

OMEGA-3 FATTY ACID SUPPLEMENTATION REDUCES BASAL TNF α BUT
NOT TOLL-LIKE RECEPTOR STIMULATED TNF α IN FULL SIZED AND MINIATURE
MARES

by

JOYCE MARIE DINNETZ

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Major Professor
J. Ernest Minton

Abstract

It has been well documented that omega-3 PUFA (**n-3 PUFA**) can confer a wide variety of health benefits to humans and animals. The current study was designed to evaluate the ability of n-3 PUFA to modulate the innate immune response in two diverse breeds of horses. Ten Quarter Horse and 10 American Miniature Horse mares were assigned to either an n-3 PUFA supplemented or control diet (5 full-sized and 5 miniature mares/treatment) for 56 d. The treatment diet was designed to deliver 64.4 mg/kg BW combined eicosapentaenoic acid (**EPA**) and docosahexaenoic acid (**DHA**) daily. Whole blood (20 mL) was collected via jugular veinipuncture into heparinized tubes on 0 d, 28 d, and 56 d. Serum PUFA analysis was conducted by gas chromatography. Peripheral blood mononuclear cell (**PBMC**) production of tumor necrosis factor α (**TNF α**) in response to toll-like receptor (**TLR**) ligands lipopolysaccharide (**LPS**), flagellin, and lipoteichoic acid (**LTA**) was estimated using an equine-specific ELISA. Peripheral blood samples from d 56 were also analyzed for total and differential leukocyte counts and subjected to flow cytometric analysis. Body type did not affect basal or TLR stimulated TNF α production. Serum PUFA analysis revealed a decrease in linoleic acid (**LA**) and substantial increases in arachidonic acid (**ARA**), EPA, DHA, and docosapentaenoic acid (**DPA**) at both d 28 and 56 in horses fed n-3 PUFA ($P < 0.0001$ for all). Dietary n-3 PUFA supplementation reduced ($P < 0.05$) un-stimulated basal, but not TLR stimulated TNF α production by PBMC's. Supplementation with n-3 PUFA did not affect total or differential leukocyte counts, nor selected cell surface markers. These results suggest that n-3 PUFA supplementation in the horse can modify circulating PUFA and alter the inflammatory response by reducing basal TNF α production. Furthermore, under conditions of the current study and considering the endpoints

evaluated, the American Miniature Horse could potentially be used as a model for full-sized horse breeds.

Key words: omega-3 fatty acid, tumor necrosis factor alpha, horse, American Miniature Horse

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Dedication

I would like to dedicate this project to my husband. Without his encouragement I could not go on. I hope I have made you very proud.

“Alone we can do so little; together we can do so much.”

-Helen Keller

CHAPTER 1 - General Review of Literature

Introduction

Polyunsaturated fatty acids (**PUFA**; those unsaturated with two or more double bonds within their hydrocarbon chain) are necessary for the health and well being of humans and animals. Those PUFA which cannot be synthesized de novo by animal cells are considered essential, and are divided into two classes based on the location of the first double bond in their hydrocarbon tail. Omega-6 (**n-6**) fatty acids (**FA**) are those PUFA with several double bonds, with the first located six carbons from the methyl end of the carbon chain. Omega-3 (**n-3**) PUFA have several double bonds within their carbon tail beginning at the third carbon from the methyl end. All members of these two FA classes are derived from their respective precursors linoleic acid (**LA**, 18:2n-6) and α -linolenic acid (**ALA**, 18:3n-3) by desaturation and elongation within the liver (Bezard et al., 1994; Allen and Harris, 2001). These PUFA are important constituents of phospholipids in cell membranes and play a major role in membrane fluidity and integrity.

Omega-3 PUFA confer several health benefits to animals and humans alike. These benefits of PUFA supplementation could translate to horses. Supplementing corn based diets with various forms of eicosapentaenoic acid (**EPA**, 20:5n-3) or docosahexaenoic acid (**DHA**, 22:6n-3) could benefit horses by replacing lost n-3 PUFA in their diet. Further research is being conducted to investigate the benefits of n-3 PUFA supplementation in the horse. The existing body of literature regarding immune and inflammatory cell function in horses receiving dietary n-3 PUFA is somewhat limited. In addition, the published literature has not accounted for potential differences in n-3 PUFA influences on diverse breeds of horses, including in the

miniature horse. Miniature horses may prove to be a useful and more economical experimental model for full-sized horses.

The intended scope of the section that follows is to review published literature of the effects of n-3 PUFA supplementation, primarily in horses, and to consider the literature that could suggest the miniature horse as a physiological model for the full-sized horse.

Fatty Acid Biosynthesis

Of the four families of PUFA, LA and ALA serve as precursors for the synthesis of other essential fatty acids in tissues, like arachidonic acid (ARA, 20:4n-6), EPA, or DHA (Bezard et al., 1994; Allen and Harris, 2001). Essential fatty acid biosynthesis is an interrelated process which occurs mainly, but not exclusively, in the liver and is comprised of several desaturation-elongation steps (Fig 1.1). The capacity of tissues or organs to synthesize n-6 or n-3 PUFA is dependent upon the concentration of desaturation and elongation enzymes. Desaturation introduces a double bond next to the carboxyl end of the carbon chain of the FA so that the first double bond is retained in its position from the methyl end. The first desaturation step (converting LA to various intermediates and ARA and ALA to stearidonic acid), catalyzed by the enzyme Δ 6-desaturase, is considered the rate limiting step in ARA and EPA synthesis (Bezard et al., 1994). Modification of the diet to increase any of the PUFA can impact synthesis of other FA (Allen and Harris, 2001). For example, a high concentration of ALA inhibits conversion of LA to γ -linolenoyl-CoA by Δ 6 desaturase because both ALA and LA are substrates for the same enzyme. The conversion of dihomo- γ -linolenoyl-CoA to ARA by Δ 5-desaturase is also inhibited by ARA, EPA, and DHA (Bezard et al., 1994). Thus, increased concentrations of a particular EFA (perhaps due to dietary supplementation) in the body can impact synthesis of another. Once

absorbed or synthesized from precursors, PUFA can be incorporated into various membranes or tissues where they can affect a wide variety of physiologic mechanisms.

PUFA Supplementation and Circulating FA Profiles and Blood

Characteristics in the Horse

Effects on circulating PUFA profiles. In recent years, several studies have investigated the potential for PUFA supplementation to modify circulating blood characteristics and fatty acid profiles in the horse. EPA and DHA must be present in blood to be incorporated into other tissues and render themselves available for incorporation into various tissues or membranes, or to act as substrates for various biological pathways like eicosanoid biosynthesis. Discrepancies lie, however, in the appropriate type and amount of FA source being fed. The richest and most abundant natural sources of EPA and DHA are found in fatty fish oils, flaxseed and flaxseed oil, and linseed oil. Caution must be taken when reviewing current PUFA supplementation studies, as FA concentration and bioavailability can vary between sources. For instance, EPA is more abundant in fish oil compared to DHA. Another important factor to include in critical analysis of current research is the time FA may take to appear in and clear from circulation. Although rapid changes in FA profiles have been seen, there is substantial variation among studies. When fed varying concentrations of EPA and DHA (0 to 40 g/d) from a marine-derived powder preparation, 16 light breed horses showed a dose-dependent increase in FA concentration in plasma (King et al., 2008). Peak plasma increases in EPA and DHA were 13x and 10x control values, respectively when horses were fed EPA plus DHA at 40g/d. EPA and DHA equilibrated at their peak levels by 7d of commencement of supplementation and declined by up to 75% by 9d after cessation of supplementation. Horses fed 10 or 20 g/d, however, were not different from

control horses, suggesting that supplementing fish oil with 30 or 40g EPA and DHA per day can rapidly and significantly increase circulating n-3 PUFA in plasma (King et al., 2008). The same protocol was used to investigate the effect of n-3 PUFA on red blood cells (**RBC**). Both EPA and DHA content increased in RBC after 23 d of feeding and remained elevated until 59 d following the end of supplementation (King et al., 2008). Flaxseed and fish oil were compared in an additional study (Vineyard et al., 2007) investigate differences in clearance of n-3 PUFA. Horses were fed 6 g/ 100kg BW total n-3 PUFA by way of either fish oil or flaxseed for 10 wk, preceding an 8 wk washout period without supplementation. After 10 wk, horses fed fish oil had increased plasma and RBC EPA, DHA, and total n-3 PUFA when compared to flaxseed or control fed horses. RBC EPA and DHA, along with plasma EPA declined to baseline levels at 5 wk in fish oil fed horses. Plasma DHA remained above baseline levels until the 8 wk sampling. Flaxseed clearance was not reported (Vineyard et al., 2007).

Another study investigated the effect of feeding fish oil and corn oil on serum fatty acid concentrations in Thoroughbreds. The menhaden fish oil source (top-dressed) delivered approximately 35 g/d of EPA and DHA, whereas the corn oil source delivered about 0.70 g/d. It was found that after 63d of supplementation, fish oil fed horses showed a significant increase in EPA and DHA, but the horses fed corn oil did not change FA concentrations. Also, the horses fed fish oil had a lower concentration of n-6 PUFA (O'Connor et al., 2007). Similar trends were seen by that same group of investigators in a previous study (O'Connor et al., 2004) where the same diet increased 12 FA compounds and caused a shift in the n-6:n3 PUFA ratio toward n-3 PUFA in serum after 63 d of supplementation. Interestingly, feeding seal blubber oil also appears to increase plasma n-3 PUFA in the horse by providing a significant EPA source. In that study, only EPA and not DHA was incorporated into phospholipids (Khol-Parisini et al., 2007). These

studies suggest that supplementing EPA and DHA as little as 35 g/d can alter FA profiles in horses which could reflect FA content in tissues.

When flaxseed oil was fed as a source of ALA for 16 wk, marked differences in serum chemistry occurred compared to the control group. EPA, but not DHA, was significantly increased in serum in supplemented horses (Hansen et al., 2002). Horses fed menhaden fish oil (3.0% by weight) compared to corn oil for 12 wk showed a 27-fold increase in EPA and a 34-fold increase in DHA in plasma (Hall et al., 2004). Interestingly, in that same study, ARA also increased by 8-fold. Synovial explants which were bathed in ALA for 24 h showed increased incorporation of ALA into membrane phospholipids, with a corresponding decrease in ARA (Munsterman et al., 2005). Lipid emulsions consisting of fish oil administered intravenously increased EPA and DHA in equine monocyte phospholipids, which remained elevated for up to 7 d (McCann et al., 2000). Arabian horses fed 15 g/d both EPA and DHA for 75 d increased their total plasma n-3 PUFA as well as their plasma DHA. Supplemented horses, in this investigation, increased their plasma n-6 PUFA and did not have an increase in plasma EPA, which could be due to the addition of their supplement to sweet feed that most likely contained corn products (Woodward et al., 2005).

Exercise related effects of n-3 PUFA supplementation. Thoroughbreds were fed a similar ration of EPA and DHA in fish oil (34 g/d) for 63 d and were subjected to exercise of increasing intensity. The results suggest that fish oil supplementation reduced heart rate during exercise (O'Connor et al., 2004), although other studies showed no difference in pulmonary function in horses fed fish oil (Khol-Parisini et al., 2007) or heart rate in horses fed soybean (n-3) oil for 28 d compared to corn oil (Howard et al., 2003). In addition, fish oil fed horses tended to have lower packed cell volume, and presumably, lower viscosity (O'Connor et al., 2004). Athletic horses

participating in event training that were supplemented with a vitamin-EPA-DHA mixture for 3 wk showed increased plasma EPA and DHA concentrations as well as a shift in the n-3: n-6 ratio, favoring n-3 (De Moffarts et al., 2007).

It is clear from the foregoing that circulating n-3 PUFA can be altered in horses using a variety of dietary approaches. This rather wide range of approaches (e.g. infusion versus feeding) and potential sources of dietary n-3 PUFA complicate conclusions that might point to a desired intake of n-3 PUFA to achieve a particular endpoint. Moreover, it is difficult to discern definitively if supplementation with sources of ALA are adequate to enrich tissues, especially inflammatory tissues, with EPA and DHA to achieve a defined effect. In addition, it is not entirely clear whether the total intake of n-3 PUFA in the form of EPA and DHA together achieve similar effects as the same total n-3 intake provided from one fatty acid or the other. Finally, it is worth noting that often, the contribution of the forage portion of the diet to n-3 and n-6 intake in equine studies has not been consistently documented and this proves to be another complicating factor.

Effects on blood characteristics. In addition to modifying the FA profile in circulation in the horse, dietary n-3 PUFA supplementation can affect serum cholesterol and plasma triglyceride concentrations. Studies in rats have shown a decrease in circulating plasma triglycerides associated with n-3 PUFA supplementation (Surette et al., 1992; Fickova et al., 1998). In horses, serum triglycerides were lowered by the conclusion of a 63 d study in which experimental subjects were supplemented with fish oil, compared to those fed corn oil. However, serum cholesterol was only lower in fish oil fed horses after 28 d (O'Connor et al., 2007). In exercising horses, serum cholesterol concentration following 28 d of soy oil supplementation was not different from horses fed the control diet, but was decreased compared to corn oil fed

horses (Howard et al., 2003). Cholesterol in serum of horses fed fish oil for 63 d was lower than controls, yet serum triglycerides were not altered by supplementation (O'Connor et al., 2004).

Plasma glucose concentrations were also affected by n-3 PUFA supplementation. At the onset of exercise, plasma glucose concentrations in horses receiving fish oil were not different from those horses fed corn oil. Plasma glucose concentration of corn oil fed horses continued to rise during recovery until 10 min after exercise, whereas glucose concentration of the fish oil treated horses leveled out 4 min after exercise (O'Connor et al., 2004). In the same study, serum insulin concentrations tended to be lower in fish oil fed exercising horses. Concern has developed over the recent influx of n-3 PUFA supplementation studies and their impact on lipid peroxidation. It has been suggested that ALA, EPA, and DHA may potentiate lipid peroxidation due to their high degree of unsaturation. Vitamin E is often added to fat supplemented diets to prevent this. Fish oil and flaxseed were supplemented to Quarter Horse yearlings to deliver 6 g/100 kg BW for 70 d, and lipid peroxidation and vitamin E status were observed. After 70 d of supplementation, yearlings fed fish oil had nearly 2-fold higher serum vitamin E concentration than control or flax fed horses. Various lipid peroxidation markers were not affected by fish oil supplementation, although increases in DHA and EPA concentrations were seen in plasma and RBC. This study indicates that dietary fish oil does not hinder vitamin E status, nor increase the likelihood of lipid peroxidation in yearling horses (White et al., 2007).

Polyunsaturated Fatty Acid Effects on Reproductive Physiology

It has been suggested that EPA and DHA compete with ARA for incorporation in phospholipid membranes, and that women with high concentrations of ARA in tissues have been shown to deliver preterm. In addition, FA status of the neonate appears to be related to gestational age (Allen and Harris, 2001). Fetal DHA accumulation occurs during the last

trimester; therefore preterm infants may have inadequate concentrations of DHA to fully support optimal neural and retinal development (DHA and ALA are required for normal brain development and visual acuity; Allen and Harris, 2001). Numerous studies suggest that eicosanoids, like prostaglandin E₂ (**PGE₂**), influence gestational length and parturition in humans and animals. Supplementation with n-3 PUFA can convert inflammatory series eicosanoids into less inflammatory forms (Henry et al., 1991; De Caterina; Funk, 2001; Calder et al., 2002; Calder, 2002a; Calder and Grimble, 2002; Hall et al., 2004). By inhibiting or reducing PGE₂ synthesis from ARA metabolism, n-3 PUFA supplementation can increase collagen synthesis in ligament fibroblasts. This could increase term length. In support of that point, results from one study showed a 70% decrease in collagen concentration in preterm women (Uldbjerg et al., 1983). Animals with high n-3: n-6 ratios in their diet have decreased prostaglandin synthesis and increased lengths of gestation (Abayasekara and Wathes, 1999). By increasing the gestational length in normal individuals or those that previously delivered preterm offspring, it is suggested that birth weight, general health, and vigor of an animal would increase. As prostaglandins affect many physiological elements associated with reproduction and fertility, modulating prostaglandin synthesis by dietary PUFAs could greatly impact reproductive physiology of various mammalian species (Abayasekara and Wathes, 1999).

In addition to altering FA profiles or other blood parameters, PUFA supplementation can alter milk content at the onset of lactation. For example, when pregnant mares were fed an n-6: n-3 ratio (corn oil vs. linseed oil) of 6:1, 16:1, or 2:1 for 112 d, at 56 d after parturition, mare's milk had the highest concentration of ALA in horses fed a ratio of 2:1 compared to those fed 16:1, and the highest ALA in mare plasma in horses fed a ratio of 2:1 compared to each of the other diets. It was also shown that foal plasma FA reflected the increase in ALA in milk of mares

fed the 2:1 n-6 to n-3 ratio (Spearman et al., 2005). Additionally, when mares were fed a marine-derived n-3 PUFA source (10.4 g EPA, 8.6 g DHA/mare daily) compared to corn oil for 60 d before through 21 d after parturition, milk EPA and DHA were increased compared to control mares at all sampling times. Foals of all PUFA-supplemented mares also had elevated concentrations of EPA and DHA in plasma, compared to control foals (Kruglik et al., 2005).

Sperm from all domestic species contains high levels of PUFA, in particular DHA and docosapentaenoic acid (**DPA**; 22:5 n-6). Unfortunately, most horse feeds are very high in precursors for n-6 PUFA whereas n-3 FA, like DHA, are low. The effect of feeding a DHA supplement on sperm quality of stallions was investigated in a thorough study by Brisko et al. (2005). Although total testicular volume did not differ, mean sperm concentration in ejaculates of stallions fed the DHA was 1.8-fold higher than the control stallions. In addition, the level of DHA/billion sperm cells was 3-fold higher, and the DHA: DPA ratio was 1.5-fold higher in semen of treated stallions compared to controls. Sperm from stallions fed the DHA supplement exhibited increased velocity and straighter trajectory after cooling. After 48 h of cooled storage, sperm from stallions fed DHA had increased percent total motility, progressive motility, and rapid motility. Similar improvements were seen when sperm from supplemented stallions were frozen and then thawed. However, motion characteristics of fresh semen were not improved by treatment. These results suggest that DHA supplementation could improve quality of semen from those stallions with marginal fertility, and also for those producers that ship semen (Brisko et al., 2005). When stallions were fed 29.1 g/d n-3 PUFA derived from fish oil for 90 d, similar results were seen (Harris et al., 2005). Plasma concentrations of n-3 PUFA in supplemented stallions increased by 19 d through 30d, and remained elevated throughout the 90 d sampling period. Increased daily spermatozoa output was seen in supplemented stallions. However, neither

motion characteristics nor the number of motile sperm were improved by supplementation (Harris et al., 2005).

PUFA and Eicosanoid Synthesis

Eicosanoids are oxygenated essential FA that serve as signaling molecules that are generated by the body as needed. They derive from FA that are cleaved from larger molecules (phospholipids and diacylglycerols) found in cellular membranes. The four classical eicosanoid families include leukotrienes, which are synthesized by the 5-lipoxygenase pathway; and prostaglandins, prostacyclins, and thromboxanes, that are synthesized by the cyclooxygenase (COX) pathway. These signaling molecules are important mediators of inflammation, and play an integral role in the innate immune response. Eicosanoids are said to be the link between the PUFA and the immune system (Calder and Grimble, 2002). Depending on the type of fatty acid substrate cleaved from cell membranes, eicosanoids can facilitate or inhibit inflammatory mediators. Cell membranes and tissues contain abundant ARA compared to other PUFA like EPA, and therefore is the most common substrate for eicosanoid synthesis (Funk, 2001; Calder, 2002a; Calder and Grimble, 2002; Tsatsanis et al., 2006).

Biosynthesis of prostaglandins, prostacyclins, and thromboxanes begins when ARA is released from membranes by phospholipases, most commonly phospholipase A₂ (Funk, 2001; Calder, 2002a). This release may occur due to tissue injury, cytokine or growth factor signals, or other stimuli (Funk, 2001). Free ARA acts as a substrate for the enzyme prostaglandin H synthase (also referred to as COX; the COX-2 isoform responsible for various inflammatory settings) and is then metabolized to an intermediate prostaglandin, PGH₂ (Figure 1.2). This intermediate can give rise to over 16 different 2-series prostaglandins and thromboxanes,

depending on available enzymes and cell type (Funk, 2001; Calder, 2002a; Calder and Grimbale, 2002).

Leukotrienes are synthesized primarily by inflammatory cells. Then, ARA, once cleaved by phospholipases, is a substrate for the enzyme 5-lipoxygenase in the nuclear envelope of these inflammatory cells. This enzyme transforms ARA to the epoxide leukotriene A₄ (LTA₄). Depending on the cellular context, LTA₄ may undergo one of three fates: hydrolysis, conjugation with glutathione, or transcellular metabolism to create bioactive eicosanoids. Hydrolytic attack of LTA₄ by LTA₄ hydrolase yields 4-series leukotrienes (LTA₄, B₄, C₄, D₄, and E₄) (Funk, 2001; Calder and Grimbale, 2002).

These 2-series prostaglandins and 4-series leukotrienes are heavily involved in modulating the intensity of inflammatory responses, and have been widely studied. The systemic effects of these pro-inflammatory eicosanoids include fever, vasodilation, increased vascular permeability, and enhanced pain caused by other mediators like histamine (Calder, 2002a; Calder and Grimbale, 2002). PGE₂ promotes immunoglobulin E production by B lymphocytes, which is a key mediator of allergic inflammation (Calder and Grimbale, 2002). LTB₄ promotes neutrophil chemotaxis and adhesion to vascular endothelium (Funk, 2001), enhances generation of reactive oxygen species, and production of tumor necrosis factor α (TNF α), IL-1, and IL-6 (Calder, 2002a; Calder and Grimbale, 2002). Both of these eicosanoid types exhibit anti-inflammatory effects however, such as suppression of lymphocyte proliferation and natural killer cell activity. Combined, these effects create a balance of pro-and anti- inflammatory reactions to mediate host immune homeostasis (Calder, 2002a; Calder and Grimbale, 2002).

Animal and human studies show that feeding fish oil results in increased proportions of EPA and DHA in inflammatory cell phospholipids which are incorporated at the expense of

ARA (Calder, 2001; Calder and Grimble, 2002). Because these FA replace ARA in cell membranes, there is less substrate for synthesis of eicosanoids from ARA. In addition, EPA inhibits ARA release from phospholipids by phospholipase A₂ and inhibits the oxygenation of ARA by COX (Calder and Grimble, 2002). EPA itself can act as a substrate for COX and 5-lipoxygenase (Figure 1.2), giving rise to structurally distinct prostaglandins and leukotrienes (3-series prostaglandins and 5-series leukotrienes) which are less potent inflammatory mediators (Calder and Grimble, 2002). For example, LTB₅ is about 10-fold less potent as a neutrophil chemoattractant as LTB₄ (Calder, 2002a; Calder and Grimble, 2002). These 3- and 5- series eicosanoids act through the same receptors and target cells as ARA derived eicosanoids, thus further competing and inhibiting the pro-inflammatory actions of 2- and 4-series eicosanoids (Calder, 2002a). These observations have led to the presumption that n-3 PUFA derived from fish oil and other sources are on the whole anti-inflammatory, and could benefit the health and well being of the horse.

PUFA and Inflammatory Gene Transcription

In addition to modulating eicosanoid synthesis, PUFA supplementation can affect inflammation and immunity at the level of inflammatory gene transcription. Downstream, effects on cytokine production, cell signaling, and inflammatory cell function can be seen when changes in gene transcription occur, specifically through nuclear factor- κ B (**NF- κ B**) signaling. Signaling through NF- κ B can be activated by a variety of bacterial products by way of toll-like receptor (**TLR**) signaling, and is inducible in all cell types (Bonizzi and Karin, 2004). These TLR recognize distinct pathogen-associated molecular patterns (**PAMPs**) and exert subsequent immune responses against a variety of pathogens (Akira and Takeda, 2004a; Akira and Takeda, 2004b). Analysis of mammalian TLRs has revealed that they recognize specific patterns of

microbial components that are conserved among pathogens, but are not found in mammals (Akira and Takeda, 2004b). Lipopolysaccharide (**LPS**) is a component of the outer membrane of gram-negative bacteria and is a TLR4 ligand. As a heterodimer TLR2 and TLR6 recognize a variety of peptidoglycans and lipoproteins and components of Gram-positive bacteria. Signaling through the TLR5 complex is triggered by flagellin, a flagellar component of Gram-negative bacteria. All three of these particular TLR complexes share different cell signaling pathways that eventually activate NF κ B and promote subsequent gene transcription of pro-inflammatory cytokines, particularly tumor necrosis factor α (**TNF α**)(Akira and Takeda, 2004a).

The TLR signaling pathways consist of a myeloid differentiation primary response gene (88) (MYD88) dependent pathway common to all TLRs, and a MYD88 independent pathway that is specific to only TLR3 and TLR4. The MYD88 dependent pathway is responsible for activation of NF- κ B and transcription of inflammatory cytokines. The activation and translocation of NF- κ B dimers is associated with increased transcription of genes encoding chemokines, cytokines, and adhesion molecules (Bonizzi and Karin, 2004) all of which are components of the classical immune response. The collection of NF- κ B dimers resides in the cytoplasm in an inactive form (as they are associated with molecules of the inhibitor (**I κ B**) family). Activation of NF- κ B results from the phosphorylation and proteolysis of the **I κ B** proteins which promotes their release and nuclear translocation of the NF- κ B factors (Figure 1.3). This activation process is mediated by the **I κ B** kinase complex. The **I κ B** kinase complex phosphorylates the **I κ Bs**, leading to their proteasome-mediated degradation. It is unknown how PUFA supplementation specifically affects this signaling pathway, although there may be a clue in the investigation of the inhibitory **I κ B** molecules (Akira and Takeda, 2004b). In addition, induction of COX-2 occurs through the NF- κ B signaling pathway commonly stimulated by LPS and other

TLR ligands (Tsatsanis et al., 2006). This highly integrated signaling cascade can be affected in multiple ways by PUFA status, leading to changes in the body's response to pathogen challenge, tissue damage, or disease.

PUFA Influence on Inflammation and Immunity in the Horse

Several recent studies have investigated the impact of dietary PUFA on immunity in the horse. Inflammation occurs as a protective response to injury or infection. Many of the characteristics of inflammation like swelling and pain must be controlled to avoid additional damage to the body by overproduction of inflammatory mediators (Calder, 2002a).

Unfortunately, in vitro animal studies are notoriously variable in this area. In addition to possible species or breed differences, variation in response to fish oil or PUFA supplementation may stem from genetic variation as pointed out by a recently published human study. Genetic differences were observed in TNF α production in response to endotoxin (Grimble et al., 2002). This variability was determined to be due to polymorphisms in the promoter regions of TNF α gene. It was found that TNF α production by peripheral blood mononuclear cells (**PBMC**) in response to endotoxin was most affected by fish oil supplementation in those individuals with high basal TNF α production (before supplementation) and those possessing the genetic variant. For the most part, those with moderate to high basal TNF α production were associated with homozygosity for the TNF β *2 allele and were the most sensitive to fish oil suppression of TNF α production. In addition, those individuals with low basal TNF α production were more likely to be sensitive to fish oil supplementation if they were heterozygous for the TNF β alleles.

Only a few studies have documented immune/inflammatory responses to n-3 PUFA in horses. When 6 healthy adult horses were fed a pelleted diet containing 8% linseed oil by weight over the course of 8 wk, peritoneal macrophages produced less TNF α in response to endotoxin in

vitro compared to pre-supplement measures. It was proposed that TNF α synthesis was altered by reduced adenylate cyclase activity, as a result of a high α -linolenic diet. This could alter formation of cyclic nucleotides that control TNF α synthesis (Morris et al., 1991). In another study by the same research team, an intravenous n-3 lipid emulsion decreased basal TNF α production by monocytes, as well as those cells which were challenged with calcium ionophore. In addition, an n-6 emulsion increased TNF α production by monocytes challenged with LPS (McCann et al., 2000). It should be noted, however, that in both of those studies (Morris et al., 1991; McCann et al., 2000) no time-matched un-supplemented control group was included. Thus, the pre-supplement samples were used as the control. In such a scenario, time confounds the result such that the effect of passage of time has the potential itself to affect responses and this cannot be separated from the effect of treatment.

Horses suffering from recurrent airway obstruction seem to benefit from PUFA supplementation as well. Compared to sunflower oil (n-6), seal blubber oil (36 g/kg/d BW) reduced the total cell and neutrophil count in pulmonary epithelial lining, reducing neutrophilic damage and inflammation (Khol-Parisini et al., 2007). Additional studies found that ALA supplementation through flaxseed (O'Neill et al., 2002) but not linseed oil (Friberg and Logas, 1999) was associated with reduced skin test lesion area in horses with culicoides hypersensitivity. The conflicting results in the linseed oil study were interesting due to moderately high ALA dosage, (37g/kg BW daily). Perhaps this result shouldn't be surprising, as the study relied on the competence of owners to ration and deliver the supplement and adhere to stringent grooming requirements. Inflammatory dermatitis may have been reduced by supplementing ALA in the diet to shift eicosanoid synthesis from pro-inflammatory, series-2 prostaglandins and series-4 leukotrienes to less-inflammatory eicosanoids. In fact, equine

peripheral blood neutrophils have reduced LTB₄ synthesis in response to dietary fish oil. At 12 weeks, neutrophils from horses fed fish oil produced 78 fold more LTB₅ (less inflammatory) than pre-dietary concentrations and 3.3 fold more than horses fed corn oil (Hall et al., 2004). Recall that LTB₄ is a neutrophil chemoattractant, and aids in recruitment of these cells to tissues for induction of inflammatory processes. In this respect, n-3 PUFA can reduce inflammation by dampening neutrophil chemotaxis. Although neutrophil eicosanoid synthesis may be modified by PUFA supplementation, supplementation with 16 g/d EPA and DHA did not affect phagocytosis-induced oxidative burst of *S.aureus* by neutrophils. It is interesting to note that antigen specific IgG in response to a vaccine was not different in PUFA supplemented horses compared to control horses (Vineyard et al., 2007). This gives some insight into the mechanism of action of PUFA on the immune system. In addition, PUFA may modulate the innate immune response without impairing humoral responses. When mares were fed fish oil supplements containing 8.6 g EPA and 10.4 g DHA daily for 60 d prior to foaling, PGE₂ synthesis by PBMC in response to LPS challenge was assessed. LPS failed to stimulate PGE₂ from cells isolated from mares supplemented with n-3 PUFA; however cells isolated from foals born to n-3 supplemented mares produced more PGE₂ in response to LPS, compared to controls (Skjolaas-Wilson et al., 2005).

Supplementation with n-3 PUFA can have a significant effect on synovial membrane production of eicosanoids in horses as well. Eight complete synovial explants from horses without existing lameness problems were divided and incubated with varying concentrations of ALA. Later, the explants were washed and incubated with LPS (or remained unchallenged). Concentration of PGE₂ in cell cultures in which explants were pretreated with 300 mg ALA was significantly reduced in response to LPS (Munsterman et al., 2005). Similar results were seen when arthritic horses were fed 15 g EPA and 19.8 g DHA for 90 d. Not only did treatment horses

have lower total white blood cell counts in synovial tissue collected from infected joints, but a reduction in plasma PGE₂ was seen (Manhart et al., 2007). It is interesting to note that the DHA supplement in this case was greater than EPA, yet still caused a reduction in eicosanoid synthesis. Besides modifying eicosanoid synthesis in this case, it is possible that the reduction in PGE₂ could occur by direct inhibition of ARA synthesis by down regulating the 5-desaturase enzyme involved in ARA production.

Intravenous administration of lipid emulsions enriched with n-3 and n-6 fatty acids modified equine monocyte phospholipid fatty acid composition and the synthesis of inflammatory mediators in vitro. Monocyte fatty acid analysis showed immediate incorporation of fatty acids into membranes which was sustained up to 7 d after infusion. Peripheral blood monocyte production of inflammatory mediator thromboxane B₂ was diminished by n-3 infusion but was either unchanged or increased by n-6 infusion (McCann et al., 2000). (Again, as noted previously, the experimental design of this study failed to include time-matched, untreated control animals).

An in vivo study investigated the effect of an ALA ration on the horse's response to endotoxin. After receiving a ration consisting of 8% linseed oil (ALA), horses were infused with *E. coli* endotoxin over the course of 30 min. Compared with a control group, the linseed oil treated group did not demonstrate any benefit in receiving ALA against endotoxemia. These results could be due to an overwhelming dose of endotoxin, or the failure of ALA to incorporate into membrane phospholipids (Morris et al., 1991).

Another, perhaps less obvious way PUFA can affect inflammation is through modulation of the fatty acid content of cell membranes and cell signaling. For instance, a number of cell signaling molecules are generated from membrane phospholipids. These phospholipids or

phospholipid-derived mediators have important roles in mediating the proteins involved in intricate cell signal transduction pathways, specifically within immune cells. Changing the fatty acid composition of these phospholipids may change their affinity for enzymes which could change immune cell responsiveness (Calder and Grimble, 2002).

Existing literature regarding the American Miniature Horse. In terms of research of the effects of n-3 PUFA supplementation in the American Miniature Horse, essentially no data has been published. However, there are some data showing variation of the effect of n-3 PUFA supplementation across horse breeds (Woodward et al., 2005; O'Connor et al., 2007) but no data comparing the miniature horse to the full-sized horse with regard to immune or inflammatory function. Overall, very little data has been reported on the general physiology of the miniature horse, and very little data is published comparing any biological function to the full-sized horse. One study, however, showed differences in breeding soundness exam parameters for the miniature stallion compared to the full-sized stallion. According to this study, miniature stallions had smaller testicles and fewer total spermatozoa in their ejaculate than what is considered normal in full-sized stallions (Paccamonti et al., 1999). It was suggested that different criteria should be used to evaluate the potential breeding soundness of miniature stallions that are commonly applied to full-sized stallions, and that the breeding soundness exam parameters for these should not be simply extrapolated from a typical full-sized stallion soundness exam. It appears that there may be some differences in reproductive physiology of miniature horses compared to full-sized horses. Therefore, research in n-3 PUFA supplementation in the miniature horse is needed to contribute to the body of knowledge that currently exists in this area.

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Figures

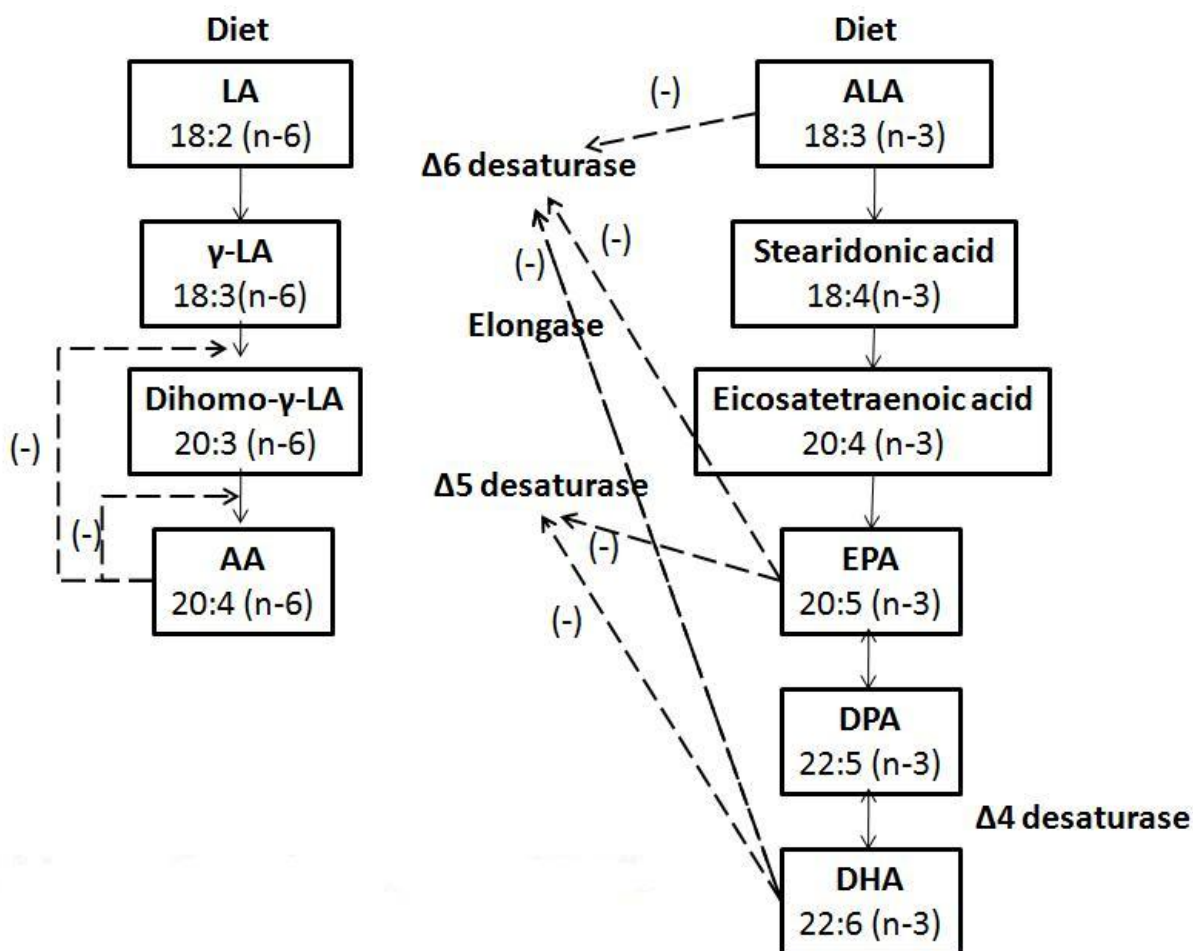


Figure 1.1. Biosynthesis of n-3 and n-6 Fatty Acids (FA) in Animal and Human Tissues. Biosynthesis of FA consists of a series of desaturation and elongation steps. Desaturation induces additional double bonds within a given carbon chain and elongation adds additional carbon atoms. The first desaturation step occurs when linoleic acid (LA, 18:2n-6) is converted to various intermediates and PUFA like arachidonic acid (AA, 20:4 n-6). Alternatively, α -linolenic acid (ALA, 18:3n-3) from the diet is converted to stearidonic acid. These two reactions are catalyzed by the enzyme $\Delta 6$ -desaturase, and are considered the rate limiting step in AA and eicosapentaenoic acid (EPA, 20:5 n-3) synthesis. Increasing the concentrations of any of the above intermediates can negatively regulate desaturase enzymes. For example, increasing amounts of AA can negatively regulate $\Delta 5$ desaturase activity for conversion of dihomogamma-LA to AA. (Redrawn from De Caterina and Basta, 2001).

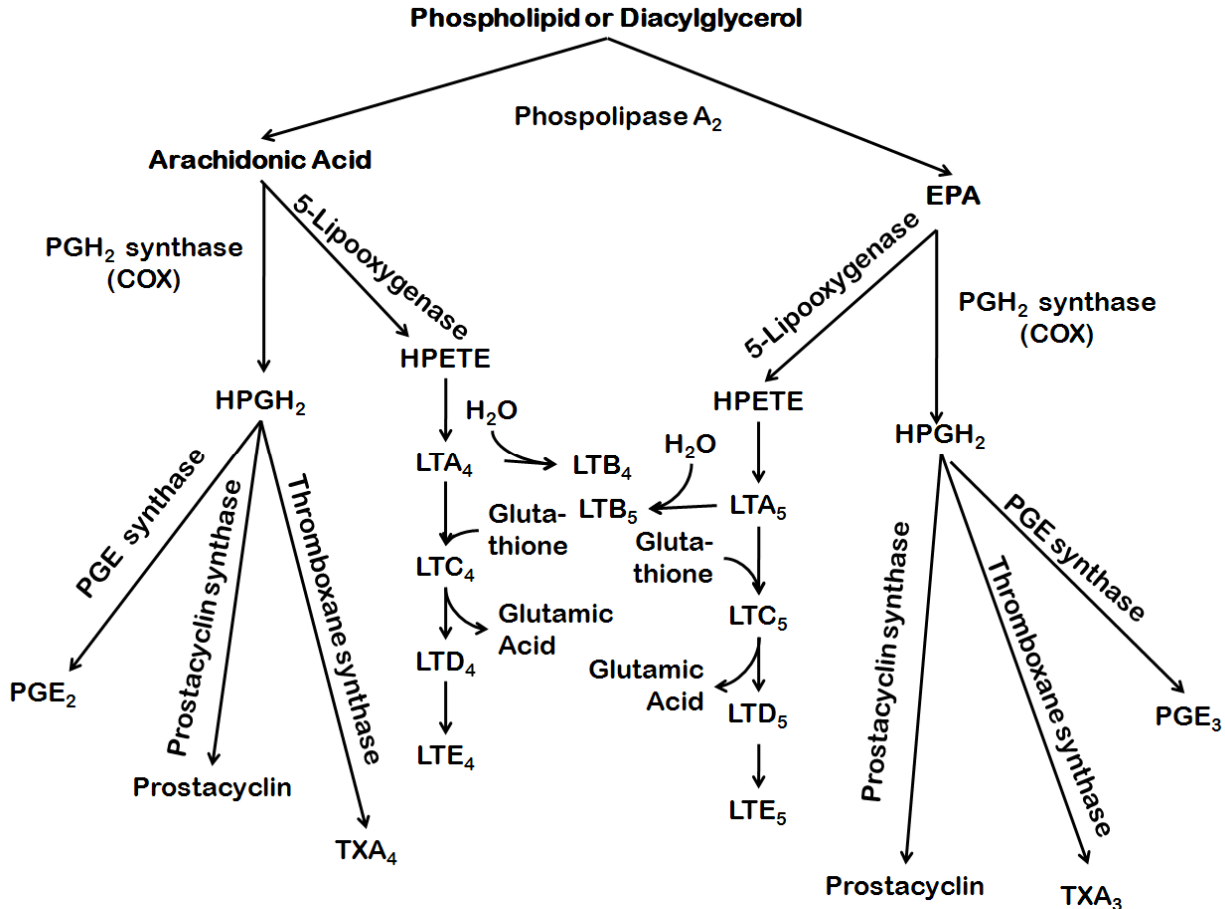


Figure 1.2 Eicosanoid synthesis from n-3 and n-6 fatty acids (FA). Biosynthesis of prostaglandins, prostacyclins, and thromboxanes begins when arachidonic acid (ARA, 20:4 n-6) is released from membranes by phospholipase A₂. Free ARA acts as a substrate for prostaglandin H synthase (COX) and is then metabolized to the intermediate HPGH₂. ARA is also a substrate for the enzyme 5-lipoxygenase which transforms ARA to the epoxide leukotriene A₄ (LTA₄). LTA₄ may undergo hydrolysis, conjugation with glutathione, or transcellular metabolism to create bioactive eicosanoids. Hydrolytic attack of LTA₄ by LTA₄ hydrolase yields 4-series leukotrienes (LTA₄, B₄, C₄, D₄, and E₄). Alternatively, eicosapentaenoic acid (EPA, 20:5 n-3) may act as a replacement substrate for ARA to create so-called anti-inflammatory eicosanoids of the 3- or 5-series. (Redrawn from Calder, 2002a).

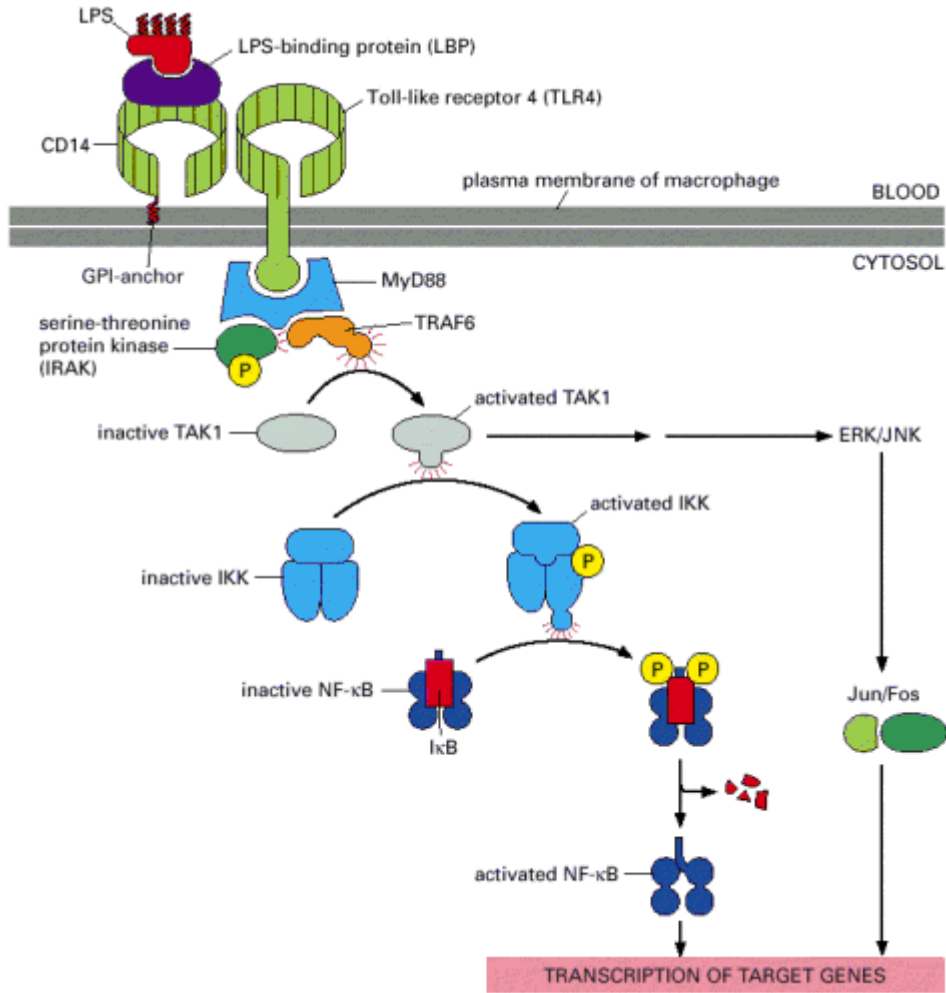


Figure 1.3 Overview of nuclear factor kappa B (NF- κ B) Inflammatory Gene Transcription. While in an inactivated state, NF- κ B is located in the cytosol complexed with the inhibitory protein I κ B. A variety of extracellular signals (Toll-like receptor 4 and lipopolysaccharide pictured) can activate the enzyme I κ B kinase (IKK) by engagement with a cell-surface receptor. IKK, in turn, phosphorylates the I κ B protein, which results in dissociation of I κ B from NF- κ B, and degradation of I κ B by a proteasome. The activated NF- κ B translocates into the nucleus where it binds to specific sequences of DNA called response elements (RE). The DNA/NF- κ B complex then recruits other proteins which transcribe downstream DNA into mRNA, which, in turn, is translated into inflammatory proteins. (From Molecular Biology of the Cell, 2007).

CHAPTER 2 - Omega-3 Fatty Acid Supplementation Reduces Basal TNF α but not Toll-like Receptor-Stimulated TNF α in Full Sized and Miniature Mares

ABSTRACT: It has been well documented that omega-3 PUFA (**n-3 PUFA**) can confer a wide variety of health benefits to humans and animals. The current study was designed to evaluate the ability of n-3 PUFA to modulate the innate immune response in two diverse breeds of horses. Ten Quarter Horse and 10 American Miniature Horse mares were assigned to either an n-3 PUFA supplemented or control diet (5 full-sized and 5 miniature mares/treatment) for 56 d. The treatment diet was designed to deliver 64.4 mg/kg BW combined eicosapentaenoic acid (**EPA**) and docosahexaenoic acid (**DHA**) daily. Whole blood (20 mL) was collected via jugular veinipuncture into heparinized tubes on 0 d, 28 d, and 56 d. Serum PUFA analysis was conducted by gas chromatography. Peripheral blood mononuclear cell (**PBMC**) production of tumor necrosis factor α (**TNF α**) in response to toll-like receptor (**TLR**) ligands lipopolysaccharide (**LPS**), flagellin, and lipoteichoic acid (**LTA**) was estimated using an equine-specific ELISA. Peripheral blood samples from d 56 were also analyzed for total and differential leukocyte counts and subjected to flow cytometric analysis. Body type did not affect basal or TLR stimulated TNF α production. Serum PUFA analysis revealed a decrease in linoleic acid (**LA**) and substantial increases in arachidonic acid (**ARA**), EPA, DHA, and docosapentaenoic acid (**DPA**) at both d 28 and 56 in horses fed n-3 PUFA ($P < 0.0001$ for all). Dietary n-3 PUFA supplementation reduced ($P < 0.05$) unstimulated basal, but not TLR stimulated TNF α production by PBMC's. Supplementation with n-3 PUFA did not affect total or differential leukocyte counts, nor selected cell surface markers. These results suggest that n-3 PUFA supplementation in the horse can modify circulating PUFA and alter the inflammatory response by reducing basal TNF α

production. Furthermore, under conditions of the current study and considering the endpoints evaluated, the American Miniature Horse could potentially be used as a model for full-sized horse breeds.

Key words: omega-3 fatty acid, tumor necrosis factor-alpha, horse, American Miniature Horse

INTRODUCTION

Long-chain omega-3 polyunsaturated fatty acids (**n-3 PUFA**) confer health benefits to humans and animals and are generally considered anti-inflammatory. Supplementation with eicosapentaenoic acid (**EPA**, C20:5n-3) or docosahexaenoic acid (**DHA**, 22:6n-3) can alter circulating PUFA profiles in horses (McCann et al., 2000; Hall et al., 2004; Woodward et al., 2005; Poland, 2006; Vineyard et al., 2007; King et al., 2008). However, far less is known regarding the functional anti-inflammatory properties of n-3 PUFA in the horse resulting from dietary supplementation. Studies evaluating dietary linseed oil (Morris et al., 1991) and infused menhaden fish oil (McCann et al., 2000) suggested that these treatments could blunt TNF α responsiveness of equine macrophages and monocytes, respectively, as estimated using a cell-based bioassay for TNF α . However, it should be noted that in both studies, untreated, time matched control animals were not included in the experimental design, thus complicating the interpretation of the results. Since TNF α has a pivotal role in mediating the immune response to pathogens (Grimble et al., 2002) and has been used as a model marker of inflammatory status, one objective was to investigate further the potential for modulating the inflammatory status in the horse with dietary n-3 PUFA. In addition, although there is interest in the miniature horse as an alternative model for full-sized horses, few direct comparisons exist in the literature, especially in regard to n-3 PUFA supplementation. Thus, another objective was to compare responses to dietary treatment in miniature and full-sized horses.

MATERIALS AND METHODS

Animal Management, Treatments, and Sampling

Horses used in this study were housed and fed at Sul Ross State University in Alpine, Texas. Ten Quarter Horse mares, 3 to 15 yr of age (435 kg average BW) and 10 American Miniature Horse mares, 7 to 19 yr of age and averaging 102 kg BW were stratified by age and assigned to either an n-3 PUFA supplemented or control group (5 full-sized and 5 miniature mares/treatment). The basal diet included a combination of 0.7 g/kg BW Coastal Bermuda Grass hay and 0.3 g/kg BW of the appropriate grain concentrate. The n-3 PUFA was from a marine-derived source (JBS United, Sheridan, IN) and delivered 64.4 mg/kg BW EPA and DHA on average per day for 56 d. The control diet was made isocaloric to the n-3 PUFA supplemented diet by the addition of choice white grease. All of the nutritional components of the control and treatment diets are described elsewhere (Furtney, 2009).

Mares were housed in individual stalls that were either 3.65 m x 3.65 m or 2.3 m x 2.3 m. They were provided an opportunity to exercise in larger dirt lots twice weekly. The hay portion of the diet for individual horses was weighed, divided in thirds and fed at 0700, 1300, and 1800. Grain was divided into two equal feedings daily at 0700 and 1800. Horses had unlimited access to water.

Horses were weighed and began an acclimation period 10 d prior to the onset of the study. Body weights were additionally monitored on d 0, 14, 28, 42 and 56 of the study. Feed was adjusted based on BW gain or loss at each 14 d interval. During the acclimation period, horses were fed hay and the control concentrate. On d 0, horses on the n-3 PUFA treatment were switched to the appropriate concentrate and remained on that diet for the following 56 d.

On d 0, 28 and 56, approximately 20 mL of whole blood was collected via jugular veinipuncture into heparinized vacutainer tubes. An additional tube of whole blood was

collected for harvest of serum. All blood samples were collected at 0600, before the horses were fed. Whole blood samples were placed on ice, packed for shipment, then transported by overnight carrier to Kansas State University.

Analyses of Blood Constituents

Upon arrival at Kansas State University, serum was harvested from clotted whole blood and frozen for fatty acid determination. All heparinized whole blood was pooled within individual animal and peripheral blood mononuclear cells (**PBMC**) were isolated (Accuspin™ Histopaque® 1077 kit; catalog A7054; procedure No. A6929 / A7054 / A0561; Sigma–Aldrich, St. Louis, MO). Isolated PBMC were plated at 10^6 cells/well in RPMI-1640 media (Invitrogen; catalog 22400-071; containing 10% fetal bovine serum (Hyclone, Logan, UT; catalog SH30070.02) and cultured in 24-well plates at 38.2 °C , 5% CO₂, for 4 h. Lipopolysaccharide (LPS; ultra pure from *S.minnesota*; InvivoGen, San Diego, CA; catalog tlr1-smpls), flagellin (from *S.typhimurium*; InvivoGen; catalog tlr1-stfla), or lipoteichoic acid, (LTA; from *S. aureus* – InvivoGen; catalog tlr1-pslta) were added to respective wells at a final concentration of 100 ng/mL. Control wells received media without toll-like receptor (TLR) ligands. Plates were incubated at above conditions for 24 h. The supernatant from each well was then aspirated and stored frozen until concentrations of TNF α were determined using an equine-specific ELISA.

The TNF α ELISA was validated in our laboratory for use with culture supernatants. The assay utilized anti-equine TNF α coating antibody (Thermo Fisher Scientific Inc. and subsidiaries, Rockford, IL; ordering code PETNFBI), anti-equine TNF α biotinylated detection antibody (Thermo Fisher Scientific Inc. and subsidiaries; ordering code PETNFABI), and recombinant equine TNF α standard (Thermo Fisher Scientific Inc. and subsidiaries; ordering code RETNFBI). When media supernatants from unstimulated equine PBMC were spiked with known amounts of

equine TNF α , the concentration measured in the ELISA averaged 99.8% of the expected concentration when corrected for basal TNF α . When media from equine PBMC stimulated with LPS were diluted 1:2 and 1:4, concentrations measured in the ELISA averaged 97.6% of undiluted samples. The standard curve ranged from 7.8 to 1,000 pg/mL and 7.8 pg/mL was considered the lower limit of detection. Within and between plate CVs averaged 8.2 and 15.9 %, respectively, for the six plates required to complete all of the samples.

Fatty acid analysis was performed on serum by capillary column gas chromatography as described previously (Poland, 2006). Although the concentration of a large number of fatty acids was determined using the gas chromatography procedure, only a subset of fatty acids were selected for statistical analysis.

Finally, at the conclusion of the study on d 56, separate tubes of heparinized whole blood were obtained. One tube was submitted to the Kansas State University Clinical Pathology laboratory for automated total and differential leukocyte determination by complete blood count. The remaining tube was submitted to the Kansas State University Flow Cytometry Laboratory for determination of selected cell populations in peripheral blood as previously described (Davis, 2004). All primary monoclonal antibodies and a general description of the cell populations labeled were provided by the supplier, VMRD, Inc., Pullman, WA. Briefly, cells were stained with monoclonal antibodies targeted to CD2; (HB88A; expressed on T lymphocytes and natural killer cells); CD4 (HB61A; expressed on T lymphocyte subsets in peripheral blood that recognize antigens associated with self-MHC class II molecules); CD5 (expressed on mature T lymphocytes); CD8 (HT14A; expressed on T lymphocytes); and B lymphocytes (two different antibodies E18A and B29A). They were incubated for 30 min, and then washed 3 times with PBS. After the initial incubation secondary antibodies included those targeted against the

appropriate Ig isotype IgG1 (Caltag IgG1/APC Cat. #M32005) or IgG2a (Caltag IgG2a/APC M32205). Following 30 min incubation, PBMCs were again washed with 0.1 M PBS and prepared for analysis by flow cytometry with the addition of 200 μ L of 0.5% paraformaldehyde. Control analysis to determine if non-specific staining was present utilized analysis of secondary antibody alone. Samples were run on the FACS Calibur (Becton Dickinson Immunocytometry system serial # E4400).

Statistical Analyses

All data were analyzed by the PROC MIXED procedure (repeated measures design) using SAS 9.1 statistical software (Cary, NC). For TNF α , data were analyzed within day of collection and within in vitro treatment (control, LPS, flagellin, or LTA). The model included effects of diet, breed and diet x breed. For serum fatty acids, the full model included fixed effects of diet, breed, time (d 0, 28, and 56), and all interactions. For total and differential leukocyte counts and for flow cytometric analysis, the model included fixed effects of diet, breed and the interaction. Main effect and interaction means were compared directly only if a significant ($P < 0.05$) F-test was observed for the respective main effect or interaction term in the analysis of variance.

RESULTS

Body type did not affect plasma n-6 or n-3 PUFA concentrations; the PBMC production of TNF α basally or in response to TLR ligands; total and differential leukocyte concentrations; or proportions of cells identified in peripheral blood by flow cytometric analysis. Therefore the data from full-sized and miniature horses were combined for all response variables.

Dietary n-3 PUFA supplementation decreased serum concentrations of linoleic acid (**LA**), whereas concentrations of arachidonic acid (**ARA**) were increased compared to CON at d

28 and 56 (Figure 2.1; $P < 0.001$ for all). Supplementation also increased serum concentrations of EPA, DHA and docosapentaenoic acid (**DPA**), but not ALA, at 28 d and 56 d (Figure 2.2 all $P < 0.001$).

Unstimulated production of TNF α by PBMC from TRT and CON horses were similar at d 0 and 28. However, basal production was reduced in TRT horses by d 56 ($P < 0.05$ compared with wells not including TLR ligands from CON horses; Figure 2.3). TNF α responses to TLR ligands LPS, flagellin and LTA were similar among dietary treatments on d 0, 28, and 56.

Total and differential leukocyte counts in blood obtained on d 56 were not affected by either n3-PUFA supplementation or breed (Figure 2.4). In addition, flow cytometric analysis did not reveal any affect of treatment or breed on cell types identified in whole blood obtained on d 56 (Figure 2.5).

DISCUSSION

It is obvious that light horse breeds that are popular worldwide are animals with relatively large body size that are costly to maintain and house as a research animal. Therefore, one of the important objectives of the current study was to determine whether horses of the American Miniature Horse breed responded to dietary supplementation with n-3 PUFA comparably to horses of the American Quarter Horse breed. Indeed, there were no instances in which breed or breed x diet interactions were statistically significant suggesting that these breeds of horses responded similarly to dietary supplementation with n-3 PUFA (on a BW basis). Although the scope of the current study was limited to responses primarily in peripheral blood and additional work in other systems is certainly warranted, results of the current study indicate that the American Miniature Horse might be a useful model for full-size horse breeds.

Another primary objective was to evaluate physiological responses to n-3 PUFA supplementation. Supplementation of mares with a combination of EPA and DHA resulted in unmistakable effects on circulating concentrations of both n-6 and n-3 PUFA. Whereas ARA was increased in serum, LA decreased in response to dietary treatment. Concentrations of EPA, DPA, and DHA were dramatically increased by supplementation with n-3 PUFA. In general, the obvious interpretation is that circulating concentrations of fatty acids of interest generally reflected their concentration within the grain portion of the diet. This conclusion is supported by relative concentrations of LA, ARA, EPA, DPA and DHA in the feed used in the current study and reported elsewhere (Furtney, 2009).

We originally anticipated that supplementation of n-3 PUFA would blunt inflammatory response of isolated PBMC as measured by production of TNF α in response to TLR ligands. This assumption was based primarily upon previous reports in which dietary linseed oil supplementation (Morris et al., 1991) and infusion of menhaden fish oil (McCann et al., 2000) reduced TNF α activity from equine peritoneal macrophages and blood-derived monocytes, respectively. Although not reported explicitly, the effect of treatment on changes in circulating n-3 PUFA in horses receiving linseed oil, it is presumed these animals were consuming greater dietary ALA and this may have enhanced conversion to EPA and DHA. It should be noted, however, that both of those studies did not include a time-matched untreated control group of horses that could have strengthened the experimental design of the studies. Even so, in the current study, it was somewhat unexpected that we observed basal but not TLR ligand-stimulated production of TNF α from PBMC to be reduced in mares supplemented with EPA and DHA. Moreover, reduction in basal TNF α in treated horses did not appear until the final blood collection on d 56.

Results of the *in vitro* production of TNF α point to at least three conclusions that warrant more detailed consideration. First, although it is not unexpected that equine cells respond to TLR challenge, it is worth noting that to our knowledge, this is the first report of TNF α production by equine PBMC in response to ligands presumed to engage TLR2 and TLR5, specifically LTA and flagellin, in addition to the more thoroughly studied TLR4 ligand LPS. In the current study, although LPS appeared to be most potent of the ligands tested in stimulating TNF α production *in vitro*, all TLR ligands resulted in greater media concentrations of TNF α compared to unstimulated control cells (note Y-axis scale in Figure 2.3; control wells compared to flagellin, LTA and LPS). Related to the first point, the second conclusion of note is that, although basal production of TNF α by PBMC was reduced by dietary n-3 PUFA supplementation, the cells in culture remained fully competent on d 56 to secrete TNF α in response to inflammatory challenge in a manner that did not differ from that of PBMC from control mares. We consider this effect to be significant in that it suggests dietary manipulation might control basal inflammation, but perhaps not compromise the ability of inflammatory/immune cells to respond appropriately when stimulated.

The final conclusion to consider in more detail is the mechanism by which basal TNF α production by PBMC was reduced by dietary n-3 PUFA. Although our data do not allow us to determine definitively the mechanism by which this effect occurred, at least four points can be made relative to that finding. The first point is that this report is the first to evaluate the effect of dietary n-3 PUFA using a very sensitive equine specific ELISA. It is possible that TNF α determined by the WEHI bioassay (Morris et al., 1991; McCann et al., 2000) could not have detected concentrations below approximately 10 pg/mL (see Figure 2.3; level of TNF α in unstimulated cells from treated horses on d 56). However, the ability to detect low levels of production *in vitro* still fails to account for the mechanism by which a reduction in TNF α could

have come about. The second point is that dietary treatment did not affect changes in total and differential leukocyte count, eliminating the possibility that a change in cell populations might account for the difference in basal TNF α production. A third consideration may relate to the degree to which EPA and/or DHA may have been incorporated into PBMC membrane phospholipids and the degree to which those changes affected basal production of TNF α . To this point, it is worth noting that serum concentrations of EPA, DHA, and DPA were all remarkably increased in horses after consuming the supplemented diet for as little as 28 d even though the reduction in basal TNF α was not statistically lower until d 56. It is possible that by increasing the concentration of unsaturated FA into membrane phospholipids, fluidity would increase by decreasing the amount of stringent “packing” of phospholipids. N-3 PUFA supplementation has been reported to increase fluidity in equine erythrocytes (De Moffarts et al., 2007; Portier et al., 2006), rat adipocytes, (Fickova et al., 1998), and murine synaptic membranes (Suzuki et al., 1998). Fluidity has the ability to affect cellular responses to agonists (McCann et al., 2000). Finally, perhaps a more likely mechanism by which basal TNF α production was reduced could be in the relative activation of nuclear factor kappa B (NF- κ B). As a well-studied inflammatory gene transcription factor, NF- κ B is known to play a role in expression of a variety of genes, including TNF α (Calder, 2002a,b; Bonizzi and Karin, 2004). Feeding mice fish oil resulted in a lower concentration of NF- κ B in the nucleus of LPS-stimulated splenocyte (Xi et al., 2001). Moreover, incubation of human monocytes with EPA decreased the activation of NF- κ B (Chen and Zao, 2001). So, changes in the NF- κ B signaling pathway could account for our observation. Still, an important caveat to note when putting our results in a physiologic context is that we investigated a singular cell population that was rather easy to access, namely the peripheral blood. Whether dietary n-3 PUFA supplementation globally reduced basal inflammatory state of

other cell types through a similar mechanism and in a broader range of tissues will be important questions for future investigations.

Finally, it is clear that neither dietary n-3 PUFA supplementation nor breed affected total and differential leukocyte populations nor the proportion of selected cell types labeled in whole blood by flow cytometric analysis. Actually, we did not expect that dietary n-3 PUFA would affect the trafficking of cell types in the periphery. However, the point we consider to have greater potential significance is that these fundamental measures used frequently in clinical settings in horses were indistinguishable between Quarter Horses and American Miniature Horses. These observations, along with others in the current study, point to the potential usefulness of the American Miniature Horse as a model for full-sized horses, at least in some settings. That said, it will be important to continue to evaluate the American Miniature Horse in other experimental settings to continue to add evidence of this breeds' utility in a broad array of equine research settings.

We conclude that feeding n3-PUFA, at the levels used in the current study; appear to reduce basal production of TNF α from PBMCs after 56 d of feeding, although cells remain responsive to inflammatory innate immune challenge. Moreover, the evidence suggests that the American Miniature Horse may be a useful experimental model for full-sized breeds of horses.

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Figures

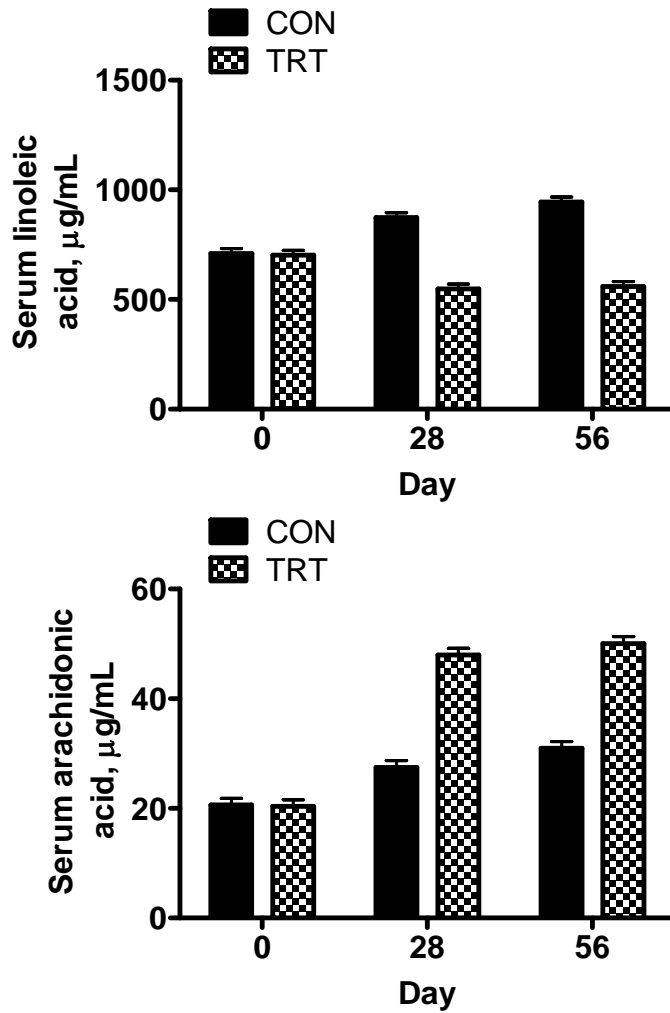


Figure 2.1 Serum concentrations of n-6 polyunsaturated fatty acids in mares supplemented with eicosapentaenoic (EPA) and docosahexaenoic acid (DHA) for 56 d. Horses were fed 64.4 mg/kg BW EPA and DHA daily (TRT) or an isocaloric control diet (CON). Each bar represents the mean (\pm SEM) of 10 mares per dietary treatment. Serum fatty acids differed between CON and TRT on d 28 and 56 ($P < 0.001$ for all).

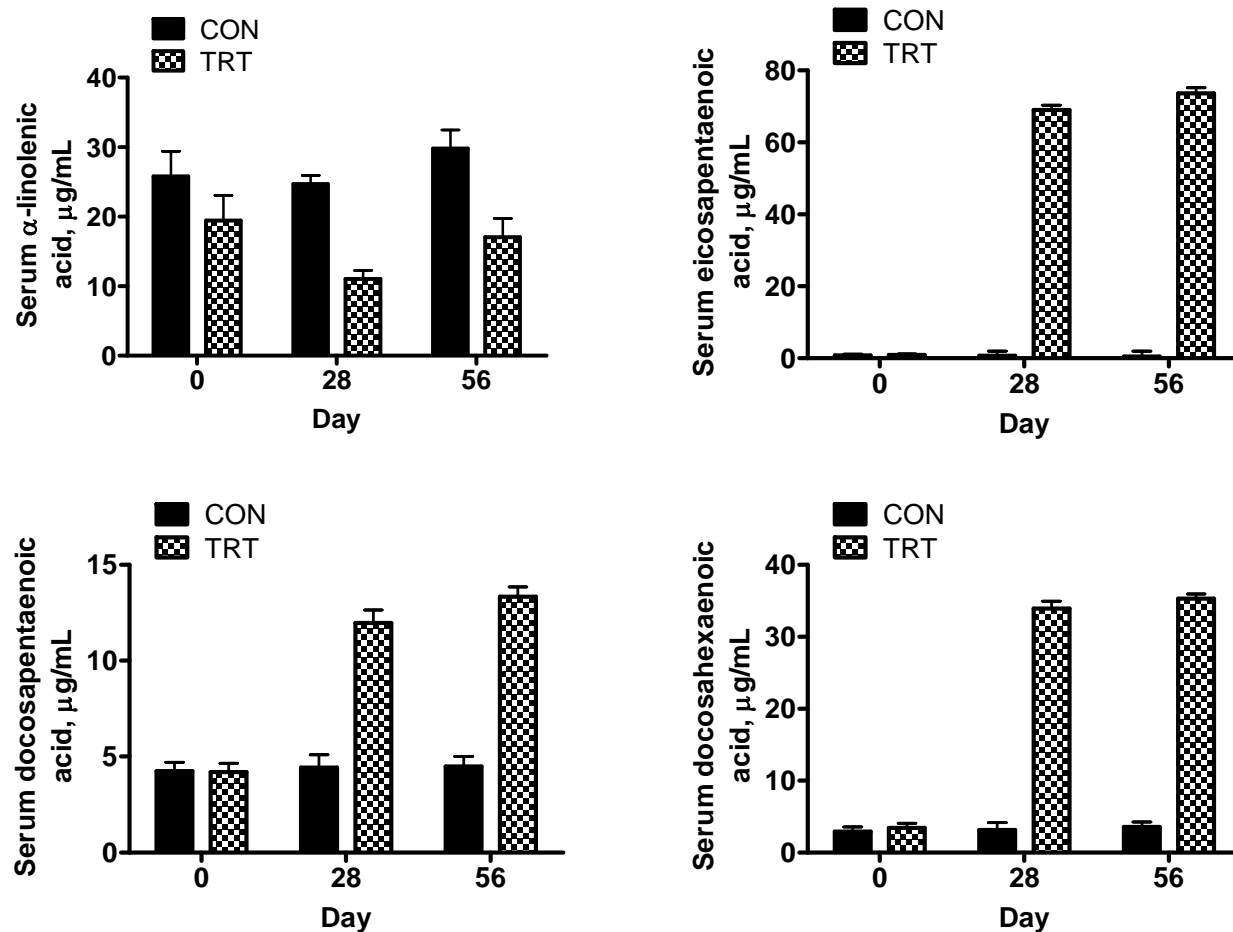


Figure 2.2 Serum concentrations of n-3 polyunsaturated fatty acids in mares supplemented with eicosapentaenoic (EPA) and docosahexaenoic acid (DHA) for 56 d. Horses were fed 64.4 mg/kg BW EPA and DHA daily (TRT) or an isocaloric control diet (CON). Each bar represents the mean (\pm SEM) of 10 mares per dietary treatment. Serum fatty acids differed between CON and TRT on d 28 and 56 ($P < 0.001$ for all).

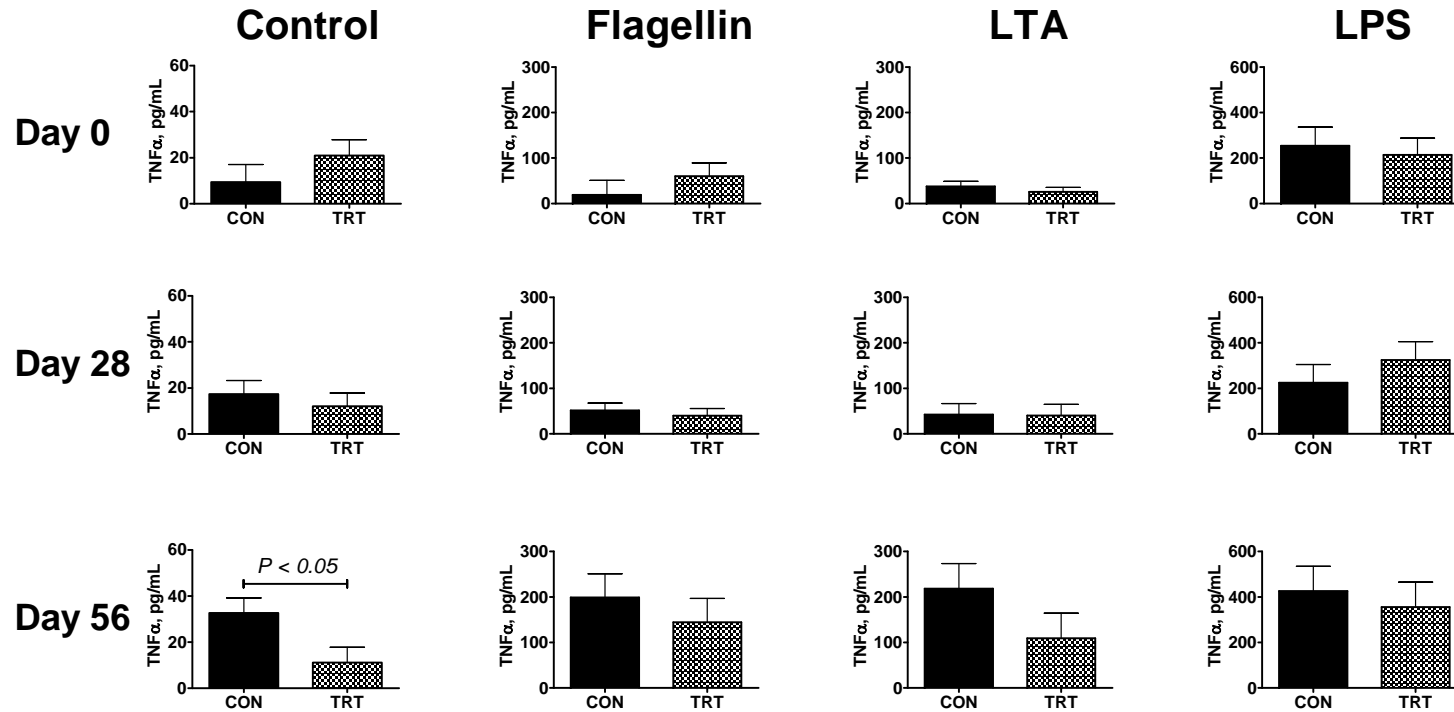


Figure 2.3 In vitro production of tumor necrosis factor alpha (TNF α) from peripheral blood mononuclear cells (PBMC) of mares supplemented with eicosapentaenoic (EPA) and docosahexaenoic acid (DHA) for 56 d. Horses were fed 64.4 mg/kg BW EPA and DHA daily (TRT) or an isocaloric control diet (CON). Isolated PBMC were incubated with toll-like receptor ligands flagellin, lipoteichoic acid (LTA), or lipopolysaccharide (LPS) at 100 ng/mL, or were exposed to control media. Cells were isolated from whole blood obtained prior to the onset of dietary treatment (d 0) and after 28 and 56 d. Bars represent the mean \pm SEM of 10 horses/treatment. At day 56, unstimulated basal production of TNF α was reduced for horses fed EPA and DHA.

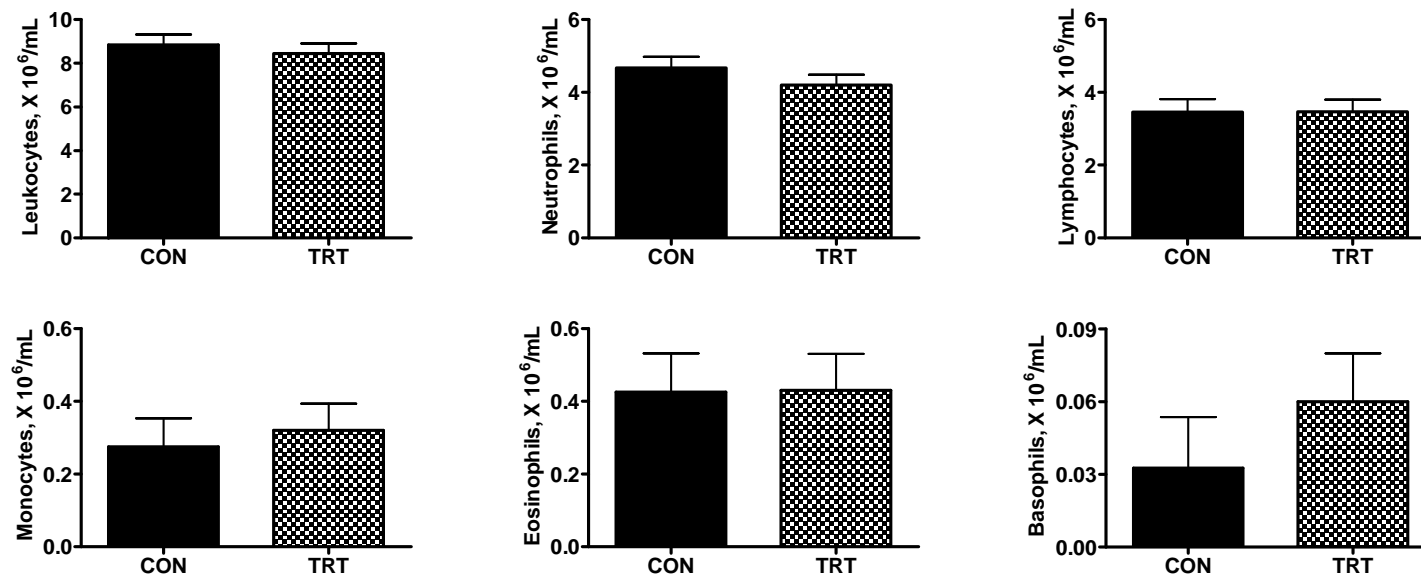


Figure 2.4 Total and differential leukocyte counts in peripheral blood of mares supplemented with eicosapentaenoic (EPA) and docosahexaenoic acid (DHA) for 56 d. Horses were fed 64.4 mg/kg BW EPA and DHA daily (TRT) or an isocaloric control diet (CON). Blood was collected for total and differential count on the final day of the study. Bars represent the mean \pm SEM of 10 horses/treatment.

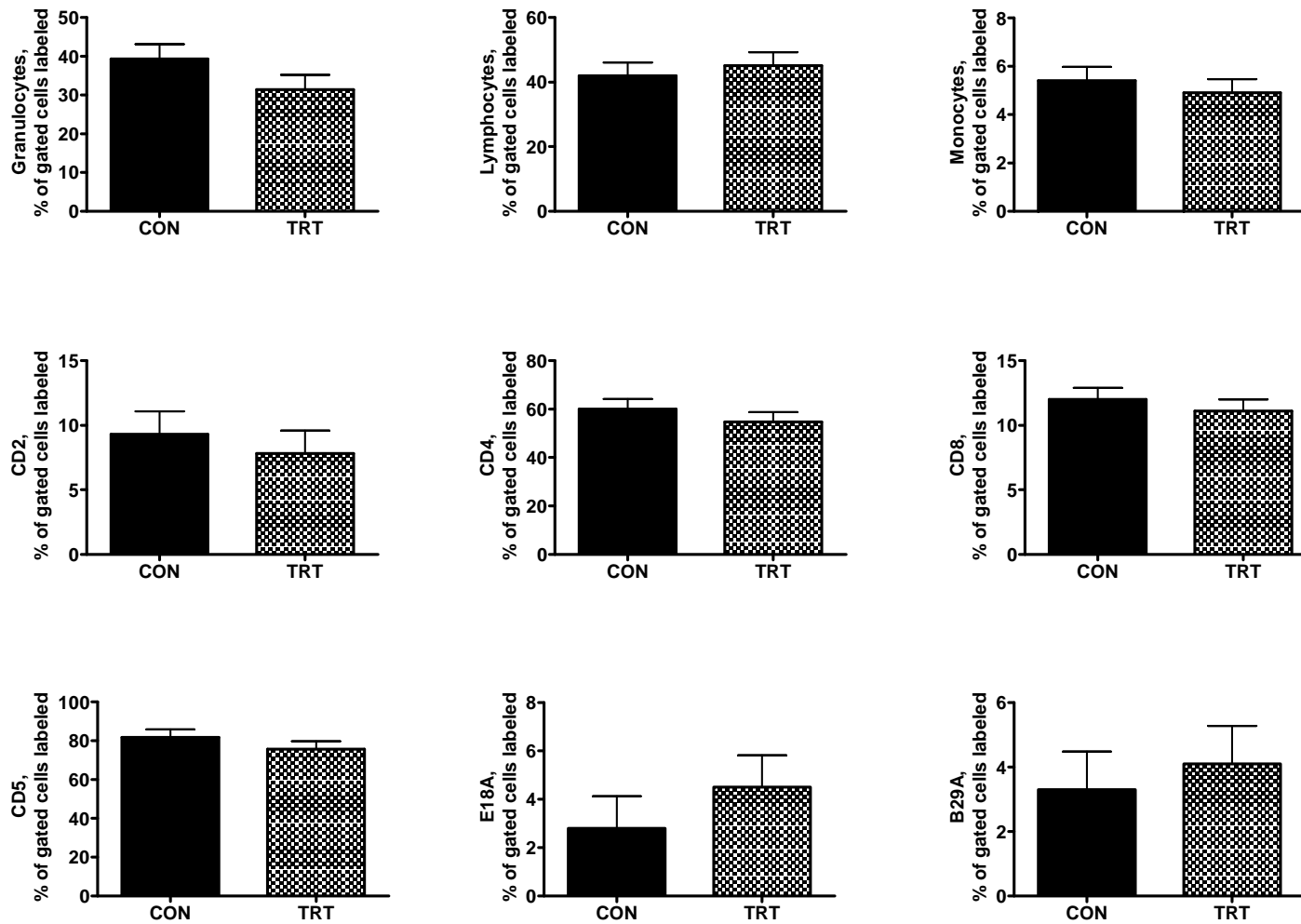


Figure 2.5 Leukocyte populations estimated by flow cytometry in peripheral blood of mares supplemented with eicosapentaenoic (EPA) and docosahexaenoic acid (DHA) for 56 d. Horses were fed 64.4 mg/kg BW EPA and DHA daily (TRT) or an isocaloric control diet (CON). Blood was collected for analysis on the final day of the study. Bars represent the mean \pm SEM of 10 horses/treatment.