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EFFECTS OF A LONG-CHAIN POLYUNSATURATED OMEGA-3 FATTY ACID SUPPLEMENT ON FATTY ACID PROFILES, LAMENESS, AND STRIDE LENGTH IN HORSES

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By

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ABSTRACT

EFFECTS OF A LONG-CHAIN POLYUNSATURATED OMEGA-3 FATTY ACID SUPPLEMENT ON FATTY ACID PROFILES, LAMENESS, AND STRIDE LENGTH IN HORSES

By

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Twelve mature and six 2-year-old Arabian horses were used to determine the effect of dietary long-chain polyunsaturated omega-3 fatty acid supplementation on fatty acids, bone markers, and lameness. Stride lengths at the walk and trot, lameness scores, and range of motion of leg joints were measured on d 0 before horses were pair-matched and fed either a treatment diet (FA) containing stabilized omega-3 fatty acids or a control diet (CO) containing corn oil for 75 d. Horses were exercised 5 d/wk, and blood samples were drawn and body weights recorded on d 0, 25, 50, and 75. Stride lengths, lameness exams, and range of motion were recorded again on d 75. Total omega-3 fatty acid concentrations were higher in FA horses than CO horses. Total omega-6 fatty acids increased from d 0 to d 25 before returning to baseline on d 75 in all horses. The ratio of omega-6:omega-3 fatty acids was lower in FA horses. Plasma EPA concentrations tended to increase over time in FA horses. FA horses had increased plasma DHA on d 25, d 50, and d 75. There was no difference in walk stride length; however, FA horses tended to have a longer trot stride after supplementation. There were no differences in osteocalcin, ICTP, PGE_2, or TNF-α. Supplementing omega-3 fatty acids increases EPA and DHA, while a trend to increase trot stride length indicates a possible decline in joint pain associated with arthritis, potentially by inhibiting inflammatory pathways.

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INTRODUCTION

Recent studies in both humans and animals have shown beneficial effects of feeding fish oils, which contain omega-3 fatty acids. Omega-3 fatty acids reduce the occurrence of lipid disorders and coronary artery disorder, and increase red blood cell deformability in humans (Leaf, 1990). In horses, fish oils reduce heart rates associated with intense exercise (O'Connor et al., 2004). Bone formation will increase in animals supplemented with an omega-3 fatty acid rich diet (Watkins et al., 1997; Reinwald et al., 2004). Furthermore, omega-3 fatty acids found in fish oils can mitigate the actions of inflammatory pathways, leading to more comfortable living conditions for people suffering from osteoarthritis (Lee et al., 1985; Curtis et al., 2000). The two relevant fatty acids that accumulate in the bloodstream are eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA).

Because EPA and DHA are essential fatty acids, dietary supplementation of fish oils is a convenient method of introducing EPA and DHA into the bloodstream. Ashes et al. (1992) reported that the amount of fish oil supplemented was proportional to the amount of omega-3 fatty acids seen in plasma fatty acid profiles. Furthermore, changes in fatty acid profiles due to diets containing omega-3 fatty acids can be observed within a few weeks of supplementation. Hall et al. (2004) noted that changes to total omega-6 and total omega-3 fatty acid concentrations changed in horses after only 6 weeks of supplementation.

Changes in fatty acid profiles bring about changes in the omega-6:omega-3 fatty acid ratio, which can affect with metabolic pathways that regulate bone formation and inflammation. Decreased omega-6:omega-3 will cause a decrease in arachidonic acid concentrations (Watkins et al., 1997), a substrate in the prostaglandin pathway. The prostaglandin pathway will lead to formation of prostaglandin E_2 (PGE_2), a hormone that plays roles in both bone development and inflammation (Watkins et al., 2000; James et al., 2000). Decreasing the amount of PGE_2 will cause an increase in the amount of bone formation as well as a decrease in inflammation, both of which are beneficial to athletic horses, especially horses that are exercised at high intensities.

Unfortunately, odor associated with fish oil supplementation necessitated the development of other means of supplying EPA and DHA. The purpose of this research is to examine the effects of a long-chain polyunsaturated omega-3 fatty acid supplement on plasma fatty acid profiles, incorporation of fatty acids into red blood cell membranes, and indicators of bone metabolism and inflammation in horses. I hypothesize supplementation of omega-3 fatty acids will increase EPA and DHA levels in blood serum and red blood cells, cause favorable changes in markers of bone metabolism and inflammation, and cause a reduced lameness grade among pair-matched horses. Additionally, omega-3 fatty acids will improve stride length among pair-matched horses, since joint pain can reduce stride length.

CHAPTER 1

REVIEW OF LITERATURE

Fatty Acids

Every year, more people are becoming aware of the roles fatty acids play in metabolic pathways and the beneficiary effects these dietary ingredients can have in the body. Fatty acids have a carboxyl end group followed by a chain of carbon atoms and have the atomic structure $C_nH_{2n+1}COOH$. They can be either saturated, containing no double bonds, or unsaturated, containing at least one double bond (Bauer, 1994). Polyunsaturated fatty acids, unsaturated fatty acids containing more than one double bond, are classified by the position of their first double bond from the methyl end: oleic acid has the first double bond at carbon nine, linoleic acid has the first double bond at carbon six, and linolenic acid has the first double bond at carbon three. Different classes of fatty acids may determine how essential a fatty acid is in the diet (Bauer, 1994); a fatty acid is considered essential when it is needed by the body but either not produced or not produced in high enough concentrations by a particular animal.

Fatty acids, while a small fraction of the normal equine diet, are gaining popularity in the realm of equine nutrition. In order to identify the fatty acids that are essential to equine, the fatty acid profiles of horses had to be determined. Luther et al. (1981) were the first to perform work on plasma fatty acid concentrations in horses using gas chromatography, a technique which produced more accurate results than any reported previously. Luther et al. (1981) showed that the highest fatty acid in equine plasma was

linolenic acid, followed by stearic acid, oleic acid, and palmitic acid. Combined, these four fatty acids made up over 85% of the total fatty acids in plasma (Luther et al., 1982). Concentrations of fatty acids are tissue specific. For example, when the same techniques were applied to harvested equine erythrocytes, the fatty acid profile changed significantly, with stearic acid making up most of the plasma fatty acid profile, followed by palmitic acid and oleic acid (Luther et al., 1982). These studies were the first to determine the fatty acid profiles of equine plasma and erythrocytes.

Free fatty acid levels not only differ between different parts of blood but also fluctuate throughout the day (Orme et al., 1994; Luther et al., 1981; Luther et al., 1982). Two studies conducted to determine the existing concentration of fatty acids in plasma (Luther et al., 1982; Orme et al., 1994) reported concentrations of particular fatty acids vary from one research trial to the next. Orme et al. (1994) stated that equine plasma contained mostly palmitic acid, followed by linoleic acid, oleic acid, stearic acid, and linolenic acid. Palmitic acid remained high throughout a normal 24-hour period; however, free fatty acid profiles showed that concentrations of oleic acid increased whereas stearic acid concentrations decreased throughout the testing period (Orme et al., 1994). These results suggest that concentrations of fatty acids fluctuate depending on time of day and fasting state in horses, and plasma fatty acid profiles can also be influenced by diet.

Omega-3 Fatty Acids

There are many classes of fatty acids; however omega-3 fatty acids have elicited considerable interest in recent years. In omega-3 fatty acids, the first double bond occurs at the third carbon from the methyl end. Omega-3 fatty acids must be supplemented to animals, as most are not synthesized in the body. Omega-3's are found mainly in chloroplasts of marine phytoplankton (Uauy-Dagach and Valenzuela, 1996), the base of the marine food chain, and as a product of some oil seeds, such as in canola, soybean, and linseed oils (Hall et al., 2004). Omega-3 fatty acids have beneficial effects on inflammation and cardiac responses in all types of animals, including humans (Leaf, 1990; Lee et al., 1985; Curtis et. al., 2000) and horses (Wilson et al., 2003; O'Connor et al., 2004). Omega-3 fatty acids are considered to be healthier for animals than their counterpart, omega-6 fatty acids. Omega-6 fatty acids have a double bond at the sixth carbon from the methyl end, and are thought to cause more damage in metabolic pathways associated with bone formation and inflammation (Watkins et al., 1997; Watkins et al., 2000; James et al., 2000).

Omega-3 fatty acids include eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), both of which are beneficial to mammals by reducing lipid disorders and coronary artery disorder, and increasing red blood cell deformability in humans (Leaf, 1990). Furthermore, omega-3 fatty acids can mitigate inflammation, leading to more comfortable living conditions for people suffering from osteoarthritis (Lee et al., 1985; Curtis et al., 2000). However, for these benefits to occur, EPA and DHA must be present in the blood stream so that they can be easily incorporated into tissues.

Studies have indicated that concentrations of fatty acids in diets are correlated to concentrations of fatty acids in plasma and in tissues, especially when considering EPA and DHA. Because the body cannot synthesize these two fatty acids, the only way to incorporate them into the body and induce a response is by eating a diet rich in foods containing omega-3 fatty acids, such as fish, or through dietary supplementation that contains EPA, DHA, or both. Kuriki et al. (2003) discovered a positive correlation between the amount of marine oils in the diet and EPA and DHA concentrations in the blood. These results show that marine oil supplementation positively affects the amount of EPA and DHA in the blood.

Dietary supplementation of fish oils is one of the most convenient methods of introducing EPA and DHA into the bloodstream. Ashes et al. (1992) discovered that serum lipid concentrations of omega-3 fatty acids were higher in sheep fed a fish oil supplement than sheep fed a control diet. In horses, six weeks of supplementation with fish oil showed an increase of plasma EPA and DHA concentrations compared with horses fed corn oil (Hall et al., 2004). The concentration of omega-3 fatty acids is also proportional to the amount of supplement fed (Ashes et al., 1992). These results are in agreement with Christensen et al. (1999), who observed that human subjects fed different quantities of EPA and DHA retained more omega-3 fatty acids when consuming the diets supplemented with 6.6 g omega-3 polyunsaturated fatty acids (PUFA) versus 2.0 g of the same supplement. Studies have also indicated that the amount of EPA and DHA in plasma directly corresponds with the ratio of these two fatty acids in the diet (Vidgren et

al., 1997), ratios that can be different depending on where the omega-3 fatty acids were obtained (Hansen et al., 2002). For example, sources of EPA and DHA are reflected within the plasma fatty acid concentrations of the horse. While marine oil increased both EPA and DHA, flaxseed oil only increased EPA; plasma concentrations of DHA were not different in horses fed flaxseed oil compared with control horses (Hansen et al., 2002). For plasma omega-3 fatty acid concentrations to change in horses, they must receive a diet that contains both omega-3 fatty acids.

Omega-3 Fatty Acids and Red Blood Cells

Not only is the concentration of plasma fatty acids altered upon supplementation; changes can also be seen in triglycerides, cholesterol esters, phospholipids, and, perhaps most importantly, erythrocytes (Vidgren et al., 1997). EPA concentrations increased in human male red blood cells with only three days of supplementation of a fish oil pill high in omega-3 fatty acids (Katan et al., 1997). After 28 days of supplementation, a half-maximal concentration was reached. The increase in omega-3 fatty acid concentration was noted despite the 120-day lifespan of red blood cells, suggesting that erythrocyte composition is influenced by the plasma fatty acid profile (Katan et al., 1997). Furthermore, Knapp et al. (1994) examined the compositional change of erythrocyte membranes when human subjects were fed EPA and DHA and discovered that these fatty acids were selectively incorporated into phospholipids located in the inner membrane of erythrocytes, which causes asymmetry of the erythrocyte lipid bilayer and membrane (Escudero et al., 1998; Knapp et al., 1994). The number of double bonds in a fatty acid will influence the asymmetry of erythrocyte bilayers (Bojesen and Bojesen, 1998).

Bojesen and Bojesen (1998) suggest that omega-3 fatty acids compete with other fatty acids for incorporation into the lipid bilayer. This competition was also noted by Katan et al. (1997), who observed that an increase in erythrocyte EPA was inversely related to a decrease in erythrocyte omega-6 fatty acids. Furthermore, Katan et al. (1997) believed that only DHA was incorporated into the inner phospholipid bilayer and that EPA could exchange between the plasma phospholipids; however, this theory has not been proven. Still, studies such as these have shown the membrane phospholipid bilayer of erythrocytes to be diverse and changing when influenced by omega-3 fatty acids.

The effect of EPA and DHA on fluidity of erythrocytes through blood vessels is closely related to the ability of the red blood cell to deform for easier passage through small blood vessels (Ho et al., 1999; Leaf, 1990). Cartwright et al. (1985) gave human subjects a maxEPA supplement (a fish oil concentration dietary supplement) for six weeks, sampling red cell filtration rate at weeks three and week six. Cartwright et al. (1985) noted that erythrocyte deformability increased throughout the study. This result is in agreement with Ernst (1989), who noted that, after four weeks of supplementation, red blood cell deformability had increased and blood viscosity declined.

Further research has linked omega-3 fatty acids to other beneficial aspects of the circulatory system of mammals. Leaf (1990) reports that supplementing diets with omega-3 fatty acids causes increased activity of endothelial cell-derived relaxing factor, a hormone that relaxes arterial smooth muscle cells and decreases the amount of arterial constriction due to vasoconstrictor agents. The larger blood vessels may allow for a

lower heart rate in humans (Christensen et al., 1999). Omega-3 fatty acid supplementation coinciding with lower heart rates has also been found in horses. O'Connor et al. (2004) supplemented horses with fish oil or corn oil for 63 days, and then exercise-tested the horses. Horses receiving the fish oil treatment had lower heart rates and packed cell volume during exercise (O'Connor et al., 2004). This could indicate that omega-3 fatty acids respond similarly in horses and in humans, allowing for lower heart rates and increased blood flow both during exercise and during rest. The benefits of EPA and DHA to the coronary arteries, red blood cells, and the circulatory system in general are numerous, and supplementing diets with these fatty acids may greatly improve the life of mammals with heart problems.

Omega-3 Fatty Acids and Bone

Omega-3 fatty acids not only benefit the heart, but they also play a role in bone formation and resorption. Both EPA and DHA play direct roles in pathways that affect the bone matrix, as determined by measuring markers of bone modeling and remodeling (Weiler and Fitzpatrick-Wong, 2002). Bone modeling is described as the continuous growth of bone through the bone cells osteoblasts and osteoclasts until mature bone structure is achieved; bone remodeling is the reshaping through bone resorption and regrowth of mature adult bone that aids in maintaining skeletal mass. The markers of these two forms of bone metabolism are becoming reliable indicators of bone turnover rates and other changes in bone due to diet and exercise. Two of these bone markers are osteocalcin (OC), an indicator of bone formation, and the carboxy-terminal pyridinoline cross-linked telopeptide region of type I collagen (ICTP), an indicator of bone

degradation. Osteocalcin is synthesized by active osteoblasts, and serum levels are positively correlated to formation of bone. Osteocalcin consists of noncollagenous proteins, unlike ICTP, which is a derivative of type I collagen, the most abundant protein in bone. While OC is released due to excess formation, ICTP is only released when bone is being degraded. When viewed together, these markers are indicators of bone metabolism.

Studies throughout the past several years have attempted to determine what effects omega-3 fatty acids have on bone metabolism. Watkins et al. (2000) used weanling rats to demonstrate that omega-3 fatty acids were positive indicators of bone formation. Furthermore, Weiler and Fitzpatrick-Wong (2002) determined that a high concentration of plasma DHA actually reduced the amount of bone resorption in piglets, although bone mass was not significantly different. This research is supported by Reinwald et al. (2004), who noted longer tibia lengths in rats descended from parents with adequate levels of omega-3 fatty acids in their diets compared to rats descended from parents with a deficient amount of omega-3 fatty acids. Furthermore, rats fed a diet adequate in omega-3 fatty acids had increased tibia and femur diameters throughout a four-week period compared to rats deficient in omega-3 fatty acids (Reinwald et al., 2004). In contrast to these two studies, Judex et al. (2000) stated that fish oil diets did not significantly affect the amount of bone formation or bone resorption in rabbits. Research by Sirois et al. (2003) supported Judex et al. (2000). Sirois et al. (2003) measured bone dimensions in male and female rats fed a fish oil diet for five weeks. In this study, there was no difference in weight, length, or width of femurs or lumbar vertebra. Still, enough

scientific evidence indicates that EPA and DHA do have a significant effect on bone matrix metabolism when supplemented in a high proportion in the diet.

The effects of omega-3 fatty acids on bone formation are mediated through the prostaglandin pathway. Prostaglandin E_2 (PGE_2) is derived from arachidonic acid (AA) and is a regulatory hormone for bone modeling (Watkins et al., 1997). Research has shown that PGE_2 shows a concentration response to bone metabolism: at low concentrations, PGE_2 stimulates bone formation, but at high concentrations, it inhibits bone formation (Watkins et al., 1997). Eicosapentaenoic acid and DHA reduce the production of PGE_2 by inhibiting arachidonic acid from binding to phospholipids (Hui et al., 1989; Watkins et al., 2000). Watkins et al. (2000) determined that supplementing rats with various ratios of omega-6:omega-3 fatty acids caused arachidonic acid concentrations to fluctuate depending on the ratio being fed. As the ratio declined, arachidonic acid concentrations also declined, as did PGE_2 production (Watkins et al., 2000). Furthermore, if a diet supplements enough EPA to replace most arachidonic acid in the blood, a less potent prostaglandin will be produced with EPA as a substrate (Mueller and Talbert, 1988). Prostaglandin E_3 (PGE_3), a derivative of EPA, is also an inhibitor of bone formation; however, PGE_3 is less bioactive than PGE_2. Furthermore, EPA is not a good metabolic substrate for PGE_3 formation (Watkins et al., 2000). The decline in PGE_2 production seems to indicate that as the amount of EPA and DHA changes within the diet, so does the rate of bone formation and the amount of bone modeling occurring in the body.

Similar results were obtained in broiler chicks fed menhaden oil rich in omega-3 fatty acids; the chicks fed menhaden oil showed a decrease in the concentration of PGE_2 precursor in bone polar lipids (Watkins et al., 1997). Additionally, Escudero et al. (1998) reported that animals supplemented with fish oil exhibited the highest amounts of EPA and DHA and the lowest amounts of arachidonic acid. Eicosapentaenoic acid incorporation leaves little room for arachidonic acid to be metabolized by phospholipids due to competitive inhibition (Katan et al., 1997; Vidgren et al., 1997), and this lack of PGE_2 precursor results in greater bone growth and bone formation in animals.

Research has also shown that omega-3 fatty acids can slow the progress of bone diseases, such as osteoporosis. Sun et al. (2004) showed that rats fed DHA or EPA had a higher fracture force and fewer clinical signs of osteoporosis than control rats. Furthermore, these rats had a higher calcium absorption rate, indicating that EPA and DHA possibly make bones stronger through actions in calcium uptake. The benefits of omega-3 fatty acids on bone appear to be numerous and need to be studied in horses, as many horses succumb to lameness caused by bone injury and arthritis each year.

Omega-3 Fatty Acids and Inflammation Associated with Arthritis

Omega-3 fatty acids are not only important in keeping bones strong, but EPA and DHA inhibit inflammation caused by different types of arthritis. Many million Americans suffer from rheumatoid arthritis, and an estimated 200,000 new cases arise each year (Horrocks and Yeo, 1999). Humans given EPA and DHA in the diet experience less pain and inflammation associated with arthritis due to the anti-

inflammatory effects of these fatty acids (Horrocks and Yeo, 1999). However, humans are not the only ones who suffer from this disease.

A survey performed in 1982 and 1983 showed lameness to be the leading cause of lost training days in horses in race training (Rossdale et al., 1985). In each barn surveyed, at least 23.3% of horses demonstrated some sort of lameness during the training period, with injuries to joints causing 14% of the problems (Rossdale et al., 1985). Equine athletes inflict loading pressures on their joints with every step in the training process, and these pressures can lead to lameness and even osteoarthritis. Osteoarthritis (OA), sometimes called degenerative joint disease (DJD), is defined as a deterioration of the articular cartilage in synovial joints. Synovial joints enable movement and transfer load. Articular cartilage is defined as an avascular tissue containing few cells but a complex extracellular matrix that absorbs shock from movement and loading. During severe OA, articular cartilage is lost, causing pain, lameness, deformed joints, and loss of use. The loss of articular cartilage is a result of increased activity of proteolytic enzymes, which break down components of the extracellular matrix (Caron, 1992). Osteoarthritis can take an expensive toll on both horse and owner, as treatments give no definitive cure and the equine's quality of life and activity are curtailed (Tung et al., 2002).

Osteoarthritis can be caused by a variety of factors. Age, conformation, and use are just some of the confounding factors that can bring about this disease (Fenton et al., 1999). Stress from longitudinal loading subjects the joint to extreme pressure (Radin et

al., 1973), and this loading pressure increases as horses increase speed and activity (Fenton et al., 1999). Trauma is also believed to be a major factor in triggering OA, and even daily normal use of the joint could generate enough force to initiate the onset of OA (Caron, 1992). Prior to degradation, cartilage can stiffen, which confirms the notion that daily use may be a factor in OA (Radin et al., 1973). Because osteoarthritis is hard to reverse once started, finding ways to protect bone and cartilage is a major concern of the equine industry.

Prostaglandins are major components of the inflammatory pathways associated with OA. Prostaglandin E_2 produced by an inflamed joint can increase pain, swelling, and redness associated with inflammation, as well as impair joint function (James et al., 2000). When OA causes pain, membrane bound fatty acids are moved and broken into eicosanoids such as PGE_2 and leukotriene B_4 (Hall et al., 2004). Excess PGE_2 will cuse a degradation of proteoglycans in articular cartilage, causing breakdown of tissues. The metabolism of AA leads to increased PGE_2, which in turn increases inflammation, pain, and breakdown of cartilage — all signs of arthritis.

However, essential fatty acids, especially EPA and DHA, inhibit PGE_2, thereby decreasing the amount of inflammation associated with OA (Ho et al., 1999). Eicosapentaenoic acid can also serve as a precursor for the inflammation pathway (Hall et al., 2004), and increased EPA concentrations will lead to decreased arachidonic acid in tissues through inhibition of the enzymatic pathway (James et al., 2000; Mueller and Talbert, 1988). The release of EPA will compete with arachidonic acid for metabolism,

and increases in EPA will produce a milder inflammatory response (Simopoulos, 2002). Eicosapentaenoic acid and DHA are coupled with decreased arachidonic acid after two weeks of dietary supplementation in human males (Mantzioris et al., 2000). However, as previously mentioned, metabolism of EPA does produce eicosanoids, but these eicosanoids are of the less inflammatory 3 and 5 series, such as PGE_3 and leukotriene B_5 (LTB_5), and are less potent than those formed by arachidonic acid (Mueller and Talbert, 1988; Bauer, 1994; Hall et al., 2004;). Although both PGE_3 and LTB_5 produce inflammatory responses, they are only produced in small amounts when EPA is the substrate, thereby diminishing inflammation caused by OA (Leaf, 1990). Therefore, supplementing a diet with omega-3 fatty acids should reduce arachidonic acid metabolism in response to an injury, causing decreased inflammation associated with this pathway.

Hall et al. (2004) researched the prostaglandin pathway in horses by feeding diets with either menhaden oil for omega-3 fatty acids or corn oil for omega-6 fatty acids. Results from this study demonstrate that horses fed fish oil had higher leukotriene B_5 concentrations, which positively correlated with an increase in plasma EPA concentrations. Additionally, Volker et al. (2000) administered diets containing different fatty acids to female rats that had an acute onset of osteoarthritis. Inflammation was measured by hock circumference and footpad thickness. Rats fed diets rich in EPA and DHA had significantly reduced inflammation in their footpads (Volker et al., 2000).

Another hormone associated with pain and arthritis is tumor necrosis factor-α (TNF-α) (Arend and Dayer, 1995). Diets that include omega-3 fatty acids can inhibit the pathway that generates TNF-α and reduce pain. Endres et al. (1989) reported that humans fed omega-3 fatty acids from fish oil decrease synthesis of TNF-α and other inflammatory stimulants. Males fed meals high in omega-3 fatty acids showed a decrease in TNF-α after only two weeks of dietary change; however, concentrations were not different from baseline after four weeks (Mantzioris et al., 2000). These contrasting concentrations are a possible indicator of how rapidly TNF-α can change even with dietary supplementation.

Fish oil reduced TNF-α concentrations in patients with rheumatoid arthritis when provided as a fatty acid supplement (Kremer et al., 1990). Recently, however, Sundrarjun et al. (2004) observed no significant differences in TNF-α in patients with rheumatoid arthritis even though there was a significant increase in EPA and DHA in plasma. Furthermore, there were no differences in the amount of swollen or painful joints in the three month treatment period (Sundrarjun et al., 2004). Work with TNF-α in horses is scarce, and research into the effects of omega-3 fatty acids on TNF-α and arthritis still needs to be performed to further understand how these fatty acids effect the inflammatory pathways in equine.

Purpose of Research

The purpose of this research is to examine the effects of EPA and DHA supplementation on plasma fatty acid profiles, incorporation of fatty acids into red blood

cell membranes, and indicators of bone metabolism and inflammation in horses. I hypothesize that supplementation of omega-3 fatty acids will increase EPA and DHA levels in blood serum and red blood cells, cause favorable changes in markers of bone metabolism and inflammation, and cause a reduced lameness grade among pair-matched horses.

CHAPTER 2:

MATERIALS AND METHODS

Animals and Management

Twelve mature Arabians (2 stallions, 4 mares, 6 geldings) and six two-year-old Arabian geldings were obtained from the Michigan State University Horse Teaching and Research Center to test the effects of long-chain polyunsaturated fatty acids on fatty acid profiles and lameness. All but the two stallions and two 2-year-olds were living in mixed grass pasture prior to the start of this project. The remaining four horses were kept in 2.7 m x 3 m stalls and allowed daily turnout in 20 m x 25 m dry lots prior to being placed on the project. The stalled horses were on a diet consisting of approximately 1 kg oats and 5 kg Timothy grass hay per day. Horses on pasture did not receive any grain supplements prior to the start of the project. All but three horses (two stallions and one mare) were being used in classes at Michigan State University. All horses were considered to be in moderate condition before the start of the trial.

Horses were kept in 2.7 m x 3 m box stalls during the duration of the project. Each horse was kept in a stall seven days per week except when the horse was being used for class or being provided with exercise. Horses were turned out for approximately five hours in 15 m x 15 m dry lots two days per week. Horses were not given access to pasture or allowed any other nutrients other than provided in their daily rations. All horses were bare-footed throughout the project. Horses were kept on the same hoof care schedule, with the same farrier trimming the horses every six to eight weeks.

Lameness Evaluations and Stride Lengths

Each mature horse underwent a lameness evaluation to determine pre-existing lameness or gait defects that might be associated with osteoarthritis and to rule out other sources of lameness such as abscesses. Lameness evaluations were performed according to the American Association of Equine Practioner's Classification (Kester, 1991) by a licensed equine practitioner on d 0 of the project. Horses were first visually appraised while being led at both the walk and trot. Next, a hoof tester was applied to the medial and lateral quarters, the toe, across the heels, from the medial frog to the lateral hoof wall, and from the lateral frog to the medial hoof wall on all four hooves to test for withdrawal from pressure as an incidence of pain. The veterinarian then palpated each leg, noting problems with the bone and soft tissue from the carpal and tarsal joint down. Any injury a horse might have received previously was noted and taken into consideration before continuing with the lameness evaluation.

Horses were scored for range of motion in their carpal, tarsal, metacarpophylangeal, and metatarsophylangeal joints by the veterinarian, and these scores were recorded. Flexion tests were performed to determine a lameness grade for each carpal, tarsal, metacarpophylangeal, and metatarsophylangeal joint. Each joint was given a score ranging from 0 (exhibiting no lameness) to 5 (exhibiting extreme lameness). Scores recorded from the flexion tests were added together to give an overall lameness score. No horse had gait deficits secondary to suspected neurological problems or muscle atrophy secondary to illness or injury.

Stride lengths of all eighteen horses were determined at both the walk and the trot using the guidelines established by Hanson et al. (1997). To measure stride lengths, a 1.5 m x 10 m patch of dry dirt was raked to erase all previous hoof prints. Horses were led at the walk and trot through the raked area, and distances between right hind toe impressions were taken. Three stride length measurements were averaged for each horse. All procedures during the lameness evaluation and measurement of stride lengths were video recorded.

Total lameness scores and stride lengths were used to stratify and pair-match the mature horses before placing them on one of two diets. The six 2-year-olds were stratified and pair-matched by stride length and prospect type (i.e. western or hunter-type horse) before being placed on one of two diets. Pair-matched horses were provided with equal exercise and turnout time throughout the duration of the project. Both lameness scores and stride lengths were performed again on d 75 to access treatment differences.

Treatments

Once horses were pair-matched, they were randomly assigned to either a treatment group (FA), which received a stabilized supplement containing 11 mg/kg BW of EPA, 22 mg/kg BW DHA, and a carrier provided by United Feeds (Sheridan, IN), or a control group (CO), which received 49 g corn oil (Table 1). The amount of corn oil was calculated to be the same isocalorically as the combination of EPA, DHA, and carrier. All horses were fed 259 g Wallace sweet feed provided by United Feeds.

Table 1. Treatment, age, sex, and osteoarthritis score of each horse on trial.

Horse No.	*Treatment*	*Age (Yrs.)*	*Sex*	*Osteoarthritis Score (d0)*
1	FA	20	S	5.5
2	FA	16	M	1.5
3	FA	7	M	2.5
4	FA	13	G	4.0
5	FA	13	G	2.5
6**	CO	12	G	4.5
7	FA	2	G	N/A
8	FA	2	G	N/A
9	FA	2	G	N/A
10	CO	20	S	10.5
11	CO	13	M	3.5
12**	FA	13	M	5
13	CO	12	G	2.5
14	CO	12	G	0.5
15	CO	11	G	3.5
16	CO	2	G	N/A
17	CO	2	G	N/A
18	CO	2	G	N/A

**Indicates pair-matched horses that switched treatment diets on 10 d of supplementation.

The supplemented feed for the treatment group was mixed and individually bagged by United Feeds. One horse refused the treatment feed and 10 mL molasses was provided to encourage eating. On d 10, the horse still refused the treatment feed, so a pair-match switch was ordered to ensure that all horses were eating properly. One other horse in the treatment group refused feed on d 14, but supplementing the diet with 10 mL molasses for 12 days was sufficient to encourage consumption of concentrate again. No other problems with palatability were seen. The control group received corn oil as top-dressing poured over their sweet feed. Horses were also fed approximately 2.5 kg Timothy grass hay twice a day at 0730 and 1630. Several horses were provided with either 1 kg, 1.4 kg, or 2.4 kg whole oats as needed to sustain weight. The amount of whole oats provided was determined by weight gain or loss and fluctuated throughout the project. Oats and hay were provided by the Michigan State University Horse Teaching and Research Center. Supplemented feed was provided once per day at 1630 for 75 d.

Exercise

Horses were exercised 5 days/week during the duration of the project. Exercise consisted of working in a horsemanship riding class or collegiate equestrian team practice (ten mature horses), a training class for 30 to 45 minutes per class session (all two-year-old horses), or on a free-flow exerciser (two stallions).

Horsemanship Classes. In horsemanship classes, students rode horses either in a stock-seat or a hunt-seat saddle. Horses underwent a warm-up that consisted of walking, trotting/jogging, and cantering/loping both directions of the arena for about 10 minutes.

After warm-up, horses were exercised by performing maneuvers being presented to the class by the instructor. Riding times increased from 25 to 45 minutes throughout the project, as well as did the difficulty of the maneuvers being presented to the horses.

Collegiate Equestrian Team Practices. Collegiate equestrian team practice consisted of riding horses for a maximum of two hours twice a week within the five days of exercise called for by the project. Practice consisted of walking (20 minutes), trotting/jogging (60 minutes), and cantering/loping (40 minutes) horses for equal times both directions of the arena. Horses were ridden either in a stock-seat or hunt-seat saddle, with disciplines being traded every week. As the project proceeded, horses increased canter/lope times and decreased trot/jog times by 10 minutes, and horses were put through more maneuvers, such as counter-canters.

Training Classes. Training classes consisted of working horses on a longe line at a walk, trot, and canter both directions for approximately 15 minutes at the beginning of the project. Once horses were ready to progress, they were subjected to training aids such as driving lines. During the later part of the project (day 50 and beyond), horses were introduced to a saddle and ridden by students. Again, exercise times increased from 25 to 45 minutes during the course of the project.

Free-flow Exerciser. When no class was scheduled, exercise consisted of working on a free-flow exerciser (Centaur Horse Walkers, Inc., Mira Loma, CA). Time on the exerciser increased every 25 days, with horses being worked for 20 minutes at 3

m/s from d 0 to 25, 30 minutes at 3 m/s from d 26 to 50, and 40 minutes at 3 m/s from d 51 to 75. Horses were then walked at 1.5 m/s for a period of 10 to 20 minutes to ensure they were cool before being returned to their stalls. The free-flow exerciser was reversed in direction mid-way through the exercise to help insure equal loading to both sides of the horses' joints. On days with inclement weather, horses were exercised at a trot (approximately 3 m/s) for the specified project time on a longe line, replacing the free-flow exerciser.

Measurements and Sample Collection

Body weights were determined on a digital scale on d 0, 25, 50, and 75. On d 0, several of the young horses were unwilling to cross the scale to get an accurate body-weight. A commercially available equine weight tape was used to approximate body weight on these horses on d 0. By d 25, all horses were willing to stand on the scale.

Blood samples were collected on d 0, 25, 50, and 75 via venipuncture of the jugular vein using a 20-gauge needle and sterile Vacutainer® tubes (Becton Dickenson). Samples were collected at 1400 to ensure a degree of fasting was met. Three 10 mL serum vials containing no additives and two 10 mL plasma vials containing K_2 EDTA as the anti-coagulant were filled during each collection day. Blood was allowed to coagulate for no more than 45 min before being centrifuged at 1,340 x g for 10 min. Once separated, plasma and red blood cells were collected separately using disposable Pasteur pipet and placed in 7 mL scintillation vials. Serum was collected using a

disposable Pasteur pipet and placed in 1.5 mL micorcentrifuge tubes. All samples were stored at -20° C for later analysis.

Laboratory Analysis

Samples were analyzed for plasma fatty acid profiles, red blood cell fatty acid profiles, PGE_2 concentrations, osteocalcin (marker of bone formation), C-terminal cross-linked teleopeptides of type I collagen (ICTP — marker of bone turnover), and tumor necrosis factor-alpha (TNF-α — marker of inflammation). Each sample was analyzed in duplicate, and replicated further if the coefficient of variation exceeded 10% of the mean. Both osteocalcin and ICTP have been previously used (Lang et al., 2001; Hoekstra et al., 1999).

Analysis of Fatty Acids in Feeds, Serum, and Red Blood Cells

Treatment feed samples were obtained from each bag of feed, and oats and hay were collected each collection day. Feed was first ground in a Wiley mill with a 2 mm screen, and 4 g of ground sample was weighed and added to a clean culture tube. For plasma and red blood cells, samples were thawed to room temperature, vortexed, and 1 mL of sample was added to a clean culture tube. To each tube, 1 mL ethanol and 1 mL hexane were added, which was capped with a Teflon septa insert, vortexed for 5 min, and centrifuged for 10 min at 1,500 x g. A positive displacement pipet was used to transfer 0.75 mL of the hexane layer into a clean tube.

One mL of hexane including a C19:0 internal standard (1 mg standard/mL hexane) and 3 mL of 10% methanolic HCL (mixed by adding 20 mL actyl chloride to 100 mL methanol) was added to each sample. Samples were layered with nitrogen gas, vortexed, capped, and placed in a 90° C water bath for 2 h.

Once removed from the water bath, samples were allowed to cool to room temperature before 10 mL of 6% potassium carbonate was added. Samples were vortexed and then centrifuged for 5 min at 950 x g, the top layer was transferred to a test tube, and approximately 1 g of sodium sulfate was added. If the sample had any color, approximately 0.5 g of activated charcoal was added to that sample. Again, samples were vortexed and then centrifuged for 5 min at approximately 950 x g. For feed samples, one mL of the hexane layer was removed with a positive displacement pipet and placed into a clean test tube. The sample was then evaporated with nitrogen gas, and 0.5 mL hexane was added to the tube. Finally, in all samples, the hexane layer was transferred to a gas chromatography 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 (MSU DCPAH) for analysis by gas chromatography.

Osteocalcin

Serum osteocalcin concentrations were determined using an enzyme immunoassay (EIA); (METRA™ Osteocalcin EIA kit, Quidel Corporation, San Diego, CA) following manufacturer's instructions. Serum from mature horses required no

dilution, while serum from 2-year-old horses required a 1:2 dilution as determined by previous work (Lang et al., 2001; Hoekstra et al., 1999). Each sample was analyzed in duplicate. Standards and controls were reconstituted with 0.5 mL of 1X wash buffer. Twenty-five μL of standard, control, or sample was added to each coated strip well, followed by the addition of 125 μL anti-osteocalcin. The plate was incubated at room temperature for 2 h, each well was washed with 300 μL of 1X wash buffer three times, and 150 μL of enzyme conjugate was added. Following 1-h incubation at room temperature, wells were again washed three times with 300 μL 1X wash buffer. Working substrate solution (150 μL) was added to each well, followed by a 40 min incubation. Once the A standard — the lowest standard — read between 1.2 and 1.6 optical density, 50 μL stop solution was added to each well and the plate was read at 405 nm optical density on a Spectra Max 340 plate reader (Molecular Devices Corp., Sunnyvale, CA).

C-Terminal Cross-Linked Teleopeptides of Type I Collagen (ICTP)

Serum C-terminal cross-linked teleopeptides of type I collagen (ICTP) was determined using a radioimmunoassay (RIA); (ICTP ^{125}I RIA Kit®, DiaSorin Inc., Stillwater, MN) following manufacturer's instructions. Each sample, total count, non-specific binding, standard, and quality control sera was run in duplicate. Tubes were set up as follows: non-specific binding tubes — 100 μL sample and 200 μL distilled water; standards — 100 μL ICTP standard and 200 μL antiserum; quality control sera and sample — 100 μL serum and 200 μL ICTP antiserum. Two hundred μL of ^{125}I ICTP was added to all tubes, including the total count tubes. Tubes were incubated for 2 h at 37° C. Separation reagent (500 μL) was added to all tubes except the total count tubes, and all

tubes were vortexed, incubated for 30 min at room temperature, centrifuged for 30 min at 2,000 x g at 4° C, and immediately decanted. Finally, tubes were placed on the 1290 GammaTrac Gamma Counting System (Tm Analytic, Elk Grove Village, IL) for determination of ICTP concentrations.

Prostaglandin E_2 Metabolite

Prostaglandin E_2 metabolite was determined using an enzyme immunoassay (EIA); (Prostaglandin E Metabolite EIA Kit, Cayman Chemical Company, Ann Arbor, MI) following manufacturer's instructions. Serum was diluted at 1:2. Dilutions were determined by testing an equine serum sample neat (no additives), spiked, and diluted at 1:2, 1:4, 1:8, and 1:10. These values were compared to manufacturer's suggestion that the sample fall between 2 and 20 pg/ml with a good correlation (less than 20%). When the samples fell within range, the corresponding dilution was chosen for all samples. The prostaglandin E metabolite (PGEM) standard was derivatized by adding 100 µL standard to 900 µL ultrapure water to yield a 40 ng/mL concentration. Fifty µL of this standard was brought to a total volume of 1 mL with EIA buffer, and 300 µL carbonate buffer was added before overnight incubation at 37° C. After incubation, 400 µL phosphate buffer and 300 µL EIA buffer were added to yield a 1,000 pg/mL concentration derivatized standard. Derivation of the samples took place by adding 500 µL of each sample to 150 µL of carbonate buffer and incubating at 37° C overnight. After incubation, 200 µL phosphate buffer and 150 µL EIA buffer were added to each sample. The standard curve was prepared by transferring 950 µL PGEM assay buffer to one tube and 500 µL PGEM assay buffer to an additional seven tubes. Fifty µL of the derivatized standard was added

to tube 1, and serially diluted over eight tubes in 500 μL aliquots. Non-specific binding, standards, and samples were analyzed in duplicate; maximum binding was analyzed in triplicate. Once all standards and samples were ready, the plate was prepared as follows: fifty μL EIA buffer and 50 μL of PGEM buffer were added to the non-specific binding wells. Fifty μL PGEM buffer was added to the maximum binding wells. Fifty μL of each standard was added to the plate in specified wells, and 50 μL of each sample was added to each additional well. Prostaglandin E Metabolite AchE tracer (50 μL) was added to each well except the total activity and blank wells, and 50 μL PGEM antiserum was added to each well except the total activity, blank, and non-specific binding wells. The plate was incubated for 18 h at room temperature. Each well was washed five times with 300 μL wash buffer before adding 200 μL Ellman's reagent. Five μL of tracer was added to the total activity wells. The plate was then covered and placed on an orbital shaker for 60 min before being read at 412 nm optical density on a Spectra Max 340 plate reader (Molecular Devices Corp., Sunnyvale, CA).

Tumor Necrosis Factor-alpha (TNF-α)

Tumor necrosis factor-alpha (TNF-α) was determined using an enzyme-linked immunosorbent assay (ELISA); (Equine TNF-α ELISA Kit, Pierce Endogen, Rockford, IL) following manufacturer's instructions. Serum was diluted at 1:8. Dilutions were determined by testing a serum sample neat, spiked, and diluted at 1:2, 1:4, 1:8, and 1:16. These values were compared to manufacturer's suggestion that the sample fall between 15.6 and 1,000 pg/ml. When the samples fell within range and recovery was the highest, the corresponding dilution was chosen for all samples. Wash buffer was prepared by

adding 50 mL of 30X wash buffer with 150 mL of ultrapure water. Reagent diluent was made by adding 2 g bovine serum albumin (BSA, Sigma) to 50 mL phosphate buffer solution to create a 4% solution. The lyophilized standard was reconstituted by adding 1 vial of standard to 1.35 mL of reagent dilutent. Standards were prepared by serial dilution: 200 μL of sample dilutent was added to seven tubes, then 200 μL of reconstituted standard was added to the first tube; 200 μL of this solution was added to the next tube; the process was repeated until all 7 standards were created. The plate was prepared by adding 50 μL sample diluent to each well. Fifty μL of standard or sample was added to each well in duplicate. The plate was covered with an adhesive plate cover and incubated for one hour at room temperature. After incubation, the plate was washed three times with wash buffer. Biotinylated antibody reagent (100 μL) was added to each well and the plate was covered and incubated for one hour at room temperature. After incubation, the plate was again washed three times with wash buffer. One hundred μL of streptavidin-HRP reagent was then added to each well, followed by a 30-minute covered incubation at room temperature and another three washes with wash buffer. Next, TMB substrate solution (100 μL) was added to each well, and an enzymatic color reaction was allowed to develop at room temperature for 30 min. After the reaction, stop solution was added to each well. The plate was then read at 450 nm and 550 nm optical density on a Spectra Max 340 plate reader (Molecular Devices Corp., Sunnyvale, CA). The 550 nm readings were subtracted from the 450 nm readings to correct optical imperfections in the microplate and give the correct absorbance levels.

Statistics

Data were analyzed using PROC MIXED in SAS 8.0. Differences between treatment, day, and treatment by day interactions were determined, with age of the horse (mature or two-year-old) used as a cofactor in the analysis. Data were normalized if there was a difference noted at d 0 of the trial period. Normalized data were analyzed for differences between treatment, day, and treatment by day interactions by SAS 8.0. Least Square Means statements were included to note treatment means, differences between means, and standard errors. Repeated days with treatment nested in horse were used for bone markers, inflammation markers, and fatty acids. There were no repeated measures for strides or erythrocytes. A X^2 test was used for lameness and range of motion scores. Tumor necrosis factor alpha, ICTP, and PGE_2 were run with compound symmetry. A P-value of less than 0.05 was considered significant, while trends were considered at P-values between 0.05 and 0.1.

CHAPTER 3

RESULTS

Body Weight

No body weight difference was detected between CO and FA horses (441 ± 10 vs. 426 ± 10 kg, P = 0.33)(Table 2). Mature horses weighed more than young horses (451 ± 9 vs. 416 ± 12 kg, P < 0.05). All horses weighed more on d 0 than on any other day of the project (453 ± 8 vs. 427 ± 8 kg, P < 0.0001). No difference in weight loss between CO and FA horses existed (29 ± 11 vs. 23 ± 11 kg, P = 0.33).

Stride Lengths

No treatment, day, or treatment*day differences were seen in walk stride lengths (P = 0.16, P = 0.22, and P = 0.93, respectively)(Table 3). There were no treatment differences in CO versus FA trot stride lengths (239 ± 6 vs. 235 ± 6 cm, P = 0.68); however, there was both a day difference (P < 0.05) and a trend for a treatment*day interaction (P = 0.08). An overall increase in trot stride lengths from d 0 to d 75 was discovered (233 ± 5 vs. 242 ± 5 cm, P < 0.05). There was no difference in trot stride lengths between CO and FA horses on d 0 (238 ± 7 vs. 228 ± 7 cm, P = 0.30). While CO horses experienced no change in trot stride length from d 0 to d 75 (238 ± 7 vs. 240 ± 7 cm, P = 0.63), a 15 cm increase from d 0 to d 75 was observed in FA horses (228 ± 7 vs. 243 ± 7 cm, P < 0.1).

Lameness

Total carpal joint scores were higher on d 0 compared to d 75 ($P < 0.05$) (Table 4). CO horses had a greater decrease from d 0 to d 75 (0.5 ± 0.1 vs. 0) than FA horses (0.2 ± 0.1 vs. 0).

When observing lameness on the left carpus joint, a day difference ($P < 0.01$) and a trend for a treatment ($P = 0.07$) and a treatment*day ($P = 0.07$) difference occurred. Both treatments had lower lameness scores on d 75 when compared to d 0 (0.3 ± 0.2 vs. 0, $P < 0.01$). The lameness score decreased in both treatments, but CO horses tended to show a larger decline from d 0 to d 75 (0.4 ± 0.1 vs. 0, $P = 0.07$) when compared to FA horses (0.1 ± 0.1 vs. 0, $P = 0.07$).

A treatment difference ($P < 0.05$) was noted in lameness score for the left metatarsalphalangeal (MTP) joint. FA horses had lower scores on d 75 compared with d 0 (0.3 ± 0.2 vs. 0.8 ± 0.2, $P < 0.05$) than CO horses (0 vs. 0.2 ± 0.2).

There were no other significant differences in lameness scores or range of motion scores between horses (Table 5).

Plasma Fatty Acid Profiles

A treatment ($P < 0.01$) and a day difference ($P < 0.05$) in total omega-3 (n-3) fatty acid concentration were shown (Table 6). Horses in the CO group had a lower total n-3 fatty acid concentration than FA horses ($P < 0.01$). There was a trend for total n-3 fatty

acid concentrations to decrease between d 0 and d 25 ($P = 0.10$). Concentrations were also lower on d 50 compared to d 0 ($P < 0.01$). Furthermore, there was a trend for total n-3 fatty acids to be lower on d 75 compared to d 0 ($P = 0.08$). There was a difference in total n-3 fatty acid concentrations on d 0; therefore, data were run with a covariate. Horses in the FA group still had higher total n-3 fatty acid concentrations than CO horses ($P<0.05$). No other differences for these total n-3 fatty acids were observed.

A day difference ($P < 0.0001$) and a treatment*day interaction ($P < 0.01$) were noted for total omega-6 (n-6) fatty acids. Overall, concentration of total n-6 fatty acids increased from d 0 to d 25 ($P < 0.0001$) and remained elevated throughout the trial period. Concentrations between d 25 and d 50 stayed constant, but the concentration decreased between d 50 and d 75 ($P < 0.01$).

There was no difference between treatments for total n-6 fatty acid concentration on d 0 ($P = 0.16$). Horses on CO treatment increased total n-6 fatty acid concentration between d 0 and d 25 ($P < 0.0001$), as did FA horses ($P < 0.01$); however, CO horses displayed a greater increase in concentration than FA horses on d 25 ($P < 0.05$) and d 50 ($P < 0.05$). Both treatments decreased on d 75, with CO horses decreasing more ($P < 0.01$) than FA horses ($P = 0.08$). There was no difference between treatments on d 75.

Treatment ($P < 0.01$), day ($P < 0.0001$), treatment*day ($P < 0.01$) differences were observed for the n-6 to n-3 fatty acid ratio (n-6:n-3). Overall, horses on the CO diet had higher n6:n3 than FA horses ($P < 0.01$). The ratio increased in all horses from d 0 to

d 25 ($P < 0.01$), and continued to increase through d 50 when compared to baseline ($P < 0.0001$). There was a tendency for the ratio to return to baseline levels on d 75 ($P = 0.07$). Furthermore, n6:n3 tended to increase from d 25 to d 50 ($P = 0.07$), but no difference between d 25 and d 75 was noted. Finally, the ratio decreased from d 50 to d 75 ($P < 0.01$).

When analyzing potential treatment by day interactions, no difference was seen between treatments on d 0 for n-6:n-3. Control horses increased n6:n3 from d 0 to d 25 ($P < 0.01$), and the increase continued to d 50 ($P < 0.0001$). The ratio tended to return to baseline levels on d 75 ($P = 0.10$). Control horses had higher ratios than FA horses on d 25 ($P < 0.01$) and d 50 ($P < 0.0001$), and tended to be higher on d 75 ($P = 0.08$). The ratio increased from d 25 to d 50 in CO horses ($P < 0.01$), but decreased from d 50 to d 75 ($P < 0.0001$). No day differences were noted within the FA group.

The concentration of EPA (20:5n3) decreased from d 0 to d 25 ($P < 0.0001$), remained the same between d 25 and d 50, and then returned to baseline on d 75 ($P < 0.0001$), resulting in an overall day difference ($P < 0.0001$).

Treatment ($P < 0.0001$), day ($P < 0.0001$), and treatment*day interaction ($P < 0.0001$) differences were noted for DHA (22:6n3). This fatty acid increased between d 0 and d 25 in FA horses and remained elevated through d 75 ($P < 0.0001$). The treatment*day interaction ($P < 0.0001$) is due to the elevated concentrations of DHA in FA horses compared to CO horses ($P < 0.0001$). Day differences were also attributed to

the increased concentration of DHA in FA horses between d 0 and d 25 ($P < 0.0001$); there were no other differences between days.

Treatment ($P < 0.05$), day ($P < 0.0001$), and treatment*day interaction ($P < 0.01$) differences were seen for arachidonic acid (20:4n6). Control horses had lower 20:4n6 concentrations than FA horses ($P < 0.05$). Concentrations were lower on d 0 than d 25, d 50, or d 75 ($P < 0.01$). There was a trend for d 25 to be higher than d 50 ($P = 0.06$), but there were no other differences between days.

When analyzing treatment*day, CO horses had increased concentrations of 20:4n6, from d 0 to d 25 ($P < 0.05$). Concentrations decreased from d 25 to d 50 ($P < 0.05$), and then remained the same from d 50 to d 25. Horses on FA treatment had increased concentrations of 20:4n6c from d 0 to d 25 ($P < 0.0001$). Concentrations remained elevated in FA horses on d 50 and d 75. Control horses had lower concentrations of AA on d 25, d 50, and d 75 ($P < 0.05$).

While the percentage of the remaining analyzed total plasma fatty acids usually changed during the course of this study (Appendix Table 1A), only fatty acids that demonstrated changes due to treatment are reported below. Treatment ($P = 0.05$) tended to be different and day ($P < 0.0001$) was different for 16:1n7t. Overall, CO horses tended to have higher 16:1n7t concentrations than FA horses ($P = 0.05$). Concentrations of 16:1n7t decreased from d 0 to d 25 ($P < 0.0001$), remained the same from d 25 to d 50, returned to baseline on d 75 ($P < 0.0001$).

A treatment difference ($P < 0.01$) and a day difference ($P < 0.05$) were observed for 18:1t9. Overall, FA horses were lower than CO horses ($P < 0.01$). There was a tendency for 18:1t9 to increase on d 25 compared to d 0 ($P = 0.09$). There was no difference between d 25 and d 50, and fatty acid levels returned to baseline on d 75.

Treatment difference tended to be different for 18:2n6 ($P = 0.07$), and there was a day difference ($P < 0.0001$) for 18:2n6. Overall, CO horses tended to have higher 18:2n6 compared to FA horses ($P = 0.07$). This fatty acid was highest on d 0 compared to all other days.

Horses in the FA group had higher concentrations of 20:3n6 than CO horses ($P = 0.05$). No differences between d 0, d 25, and d 50 were noted. However, concentration of 20:3n6 increased throughout the treatment period ($P < 0.05$), with d 75 being higher than d 0 and d 25 ($P < 0.05$), and d 75 tended to be higher than d 50 ($P = 0.06$).

A treatment ($P < 0.05$) and a day ($P < 0.01$) difference was observed for 20:3n3. FA horses had higher concentrations of 20:3n3 than CO horses ($P < 0.05$). Day 0 was lower than d 25 ($P < 0.05$) and tended to be lower than d 50 ($P = 0.06$); however d 0 tended to be higher than d 75 ($P = 0.09$).

A treatment ($P < 0.05$), a day ($P < 0.01$), and a treatment*day interaction ($P < 0.01$) difference was observed for 22:2n6. These differences are due to an increase in the

CO horses on d 75 ($P < 0.0001$). Due to this high increase, FA horses had lower concentrations of 22:2n6 than CO horses ($P < 0.05$).

A treatment difference ($P < 0.0001$) was noted for 24:0. Horses in FA group had higher concentrations than CO horses ($P < 0.0001$).

Osteocalcin

Treatment tended to be different ($P = 0.06$) in osteocalcin concentrations, and there were also differences in age ($P < 0.0001$) and day ($P < 0.05$) (Table 7). Horses on FA treatment tended to have higher ($P = 0.06$) osteocalcin concentrations overall; there was no difference on d 0. Young horses had higher osteocalcin concentrations than mature horses ($P < 0.0001$). Osteocalcin was higher on d 50 ($P < 0.05$) than d 0 across both treatments. Day 50 had higher ($P < 0.01$) osteocalcin concentrations than d 25. Day 75 tended to have higher ($P = 0.07$) osteocalcin concentrations than d 25.

ICTP

Serum ICTP concentrations displayed an age ($P < 0.0001$), day ($P < 0.0001$), and age*day interaction ($P < 0.01$) difference, but there was no treatment difference ($P = 0.89$)(Table 7). Young horses had higher ICTP concentrations than mature horses ($P < 0.0001$) (Table 8). Concentrations of ICTP were lower on d 75 compared with all other sample days ($P < 0.0001$). Concentrations of ICTP of mature horses decreased from d 0 to d 75 and were lower on d 75 versus d 0 ($P < 0.0001$). Concentrations of ICTP of

young horses increased between d 0 and d 50 ($P < 0.05$) and decreased from d 50 to d 75 ($P < 0.05$)

PGE_2 Metabolite

No treatment or day differences for PGE_2 ($P = 0.51$ and $P = 0.87$, respectively)(Table 7) were noted; however, an age difference ($P < 0.01$) was observed. Young horses had lower ($P < 0.01$) PGE_2 concentrations than mature horses.

TNFα

There were no treatment, age, or day differences ($P = 0.28$, $P = 0.33$, and $P = 0.51$, respectively) in TNFα concentrations between any groups of horses (Table 7).

Table 2. Mean body weights (kg) and the SEM of horses on either control or treatment diet on d 0, 25, 50, and 75.

Measurement	*Treatment*	*0*	*25*	*50*	*75*	*SEM*
Body Weight	CO	462	433	433	433	11
(kg)	FA	444	421	420	422	11
	Mean	453[a]	427[b]	426[b]	428[b]	8

[ab] Values within a row lacking common superscripts differ in day values ($P < 0.05$).

Table 3. Mean walk stride length (cm) and trot stride length (cm) of horses on control or treatment diet pre- and post-trial.

		Day		
Measurement	*Treatment*	*0*	*75*	*SEM*
Walk Stride	CO	168	172	4
(cm)	FA	161	166	4
Trot Stride	CO	238	240	7
(cm)	FA	228	243[†]	7
	Mean	233[a]	242[b]	5

[†] FA horses had increased trot stride lengths from d 0 to d 75 ($P < 0.10$).
[ab] Values within a row lacking common superscripts differ in day values ($P < 0.05$).

Table 4. Mean lameness scores of horses on control or treatment diet pre- and post-trial.

Joint	*Treatment*	*Day* 0	*Day* 75	*SEM*	X^2 *P Value*
Right Carpus	CO	0.08	0.00	0.06	P = 0.58
	FA	0.08	0.00	0.06	
Right MCP	CO	1.00	0.83	0.28	P = 0.15
	FA	0.33	0.17	0.28	
Right Tarsus	CO	0.67	0.83	0.38	P = 0.82
	FA	0.67	0.33	0.38	
Right MTP	CO	0.25	0.42	0.24	P = 0.97
	FA	0.33	0.33	0.24	
Left Carpus†	CO	0.42	0.00	0.09	P < 0.01
	FA	0.08	0.00	0.09	
Left MCP	CO	1.08	0.75	0.31	P = 0.62
	FA	0.50	0.75	0.31	
Left Tarsus	CO	0.50	0.33	0.22	P = 0.76
	FA	0.67	0.50	0.22	
Left MTP*	CO	0.17	0.00	0.23	P < 0.10
	FA	0.83	0.33	0.23	
Total Right	CO	2.00	2.08	0.77	P = 0.64
	FA	1.42	0.83	0.77	
Total Left	CO	2.17	1.08	0.70	P = 0.68
	FA	2.08	1.58	0.70	
Total Carpus†	CO	0.50	0.00	0.12	P < 0.05
	FA	0.17	0.00	0.12	
Total MCP	CO	2.08	1.58	0.50	P = 0.27
	FA	0.83	0.92	0.50	
Total Tarsus	CO	1.17	1.17	0.48	P = 0.90
	FA	1.33	0.83	0.48	
Total MTP	CO	0.42	0.42	0.33	P = 0.35
	FA	1.17	0.67	0.33	

Table 4 (cont'd).

Overall	CO	4.17	3.17	1.23	P = 0.79
	FA	3.50	2.42	1.23	

† Overall day difference, regardless of treatment: $P < 0.05$.
* Overall treatment difference, regardless of day: $P < 0.05$

Table 5. Mean range of motion scores of horses on control or treatment diet pre- and post-trial.

Joint	*Treatment*	*Day* 0	*Day* 75	*SEM*	X^2 *P Value*
R Carpus ROM	CO	0.98	1.00	0.01	P = 0.41
	FA	1.00	1.00	0.01	
R MCP ROM	CO	0.92	0.80	0.04	P < 0.10
	FA	0.97	0.92	0.04	
R Tarsus ROM	CO	1.00	1.00	0.00	P = --
	FA	1.00	1.00	0.00	
R MTP ROM	CO	1.00	1.00	0.00	P = --
	FA	1.00	1.00	0.00	
L Carpus ROM	CO	1.00	1.00	0.00	P = --
	FA	1.00	1.00	0.00	
L MCP ROM	CO	0.87	0.77	0.05	P = 0.10
	FA	0.95	0.88	0.05	
L Tarsus ROM	CO	1.00	1.00	0.00	P = --
	FA	1.00	1.00	0.00	
L MTP ROM	CO	1.00	1.00	0.00	P = --
	FA	1.00	1.00	0.00	
Right ROM	CO	3.90	3.80	0.05	P = 0.13
	FA	3.97	3.92	0.05	
Left ROM	CO	3.87	3.77	0.05	P = 0.10
	FA	3.95	3.88	0.05	
Carpus ROM	CO	1.98	2.00	0.01	P = 0.41
	FA	2.00	2.00	0.01	
MCP ROM	CO	1.78	1.57	0.09	P < 0.10
	FA	1.92	1.80	0.09	
Tarsus ROM	CO	2.00	2.00	0.00	P = --
	FA	2.00	2.00	0.00	

Table 5 (cont'd).

MTP ROM	CO	2.00	2.00	0.00	P = --
	FA	2.00	2.00	0.00	
Overall ROM	CO	7.77	7.57	0.09	P.10
	FA	7.92	7.80	0.09	

Table 6. Mean percentage (%) of total n-3, total n-6, n-6:n-3 ratio, EPA, DHA, and AA in CO or FA horses on d 0, 25, 50, and 75.

Fatty Acid	*Treatment*	*Day* 0	25	50	75	*Std. Error*
Total N3	CO*	4.58	3.84	2.63	3.82	0.57
	FA	6.38	5.52	5.29	5.40	0.57
	Mean	5.48^{b}	4.68^{ab}	3.96^{a}	4.61^{ab}	0.40
Total N6	CO	41.3^{a}	49.5^{c}	50.3^{c}	45.9^{b}	0.99
	FA	43.3^{a}	46.6^{b}	46.7^{b}	44.6^{ab}	0.99
	Mean	42.3^{a}	48.0^{c}	48.5^{c}	45.2^{b}	0.70
N6:N3 Ratio	CO*	9.73^{a}	15.6^{b}	20.1^{c}	12.5^{ab}	1.35
	FA	7.64^{a}	9.78^{a}	9.58^{a}	9.09^{a}	1.35
	Mean	8.68^{a}	12.68^{bc}	14.85^{c}	10.81^{ab}	0.96
20:5n3c EPA	CO	0.50	0.08	0.10	0.41	0.08
	FA	0.43	0.11	0.14	0.56	0.08
	Mean	0.46^{b}	0.09^{a}	0.12^{a}	0.48^{b}	0.06
22:6n3c DHA	CO	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.12
	FA	0.00^{a}	1.90^{c}	1.74^{bc}	1.61^{b}	0.12
	Mean	0.00^{a}	0.95^{b}	0.87^{b}	0.80$_{b}$	0.08
20:4n6c AA	CO*	0.74^{a}	1.04^{b}	0.73^{a}	0.90^{ab}	0.16
	FA	0.75^{a}	1.66^{b}	1.57^{b}	1.58^{b}	0.16
	Mean	0.75^{a}	1.35^{b}	1.15^{b}	1.24^{b}	0.11

* Overall treatment difference, regardless of day: $P < 0.05$.

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

Table 7. Mean osteocalcin, ICTP, PGE2, and TNFa of CO and FA horses on d 0, 25, 50, and 75.

Measurements	*Treatment*	*Day* 0	25	50	75	*Std. Error*
Osteocalcin† (ng/ml)	CO	14.9	13.9	20.3	19.7	2.41
	FA	19.3	17.6	24.1	20.4	2.41
	Mean	17.1[a]	15.8[a]	22.2[b]	20.0[ab]	1.69
ICTP (µg/l)	CO	8.27	8.38	8.26	7.20	0.26
	FA	8.16	8.31	8.37	7.11	0.26
	Mean	8.22[a]	8.34[a]	8.31[a]	7.16[b]	0.19
PGE_2 (pg/ml)	CO	12.9	13.1	12.6	12.1	1.07
	FA	12.6	11.5	11.6	12.2	1.07
TNFα (ng/ml)	CO	434	344	315	392	239
	FA	739	640	914	547	239

† Overall treatment tended to be different, regardless of day: $P < 0.10$.
[abc] Values within a row lacking common superscripts differ in day values ($P < 0.05$).

Table 8. Mean ICTP concentrations of mature versus young horses on d 0, 25, 50, and 75.

Measurements	*Age*	*Day* 0	25	50	75	*Std. Error*
ICTP (µg/l)	Mature	5.79[c]	6.06[c]	5.28[b]	4.50[a]	0.22
	Young*	10.65[b]	10.63[b]	11.35[c]	9.82[a]	0.31

[abc] Values within an age lacking common superscripts differ ($P < 0.05$).
* Young horses had higher ICTP concentration, regardless of treatment: $P < 0.001$.

CHAPTER 4

DISCUSSION

All horses on the trial did show a decrease in body weight during the first 25 days of the new diet; however, body weight did not differ between treatments. As expected, mature horses weighed more than young horses, considering that the young horses are growing and have not yet reached a mature weight. The decrease in body weight was likely due to the fact that mature horses were taken off free-choice pasture and placed into stalls with a simultaneous increase in exercise.

Horses tended to show a treatment difference when analyzing trot stride length from the beginning of the study to its completion, with FA horses having an overall greater increase than CO horses. The trot stride length of the CO horses remained relatively unchanged while the FA horses increased trot stride length by 15 cm, a large increase when considering that horses tend to reflect soundness within their stride length. Hanson et al. (1997) substantiates using stride length as a measure of lameness and osteoarthritic pain; their study showed that horses placed on an anti-inflammatory supplement for OA increased their stride length, which they concluded was an indicator of less pain.

Omega-3 fatty acids inhibit proinflammatory eicosanoids; therefore, inflammation, pain, and signs of lameness will be reduced with supplementation (Hall et al., 2004). Omega-3 fatty acids may provide relief from pain associated with

inflammation of the joints. Eicosapentaenoic acid inhibits the metabolic pathway of arachidonic acid (AA)(James et al., 2000), thereby allowing production of prostaglandins with a decreased inflammatory response (Hall et al., 2004; Simopoulos, 2002; Mueller and Talbert, 1988). One explanation for the increase trot stride length in FA treated horses is that they had decreased localized inflammation and consequently decreased joint pain. Horses experiencing pain tend to shorten their stride to decrease weight bearing on the affected leg. However, with supplementation of omega-3 fatty acids, horses tended to extend their stride, a potential sign of less pain within joints. Another argument is that horses reached a higher level of fitness; however, horses were pair-matched and treated to the same levels of exercise. One could argue that FA horses had an increased level of muscle fitness, but there was no evidence indicating that omega-3 fatty acids were absorbed into tissues.

There was not an overall decrease in lameness scores in any horses on the trial, nor was there any difference in range of motion values between treatments. All horses had a decreased lameness score in the left carpal and total carpal joints. Control horses experienced a greater decrease in numerical scores; however, all scores returned to 0, indicating no lameness was observed. Lameness scores cannot show marked improvements beyond "no lameness", thus it can be misleading to declare that control horses improved more than treatment horses since all horses improved as much as possible. Horses on the FA treatment tended to show an increase in trot stride length, although lameness scores did not provide clues as to why this happened. It should be

noted that the lameness scale can only be measured in half point increments, so anything under 0.5 could be considered insignificant.

Horses fed the treatment containing omega-3 fatty acids had a higher concentration of omega-3 fatty acids in plasma. This agrees with the findings of Ashes et al. (1992), who reported that serum lipid levels of omega-3 fatty acids were higher in sheep fed a fish oil supplement. In horses, plasma fatty acid profiles indicate that different amounts of omega-3 fatty acid supplementation will yield the same result; O'Connor et al. (2004) fed omega-3 fatty acids at 60 mg/kg BW and noted changes in horses' heart rates due to omega-3 fatty acids, and Spearman et al. (2005) used as much as 160 g of omega-3 fatty acids in testing effects on plasma composition in foals. Furthermore, as omega-3 supplementation rose in the horse, overall plasma omega-3 fatty acid concentration would change linearly with the amount supplemented (Spearman et al., 2005; Hall et al., 2004). The dietary supplement in this study contained only 33 mg/kg BW total of EPA and DHA, which indicates that even small amounts of these two omega-3 fatty acids can cause an increase in total omega-3 fatty acids in equine plasma profiles.

Because this study provided corn oil, which is high on omega-6 fatty acid, to CO horses, there was an increase in the concentration of plasma omega-6 fatty acids from d 0 to d 75 in CO horses, with no difference in plasma omega-6 fatty acids found in FA horses. This is contrary to the findings of Hall et al. (2004), who noted that omega-6 fatty acid concentrations decreased by 24% in horses fed menhaden oil. Again, the

reason for the lack of change in the FA horses is probably due to the small amount of EPA and DHA within the supplemented feed. Horses on the CO diet were receiving a large amount of omega-6 fatty acids from the corn oil, which is reflected in the omega-6 fatty acids in plasma.

This study did record a lower ratio of omega-6:omega-3 fatty acids in FA horses on d 75, which will allow more incorporation of omega-3 fatty acids into tissues (Bojesen and Bojesen, 1998). Due to the composition of erythrocyte membranes, fatty acids must compete for incorporation into red blood cells for transport to other tissues (Knapp et al., 1994; Bojesen and Bojesen, 1998). A lower ratio indicates that the percentage of omega-6 fatty acids and omega-3 fatty acids is growing closer. As this ratio lowers, more omega-3 fatty acids are present in the plasma for incorporation into erythrocytes (Bojesen and Bojesen, 1998). However, the current study noted no changes in erythrocyte fatty acid composition due to treatment. There were no increases in EPA or DHA within erythrocytes, and no treatment differences were discovered on any fatty acid within the red blood cell. These results also do not support findings of Katan et al. (1997), which state that incorporation into red blood cells can be seen as soon as three days after supplementation begins. The benefits of having omega-3 fatty acids present within the erythrocyte membrane are still being examined in horses, but it is believed that incorporating these fatty acids into red blood cells will enhance performance by allowing horses to work at the same intensity while keeping a lower heart rate due to increased erythrocyte deformability (O'Connor et al., 2004). Human studies have shown that incorporating omega-3 fatty acids into the diet increases red blood cell deformability and

causes blood vessels to dilate, thereby slowing heart rates (Leaf, 1990; Christensen et al., 1999). With a decreased heart rate, athletes are able to perform intense exercise for longer periods of time.

Not only is a decreased omega-6:omega-3 ratio beneficial to the circulatory system, but Watkins et al. (2002) reported that as the omega-6:omega-3 ratio declined, so did the amount of AA in bone due to increased concentrations of EPA. Watkins et al. (2002) supplemented four diets with omega-6:omega-3 ratios of 23.8, 9.8, 2.6, and 1.2, with the latter two diets showing the most decline in concentrations of AA. In rats fed the 9.8 ratio diet, which is similar to our ratio of 10, there were no differences in the amount of AA in bone when compared with control rats. It is likely that a n-6:n-3 ratio closer to 2.6 would show the same decreases in AA in bone in horses. Furthermore, the decline in AA led to a decreased concentration of PGE_2 in bone (Watkins et al., 2002), which in turn can affect bone modeling (Watkins et al., 1997). Prostaglandin E_2 will stimulate bone growth at concentrations as low as 91.9 ng/g protein (Watkins et al., 1997), a feat that can be accomplished with a low omega-6:omega-3 ratio. Young horses on this study had lower PGE_2 concentrations compared to mature horses. Treatment did not play a factor in this difference. This age difference is possibly due to PGE_2 effects on bone growth. The whole relationship between omega-6:omega-3 fatty acid ratio and PGE_2 has not yet been proven in horses. Results from this study showed no correlation between omega-6:omega-3 and PGE_2; however, the supplement fed contained a higher ratio than those used in previous studies (Watkins et al., 2002; Watkins et al., 1997).

The present study did observe changes within concentrations of AA in horses. Horses on the FA treatment diet had higher concentrations of AA than horses on the control diet. Previous studies have also shown that horses fed fish oil had an increase in plasma concentrations of AA after initial supplementation (Hansen et al., 2002; Hall et al., 2004). One would believe that increasing the amount of omega-6 fatty acids being supplied to the diet would cause an increase in AA, considering that it is an omega-6 fatty acid. However, the present study showed that even though all horses had an increase in AA during the first 25 days, only horses on the FA treatment diet had elevated levels of AA throughout the rest of the 75 d trial. High concentrations of EPA will compete with AA for metabolism in the prostaglandin pathway (Simopoulos, 2002; Bauer, 1994; Mueller and Talbert, 1988). If EPA was being metabolized into PGE_3, that might inhibit AA from binding to phospholipids and completing metabolism to PGE_2 (Watkins et al., 2002). This would leave more AA within the bloodstream and in plasma fatty acid profiles. Therefore, because CO horses might still have been able to convert AA into PGE_2, their fatty acid profiles indicated decreased concentrations of AA on d 50 and d 75, while FA horses maintained an increased amount of AA in the blood. Furthermore, Hall et al. (2004) postulated that the increase in plasma AA concentrations in horses was due to the presence of AA within the fish oil supplement. This is possibly the case for our trial also, as the omega-3 fatty acid supplement had a higher concentration of AA than the control feed.

Most diets supplemented with omega-3 fatty acids contain more EPA than DHA (O'Connor et al., 2004; Sundrarjun et al., 2004; Hall et al., 2002; Watkins et al., 2002).

This is not the case with our diet, as DHA exceeded EPA concentrations two fold. Because EPA is the competitive inhibitor of AA, higher concentrations of this omega-3 fatty acid will cause beneficiary effects in the inflammatory pathways (Watkins et al., 2002), while DHA will not elicit the same responses. Further, the diet in this study had only 11 mg/kg BW of EPA compared to 36 mg/kg BW in the O'Connor et al. study (2004). When comparing concentrations of DHA, this study contained only 22 mg/kg BW, while O'Connor et al. (2004) had 27 mg/kg BW. This lower amount of both EPA and DHA could cause the lack of bone responses that have been shown in other animals (Watkins et al., 2002; Watkins et al., 1997). To elicit a more positive response of both bone markers and markers of inflammation, more EPA would need to be added to the diet.

Increased concentrations of EPA have been associated with supplementing fish oils in the diet (Kruglik et al., 2005; Hall et al., 2004; Watkins et al., 2000). The current study found no such association between feeding omega-3 fatty acids and EPA. Instead, the concentration of plasma EPA remained unchanged throughout the trial, no matter which diet the horses were receiving. Even without an increase in EPA, FA horses showed an increase in total omega-3 fatty acids. One would believe that because total omega-3 fatty acids increased, the concentration of EPA would have also increased due to the low amount of EPA within the supplement. However, the LC PUFA supplement may not have had a large enough quantity of EPA to cause metabolic responses. More research would have to be performed to establish whether the amount of EPA within the omega-3 fatty acid supplement could bring about these changes. As little as 9 g/100 kg

body weight has demonstrated lower prostaglandin levels (Watkins et al., 2000); however, no set amount of EPA or DHA supplementation has been recorded and indicated to bring about changes within the prostaglandin pathway in horses.

In the present study, plasma DHA concentrations increased only in horses fed the FA diet. Docosahexaenoic acid concentrations increased significantly in FA horses from d 0 to d 25, and remained elevated throughout the duration of the trial. Similar results of increased DHA with incorporation of fish oil into the diet have been found (Kruglik et al., 2005; Hall et al., 2004; Watkins et al., 1997). Because DHA cannot be synthesized within the body, even a small amount of the fatty acid can affect plasma concentrations.

With increased omega-3 fatty acids within plasma fatty acid profiles, benefits of EPA and DHA can be seen in increased bone formation and decreased inflammation. Omega-3 fatty acids have beneficial effects on bone growth and remodeling (Watkins et al., 1997); these can be analyzed by measuring osteocalcin and ICTP concentrations with blood serum. Osteocalcin is a marker of bone formation and ICTP is a marker of bone resorption; together these two markers show changes within bone matrix. Increased osteocalcin combined with stationary or decreased ICTP will lead to bone growth, while increased ICTP combined with stationary or decreased osteocalcin will cause bone resorption. Horses on the treatment diet tended to have higher concentrations of osteocalcin; however, when data were normalized, no treatment differences existed, so one cannot conclude that supplementing with LC PUFAs will cause an increase in this marker of bone formation. Young horses had higher osteocalcin and ICTP

concentrations than mature horses in bone. Young horses are still growing and their bones have not yet reached a state of maturity; therefore, their bones are undergoing more formation and resorption. Many factors could be involved with the increases in osteocalcin and ICTP concentrations on differing days of the project, including increased loading pressures provided by increasing exercise throughout the trial. Woo et al. (1981) discovered that loading pressures correspond to bone metabolism, with increased loading pressures causing an increase in bone formation. Further, Hiney et al. (2004) showed that even small bouts of intense exercise would cause an increase in the mechanical properties of bone. This research shows that with increased exercise, bone formation will occur; thus, as horses on this project went from no exercise in the three months prior to the study to exercising daily, their osteocalcin concentrations reflected the increase in bone formation due to the increase in loading.

Both OC and ICTP markers in this study are not in accordance with reports by Watkins et al. (2000) that stated that omega-3 fatty acid supplementation increases bone formation. It should be noted that the Watkins et al. (2000) study did not find treatment differences in serum OC, and there were no measurements of ICTP, bone thickness, or bone strength; instead, levels of PGE_2 and arachidonic acid were taken into consideration due to their activity in the development of bone. Furthermore, Watkins et al. used chicks (1997) and rats (2000) in studies; therefore, horses will probably utilize fatty acids differently than what was discovered in their research. Most research in livestock has shown that it is the type of exercise and loads placed upon the skeleton that will increase

bone density (Hiney et al., 2004; Nielsen et al., 2002), and not necessarily dietary supplementation.

Omega-3 fatty acids also play a role in the metabolic pathway associated with inflammation caused by arachidonic acid. Wilson et al. (2003) determined that horses fed a diet containing omega-3 fatty acids had a lower inflammatory response with exercise than did horses fed a diet supplemented with omega-6 rich corn oil. Lower inflammation was correlated to decreased concentrations of the inflammatory response marker fibrinogen. Furthermore, humans fed a high omega-3 fatty acid diet decreased synthesis of TNF-α and other inflammatory stimulants after only two weeks of supplementation (Endres et al., 1989). The current study found no differences in TNF-α concentrations between any groups of horses. The lack of response might be due to increased AA within the FA treatment. Arachidonic acid is associated with increased levels of PGE_2, which is associated with pain, swelling, and joint loss (Hall et al., 2004; James et al., 2000; Mueller and Talbert, 1988). Increased AA would have a stimulatory effect on levels of TNF-α, as both indicate inflammation.

In conclusion, this study found that horses fed a diet including only 15 g of EPA and DHA had a tendency to increase their trot stride length over a 75 d trial, even though no differences were found in lameness scores or in joint range of motion that would indicate that such an increase would take place. Further, horses fed the LC PUFA diet had increased concentrations of plasma DHA and AA, but no changes in plasma EPA. There were no treatment differences in bone markers of formation or resorption, nor were

there differences in serum markers of inflammation. Feeding a diet supplemented with LC PUFAs may improve overall joint health, despite the lack of markers, if one considers that horses with unsoundness tend to have shorter strides, while horses fed LC PUFAs had a tendency to increase stride length.

CHAPTER 5

IMPLICATIONS

This study found that horses fed a diet consisting of 15 g of EPA and DHA had a tendency to increase their trot stride length by 15 cm over a 75 d trial. This increase in trot stride length occurred even though no lameness differences were found between treatments and range of motion scores remained consistent from d 0 to d 75. Trot stride length was likely increased due to horses experiencing less pain associated with osteoarthritis during movement of the joint.

Further, horses fed the omega-3 fatty acid diet had increased concentrations of plasma DHA and AA, but no changes in plasma EPA. Docosahexaenoic acid is not produced by the body, so all changes were due to supplementation. The increase in AA was most likely due to an increased level of AA within the supplement. Arachidonic acid is known to be associated with bone development (Watkins et al., 2000) and inflammation (James et al., 2000). However, increased levels of omega-3 fatty acids reduce the occurrence of inflammation and increase the performance of horses (Wilson et al., 2003). There were no treatment differences in bone markers of formation or resorption, nor were there differences in serum markers of inflammation within this study. A supplement that provides the benefits of EPA and DHA while keeping levels of AA at a low rate would increase the chances of observing increases in bone formation and decreases in inflammation.

Feeding a diet supplemented with omega-3 fatty acids may still improve overall joint health, despite the lack of markers, if one considers that horses with unsoundness tend to have shorter strides, while horses fed omega-3 fatty acids had a tendency to increase stride length by 15 cm during the trial period. Horses with unsoundnesses would not only benefit from omega-3 fatty acids; any horse that competes in an activity where stride length is important would also benefit from supplementing omega-3 fatty acids. When races come down to a fraction of second between winning and losing, an increase in stride length can have a benefit to those horses competing in high intensity races.

More research is needed to determine how the omega-3 fatty acids caused an increase in stride length in horses. For example, discoveries need to be made about where the beneficiary effects took place—in the muscle or in the joint. Furthermore, research on how movement changed with supplementation could help determine what caused horses fed omega-3 fatty acids to be able to stretch joints more for increases in strides. Research should also be performed on different levels of omega-6:omega-3 ratios and the benefits these lower ratios have on bone growth. Watkins et al. (2000) showed that a low ratio will cause an increase in bone formation; therefore, until this conclusion has been completely researched, the omega-6:omega-3 fatty acid ratio should not be overlooked as a way to influence bone growth.

Research should also be performed on the effects of omega-3 fatty acids on inflammation in horses. While this study did not show a decrease in markers of inflammation, the work of Wilson et al. (2003) did note that omega-3 fatty acids

decreased inflammation markers and improved performance of horses. Many aspects of our supplement could have had an affect on the results of this study, including a higher than desirable omega-6:omega-3 fatty acid ratio and high levels of AA within the supplement. Controlling these two elements by decreasing both the ratio and AA could possibly bring about different results than those measured here.

Watkins et al. (2000) states that supplementing with omega-3 fatty acids increased EPA, DHA and bone formation, and decreased omega-6:omega-3 ratio, PGE_2, and AA. Our results in horses agree with only the increase in DHA and decrease in omega-6:omega-3 ratio; however horses receiving omega-3 fatty acids did experience an increase in trot stride lengths. Therefore, we conclude that supplementing with omega-3 fatty acids will cause increased total omega-3 fatty acids, DHA, and trot stride length in horses. No difference between treatments was observed in EPA, bone markers, nor markers of inflammation.

APPENDIX

CHAPTER 4: RESULTS

Lameness

A trend for a treatment (P=0.05) difference was seen in overall ROM. Treatment horses tended to have a higher (7.86 ± 0.06, P=0.05) overall ROM than CO horses (7.67 ± 0.1)(Table 4).

A day difference (P<0.05) was observed when comparing total carpal joint scores. Scores were higher on d 0 compared to d 75 (P<0.05). Control horses had a greater decrease (0 vs. 0.50 ± 0.12) on d 75 than d 0 when compared to FA horses (0 vs. 0.17 ± 0.12).

A day (P<0.01), a trend for a treatment (P=0.07), and a treatment*day interation (P=0.07) difference was noted in lameness on the left carpus joint. Both treatments had lower (0 vs. 0.25 ± 0.24, P<0.01) lameness scores on d 75 when compared to d 0. Both treatments lowered the lameness score, but CO horses tended to show a larger decline (0 vs. 0.42 ± 0.09, P=0.07) in lameness scores on d 75 compared to d 0 than FA horses (0 vs. 0.83 ± 0.09, P=0.07).

There was a treatment (P<0.05) difference in lameness scored of the left metatarsalphalangeal (MTP) joint. FA horses decreased more (0.33 vs. 0.83 ± 0.23, P<0.05) in lameness score on d 75 compared with d 0 than CO horses (0 vs. 0.17 ± 0.23).

Right metacarpalphalangeal (MCP) range of motion (ROM) tended to show a treatment (P=0.07) and day (P=0.07) difference. Control horses tended to have a larger decrease (0.80 vs. 0.92 ± 0.04, P=0.07) in ROM on d 75 compared with d 0. Horses on the FA diet, however, also had decreased (0.92 vs. 0.97 ± 0.04) ROM in the right MCP, but it was not as pronounced as in CO horses.

Left MCP ROM tended to have a treatment (P=0.06) difference. Both treatments showed a tendency to decrease (P=0.06) in ROM scores on d 75 compared to d 0, but CO horses decreased more (0.77 vs. 0.87 ± 0.05) than FA horses (0.88 vs. 0.95 ± 0.05).

Total left joint ROM tended to have a treatment (P=0.06) difference. CO horses had a tendency to have lower (3.77 vs. 3.87 ±0.05) left ROM scores on d 75 than d 0 when compared to FA horse (3.88 vs. 3.95 ± 0.05).

Range of motion for total MCP had trends for both treatment (P=0.05) and day (P=0.07) differences (Table 5). CO horses had a more pronounced decrease (1.57 vs. 1.78 ± 0.09) in ROM on d 75 than d 0, compared to FA horses, whose scores were (1.80 vs. 1.92 ± 0.09) on d 75 compared to d 0.

There were no other differences in lameness scores or range of motion scores between horses.

Plasma Fatty Acid Profiles

No differences were seen in the following fatty acids: 18:1c6, 18:1t10, 18:3n6, 20:1n12, 20:1n15, 20:2n6, 22:3n3, 22:4n6, 22:5n3, and 24:1n9 (Table 1A).

A day difference was observed for 16:0 ($P<0.01$). This fatty acid increased form d 0 to d 25 (13.80 % vs. 14.54 % ± 0.30, $P<0.05$), remained the same from d 25 to d 50, and returned to baseline values on d 75 (14.69 % vs. 13.31 % ± 0.30, $P<0.01$).

A day difference was noted for 16:1n7c ($P<0.01$). This fatty acid was lower in d 50 compared to d 0 (0.96 % vs. 0.70 % ± 0.11, $P<0.05$), on d 50 compared to d 25 (1.10 % vs. 0.70 %, $P>0.01$), and on d 75 compared to d 25 (1.10 % vs. 0.80 % ± 0.11, $P<0.05$).

A trend for a treatment difference ($P=0.05$) and a day difference ($P<0.0001$) for 16:1n7t was seen. CO horses tended to have lower 16:1n7t concentrations than FA horses (0.34 % vs. 0.21 % ± 0.04, $P=0.05$). Concentrations of this fatty acid decreased from d 0 to d 25 (0.43 % vs. 0.05 % ± 0.04, $P<0.0001$), remained the same from d 25 to d 50, and increased on d 75 to baseline concentrations (0.10 % vs. 0.52 % ± 0.04, $P<0.0001$).

A day difference existed for 17:0 ($P<0.0001$). This fatty acid decreased on d 25 and d 50 and then returned to baseline values on d 75 (0.55 %, 0.24 %, 0.25 %, and 0.47 % ± 0.05, respectively, $P<0.001$).

A day difference was observed for 17:1n7 ($P<0.05$). This fatty acid decreased from d 0 to d 25 (0.05 % vs. 0.00 % ± 0.01, $P<0.01$), and was lower on d 50 compared to d 0 (0.00 % vs. 0.05 % ± 0.01, $P<0.05$). No differences between d 25 and d 50, or d 50 and d 75 were noted, yet d 25 tended to be lower than d 75 (0.00 % vs. 0.04 % ± 0.01, $P=0.06$).

A day difference was shown for 18:0 ($P<0.0001$). This fatty acid increased from d 0 to d 25 (14.44 % vs. 15.32 % ± 0.35, $P<0.01$), decreased and returned to baseline on d 50 (15.32 % vs. 14.11 % ± 0.35, $P<0.01$), and decreased on d 75 (14.11 % vs. 13.31 % ± 0.35, $P<0.05$). When data was normalized, there tended to be a treatment difference, with CO horses maintaining a higher concentration of 18:0 compared to FA horses (0.25 % vs. –0.65 %, $P=0.08$).

Normalizing data for 18:1c11 caused a trend for treatment differences to develop, where CO horses had a lower concentration of 18:1c11 compared to FA horses (-0.03 % vs. 0.17 %, $P=0.06$).

A day difference existed for 18:1c9 ($P<0.0001$). This fatty acid decreased from d 0 to d 25 (12.64 % vs. 8.99 % ± 0.50, $P<0.0001$), and there was no difference between d 25, d 50, and d 75.

A day difference was seen for 18:1t11 ($P<0.05$). No differences existed between d 0, d 25, and d 50; however, 18:1t11 increased on d 75 compared to all other days (0.00 % vs. 0.04 % ± 0.01, $P<0.01$).

A day difference for 18:1t6-8 ($P<0.0001$). This fatty acid decreased from d 0 to d 25 (0.32 % vs. 0.01 % ± 0.04, $P<0.0001$), remained the same between d 25 and d 50, and increased to return to baseline values on d 75 (0.02 % vs. 0.26 % ± 0.04, $P<0.0001$). When data was normalized, there tended to be a treatment difference, where CO horses had a higher concentration of 18:1t6-8 than FA horses (–0.31 % vs. –0.12 %, $P=0.05$).

Treatment ($P<0.01$) and day differences ($P<0.05$) were observed for 18:1t9. Overall, FA was lower than CO (0.01 % vs. 0.13 % ± 0.02, $P<0.01$). There was a tendency for the fatty acid to increase on d 25 compared to d 0 (0.00 % vs. 0.09 % ± 0.04, $P=0.09$). There was no difference between d 25 and d 50, and fatty acid levels returned to baseline on d 75 (0.15 % vs. 0.04 % ± 0.04, $P<0.05$). Day 0 was lower than d 50 (0.00 % vs. 0.15 % ± 0.04, $P<0.01$), and d 25 was not different from d 75.

There tended to be a treatment difference for 18:2n6 ($P=0.07$), and there was a day difference ($P<0.0001$) for 18:2n6. Fatty acid 18:2n6 tended to be higher in CO compared to FA (45.61 % vs. 43.76 % ± 0.67, $P=0.07$). This fatty acid was highest on d 0 compared to all other days (41.40 % vs. 46.52 %, 47.11 %, and 43.72 % ± 0.70, respectively, $P<0.01$). There was no difference between d 25 and d 50; however, the concentration rose again on d 75 ($P<0.01$).

A day difference was shown for 18:3n3 ($P<0.0001$). This fatty acid decreased from d 0 to d 25 (3.70 % vs. 1.67 % ± 0.33, $P<0.0001$). The concentration remained below baseline levels on both d 50 (3.70 % vs. 1.14 % ± 0.33, $P<0.0001$) and d 75 (3.70 % vs. 2.26% ± 0.33 $P<0.01$). No concentration differences existed between d 25 and d 50 or between d 25 and d 75; however, concentrations increased again between d 50 and d 75 (1.14 % vs. 2.26 % ± 0.33, $P<0.01$).

When data was normalized, there was a treatment difference; CO horses maintained a higher concentration of 18:3n3 than FA horses (–1.10 % vs. –2.92 %, $P<0.01$) due to high concentrations of this fatty acid in FA horses on d 0.

A trend for a day difference was observed ($P=0.10$) for 20:0. Day 0 tended to be higher than d 25 (0.59 % vs. 0.45 % ± 0.07, $P=0.08$), and d 25 was lower than d 75 (0.45 % vs. 0.64 % ± 0.07, $P<0.05$).

When data was normalized there was a treatment difference ($P<0.05$) the two groups. The fatty acid 20:0 decreased in CO horses while increasing in FA horses (–0.23 % vs. 0.11 %, $P<0.05$).

A day difference was observed for 20:1n9 ($P<0.0001$). This fatty acid increased from d 0 to d 25 (0.00 % vs. 1.52 % ± 0.23, $P<0.0001$), and there was no difference

between d 25 and d 50. The concentration of this fatty acid decreased and returned to baseline on d 75 (1.25 % vs. 0.00 % ± 0.23, P<0.0001).

A trend for a treatment difference (P=0.05), and a day difference (P<0.05) were noted for 20:3n6. Horses in the FA group tended to have higher concentrations of this fatty acid than CO horses (0.13 % vs. 0.04 % ± 0.03, P=0.05). No differences were seen between d 0, d 25, and d 50. However, concentration of 20:3n6 increased throughout the treatment period, with d 75 being higher than d 0 and d 25 (0.16 % vs. 0.03 % and 0.08 % ± 0.03, respectively, P<0.05), and d 75 tended to be higher than d 50 (0.16 % vs. 0.09 % ± 0.03, P=0.06).

Treatment (P<0.05) and day differences (P<0.01) were observed for 20:3n3. FA horses had higher concentrations of this fatty acid than CO horses (1.10 % vs. 0.73 % ± 0.11, P<0.05). Day 0 was lower than d 25 (0.79 % vs. 1.25 % ± 0.15, P<0.05) and tended to be lower than d 50 (0.79 % vs. 1.19 % ± 0.15, P=0.06); however d 0 tended to be higher than d 75 (0.79 % vs. 0.44 % ± 0.15, P=0.09). No difference between d 25 and d 50 was seen; however, d 25 was higher than d 75 (1.25 % vs. 0.44 % ± 0.15, P<0.01), and d 50 was also higher than d 75 (1.19 % vs. 0.44 % ± 0.15, P<0.01).

A day difference was observed for 22:0 (P<0.05). The concentration of this fatty acid was lower the first three collection periods before increasing from d 50 to d 75 (0.16 % vs. 0.30 % ± 0.04, P<0.05).

A day difference was shown for 22:1n9 ($P<0.0001$). This fatty acid decreased from d 0 to d 25 (0.89 % vs. 0.09 % ± 0.12, $P<0.0001$), and remained low from d 25 to d 50. The fatty acid then increased to higher than baseline concentrations from d 50 to d 75 (0.04 % vs. 1.30 % ± 0.12, $P<0.0001$). Concentrations on d 75 were higher than concentrations on d 0 (1.30 % vs. 0.89 % ± 0.12, $P<0.005$).

There was a treatment difference ($P<0.05$), a day difference ($P<0.01$), and a difference in the interaction of treatment*day ($P<0.01$) for 22:2n6. All these differences had to do with the increase in concentration of this fatty acid in the CO horses on d 75 (0.00 % vs. 0.10 % ± 0.01, $P<0.0001$). All other days and interactions are the same. Because of this high increase, FA horses had lower concentrations of 22:2n6 than CO horses (0.00 % vs. 0.03 % ± 0.01, $P<0.05$).

A treatment difference ($P<0.0001$) was noted for 24:0. Horses in FA group had higher concentrations than CO horses (0.84 % vs. 0.15 % ± 0.09, $P<0.0001$). Upon normalization, all other differences ceased to exist.

Erythrocyte Fatty Acid Profiles

No differences in treatment, day, or treatment*day interaction were observed for the following fatty acids: 16:1n7c, 17:0, 17:1n7c, 18:1c6, 18:1t10, 18:1t11, 18:1t6-8, 18:1t9, 18:2n6c, 18:3n3c, 18:3n6c, 20:0, 20:1n12c, 20:1n15c, 20:1n9c, 20:2n6c, 20:3n6c, AA, EPA, 22:0, 22:1n9c, 22:2n6c, 22:3n3c, 22:4n6c, DHA, 24:0, 22:6n3 (Table 2A).

Fatty acid 16:0 decreased in both treatments ($P < 0.01$) from d 0 to d 75. Conversely, the fatty acid 16:1n7t increased in both treatments ($P < 0.01$) from d 0 to d 75. However, 18:0 decreased in both treatments ($P < 0.05$) from d 0 to d 75.

Treatment*day tended to be different for 18:1c11 ($P = 0.06$). Horses on the FA diet had increased concentrations of 18:1c11 on d 75 compared to d 0 ($P < 0.05$).

The fatty acid 18:1c9 tended to decrease in both treatments ($P = 0.05$) from d 0 to d 75, and 20:3n3c also tended to decrease in both treatments ($P = 0.07$) from d 0 to d 75. However, 24:1n9c tended to increase ($P = 0.08$) from d 0 to d 75.

Table 1A. Mean percentage (%) of total plasma fatty acids in CO or FA horses on d 0, 25, 50, and 75.

Fatty Acid	*Treatment*	*Day* 0	25	50	75	*Std. Error*
	CO	12.8	13.5	13.9	12.3	0.42
16:0	FA	14.8	15.5	15.5	14.4	0.42
	Mean	13.8[a]	14.5[b]	14.7[b]	13.3[a]	0.30
	CO	0.98	1.01	0.56	0.70	0.16
16:1n7c	FA	0.93	1.20	0.84	0.90	0.16
	Mean	0.96[b]	1.11[b]	0.70[a]	0.80[a]	0.11
	CO	0.59	0.09	0.11	0.58	0.06
16:1n7t	FA	0.28	0.00	0.10	0.47	0.06
	Mean	0.43[b]	0.05[a]	0.10[a]	0.52[b]	0.04
	CO	0.60	0.16	0.25	0.45	0.07
17:0	FA	0.49	0.32	0.26	0.49	0.07
	Mean	0.55[b]	0.24[a]	0.26[a]	0.47[b]	0.05
	CO	0.05	0.00	0.01	0.03	0.02
17:1n7	FA	0.06	0.00	0.00	0.04	0.02
	Mean	0.05[c]	0.00[a]	0.01[ab]	0.04[bc]	0.01
	CO	14.1	15.5	14.4	13.2	0.50
18:0	FA	14.8	15.1	13.8	13.4	0.50
	Mean	14.4[b]	15.3[c]	14.1[b]	13.3[a]	0.35
	CO	0.65	0.73	0.51	0.63	0.11
18:1c11	FA	0.65	0.89	0.78	0.77	0.11
	Mean	0.65	0.81	0.65	0.70	0.07
	CO	12.3	9.12	9.66	8.97	0.70
18:1c9	FA	12.9	8.86	9.30	8.78	0.70
	Mean	12.6[b]	8.99[a]	9.48[a]	8.88[a]	0.50
	CO	0.00	0.00	0.00	0.06	0.02
18:1t11	FA	0.00	0.00	0.00	0.03	0.02
	Mean	0.00[a]	0.00[a]	0.00[a]	0.04[b]	0.01
	CO	0.45	0.03	0.03	0.34	0.06
18:1t6-8	FA	0.19	0.00	0.02	0.18	0.06
	Mean	0.32[b]	0.01[a]	0.02[a]	0.26[b]	0.04

Table 1A (cont'd).

	CO*	0.00	0.18	0.26	0.07	0.05
18:1t9	FA	0.00	0.00	0.03	0.00	0.05
	Mean	0.00^{a}	0.09^{bc}	0.15^{c}	0.04^{ab}	0.04
	CO	40.4	48.2	49.3	44.6	0.99
18:2n6	FA	42.5	44.8	45.0	42.8	0.99
	Mean	41.4^{a}	46.5^{c}	47.1^{c}	43.7^{b}	0.70
	CO	2.93	2.03	1.00	2.43	0.46
18:3n3	FA	4.48	1.31	1.27	2.10	0.46
	Mean	3.70^{c}	1.67^{ab}	1.14^{a}	2.26^{b}	0.33
	CO	0.65	0.27	0.42	0.58	0.10
20:0	FA	0.53	0.63	0.60	0.70	0.10
	Mean	0.59	0.45	0.51	0.64	0.07
	CO	0.00	1.14	1.29	0.00	0.33
20:1n9	FA	0.00	1.90	1.21	0.00	0.33
	Mean	0.00^{a}	1.52^{b}	1.25^{b}	0.00^{a}	0.23
	CO*	0.18	0.19	0.28	0.14	0.05
20:2n6	FA	0.02	0.00	0.03	0.00	0.05
	Mean	0.10	0.10	0.15	0.07	0.04
	CO	0.02	0.00	0.05	0.10	0.05
20:3n6	FA	0.03	0.15	0.12	0.23	0.05
	Mean	0.03^{a}	0.08^{a}	0.09^{ab}	0.16^{b}	0.03
	CO*	0.63	1.05	0.95	0.30	0.21
20:3n3	FA	0.95	1.45	1.42	0.59	0.21
	Mean	0.79^{ab}	1.25^{c}	1.19^{bc}	0.44^{a}	0.15
	CO	0.17	0.12	0.12	0.28	0.06
22:0	FA	0.13	0.13	0.20	0.33	0.06
	Mean	0.15^{a}	0.13^{a}	0.16^{a}	0.30^{b}	0.04
	CO	0.97	0.00	0.00	1.35	0.18
22:1n9c	FA	0.80	0.19	0.08	1.26	0.18
	Mean	0.89^{b}	0.09^{a}	0.04^{a}	1.30^{c}	0.12
	CO*	0.00^{a}	0.00^{a}	0.00^{a}	0.10^{b}	0.01
22:2n6	FA	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.01
	Mean	0.00^{a}	0.00^{a}	0.00^{a}	0.05^{b}	0.01

Table 1A (cont'd).

	CO	0.52	0.69	0.58	0.69	0.10
22:3n3	FA	0.51	0.76	0.71	0.55	0.10
	Mean	0.52	0.72	0.64	0.62	0.07
	CO	0.00	0.00	0.00	0.00	0.01
22:5n3	FA	0.00	0.00	0.02	0.00	0.01
	Mean	0.00	0.00	0.01	0.00	0.00
	CO	0.29	0.00	0.05	0.27	0.12
24:0	FA	0.00	1.17	1.04	1.14	0.12
	Mean	0.14^{a}	0.59^{b}	0.55^{b}	0.71^{a}	0.09

* Overall treatment difference, regardless of day: $P<0.05$.
[abc] Values within a treatment lacking common superscripts differ ($P<0.05$).

Table 2A. Mean percentage (%) of total erythrocyte fatty acids in CO or FA horses on d 0, and 75.

Fatty Acid	*Treatment*	*Day*		*Std. Error*	*P value Trt*day*
		0	*75*		
16:0	CO	9.67	7.47	1.87	P = 0.75
	FA	7.81	5.98	1.87	
	Mean	8.74[a]	6.73[b]	1.32	
16:1n7c	CO	0.81	0.76	0.29	P = 0.35
	FA	0.44	0.72	0.29	
16:1n7t	CO	0.36	1.40	0.29	P = 0.28
	FA	0.47	0.96	0.29	
	Mean	0.42[a]	1.18[b]	0.21	
17:0	CO	0.53	0.58	0.29	P = 0.96
	FA	0.59	0.63	0.59	
17:1n7c	CO	0.14	0.10	0.08	P = 0.48
	FA	0.00	0.09	0.08	
18:0	CO	6.95	5.47	1.58	P = 0.85
	FA	6.01	4.71	1.58	
	Mean	6.48[a]	5.09[b]	1.12	
18:1c11	CO	0.09	0.06	0.07	P = 0.06
	FA	0.00	0.15	0.07	
18:1c6	CO	0.12	0.35	0.32	P = 0.20
	FA	0.58	0.00	0.32	
18:1c9	CO	12.6	10.1	2.30	P = 0.53
	FA	11.3	10.0	2.30	
18:1t10	CO	0.56	0.52	0.47	P = 0.57
	FA	0.48	0.89	0.47	
18:1t11	CO	0.68	0.69	0.46	P = 0.27
	FA	1.49	0.45	0.46	
18:1t6-8	CO	2.45	1.00	0.80	P = 0.36
	FA	0.25	0.37	0.80	

Table 2A (cont'd).

18:1t9	CO	0.35	0.07	0.26	P = 0.86
	FA	0.39	0.00	0.26	
18:2n6c	CO	2.58	5.81	1.24	P = 0.33
	FA	8.56	7.70	1.24	
18:3n3c	CO	1.56	0.00	0.72	P = 0.33
	FA	0.39	0.20	0.72	
20:0	CO	0.47	0.37	0.19	P = 0.87
	FA	0.37	0.23	0.19	
20:1n9c	CO	0.17	0.05	0.13	P = 0.94
	FA	0.18	0.05	0.13	
20:2n6c	CO	1.31	1.58	0.67	P = 0.36
	FA	2.26	1.70	0.67	
20:3n6c	CO	0.06	0.00	0.02	P = 0.33
	FA	0.00	0.00	0.02	
20:3n3c	CO	5.72	4.59	1.64	P = 0.38
	FA	8.62	5.50	1.64	
20:4n6c	CO	0.10	0.18	0.15	P = 0.62
	FA	0.00	0.23	0.15	
20:5n3c	CO	4.45	4.32	1.27	P = 0.69
	FA	3.29	2.77	1.27	
22:0	CO	0.70	0.61	0.25	P = 0.14
	FA	0.22	0.47	0.25	
22:1n9c	CO	2.57	0.00	1.29	P = 0.33
	FA	0.00	0.00	1.29	
22:3n3c	CO	1.10	1.07	0.37	P = 0.41
	FA	0.58	0.85	0.37	
24:0	CO	0.06	0.00	0.03	P = 0.33
	FA	0.00	0.00	0.03	
22:6n3c	CO	0.10	0.00	0.05	P = 0.25
	FA	0.00	0.02	0.05	

Table 2A (cont'd).

24:1n9c	CO	0.00	0.55	0.20	P = 0.36
	FA	0.00	0.17	0.20	

[abc] Values within a row lacking common superscripts differ in day values ($P < 0.05$).

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