

EFFECT OF TYPE AND AMOUNT OF OMEGA-3 FATTY ACIDS IN THE DIETS OF
EXERCISING HORSES

By

Alaina Parsons

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Animal Science

2011

ABSTRACT

EFFECTS OF TYPE AND AMOUNT OF OMEGA-3 FATTY ACIDS IN THE DIETS OF EXERCISING HORSES

By

Alaina Michelle Parsons

The objective of this study was to determine the effect of the supplementation of differing sources of dietary long-chain polyunsaturated omega-3 fatty acids on health and performance parameters of exercising horses. Twenty-four horses were used in a repeated 3 x 3 Latin square design with 21-d periods. During each period each horse received one of three diets that differed in omega-3 fatty acid profiles: a soybean oil source (SOY), a flaxseed oil source (FLAX), or an algae source (ALG). Horses were exercised 5 d/wk for a minimum of 20 min, with body weights and body condition scores recorded every 7 d, while blood samples, heart rates, and stride lengths were measured on days 0, 21, 42, and 63. Blood samples were analyzed for various inflammatory markers and for a complete plasma fatty acid profile. Heart rates were recorded during a 30-min exercise bout and stride lengths were measured at the walk and trot before and after exercise. No treatment differences were observed for stride lengths, heart rates, total plasma omega-3, omega-6, and docosahexaenoic acid concentrations, or several inflammatory markers measured in plasma and serum. Plasma eicosapentaenoic acid concentrations were higher in horses on the SOY diet (0.45 ± 0.07 percent of total plasma lipid) than horses on FLAX and ALG ($P < 0.05$) and plasma α -linolenic acid was altered in response to treatment ($P < 0.05$) with horses on FLAX having the greatest percentage. Serum interleukin-10 concentrations were lower in SOY than in ALG ($P < 0.05$). The results of this study indicate that mature exercising horses free from osteoarthritis do not exhibit physiological anti-inflammatory benefits when supplemented with a low dose of omega-3 fatty acids over a 21-d period.

ACKNOWLEDGEMENTS

I would like to acknowledge and thank the members of my committee (Dr. Nielsen, Dr. Orth, Dr. Geor, and Dr. Schott) for their support of this project. The guidance and knowledge that they have supplied has truly enhanced my learning experience here. It has been such a great opportunity to work with a group as talented as this. I would also like to thank Roy Hall and Cooperative Research Farms for their support of this research, as well as several faculty members at North Carolina State University for their encouragement to attend graduate school and their support throughout this experience.

This project involved the assistance of so many individuals. Foremost, I would like to thank Cara Robison for pretty much rocking my world. Her support and contributions in all stages of this project were unparalleled, and her encouragement in times of frustration was unmatched (and she makes a pretty mean “Mussolini” pasta!). Credit is certainly due to all of the undergraduates who made it possible to exercise 120 horses a week, as well as other staff members who lent support in this project by helping me learn new techniques and invade their lab space, especially Jim Liesman, Dave Main, Justin Zyskowski, and Courtney Preseault.

I know that everyone always thanks their fellow grad students, but the MSU grads truly hold a special place in my heart. Their unwavering support, awe-inspiring volleyball skills, and the ever-encouraging “awkward hallway wave” have truly made my time here at MSU the great experience that it has been. I would like to especially thank Landon Cross, Sarah Elzinga, and Becca Rockwell for helping me keep my head on straight and for their constant support, love, and encouragement. I’m pretty sure that all of our adventures, particularly all of the pool-side dates, were the primary contributing factor to the retention of my sanity during the past year.

And to my roommates, Keri Jewell and Katie Murtha: thank you for not kicking me out of the apartment during this process. Thank you for putting up with my crazy hours and scatter-brained tendencies, but most importantly, thank you for your love and support in all the ups and downs and crazy antics that we have endured together over the past two years.

And finally I would like to thank my family (mom, pops, Ben, and Ryan) for their incredible love and support. Even though I know that they hate the fact that I live so far away, their daily encouragement has always been clearly felt. I have been truly blessed to have had this opportunity, and I am so grateful for the experiences, friendships, and skills that I have acquired here.

TABLE OF CONTENTS

LIST OF TABLES	viii
LIST OF FIGURES	ix
CHAPTER 1	
INTRODUCTION	1
CHAPTER 2	
REVIEW OF THE LITERATURE	2
Introduction	2
Polyunsaturated Fatty Acids.....	3
Classifications.....	3
Metabolism and Dietary Sources	3
The Omega-6 to Omega-3 Ratio.	6
Omega-3 Fatty Acids in Phospholipids.....	7
Phospholipid Incorporation	7
Cell Signaling	8
Metabolism.	8
Inflammatory and Immune Responses and PUFA.....	9
Introduction.....	9
Immune and Inflammatory Responses	9
Eicosanoids	10
Biological Effects.....	14
Metabolic Effects.....	14
Arthritis.....	15
Exercise.....	15
Insulin	15
Lipid in Equine Diets	16
Omega-3 Supplementation and Metabolism	16
Equine Lipid Profiles.	16
Biological Effects	18
Inflammation and Omega-3 Supplementation in Horses	19
Immunity and Inflammatory Responses	19
Arthritis and Exercise	21
DHA-Rich Microalgae	22
Conclusions	23
CHAPTER 3	
MATERIALS AND METHODS.....	25
Animal Management	25
Lameness Evaluations	25
Experimental Design and Diets	26

Treatments	27
Exercise	28
Horsemanship Classes	28
Training Classes.....	28
Lunge Line Exercise	29
Measurements and Sample Collection	29
Blood Collection and Handling	29
Stride Length Measurement.....	30
Heart Rate Measurement	30
Laboratory Analyses	31
Analysis of Fatty Acids in Feeds and Plasma.....	31
Prostaglandin E ₂	32
Tumor Necrosis Factor-alpha	32
Interleukin 10.....	33
Interferon-gamma	33
Statistical Analyses	33
 CHAPTER 4	
RESULTS	39
Body Weight	39
Stride Lengths	39
Heart Rates	40
Plasma Fatty Acid Profiles.....	42
Total Protein and Fibrinogen.....	43
Prostaglandin E ₂	44
Tumor Necrosis Factor- α	44
Interleukin 10	45
Interferon- γ	45
 CHAPTER 5	
DISCUSSION	50
 CHAPTER 6	
SUMMARY AND CONCLUSIONS	63
 APPENDIX A.....	67
FAMES Methylation Procedure.....	67
 APPENDIX B	69
Prostaglandin E ₂ ELISA Procedure	69
 APPENDIX C	71
Tumor Necrosis Factor-alpha ELISA Procedure	71
 APPENDIX D.....	73

Interleukin 10 ELISA Procedure	73
APPENDIX E	75
Interferon-gamma ELISA Procedure	75
APPENDIX F.....	77
Tumor Necrosis Factor-alpha Assay Reagent Preparation:	77
APPENDIX G.....	79
Equine Interleukin 10 Assay and Equine Interferon-gamma Assay Reagent Preparation:..	79
LITERATURE CITED	82

LIST OF TABLES

Table 3.1.	Latin square design used for the experiment, where ALG denotes algae-based supplement, FLAX denotes flaxseed oil-based supplement, and SOY denotes soybean oil-based supplement. Each block of three horses was randomly assigned to either Group 1 or Group 2 and fed in the supplement order provided below.	35
Table 3.2.	Block, group, classification, age, sex, and osteoarthritis score for each horse on trial.	36
Table 3.3.	Delivery (g/d) of n-3, n-6, α -linolenic acid (LNA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) as a protected fatty acid supplement volume of 908 g/d as fed.	37
Table 3.4.	Selected fatty acids supplied by each feedstuff.	38
Table 4.1.	Body weight (kg) and body condition score (BCS) for all horses within treatment diets.	46
Table 4.2.	Mean walk stride length (cm) and trot stride length (cm) of horses. Stride lengths were measured before and after a 20-min specified exercise on the lunge line to determine a baseline at d 0 and following 21-d supplementation periods for all treatments.	46
Table 4.3.	Mean heart rate (HR) measurements at baseline (d 0) and following 21-d supplementation periods for all treatments. Heart rates are provided at rest, the maximum HR during a 20-min specified lunge line exercise, and specific heart rates post-exercise during a 10-min recovery period: immediately post-exercise (HR 0), 1 min post-exercise (HR 1), 2.5 min post-exercise (HR 2.5), 5 min post-exercise (HR 5), and 10 min post-exercise (HR 10).	47
Table 4.4.	Effect of feeding horses diets supplemented with three differing sources and content of n-3 fatty acids on plasma fatty acid profiles after 21-d supplementation periods and at baseline values (d 0).	48
Table 5.1.	Comparison of n-3 supplementation sources, amounts of fatty acids, and length of supplementation (d) across equine studies.	51

LIST OF FIGURES

Figure 2.1. Metabolism of n-6 and n-3 fatty acids (Calder, 2001).	5
Figure 2.2. Synthesis of eicosanoids from arachidonic acid and sites of inhibition by eicosapentaenoic acid. ARA, arachidonic acid; COX, cyclo-oxygenase; EPA, eicosapentaenoic acid; HETE, hydroxyeicosatetraenoic acid; HPETE, hydroperoxyeicosatetraenoic acid; LOX, lipoxygenase, LT, leukotriene; PG, prostaglandin, TX, thromboxanes (Calder, 2002).	12
Figure 4.1. The response of hematological variables to n-3 treatments of soybean oil (SOY), flaxseed oil (FLAX), or algal DHA (ALG) in equine plasma.....	49

LIST OF ABBREVIATIONS

Alpha-linolenic acid.....	ALA
Arachidonic acid.....	ARA
Cyclooxygenase.....	COX
DHA-rich microalgae.....	DRM
Docosahexaenoic acid.....	DHA
Docosapentaenoic acid.....	DPA
Eicosapentaenoic acid.....	EPA
Fatty acids.....	FA
Interferon-gamma.....	INF- γ
Interleukin 1.....	IL-1
Interleukin 6.....	IL-6
Interleukin 10.....	IL-10
Linoleic acid.....	LA
Lipopolysaccharide.....	LPS
Lipoxygenase.....	LOX
Omega-3.....	n-3
Omega-6.....	n-6
Polyunsaturated fatty acids.....	PUFA
Prostaglandin E ₂	PGE ₂
Tumor necrosis factor-alpha.....	TNF- α

CHAPTER 1

INTRODUCTION

Recently research in the area of fat supplementation in the diets of horses has been increasing, particularly concerning long-chain polyunsaturated fatty acids, with omega-3 and omega-6 fatty acids of particular interest. Many exercising horses suffer from disease and unsoundness related to joint health, and current research suggests that oral omega-3 fatty acid supplementation may have beneficial anti-inflammatory effects to mediate these issues. However, research has been conducted over varying lengths, amounts, and sources of fatty acid supplementation, with inconsistent physiological responses to treatment. The purpose of the following study is to determine the effect of supplementation of differing sources and amounts of dietary long-chain polyunsaturated omega-3 fatty acids across 21-d periods on health and performance parameters of exercising horses. Greater insight into the biological effects observed at an optimal level and source of omega-3 supplementation could contribute to the development of feed recommendations to mediate inflammatory joint factors that can be associated with lameness among horses and therefore improve their potential for performance. We therefore hypothesize that when supplied with supplemental sources of omega-3 fatty acids, plasma fatty acid concentrations will show a change in relation to the fatty acid sources from each diet after a 21-d period. We also hypothesize that horses supplied omega-3 fatty acids from an algal source, when compared to a soybean oil and flaxseed source, will show the greatest increase in plasma docosahexaenoic acid concentrations as well as increased stride lengths and decreased heart rates during exercise as a result of a decrease of pro-inflammatory cytokines and eicosanoids as measured in the plasma and serum.

CHAPTER 2

REVIEW OF THE LITERATURE

Introduction

Fat is commonly supplemented to both human and animal diets to increase the energy density of the diet. Recently, research concerning individual dietary fatty acids (FA) and their role as biological regulators has increased. These FA, particularly the polyunsaturated fatty acids (PUFA), are important constituents of phospholipids in cell membranes and play a major role in the structure and function of the membrane (Lands, 2005). The FA composition of the membrane also influences the eicosanoids that are produced and used for intercellular signaling. Omega-3 (n-3) PUFA confer several health benefits to animals and humans alike, and therefore have many potential uses in equine nutrition. In other animal species, n-3 supplementation from fish oil sources increases the serum concentration of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in proportion to the amount of fish oil fed (Bjerve et al., 1993; Ashes et al., 1992). Additionally, eicosanoids produced from n-3 FA precursors are less inflammatory than those produced from omega-6 (n-6) FA precursors. This alteration in the type of inflammatory products produced may be useful in treating inflammatory diseases. Omega-3 supplementation can reduce the risk of cardiovascular disease, cancer, chronic airway disease, chronic arthritis, gastrointestinal disease, and other inflammatory and autoimmune diseases in humans (Simopoulos, 2008; Goldberg and Katz, 2007; Knapp, 1995).

Compared to other species, relatively few studies have been conducted regarding the effects of dietary n-3 FA on inflammatory and immune cell functions *in vivo* concerning the horse. This review of published literature will examine the metabolism of n-3 and n-6 FA from

dietary sources and the role of these FA as potential modulators of inflammatory pathways. The effects of supplementation in the equine model will also be reviewed, with a focus on blood lipid profile modifications as well as exercise and inflammatory responses.

Polyunsaturated Fatty Acids

Classifications. Fatty acids are composed of unbranched hydrocarbon chains with an even number of carbons and are classified as short chain (4 carbon atoms), medium chain (6 to 12 carbon atoms), or long chain (14 or more carbon atoms). Depending on the presence and number of double bonds in the aliphatic carbon chain of the molecule, FA can also be classified as saturated (no double bonds), monounsaturated (one double bond), or polyunsaturated (more than one double bond). Polyunsaturated fatty acids are further classified according to the position of the first double bond when counting from the methyl end and consist of two major classes: the n-3 FA, which exhibit a first double bond positioned between the third and fourth carbons, and the n-6 FA, with the first double bond located between the sixth and seventh carbons.

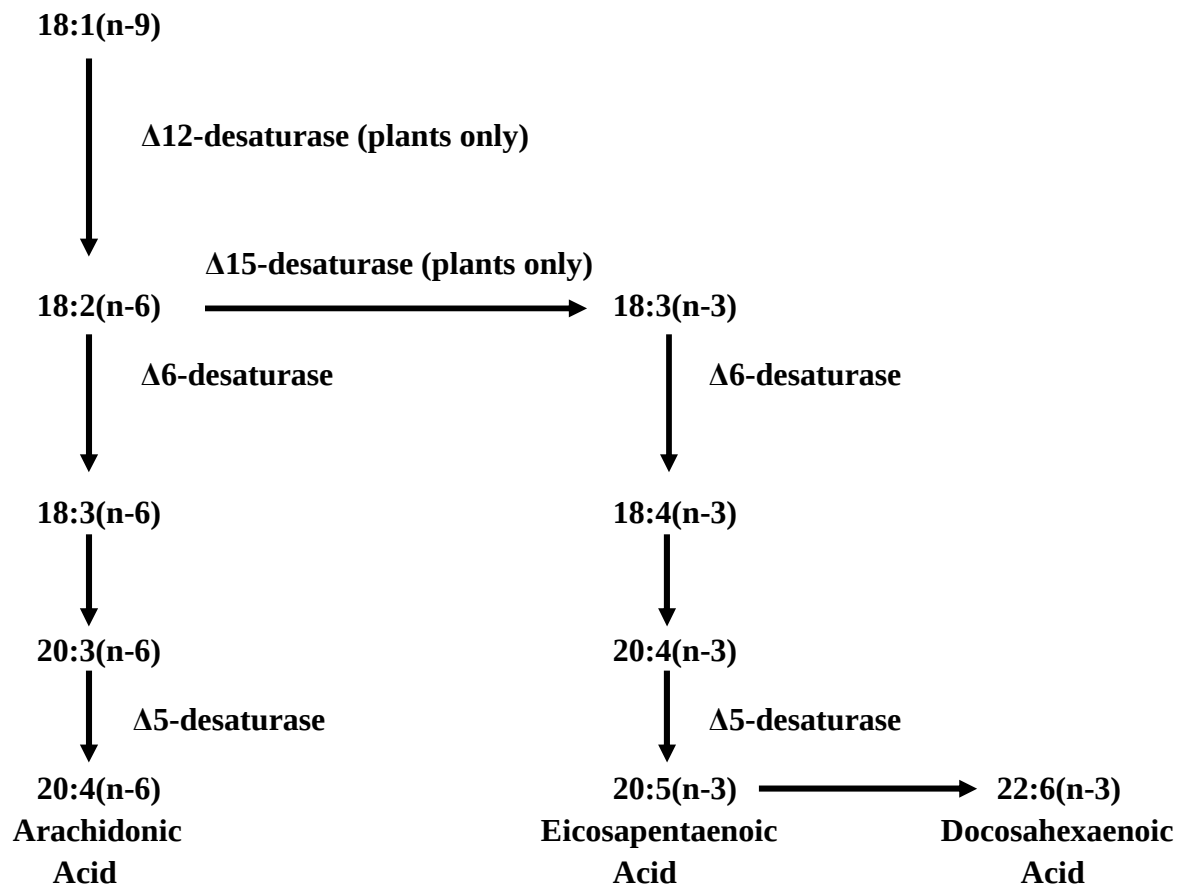
Metabolism and Dietary Sources. All members of the n-3 and n-6 classes of PUFA are derived from their respective precursors. Linoleic acid (LA; 18:2n-6) is desaturated and elongated to yield arachidonic acid (ARA; 20:4n-6) and other long chain PUFAs, while α -linolenic acid (ALA; 18:3n-3) is desaturated and elongated to yield EPA (20:5n-3) and docosapentaenoic acid (DPA; 22:5n-3). Docosapentaenoic acid can be retroconverted to EPA and further metabolized to DHA (20:6n-3). The 20-carbon FA can also be made by retroconversion of longer chain FAs obtained in the diet (Figure 2.1). The precursor molecules

of LA and ALA compete for the same desaturase and elongation enzymes to form the polyunsaturates, principally ARA and DHA. These end products are incorporated into the 2-position of phospholipids via *de novo* synthesis and acylation-deacylation mechanisms (Neuringer et al., 1988).

Within each series of FA, elongation and desaturation occur without altering the methyl end of the molecule. Both LA and ALA are essential FA in mammals, as they lack the desaturases necessary to convert LA from short chain FA precursors and are unable to interconvert n-6 and n-3 FA. These enzymes are found in plants, making some plant tissues and oils rich sources of the 18-carbon FA. Linoleic acid is found in seeds of most plants, except coconut, cocoa, and palm, and is a primary component of maize, sunflower, safflower, and soybean oils, contributing over 50% and often up to 80% of the FA content (Calder, 2001a). Rapeseed, canola, and soybean oils are good sources of ALA, contributing between 5% and 15% of the FA present. Flaxseed oil is the richest source of ALA, contributing as much as 60% of the FA content (Calder, 2001a). Alpha-linolenic acid is also found in the chloroplasts of green leafy vegetables, in animal fat, and in some nuts.

The longer chain members of the n-6 and n-3 families are obtained directly from animal foods of land or marine origin. Fish and other marine animals are a particularly rich source of the long chain PUFA, typically containing approximately 18% EPA and 12% DHA (Cleland et al., 2006). Many marine plants, especially the unicellular algae in phytoplankton, elongate and desaturate ALA to yield EPA and DHA. These PUFA then get transferred through the food chain, accumulating in the tissues of some marine mammals and fish, particularly “oily” fish, such as herring, mackerel, and tuna (Calder, 2001a).

Figure 2.1. Metabolism of n-6 and n-3 fatty acids (Calder, 2001).



While the relationship between ALA intake and EPA incorporation has been shown to be linear, several studies consistently demonstrate that increased consumption of ALA does not result in increased proportions of DHA in plasma or cell lipids (Burdge and Calder, 2005). Although plant sources can theoretically provide EPA and DHA via desaturation and elongation of ALA, this conversion of ALA to long chain PUFA appears to be limited in human subjects (Simopolous 2008). Overall these studies demonstrate that although chronically increased consumption of ALA does result in conversion to EPA, and therefore enhancement of EPA concentration in plasma and cell membranes, and the estimated proportion of ALA entering this pathway and being converted to EPA is low, and lies between 8% and 10% in humans. The extent of conversion of ALA to DHA is less clear, with most studies reporting estimated fractional conversion at <0.05%, an amount insufficient to increase the concentrations of DHA in membranes (Williams and Burdge, 2006).

The Omega-6 to Omega-3 Ratio.

As $\Delta 6$ -desaturation is the rate-limiting step in the bioconversion pathway, high dietary intakes of n-6 FA have been proposed to be a limiting factor in the conversion of ALA to its long-chain polyunsaturates. When increasing the amount of dietary n-3 FA relative to n-6 FA in the diet, n-3 FA are preferentially incorporated into cell membrane phospholipids (Calder, 2001b). Thus modifications of the diet that result in the increase of a particular FA can impact synthesis of another. For example, a high concentration of ALA inhibits conversion of LA because both are substrates for the same $\Delta 6$ -desaturase enzyme. The conversion executed by the $\Delta 5$ -desaturase is also inhibited by ARA, EPA, and DHA (Bezard et al., 1994). Therefore the

ratio of n-6 to n-3 in the diet becomes extremely important, as the relative abundance of these FA in the diet affects the relative intensity of a particular response by tissue (Lands, 2005).

The suggested dietary ratios range from 4 to 10 for humans (Albertazzi and Coupland, 2002; Sugano, 1996). An imbalanced ratio can have adverse consequences, as mammalian cells cannot interconvert n-6 and n-3 FA. Excessive amounts of n-6 promotes the pathogenesis of many diseases, including cardiovascular disease, cancer, and inflammatory and autoimmune diseases, while increased levels of n-3 FA (and hence a lower n-6 to n-3 ratio) exert suppressive effects. Additionally, an imbalance of n-6 and n-3 in the peripheral blood causes an overproduction of pro-inflammatory cytokines (Simopoulos, 2008).

Omega-3 Fatty Acids in Phospholipids

Phospholipid Incorporation. While it is known that differing classes of FA will elicit differing metabolic effects in tissues, they must first be present in the blood to allow for incorporation. When FA are supplemented in the diet, they are incorporated into the cell membrane, with the concentration of PUFA in tissue consequently mimicking the dietary FA when consumed in adequate quantities in a habitual dietary pattern. This is where the chemical differences between n-3 and n-6 fatty acids are important, as their unique chemical structures elicit different biological effects. For example, ALA and EPA are found in triglycerides, cholesteryl esters, and in phospholipids, while DHA is mostly found in phospholipids (Simopoulos, 2008) and is one of the most abundant components of the brain's structural lipids. High concentrations of DHA are also found in the cerebral cortex, retina, testes, and sperm (Brinsko et al., 2005; Harris et al., 2005; Mueller and Talbert, 1988; Neuringer et al., 1988).

Cell Signaling. Once in the tissue, n-6 and n-3 FA form specific lipid-protein membrane complexes that are needed for cellular structures at specific stages of tissue differentiation and development, particularly during brain and retina development (Lands, 2005; Neuringer et al., 1988). Fatty acids released from membrane phospholipids by cellular phospholipases, or those made available to the cell from the diet or other aspects of the extracellular environment, are important signaling molecules. They can act either as secondary messengers or substitute for classic secondary messengers of the inositide phospholipid and the cyclic adenosine monophosphate signal transduction pathways (Simopoulos, 2008). Omega-3 and n-6 FA can also act as precursor molecules by forming hormone-like self-healing autacoids, such as prostaglandins, leukotrienes, and thromboxanes, which signal important adjustments of healthy tissue in response to stimuli (Lands, 2005).

Metabolism. Polyunsaturated fatty acids also have a role in the regulation of hepatic gene expression. As the liver plays a central role in whole body carbohydrate and lipid metabolism, several metabolic pathways may be regulated by PUFA through changes in the activity or abundance of transcription factors that target carbohydrate, FA, cholesterol, and bile acid metabolism. The principle action of n-3 FA on hepatic metabolism involves a shift from lipid synthesis and storage to lipid oxidation. This mechanism may contribute to the regulation of blood levels of triglycerides and cholesterol, which are important risk factors for chronic inflammatory diseases (Jump, 2002). Additionally, long chain PUFA supplementation can affect inflammation and immunity at the level of inflammatory gene transcription, leading to a highly integrated signaling cascade that can be affected in multiple ways by PUFA status, leading to changes in the body's response to tissue damage, pathogen challenge, or disease (Calder, 2001b).

Inflammatory and Immune Responses and PUFA

Introduction. The fatty acid composition of inflammatory and immune cells is sensitive to change according to the ratio of n-6 and n-3 FA in the body, and therefore provides a link between dietary PUFA intake and inflammation. Among all of the FA classes, it is the n-3 PUFA that possess the most potent immunomodulatory activities, with EPA and DHA being more biologically potent than ALA (Calder, 2001b). An increase in EPA results in partial replacement of ARA in cell membranes, which leads to a decreased production of ARA-derived mediators (pro-inflammatory prostaglandins, leukotrienes, and related compounds). Eicosapentaenoic acid is also a substrate that gives rise to mediators that often have different biological actions or potencies than those formed from ARA (Calder, 2001b).

Immune and Inflammatory Responses. Communication within the immune system is brought about by direct cell-to-cell contact involving adhesion molecules and by the production of chemical messengers, chief among these being proteins called cytokines. Cytokines, such as tumor necrosis factor-alpha (TNF- α), interleukin 1 (IL-1), and interleukin 6 (IL-6), act to regulate cellular activity and have multiple activities on different cell types. Cytokines act by binding to specific receptors on the cell surface and induce changes in growth, development, or activity of the target cell. In addition, these cytokines mediate the systemic effects of inflammation. Production of appropriate amounts of TNF- α is clearly beneficial in response to infection, but chronic overproduction can be dangerous, as it is implicated in causing some of the pathological responses that occur in inflammatory conditions. These pro-inflammatory cytokines are antagonized by anti-inflammatory cytokines, such as interleukin 10 (IL-10; Calder, 2001b). Other inflammatory chemical messengers, such as prostaglandin E₂ (PGE₂), have a

number of pro-inflammatory effects, including the induction of fever and erythema and enhancing pain. Prostaglandin E₂ is also immunosuppressive, as it inhibits production of TNF- α , interleukins, and interferon-gamma (INF- γ).

Several animal studies demonstrate that dietary n-3 supplementation may improve immune function. These findings indicate that diets rich in EPA plus DHA modulate aspects of the immune response *in vivo* (Calder, 2001b), as these are the n-3 PUFA that exhibit the most potent anti-inflammatory and antioxidant effects (Bloomer et al., 2009; Simopoulos, 2008). As inflammation forms the foundation for many chronic diseases, n-3 FA supplementation could offer a form of prevention.

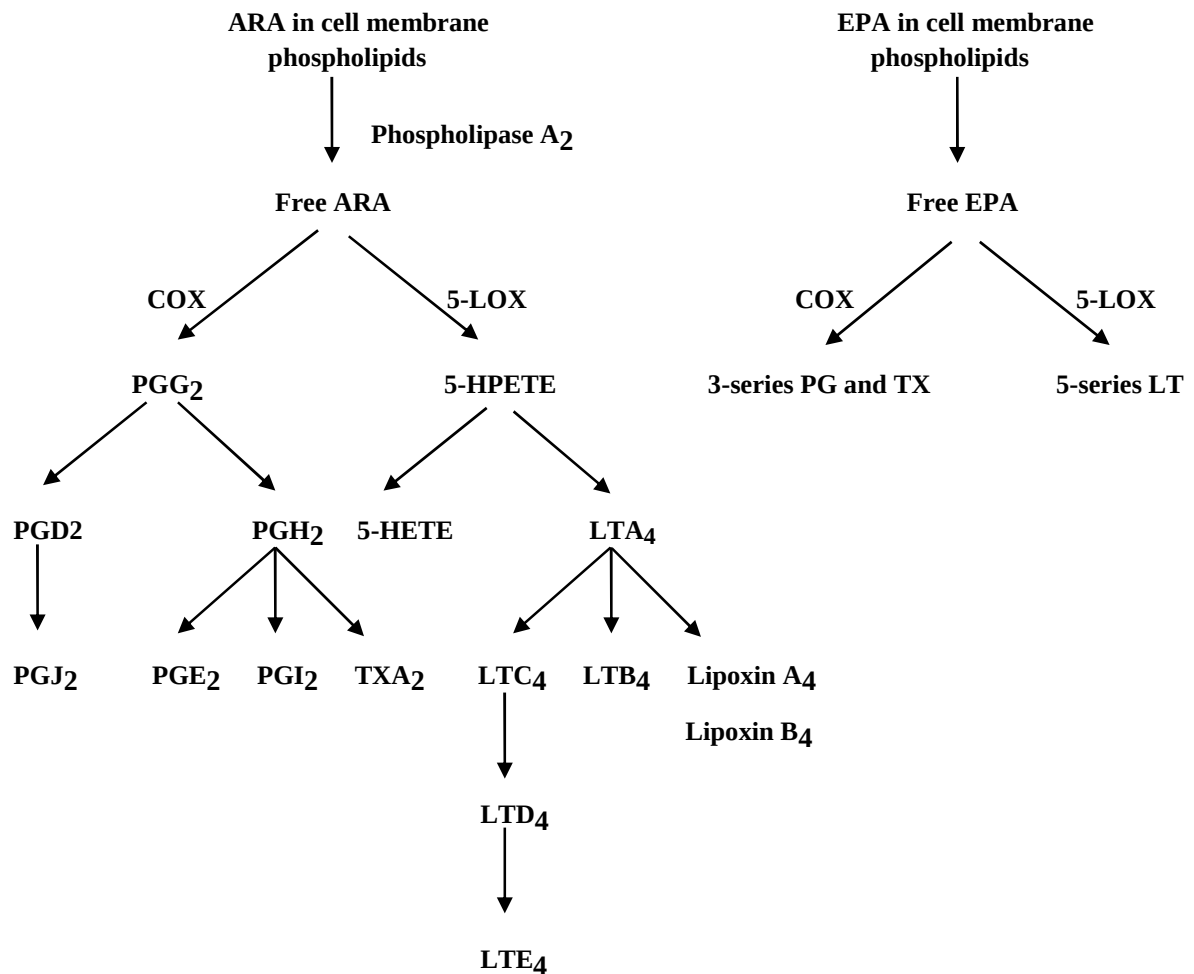
Eicosanoids. Eicosanoids are a secondary group of chemical messengers, synthesized from PUFA, that act within the immune system and are released in response to a cellular stimulus. They exert complex control over many bodily systems, primarily concerning the areas of inflammation and immunity, and provide a link between the PUFA and the inflammatory pathways. Depending on the type of fatty acid substrate cleaved from cell membranes, eicosanoids can facilitate or inhibit inflammatory mediators. Eicosanoids include the prostaglandins, thromboxanes, and leukotrienes. The two essential FA families are converted into two distinct families of eicosanoids, with those produced from n-6 fatty acids considered to be pro-inflammatory, while those produced from n-3 fatty acids have reduced less pro-inflammatory activity. The conversion of tissue ARA controls the signaling of many eicosanoid-mediated pathways, with EPA providing the most important competing substrate. The competition of these fatty acid precursors during metabolism affects the ratios and types of

eicosanoids likely to be formed, and will therefore alter the body's metabolic function (Lands, 2005; Calder, 2001b).

Generally most immune cell membranes contain large amounts of ARA (when compared to EPA), thus ARA is usually the principal precursor for eicosanoid synthesis. Arachidonic acid gives rise to such a range of mediators that have opposing effects to one another that the overall physiological effect will be governed by the concentration of those mediators. Metabolism of ARA via cyclooxygenase (COX) enzymes gives rise to the 2-series prostaglandins and thromboxanes, whereas 4-series leukotrienes are produced from ARA by lipoxygenase (LOX) metabolism. Overproduction of ARA derived eicosanoids has been implicated in many inflammatory and autoimmune disorders (Albertazzi and Coupeland, 2002). As EPA competes with ARA for the active sites on these enzymes, increased phospholipid n-3 FA causes the anti-inflammatory products of n-3 FA metabolism, (3-series prostaglandins and 5-series leukotrienes) to increase, with an analogous decrease in the pro-inflammatory products of n-6 FA (Calder, 2001b).

Biosynthesis of prostaglandins and thromboxanes begins when ARA is released from membranes by phospholipases. This release may occur due to tissue injury, cytokine or growth factor signals, or other stimuli. Free ARA acts as a substrate for COX and is then metabolized to an intermediate prostaglandin, which can then give rise to over 16 different 2-series prostaglandins and thromboxanes depending on available enzymes and cell type (Figure 2.2; Calder, 2002). Leukotrienes are synthesized primarily by inflammatory cells. Then ARA, once cleaved from the membrane, is a substrate for LOX, which is found in the nuclear envelope of certain inflammatory cells. Arachidonic acid is then transformed to an epoxide leukotriene,

Figure 2.2. Synthesis of eicosanoids from arachidonic acid and sites of inhibition by eicosapentaenoic acid. ARA, arachidonic acid; COX, cyclo-oxygenase; EPA, eicosapentaenoic acid; HETE, hydroxyeicosatetraenoic acid; HPETE, hydroperoxyeicosatetraenoic acid; LOX, lipoxygenase, LT, leukotriene; PG, prostaglandin, TX, thromboxanes (Calder, 2002).



which will then be either hydrolyzed, conjugated with glutathione, or undergo transcellular metabolism to create bioactive eicosanoids (Calder, 2002).

Animal and human studies show that feeding diets rich in long chain PUFA results in increased proportions of EPA and DHA in inflammatory cell phospholipids which are incorporated at the expense of ARA (Calder, 2001a), as EPA inhibits ARA release from phospholipids and inhibits the oxygenation of ARA by COX (Calder, 2002). Eicosapentaenoic acid itself can act as a substrate for COX and 5- LOX (Figure 2.2), giving rise to structurally distinct prostaglandins and leukotrienes which are less potent inflammatory mediators. These 3- and 5- series eicosanoids act through the same receptors and target cells as ARA derived eicosanoids, thus further competing against and inhibiting the pro-inflammatory actions of 2- and 4-series eicosanoids (Calder, 2002). These observations have led to the presumption that n-3 FA derived from fish oil and other sources are on the whole anti-inflammatory, and could benefit the health and well being of the horse. Omega-3 FA supplementation decreased these mediators, namely the production of PGE₂, 4-series leukotrienes, and the pro-inflammatory cytokines (especially TNF- α).

Many studies have specifically examined the effects of n-3 supplementation on TNF- α production. Rodents supplemented with ALA via linseed oil have increased production of TNF- α by resident macrophages, but show no effect on TNF- α by inflammatory macrophages (Hubbard et al., 1994; Turek et al., 1991). Animal and human studies show that *ex vivo* production of TNF- α following lipopolysaccharide (LPS) stimulation of monocytes is reduced following introduction of n-3 FA (Browning et al., 2003; Harbige et al., 2003; Calder, 2001b). In animal studies using primarily EPA for supplementation, lower peak serum TNF- α

concentrations are observed (Sadeghi et al., 1999; Hayashi et al., 1998; Tashiro et al., 1998) while others have reported that dietary fish oil decreases the expression of interferon gamma (IFN- γ) on murine peritoneal macrophages (Byleveld et al., 1999; Feng et al., 1999). After supplementation with EPA and DHA, decreased resting levels of inflammatory biomarkers are seen in exercise-trained men (Bloomer et al., 2009).

Biological Effects

Metabolic Effects. These n-6 and n-3 PUFA competitive interactions affect health either via influences in membrane structures, and gene expression in addition to eicosanoid actions (Lands, 2005). Omega-3 FA supplementation can have a number of beneficial metabolic effects in several species, particularly associated with the previously described anti-inflammatory effects of EPA and DHA, which may be beneficial in combating many chronic inflammatory diseases including coronary heart disease, diabetes, arthritis, cancer, osteoporosis, mental health disorders, dry eye disease, and age-related macular degeneration (Simopoulos, 2008; MacLean et al., 2006). A large body of evidence exists, including human experimental studies, animal experimental studies, and cell culture studies, to suggest beneficial effects of long chain n-3 PUFA on the molecular, cellular, and whole-body pathogenic processes of atherosclerosis and thrombosis, suggesting that the consumption of EPA and DHA may decrease the risk of cardiovascular disease (Calder, 2006). As n-3 FA increase erythrocyte deformability and are incorporated into the membranes (De Moffarts, et al. 2007; Portier et al. 2006), they could play a role in reducing risks for these diseases. Additionally, dietary supplementation of n-3 FA have a cholesterol-reducing effect in animals (O'Connor et al., 2004; Warner et al., 1989), may influence both bone formation and bone resorption in rats (Li et al., 1999), and may also have

potential as modulators of respiratory diseases involving chronic inflammatory and infectious processes (Knapp, 1995).

Arthritis. The responses concerning inflammatory mediators following n-3 FA supplementation have been an area of interest concerning the alleviation of joint pain, as supplementation with n-3 FA can have a significant effect on synovial membrane production of eicosanoids. Controlled trials have demonstrated the efficacy of n-3 FA in reducing joint pain associated rheumatoid arthritis (Goldberg and Katz, 2007). However, it remains to be determined empirically whether the ability of n-3 FA to reduce pain is due to either suppression of the inflammation underlying arthritic conditions, or via direct effects on prostaglandins or possibly cytokines in the spinal cord dorsal horn (Goldberg and Katz, 2007).

Exercise. As the inflammatory response is increased in response to strenuous exercise, the anti-inflammatory properties of n-3 FA may have beneficial effects during exercise. A combination of decreased blood lipid concentrations, increased erythrocyte membrane fluidity and increased insulin sensitivity as a result of n-3 supplementation have the potential to beneficially alter several physiological responses to exercise, such as decreasing heart rate (O'Connor et al., 2004; Simopoulos, 1991; Mueller and Talbert, 1988).

Insulin. Omega-3 FA supplementation also increases insulin sensitivity across a variety of species. Miniature pigs supplemented with n-3 FA have increased insulin sensitivity in response to an intravenous glucose tolerance test (Behme, 1996). These results are in agreement with findings from similar studies conducted using rats. Chicco et al. (1996) found that rats supplemented with fish oil comprising 7% of their diet also exhibit increased insulin sensitivity in response to a glucose tolerance test. In an additional study, rats supplemented with a similar

quantity of n-3 FA for 6 wk exhibit an increase in n-3 FA concentration in the skeletal muscle and sarcolemma, combined with a 14-fold increase in insulin binding (Liu et al., 1994). These researchers suggested that the increasing amount of n-3 FA in the cell membrane might alter the action of insulin and insulin receptors, therefore resulting in an increase of insulin sensitivity. Increased insulin sensitivity decreases the fasting blood free fatty acids and triglyceride levels in humans (McCarty, 1998).

Lipid in Equine Diets

Omega-3 Supplementation and Metabolism. Regarding the diets of exercising horses, fat has traditionally been added as a means of increasing energy density. Different methods can be used to supplement fat in the equine diet, with common fat sources being oils from soybean, corn, sunflower, olive, flaxseed, canola, and fish. All of these sources will increase energy density equally, although their fatty acid profiles vary. However, the most effective n-3 supplementation is done using marine-based oils, as these are the only oils that naturally contain significant amounts of EPA and DHA, and will therefore elicit the greatest biological effects (Lands, 2005). As seen in other species, horses also have a limited ability to convert dietary ALA to significant amounts of EPA or DHA. When horses were supplemented with equal amounts of n-3 FA from either flaxseed or fish oil, plasma EPA and DHA content only increased in horses fed encapsulated fish oil (Vineyard et al., 2009; Siciliano et al., 2003), indicating an inability to obtain sufficient quantities of EPA or DHA from a land-based food source.

Equine Lipid Profiles. While FA are a small fraction of the normal equine diet, their popularity in equine supplementation is increasing, with several studies investigating the

potential for PUFA to modify circulating blood characteristics and FA profiles in the horse. Luther et al. (1981) were the first to perform work concerning plasma FA concentrations in horses using gas chromatography, producing more accurate results than any previously reported. Their results showed that ALA has the highest concentration in equine plasma, followed by stearic acid (18:0), oleic acid (18:1n-9), and palmitic acid (16:0). Combined, these four FA make up over 85% of the total plasma FA profile. Concentrations of FA are tissue specific, as seen when the same analytical technique was applied to harvested equine erythrocytes and the FA profile changed significantly. In erythrocytes, stearic acid exists in the highest percentage, followed by palmitic and oleic acid (Luther et al., 1982). Free FA levels not only differ among parts of the blood, but also fluctuate throughout the day (Orme et al., 1994; Luther et al., 1982; Luther et al., 1981), with concentrations of oleic and stearic acid varying. These results suggest that concentrations of FA fluctuate depending on the time of day, prandial state, and dietary influences in horses.

Dietary supplementation of fish oils increases the concentrations of EPA and DHA in equine FA profiles proportionally to the quantity fed (McCann et al., 2000; Morris et al., 1991). In a study conducted by Hall et al. (2004b), horses were supplemented for 14 wk with either fish oil or corn oil. Horses fed fish oil had increased plasma concentrations of EPA and DHA compared to horses fed corn oil, accompanied by a corresponding decreased LA and ALA concentration. O'Connor et al. (2007) supplied exercising horses with a diet of 324 mg oil/kg BW from either a corn oil source or a menhaden fish oil source for 63 d and observed that horses receiving the fish oil treatment had increased serum concentrations of both EPA and DHA. Several studies are in agreement with this, showing a positive correlation of the n-6 to n-3 ratio in plasma and serum according to diet in horses fed n-3 supplements from marine sources

compared to land based n-3 sources (Vineyard et al., 2009; King et al., 2008; De Moffarts et al., 2007; Khol-Parisini et al., 2007). Plasma EPA and DHA levels increase as soon as three days following supplementation with n-3 FA in a dose-responsive manner, with a peak level obtained by seven days when offered at the amount of 40 g/d of EPA/DHA (King et al., 2008).

Biological Effects. Once incorporated into cell membranes, n-3 FA can have many beneficial metabolic effects in the horse. Mares fed a marine-derived n-3 FA source containing 10.4 g EPA and 8.6 g DHA for a period of 60 d prior to parturition through 21 d post-parturition had increased milk EPA and DHA at all sampling times. Foals of all PUFA-supplemented mares had elevated concentrations of EPA and DHA in plasma compared to control mares (Kruglik et al., 2005). Concerning sperm output and dynamics, beneficial effects have also been observed with n-3 supplementation in breeding stallions. Supplementation with long chain PUFA increases sperm output and the percentage of normal sperm (Harris et al., 2005) and also increases semen DHA concentrations, improves sperm motility (when cooled and stored for 48 hours), and improves total and progressive motility in stallions (Brinsko et al., 2005).

Beneficial changes in response to exercise following n-3 supplementation have also been shown in both horses as well as humans. Following exercise in humans, a decrease in resting oxidative stress, inflammatory biomarkers, and an increase in maximal oxygen consumption occurs when subjects are treated chronically with EPA and DHA (Calder, 2001b; Mori and Beilin, 2004; Brilla and Landerholm, 1990). Several beneficial effects following n-3 supplementation in exercising horses have also been observed, including an increase in trot stride length (Woodward et al., 2007), a decrease in serum cortisol following a standardized exercise test (Howard et al., 2003), and reduced heart rates associated with intense exercise when supplemented with a fish oil source (O'Connor et al., 2004). Conversely, horses fed soybean oil

for 28 d show no difference in heart rate during exercise when compared to a control group of corn oil fed horses (Howard et al., 2003). The decrease in heart rate may potentially be attributed to an increase in fluidity in the erythrocyte membrane as a result of EPA and DHA incorporation (O'Connor et al., 2004). Two additional studies have been done examining the effects of n-3 FA on erythrocyte deformability in horses (De Moffarts et al., 2007; Portier et al., 2006), with horses on a fish oil diet having increased concentrations of EPA and DHA in erythrocyte membranes that delayed the onset of an exercise-induced decrease in erythrocyte membrane fluidity. Further, exercising rats receiving dietary fish oil also exhibit decreased resting heart rates, blood pressure, aortic flow, and cardiac output due to a decrease in vascular resistance (Demaison et al., 2000; Lortet and Verger, 1995).

When O'Connor et al. (2004) examined the effects of fish oil supplementation on exercising horses, horses in the treatment group had lower plasma glucose during recovery after a standardized exercise test and tended to have higher glucose to insulin ratios, suggesting an increase in insulin sensitivity. Following a 12-h fast, the fish oil treated horses also had lower free FA concentrations and lower serum glycerol concentrations than the control group.

Inflammation and Omega-3 Supplementation in Horses

Immunity and Inflammatory Responses. While the ability of n-3 FA to mediate inflammatory and immune responses has been well documented in other animals, only a few studies have examined these factors in horses. When 6 healthy adult horses were fed a diet containing 8% linseed oil by weight over an 8 wk period, peritoneal macrophages produced less

TNF- α in response *in vitro* when stimulated with endotoxin compared to pre-supplement measures. It was proposed that TNF- α synthesis was altered by reduced adenylate cyclase activity as a result of a high ALA diet. This could alter formation of cyclic nucleotides that control TNF- α synthesis (Morris et al., 1991). In another study by the same research team, an intravenous n-3 lipid emulsion decreased basal TNF- α production by monocytes, as well as those cells which were challenged with calcium ionophore. In addition, an n-6 emulsion increased TNF- α production by monocytes challenged with LPS (McCann et al., 2000). In mature horses supplemented with fish oil, EPA and DHA serum levels increase, a 78-fold increase in the production of leukotrienes B₅ in neutrophils can be observed, and a decrease of PGE₂ is seen in bronchoalveolar lavage fluid cells (Hall et al., 2004b). This same research team also saw that equine peripheral blood neutrophils have reduced leukotriene B₄ synthesis in response to dietary fish oil (Hall et al., 2004a). Many other studies have demonstrated the ability of fish oil to act as a PGE₂ antagonist, however, it is important to note that the overall outcome of dietary n-3 supplementation cannot be predicted solely on the basis of abrogation of PGE₂–mediated effects.

Dietary n-3 supplementation may also improve immune function in both yearling and mature horses. Yearling horses supplemented with a fish oil/olive oil blend for 42 d had increased plasma and red blood cell concentrations of EPA and DHA, with a corresponding decrease in PGE₂ production. Yearlings were given a tetanus booster midway through the trial, and when tested at the end of supplementation, horses on the n-3 diet had higher tetanus titers than those on the control diet (Vineyard et al., 2009). While studies in humans have linked n-3s

with a reduction of bronchial inflammation associated with asthma (Nagakura et al., 2000; Broughton et al., 1997), horses fed fish oil showed no differences in pulmonary function (Khol-Parisini et al., 2007). However, the horses on this study were evaluated for a period of 20 wk, and these researchers suggest that the influence of time, environment, and diet interfered with each other or contributed to the lack of clinical or lung functions due to changes in diet.

Arthritis and Exercise. Arthritis is a condition of acute or chronic inflammation of a joint and is a condition that can have adverse implications in the performance horse. As with humans, the effect of supplementation of long chain PUFA on indicators of arthritis in horses has been examined. Following 75 d of supplementation with fish oil, horses that had been given dietary n-3 exhibited an increase in plasma DHA levels with no changes in PGE₂ metabolite or TNF- α (Woodward et al., 2007). However, these horses exhibited longer stride lengths at the trot following supplementation with fish oil, suggesting a decrease in joint pain. Despite lack of cytokine and prostaglandin changes following oral supplementation, dietary n-3 PUFA can reduce the pain associated with arthritis, although the mechanisms by which this occurs are not known (Goldberg and Katz, 2007).

Munsterman et al. (2005) conducted a study that tested the effects of n-3 FA on synovial explants from horses following LPS stimulation with an inflammatory agent. Explants treated with ALA exhibit an increased concentration of ALA in the cell membranes in linear relationship to the ALA concentration in the media, as well as a decrease in production of PGE₂ (Munsterman et al., 2005). Similar results were seen in an *in vivo* study that examined the PUFA supplementation of mature horses with arthritis in the leg and foot joints. Horses were grouped according to severity of arthritis, affected joints, and age, and were supplemented with either a

control diet or a diet of 15 g EPA and 19.8 g DHA for 90 d, with synovial fluid collected from an arthritic joint every 30 d. Following supplementation, treatment group horses exhibited lower synovial fluid white blood cell counts and lower plasma PGE₂ (Manhart et al., 2009).

DHA-Rich Microalgae

While incorporation of EPA and DHA has been clearly seen with fish oil supplementation in humans and animals, issues with palatability are often reported. Additionally, fish oil contains varying amounts of ARA, which is a mediator of several pathways and can interfere with the influences of n-3 FA. Due to these issues, commercial production of DHA-rich microalgae (DRM) from *Schizochytrium* sp. has grown in popularity as an n-3 supplement. *Schizochytrium* sp. is a heterotrophic eukaryotic organism that makes commercial amounts of PUFA by fermentation. It makes both DHA and DPA, but does not produce ARA (Lippmeier et al., 2009), and therefore eliminates the issue of potential interference regarding ARA metabolic pathways during supplementation. Microalgae are the original source of DHA in the marine food chain, and previous research has shown that DRM is a safe and stable source of DHA that can be effectively used to enrich poultry products, pork products (Abril et al., 2003), and milk produced from dairy cattle (Franklin et al., 1999; Barclay et al., 1998; Barclay et al., 1994).

While issues with supplement palatability and consumption have been observed with fish oil in horses (Woodward et al., 2007), an alternative source of DHA through a DRM based supplement has the probability of delivering the same quantities of n-3 FA without compromising intake values or complications due to ARA. To the author's knowledge, DRM supplementation in the horse has yet to be investigated.

Conclusions

While supplementation of PUFA has been extensively researched in studies involving both animal and human subjects, research in the horse is limited, especially with regards to both the effects of a DRM source of DHA and the effects of oral supplementation on exercise in the horse. Although it is clear that EPA and DHA supplementation has the ability to alter the circulating FA profile of the horse as well as elicit biological and metabolic effects, not enough data currently exists to create feeding guidelines for the horse regarding n-3 supplementation. Previous trials have used varying quantities over varying periods of time, initially based off of the suggested dietary ratios for human consumption, and have consequently seen varying responses in tissue concentrations. King et al. (2008) suggests that 30 to 35 g of EPA and DHA per day for a 500 kg horse would be ideal, while other studies have used an n-3 supplementation rate of around 30 g total n-3/d and seen beneficial effects. The proposed study has a focus on discerning the effects of n-3 supplementation in the exercising horse when supplying a low quantity of DHA over a short period (when compared to previous research in the horse), which will contribute to the increased understanding of DHA incorporation into the body.

Many of these effects, particularly the anti-inflammatory properties of EPA and DHA, could influence performance capability in the horse. The ability to influence parameters such as stride length through dietary supplementation would obviously have a tremendous impact on the equine industry as a whole, and certainly warrant further investigation following the Woodward et al. (2007) study which demonstrated a tendency for stride length changes. Additionally, as a DRM source of DHA has not yet been examined in the horse, gathering information regarding its ability to influence metabolic changes in the horse could encourage opportunities for further

research and will allow for information regarding the influence of DHA over a comparatively shorter time period.

As different species tend to respond similarly to n-3 FA supplementation, this allows for comparison of results between studies and for the application of hypotheses for further investigations that could uncover additional benefits of DHA supplementation from a DMR source in the exercising horse, as well as other species. This could have several positive applications concerning the alleviation of inflammation, benefitting the equine industry.

CHAPTER 3

MATERIALS AND METHODS

Animal Management

Twenty-four mature Arabians (15 mares, 9 geldings) with an average body weight of 434 $\pm$ 94 kg were used in this study. All methods were approved by the Michigan State University Institutional Animal Care and Use Committee (approval number 11/09-173-00). All animals were housed in mixed-grass pastures at the Michigan State University Horse Teaching and Research Center with free access to grass hay, water, and a trace mineralized salt block prior to the start of the experiment and did not receive any grain supplementation. All horses were being used in classes at Michigan State University, with the exception of five horses who were not receiving exercise. All horses were considered to be in moderate fitness condition at the start of the trial.

Horses were individually housed in 2.7 m x 3 m box stalls bedded with wood shavings while on the 63-d study conducted from January 18th through March 28th, 2010, with the exception of being used for classes or being provided with exercise. Horses were turned out for approximately one hour in 15 m x 5 m dry lots 2 d/wk and were not given access to pasture or any nutrients other than those provided in their daily rations.

Lameness Evaluations

Each horse underwent a lameness evaluation prior to the start of the study to determine pre-existing lameness or gait defects that might affect stride length. Lameness evaluations and

flexion tests were performed according to the American Association of Equine Practitioners Classification (Kester, 1991) by licensed equine practitioners three days prior to the start of the trial. Horses were visually evaluated at the walk and the trot. Each leg was then palpated, searching for bone or soft tissue abnormalities from the carpal and tarsal joints downward, with any preexisting conditions noted and taken into consideration. Flexion tests were performed to determine a lameness grade for each carpal, tarsal, metacarpophylangeal, metatarsophylangeal, and femoropatellar joints, with scores recorded. Each joint was given a score ranging from 0 (no lameness exhibited) to 5 (extreme lameness exhibited). Scores recorded from flexion tests were added together to give an overall lameness score, which was used to assign horses to treatment blocks. None of the horses exhibited gait defects secondary to suspected neurological problems or muscle atrophy secondary to injury or illness.

Experimental Design and Diets

This study used a repeated 3 x 3 Latin square design with 21-d feeding periods. During each period, each treatment was fed to eight horses, such that by the end of the study each horse had received all treatments (Table 3.1). Horses were blocked by gender, age, and lameness score. Each block, consisting of three horses, was randomly assigned to one of two groups. Within each block, each horse was randomly assigned a classification of either A, B, or C to determine the order in which treatments would be fed to eliminate any possible treatment carry-over effects (Table 3.2). Horses within the same block were provided with equal exercise and turnout time throughout the project. Prior to the start of the study and on the last day of each

period, blood samples, stride lengths, and heart rate changes surrounding a 20-min exercise protocol were measured.

Horses were weighed using a digital floor scale and were given a body condition score (BCS) at d -4 prior to the start of the trial, and then weekly for the duration of the project. The BCS was determined using the Henneke system (Henneke et al., 1983), with observations from multiple researchers averaged to obtain the most accurate score. Late bloom timothy grass hay from the same cutting was provided by the Michigan State University Horse Teaching and Research Center and was offered at 1.5% (as fed) of each horse's body weight. A textured feed (Kalmbach Feeds, Inc., Upper Sandusky, OH) was offered at a total of 0.5% (as fed) of each horse's body weight when combined with 908 g/d of treatment supplement. Hay and textured feed amounts were adjusted individually throughout the project to maintain starting body weight (BW) and BCS as needed based on weekly evaluations.

Treatments

One week prior to the start of the experiment horses were brought into stalls and acclimated to a diet of approximately 2.2 kg textured feed and approximately 6.5 kg grass hay. Both grass hay and textured feed were split between two feedings at 0700 and 1600 and horses had unlimited access to water and a trace mineralized salt block.

During each period, each horse received one of three treatments, all in the form of a stabilized pelleted supplement providing differing amounts of omega-3 fatty acids from differing sources: α -linolenic acid from a soybean oil source (SOY; 4 g/d), α -linolenic acid from a flaxseed oil source (FLAX; 38 g/d), or docosahexaenoic acid from an algae source (ALG; 28

g/d). The supplements were balanced to be isocaloric and isonitrogenous and were mixed and bagged separately by Kalmbach Feeds, Inc. (Upper Sandusky, OH). Supplements were offered top-dressed at 908 g/d onto the textured feed (Table 3.3). Orts were collected and recorded before each feeding. Two horses refused the ALG treatment on the first two days of supplementation. To encourage consumption, the offered amount was reduced to 227 g/d, and then was incrementally increased to 908 g/d over a 3-d period. No other issues with palatability were observed.

Exercise

Horses were exercised 5 d/wk during the duration of the project. Exercise consisted of a specified lunge line exercise protocol or participation in horsemanship or training classes. Exercise was initiated on d -5 at the start of the acclimation week.

Horsemanship Classes. In horsemanship classes, horses were worked either hunt seat or western pleasure style. Horses were lunged at the walk, trot/jog, and canter/lope both directions for approximately 10 min prior to riding. An additional warm-up period under saddle followed, consisting of a total of 10 min of work at all three gaits both directions of the arena. Horses were then exercised by performing maneuvers presented to the class as per the instructor. Riding times increased from 25 to 45 min throughout the project, with the difficulty of maneuvers increasing according to rider skill.

Training Classes. Training classes consisted of working horses on a lunge line at the walk, trot, and canter both directions for approximately 15 min at the start of the trial. As horses progressed with training, they began work in training aids, such as driving lines and side reins,

and were later introduced (d 40 and beyond) to a saddle and ridden by students. Exercise times again increased from 25 to 45 min throughout the project.

Lunge Line Exercise. For horses not used in classes and for days when classes were not scheduled, horses were exercised for 20 min on a lunge line. Horses were lunged according to the following protocol: 5 min walk, 5 min trot, 5 min canter, reverse of direction, 5 min trot, and 5 min walk.

Measurements and Sample Collection

Blood Collection and Handling. Blood samples were collected following a 12 h fast prior to the start of the study and on the last day of each of the three feeding periods via jugular venipuncture using a 20 G needle and vacuum tubes (BD Vacutainer®, BD, Franklin Lakes, NJ) to be analyzed for fibrinogen, total protein, interferon-gamma (INF- γ), tumor necrosis factor-alpha (TNF- α), interleukin 10 (IL-10), prostaglandin E₂ (PGE₂), and a complete fatty acid profile. One 10-mL serum vial containing no additives, one 10-mL serum vial containing indomethacin solution (for PGE₂ analysis) and three 10-mL plasma vials containing K₃EDTA as the anti-coagulant were filled during each collection day. Indomethacin solution was prepared by making a stock solution containing 0.001 g indomethacin, 300 μ L ethanol, and 700 μ L PBS. The stock solution was added to serum vials at a concentration of 10 μ g indomethacin/mL serum. Tubes were inverted several times to assure thorough mixing with contents. Immediately following collection, one EDTA plasma vial was taken to the Clinical Pathology Laboratory in the Diagnostic Center for Population and Animal Health (Lansing, MI) and analyzed for

fibrinogen and total protein. Total protein was determined using physical analytical methods (Todd et al., 1974) and fibrinogen was analyzed according to previously established methods (Williard et al., 1989; Jain, 1986). All other samples were allowed to coagulate for 30 min prior to centrifugation at 3,000 x g for 15 min (GS-6KR Centrifuge, Beckman, Fullerton, CA). Serum or plasma was pipetted off the top, placed into duplicate microcentrifuge tubes, and then frozen at -20° C until further analysis.

Stride Length Measurement. Stride lengths were measured on d 0, 21, 42, and 63. On these collection days, each horse performed a 20-min exercise on the lunge line according to previously described protocol. Stride lengths were determined at both the walk and the trot, both immediately before and immediately following exercise, using previously established guidelines (Woodward et al., 2007; Hanson et al., 1997). A 2 m x 10 m area of dry dirt on a level arena surface was raked to erase any previous hoof prints. Horses were led at the walk and the trot through the raked area on a loose lead to ensure natural head carriage and stride length by minimizing possible interference by the handler. The same handler led all horses for all collection days to maximize consistency. Prior to recording measurements, each horse was led through to allow for desensitization and prevent interference with accurate measurements due to tenseness or shying. Three full stride lengths were measured from right hind toe impressions. This measurement was repeated three times for a total of nine measurements averaged for each gait. All procedures during the measurement of stride lengths were video recorded.

Heart Rate Measurement. Prior to the start of the study and on the last day of each period, each horse performed a 20-min exercise on the lunge line according to previously described protocol. During exercise, heart rate was monitored using an equine heart rate monitor (Polar Electro Oy, Kempele, Finland), with electrodes placed in the hollow of the left withers

and behind the elbow on the left side via an adjustable elastic girth (Polar Electro Oy, Kempele, Finland) and conductive ultrasound gel was used to enhance detection of heart rate by the electrodes. Heart rate was recorded using the equine CS600X Training Computer and analyzed using Polar Equine Software (Polar Electro Oy, Kempele, Finland).

Immediately on completion of exercise, each horse stood quietly in the arena for a 10-min recovery period. Resting heart rates were recorded prior to exercise, during exercise, and during the recovery period. Maximum heart rate during exercise, heart rate at the end of exercise, 1 min post exercise, 2.5 min post exercise, 5 min post exercise, and 10 min post exercise were recorded. Total area under the curve during the 10-min post exercise recovery period was also determined.

Laboratory Analyses

Blood samples were analyzed for plasma fatty acid profiles and serum INF- γ , TNF- α , IL-10, and PGE₂ concentrations. Each sample was analyzed in duplicate, and replicated further if the coefficient of variation exceeded 10%. Prostaglandin E₂ and TNF- α assays in equine serum have been previously used in clinical studies (Woodward et al., 2007). Other assays are being validated by this laboratory. A quality control sample of pooled serum was analyzed with each INF- γ , TNF- α , and IL-10 assay.

Analysis of Fatty Acids in Feeds and Plasma. All hay bales were core-sampled prior to each period and textured feed and treatments were sampled continuously throughout the experiment. Feed samples for each period were pooled and analyzed separately (Table 3.4).

Feed samples were ground through a 1-mm screen (Cyclotec 1093 Sample Mill, Foss, Eden Prairie, MN), then 2.5 g of ground sample was weighed into a clean culture tube. Plasma samples were thawed to room temperature, vortexed, and 1 mL of sample was added to a clean culture tube. To extract the lipid in samples, 1 mL methanol, 1 mL hexane, and 1 mL deionized water were added to each tube, the tubes capped with a Teflon septa insert, vortexed for 10 min, and centrifuged for 10 min at 950 x g. A positive displacement pipette was used to transfer the top layer of approximately 0.75 mL hexane into a clean, previously weighed, culture tube. Samples were then evaporated under a nitrogen gas stream and weighed to determine lipid content. Lipid extracts were then transmethylated to form fatty acid methyl esters (FAME) using the procedure described by Woodward et al. (2007) (Appendix A).

Prostaglandin E₂. Prostaglandin E₂ was determined using an enzyme-linked immunosorbent assay (ELISA; Prostaglandin E₂ ELISA Kit, Thermo Scientific Pierce Protein Research Products, Rockford, IL) following the manufacturer's instructions. Serum dilution factor was determined based on the procedures described in Woodward et al. (2007), with slight modifications. Preliminary testing at a dilution of 1:2 showed several results that were near the lower limit of the standard curve. Dilution was therefore adjusted to 1:1.25, which provided readings falling within the standard curve with good correlation (less than 15%; Appendix B).

Tumor Necrosis Factor-alpha. Tumor necrosis factor-alpha was determined using an ELISA (Equine TNF- α Screening Kit, Thermo Scientific Pierce Protein Research Products, Rockford, IL) following the manufacturer's instructions. Serum was diluted at 1:8. Dilutions were determined by testing a serum sample diluted at 1:2, 1:4, 1:8, and 1:16. These values were compared to the manufacturer's suggestion that the samples fall between 15.6 and 1,000 pg/mL.

When the samples fell within the range and recovery was the highest, the corresponding dilution was chosen for all samples (Appendix C).

Interleukin 10. Interleukin 10 was determined using an ELISA (Equine IL-10 DuoSet ELISA, R&D Systems, Inc., Minneapolis, MN) following the manufacturer's instructions. Serum was filtered (Millex®-GP 0.22 µm filter unit, Millipore, Bedford, MA) and diluted on plate at 1:4 with reagent diluent. Dilutions were determined by testing a neat (no additives) serum sample and testing dilutions at 1:2, 1:4, 1:8, 1:10, 1:12.5, 1:16, 1:25, 1:32, 1:50, and 1:64. These values were compared to the manufacturer's suggestion that the samples fall between 39 and 20,000 pg/mL. When the samples fell within the range and recovery was the highest, the corresponding dilution was chosen for all samples (Appendix D).

Interferon-gamma. Interferon-gamma was determined using an ELISA (Equine INF-γ DuoSet ELISA, R&D Systems, Inc., Minneapolis, MN) following the manufacturer's instructions. Serum was filtered (Millex®-GP 0.22 µm filter unit, Millipore, Bedford, MA) and diluted on plate at 1:10 with reagent diluent. Dilutions were determined by testing a serum sample spiked and diluted neat (no additives), and at dilutions of 1:2, 1:4, 1:8, 1:10, 1:12.5, 1:16, 1:25, 1:32, 1:50, and 1:64. These values were compared to the manufacturer's suggestion that the samples fall between 31 and 4,000 pg/mL. When the samples fell within the range and recovery was the highest, the corresponding dilution was chosen for all samples (Appendix E).

Statistical Analyses

The study was a 3x3 Latin square design with 8 horses per 21-d period, and was balanced for carryover effects. Analysis was done with SAS 9.2 (SAS Inst. Inc., Cary, NC) using the

PROC MIXED procedure. Terms in the model statement included horse, supplement, gender, age, and the interaction of supplement and age. Horse and period were set as repeated measures. Means were separated using the PDIFF option within SAS and were adjusted using Tukey's test.

Additional statistics were analyzed to determine differences from pre-experiment values to end of period values for all variables. This was to allow for a comparison of each supplement to pre-supplemented values, using each horse as its own control, and was accomplished by subtracting pre-experimental values from end of period values. These values were then analyzed using the PROC MIXED procedure in SAS using the same statistical program as previously described. For all procedures, significance was declared at $P < 0.05$ and a trend toward a significant difference at $0.05 < P < 0.10$, with least squares means presented throughout.

Table 3.1. Latin square design used for the experiment, where ALG denotes algae-based supplement, FLAX denotes flaxseed oil-based supplement, and SOY denotes soybean oil-based supplement. Each block of three horses was randomly assigned to either Group 1 or Group 2 and fed in the supplement order provided below.

<i>Period</i>	<i>Group 1^a Supplement</i>			<i>Group 2^b Supplement</i>		
	<i>Horse A</i>	<i>Horse B</i>	<i>Horse C</i>	<i>Horse A</i>	<i>Horse B</i>	<i>Horse C</i>
d 0 to d 21	ALG	FLAX	SOY	ALG	FLAX	SOY
d 21 to d 42	SOY	ALG	FLAX	FLAX	SOY	ALG
d 42 to d 63	FLAX	SOY	ALG	SOY	ALG	FLAX

^aBlocks of horses in group 1: n=4

^bBlocks of horses in group 2: n=4

Table 3.2. Block, group, classification, age, sex, and osteoarthritis score for each horse on trial.

<i>Block No.</i>	<i>Horse No.</i>	<i>Group, Classification</i>	<i>Age (yr)</i>	<i>Sex</i>	<i>Osteoarthritis Score</i>
1	2	1, A	11	M	3.5
1	3	1, B	11	M	1
1	4	1, C	9	M	1.5
2	1	2, B	7	M	0
2	5	2, C	9	M	0
2	6	2, A	10	M	1.5
3	7	1, B	4	M	0
3	8	1, A	4	M	0
3	9	1, C	4	M	0
4	10	1, A	3	M	0.5
4	11	1, C	3	M	0
4	13	1, B	3	M	0
5	12	2, B	3	M	0.5
5	14	2, C	3	M	0.5
5	15	2, A	3	M	1.5
6	16	2, C	4	G	0
6	17	2, A	4	G	0
6	18	2, B	4	G	0
7	20	1, B	18	G	3.5
7	21	1, A	18	G	4
7	23	1, C	17	G	3
8	19	2, C	18	G	2
8	22	2, B	18	G	2
8	24	2, A	12	G	0.5

Table 3.3. Delivery (g/d) of n-3, n-6, α -linolenic acid (LNA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) as a protected fatty acid supplement volume of 908 g/d as fed.

<i>Fatty Acid</i>	<i>Supplement</i>		
	<i>SOY</i>	<i>FLAX</i>	<i>ALG</i>
Total n-3 (g)	4.01	37.76	31.48
Total n-6 (g)	27.84	27.66	19.36
n-6:n-3	6.93	0.73	0.61
ARA (g)	ND	ND	ND
ALA (g)	3.55	37.68	2.65
EPA (g)	ND	ND	0.54
DHA (g)	ND	ND	27.86

ND: Not detectable.

Table 3.4. Selected fatty acids supplied by each feedstuff.

<i>Measurement^a</i>	<i>Textured Feed^b</i>	<i>Timothy Hay</i>	<i>Treatments</i>		
			<i>SOY^b</i>	<i>FLAX^b</i>	<i>ALG^b</i>
DM	89.75	90.12	91.28	91.82	91.41
Crude Fat	10.62	3.2	6.58	10.29	9.31
C14:0	0.15	0.54	0.25	0.15	6.71
C16:0	12.69	24.01	13.03	7.99	21.27
C18:0	4.40	1.79	3.37	2.95	1.48
C24:0	0.18	ND	0.16	0.14	0.30
Sum SFA ^c	17.84	27.77	17.26	11.45	31.46
C18:2(n-6)	49.62	18.83	49.68	29.22	22.24
C18:3(n-6)	ND	ND	0.42	0.35	0.34
C18:3(n-3)	7.83	17.26	6.75	40.33	3.13
C20:4(n-6)	ND	ND	ND	ND	ND
C20:5(n-3)	ND	0.38	ND	ND	0.64
C22:5(n-3)	0.03	1.72	0.52	0.04	0.21
C22:6(n-3)	ND	ND	ND	ND	32.95
(n-6) : (n-3)	6.77	0.54	6.93	0.73	0.61
Sum (n-6) ^d	50.17	19.27	52.87	29.61	22.90
Sum (n-3) ^e	7.41	35.81	7.63	40.41	37.24

^aFatty acids are listed as a percentage of the lipid in the sample.

^bObtained from Kalmbach Feeds, Inc. (Upper Sandusky, OH).

^cSum of the saturated fatty acids: 14:0 + 15:0 + 16:0 + 18:0 + 20:0 + 22:0 + 24:0.

^dSum of the (n-6) fatty acids.

^eSum of the (n-3) fatty acids.

^fND = not detectable.

CHAPTER 4

RESULTS

Body Weight

No difference in BW was seen between d 0 and d 63 for all horses (430 ± 94 kg vs. 440 ± 94 kg, $P = 0.45$). A difference was observed between BCS at d 0 and d 63 (6 ± 0 vs. 6 ± 0 , $P < 0.05$). Regardless of treatment, no differences were seen for BW or BCS across each 21-d period ($P = 0.58$ and $P = 0.60$, respectively; Table 4.1).

Stride Lengths

Walk and trot stride lengths were measured both before and after a specified 20-min exercise on the lunge line. Baseline measurements were taken on d 0 and following each 21-d supplementation period. Stride length data is presented in Table 4.2. Baseline measurements at the walk pre-exercise and post-exercise did not differ as a result of age ($P = 0.38$ and $P = 0.79$, respectively). When evaluating the change in stride length at the walk from pre- to post-exercise on d 0, no difference was seen as a result of age ($P = 0.81$). Baseline trot stride lengths pre-exercise were not different due to age ($P = 0.36$). When analyzing stride length change from pre- to post-exercise on d 0, no difference was seen as a result of age ($P = 0.68$).

When stride lengths were measured following n-3 FA supplementation periods, no effects of treatment, age, or treatment $\times$ age interaction were seen at the walk pre-exercise ($P = 0.91$, $P = 0.72$, and $P = 0.89$, respectively) or post-exercise ($P = 0.20$, $P = 0.71$, and $P = 0.99$,

respectively). Stride length change from pre- to post-exercise at the walk also did not differ due to treatment, age, or treatment $\times$ age interaction ($P = 0.45$, $P = 0.88$, and $P = 0.92$, respectively).

When trot stride lengths following supplementation with n-3 FA sources were measured, no treatment, age, or treatment $\times$ age interaction effects were seen pre-exercise ($P = 0.61$, $P = 0.28$, and $P = 0.86$, respectively) or post-exercise ($P = 0.33$, $P = 0.40$, and $P = 0.59$, respectively). Stride length change from pre- to post-exercise at the trot did not differ due to treatment, age, or treatment $\times$ age interaction ($P = 0.74$, $P = 0.10$, and $P = 0.33$, respectively).

When stride lengths from across each supplementation period were analyzed in comparison to baseline measurements, no differences for treatment, age, or treatment $\times$ age interactions were seen at the walk pre-exercise ($P = 0.95$, $P = 0.75$, and $P = 0.89$, respectively) or post-exercise ($P = 0.21$, $P = 0.59$, and $P = 0.99$, respectively). Again, no differences were seen when analyzing stride length change from pre- to post-exercise for treatment, age, or as a result of a treatment $\times$ age interaction ($P = 0.50$, $P = 0.54$, and $P = 0.95$, respectively). Trot stride lengths continued to show no difference for treatment, age, or treatment $\times$ age interactions for pre-exercise ($P = 0.59$, $P = 0.68$, and $P = 0.87$, respectively), post-exercise ($P = 0.36$, $P = 0.16$, and $P = 0.55$, respectively), or when analyzing the change from pre- to post-exercise ($P = 0.71$, $P = 0.10$, and $P = 0.63$, respectively).

Heart Rates

Resting heart rate (HR) was recorded prior to the onset of exercise. Baseline resting HR did not differ between horses as a result of age ($P = 0.91$) and was 34 ± 1 beats/min. When measured following n-3 FA supplementation periods, resting HR did not differ as a result of

treatment, age, or treatment $\times$ age interaction ($P = 0.73$, $P = 0.33$, and $P = 0.14$, respectively; Table 4.3). When resting HR from each period of supplementation were analyzed in comparison to baseline values, no difference as a result of treatment, age, or treatment $\times$ age interaction was observed ($P = 0.53$, $P = 0.78$, and $P = 0.59$, respectively).

Heart rates were monitored during a specified 20-min lunge line exercise while the maximum HR during exercise was recorded. Baseline maximum HR differed between horses as a result of age ($P < 0.05$), ranging from 164 ± 2 beats/min (18-yr old horses) to 185 ± 3 beats/min (10-yr old horses). When measured following supplementation, maximum HR did not differ as a result of treatment, age, or treatment $\times$ age interaction ($P = 0.46$, $P = 0.14$, and $P = 0.69$, respectively). When results from each supplementation period were analyzed in comparison to baseline values, maximum HR did not differ as a result of treatment, age, or treatment $\times$ age interactions ($P = 0.31$, $P = 0.37$, and $P = 0.55$, respectively).

Following exercise, HR was monitored continuously for a 10-min recovery period. Specific heart rates were recorded and analyzed: immediately post-exercise (HR 0), 1 min post-exercise (HR 1), 2.5 min post-exercise (HR 2.5), 5 min post-exercise (HR 5), and 10 min post-exercise (HR 10). The total area under the curve for the entire 10-min recovery period was also calculated and analyzed (HR AUC). Baseline recovery HR measurements did not differ as a result of age (Table 4.3), and following n-3 FA supplementation, all recovery HR measurements showed no differences as a result of treatment, age, or treatment $\times$ age interaction (Table 4.3). When recovery HR measures from each period were compared to baseline values, no differences were seen in total HR AUC based on treatment, age, or treatment $\times$ age interaction ($P = 0.15$, $P = 0.62$, and $P = 0.19$, respectively).

Plasma Fatty Acid Profiles

Plasma FA profiles are presented as a percentage of total plasma lipid concentration (Table 4.4). Plasma n-6 and n-3 FA composition between horses at baseline measurements did not differ, with the exception of a trend for α -linolenic acid to be highest in 3-yr old horses and lowest in 4-yr old horses (4.38 ± 1.88 % vs. 2.81 ± 1.85 %; $P < 0.10$). Before beginning supplementation, linolenic acid made up the largest percentage of total plasma PUFA in all horses. After supplementation with all treatments, this observation remained the same.

Baseline values for the total n-3 and total n-6 content of the plasma FA profile did not differ ($P = 0.87$ and $P = 0.94$, respectively). No differences were observed across supplementation periods for total n-3 or total n-6 ($P = 0.64$ and $P = 0.24$, respectively). When compared to baseline values, total n-3 did not differ as a result of treatment or age ($P = 0.60$ and $P = 0.74$, respectively); however, a trend for a treatment $\times$ age interaction existed ($P < 0.10$). No differences were seen in total n-6 when compared to baseline measures based on treatment, age, or as the result of a treatment $\times$ age interaction ($P = 0.15$, $P = 0.77$, and $P = 0.77$, respectively).

The calculated ratio of n-6 to n-3 FA in plasma was not different between horses at baseline measures ($P = 0.94$) or across supplementation periods as a result of treatment, age, or treatment $\times$ age interaction ($P = 0.64$, $P = 0.56$, and $P = 0.17$, respectively). When analyzing the change across each treatment in comparison to baseline values, there continued to be no differences ($P = 0.61$, $P = 0.88$, and $P = 0.22$, respectively).

On d 0, the mean basal concentrations of the primary n-3 FA of interest, EPA and DHA, did not differ based on age ($P = 0.98$ and $P = 0.88$, respectively). A treatment effect was observed for EPA plasma levels, with horses on SOY having higher EPA levels (0.45 ± 0.07

percent of total plasma lipid) than horses on FLAX and ALG ($P < 0.05$; Table 4.4).

Docosahexaenoic acid levels remained unchanged as a result of treatment or age ($P = 0.62$ and $P = 0.46$, respectively), but a treatment $\times$ age interaction was observed ($P < 0.05$). When compared to baseline values across treatments, EPA continued to exhibit a treatment effect ($P < 0.05$), with horses on the SOY treatment showing approximately a forty percent increase in EPA levels, FLAX having an approximate forty percent decrease from baseline values, and ALG showing approximately a one percent decrease. When compared to baseline measures, DHA remained unchanged as a result of treatment or age ($P = 0.63$ and $P = 0.79$, respectively), but did show a trend for a treatment $\times$ age interaction ($P < 0.10$). Plasma α -linolenic acid was also altered in response to treatment ($P < 0.05$), with horses on the FLAX treatment having a greater percentage than horses on either the SOY or ALG treatments (Table 4.4).

Total Protein and Fibrinogen

Plasma total protein and fibrinogen were measured at d 0 to obtain a baseline value and following each supplementation period. At baseline there was no difference in plasma total protein as a result of age ($P = 0.61$). Following supplementation, plasma total protein increased in horses on the SOY treatment when compared to both FLAX and ALG (6.80 ± 0.07 g/dL; $P < 0.05$). When analyzed in comparison to baseline values, a treatment effect continued to exist ($P < 0.05$) and a trend for an age $\times$ treatment interaction was still present ($P < 0.10$).

Plasma fibrinogen levels did not differ in horses at baseline as a result of age ($P = 0.69$). Following supplementation, plasma fibrinogen levels were not different as a result of treatment, age, or a treatment $\times$ age interaction ($P = 0.47$, $P = 0.49$, and $P = 0.50$, respectively; Figure 4.1).

When analyzed in comparison to baseline values on d 0, there continued to be no differences ($P = 0.49$, $P = 0.51$, and $P = 0.53$, respectively).

Prostaglandin E_2

When serum PGE_2 was analyzed via ELISA on d 0, baseline values did not differ due to age ($P = 0.46$). Following n-3 FA supplementation, PGE_2 concentrations did not differ between horses as a result of treatment, age, or an age $\times$ treatment interaction ($P = 0.34$, $P = 0.59$, and $P = 0.43$, respectively; Figure 4.1). When changes during each 21-d period were compared to baseline values, no differences were seen for treatment, age, or as the result of a treatment $\times$ age interaction ($P = 0.62$, $P = 0.23$, and $P = 0.71$, respectively).

Tumor Necrosis Factor- α

Serum TNF- α concentrations did not differ in baseline values as a result of age ($P = 0.38$). Following n-3 FA supplementation, TNF- α concentrations were not different as a result of treatment, age, or treatment $\times$ age interaction ($P = 0.26$, $P = 0.79$, and $P = 0.31$, respectively; Figure 4.1). When comparing changes from each 21-d period to baseline values, no differences were seen for treatment, age, or as the result of a treatment $\times$ age interaction ($P = 0.16$, $P = 0.66$, and $P = 0.74$, respectively).

Interleukin 10

At baseline on d 0, no differences were observed as a result of age in serum IL-10 concentrations ($P = 0.51$). Serum concentrations differed as a result of treatment, with SOY having lower concentrations of IL-10 than ALG ($4,490 \pm 5,080$ pg/mL vs. $10,610 \pm 4,100$ pg/mL; $P < 0.05$). However, there was no effect noted as a result of age or due to a treatment $\times$ age interaction ($P = 0.48$ and $P = 0.35$, respectively; Figure 4.1). When analyzing changes across each 21-d period in comparison to baseline values, no differences were seen for treatment, age, or as the result of a treatment $\times$ age interaction ($P = 0.21$, $P = 0.45$, and $P = 0.40$, respectively).

Interferon- γ

Serum INF- γ concentrations were not different among all horses in baseline values as a result of age ($P = 0.42$). Following n-3 FA supplementation, INF- γ concentrations did not differ as a result of treatment, age, or treatment $\times$ age interaction ($P = 0.46$, $P = 0.34$, and $P = 0.62$, respectively; Figure 4.1). When changes across each supplementation period were compared to baseline values, again no differences were seen for treatment, age, or as the result of an age $\times$ treatment interaction ($P = 0.55$, $P = 0.51$, and $P = 0.90$, respectively).

Table 4.1. Body weight (kg) and body condition score (BCS) for all horses within treatments.

<i>Measurement</i>		<i>Treatment</i>			<i>P-value</i>
		<i>SOY</i>	<i>FLAX</i>	<i>ALG</i>	
Body weight (kg)	Starting Weight*	436 ± 94	436 ± 94	439 ± 94	0.97
	Change [†]	5.0 ± 1.8	4.9 ± 1.8	0.2 ± 1.8	0.12
BCS	Starting BCS*	6 ± 0	6 ± 0	6 ± 0	0.71
	Change [†]	0 ± 0	0 ± 0	0 ± 0	0.29

*Representative of weight and BCS on the first day of supplementation of each respective treatment.

[†]Representative of the change in body weight or BCS across each 21-d period while on each respective treatment.

Table 4.2. Mean walk stride length (cm) and trot stride length (cm) of horses. Stride lengths were measured before and after a 20-min specified exercise on the lunge line to determine a baseline at d 0 and following 21-d supplementation periods for all treatments.

<i>Measurement</i>		<i>Baseline</i>	<i>Treatment</i>			<i>P-value</i>
		<i>Day 0</i>	<i>SOY</i>	<i>FLAX</i>	<i>ALG</i>	
Walk Stride (cm)	Pre-exercise	158 ± 2	161 ± 2	162 ± 2	161 ± 2	0.91
	Post-exercise	157 ± 2	161 ± 2	160 ± 2	162 ± 2	0.20
	Change*	-1 ± 1	-1 ± 1	-2 ± 1	0 ± 1	0.45
Trot Stride (cm)	Pre-exercise	189 ± 3	192 ± 5	194 ± 5	194 ± 5	0.61
	Post-exercise ¹	192 ± 3	190 ± 5	194 ± 5	193 ± 5	0.33
	Change*	3 ± 2	-2 ± 2	0 ± 2	-2 ± 2	0.74

*Representative of the mean change in stride length for all horses from pre- to post-exercise.

¹Post-exercise baseline trot measurements showed a trend for a difference for age ($P < 0.10$).

Table 4.3. Mean heart rate (HR) measurements at baseline (d 0) and following 21-d supplementation periods for all treatments. Heart rates are provided at rest, the maximum HR during a 20-min specified lunge line exercise, and specific heart rates post-exercise during a 10-min recovery period: immediately post-exercise (HR 0), 1 min post-exercise (HR 1), 2.5 min post-exercise (HR 2.5), 5 min post-exercise (HR 5), and 10 min post-exercise (HR 10).

<i>Measurement*</i>	<i>Baseline</i>	<i>Treatment</i>			<i>P-value</i>
	<i>Day 0</i>	<i>SOY</i>	<i>FLAX</i>	<i>ALG</i>	
Resting HR	34 ± 1	34 ± 1	35 ± 1	35 ± 1	0.73
Maximum HR ¹	174 ± 5	160 ± 6	157 ± 6	164 ± 6	0.46
HR 0	66 ± 3	66 ± 2	67 ± 2	69 ± 2	0.50
HR 1	59 ± 3	56 ± 3 ^{ab}	55 ± 3 ^a	61 ± 3 ^b	0.05
HR 2.5	50 ± 2	46 ± 2	49 ± 2	50 ± 2	0.32
HR 5	45 ± 2	44 ± 2	45 ± 2	48 ± 2	0.31
HR 10	41 ± 2	40 ± 2	42 ± 2	42 ± 2	0.46

*Heart rates are expressed as beats/min.

¹ An overall decrease in maximum HR was observed from d 0 to d 63 of the study ($P < 0.05$).

^{abc} Values lacking common superscripts differ ($P < 0.05$).

Table 4.4. Effect of feeding horses diets supplemented with three differing sources and content of n-3 fatty acids on plasma fatty acid profiles after 21-d supplementation periods and at baseline values (d 0).

<i>Fatty Acid*</i>	<i>Baseline</i>	<i>Treatment</i>				<i>P-value</i>
	<i>Day 0</i>	<i>SOY</i>	<i>FLAX</i>	<i>ALG</i>	<i>Pooled SEM</i>	
C18:2(n-6)	34.77	34.44	42.01	36.17	3.54	0.20
C18:3(n-6)	0.73	1.02	0.87	0.74	0.10	0.12
C18:3(n-3) ¹	3.85	3.19 ^{ab}	4.06 ^a	2.72 ^b	0.44	0.02
C20:4(n-6)	0.66	0.58	0.39	0.67	0.11	0.18
C20:5(n-3)	0.24	0.45 ^a	0.18 ^b	0.24 ^b	0.07	0.03
C22:5(n-3)	1.83	1.71	2.31	1.84	0.50	0.66
C22:6(n-3) ²	5.46	7.04	5.18	5.88	2.11	0.62
Sum (n-6) ³	36.16	36.04	43.26	37.58	3.62	0.24
Sum (n-3) ⁴	11.08	12.39	12.35	10.70	1.50	0.64
Ratio (n-6): (n-3)	0.36	0.42	0.37	0.33	0.07	0.64

*Plasma fatty acids are given as a percentage of total plasma lipid content.

^{abc} Means in the same row not sharing a common superscript differ ($P < 0.05$)

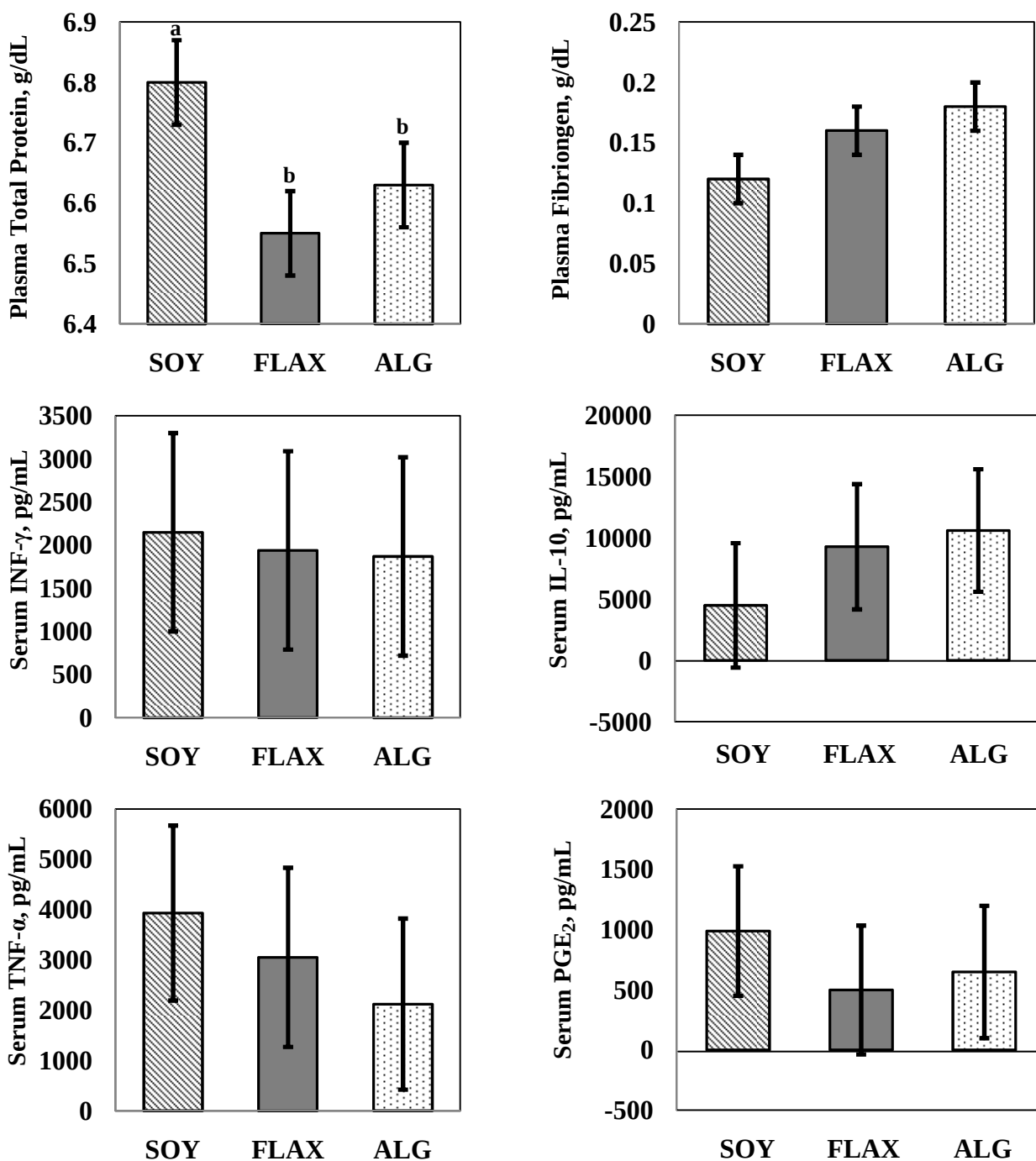
¹ Trend for an age effect on d 0 ($P < 0.10$).

² Age $\times$ treatment effect ($P < 0.05$).

³ Sum of n-6: C18:2(n-6), C18:3(n-6), C20:2(n-6), C20:3(n-6), C20:4(n-6), C22:2(n-6), and C22:4(n-6).

⁴ Sum of n-3: C18:3(n-3), C20:3(n-3), C20:5(n-3), C22:3(n-3), C22:5(n-3), and C22:6(n-3).

Figure 4.1. The response of hematological variables to n-3 treatments of soybean oil (SOY), flaxseed oil (FLAX), or algal DHA (ALG) in equine plasma.



^{abc} Values lacking common superscripts differ ($P < 0.05$).

CHAPTER 5

DISCUSSION

The effects of n-3 supplementation have been studied extensively in a variety of animals, and to a lesser extent in the horse. Based on the FA composition of the n-3 supplements used in this study (Table 3.4), the amount of total n-3 FA intake for a 500-kg horse would be approximately either 4 g/d if consuming the SOY treatment, 38 g/d if consuming the FLAX treatment, or 31 g/d if consuming the ALG treatment. It can be seen in Table 3.4 that the ALG supplement was the only significant source of EPA and DHA in this study.

In comparison to other studies using the horse as a model, the total amount of n-3 FA, as well as the amount of DHA and EPA provided by our treatments was generally lower and our 21-d supplementation period for each treatment was shorter than those previously reported (Table 5.1). While previous research has indicated that dietary supplementation of marine-derived sources of EPA and DHA can rapidly and significantly increase the circulating concentrations of these n-3 FA when orally supplemented in significant quantities, the results of this study did not show a change in response to treatment in plasma concentrations of DHA, total n-3, or total n-6 (and consequently no change in the n-6 to n-3 ratio).

Stride lengths at the walk and trot both before and immediately following exercise did not differ in horses at baseline values as a result of age. Across all treatments, no differences were seen for stride length values at either gait and no difference was seen when stride length changes from d 0 to d 63 were evaluated. These findings are in contrast to those reported by Woodward et al. (2007), who saw a tendency for an increase in trot stride length in horses supplemented with 19.4 g/d of n-3 FA from a fish oil source for 75 d compared to horses supplemented with

Table 5.1. Comparison of n-3 supplementation sources, amounts of fatty acids, and length of supplementation (d) across equine studies.

<i>Study</i>	<i>n-3 Source</i>	<i>Supp. Length</i>	<i>EPA</i> ¹	<i>DHA</i> ¹	<i>Total n-3</i> ¹	<i>Results</i>
Brinsko et al., 2005	Fish oil	98 d	17.5	3.5	70	Increased sperm concentrations, higher average velocity, increased DHA concentrations in semen fatty acids
De Moffarts et al., 2007	Fish oil	21 d	20.0	10.0	NR	Exercise induced decrease of erythrocyte membrane fluidity associated with changes in blood oxidative balance
Hall et al., 2004 a, b	Fish oil	98 d	22.9	19.6	49.6	Plasma FA changes, modulation of inflammatory responses in bronchoalveolar lavage fluid cells and leukotrienes
Harris et al., 2005	Marine	90 d	NR	NR	29.1	Increased daily sperm output, normal morphology, and DHA concentrations in sperm plasma membrane
King et al., 2008	Marine	28 d	0, 10.0, 20.0, 40.0 ²	NR	4.8, 17.2, 29.2, 53.1	Plasma FA response is dose dependent, EPA and DHA timelines to appear and clear from plasma
Khol-Parisini et al., 2007	Seal blubber oil	70	NR	NR	35.5	Leukocyte FA changes, pulmonary epithelial lining fluid immune changes, no changes in pulmonary function or clinical signs
Manhart et al., 2009	NR	90 d	15.0	19.8	NR	N-3 treated horses had lower synovial WBC concentrations
O'Connor et al., 2004, 2007	Fish oil	63 d	20.1	15.2	39.8	Serum FA changes, lower heart rates, serum cholesterol, and serum glycerol during exercise, increase in insulin sensitivity
Parsons et al., 2011	Algae	21 d	0.5	27.9	31.5	No plasma FA changes, no physiological or metabolic changes
Portier et al., 2006	Fish oil	28 d	20.0	10.0	NR	Decrease in erythrocyte membrane fluidity induced by exercise

Table 5.1 (cont'd)

Vineyard et al., 2009 ³	Fish oil, flaxseed	70 d	NR	NR	23.5	Plasma and RBC FA changes (greater impact with fish oil), enhanced early inflammatory responses to phytohemagglutinin injections
Woodward et al., 2007	Fish oil	75 d	1.4	3.2	19.4	Plasma FA changes, tendency for increase of trot stride length

¹Measurements are given in g/d consumed per horse.

²Combination of EPA and DHA.

³Amounts given represent concentrations in fish oil.

NR: Not reported/unable to determine from provided information.

corn oil. While these researchers were supplementing a lower amount of DHA than our trial, they observed changes in plasma FA profiles that reflected their n-3 dietary sources. A favorable anti-inflammatory response as a result of n-3 supplementation cannot be elicited unless the n-3 FA are present in the blood to be delivered to the body, consequently enhancing the potential for their incorporation into cell membranes. As Woodward et al. (2007) witnessed an increase in plasma DHA, as well as a decrease in the n-6 to n-3 ratio, this suggests that the potential incorporation of DHA into cell membranes may have been involved in their observation of increased trot stride lengths, whereas our study did not note any changes in plasma levels for these same variables. Stride lengths reported by Woodward et al. (2007) were overall greater at both the walk and the trot when compared to our study (approximately 168 ± 4 cm vs. 160 ± 2 cm and approximately 237 ± 7 cm vs. 194 ± 5 cm).

While horses tend to reflect soundness within their stride length (Rogers et al., 2005; Toutain and Cester, 2004; White et al., 2003), this can potentially be used as a measure of lameness and osteoarthritic pain (Hanson et al., 1997). However, Woodward et al. (2007) noted that while an increase in trot stride length was seen, no definite answer for this increase could be reached from the study due to a lack of difference in overall lameness scores or markers of inflammation due to treatment.

A recent study by Headley et al. (2011) examined the effects of supplementation of 50 g/d of conjugated linoleic acid over a 6-wk period on markers of stride length in sedentary horses. No differences in walk or trot stride length were observed in this study, suggesting that supplementation with higher quantities of n-6 FA has a limited ability to affect stride length in sound, sedentary horses. Horses on the SOY treatment were receiving the highest n-6 to n-3 ratio in this study. The addition of one treatment (SOY) that had a substantially higher n-6 to n-3

ratio than the others in this repeated Latin square design may have negated the potential positive effects that a low dose of n-3 FA may have had on stride length.

The lack of change in stride length seen in the horses in our study is likely due to both the absence of circulatory n-3 FA combined with the absence of osteoarthritic disease and ensuing inflammation and unsoundness in our experimental model. The horses used by Woodward et al. (2007) exhibited overall higher lameness evaluation scores than the horses used in this study. While our horses varied in age and lameness scores, and were grouped accordingly, no differences in stride lengths could be seen as a result of age, group, treatment $\times$ age, or treatment $\times$ group interactions, suggesting that our horses had insignificant inflammation and pain to effect stride length (Hanson et al., 1997).

No differences were observed in this study concerning resting heart rate, maximum heart rate reached during exercise, or heart rate recovery post-exercise as a result of treatment. These results do not reflect a previously conducted trial by O'Connor et al. (2004), who saw that horses receiving fish oil delivering 17 g/d EPA and 13 g/d DHA for 63 d had lower heart rates during exercise compared to those receiving corn oil. This study also showed an increase in plasma EPA and DHA (O'Connor et al., 2007) and an increase in erythrocyte deformability in both the treatment and control diets, which has been cited as a mechanism to lower heart rate (Oostenburg et al., 1997; Cartwright et al., 1985). When compared to our study, the exercise protocol used by O'Connor et al. (2004) was conducted over a longer period of time and at a higher intensity. The standardized exercise test used by O'Connor et al. (2004) was also performed at a higher intensity than the exercise test used in our study.

It is likely that no resting, maximum, or recovery heart rate differences were observed as a result of treatment in this study due to DHA and EPA invariance in the circulatory profiles combined with a light exercise training program. Although baseline maximum heart rates differed as a result of age, with older horses having lower maximum heart rates than younger horses, this was most likely due to behavioral differences exhibited by the horses on the baseline testing day. Generally, older horses were much calmer during the lunging exercise on the first collection day, whereas younger (and less trained) horses generally reacted more strongly, with inconsistency primarily at the canter, including bucking, shying, and working with excessive speed, all of which can result in a greater increase in heart rate than a horse performing a relaxed and consistent gait.

The plasma FA profile will change to reflect dietary FA influences, and in this study a treatment difference was observed with ALA plasma concentrations, with horses on the FLAX treatment having higher plasma concentrations of ALA than horses on the ALG treatment. This response reflected the dietary intake of ALA, with FLAX horses consuming 38 g/d of ALA and ALG horses consuming 3 g/d of ALA and is in agreement with other studies examining dietary influence of ALA in the circulatory profiles of the horse (Vineyard et al., 2009; Hall et al., 2004b; Hansen et al., 2002). However, other studies using the horse as a model have conversely not observed a change in plasma ALA as a result of dietary n-3 supplementation (King et al., 2008; Woodward et al., 2007; O'Connor et al., 2004). The discrepancy for these results could lie in both differing sources of n-3 as well as a potential seasonal influence of FA circulating concentrations as suggested by King et al. (2008). Their study noted that plasma concentrations of 22 different FA increased or decreased in all treatment groups, including controls, and suggested that this was due in part to a change in the FA profiles of seasonal forages. Non-

supplemented horses in their experiment consumed a diet considered to be typical for the average management practices in the United States (fresh or cured grass forage plus <0.5 kg of whole oats), which resulted in a plasma n-6 to n-3 FA ratio of approximately 7.5 during the winter and approximately 6 during the spring. Changes in the ALA concentrations in varying forages, independent of supplementation, could be responsible for the variation of ALA plasma responses to treatment in these studies.

Additionally, a treatment effect was seen for plasma EPA in this study, with horses on the SOY treatment exhibiting a higher concentration of EPA than horses on the FLAX or ALG treatment, with concentrations that were higher than baseline values. This is a surprising discovery, since all treatments were low in EPA, with the ALG treatment delivering the highest quantity of EPA, at a very low amount of 0.5 g/d, and the SOY and FLAX treatments showing non-detectable quantities of EPA. This shows that a potential elongation of ALA may have occurred, yielding an increase in plasma EPA. Mantzioris et al. (1994) found that tissue EPA concentrations increase in a linear fashion after dietary ALA supplementation. This idea is further supported by the work of Hansen et al. (2002), where they found plasma concentration of EPA to be markedly increased in horses supplemented with flaxseed oil in comparison to baseline measures. The horses in the Hansen et al. study had no other dietary sources of EPA, so it can be assumed that amounts detected in tissue were the result of bioconversion. However, the difference in EPA concentrations as a result of treatment in our study is most likely not due to bioconversion. If bioconversion from ALA was the case, then EPA levels should have been highest in the FLAX supplement, which supplied a significantly higher quantity of ALA than either the SOY or the ALG supplement. Additionally, the efficiency of the equine model to elongate ALA to EPA within the body is still questioned (Vineyard et al., 2009; Siciliano et al.,

2003), and it would therefore be difficult to conclude that such a small quantity of EPA present in the circulatory FA profile was absolutely the result of the bioconversion of a small oral quantity of ALA from the diet. It is more likely that, due to the small values of EPA reported here, solvent interference or misidentification of peaks by the GC software is responsible for this difference.

While plasma levels of DHA remained unchanged across treatments, a trend for an age x treatment interaction was seen. Upon closer examination, this trend only showed differences between ages in regards to the SOY and FLAX treatment, both of which contained non-detectable amounts of DHA. While there is the limited ability of the body to convert ALA to EPA, elongation and desaturation of ALA to form DHA is estimated to be fractional, at < 0.05 percent, which is an amount insufficient to increase the concentration of DHA in plasma and other membranes (Williams and Burdge, 2006; Siciliano et al., 2003), suggesting that the trend for this interaction is again due to sampling and processing error rather than bioconversion of ALA.

In contrast to previous studies, we did not see a treatment difference in circulating levels of DHA, total n-3, total n-6, or the n-6 to n-3 ratio. Several possibilities exist for these observations. The horses in this study were being supplied a uniform amount of supplement. As our horses varied widely in body weight, the amount of FA that was being provided on a $\text{mg}\cdot\text{kg}^{-1}$ BW basis also varied. For example, regarding horses on the ALG treatment, the lightest horse weighed 354 kg, and would therefore be supplied with approximately $79 \text{ mg}\cdot\text{kg}^{-1}$ BW DHA, while the heaviest horse, weighing 546 kg, would be supplied with approximately $51 \text{ mg}\cdot\text{kg}^{-1}$ BW DHA. The average 434 kg horse would have received $64 \text{ mg}\cdot\text{kg}^{-1}$ BW DHA on the ALG

treatment. This variance makes it difficult to evaluate these horses equally. However, no correlation was seen between body weight and DHA levels ($r^2 = -0.14$, $P = 0.27$), total n-3 levels ($r^2 = -0.12$, $P = 0.34$), or when evaluating the n-6 to n-3 ratio ($r^2 = -0.14$, $P = 0.25$), indicating that regardless of weight, horses were receiving a deficient amount of n-3 FA to elicit plasma changes over a 21-d period. The combination of a shorter supplementation period and lower dose of n-3 FA when compared to previous literature resulted in very minute changes in the plasma profile, with a small quantity of various FA being displayed in the plasma. At such small quantities, these values could easily be altered by sample contamination, incomplete extraction and methylation of FAMES, or misidentification of peaks by the GC software. This is further supported by our baseline data. Although we know little about the naturally occurring concentrations of n-3 in the horse, the primary source of DHA must come from a marine source in the diet. When our baseline values are examined, there is a mean basal concentration of DHA present in the plasma. While this value is low, it should be at non-detectable levels, indicating that there is some degree of error in the processing and analysis.

Others have fed an amount of DHA from a fish oil source in similar quantities to what was supplied by the ALG treatment in this trial (Vineyard et al., 2009; King et al., 2008; Woodward et al., 2007) and have seen increases in DHA in the plasma profile. As DHA cannot be synthesized in the body, even a small amount of the FA can affect plasma concentrations, as especially seen in the Woodward et al. (2007) study. However, all of these studies fed low quantities of DHA for periods of time much greater than the 21-d periods of our study (Table 5.1). Although overall plasma n-3 FA concentration will change linearly with the amount supplemented (King et al., 2008; Spearman et al., 2005; Hall et al., 2004b), these studies have

been conducted with much higher overall n-3 FA and DHA/EPA supplementation than the data represented here.

King et al. (2008) saw an increase in circulating EPA and DHA in mares supplemented with varying amounts of both FA following 3 d of supplementation, with a dose-related peak level by 7 d of feeding. Plasma EPA and DHA demonstrated a steep decline from peak values 9 d after cessation of feeding, were elevated for up to 2 wk post supplementation, and were not different than pre-supplemented levels at 42 d post-supplementation. This rate of plasma clearance generally agrees with results of fish oil supplementation of yearling horses (Vineyard et al., 2009), where plasma DHA, but not EPA, remained elevated beyond baseline through 5 wk post-supplementation. Harris et al. (2005) reported a longer time (30 d) to attain peak n-3 FA plasma concentrations after supplementation with a similar compound, but in a smaller population of horses. As EPA and DHA plasma values were not reflected in this study, it can be assumed that when feeding low quantities of these n-3 FA, they must be fed for longer than 21 d to see significant changes in the plasma profile.

It is possible that feeding similar quantities of various FA may require a wash-out period to eliminate the possibility of a carry-over effect from the previous treatment. Although our model was statistically tested for any existing carry-over effect (none were observed), previous literature suggests otherwise. In the King et al. (2008) study, it took a 24-d period to increase the total n-3 concentration (and consequently reduce the n-6 to n-3 ratio) in the plasma. Additionally EPA and DHA levels have existed at elevated levels from baseline measurements for up to 2-wk post-supplementation (King et al., 2008) or 5-wk post-supplementation (Vineyard et al., 2009) in horses. With residual levels reported at even low dosages of DHA and EPA, it is possible that when using a repeated Latin square design with short repeated periods, a low quantity of DHA

was not being offered long enough for consumption to make an impact on plasma concentration of DHA and was potentially being influenced by residual effects from the other treatments on the trial.

To the author's knowledge, this is the first study to examine the effects of DHA from a DRM source in the horse. Docosahexaenoic acid from algal sources effectively enriches poultry and pork products as well as milk produced from dairy cows (Abril et al., 2003; Franklin et al., 1999; Barclay et al., 1998). Few, if any, studies have looked at the effect of DHA supplied from a microalgae source on plasma lipid profiles in production animals. Algal DHA sources increase plasma DHA and total n-3 FA in circulatory profiles in human vegetarians (Conquer and Holub, 1996), however little is known as to the effects of a DRM source of DHA in an animal model similar to the horse. It could be possible that the bioavailability for absorption of algal DHA is slightly differently than DHA offered in the source of fish oil or seal blubber, as previously reported, and therefore needs to be supplied in a higher concentration to have the same biological effectiveness.

A lower n-6 to n-3 ratio allows for n-3 to be preferentially incorporated into tissues, and as this ratio lowers, more n-3 FA are present in the plasma for incorporation into erythrocytes (Bojensen and Bojensen, 1998). As no differences in this ratio were observed in this study, the likelihood of n-3 incorporation into bodily tissues and membranes is unlikely. This ratio change is normally attributed to a decline in the percentage of n-6 FA rather than a substantial increase in n-3 FA. King et al. (2008) suggests that feeding a level of 30 to 35 g/d of EPA/DHA would be sufficient to shift the n-6 to n-3 ratio to a n-3 favorable state. As our treatments supplied a maximum of 28.4 g/d of EPA/DHA, the lack of detection of a ratio shift would support this suggestion.

Plasma fibrinogen is a positive marker in the acute phase of inflammation (Auer et al., 1989). In this study, no differences were seen in fibrinogen changes as a result of treatment. The horses on our study were on a light exercise program that was not designed at an intensity to induce inflammation. Additionally, n-3 was not present in circulation for incorporation into these metabolic pathways. Plasma total protein in the horse may increase by greater than 25% in response to inflammation, infection or trauma. As a result, these proteins are used as quantitative markers in general health screenings, diagnosis of disease, and as prognostic indicators (Eckersall, 2008). In this study an overall increase in plasma total protein was seen for horses on the SOY treatment when compared to the ALG and FLAX treatments. Numerically, these changes are minute when realizing that the average plasma protein concentration in healthy adult horses ranges from approximately 6.0 to 8.5 g/dL (Duncan and Prasse, 1986). As fibrinogen, included in the total plasma protein count and representing the greatest proportion of plasma protein synthesized during an acute phase inflammatory response, levels were not determined to be different, the difference in total protein levels is not noteworthy in the context of this research.

Regarding other inflammatory metabolites, we saw no changes in PGE₂, TNF- α , or INF- γ . The values recorded for all of these makers were consistently low, with several being too low to read accurately on the standard curve. As these values were so low, our standard errors were markedly high. As overproduction of these markers were not present in our horses in general, this may suggest that they did not have a need for a reduction of them, hence one potential reason why we did not observe a treatment effect on this study. However, IL-10 showed no differences at baseline measurements, but did show treatment differences, with IL-10 concentrations in serum being lower for the SOY treatment than the ALG treatment. However, when analyzed as compared to baseline values, no treatment effect existed. Additionally, IL-10

is a known inhibitor of both TNF- α and INF- γ , so it would be expected that a difference observed in IL-10 concentration would also reflect a difference in TNF- α production (Calder, 2001b). As no treatment differences were seen for either of these metabolites, it is likely that an accuracy error occurred in regards to the ELISA, as there was not an overproduction of IL-10. This can be supported by the high standard errors seen with this analysis.

For all of the metabolites measured, fasting blood samples were taken following a 12-h period where the horses were in stalls overnight. As these inflammatory markers are whole-body indicators of inflammation, they are rapidly cleared from the blood in times where inflammation is not present. As our horses were on a light exercise schedule which was not strenuous enough to induce inflammation, it is likely that these metabolites were simply not present in detectable quantities at the time of collection. The ELISAs used for analysis are designed to evaluate high levels of these metabolites, and as most of our measures fell near the bottom end of the standard curve (or were non-detectable), these resulted in high standard errors for all assays performed.

In conclusion, this study found that feeding either 4 g/d, 38 g/d, or 31 g/d of total n-3 with varying FA profiles for 21-d periods does not elicit changes in the plasma FA profile of the horse. Additionally, feeding these quantities of n-3 and n-6 FA over a total 63-d period without a wash-out period did not result in plasma FA concentrations, total n-3 or n-6 concentrations, or alter the n-6 to n-3 ratio. Further, no changes in stride lengths, heart rates, or inflammatory metabolites in both plasma and serum showed no treatment differences, likely as a result of the absence of n-3 FA in the plasma, and therefore other cell membranes within the body, and due to a lack of inflammatory disease as a result of osteoarthritis or exercise in our model.

CHAPTER 6

SUMMARY AND CONCLUSIONS

Our research has shown that the mature exercising horse does not show physiological responses to a supply of 4 g/d, 38 g/d, or 31 g/d of total n-3 with varying FA profiles over 21-d periods. No changes in the plasma FA profile of the horse, stride length at the walk or trot, heart rate before, during, or post-exercise, or inflammatory metabolites in both plasma and serum were elicited as a result of n-3 supplementation.

Previous research has suggested feeding a minimum of 30 to 35 g/d of EPA and DHA to elicit a rapid change in the plasma profile of the horse (King et al., 2008). Our study supports this claim, as no changes in plasma profiles after a 21-d period were observed in horses being supplemented with 28.4 g/d of EPA and DHA. Based on the results from this experiment, it would be recommended that if feeding EPA/DHA at an amount lower than 30 to 35 g/d, a supplementation period of longer than 21-d would be required to observe differences in the plasma FA concentrations as a reflection of diet. For an experimental design similar to the work presented here, it would be recommended that a higher dose of EPA/DHA be offered.

As overproduction of inflammatory markers was not present in our horses in general, this suggested that they did not have a need for the reduction of anything, regardless of n-3 supplementation. To better test the effects of n-3 supplementation on joint health in exercising horses, inflammation as a result of exercise should be stimulated, with a more intense exercise protocol than that exhibited here. In hindsight, a post-exercise blood sample rather than a resting blood sample may have provided a better indicator of inflammatory responses or changes during

exercise to allow for more accurate detection by the ELISAs and would be recommended for future studies.

To our knowledge, this is the first study to supplement n-3 FA in the form of DRM to exercising horses. As nothing is known about potential differences in bioavailability of a DRM source of DHA in the horse, additional research is needed with an amount greater than 28.4 g/d for a 21-d period of EPA/DHA to validate the effects of a DRM DHA source on the metabolic effects of DHA in the horse.

More research into n-3 effects on joint health would have to be performed before conclusions regarding the effect of n-3 on stride length could be decisively made. While others have observed stride length differences (Woodward et al., 2007), these were not reflected in our study, but this was likely due to an inadequate supply of EPA and DHA to elicit changes in synovial tissues. Further studies should examine the effect EPA and DHA on stride length when supplied in an amount of 28.4 g/d over a continuous period greater than 21 d to allow for changes in the chondrocyte. Also, as our horses showed no overproduction of inflammatory markers, a more accurate measure of the effect of n-3 supplementation on stride length may be determined by evaluating horses that would be suspected to have elevated levels due to injury or disease to witness possible improvements or influences of a n-3 dietary source.

Additionally, further research investigating the time for FA to appear and clear from circulation would be beneficial. Although rapid changes in FA profiles have been seen, there is substantial variation among studies and varying amounts of supplementation have been used. Unfortunately, *in vivo* animal studies are notoriously variable in this area. In addition to possible species or breed differences, variation in response to fish oil or PUFA supplementation may stem

from genetic variation as pointed out by a recently published human study. Genetic differences were observed in TNF- α production in response to endotoxin (Grimble et al., 2002). This variability was determined to be due to polymorphisms in the promoter regions of TNF- α gene. Identifying these potential differences in clearance and appearance rates would allow us to better identify a minimum value of EPA/DHA supplementation in the mature, healthy horse over a certain time period.

APPENDICES

APPENDIX A

FAMES Methylation Procedure

Following the lipid extraction procedure described using 0.5 g of ground feed sample, or 1 mL plasma, samples were methylated to obtain FAMES. One milliliter of hexane including a C19:0 internal standard (1 mg standard/mL hexane) and 3 mL of 10% methanolic HCl (prepared by adding acetyl chloride to methanol at a 1:5 ratio) were added to each sample. Samples were layered with nitrogen gas, capped, vortexed, and placed into a 90° C water bath for 2 h. Once removed from the water bath, samples were cooled to room temperature and 10 mL of a 6% potassium carbonate solution (prepared by adding 60 g K₂CO₃ to 1 L deionized water) were added. Samples were vortexed and centrifuged for 5 min at 950 x g, the top layer transferred to a clean borosilicate test tube, and approximately 1 g sodium sulfate was added to each sample. If the sample contained color, approximately 0.5 g of activated charcoal was added to it. Again, samples were vortexed and centrifuged for 5 min at 950 x g. The hexane layer was transferred to a previously weighed test tube, evaporated under a nitrogen gas stream, and reweighed to determine lipid content. Samples were then reconstituted with hexane so that the concentration ranged between 0.25 to 0.40 g lipid/mL hexane.

The reconstituted samples were vortexed and transferred to a gas chromatography (GC) auto sampler vial and sealed with Teflon septa inserts. Samples were stored at -20° C until transferred to the Michigan State University Diagnostic Center for Population and Animal Health for analysis by GC (Clarus 500 with an autosampler; PerkinElmer, Waltham, MA) equipped with a flame ionization detector, and a 100-m SP-2560 fused silica capillary column (0.25 mm i.d. x

0.1 μm film thickness; Supelco, Bellefonte, PA). Chromatograms were obtained and analyzed using Empower Chromatography Data Software (Waters Corp., Milford, MA) and were examined for the presence of FAME by comparing the peak retention times. The individual FAME results were calculated as the concentration (mg/mL) in plasma and as a percentage of the total plasma FA. The fatty acids summed to obtain total n-3 FA are as follows: C18:3n3, C20:3n3, C20:5n3, C22:3n3, C22:5n3, and C22:6n3c. The fatty acids summed to obtain total n-6 FA are as follows: C18:2n6, C18:3n6, C20:2n6, C20:3n6, C20:4n6, C22:2n6, and C22:4n6c.

APPENDIX B

Prostaglandin E₂ ELISA Procedure

Prostaglandin E₂ was determined using an enzyme-linked immunosorbent assay (ELISA; Prostaglandin E₂ ELISA Kit, Thermo Scientific Pierce Protein Research Products, Rockford, IL) following the manufacturer's instructions. Serum dilution factor was determined based on the procedures described in Woodward et al. (2007), with slight modifications. Preliminary testing at a dilution of 1:2 showed several results that were near the lower limit of the standard curve. Dilution was therefore adjusted to 1:1.25, which provided readings falling within the standard curve with good correlation (less than 15%)

The standard curve was prepared by transferring 900 µL reagent diluent to one tube and 500 µL reagent diluent to an additional eight tubes. One hundred microliters of stock PGE₂ standard (50,000 pg/mL) was added to the first tube and serially diluted over the remaining eight tubes in 500 µL aliquots. The concentrations of PGE₂ in the diluted standard tubes were 5,000, 2,500, 1,250, 625, 313, 156, 78, 39, and 19 pg/mL, respectively. Once all standards and samples were ready, the plate was prepared as follows: 150 µL of reagent diluent was added to the non-specific binding well, 100 µL of reagent diluent was added to the maximum binding well, 100 µL of each standard was added to specified wells, and 100 µL of each sample was added to each additional well. Samples were then diluted with 100 µL of reagent diluent on plate (1:1.25 dilution factor). Fifty microliters of Alkaline Phosphatase conjugated PGE₂ (PGE₂-AP) were

added to each well, excluding the total activity and blank wells, and 50 μL of PGE_2 antibody were added to each well except the blank, total activity, and non-specific binding wells. The plate was then covered with a plate sealer and incubated at room temperature (22 to 25° C) on a plate shake for 2 h at approximately 500 rpm. The contents of all wells were then aspirated, washed four times with 400 μL of wash buffer, aspirated again, and firmly tapped on a lint free paper towel to remove any remaining wash buffer. Five microliters of PGE_2 -AP was added to the total activity well and 200 μL of substrate solution was added to every well. The plate was then covered and incubated for 45 min at room temperature before being read at 405 nm optical density on a Spectra Max 340 plate reader (Molecular Devices Corp., Sunnyvale, CA). Fifty μL of stop solution was added to every well and the plate was immediately read at 580 nm.

APPENDIX C

Tumor Necrosis Factor-alpha ELISA Procedure

Tumor necrosis factor-alpha was determined using an ELISA (Equine TNF- α Screening Kit, Thermo Scientific Pierce Protein Research Products, Rockford, IL) following the manufacturer's instructions. Serum was diluted at 1:8. Dilutions were determined by testing a serum sample diluted at 1:2, 1:4, 1:8, and 1:16. These values were compared to the manufacturer's suggestion that the samples fall between 15.6 and 1,000 pg/mL. When the samples fell within the range and recovery was the highest, the corresponding dilution was chosen for all samples (Appendix C). Flat bottom 96-well plates were used (MaxiSorpTM, Nalge Nunc International, Rochester, NY) and were coated with coating antibody (CA) prior to use. Coating antibody was diluted 1:100 in a 0.2 M carbonate/bicarbonate buffer, pH 9.4 (110 μ L CA added to 10.89 mL of buffer; Appendix A) and 100 μ L was added to each well. The plate was covered with a plate sealer and incubated at room temperature for 18 h, the CA solution aspirated, and 300 μ L of blocking buffer (4% BSA, 5% sucrose in PBS) added to each well. The plate was covered, incubated for 1 h at room temperature on a plate shaker at 500 rpm, and blocking buffer was then aspirated.

The lyophilized standard was reconstituted with 1.12 mL of reagent diluent to produce a stock standard with a concentration of 10,000 pg/mL. The standard curve was prepared by transferring 900 μ L reagent diluent to one tube and 500 μ L reagent diluent to an additional six tubes. One hundred microliters of stock TNF- α standard was added to tube 1 and serially diluted over the remaining six tubes in 500 μ L aliquots. The concentrations of TNF- α in the diluted

standard tubes were 1,000, 500, 250, 125, 62.5, 31.25, and 15.6 pg/mL, respectively. Once all standards and samples were ready, the plate was prepared as follows: 100 μ L of each standard was added to specified wells, 100 μ L of reagent diluent were added to the blank wells, and 12.5 μ L of each sample was added to each additional well. Samples were then diluted with 87.5 μ L of reagent diluent on plate. The plate was covered with a plate sealer, incubated for 2 h on a plate shaker, the contents of all wells were aspirated, the plate washed five times with 400 μ L of wash buffer, wells aspirated again, and firmly tapped on a lint free paper towel to remove any remaining wash buffer.

Biotinylated antibody reagent was diluted with reagent diluent to prepare a 1:100 μ g/mL concentration and was added (100 μ L) to each well, the plate sealed and incubated for 1 h at room temperature on a plate shaker at 500 rpm, the contents aspirated, and the plate was washed five times with wash buffer. One hundred microliters of streptavidin-HRP reagent (diluted 1:400 in reagent diluent) was added to each well, followed by a 30-min covered incubation at room temperature and another five washes with wash buffer. Next, TMB substrate solution (100 μ L) was added to each well and an enzymatic color reaction was allowed to develop in the dark at room temperature for 20 min before being read at 650 nm optical density on a Spectra Max 340 plate reader (Molecular Devices Corp., Sunnyvale, CA). One hundred microliters of stop solution was added to every well and the plate was immediately read at 450 nm and 550 nm optical density. The 550 nm readings were subtracted from the 450 nm readings to correct optical imperfections in the microplate and give the correct absorbance levels.

APPENDIX D

Interleukin 10 ELISA Procedure

Interleukin 10 was determined using an ELISA (Equine IL-10 DuoSet ELISA, R&D Systems, Inc., Minneapolis, MN) following the manufacturer's instructions. Serum was filtered (Millex®-GP 0.22 µm filter unit, Millipore, Bedford, MA) and diluted on plate at 1:4 with reagent diluent. Dilutions were determined by testing a neat (no additives) serum sample and testing dilutions at 1:2, 1:4, 1:8, 1:10, 1:12.5, 1:16, 1:25, 1:32, 1:50, and 1:64. These values were compared to the manufacturer's suggestion that the samples fall between 39 and 20,000 pg/mL. When the samples fell within the range and recovery was the highest, the corresponding dilution was chosen for all samples. Flat bottom 96-well plates were used (MaxiSorp™, Nalge Nunc International, Rochester, NY) and were coated with CA (goat anti-equine IL-10) prior to use. Capture antibody was reconstituted with 1 mL 1X PBS solution to produce a stock solution (concentration of 144 µg/mL) and then a working concentration of 0.8 µg/mL (61.1 µL of CA added to 10.939 µL 1X PBS solution; Appendix B). One hundred microliters of CA was added to each well, the plate was covered with a plate sealer, incubated at room temperature for 18 h, the CA solution aspirated, and then washed five times with 400 µL per well of wash buffer (0.05% Tween-20 in PBS). Plates were then blocked by adding 300 µL of reagent diluent (1% BSA in PBS) to each well, the plate covered and incubated for 75 min on a plate shaker, aspirated, then washed five times with 400 µL per well of wash buffer.

The standard (20,000 pg/mL recombinant equine IL-10) was reconstituted with 0.5 mL of reagent diluent to produce a stock standard. The ten point standard curve was prepared by 2-fold

serial dilutions in reagent diluent. The concentrations of IL-10 in the diluted standard tubes were 20,000, 10,000, 5,000, 2,500, 1,250, 625, 313, 156, 78, and 39 pg/mL, respectively. Once all standards and samples were ready, the plate was prepared as follows: 100 μ L of each standard was added to specified wells, 100 μ L of reagent diluent were added to the blank wells, and 25 μ L of each sample was added to each additional well. Samples were then diluted with 75 μ L of reagent diluent on plate, the plate covered with a plate sealer, incubated for 2 h on the plate shaker at 500 rpm, well contents aspirated, washed five times with 400 μ L of wash buffer, aspirated, and firmly tapped on a lint free paper towel to remove any remaining wash buffer.

Biotinylated antibody reagent was diluted with reagent diluent to prepare a 100 ng/mL working concentration (61.1 μ L of antibody into 10.939 mL of reagent diluent) and was added (100 μ L) to each well, the plate sealed and incubated for 2 h on the plate shaker, contents aspirated, the plate washed 5 times with wash buffer, and wash buffer aspirated. One hundred microliters of streptavidin-HRP reagent (made by adding 55 μ L streptavidin-HRP reagent to 10.945 mL reagent diluent) was added to each well, followed by a 20-min covered incubation on the plate shaker and another five washes with wash buffer. Next, the working substrate solution (prepared by mixing equal parts of Color Reagent A and Color Reagent B) was added to each well (100 μ L), and an enzymatic color reaction was allowed to develop in the dark at room temperature for 20 min before being read at 620 nm optical density on a Spectra Max 340 plate reader (Molecular Devices Corp., Sunnyvale, CA). Fifty microliters of stop solution was added to every well and the plate was immediately read at 450 nm and 570 nm optical density. The 570 nm readings were subtracted from the 450 nm readings to correct optical imperfections in the microplate and give the correct absorbance levels.

APPENDIX E

Interferon-gamma ELISA Procedure

Interferon-gamma was determined using an ELISA (Equine INF- γ DuoSet ELISA, R&D Systems, Inc., Minneapolis, MN) following the manufacturer's instructions. Serum was filtered (Millex®-GP 0.22 μ m filter unit, Millipore, Bedford, MA) and diluted on plate at 1:10 with reagent diluent. Dilutions were determined by testing a serum sample spiked and diluted neat (no additives), and at dilutions of 1:2, 1:4, 1:8, 1:10, 1:12.5, 1:16, 1:25, 1:32, 1:50, and 1:64. These values were compared to the manufacturer's suggestion that the samples fall between 31 and 4,000 pg/mL. When the samples fell within the range and recovery was the highest, the corresponding dilution was chosen for all samples. Flat bottom 96-well plates were used (MaxiSorpTM, Nalge Nunc International, Rochester, NY) and were coated with CA (goat anti-equine INF- γ) prior to use. Capture antibody was reconstituted with 1 mL 1X PBS solution to produce a stock solution (concentration of 72 μ g/mL) and then a working concentration of 0.4 μ g/mL (61.1 μ L of CA added to 10.939 μ L 1X PBS solution; Appendix B). One hundred microliters of CA was added to each well, the plate was covered with a plate sealer, incubated at room temperature for 18 h, the CA solution aspirated, and then washed five times with 400 μ L per well of wash buffer (0.05% Tween-20 in PBS). Plates were then blocked by adding 300 μ L of reagent diluent (1% BSA in PBS) to each well, the plate covered and incubated for 75 min on a plate shaker, aspirated, then washed five times with wash buffer.

The standard (4,000 pg/mL recombinant equine INF- γ) was reconstituted with 0.5 mL of reagent diluent to produce a stock standard. The eight-point standard curve was prepared by 2-

fold serial dilutions in reagent diluent. The concentrations of INF- γ in the diluted standard tubes were 4,000, 2,000, 1,000, 500, 250 125, 62, and 31 pg/mL, respectively. Once all standards and samples were ready, the plate was prepared as follows: 100 μ L of each standard was added to specified wells, 100 μ L of reagent diluent were added to the blank wells, and 10 μ L of each sample was added to each additional well. Samples were then diluted with 90 μ L of reagent diluent on the plate, the plate covered with a plate sealer and incubated for 2 h on the plate shaker at 500 rpm, wells aspirated, washed five times with wash buffer, aspirated again, and firmly tapped on a lint free paper towel to remove any remaining wash buffer.

Biotinylated antibody reagent was diluted with reagent diluent to prepare a 400-ng/mL working concentration (61.1 μ L of antibody into 10.939 mL of reagent diluent) and was added (100 μ L) to each well, with the plate then sealed and incubated for 2 h on the plate shaker. After incubation, the contents were again aspirated and the plate was washed five times with wash buffer. One hundred microliters of streptavidin-HRP reagent (made by adding 55 μ L streptavidin-HRP reagent to 10.945 mL reagent diluent) was added to each well, followed by a 20-min covered incubation on the plate shaker and another five washes with wash buffer. Next, the working substrate solution (prepared by mixing equal parts of Color Reagent A and Color Reagent B) was added to each well (100 μ L), and an enzymatic color reaction was allowed to develop in the dark at room temperature for 20 min before being read at 620 nm optical density on a Spectra Max 340 plate reader (Molecular Devices Corp., Sunnyvale, CA). Fifty microliters of stop solution was added to every well and the plate was immediately read at 450 nm and 570 nm optical density. The 570 nm readings were subtracted from the 450 nm readings to correct optical imperfections in the microplate and give the correct absorbance levels.

APPENDIX F

Tumor Necrosis Factor- α Assay Reagent Preparation:

Equine TNF- α Screening Kit, Thermo Scientific Pierce Protein Research Products, Rockford, IL

Carbonate/bicarbonate Buffer Preparation (0.2 M buffer, pH 9.4):

1. Measure 21.2 g Na_2CO_3 into 1 L deionized water.
2. Measure 16.8 g NaHCO_3 into a separate 1 L flask of deionized water.
3. Mix 19 mL of the Na_2CO_3 solution and 81 mL of the NaHCO_3 solution.
4. The pH of this solution should be close to 9.3. Adjust to 9.4, adding a bit more NaOH as needed to make the solution more basic.

Storage and Stability:

- Store at 2 to 10° C. Warm to room temperature prior to using. Stable for one month.

Wash Buffer Preparation (0.05% Tween-20 in D-PBS, pH 7.4):

1. Prepare 10X PBS stock solution.
 - a. Combine the following: 80 g NaCl, 2 g KCl, 11.5 g Na_2HPO_4 , and 2.04 g KH_2PO_4 . Bring to a volume of 1 L using deionized water.
2. Prepare wash buffer 10X stock solution.
 - a. Combine 49.75 mL 10X PBS stock solution with 250 μL Tween-20. Store in 50 mL conical tubes at 2 to 10°C.

3. Prepare wash buffer.

- a. Thaw wash buffer 10X stock wash buffer to room temperature. In a clean glass or plastic container dilute the wash buffer concentrate to 1:30 with distilled or deionized water. Add 16.7 mL of wash buffer to 483.3 mL of water. Mix thoroughly.

Storage and Stability:

- Make fresh daily. Approximately 500 mL of wash buffer is needed per plate per assay.

Reagent Diluent Preparation (4% BSA in D-PBS, pH 7.4):

1. Prepare 1X PBS solution.

- a. Combine the following: 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂PO₄, and 1.5 mM KH₂PO₄. Bring to a volume of 1 L using deionized water.

2. Prepare 4% BSA solution.

- a. Add 2 g BSA to 50 mL 1X PBS solution.

Storage and Stability:

- Store at 2 to 10°C for up to a month. Approximately 50 mL is needed per plate per assay.

APPENDIX G

Equine Interleukin 10 Assay and Equine Interferon-gamma Assay Reagent Preparation:

Equine IL-10 DuoSet ELISA, R&D Systems, Inc., Minneapolis, MN

Equine INF- γ DuoSet ELISA, R&D Systems, Inc., Minneapolis, MN

1X PBS Solution Preparation

1. Combine the following: 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂PO₄, and 1.5 mM KH₂PO₄.
2. Bring to a volume of 1 L using deionized water.

Wash Buffer Preparation (0.05% Tween-20 in PBS, pH 7.4):

1. Prepare 10X PBS stock solution.
 - a. Combine the following: 80 g NaCl, 2 g KCl, 11.5 g Na₂HPO₄, and 2.04 g KH₂PO₄. Bring to a volume of 1 L using deionized water.
2. Prepare wash buffer 10X stock solution.
 - a. Combining 49.75 mL 10X PBS stock solution with 250 μ L Tween-20. Store in 50 mL conical tubes at 2 to 10°C.
3. Prepare wash buffer.

- a. Thaw wash buffer 10X stock wash buffer to room temperature. In a clean glass or plastic container dilute the wash buffer concentrate to 1:9 with deionized water by adding 50 mL of 10X wash buffer stock solution to 450 mL of deionized water.
Mix thoroughly.

Storage and Stability:

- Make fresh daily. Approximately 500 mL of wash buffer is needed per plate per assay.

Reagent Diluent Preparation (1% BSA in PBS, pH 7.4):

1. Combine 10X PBS stock solution with deionized water at a 1:9 concentration (ex. 50 mL 10X PBS stock solution and 450 mL deionized water).
2. Add 5 g BSA, stirring slowly to avoid excessive foaming.

sStorage and Stability:

- Store at 2 to 10°C for up to a month. Approximately 50 mL is needed per plate per assay.

LITERATURE CITED

LITERATURE CITED

- Abril, R., J. Garrett, S. G. Zeller, W. J. Sander, and R. W. Mast. 2003. Safety assessment of DHA-rich microalgae from *Schizochytrium* sp. Part V: target animal safety/toxicity study in growing swine. *Regul. Toxicol. Pharmacol.* 37(1):73-82.
- Albertazzi, P., and K. Coupland. 2002. Polyunsaturated fatty acids. Is there a role in postmenopausal osteoporosis prevention? *Maturitas.* 42(1):13-22.
- Ashes, J. R., B. D. Siebert, S. K. Gulati, A. Z. Cuthbertson, and T. W. Scott. 1992. Incorporation of n-3 fatty acids of fish oil into tissue and serum lipids of ruminants. *Lipids* 27:629-631.
- Auer, D. E., J. C. NG, H. L. Thompson, S. Inglis, and A. A. Seawright. 1989. Acute phase response in horses: changes in plasma cation concentrations after localized tissue injury. *Vet. Record.* 124:235-239.
- Barclay, W. R., K. M. Meager, and J. R. Abril. 1994. Heterotrophic production of long chain omega-3 fatty acids utilizing algae and algae-like microorganisms. *J. Appl. Phycol.* 6:123-129.
- Barclay, W. R., R. Abril, P. Abril, C. Weaver, and A. Ashford. 1998. Production of DHA from microalgae and its benefits for use in animal feeds. *World Rev. Nutr. Diet.* 83:61-76.
- Behme, M. T. 1996. Dietary fish oil supplementation enhances insulin sensitivity in miniature pigs. *J. Nutr.* 126:1549-1553.
- Bezard, J., J. P. Blond, A. Bernard, and P. Clouet. 1994. The metabolism and availability of essential fatty acids in animal and human tissues. *Reprod. Nutr. Dev.* 34:539-568.
- Bjerve, K. S., A. M. Brubakk, K. J. Fougner, H. Johnsen, K. Midthjell, and T. Vik. 1993. Omega-3 fatty acids: Essential fatty acids with important biological effects, and serum phospholipid fatty acids as markers of dietary omega 3-fatty acid intake. *Am. J. Clin. Nutr.* 57:801S-805S.
- Bloomer, R. J., D. E. Larson, K. H. Fisher-Wellman, A. J. Glin, and B. K. Schilling. 2009. Effect of eicosapentaenoic and docosahexaenoic acid on resting and exercise-induced inflammatory and oxidative stress biomarkers: a randomized, placebo controlled, crossover study. *Lipids Health Dis.* 8(36):1476-1511.
- Bojensen, I. N. and E. Bojensen. 1998. Nature of the elements of transporting long-chain fatty acids through the red cell membrane. *J. Membrane Biol.* 163:169-181.
- Brilla, L. B., and T. E. Landerholm. 1990. Effect of fish oil supplementation and exercise on serum lipids and aerobic fitness. *J. Sports Med. Phys. Fitness* 30:173-180.

- Brinsko, S. P., D. D. Varner, C. C. love, T. L. Blanchard, B. C. Day, and M. E. Wilson. 2005. Effect of feeding a dha-enriched nutraceutical on the quality of fresh, cooled and frozen stallion semen. *Theriogenology* 63:1519-1527
- Broughton, K. S., C. S. Johnson, B. K. Pace, M. Liebman, and K. M. Kleppinger. 1997. Reduced asthma symptoms with n-3 fatty acid ingestion are related to 5-series leukotriene production. *Am. J. Clin. Nutr.* 65:1011-17.
- Browning, L. M. 2003. n-3 Polyunsaturated fatty acids, inflammation and obesity-related disease. *Proc. Nutr. Soc.* 62:447–453.
- Burdge, G. C., and P. C. Calder. 2005. α -Linolenic acid metabolism in adult humans: the effects of gender and age on conversion to longer-chain polyunsaturated fatty acids. *Eur. J. Lipid Sci. Technol.* 107:426–439.
- Bylevel, P.M., G. T. Pang, R. L. Clancy, and D. C. K. Roberts. 1999. Fish oil feeding delays influenza virus clearance and impairs production of interferon- γ and virus-specific immunoglobulin in lungs of mice. *J. Nutr.* 129:328–335.
- Calder, P. C. 2001a. N-3 polyunsaturated fatty acids, inflammation, and immunity: pouring oil on troubled waters or another fishy tale? *Nutr. Res.* 21:309-341.
- Calder, P. C. 2001b. Polyunsaturated fatty acids, inflammation, and immunity. *Lipids.* 36(9):1007-1024.
- Calder, P. C. 2002. Dietary modification of inflammation with lipids. *Proc. Nutr. Soc.* 61:345-358.
- Calder, P. C. 2006. N-3 polyunsaturated fatty acids, inflammation, and inflammatory diseases. *Am. J. Clin. Nutr. Suppl*(83):1505S-1519S.
- Carter, R., K. H. Treiber, R. J. Geor, L. W. Douglass, and P. A. Harris. 2009. Prediction of incipient pasture associated laminitis from hyperinsulinaemia, hyperleptinaemia and generalized and localized obesity in a cohort of ponies. *Equine Vet. J.* 41:171-178.
- Cartwright, I. J., A. G. Pockley, J. H. Galloway, M. Greaves, and F. E. preston. 1985. The effects of dietary n-3 polyunsaturated fatty acids on erythrocyte membrane phospholipids, erythrocyte deformability and blood viscosity in healthy volunteers. *Artherosclerosis.* 55:267-281.
- Chicco, A., M. E. D'Alessandro, L. Karabatas, R. Gutman, and Y. B. Lombardo. 1996. Effect of moderate levels of dietary fish oil on insulin secretion and sensitivity, and pancreas insulin content in normal rats. *Ann. Nutr. Metab.* 40:61–70.

- Cleland, L. G., M. J. James, and S. M. Proudman. 2006. Fish oil: what the prescriber needs to know. *Arthritis Res. Ther.* 8:202-210.
- Conquer, J. A. and B. J. Holub. 1996. Supplementation with an algae source of docosahexaenoic acid increases (n-3) fatty acid status and alters selected risk factors for heart disease in vegetarian subjects. *J. Nutr.* 126:3032-3039.
- Demaison, L., J. Blet, J. P. Sergiel, S. Gregoire, and D. Argaud. 2000. Effect of dietary polyunsaturated fatty acids on contractile function of hearts isolated from sedentary and trained rats. *Reprod. Nutr. Dev.* 40:113-125.
- De Moffarts, B., K. Portier, N. Kirschvink, J. Coudert, N. Fellman, E. van Erck, C. Letellier, C. Motta, J. Pincemail, T. Art, and P. Lekeux. 2007. Effects of exercise and oral antioxidant supplementation enriched in (n-3) fatty acids on blood oxidant markers and erythrocyte membrane fluidity in horses. *Vet. J.* 174:113-121.
- Duncan J. and Prasse K. Reference values. In: *Veterinary laboratory medicine*. 2nd ed. Ames: Iowa State University Press, 1986;227-234.
- Eckersall P. Proteins, proteomics, and the dysproteinemias. In: Kaneko J, Harvey J, Bruss M, eds. *Clinical biochemistry of domestic animals*. 6th ed. Boston: Academic Press. Elsevier, 2008;117-155.
- Feng, C., D. H. Keisler, and K. L. Fritsche. 1999. Dietary omega-3 polyunsaturated fatty acids reduce IFN-gamma receptor expression in mice. *J. Interferon Cytokine Res.* 19:41-48.
- Franklin, S.T., K. R. Martin, R. J. Baer, D. J. Schingoethe, and A. R. Hippen. 1999. Dietary marine algae (*Schizochytrium* sp.) increases concentrations of conjugated linoleic, docosahexaenoic and transvaccenic acids in milk of dairy cows. *J. Nutr.* 129:2048-52.
- Goldberg, R. J., and J. Katz. 2007. A meta-analysis of the analgesic effects of omega-3 polyunsaturated fatty acid supplementation for inflammatory joint pain. *Pain.* 129:210-223.
- Grimble, R. F., W. M. Howell, G. O'Reilly, S. J. Turner, O. Markovic, S. Hirrell, J.M. East, and P.C. Calder. 2002. The ability of fish oil to suppress tumor necrosis factor alpha production by peripheral blood mononuclear cells in healthy men is associated with polymorphisms in genes that influence tumor necrosis factor alpha production. *Am. J. Clin. Nutr.* 76: 454-459.
- Hall, J. A., R. J. Van Saun, S. J. Tornquist, J. L. Gradin, E. G. Pearson, and R. C. Wander. 2004a. Effect of type of dietary polyunsaturated fatty acid supplement (corn oil or fish oil) in immune response in healthy horses. *J. Vet. Intern. Med.* 18:880-886.

- Hall, J. A., R. J. Van Saun, and R. C. Wander. 2004b. Dietary (n-3) fatty acids from menhaden fish oil alter plasma fatty acids and leukotrienes b synthesis in healthy horses. *J. Vet. Intern. Med.* 18:871-879.
- Hansen, R. A., C. J. Savage, K. Reidlinger, J. L. Traub-Dargatz, G. K. Ofilvie, D. Mitchell, and M. J. Fettman. 2002. Effects of dietary flaxseed oil supplementation on equine fatty acid concentrations and whole blood platelet aggregation. *J. Vet. Intern. Med.* 16:457-463.
- Hanson, R. R., L. R. Smalley, G. K. Huff, S. White, and T. A. Hammad. 1997. Oral treatment with a glucosamine-chondroitin sulfate compound for degenerative joint disease in horses: 25 cases. *Equine Practice.* 19(9):16-22.
- Harbige, L. S. 2003. Fatty acids, the immune response, and autoimmunity: a question of n-6 essentiality and the balance between n-6 and n-3. *Lipids.* 38:323–341.
- Harris, M. A., L. H. Baumgard, M. J. Arns, and S. K. Webel. 2005. Stallion spermatozoa membrane phospholipid dynamics following dietary n-3 supplementation. *Anim. Reprod. Sci.* 89:234-237.
- Hayashi, N., T. Tashiro, H. Yamamori, K. Takagi, Y. Morishima, Y. Otsubo, T. Sugiura, K. Furukawa, H. Nitta, N. Nakajima, N. Suzuki, and I. Ito. 1998. Effects of intravenous omega-3 and omega-6 fat emulsion on cytokine production and delayed type hypersensitivity in burned rats receiving total parenteral nutrition. *J. Parenter. Enteral Nutr.* 22:363–367.
- Headley, S., J. Coverdale, T. Jenkins, L. Boone, J. Sharp, and K. Vernon. 2011. Dietary supplementation of conjugated linoleic acid in horses. *J. Equine Vet. Sci.* 31:230.
- Henneke, D., G. Potter, J. Kreider, and B. Yates. 1983. Relationship between condition score, physical measurements and body fat percentage in mares. *Equine Vet. J.* 15:371-372.
- Hoffman, R. M., J. P. Kayser, R. M. Lampley, and J. C. Haffner. 2011. Dietary fish oil supplementation affects plasma fatty acids and glycemic response but not insulin sensitivity in horses. *J. Equine Vet. Sci.* 31:252-253.
- Howard, A. D., G. D. Potter, E. M. Michael, P. G. Gibbs, D. M. Hood, and B. D. Scott. 2003. Heart rates, cortisol, and serum cholesterol in exercising horses fed diets supplemented with omega-3 fatty acids. Pages 41-46 in *Proc. 18th Equine Nutr. Physiol. Soc. Symp.*, East Lansing, MI.
- Hubbard, N.E., R. S. Chapkin, and K. L. Erickson. 1994. Effect of dietary linseed oil on tumoricidal activity and eicosanoid production in murine macrophages. *Lipids.* 29:651–655.
- Jain, N.C. 1986. *Schalm's Veterinary Hematology*, Fourth edition. Philadelphia, PA.

- Jump, D. B. 2002. Dietary polyunsaturated fatty acids and regulation of gene transcription. *Curr. Opin. Lipidol.* 13:155-164.
- Kester, W. O. 1991. Definition and Classification of Lameness. Page 19 in *Guide for Veterinary Service and Judging of Equestrian Events*, 4th ed. American Association of Equine Practitioners, Lexington, KY.
- Khol-Parisini, A., R. van den Hoven, S. Leinker, H. W. Hulan, and J. Zentek. 2007. Effects of feeding sunflower oil or seal blubber oil to horses with recurrent airway obstruction. *Can. J. Vet. Res.* 71:59-65.
- King, S. S., A. A. Abughazeleh, S. K. Webel, and K. L. Jones. 2008. Circulating fatty acid profiles in response to three levels of dietary omega-3 fatty acid supplementation in horses. *J. Anim. Sci.* 86:1114-1123.
- Knapp, H. R. 1995. Omega-3 fatty acids in respiratory diseases: a review. *J. Am. College Nutr.* 14:18-23.
- Kruglik, V. L., J. M. Kouba, C. M. Hill, K. A. Skjolaas-Wilson, C. Armendariz, J. E. Minton, and S. K. Webel. 2005. Effect of feeding protected n-3 polyunsaturated fatty acids on plasma and milk fatty acid levels and IgG concentrations in mares and foals. Pages 135-136 in *Proc. 19th Equine Soc.*
- Lands, W. E. M., 2005. *Fish, omega 3 and human health*. 2nd ed. Amer. Oil. Chemists Soc., Champaign, Ill.
- Li, Y., M. F. Seifert, D. M. Ney, M. Grahn, A. L. Grant, K. G. Allen, and B. A. Watkins. 1999. Dietary conjugated linoleic acids alter serum IGF-1 and IGF binding protein concentrations and reduce bone formation in rats fed (n-6) or (n-3) fatty acids. *J. Bone Miner. Res.* 14:1153-1162.
- Liu, S., V. E. Baracos, H. A. Quinney, and M. T. Clandinin. 1994. Dietary omega-3 and polyunsaturated fatty acids modify fatty acyl composition and insulin binding in skeletal muscle sarcolemma. *Biochem. J.* 299:831-837.
- Lippmeier, J. C., K. S. Crawford, C. B. Owen, A. A. Rivas, J. G. Metz, and K. E. Apt. 2009. Characterization of both polyunsaturated fatty acid biosynthetic pathways in *Schizochytrium* sp. *Lipids.* 44:621-630.
- Lortet, S., and P. Verger. 1995. Alteration of cardiovascular function in trained rats fed with fish oil. *Int. J. Sports Med.* 16:519-521.
- Luther, G. D., H. U. Cox, and G. T. Dimopoulos. 1981. Fatty acid composition of equine plasma. *Am. J. Vet. Res.* 42(1):91-93.

- Luther, G. D., H. U. Cox, and G. T Dimopoulos. 1982. Fatty acid composition of equine erythrocytes. *Am. J. Vet. Res.* 43(6):1006-1008.
- MacLean, C. H., S. J. Newberry, and W. A. Mojica. 2006. Effects of omega-3 fatty acids on cancer risk: a systematic review. *J. Am. Med. Assoc.* 295:403-415
- Manhart, D. R., B. D. Scott, P. G. Gibbs, J. A. Coverdale, E. M. Eller, C. M. Honnas, and D. M. Hood. 2009. Markers of inflammation in arthritic horses fed omega-3 fatty acids. *Prof. Anim. Sci.* 25:155-160.
- Mantzioris, E., M. J. James, R. A. Gibson, and L. G. Cleland. 1994. Dietary substitution with an a-linolenic acid-rich vegetable oil increases eicosapentaenoic acid concentrations in tissues. *Am J Clin Nutr.* 59:1304 - 1309.
- McCann, M. E. , J. N. Moore, J. B. Carrick, and M. H. Barton. 2000. Effect of intravenous infusion of omega-3 and omega-6 lipid emulsions on equine monocyte fatty acid composition and inflammatory mediator production in vitro. *Shock.* 14:222-228.
- McCarty, M. F. 1998. Complementary measures for promoting insulin sensitivity in skeletal muscle. *Med. Hypoth.* 51:451-464.
- Mori, T. A., and L. J. Beilin. 2004. Omega-3 fatty acids and inflammation. *Curr Atheroscler Rep.* 6:461-467.
- Morris D. D. , M. M. Henry, J. N. Moore, and J. K. Fischer. 1991. Effect of dietary alpha linolenic acid on endotoxin-induced production of tumor necrosis factor by peritoneal macrophages in horses. *Am J Vet Res.* 52:528-532.
- Mueller, B. A., and R. L. Talbert. 1988. Biological mechanisms and cardiovascular effects of omega-3 fatty acids. *Clin. Pharm.* 7:795-807.
- Munsterman, A. S., A. L. Bertone, T. A. Zachos, and S. E. Weisbrode. 2005. Effects of the omega-3 fatty acid, alpha-linolenic acid, on lipopolysaccharide-challenged synovial explants from horses. *Am. J. Vet. Res.* 66:1503-1508.
- Nagakura, T., S. Matsuda, K. Shichijvo, H. Sugimoto, and K. Hata. 2000. Dietary supplementation with fish oil rich in n-3 polyunsaturated fatty acids in children with bronchial asthma. *Eur. Respir. J.* 16:861-865.
- Neuringer, M., G. J. Anderson, and W. E. Connor. 1988. The essentiality of n-3 fatty acids for the development and function of the retina and brain. *Annu. Rev. Nutr.* 8:517-541.
- NRC. 2007. Nutrient requirements of Horses. 6th rev. ed. Natl. Acad. Press, Washington, DC.

- O'Connor, C.I., L.M. Lawrence, A.C. St. Lawrence, K.M. Jnicki, L.K. Warren, and S. Hayes. 2004. The effect of dietary fish oil supplementation on exercising horses. *J. Anim. Sci.* 82:2978-2984.
- O'Connor, C. I., L. M. Lawrence, and S. H. Hayes. 2007. Dietary fish oil supplementation affects serum fatty acid concentrations in horses. *J. Anim. Sci.* 85:2183-2189.
- Oostenburg, G. S., R. P. Mensink, M. R. Hardeman, T. De Vries, F. Brouns, and G. Hornstra. 1997. Exercise performance, red blood cell deformability, and lipid peroxidation: effects of fish oil and vitamin E. *Eur. J. Appl. Physiol.* 83:746-752.
- Orme, C. E., M. Dennett, and R. C. Harris. 1994. Variation in the concentration of long chain free fatty acids in equine plasma over 24 hours. *Brit. Vet. J.* 150(4):339-347.
- Portier, K. B., B. De Moffarts, N. Fellman, N. Kirschvink, C. Motta, C. Letellierw, A. Ruelland, E. van Erck, P. Lekeux, and J. Couder. 2006. The effects of dietary n-3 and antioxidant supplementation on erythrocyte membrane fatty acid composition and fluidity in exercising horses. *Equine Vet. J. Suppl.* 279-284.
- Rogers C., E. Firth, and B. Anderson. 2005. Musculoskeletal responses of two-year old Thoroughbred horses to early training. *New Zealand Vet.J.* 53:95–100.
- Ross, T. N., G. D. Potter, E. M. Eller, B. D. Scott, P. G. Gibbs, and H. W. Sampson. 2007. Evaluation of inflammatory markers in yearlings fed various sources of polyunsaturated fatty acids. Page 104 in *Proc. 20th Equine. Soc.*.
- Ross, T., T. Hess, D. Hansen, T. Engle, N. Schauermaann, and J. Rexford. 2011. Synovial prostaglandin E₂ concentrations in horses fed long chain polyunsaturated fatty acids: an inquiry study. *J. Equine Vet. Sci.* 31:270.
- Sadeghi, S., F. A. Wallace, and P. C. Calder. 1999. Dietary lipids modify the cytokine response to bacterial lipopolysaccharide in mice. *Immunology.* 96:404–410.
- Siciliano, P. D., S. K. Webel, L. S. Brown, L. K. Warren, T. E. Engle, and P. D. Burns. 2003. Effect of n-3 polyunsaturated fatty acid source on plasma fatty acid profiles of horses. *J. Anim. Sci. (Suppl. 1)* 81:72.
- Simopoulos, A. P. 1991. Omega-3 fatty acids in health and disease and in growth and development. *Am. J. Clin. Nutr.* 54:438-463.
- Simopoulos, A. P. 2008. The importance of the omega-6/omega-3 fatty acid ratio in cardiovascular disease and other chronic diseases. *Exp. Biol. Med (Maywood)* 233:674-688.

- Spearman, K. R., E. A. Ott, J. Kivipelto, and L. K. Warren. 2005. Effect of fatty acid supplementation of the mare on mil and foal plasma composition and foal growth. Pages 3-4 in Proc. 19th Equine Sci. Soc. Symp., Tuscon, AZ. Equine Sci. Soc., Champagne, IL.
- Suagee, J. K., A. O. Burk, R. W. Quinn, T. G. Hartsock, and L. W. Douglass. 2011. Effects of diet and weight gain on circulating tumor necrosis factor- α concentrations in Thoroughbred geldings. *J. Anim. Physiol. Anim. Nutr.* 95:161-170.
- Sugano M. 1996. Characteristics of fats in Japanese diets and current recommendations. *Lipids.* 31(Suppl. 1):283-286.
- Sundrarjun, T., S. Komindr, N. Archararit, W. Dahlan, O. Puchaiwatananon, S. Angthararak, U. Udomsuppayakul, and S. Chuncharunee. 2004. Effects of n-3 fatty acids on serum interleukin-6, tumour necrosis factor- α , and soluble tumour necrosis factor receptor p55 in active rheumatoid arthritis. *J. Intern. Med. Res.* 32:443-454.
- Tashiro, T., H. Yamamori, K. Takagi, N. Hayashi, K. Furukawa, and N. Nakajima. 1998. n-3 versus n-6 polyunsaturated fatty acids in critical illness. *Nutrition.* 14:551-553.
- Todd, J. C., A. H. Sanford, I. Davidsohn, and J. B. Henry. 1974. Clinical diagnosis by laboratory Methods. Philadelphia, PA.
- Toutain, P., and C. Cester. 2004. Pharmacokinetic-pharmacodynamic relationships and dose response to meloxicam in horses with induced arthritis in the right carpal joint. *Am. J. Vet. Res.* 65:1533-1541.
- Turek, J.J., I. A. Schoenlein, and G. D. Bottoms. 1991. The effect of dietary n-3 and n-6 fatty acids on tumor necrosis factor- α production and leucine aminopeptidase levels in rat peritoneal macrophages. *Prostaglandins Leukot. Essent. Fatty Acids.* 43:141-149.
- Vick, M. M., A. A. Adams, B. A. Murphy, D. R. Sessions, D. W. Horohov, R. F. Cook. B. J. Shelton, and B. P. Fitzgerald. 2007. Relationships among inflammatory cytokines, obesity, and insulin sensitivity in the horse. *J. Anim. Sci.* 85:1144-1155.
- Vineyard, K. R., L. K. Warren, and J. Kivipelto. 2009. Effect of dietary omega-3 fatty acid source on plasma and red blood cell membrane composition and immune function in yearling horses. *J. Anim. Sci.* 88(1)248-257.
- Warner, J. G., I. H. Ullrich, M. J. Albrink, and R. A. Yeater. 1989. Combined effects of aerobic exercise and omega-3 fatty acids in hyperlipidemic persons. *Med. Sci. Sports Exerc.* 21:498-505.
- Watkins, B., Y. Li, K. Allen, W. Hoffman, and M. Seifert. 2000. Dietary ratio of (n-6)/(n-3) polyunsaturated fatty acids alters the fatty acid composition of bone compartments and biomarkers of bone formation in rats. *J. Nutr.* 130:2274-2284.

- White, G., T. Stites, E. Jones, and S. Jordan. 2003. Efficacy of intramuscular chondroitin sulfate and compounded acetyld-glucosamine in a positive controlled study of equine carpalis. *J. Equine Vet. Sci.* 23:295–300.
- Williams, C. M., and G. Burdge. 2006. Long-chain n-3 PUFA: plant v. marine sources. *Proc. Nutr. Soc.* 65:42-50.
- Williard, M.D., H. Tvedten, and G. H. Turnwald. 1989. *Small Animal Clinical Diagnosis By Laboratory methods.* Philadelphia, PA.
- Woodward, A. D., B. D. Nielsen, C. I. O'Connor, C. D. Skelly, S. K. Webel, and M. W. Orth. 2007. Supplementation of dietary long-chain polyunsaturated omega-3 fatty acids high in docosahexaenoic acid (dha) increases plasma dha concentration and may increase trot stride length in horses. *Equine and Compar. Exer. Physiol.* 4:71-78.