating TNFα with the development of insulin resistance (Hotamisligil et al., 1993; Kushibiki et al., 2001). In addition, research indicates that the relationship between inflammation and insulin resistance is due to their links with obesity. A study in obese mice lacking TNFα function demonstrated increased insulin sensitivity (Uysal et al., 1997). Regression models in horses indicate an influence of TNFα mRNA and protein on insulin sensitivity when variables representing obesity (BCS or %fat) are included (Vick et al., 2007). The relationship of inflammation and insulin sensitivity seems to be dependent on obesity. Strong correlations between minimal model insulin resistance markers (SI and AIRg) and BCS in this study provide further evidence that obesity is linked with increased insulin resistance (Table 4.2). The weak association between TNFα and BCS may explain why correlations between inflammation and the insulin resistance markers were not found.

Conclusion

Age had a positive relationship with TNFα concentrations reinforcing the notion of inflamm-aging. A lack of correlation between inflammation levels and insulin

resistance parameters or obesity markers along with weekly variation may be due to a more prominent effect of an outside factor such as reproductive cycle on systemic inflammation levels in mares. Future inflammation research in mares should include reproductive records. In addition, research is needed in horses to determine if and at what level do environmental stresses due to climate influence the immune system. Previous research has shown an effect of n-3 PUFA supplementation on inflammatory concentrations not found in this study. Therefore, the effect of n-3 PUFA was unclear in this study which may be because stimulation of the immune system is needed in order to detect changes in inflammation.

FIGURES AND TABLES

Figure 1.1: Summary of n-6 and n-3 PUFA conversion

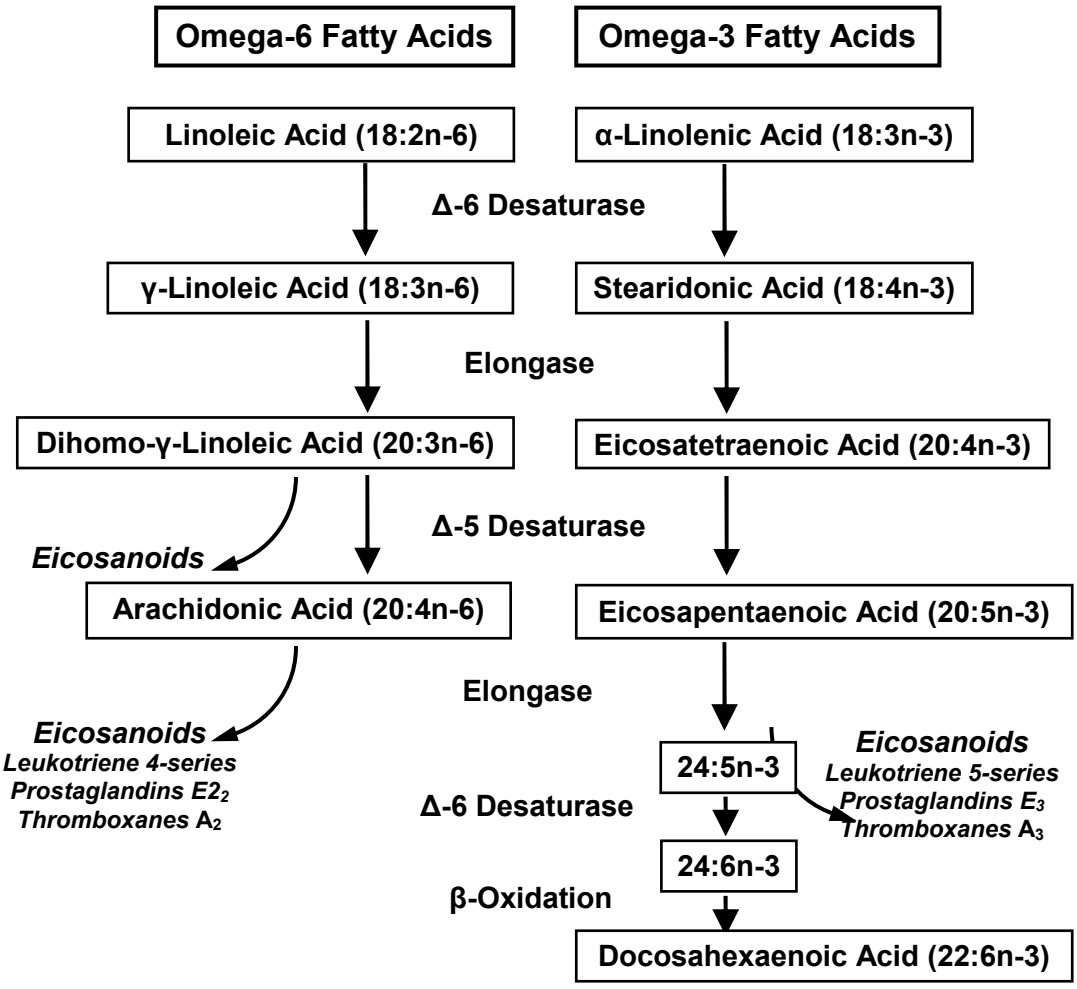
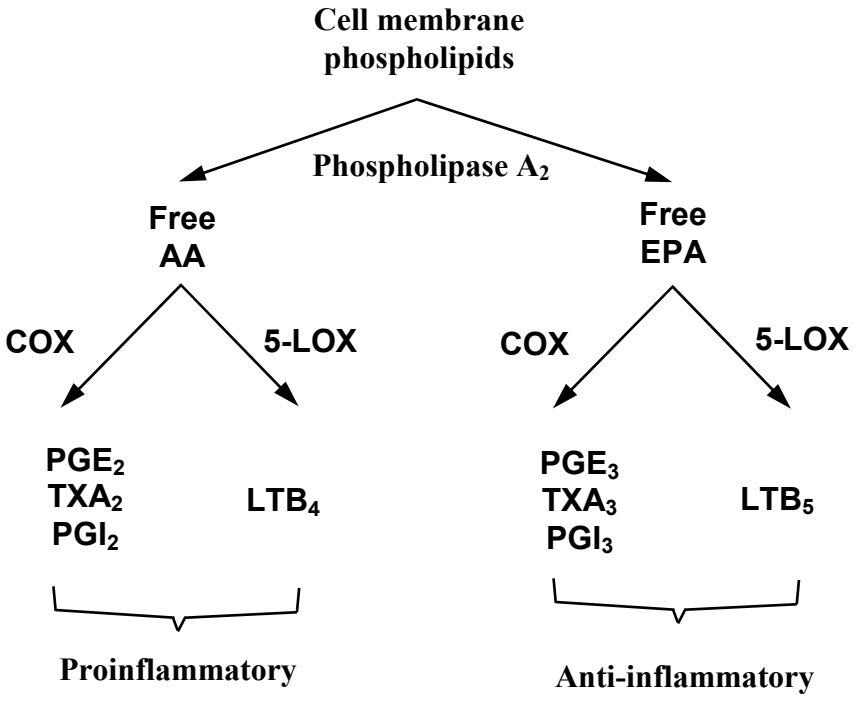


Image obtained from Oregon State University

Figure 1.2: Summary of n-6 and n-3 PUFA eicosanoid production



Modified from cited literature (Fetterman and Zdanowicz, 2009)

Table 2.1: Treatment Group Means and Ranges for Age, Body Condition Score (BCS) and Weight at Baseline

	Age		BCS (1-9)		Weight (kg)	
TRT	**Mean**	**Range**	**Mean**	**Range**	**Mean**	**Range**
FISH	9 ± 2.2	7 – 12	6.8 ± 0.79	5.0 – 8.0	595.5 ± 48.0	538.6 – 665.9
FLAX	9 ± 1.8	5 – 14	6.9 ± 0.79	6.0 – 7.5	573.7 ± 36.3	525.0 – 631.8
CON	9 ± 1.6	7 – 12	6.5 ± 0.65	5.5 – 7.5	588.0 ± 42.8	536.4 – 672.7

Table 2.2: Nutritional Analysis of Diets

Item	Hay	Barley	Magnitude™	Nutra-Flax™
DM %	91.20	89.98	95.30	93.20
DE (Mcal/kg)	1.07	3.65	2.02	2.23
Crude Protein %	20.60	12.65	12.30	21.80
ADF %	33.40	7.65	9.00	17.10
NDF %	42.80	19.27	14.90	25.20
Fat %	1.40	2.59	22.50	39.00
WSC %	7.70	7.05	5.40	3.80
ESC %	5.20	2.14	3.80	1.30
Starch %	4.20	54.62	39.20	4.60
Non Fiber Carb %	25.40	64.42	45.70	23.50

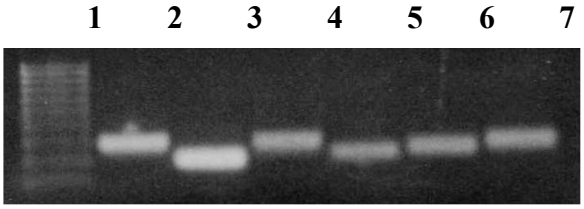
Table 2.3: Polyunsaturated Fatty Acid Composition of Diets as a % of Total Fat

Fatty Acid	Hay	Barley	Magnitude™	Nutra-Flax™
C18:2n-6	16.98	53.60	5.74	15.69
C18:3n-3	35.92	4.25	2.18	56.52
C20:4n-6	-	-	-	-
C20:5n-3	-	-	7.94	-
C22:5n-3	-	-	1.72	-
C22:6n-3	-	-	27.64	-

Table 3.1: Target and Housekeeping Gene Primer Sequences and Gene Segment Sizes

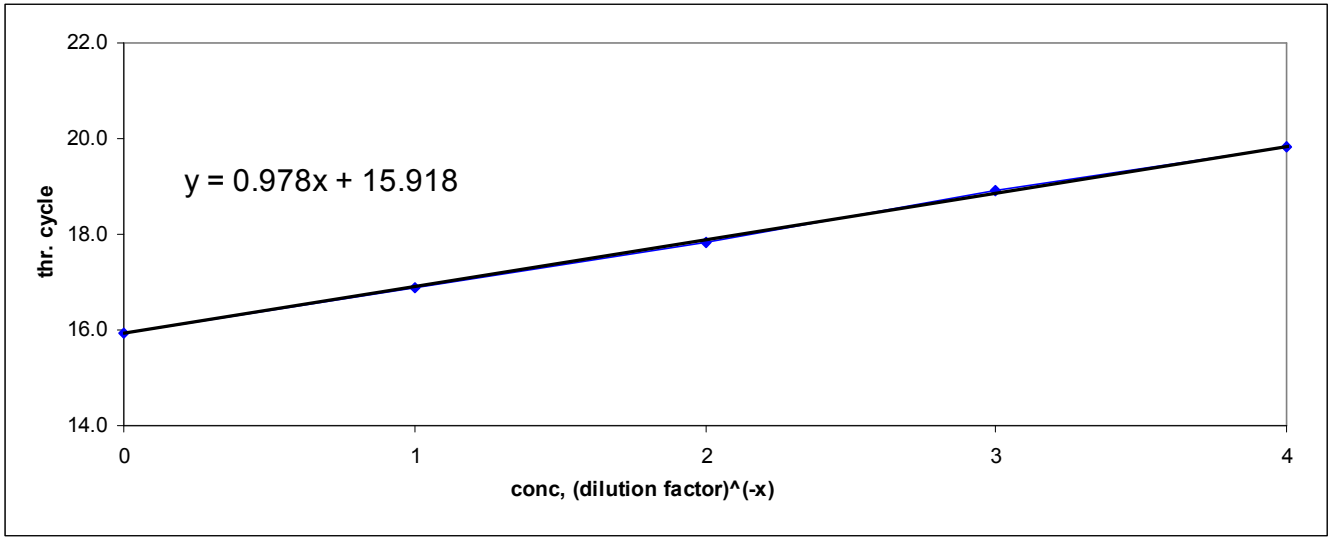
Type	Gene	Forward Primer	Reverse Primer	Size (bp)
Target	**TNFα**	GATCATCTTCTCGARACCCCA	TTGATGGCAGAGAGGAGGTT	289
	IL-1	TCGTGTCARATCATTGTGGCT	ACAGCACCAGGGATTTATGG	216
	IL-6	ARATCACCACTGGTCTTTCGG	GCTTCGARAGGATGAGGTGAG	247
	IL-10	GAGARACAGCTGCACCCACTT	CTCACTCGGAGGGTCTTCAG	287
House Keeping	**18S**	GTGGAGCGATTTGTCTGGTT	CGCTGAGCCAGTCAGTGTAG	200
	GAPDH	CGATGCTGGTGCTGARATATG	GTCTTCTGGGTGGCAGTGAT	297
Redesigned	**IL-6**	CCCCACCCCACTACCCCTGG	GGCAGGTCTCCTGATTGARACCCAGA	248
	IL-10	GCTCCATGACCTCCGAGCCG	CCCCCAGGGAGTTCAGGTGCT	221

Figure 3.1: Housekeeping and Target Gene PCR Products



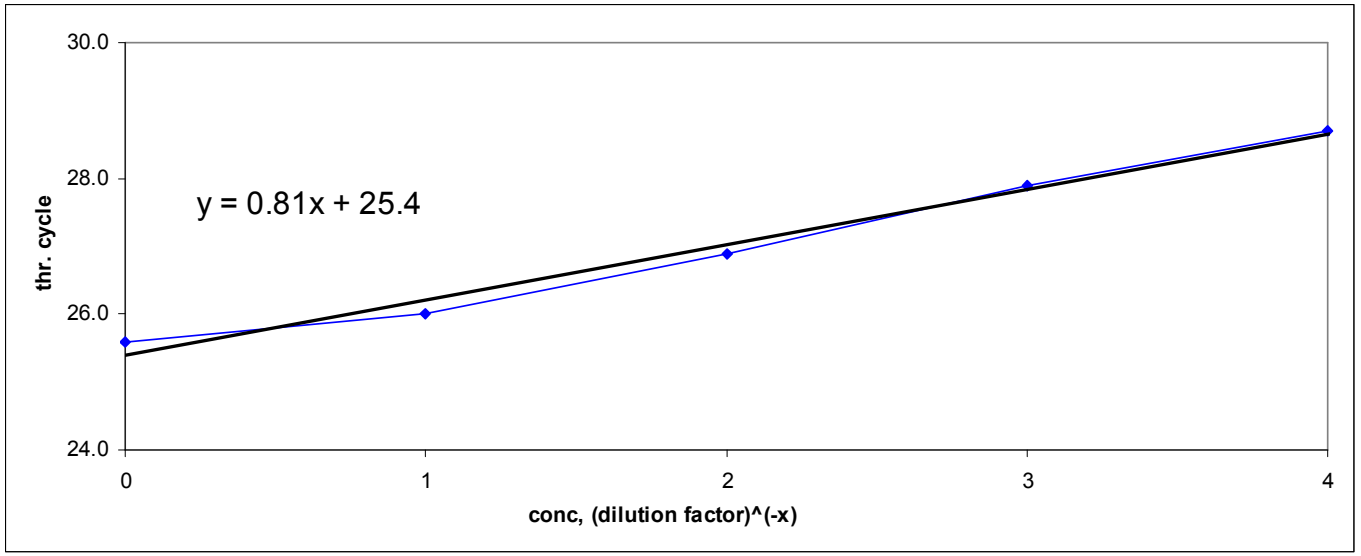
Lane 1, 100bp Ladder; lane 2, GAPDH; lane 3, 18S; lane 4, TNFα; lane 5, IL-1; lane 6, IL-6; lane 7, IL-10.

Figure 3.2: 18S Primer Efficiency with Trend Line



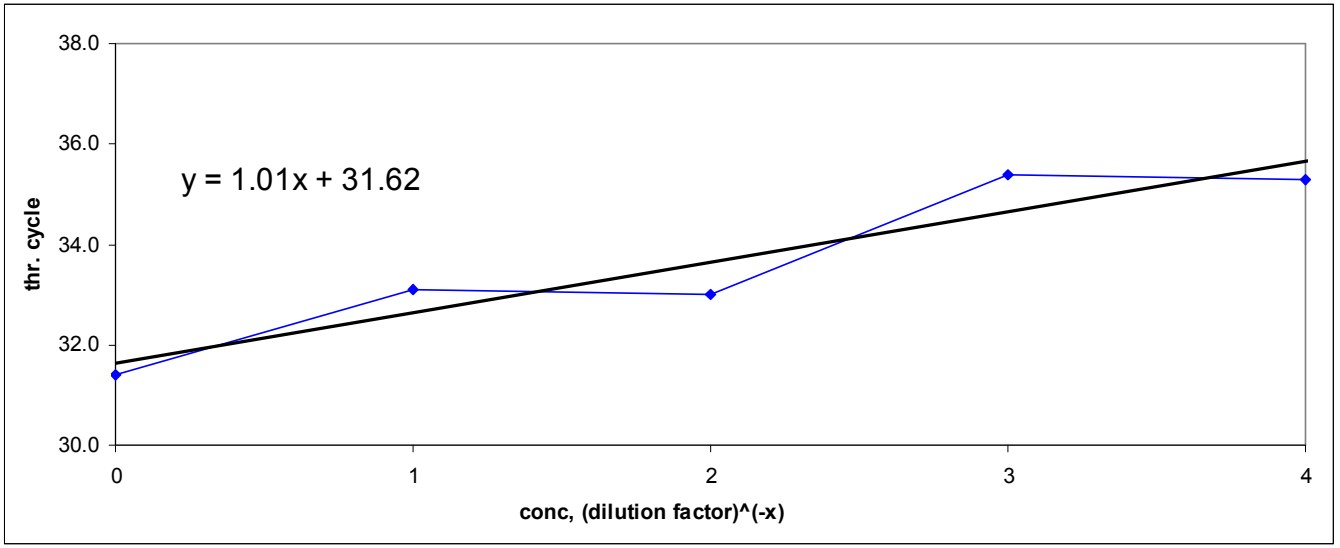
Trendline and equation (black),

Figure 3.3: GAPDH Primer Efficiency with Trend Line



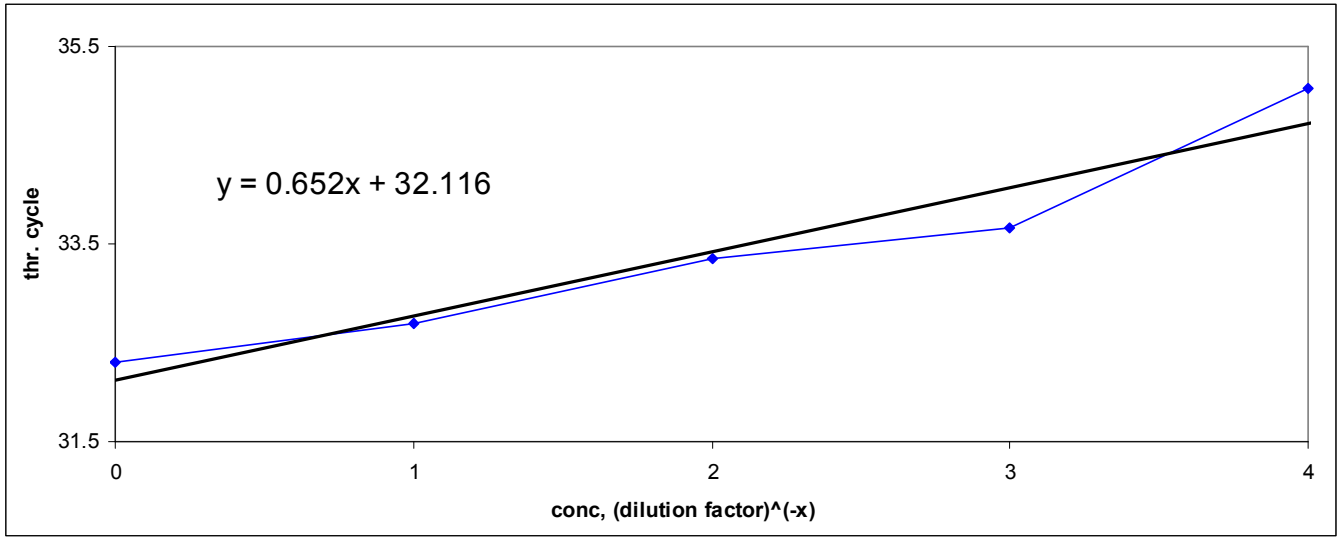
Trendline and equation (black),

Figure 3.4: TNFα Primer Efficiency with Trend Line



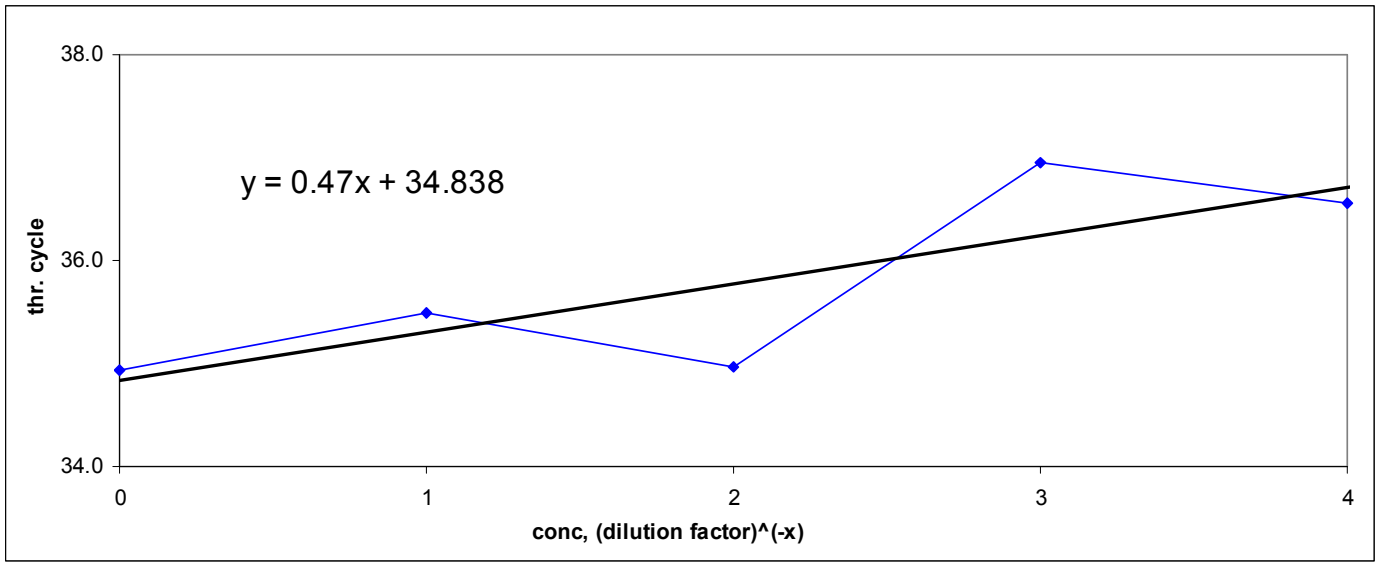
Trendline and equation (black),

Figure 3.5: IL-1 Primer Efficiency with Trend Line



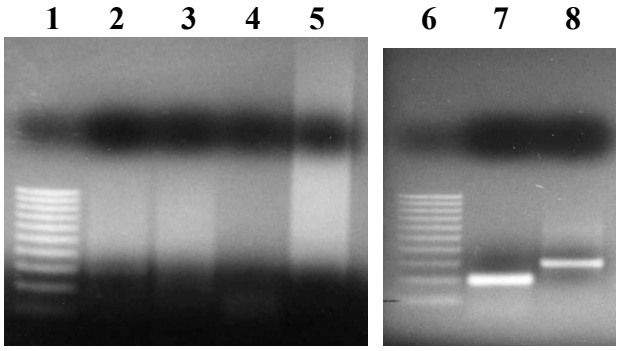
Trendline and equation (black),

Figure 3.6: IL-10 Primer Efficiency with Trend Line



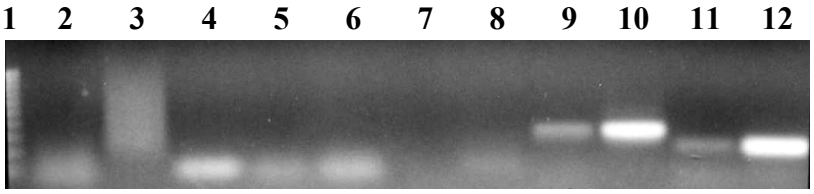
Trendline and equation (black),

Figure 3.7: Electrophoresis of product from each primer set



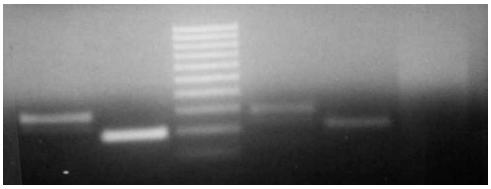
PCR products from reactions showing melting peaks inconsistent with initial primer sequencing. Lane 1 and 6, 100 bp ladder; lane 2, TNFα; lane 3, IL-1; lane 4, IL-6; land 5, IL-10; lane 7, 18S; lane 8, GAP.

Figure 3.8: Electrophoresis of primer products on cDNA samples or nuclease-free water



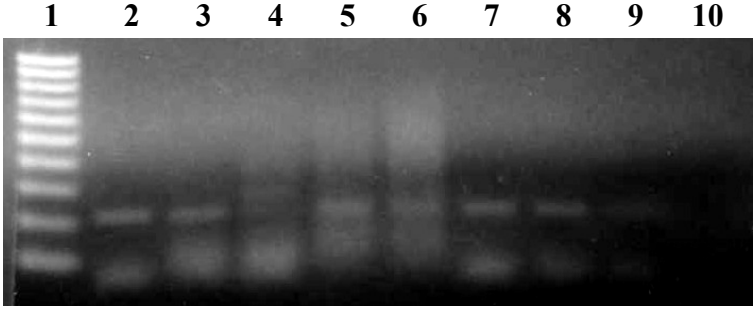
PCR product of 25 µL reactions with or without cDNA. Lane 1, 100 bp ladder; lane 2, IL-10 (H_20); lane 3, IL-10 (cDNA); lane 4, IL-6 (cDNA); lane 5, IL-1 (H_20); lane 6, IL-1 (cDNA); lane 7, TNFα (H_20); lane 8, TNFα (cDNA); lane 9, GAP (H_20); lane 10, GAP (cDNA); lane 11, 18S (H_20); lane 12, 18S (cDNA).

Figure 3.9: Electrophoresis of primer set products



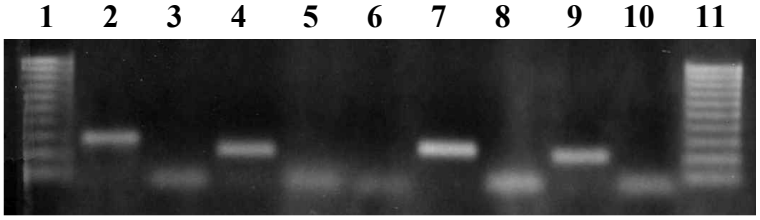
PCR reactions at 65°C for 45 cycles. Lane 1, GAP; lane 2, 18S; lane 3, 100 bp ladder; lane 4, TNFα; lane 5, IL-1; lane 6, IL-10.

Figure 3.10: Temperature gradient (56°C to 70°C) on primer set IL-1



Aliquots of cDNA run in a 25 µL reaction at nine different temperatures to determine ideal PCR temperature for clean amplification. Lane 1, 100 bp ladder; lane 2, 56.9°C; lane 3, 57.2°C; lane 4, 58.1°C; lane 5, 59.6°C; lane 6, 61.4°C; lane 7, 63.5°C; lane 8, 65.7°C; lane 9, 67.9°C; lane 10, 69.9°C.

Figure 3.11: Selective reactions from final whole plate runs separated by melting peaks



Lane 1 and 11, 100 bp ladder; lane 2, TNF (89°C); lane 3, TNF (80°C); lane 4, IL-1 (84°C); lane 5, IL-1 (76°C /78°C); lane 6, IL-1 (75°C); lane 7, IL-6 (84°C); lane 8, IL-6 (83°C); lane 9, IL-10 (87°C); lane 10, IL-10 (82°C).

Figure 4.1: Log Transformed Mean Concentrations of TNFα Over Time

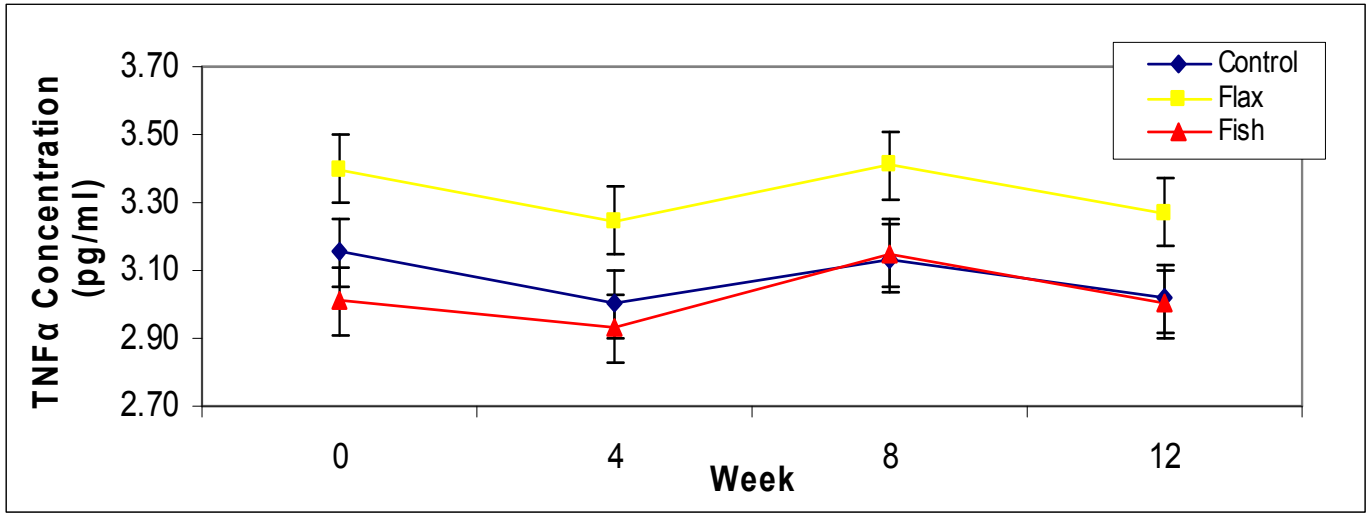


Table 4.1: Log Transformed TNFp Means Unadjusted or Adjusted by Baseline (Week 0)

			TRT				P <	
	TIME	**Overall**	**FISH**	**FLAX**	**CON**	**SEM**	**Trt**	**Trt*Time**
Unadjusted Means	**Overall**	-	2.724	3.099	2.917	0.166	-	-
	W0	2.992	2.794	3.137	3.046	0.177	-	-
	W4	2.796	2.473	3.057	2.858	0.177	-	-
	W8	3.062	2.977	3.184	3.026	0.177	-	-
	W12	2.803	2.651	3.020	2.739	0.177	-	-
Adjusted Means	**Overall**	-	2.882	2.954	2.825	0.105	0.678	0.009
	W 4	2.796_{a}	2.655_{a}	2.924_{ab}	2.808_{a}	0.116	-	0.478
	W 8	3.062_{b}	3.159_{b}	3.051_{a}	2.977_{b}	0.116	-	0.647
	W 12	2.803_{a}	2.833_{a}	2.888_{b}	2.689_{a}	0.116	-	0.749
P <	**Time**	0.001	-	-	-	-	-	-
	Trt*Time	0.009	0.091	0.723	0.253	-	-	-

[a, b] Values within columns lacking common superscripts differ by $P \leq 0.05$

Figure 4.2: Baseline Adjusted Mean Concentration of TNFα Over Time

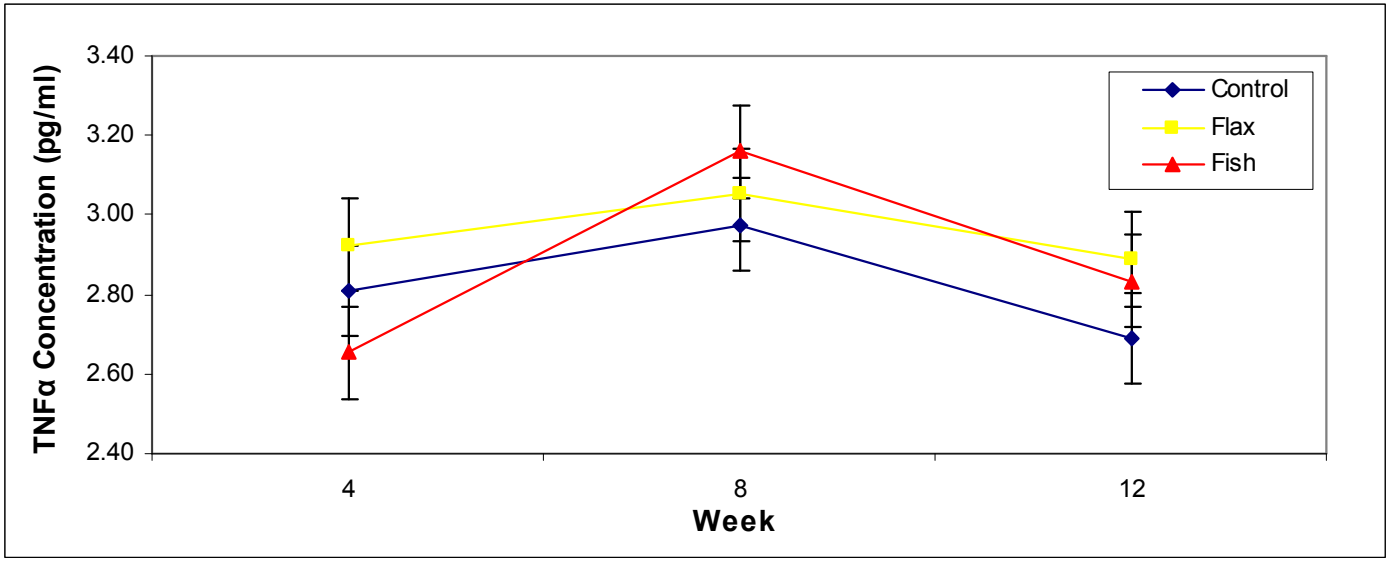
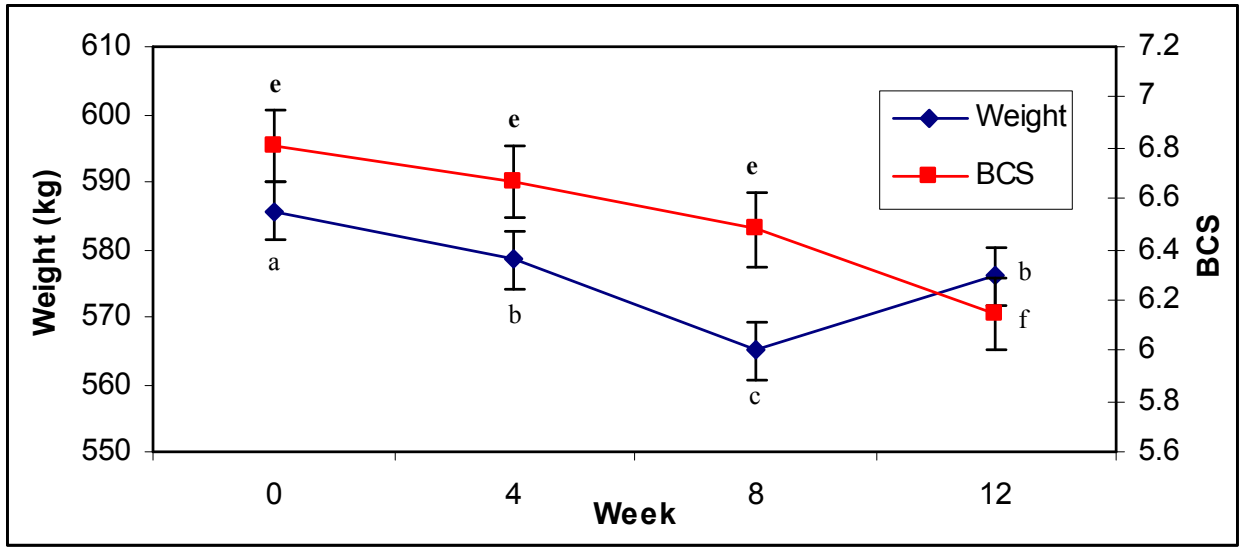


Table 4.2: Pearson Correlation Coefficients for Individual Pairs of Items Measured

Item	Age	BCS	Weight	SI	AIRg	RISQI	MIRG	IR
TNFα, pg/mL[1]	0.235**	-0.193*	-0.288**	NS	NS	NS	NS	NS
Age, year		0.337***	0.268**	-0.195*	NS	0.230**	NS	NS
BCS			0.667***	-0.381***	0.313***	NS	NS	0.219**
Weight, kg				NS	NS	NS	NS	NS
SI					-0.391***	-0.240**	0.572***	0.295***
AIRg						NS	-0.319***	-0.489***
RISQI							NS	0.188*
MIRG								-0.190*

[1]Log transformed data used for correlations
*P < 0.10, **P < 0.05, ***P < 0.01

Figure 4.3: Mean Weight and BCS by Week Across All Treatments



[a, b, c] Weight points lacking common letters differ by $P \leq 0.05$
[e, f] BCS points lacking common letters differ by $P \leq 0.05$

Table 4.3: Mean BCS, Weight Across All Treatments

WEEK	BCS	Weight (kg)
0	6.74 ± 0.85^{a}	585.7 ± 41.51^{a}
4	6.74 ± 0.75^{a}	578.5 ± 36.38^{b}
8	6.57 ± 0.78^{a}	565.0 ± 36.59^{c}
12	6.38 ± 0.82^{b}	576.1 ± 36.81^{b}

[a, b, c] Values within columns lacking common superscripts differ by $P \leq 0.05$

Table 4.4: Climate Values During the Month Prior to Each Sampling

Month (Week)	Mean Temperature	Total Precipitation	Total Snowfall
March (4)	42°F	.93”	9.2”
April (8)	46°F	4.44”	8.1”
May (12)	59°F	2.24”	0”

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