Hempseed oil as a novel source of polyunsaturated fatty acids and its effect on inflammation in sedentary horses

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Abstract

Chronic, low-grade inflammation is a contributing factor in diseases that impact the health and well-being of horses. Pharmaceutical treatments reduce inflammation, but their use results in negative digestive and kidney disturbances. Polyunsaturated fatty acids (PUFA) play a role in mitigating the inflammatory response and are therefore explored as a dietary approach to attenuate inflammation. γ-Linolenic acid (GLA) is a unique PUFA that when supplemented in the diet can increase the production of anti-inflammatory eicosanoids; however, it is uncommon in the dietary components normally fed to horses. Interest in industrial hemp (*Cannabis sativa L.*) as a novel source of PUFA stems from the presence of GLA and the potential to reduce inflammation; although, concerns over cannabinoid contamination limit its acceptance. Six Thoroughbred geldings were used in a crossover study with two 63-d periods to measure PUFA metabolism, inflammatory biomarkers, and cannabinoid accumulation in response to hempseed oil (HSO) fed to sedentary horses compared to controls (CON). Treatment diets were offered for the first 35 d of each period and then all horses resumed a uniform feeding rate from d 36 to 63. Serum and synovial fluid PUFA reflected dietary intake. GLA was greater in serum (0.465 vs. 0.046; $P < 0.0001$) and synovial fluid (0.270 vs. 0; $P < 0.0001$) in horses fed HSO compared to CON. This contributed to greater dihomo-γ-linolenic acid (DGLA) conversion in serum (0.287 vs. 0.195; $P < 0.0001$) and synovial fluid (0.348 vs. 0.262; $P < 0.04$) but not arachidonic acid (AA). Serum GLA returned to baseline concentrations by two weeks post-supplementation, but no treatment x time effect was observed for synovial fluid. HSO did not affect FA in muscle; it is

likely the length or quantity of supplementation was inadequate to see changes in muscle PUFA. HSO increased serum interleukin 1β (IL1β; $P = 0.01$) but there was no treatment by time interaction ($P = 0.62$). No other inflammatory biomarkers were influenced by treatment. Stride length was not affected by HSO supplementation but was inversely correlated ($P \leq 0.01$) with synovial fluid prostaglandin E2 (PGE2; $r = -0.56$), and positively correlated with serum tumor necrosis factor α (TNFα; $r = 0.58$), serum IL6 ($r = 0.61$), and serum IL1β ($r = 0.65$). Cannabinoids were measured in the HSO supplement, but no cannabinoids were detected in plasma or synovial fluid of horses fed HSO when tested to a 50-ppb limit of detection. These results demonstrate the suitability of HSO as a novel source of PUFA and, more specifically, as a source of GLA without further increasing AA, however, implications for its effect on inflammation require further evaluation.

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General Audience Abstract

Inflammation contributes to diseases in the horses that reduce their health and well-being. Anti-inflammatory drugs reduce inflammation but are associated with negative health effects including gastric ulcer formation and kidney damage. Diet can influence the inflammatory response and is therefore targeted to moderate inflammation. Specific dietary targets include polyunsaturated fatty acids (PUFA). Hempseed (Cannabis sativa L.) oil (HSO) contains a unique and uncommon dietary PUFA, γ-Linolenic acid (GLA), which can increase the production of anti-inflammatory biomolecules. The goal of this research was to measure PUFA accumulation, specifically GLA, in horses fed HSO for 35 d and then clearance for 28 d post-supplementation. Additionally, we looked at inflammatory markers to determine the effect on inflammation in sedentary horses. Finally, we measured cannabinoids to evaluate if the low level of cannabinoid contamination found in HSO transfers to horse plasma and synovial fluid. To accomplish these goals, we conducted a feeding trial from May 2022 to September 2022 using six Thoroughbred geldings in a cross over study with two 63 d periods. HSO was supplemented the first 35 d of each period and then removed. Serum and synovial fluid PUFA reflected dietary PUFA. Inflammatory biomarkers had a mixed response that could be influenced by additional, unknown factors. The low-level of cannabinoids in the HSO supplement were not detected in plasma or synovial fluid. HSO shows promise as a novel source of PUFA, specifically GLA, without concerns of cannabinoid contaminants.

Dedication

To Mickie, Jack, Blues, and Momo…

…and to all the horses who made this possible and all the horses this work might help.

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List of Abbreviations

ALA	α-linolenic acid
AA	arachidonic acid
AUC	area under the curve
BCS	body condition score
BW	body weight
COX	cyclooxygenase
d	day(s)
DGLA	dihomo-γ-linolenic acid
DHA	docosahexaenoic acid
DM	dry matter
DPA	docosapentaenoic acid
EPA	eicosapentaenoic acid
ELISA	enzyme linked immunosorbent assay
ETA	eicosatetraenoic acid
FA	fatty acid
GLA	γ-linolenic acid
HSO	hempseed oil
IL-1β	interleukin-1β
IL-6	interleukin-6
LOD	limit of detection
LA	linoleic acid
MAG	monoacylglycerides
NEFA	non-esterified fatty acids
NSAIDs	nonsteroidal anti-inflammatory drugs
ppb	parts per billion
ppm	parts per million
PBS	phosphate buffered saline

PUFA	polyunsaturated fatty acids
PG	prostaglandin
PGE2	prostaglandin E2
SDA	stearidonic acid
TAG	triacylglycerides
TNFα	tumor necrosis factor-α

Chapter 1
Introduction

Low grade, chronic inflammation is a contributing factor in numerous disease states (e.g., joint issues, metabolic disease, and gastrointestinal disease, etc.) that can lead to irreversible damage and reduced comfort, and contribute to poor quality of life in the horse (Menarim et al. 2020; Zak et al. 2020). Prevalent in nearly 50% of the equine population, excessive adipose tissue present in overconditioned or obese horses can contribute to an increased circulating inflammatory markers (Thatcher et al. 2012; Suagee et al. 2013). This is also seen in the normal aging process in what is termed "inflamm-aging" (Adams et al. 2008). Inflammation can be managed using traditional medicine (i.e., NSAIDs), however, long term use to mitigate chronic issues can have unintentional and negative consequences such as gastrointestinal ulceration and kidney damage (Flood and Stewart 2022). Finding ways to mitigate inflammation and prevent these negative effects is a more ideal solution and one sought by owners as an alternative to pharmaceuticals.

One such way is through nutritional modification of the diet, specifically targeting polyunsaturated fatty acids (PUFA). PUFA, and fats in general, are well known for their role in supplying energy in the diet and as stored excess energy in the form of adipose tissue. Fat is important for the absorption of fat-soluble vitamins from the lumen of the small intestine. Additionally, PUFA are an integral part of cellular membranes, but the feature of most interest is their role in cell signaling, specifically eicosanoid production (Watkins et al. 2005). PUFA are long chain fatty acids and considered essential in human diets; however, a true deficiency has never been induced in the horse. Nonetheless, they do not naturally occur within the horse, and

thus are assumed to be necessary components of the diet, although minimum dietary intake recommendations exist only for linoleic acid (LA; NRC 2007).

On pastures or range, horses generally spend 16 hours throughout the day consuming forage in multiple, small grazing bouts. While forages are relatively low in overall fat content, they are high in omega 3 PUFA, which stimulate the body's anti-inflammatory response (Calder 2013b). As the horses' role transitioned towards work and sport, energy dense grain feeding was introduced to meet performance demands. Most grains have an inverted omega 6:3 ratio compared to grass in which omega 6 outweighs omega 3. Work to evaluate the balance of omega 6 to omega 3 fatty acids has primarily focused on increasing dietary omega 3 FA levels. This can be accomplished by either plant- (e.g., flax; *Linum usitatissimum*) or marine-based sources (e.g., fish oil or algae). Plant-based sources are high in the parent omega 3 fatty acid, α-linolenic acid (ALA), but conversion rates to metabolically active PUFA are low in horses (Hess et al. 2012). Marine based sources are high in the direct precursors of anti-inflammatory mediators, but palatability can be an issue, and using these sources may also present sustainability challenges. γ-Linolenic acid (GLA), is a potential alternative to these PUFA sources. Although an omega 6 FA, dietary GLA supplementation promotes anti-inflammatory actions by stimulating the synthesis of anti-inflammatory prostanoids which compete with and therefore inhibit conversion of arachidonic acid (AA) to pro-inflammatory eicosanoids (Kapoor and Huang 2006). While GLA is not present in modern commodity grains and oilseeds, the FA occurs in relatively high concentrations in industrial hemp (*Cannabis sativa* L.).

Commercial interest in industrial hemp production has grown following the crop's legalization with the 2018 Farm Bill (Abernathy 2019; NASS 2023). Industrial hemp has been grown for millennia and historically used in the diets of horses (De Briyne et al. 2021). However,

production in the USA (historically for low quality fibers, seed oils, and bird feed) was greatly restricted by the Marihuana Tax Act of 1937 (Musto 1972) and subsequently criminalized with the Controlled Substance Act (P.L. 91-513) (91st Congress of the U.S 1970). This occurred as no distinction was made between industrial hemp (i.e., cannabis forms with low levels of the psychotropic compound Δ9 tetrahydrocannabinol (THC)) and marijuana (psychotropic forms of cannabis with high levels of THC). Although cannabis forms vary substantially in morphology and chemistry, hemp and marijuana are differentiated solely on the basis of their cannabinoid content (Fike 2019). That is, the distinction between hemp and marijuana is a human construct based in law. In the U.S.A., industrial hemp must have no more than 0.3% THC with no limits on non-psychotropic cannabinoids such as cannabidiol (CBD). European law initially set this threshold at 0.2% THC but has since adopted 0.3% THC limits (Sabaghi 2021).

During its nearly 80-year hiatus as an illegal crop, many other commonly supplemented feedstuffs were grandfathered into the AAFCO "Official Publication" of approved feed ingredients. Even though hempseed can be found in historical animal nutrition reference guides (Linton 1927; Morrison 1956) and they don't contain cannabinoids, their association with cannabinoids has led to their continued restriction from production animal and equine diets. Concerns regarding the use of hemp in equine diets are based on the potential performance enhancing effects of cannabinoids thus providing an edge to competitors. To rehabilitate hempseed as a feed resource, research will be needed that demonstrates its nutritional value and safety.

This research explores the potential nutritional use of hempseed oil in equine diets. The general hypothesis for this research was that the fatty acids supplied by hempseed oil would

positively influence fatty acid profiles and reduce overall inflammation in the horse and carpal joint and lead to increased comfort as demonstrated by improved stride length.

Our goal was to evaluate the effects of hempseed oil in the horse's diet by addressing the following objectives:

1. Measure fatty acid profiles of blood, muscle, and synovial fluid in horses fed diets containing supplemental hempseed oil for 35 d and an additional 28 d post-supplementation.

2. Assess inflammatory biomarkers and stride length in sedentary horses supplemented with hempseed oil.

3. Determine if residual cannabinoids from hempseed oil are absorbed and transferred to blood and synovial fluid in the horse.

Chapter 2
Literature Review

Lipids Overview

Lipid structure and nomenclature

Lipids are a large class of molecules that are not as simple to define chemically as proteins and carbohydrates due to their complex and heterogeneous nature. Fats, a lipid subgroup, are with few exceptions water insoluble organic compounds with primary biological functions that include energy storage, cellular membrane structure, and cell signaling. Fats can be further classified as phospholipids (PL) or triacylglycerols (TAG) based on their structure and constituents. Both molecular types share structural similarities that include a three-carbon glycerol "backbone" attached to either two fatty acids (FA) in the case of PL or three FA in the case of TAG. PL substitute phosphate in place of a single FA on the third carbon of the glycerol backbone. The phosphate group can be attached to choline, serine, or ethanolamine, which provide the PL with amphipathic properties. Thus, PL can self-associate in large macromolecular complexes in an aqueous environment, which is the basis for membrane formation. In contrast, TAG are extremely hydrophobic and do not dissolve in water. This property causes them to coalesce with one another and pack densely together. This is a beneficial feature for storage, and as such TAG are the primary storage form of lipids in both plants and animals.

FA are made up of hydrophilic carboxyl heads followed by a chain of hydrophobic carbon atoms that can be fully saturated with hydrogen atoms or contain one or more double bonds. The length of the hydrocarbon tail dictates if a FA is classified as short (volatile), medium, long, or very long chain. The degree of unsaturation dictates whether they are further classified as mono- or polyunsaturated fatty acids (MUFA or PUFA). The carboxyl carbon is referred to as the (Δ) delta carbon, whereas the methyl carbon is the omega (ω or n) carbon. The

latter gives rise to readily recognized nomenclature based on the position of the first double bond in unsaturated FA with relation to the ω carbon. Since multiple naming systems exist, common names and shorthand notation will be used where applicable to both maintain consistency with commonly published literature and to increase readability.

Digestion and metabolism of dietary fat

In simple stomached mammals, fatty acid composition within tissues is influenced by and therefore typically reflects dietary intake. Dietary fats are mostly present as TAG and require enzymatic assistance to be digested and absorbed for use by the body. For horses, lipid absorption occurs in the small intestine, although digestion begins with chewing, which reduces lipid particle size. Once swallowed, gastric lipase and the churning action of the stomach physically emulsifies the lipids which in turn facilitates suspension. The fat emulsion then enters the duodenum where it mixes with bile salts and pancreatic juices. Horses are unique compared to other mammals in that they do not have a gall bladder and instead constantly secrete bile salts directly into the small intestines. Bile salts, working in conjunction with co-lipase and lipase enzymes, further emulsify and digest fats. The cleaved FA from TAG (non-esterified FA, or NEFA) and monoacylglycerides (MAG), along with cholesterol, and fat-soluble vitamins, become constituents of micelles formed with a PL monolayer membrane. The micelle structure facilitates transport through the epithelial lining of the GI tract. Once absorbed into the cells lining the gut, FA are rapidly reassembled in the endoplasmic reticulum into TAG and packaged within the enterocyte into TAG-rich lipoproteins for transport. These aggregates are excreted into the lymphatic system, bypassing the liver, and entering directly into the bloodstream via the thoracic duct. Once in the bloodstream, TAG-rich lipoproteins are directed to either adipose

tissue for storage when in the fed state or skeletal or cardiac muscle for energy use in the fasted state.

Short and medium chain FA are absorbed directly from the lumen of the small intestine and enter the bloodstream to the liver using the hepatic portal vein where they can be metabolized for energy. Similar to lipid absorption from the small intestine, circulating long and very long chain FA must be broken down into NEFA and MAG for cellular uptake. Once inside the cell, NEFA can be reassembled into TAG for storage, directed toward the mitochondria for energy production, or incorporated into cellular membranes. Lipid turnover is constant, therefore, FA in both TAG and PL are repeatedly replaced whether due to damage repair or cellular signaling on a consistent basis.

Fatty acid synthesis and degradation

Fatty acids found in the body arise from dietary (exogenous) sources or from *de novo* synthesis (endogenous). *De novo* lipogenesis (DNL) is stimulated by excess energy intake. DNL can occur in either liver or adipose tissues depending on substrate preferences. Liver is the primary site of DNL in humans, whereas adipose tissue is believed to be the primary site in horses, which is similar to ruminants, pigs, and rabbits (Suagee et al. 2010). Both glucose and acetate can be used as substrates for DNL, but acetate from cecal fermentation is the primary carbon source in equine adipose tissue. Acetate is converted to acetyl CoA by acetyl CoA synthetase. FA synthesis is then regulated by two important enzymes, acetyl-CoA carboxylase, and fatty acid synthase with palmitate as the end-product FA. These products can be modified by elongation and desaturase enzymes to produce additional FA products.

FA degradation can occur through three pathways (α, β, or ω) depending on which carbon is targeted for oxidation. β-oxidation is the most common of the three, and the primary

site of β-oxidation is in the mitochondria. However, to enter the mitochondria, FA (>10C) must be converted to fatty acyl-CoA and then transported from the cytosol of the cell into the mitochondrial matrix using the carnitine shuttle system to facilitate this action. Short chain FA (<10C) can simply diffuse without the use of the shuttle (Gurr et al. 2016). Once inside the mitochondria, β-oxidation occurs through a series of four steps, which results in the cleavage of a 2-C acetyl-CoA every cycle. This process continues until all carbons of the FA are turned into acetyl-CoA, which can then enter the TCA cycle to yield ATP or energy.

Polyunsaturated Fatty acids

Polyunsaturated fatty acids draw the most attention from a nutritional aspect due to their role in mediating inflammation. Plant derived PUFAs in equine diets are primarily made up of LA and ALA, the parent FA for the omega 6 and omega 3 PUFA series, respectively. These FA are so named due to the location of their first double bond from the terminal ω carbon. In humans and other mammals, LA and ALA are considered essential fatty acids (EFA) and thus required in the diet (Di Pasquale 2009). Although a true deficiency has not been described in the horse (Sallmann et al. 1991), neither have the desaturase enzymes required to produce the 18C PUFA been found. Thus, while technically not deemed “essential” in horses they are treated as such in the diet.

LA and ALA are precursors for a variety of inflammatory mediators and must be converted through a series of desaturase and elongase steps to metabolically active FA (fig.1). Both FA families compete for the same enzymes, which preferentially favor omega 3 FA over omega 6. However, the enzymes often are overwhelmed by the large supply of dietary LA. The initial Δ6-desaturase driven conversion step for both LA and ALA is considered the key rate limiting step in the PUFA pathway (Balić et al. 2020).

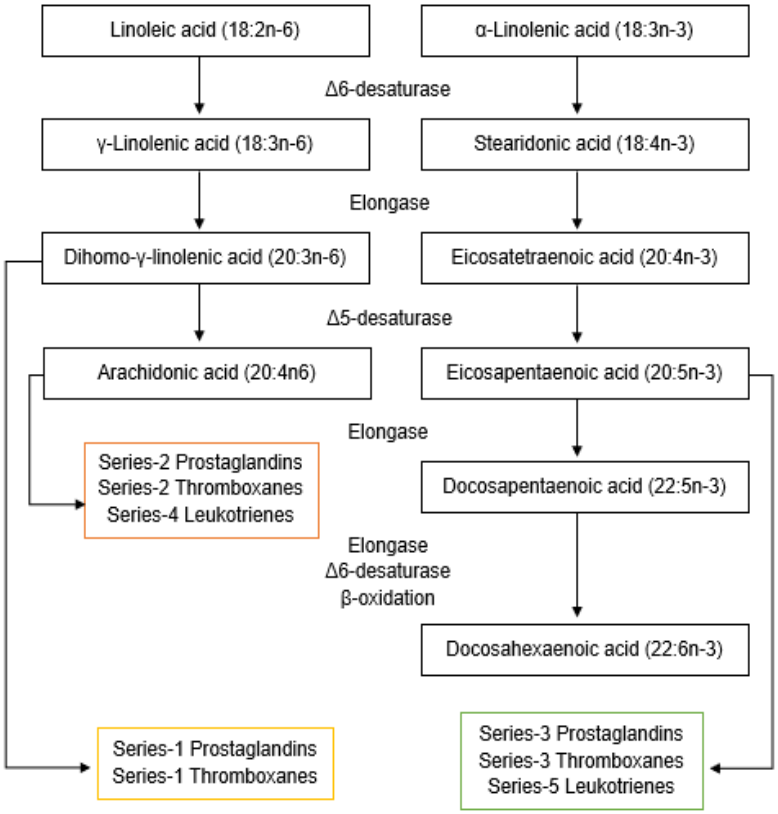

Figure 1. ω6 and ω3 fatty acid metabolism with associated eicosanoids

Omega 6 fatty acids

Omega 6 series FA are often described as pro-inflammatory. LA is converted to γ-linolenic acid (GLA) which is subsequently converted to dihomo-γ-linolenic acid (DGLA) and then to arachidonic acid (AA). AA is the predominant FA found in cellular membranes and primarily known for its role as a precursor to pro-inflammatory eicosanoids such as prostaglandin E2 (PGE2) (Innes and Calder 2018). As mentioned above, the conversion of LA to GLA using Δ6-desaturase is a slow process. However, because LA is prominent in equine diets (and thus outcompetes other FA sources for access to the desaturase), sufficient AA is synthesized. Carnivores can directly consume AA in the diet, but for horses and other herbivores, the primary omega 6 FA consumed is LA.

GLA, which is generally limited in the diet, is rapidly converted to DGLA, and can then be used in the synthesis of anti-inflammatory eicosanoids (Kapoor and Huang 2006). Supplemental GLA bypasses the initial omega 6 metabolism step, inhibiting further conversion of LA and increasing desaturase availability for ALA, therefore indirectly increasing conversion of omega 3 FA as well (Fokkema et al. 2000). Although a small portion of DGLA is converted to AA, that reaction is slow and similarly influenced by enzyme availability. In addition, eicosanoids produced from DGLA have an inhibitory effect on further conversion to AA (Sergeant et al. 2016). Few plant species possess the Δ6-desaturase enzyme necessary to insert the double bond on the carboxyl side of existing bonds. Indeed, GLA does not accumulate in forages or modern commodity grains and oilseeds, nor in other feed ingredients typically used for horse diets (Gurr et al. 2016). True FA dietary need has not been established in the horse but a LA intake of 0.5% dry matter (DM) for horses is recommended based on work in other species (NRC 2007).

Omega 3 fatty acids

Omega 3 series FA are touted for their anti-inflammatory properties. ALA is converted to stearidonic acid (SDA) by Δ6-desaturase, and then further converted to biologically relevant eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) using the same series of enzymatic steps as omega 6 FA. EPA gives rise to eicosanoids that are considered anti-inflammatory. Additionally, EPA and DHA serve as building blocks for resolvins, PUFA metabolites that also play a role in terminating inflammation (Calder 2013a).
Direct supplementation of SDA can more effectively increase tissue-level SDA since the slow, Δ6-desaturase step converting ALA is bypassed; but similar to GLA, SDA presence in typical food sources is limited (Gurr et al. 2016). EPA and DHA are produced by photosynthetic marine

and freshwater microalgae, which horses do not naturally consume. No recommendations for omega 3 intake are recognized for the horse, but, like humans, the omega 6:3 ratio could prove to be important as a means of regulating and reducing inflammation and disease (Bang et al. 1976).

Omega 6:3 ratio

Dietary omega 3 intake was linked with reduced risk of cardiovascular disease in a population of Greenland Eskimos almost 50 years ago (Bang et al. 1976). This sparked a dramatic increase in interest surrounding omega 6:3 PUFA ratios in human diets and the associated benefits of diets with lower omega 6:3 ratio. Similar work has been conducted with horses (Harris 1998), although most of this research has focused on increasing omega 3 intake and not determining the ideal omega 6:3 ratio. Modern diets have relied heavily on starch-rich grains to meet the energy demands of working horses. However, more recent recognition of the ill health effects associated with consuming large amounts of nonstructural carbohydrates (NSC) (i.e., equine metabolic syndrome) has led researchers and nutritionists to recommend greater inclusion of fat as a dietary energy source (Harris 1998). However, the source of fat and the associated omega 6:3 ratios were overlooked, and the diets in early studies were heavily fortified with omega 6-rich grains (e.g., corn, oats, barley) and resultant high omega 6:3 ratios. Omega 6 FA are important for horse metabolism beyond their role in inflammation regulation. Thus, the goal is not to eliminate omega 6 from the diet but to determine the ideal balance between omega 6 and omega 3 FA.

Fat sources in horse diets

Forages are typically low in total fat content (<4% DM) and consist of a mixture of simple lipids (e.g., di- and triacylglycerol), NEFA, waxes, and complex lipids (e.g., phospholipids) (Warren and Vineyard 2013). However, the majority of fat in forage is relatively

high in PUFA, specifically omega 3 ALA. In contrast, added dietary fat is most often provided from grain sources and seed oils which are rich in TAG. These sources vary in composition, with seed oils tending both to be greater in PUFA and to have elevated omega 6. Among oil sources, flaxseed oil is commonly supplemented for its omega 3 content.

Fats derived from forages have a lower level of digestibility (~50%) due to the presence of terpenes, waxes, and other pigments that interfere with digestion (Fonnesbeck et al. 1967; Sturgeon et al. 2000). Fats in grains are more digestible (~75%) but can still be inhibited by fibrous seed coats and other constituents that limit enzymatic access (Warren and Vineyard 2013). Given these limitations, it can be more efficient to feed fat by directly supplementing oil from processed grains and oilseeds. Equine digestion of added fats provided as top-dressed oils, powdered/pelleted supplements, or fatty acid supplements ranges from 88 to 95 percent (Kronfeld et al. 2004).

Benefits of feeding fats to horses

Increasing dietary fat has many advantages for horses. Aside from the inflammatory response (which will be discussed below), research has demonstrated several potential benefits associated with the addition of fats to equine diets. Fat is an efficient energy source, providing 2.25x the amount of energy that carbohydrates and protein produce. For working horses at the upper limit of DM intake capacity, feeding fats provides a way to increase caloric density (and thus meet caloric needs) without requiring increased DM intake (Harris 1998). Horses evolved to capture their nutrient needs from grazing grasses, but as energy demands have increased due to work, sport, or recreation so has the incidence of grain feeding. Many of the commonly supplemented grains (i.e., corn, oats, barley) are high in NSC (i.e., starch and sugar) which are associated with negative digestive and metabolic consequences in horses (Kronfeld and Harris

2003). Fats are an alternative energy source that replaces the calories normally provided by NSC, reducing those risks. Additionally, replacing NSC with fat has been shown to increase insulin sensitivity (Hoffman et al. 2003; Treiber et al. 2005; Quinn et al. 2008).

Fat is considered a cool or less excitable form of energy compared to carbohydrates (Holland et al. 1996; McKenzie et al. 2003). Once adapted to fat metabolism, horses exercising at low to moderate intensity can utilize fat more efficiently for more sustained energy needs. Even though horses do not have a gallbladder, fat can be consumed at up to 20% of the overall diet without ill effect (Kronfeld et al. 2004), however, source of fat should be considered because some fats (i.e., soybean oil) fed to horses can reduce fiber digestibility (Jansen et al. 2000, 2002).

Overview of inflammation in the horse

Inflammation is a vital part of the body's response to injury and disease; however, unregulated inflammation can wreak havoc on the body, leading to compounded disease states and conditions (Carl 2002). Acute inflammation is a healthy and necessary response to injury and infection; it elicits a cascade of inflammatory molecules to either fight off invaders or to break down damaged tissue in preparation for tissue healing and repair. This response is accompanied by the cardinal signs of acute inflammation: swelling, heat, pain, and redness (Rocha e Silva 1978). Following these initial responses, anti-inflammatory molecules are released which prompt a reversal of inflammation and allow tissues to begin the healing process. Acute inflammation that does not resolve is termed chronic inflammation; this response is not well understood but is known to cause tissue damage resulting in disease (Calder 2013b).

Eicosanoids

Eicosanoids are potent lipid-based signaling molecules that are synthesized from the oxidation of 20C FA by cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450

enzymes (Dennis and Norris 2015). Phospholipase A2 (PLA2) hydrolyzes FA from PL in response to a trigger (e.g., injury or infectious agent), which is then oxidized by the enzymes listed above to metabolically active biomolecules (Burke and Dennis 2009). PUFA precursors of primary interest include omega 6 DGLA and AA and omega 3 EPA. COX enzymes have been studied in greatest detail due to their role in inflammatory signaling pathways. Two major COX isoforms, COX1 and COX2, exist which give rise to cyclic endoperoxides from which prostaglandins (PG) and thromboxanes are synthesized (Park et al. 2006). Both COX isoforms can give rise to inflammatory mediators but typically COX1 is associated with general physiological responses whereas COX2 appears to be the dominant source of PG formation in inflammation (Ricciotti and Fitzgerald 2011).

PGs were the first biologically active eicosanoid identified, and of these, the 2-series PG are the most notable and prominent (Gurr et al. 2016). COX stimulation of AA oxidation leads to the production of PGE2. PGE2 is considered pro-inflammatory but has a host of homeostatic functions as well, such as blood pressure, gastrointestinal integrity, and fertility regulation. Dysregulated PGE2 synthesis or degradation has been associated with a wide range of pathological conditions and is of particular interest in inflammation because it is involved in all processes leading to the cardinal signs of inflammation (Ricciotti and Fitzgerald 2011). In addition to PGE2, COX also metabolizes DGLA and EPA to produce 1- and 3-series PG, respectively, which have anti-inflammatory properties and competitively inhibit PGE2 (Wang et al. 2012).

Cytokines

Cytokines, small protein messengers active in cell signaling, serve an important role combating invading pathogens along with regulating and resolving inflammation. The acute

inflammatory response occurs in two phase, the initial proinflammatory phase produces mediators which target and destroy the threat and clear away dead tissue. This is followed by the second release of inflammation resolving mediators that allow the process of tissue repair and healing to begin (Calder and Grimble 2002; Ricciotti and Fitzgerald 2011). Proinflammatory cytokines influence the immune response by upregulating inflammation (Calder 2001). The most potent stimulatory cytokines include tumor necrosis factor (TNFα), interleukin 1β (IL-1β), and interleukin 6 (IL-6) for their direct role in upregulating inflammation. TNFα, IL-1β, and IL-6 are produced by monocytes and macrophages in response to an invading threat to the body. They then attract and activate additional immune cells to the site of infection, which stimulates the release of additional cytokines (Calder and Grimble 2002; Grimble 2005). Anti-inflammatory cytokines antagonize the actions of proinflammatory cytokines which lead to the resolution of inflammation.

Inflammation in the horse

Inflammation is implicated in several diseases such as osteoarthritis, gastrointestinal disease, and equine metabolic syndrome in the horse (Ross-Jones et al. 2016; Elzinga et al. 2016; Stewart et al. 2017). Osteoarthritis, one of the leading causes of lameness, is associated with inflammation in the joint and often leads to early horse retirement (Oke and Mcilwraith 2010). Elevated concentrations of inflammatory cytokines (IL-6, IL-1β, and TNFα) and PGE2 in synovial fluid are markers of joint disease (Bertone et al. 2001; Ma et al. 2017).The associated inflammation causes tissue degradation within the joint, inducing pain, and reducing quality of life (Baccarin et al. 2022).

Excessive adipose tissue increases inflammatory cytokine production producing a low grade constant inflammatory assault; therefore, obesity gives rise to an elevated state of

inflammation (Adams et al. 2009). In humans, this is associated with diseases such as diabetes (Hotamisligil et al. 1995). Similar associations are seen in obese horses with equine metabolic disease. Proinflammatory cytokines TNFα and IL-1β are elevated in adipose tissue of obese horses with equine metabolic syndrome (Vick et al. 2008; Reynolds et al. 2019). Advanced age is also associated with low grade chronic inflammation in what is termed "inflamm-aging". Inflamm-aging is characterized by an increase in circulating cytokines including TNFα and IL-1β, although the cause of which is not well understood (Adams et al. 2008, 2009). Mitigating the effects of inflammation could reduce irreparable tissue damage, increasing comfort and longevity, and improving overall quality of life in horses.

Addressing inflammation

Pharmaceutical solutions have been developed to combat the effects of inflammation; however, these interventions may come with negative side effects that add to the direct cost of medical treatment. While these therapies manage the symptoms of inflammation (e.g., pain), they do not eliminate the cause, which can allow the horse to overexert itself causing more damage (Jacobs et al. 2022). Thus, nutritional approaches that can preclude or reduce the need for such therapies are being evaluated.

Increasing dietary omega 3 FA to compete with omega 6 FA for membrane incorporation is often proposed as a means of reducing the equine inflammatory response. Indeed, increasing membrane omega 3 at the expense of omega 6, (specifically AA), reduces PGE2 production (Calder 2008). Currently proposed solutions can be described as proactive measures (preventing inflammation) or reactive (resolving inflammation once already present). Some measure of balancing both approaches could prove useful.

Pharmaceutical treatments available include the use of either selective or non-selective non-steroidal anti-inflammatory drugs (NSAIDs) which target COX1 and COX2 enzymes to inhibit the production of proinflammatory mediators (Mercer et al. 2023). Although effective at alleviating pain and reducing inflammation, the use of non-selective NSAIDs (e.g., phenylbutazone and flunixin meglumine) may have gastrointestinal and renal consequences (Flood and Stewart 2022). Newer, selective COX2 drugs that have similar efficacy to non-selective NSAIDs have been evaluated, but their use is still reactive to active inflammation. Additionally, long term effects of COX2 inhibition have not been thoroughly evaluated. One study showed that both non-selective and selective COX2 inhibitors reduced microbial diversity similarly in the equine microbiome which could negatively impact overall health (Whitfield-Cargile et al. 2018). Minimizing long term reliance on pharmaceuticals is considered desirable by owners (Pusillo and Purevjav 2014), and feeds or dietary supplements with increased PUFA content are a primary target given their role in inflammation attenuation.

PUFA supplements for horses are provided from either plant or marine sources. Flaxseed is the primary source of plant-based omega 3 currently available. It is rich in ALA and has an omega 6:3 ratio of 0.2:1. Flaxseed should be ground prior to feeding to increase digestibility or fed as an oil, but the high levels of omega 3, specifically ALA, make it unstable and susceptible to rancidity, posing a challenge to incorporate it into feeds given its low shelf-life stability (Tańska et al. 2016). Whether supplementing ALA influences inflammation remains a question. Incorporating flaxseed oil at 10% of the concentrate diet influenced fatty acid profile in serum but had no effect on platelet aggregation (Hansen et al. 2002). Furthermore, supplementing horses with 38 g ALA from flaxseed did not elicit a reciprocal increase in EPA and DHA or a decrease in PGE2 (Ross-Jones et al. 2014). Although beneficial for hair coat and skin condition,

supplementing omega 3 FA may not be effective at increasing anti-inflammatory potential (Goh et al. 2004).

Marine-based sources (i.e., fish and algae) of omega 3 FA provide metabolically active EPA and DHA, thus bypassing the slow enzymatic reactions from ALA. This increases those specific FA in horse tissues (Hess et al. 2012), but it has not always translated into effective inflammatory control (Ross-Jones et al. 2016). There are palatability and sustainability concerns with fish-based supplements (Woodward et al. 2005; Hess et al. 2012). Less is known about incorporating microalgae into horse diets, but such studies are underway (Gu et al. 2022). In either case, marine-based supplements would only be suitable to feed in small quantities to target specific FA, and thus are not candidates as alternatives to the current seed oils used to fortify energy density in horse rations.

Horses supplemented with GLA exhibit an increase in DGLA but not AA; furthermore, GLA was associated with reduced inflammatory status (Craig et al. 1997; McNiven and Rodriguez 2016). However, direct supplementation of GLA through equine diets is uncommon. GLA can be obtained from plant-based sources such as evening primrose (*Oenothera biennis*), borage seed oil (*Borago officinalis*), blackcurrent seed oil (*Ribes nigrum*), Ahiflower oil (*Buglossoides arvensis*), and genetically modified GLA safflower oil (*Carthamus tinctorius*) (Flider 2013; Guil-Guerrero et al. 2020). However, the majority of these oils are not produced at scale and therefore tend to be cost-prohibitive to incorporate at quantities required to see benefit. One promising source of GLA – should it gain pass regulatory approval– is industrial hempseed oil (*Cannabis sativa*) (Guil-Guerrero et al. 2020).

Brief review of the history of hemp

A native of Asia, industrial hemp has been known to and used by humans for thousands of years (Fike 2019). Historical accounts have demonstrated its versatility, including its use in livestock and horse feeds (Linton 1927). However, the crop was heavily regulated from the late 1930s and subsequently legislated out of use with the Controlled Substance Act (91st Congress of the U.S 1970). This law made no distinction between psychotropic (marijuana, containing THC) and non-psychotropic (industrial hemp) forms of the species. Following a brief flourishing of hemp production in the 1940s for the war effort, the crop was effectively not grown for 70 years, until research and production were once again legalized through federal legislation (Congressional Research Service 2021). With legalization came a revitalized interest in the diverse properties and numerous uses that hemp has to offer.

Anatomy and physiology of the hemp plant

Although hemp is perhaps best known as a fiber source, it was grown historically in early China as both a fiber and a food crop. In time, its use as a food resource was supplanted by modern grains and oilseeds (Fike 2019). It is interesting, then, that the West's newfound focus on hemp as a potential oilseed crop has occurred largely on the basis of the seed's considerable nutritional value.

Hempseed's nutritional profile makes it an appealing option for inclusion in mammalian and avian diets. With nearly 30% fat of ~80% PUFA and a desirable omega 6:3 ratio of 3:1, it is considered ideal for human nutrition, however, no such recommendations exist for horses. Moreso, that hempseed contains GLA and SDA, PUFA not commonly found in commercial seed oils, (Matthäus and Brühl 2008) makes the seed oil even more valuable. Concerns that cannabinoids may enter the feed supply as a contaminant during harvest and processing have led

to continued restrictions on the use of hempseed and its byproducts in equine feeds. Cannabinoids are produced in glandular trichomes that cover almost every part of the plant but are heavily concentrated on the upper leaves and flowers. They are especially dense on the bracts, specialized leaves that surround the developing seed (Mahlberg and Eun 2004). Cannabinoids are excreted from trichomes as sticky resin and can easily adhere to and contaminate other substances. During harvest and processing, this resin can accumulate on equipment or the hempseed itself as all of these materials flow through the system. Thus, there is potential to transfer cannabinoids to hempseed and its products (i.e., oil and meal; Matthäus and Brühl 2008). Harvest, handling, and processing methods vary widely; therefore, drawing consistent conclusions about residual cannabinoid presence is challenging. Processors have indicated that if cannabinoids continue to remain a concern, seed washing protocols could potentially be implemented (Tweet, personal communication). However, such measures likely will prove prohibitive – at least for as a commodity livestock or equine feed.

Given the contaminant concerns, little research exists to guide the use of hempseed-based products for animal nutrition. Perhaps ironically, most recent hemp-livestock nutrition research has focused on the feed value of spent hemp flowers from cannabinoid processing (Kleinhenz et al. 2020, 2022; Ates 2021; Parker et al. 2022). Although early feed guides (Linton 1927; Morrison 1956) do consider the nutritional attributes of hempseed, and feeding trials with cattle have been reported (Hessle et al. 2008; Turner et al. 2008; Chakrabarty et al. 2022; Winders et al. 2022, 2023; Smith et al. 2023), no published research exists that directly evaluates hempseed oil (HSO) use in horses. Knowledge of cannabinoid residuals accumulation must be inferred from work feeding hempseed meal (HSM), the left-over products from HSO production, and directly supplementing cannabinoids.

Cannabinoids

Over 500 bioactive compounds have been identified in the industrial hemp plant, of which 125 are identified as cannabinoids (Radwan et al. 2021). While all bioactive compounds have the ability to physiologically influence the consumer, primary focus is on THC and CBD Although potentially beneficial for human and animal health and wellness, concerns about their possible presence in feed has led to much controversy around tissue accumulation. Accumulation in meat, milk, and eggs intended for human consumption are the major concerns in livestock. Cannabinoid concerns in horses are based on a lack of safety data associated with horses consuming hempseed products contaminated with cannabinoids acquired during processing. As such, federal feed restrictions are in place for horses and other companion animals even though they are not consumed in the United States. Additionally, several equine sports authorities have placed CBD and THC on lists of banned medications with a zero-tolerance policy (AQHA 2019; USEF 2019; FEI 2022). Little direct research of hempseed products in horse diets is available, and responses must therefore be predicted from work with other species.

Cattle fed a finishing diet with 20% HSM for 111 days consumed 22.7 mg cannabinoids/day but had no detectable CBD or THC concentrations in plasma. Cannabinoids were detectable in urine at a single time point but were detectable in adipose tissue at multiple timepoints. Additionally, CBC acid and THC acid, the active forms of cannabinoids, were detected sporadically in plasma and urine but were no longer detectable one day after withdrawal from the HSM diet (Smith et al. 2023). Dairy steers that consumed 3.15 g cannabinoids/day had detectable CBD concentrations in 55% of plasma samples, while CBD acids were detectable in all plasma samples of hemp-fed cattle (Kleinhenz et al. 2022). Kleinhenz et al. (2022) fed a variety grown for flower production and provided no indication of when or exactly what part of

the plant was harvested and fed. Wagner et al. (2022) fed low- and high-cannabinoid (0.84 and 1.68 kg DM/cow/day, respectively) hemp silage to lactating dairy cattle and measured significant levels of CBD and THC in milk. Four of six sheep fed diets with 42% hemp for 22 d had measurable cannabinoid acids in subcutaneous fat tissue on the final day of feeding. However, these metabolites were observed in fat from all six sheep 35-d post-feeding and persisted in one sheep for 140 d post-feeding (Stevens et al. 2022). Similarly, sheep fed a diet including either 28% or 56% pelleted whole hemp plant had detectable quantities of THC in subcutaneous fat (Krebs et al. 2021). The rumen may influence the availability of cannabinoids, therefore, the applicability of this research in the context of horses may be limited.

Since cannabinoids are lipophilic and the majority of fat has been removed from HSM, the potential for cannabinoid contamination would seem likely to be greater in HSO. Oral bioavailability of CBD dosed at 10 mg/kg reached a peak concentration of 55 ng/mL but was only estimated to be 14% bioavailable and required a lipid carrier to enhance absorption (Sánchez de Medina et al. 2023). Horses dosed at 0.3 mg/kg CBD infused in oil had peak CBD concentrations of 0.51 ng/mL (Cohen et al. 2021). Horses dosed at 0.5 mg/kg CBD in sesame (*Sesamum inidcum* L.) oil reached a peak concentration of 1.69 ng/mL (Ryan et al. 2021). Senior horses dosed with 2 mg CBD/kg BW had peak plasma CBD concentrations of 18.5 ng/mL (Turner et al. 2022). CBD was measured intermittently in synovial fluid after 5 weeks of daily administration of 1.5 mg CBD/kg BW (Yocom et al. 2022). Cannabinoids were not detected in horses dosed with 0.12 mg CBD/kg BW and 0.13 mg CBD/kg BW (Leise et al. 2023).

One study has suggested that form of administration has an effect on cannabinoid (here, CBD) uptake in horses. Cannabinoids supplied from pellets had greater absorption compared to oil preparations (Draeger 2020). It has been speculated that horses metabolize cannabinoids

faster than other species, therefore, cannabinoid residuals in hempseed oil may not be a concern when fed to horses. In summary, these studies show that consistent dosing at high concentrations are required to achieve a steady state within plasma.

Summary

Chronic inflammation is a threat to horse health and comfort. The unique fatty acids in industrial hemp could provide an alternative dietary strategy to mitigating inflammation. However, concerns over cannabinoid contamination must be addressed to assess the safety of feeding hempseed oil in horses. Alternative fatty acid sources as a way to mitigate inflammation should be explored.

Chapter 3

Polyunsaturated fatty acid profiles in plasma, muscle, and synovial fluid in response to supplemental hempseed oil in the diet of horses

Abstract

Dietary polyunsaturated fatty acids (PUFA) are targeted for their role in inflammation attenuation. Industrial hemp (*Cannabis sativa L.*) has a unique PUFA profile that contains γ-linolenic acid (GLA), an omega 6 PUFA, purported to have anti-inflammatory benefits when consumed in the diet. The goal of this study was to evaluate the influence hempseed oil had on FA composition in plasma, synovial fluid, and muscle in horses. Six Thoroughbred geldings (11 ± 1.3 yr) were used in a crossover study with two, 63-d periods. Horses were maintained on 0.4 ha with ad libitum access to water and white salt. Horses were assigned one of two isocaloric treatment diets consisting of 1.3% BW grass hay and either a basal diet of 0.5% BW concentrate (CON) or basal diet top dressed with 168 mL hempseed oil (HSO) delivering 5 g GLA. Horses were fed their respective treatment diets for the first 35 d of each period and then all horses resumed the basal diet from d 36 to 63. Horses were weighed and body condition score (BCS) assessed weekly. Blood samples were obtained weekly to determine plasma FA composition. Muscle biopsies and synovial fluid were collected on d 0, d 35 and d 63 of each period. FA composition was determined using gas chromatography. Data were analyzed using Proc MIXED with repeated measures in SAS Studio (v.15.1 SAS Institute, Inc., Cary, NC).

Treatment did not affect BW or BCS. Plasma GLA was increased by three weeks of HSO supplementation and returned to baseline by two weeks post-supplementation ($P < 0.0001$). Synovial fluid GLA was detected by d 35 in horses fed HSO but not at any other timepoint ($P < 0.0001$). HSO also increased ($P \leq 0.04$) plasma and synovial fluid dihomo-γ-linolenic acid (DGLA) that persisted post-supplementation. Plasma FA reflected dietary FA intake with

increased ($P \leq 0.0006$) linoleic acid (LA), eicosadienoic acid (EDA), total omega 6 FA, and omega 6:3 ratio and decreased ($P < 0.0001$) α-linolenic acid (ALA) and total omega 3 FA. Adding HSO to diets tended to increase DGLA (P = 0.10) and decrease ALA (P = 0.09) and total omega 3 FA (P = 0.08) in muscle. These data indicate fatty acid composition in plasma, synovial fluid, and muscle can be influenced by targeted supplementation with HSO. The implications of such alterations remain to be elucidated.

Keywords: Industrial hemp, γ-linolenic acid, polyunsaturated fatty acids, horse

Introduction

Polyunsaturated fatty acids (PUFA) serve several important roles in the body. These include supplementing and storing energy, transporting fat-soluble vitamins, and serving as integral cellular membrane components. However, more recent interest has focused on their role in cell signaling and eicosanoid production (Watkins et al. 2005).

Plant-derived PUFAs in equine diets primarily consist of alpha-linolenic acid (ALA) and linoleic acid (LA), precursors for the downstream metabolically active omega 3 and omega 6 series of PUFA, respectively. LA is converted to γ-linolenic acid (GLA) by the Δ6-desaturase enzyme, then subsequently elongated to dihomo-γ-linolenic acid (DGLA). DGLA is converted to arachidonic acid (AA) by the Δ5-desaturase enzyme (Gurr et al. 2016). Similarly, ALA is enzymatically converted to biologically relevant eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Calder 2013b).

Both omega 6 and omega 3 FA are precursors for eicosanoids, potent signaling molecules (Dennis and Norris 2015). Of significant note is the prostaglandin (PG) family for its role in modulating the inflammatory response (Calder 2006). Omega 6 FA are primarily considered pro-inflammatory since AA oxidation to PGE2 produces a strong inflammatory response. Yet DGLA, an omega 6 FA, and EPA produce anti-inflammatory eicosanoids PGE1 and PGE3, respectively (Saini and Keum 2018).

FA composition in tissues reflects FA in the diet and thus can be altered through dietary enrichment (Calder 2008). Supplemented omega 3 FA compete with omega 6 FA for incorporation into cellular membranes in a dose-dependent fashion. AA is the most prevalent FA in cell membranes, therefore omega 3 FA incorporation occurs at the expense of AA (Calder 2008). This influences substrate availability for eicosanoid synthesis which could potentially

decrease the inflammatory response (Kang and Weylandt 2008). Both omega 6 and omega 3 FA compete for the same desaturase and elongase enzymes, thus, the initial desaturase driven conversion step for both LA and ALA is considered the key rate limiting step in the PUFA pathway (Balić et al. 2020).

Supplementing GLA precludes the need for LA conversion. Theoretically this increases desaturase availability for ALA, potentially facilitating increased conversion to omega 3 FA (Fokkema et al. 2000). Dietary GLA is converted to DGLA, of which a small portion is converted to AA, but that reaction is slow and similarly influenced by enzyme availability. In addition, eicosanoids produced from DGLA have an inhibitory effect on further conversion to AA (Sergeant et al. 2016). This has been shown in horses supplemented with GLA which exhibited increased plasma DGLA but not AA (Craig et al. 1997; McNiven and Rodriguez 2016). GLA, however, does not accumulate in forages, grains, or oilseeds typically used in equine diets (Gurr et al. 2016).

Recent interest in hemp (*Cannabis sativa* L.) seed oil (HSO), a novel PUFA source, has arisen due to its unique FA profile. HSO contains greater total omega 3 PUFA compared to common commodity seed oils, with an omega 6:3 ratio of 3:1 (Farinon et al. 2020). Additionally, HSO possesses the enzymes required to produce and therefore contains GLA (Gurr et al. 2016). We hypothesized that manipulating dietary FA may lead to beneficial alterations in tissue FA profiles in the horse which could promote a reduced inflammatory response. Therefore, our overall goal was to determine if oral supplementation of HSO for 35 d would result in detectable changes in fatty acid composition in plasma, synovial fluid, and muscle.

Materials and Methods

Use of animals for this experiment was approved by the Virginia Tech Institutional Animal Care and Use Committee. This study was conducted at the Virginia Tech Middleburg Agricultural Research and Extension Center in Middleburg, VA from May to September 2022.

Horses. Six mature, Thoroughbred geldings (11 ± 1.3 yr) were used in a crossover study with two consecutive 63-d periods. Average body weight (BW) was 567 ± 11 kg and body condition score (BCS; Henneke et al. 1983) was 5.58 ± 0.27. Horses were maintained on a 0.4-ha lot with access to shelter and provided water and white salt *ad libitum*. The lot was mowed to 2.54 cm weekly to minimize grass intake. Horses were acclimated to housing 14 d prior to the start of the study.

Treatments. Horses were randomly assigned to one of two treatment diets for the first 35 d of each period. The control was a basal diet of concentrate offered at 0.5% BW (CON) and the treatment diet was the basal diet top dressed with 84 mL of HSO (for a total of 168 mL daily; HPS Food & Ingredients; Saskatoon, SK). Treatment diets were offered in two evenly split meals (AM and PM). The HSO inclusion rate was established to deliver 5 g GLA/d (Table 3.1). HSO was gradually introduced over 7 d, to minimize digestive upset, and the full inclusion rate maintained for 28 d. On d 36, HSO was removed from the diet and both treatment groups resumed the uniform basal feeding rate until d 63 for post-supplementation observation. Diets were designed to be isocaloric and meet the nutrient requirements for an adult horse at maintenance (NRC 2007). See Table 3.2 for nutrient profiles of feedstuffs. HSO concentrate meals were reduced by 0.42 kg to remain isocaloric with the CON diet. All horses were offered 2nd cut orchardgrass (*Dactylis glomerata*) hay at 1.3% BW. Following the first trial period, horses were switched to the opposite treatment and the study was repeated.

Sampling. Horses were weighed and BCS was assessed weekly. Blood samples, collected via jugular venipuncture, were collected weekly prior to the AM meal. Samples were collected in 10-mL lithium heparin and serum vacutainer tubes. Samples were immediately placed on ice until processing within 1-hr of sampling. Serum samples were allowed to clot at room temperature for 1 hr. Sample tubes were centrifuged at 1500 x g at 4^{o}C for 15 min. Serum and plasma samples were aliquoted into 1.5-mL microcentrifuge tubes and stored at -80^{o}C until analyzed.

Skeletal muscle and synovial fluid were collected d 0, d 35 and d 63 of each period. Animals were sedated (detomidine hydrochloride; 0.1 mg/100 kg; Zoetis Inc., Kalamazoo, MI) and the skin atop the middle gluteal muscle and Carpal joint was shaved and surgically sterilized. A local anesthetic (lidocaine; Aspen Veterinary Resources, Liberty, MO) was administered subcutaneously approximately 1/3 between the tuber coxae and tuber ischii and a small incision (~1 cm) was made. Muscle biopsies were taken using a 14 G 'Tru-Cut' tissue biopsy needle (CareFusion; McGaw Park, IL) and immediately frozen and stored at -80^{o}C. Synovial fluid was aspirated from the Carpal joint of each horse using routine arthrocentesis procedures and stored at -80^{o}C until analysis. Sampling sites were monitored for 7 d post-collection for signs of inflammation and infection.

Feed stuff analysis. Compiled subsamples of concentrate and hay were dried at 55^{o}C in a forage drying oven and ground through a Wiley Mill (Thomas Scientific, Swedesboro, NJ) to pass a 1-mm sieve. Subsamples were submitted to a commercial lab (Equi-analytical, Ithaca, NY) for nutrient analysis. Concentrate, hay, and oil subsamples were analyzed for Vitamin E (NP Analytical Laboratories, St. Louis, MO). For each feedstuff, FA content was determined as follows (Jenkins 2010). In a glass screw top tube, 0.5 g of each feedstuff was added along with

0.5 mL of C19:0 internal standard. Following, 2 mL of 0.5 M sodium methoxide was added, and samples were capped tightly and vortexed lightly. Samples were then incubated at 50^{o}C for 10 min. After removal from heat, samples were allowed to cool for 5 min, then 3 mL of a 5% methanolic HCl solution was slowly added. Samples were recapped and incubated at 80^{o}C for 10 min. Samples were allowed to cool for 7 min prior to adding 1 mL hexane and 7.5 mL 6% K_2CO_3. Samples were centrifuged at 1000 x g for 10 min. The upper layer was transferred to a fresh tube and a pinch of activated charcoal was added. After sitting for 1-hr, samples were centrifuged at 1000 x g for 5 min and the top layer transferred to a gas chromatography (GC) vial and stored at -20^{o}C until analysis.

Plasma Fatty Acid Extraction. In a 125-mm glass tube, 200 μL of C19:0 internal standard was added to 1.3 mL of each plasma sample. The tube was vortexed, then 4.5 mL of 3:2 hexane-isopropanol solution was added. The tube was vortexed again for 30 sec and 3 mL of sodium sulfate solution (1 g Na_2SO_4/15 mL DI H_2O) was added. The tube was vortexed for 30 sec and then centrifuged at 1000 x g for 5 min to allow the phases to separate. The upper hexane phase was transferred via a Pasteur pipette to another 125-mm glass tube containing 1 g of anhydrous Na_2SO_4. In the original tube, 3 mL of 7:2 hexane-isopropanol solution was added, the tube vortexed for 30 sec and then centrifuged at 1000 x g for 5 min. The upper hexane phase was added to the second tube and incubated on the bench top for 30 min. The solution was pipetted to a third 100-mm glass tube and placed under nitrogen flow (N-EVAP 111, Organomotion; Berlin, MA) in a water bath at 40^{o}C until dry. Following this step, tubes either were capped under nitrogen and stored at -20^{o}C or proceeded with methylation.

Muscle Fatty Acid Extraction. Muscle samples along with C19 internal standard were added to 12 x 75-mm polypropylene snap cap tubes and 1.8 mL of 3:2 hexane-isopropanol was

added prior to homogenizing with a laboratory homogenizer (PRO200, PRO Scientific; Oxford, CT). Following this, the homogenate was transferred to a new tube and 1 mL of sodium sulfate solution (1 g Na_2SO_4/15 mL DI H_2O) was added. The mixture was vortexed twice for 30 sec and then centrifuged at 1,000 x g for 5 minutes. The upper phase was transferred to a fresh tube containing 0.5 g anhydrous Na_2SO_4. The sample tube was extracted a second time using 1 mL 7:2 hexane-isopropanol and the upper phase added to the tube containing Na_2SO_4 and allowed to stand for 30 minutes on the bench top. The solvent was transferred to a fresh screw top tube and placed under nitrogen flow in a water bath at 40^{o}C until dry. Samples proceeded to the methylation steps listed below.

Synovial Fluid Fatty Acid Extraction. In a glass screw top tube, 0.5 mL of each synovial fluid sample and 100 uL of C19:0 internal standard were added. Following this, 9.1 mL extraction reagent (2:1:0.6 methanol-chloroform-water) was added to make the ratio of solvents 2:1:0.8. Tubes were capped under nitrogen and stored at 4^{o}C overnight. Samples were centrifuged the next day at 2,000 x g for 10 min and supernatant transferred to a fresh tube. Chloroform (2.5 mL) was added to change the solvent ratio to 2:2:0.8, then water (2.5 mL) was added to change the solvent ratio to 2:2:1.8. Samples were vortexed for 30 sec then centrifuged at 2000 x g for 10 min. The upper layer and interface were removed and discarded. The lower phase was transferred to a fresh tube and placed under nitrogen flow in a water bath at 40^{o}C until dry. Samples proceeded to the methylation steps listed below.

Methylation and Gas Chromatography Analysis. All sample types, except feed, followed the same methylation steps as follows. Once dry, 1 mL toluene was added and sample tubes vortexed for 30 sec. Then, 2mL of 1% sulfuric acid in methanol were added to the samples and the tubes were capped and vortexed for 30 sec. Following overnight incubation (50^{o}C), 5 mL 5%

NaCl solution and 3 mL hexane were added to tubes. Tubes were vortexed and centrifuged at 1000 x g for 5 min. The upper hexane phase was transferred to a new 125-mm glass tube. The original sample was reextracted with an additional 3 mL hexane as described above and then 4 mL 2% $KHCO_3$ solution was added to the extracts. The tube was vortexed and centrifuged if a clear phase separation layer was not obvious. The upper hexane phase was transferred to a new tube containing 1 g of anhydrous Na_2SO_4 and incubated on the bench top for 30 min. After incubation, the extract was again transferred to a new 100-mm glass tube and placed under nitrogen flow in a water bath at 40^oC until dry. Once dry, 1 mL hexane was added and the tube was vortexed briefly. The final hexane solution was transferred to a labeled GC vial for analysis.

Fatty acid methyl esters were analyzed by GC (Agilent GC System 6890N, Agilent Technologies; Santa Clara, CA) using a DB-225 column (30 m x 250-µm i.d. x 0.15-µm thickness, Agilent Technologies; Santa Clara, CA). The oven temperature was initially set at 150°C for 1 min and increased at 1.5°C/min to 210°C and maintained for 41 min. Inlet temperature was 225°C and flame-ionization detector temperature was 275°C, the split ratio was 100:1, and a 1-µL injection volume was used. Gas pressure through the column was constant at 100.7 kPa with hydrogen carrier gas. Hydrogen flow to the detector was 30 mL/min, airflow was 400 mL/min, and the flow of nitrogen makeup gas was 30 mL/min.

Peaks were identified using specific standards (Pure Methyl Ester Standards 68D and 91, Nu-Check Prep Inc), then converted to a percentage based on the total fatty acid (TFA) content. Although numerous fatty acids including palmitic acid (PA), stearic acid (SA), and oleic acid (OA) were also quantified, this data analysis focused on the PUFA: LA, GLA, eicosadienoic acid (EDA), DGLA, AA, ALA, stearidonic acid (SDA), eicosatetraenoic acid (ETA), EPA, and DHA.

Statistical analysis. Individual FA data (represented as g of FA per 100 g of TFA ± SEM) were analyzed using Proc MIXED with repeated measures in SAS Studio (v.15.1 SAS Institute, Inc., Cary, NC). The model included period, treatment, time, and treatment x time interaction as fixed effects, horse x period within date as a repeated effect, and d 0 as a covariate. Data were log transformed if needed and back transformed for reporting. Differences were considered significant at $P < 0.05$.

Results

No significant changes in BW or BCS were observed throughout the study. Actual daily intake of dietary FA is listed in Table 3.3. There were no complications from the arthrocentesis or muscle biopsy procedures. HSO supplementation increased ($P < 0.0001$) several plasma FA (Table 3.4). Horses fed HSO had greater plasma LA, EDA, GLA, and DGLA ($P < 0.0001$), whereas plasma AA was the only omega 6 FA that did not differ between treatments. Plasma ALA, the only individual omega 3 FA affected, was lower in horses that consumed HSO compared to CON (1.55 ± 0.03 vs. 1.75 ± 0.03 g/100 g TFA, respectively). Plasma GLA was greater ($P < 0.0001$) in horses fed HSO compared to CON at d 35 (0.465 ± 0.015 vs 0.046 ± 0.015, respectively) but did not differ by d 49, two weeks post-supplementation, (0.09 ± 0.02 vs 0.04 ± 0.02, respectively). Plasma DGLA increased over time, peaking at d 35, and decreased at a similar rate during the post-supplementation period, but the Trt x Time interaction was not as strong ($P = 0.08$) as observed for GLA. Sampling date (i.e., time) had a significant effect ($P \leq 0.05$) on all individual FA except AA, which increased total omega 6 and omega 6:3 ratios.

Fewer changes in SF and muscle FA were observed (Tables 3.5 and 3.6, respectively). GLA was detected in SF on d 35 of HSO supplementation (0.27 ± 0.03 g/100 g TFA) but not at any other time point (trt x time interaction; $P < 0.0001$). Horses supplemented with HSO had a

corresponding increase ($P = 0.04$) in SF DGLA compared with those fed CON (0.31 ± 0.04 vs. 0.19 ± 0.04 g/100g TFA), but there was no treatment x time interaction. No other SF FA were influenced by HSO supplementation. Muscle ETA was lower (P = 0.003) in horses fed HSO than horses fed CON (0.125 ± 0.004 vs. 0.138 ± 0.004). Muscle ALA and total omega 3 tended ($P \leq 0.09$) to be greater in horses fed CON while DGLA in muscle tended (P = 0.10) to be greater in horses fed HSO.

Discussion

HSO is a novel source of PUFA, especially GLA, which is not found in grain and seed oils commonly supplemented in equine diets. Excitement around the nutritional possibilities of hemp has increased dramatically since its legalization in the 2018 Farm Bill. However, much of the hemp-based research effort with horses has focused on the effect of cannabinoid use. To the best of this author's knowledge, this is the first study of HSO-supplementation for horses reported in the literature.

Neither BW nor BCS were influenced by treatment diet, indicating that diets remained isocaloric throughout the study period. The flavor of HSO is described as nutty, with a distinct aromatic profile (Cerino et al. 2021). Although horses can have selective palates (Goodwin et al. 2005), no HSO refusals were observed, and all offered concentrate was readily consumed. A true preference test was not performed, but this study empirically demonstrated that horses found HSO acceptable and palatable.

The results from this research indicate that dietary supplementation of HSO in horses can rapidly increase GLA and DGLA concentrations in serum and synovial fluid. Serum omega 6 FA, apart from DGLA, generally reflected the direct effect of dietary intake (Table 3.3) of those FA. It is likely the increase in DGLA that was observed came from converted GLA since there

were no sources of DGLA in the diet. Although plasma and SF GLA were converted to DGLA, an increase in AA was not observed in either tissue type. Similar results were reported for horses supplemented with GLA in which circulating DGLA was increased but AA was not affected (Craig et al. 1997; McNiven and Rodriguez 2016). This might be explained by the differential expression of enzymes in various cell types and the effect on the rate in which DGLA is desaturated to AA (Sergeant et al. 2016).

Varying enzyme expression is further highlighted by looking at the poor conversion of dietary LA and ALA in horses. Direct supplementation of EPA and DHA increases those omega 3 FA in SF, yet supplementing ALA does not lead to similar elevations. This differential response suggests that enzymes for converting ALA to downstream metabolites may be limited in SF (Ross-Jones et al. 2014). Similar results were seen in horses supplemented with either LA or GLA in which no increases in GLA or DGLA were observed in the LA supplemented group, however, increases in both FA were measured in horses supplemented directly with GLA (Pagan and Hauss 2023).

Serum ALA was reduced in the HSO group even though ALA intake was greater (Table 3.3); indicating that, perhaps, HSO supplemented horses had greater conversion to the long-chain omega 3 derivatives. However, no other omega 3 FA were affected by treatment. Total fat intake was greater in HSO treatment horses with greater LA but also greater ALA intake resulting in a lower omega 6:3. Studies increase dietary omega 3 using ALA which does not increase EPA and DHA (Hess et al. 2012; Ross-Jones et al. 2014; Patoux and Istasse 2016). This increase in ALA may potentially reduce omega 3 availability and partially explain the lack of consistent effect when supplementing ALA. Tracer studies by Vermunt et al. (2000) indicated that greater dietary ALA increased the metabolic oxidation of ALA instead of the conversion to EPA and DHA.

Additionally, ALA incorporation into phospholipids decreased with greater inclusion of dietary ALA (Goyens et al. 2006). Similar reductions were measured in muscle ETA and a trend for lower ALA in the HSO group but no effect on EPA and DHA. Therefore, the reduction in omega 3 FA in serum and muscle may be due to increased metabolic oxidation of omega 3 FA and warrants further evaluation in future studies.

Overall limited changes in muscle FA profile suggest that 35 d of HSO supplementation may not have been long enough to induce measurable differences in this tissue. One study reported that horses supplemented with GLA from *Buglossoides arvensis* (Ahiflower) oil for 60 d had greater combined muscle EPA+DHA levels compared to baseline but they did not specify if this was at d 30 or d 60 (Jacobs and Gordon 2023). Another study evaluated increasing rates of GLA supplementation effect on DGLA composition in red blood cell (RBC). RBC DGLA significantly differed after 3 months in horses supplemented with 1.9 g GLA/d, whereas horses supplemented with 7.3 g GLA/d differed after 1 month. However, comparisons were not reported to determine if peak concentrations were different between treatments (Hauss et al. 2023). King et al. (2008) fed diets with three different concentrations of an omega 3 FA supplement and reached steady state in plasma with all three by 7 d. They also observed a linear increase in response to increasing dietary FA concentrations. These results may indicate that longer supplementation is required to increase incorporation into muscle cell membranes. Alternatively, supplementing a greater quantity of HSO may be needed to induce noticeable muscle FA changes in a rapid manner.

Most published studies have evaluated PUFA supplemented for 70 to 90 d but provide no clear indication of the optimal length of time or supplementation rate required to reach peak incorporation (Woodward et al. 2007; Vineyard et al. 2010; Ross-Jones et al. 2014). Even less

provide data on FA clearance. Plasma and SF DGLA remained elevated longer than GLA after HSO was removed from the diet. This may reflect a slower or extended rate of conversion of GLA to DGLA in horses. Sustained elevated plasma DGLA concentrations could support greater incorporation into membrane phospholipids; thus providing increased availability of substrates for reducing the inflammatory response (Calder 2008). However, the clearance rate of plasma FA observed in this study contrast with results from previous FA supplementation studies in which six to eight weeks were required for plasma FA to return to baseline (King et al. 2008; Vineyard 2008). In yet another study, RBC EPA returned to baseline by 3-mo post-supplementation but DHA had still not returned (Hauss and Pagan 2023). No other studies have evaluated the rate of clearance of GLA in horses.

It should be noted that sampling time significantly influenced several FA measured in plasma and muscle. There were few treatment x time interactions, and those observed generally reflect the rise and fall of FA in response to inclusion and removal from the diet. Time only effects may reflect our capacity to control grass intake during the study. Although caution was taken to minimize grass intake by frequent mowing, periodic grazing was observed in all horses especially in Period 1 when grasses were most actively growing. Grasses are low in total fat content but contain a greater amount of omega 3 FA, specifically ALA, compared to omega 6. Thus, the effect of time could reflect grass intake and its effect on overall dietary FA supply.

Conclusions

Fatty acid metabolism in horses is poorly understood. The work that is available is often confounded by varying study designs and FA supplement rates. This work aligns with prior studies highlighting that FA profiles in the horse reflect dietary intake. Supplementing horses with HSO for 35 d increased GLA and DGLA profiles in equine plasma and synovial fluid.

While biological relevance requires more research, elevated GLA levels could potentially influence inflammation signaling molecules and the consequent inflammatory response.

Additionally, these results demonstrate that PUFA in SF can be manipulated in as little as two weeks. This information could be useful for future study designs where funding longer studies is becoming challenging. However, studies using horses with varied activity levels and different quantities of HSO are needed to determine peak incorporation and ideal supplementation rates.

Table 3.1. Fatty acid profiles of hay, concentrate, and hempseed oil.

Fatty Acid*	Hay	Concentrate	Hempseed Oil
PA; 16:0	21.6	12.7	5.7
SA; 18:0	2.9	3.3	2.6
OA; 18:1,c9	6.8	20.3	11.2
LA; 18:2n6	27.4	53.1	54.9
GLA; 18:3n6	nd†	nd	3.5
EDA; 20:2n6	nd	nd	0.1
DGLA; 20:3n6	nd	nd	nd
AA; 20:4n6	nd	nd	nd
ALA; 18:3n3	20.9	6.4	17.2
SDA; 18:4n3	nd	nd	1.2
ETA; 20:4n3	0.2	nd	nd
EPA; 20:5n3	0.2	0.1	nd
DHA; 22:6n3	nd	nd	nd
Total Omega 6	27.4	53.1	56.4
Total Omega 3	21.2	6.4	18.4
Omega 6:3 ratio	1.3	8.2	3.2

*Fatty acids expressed as a percentage of total fatty acids.

†nd= not detected

Table 3.2. Nutrient profiles of hay, concentrate, and hempseed oil.

Nutrients	Hay	Concentrate	Hempseed Oil
CP (%)	13.3*	13.8	
Lignin (%)	6.2	3.1	
ADF (%)	40.2	15.9	
NDF (%)	64.8	30.9	
WSC (%)	4.6	14.9	
ESC (%)	4.0	11.6	
Starch (%)	0.6	8.2	
EE (%)	3.6	13.2	99
Ash (%)	7.0	10.0	
Vit. E[1]	6.36	24.5	1.56

*All values presented on a dry matter basis.

[1]Vitamin E = α-Tocopherol acetate (mg/100g)

Table 3.3. Average[1] daily dry matter intake of total fat and fatty acids for hay and treatment diets.

Nutrients	Hay[2]	CON[3]	HSO[4]
Total dietary fat (g)	265.8	375.4	480.1
	— Individual fatty acids —		
PA[5]; 16:0 (g)	57.4	47.8	50.3
SA[6]; 18:0 (g)	7.8	12.5	14.9
OA[7]; 18:1,c9 (g)	18.0	76.2	83.3
LA; 18:2n6 (g)	72.9	199.2	257.7
GLA; 18:3n6 (g)	0	0	5.5
EDA; 20:2n6 (g)	0	0	0.1
ALA; 18:3n3 (g)	55.4	23.9	47.4
SDA; 18:4n3 (g)	0	0	1.9
ETA; 20:4n3 (g)	0.4	0	0
EPA; 20:5n3 (g)	0.6	0.2	0
DHA; 22:6n3 (g)	0	0	0
Total Omega 6 (g)	72.9	199.2	263.3
Total Omega 3 (g)	56.4	24.2	49.4
Omega 6:3 ratio	1.3	8.2	5.3

[1]Estimated for average BW of 568 kg
[2]Hay = 1.3% BW 2nd cut orchardgrass hay
[3]CON = basal diet of 0.5% BW of concentrate feed
[4]HSO = basal diet minus 0.42 kg, top-dressed w/168 mL of HSO
[5]PA = palmitic acid
[6]SA = stearic acid
[7]OA = oleic acid

Table 3.4. Plasma fatty acid profiles in horses supplemented with hempseed oil (HSO) or no supplementation (CON) for 35 days and during the 28-day post-supplementation observation period.

Fatty Acid*	Treatment	Supplemental					Post-supplemental				SEM	P-values		
		d 7	d 14	d 21	d 28	d 35	d 42	d 49	d 56	d 63		Trt	Time	Trt*Time
LA; 18:2n6														
	Control	54.8	55.2	55.7	55.8	55.9	56.0	56.4	55.8	55.8	0.46	<0.0001	0.04	0.51
	HSO	55.8	56.6	57.5	56.8	57.8	56.5	56.7	56.0	57.1	0.46			
GLA; 18:3n6														
	Control	0.049^{d†}	0.053^{d}	0.050^{d}	0.057^{d}	0.046^{d}	0.048^{d}	0.046^{d}	0.064^{d}	0.047^{d}	0.015	<0.0001	<0.0001	<0.0001
	HSO	0.226^{c}	0.400^{b}	0.420^{ab}	0.491^{a}	0.465^{ab}	0.167^{c}	0.087^{d}	0.084^{d}	0.061^{d}	0.015			
EDA; 20:2n6														
	Control	0.352	0.384	0.391	0.398	0.392	0.387	0.399	0.412	0.422	0.018	<0.0001	0.04	0.38
	HSO	0.383	0.421	0.456	0.461	0.439	0.409	0.403	0.442	0.402	0.018			
DGLA; 20:3n6														
	Control	0.200	0.203	0.212	0.203	0.195	0.200	0.201	0.190	0.201	0.013	<0.0001	0.02	0.08
	HSO	0.213	0.252	0.280	0.280	0.287	0.268	0.236	0.227	0.229	0.013			
AA; 20:4n6														
	Control	0.754	0.748	0.745	0.745	0.747	0.752	0.763	0.726	0.765	0.018	0.83	0.54	0.58
	HSO	0.756	0.763	0.775	0.709	0.744	0.742	0.738	0.759	0.776	0.018			
ALA; 18:3n3														
	Control	2.20	1.91	1.85	1.75	1.61	1.70	1.76	1.45	1.56	0.11	<0.0001	0.0002	0.41
	HSO	1.81	1.59	1.42	1.52	1.58	1.49	1.54	1.36	1.59	0.11			
DHA; 22:6n3														
	Control	0.036	0.049	0.048	0.025	0.037	0.039	0.045	0.027	0.041	0.004	0.55	0.0003	0.22
	HSO	0.038	0.041	0.040	0.028	0.046	0.037	0.031	0.032	0.042	0.004			
Total Omega 6														
	Control	56.1	56.6	57.0	57.2	57.3	57.4	57.8	57.2	57.3	0.51	<0.0001	0.05	0.29
	HSO	57.4	58.4	59.5	58.7	59.8	58.1	58.1	57.5	58.5	0.51			
Total Omega 3														
	Control	2.32	1.99	1.92	1.86	1.69	1.77	1.79	1.52	1.62	0.12	<0.0001	0.0003	0.34
	HSO	1.87	1.65	1.48	1.58	1.66	1.55	1.60	1.44	1.65	0.12			
Omega 6:3 ratio														
	Control	26.0	30.7	30.4	33.6	36.0	34.5	33.7	38.7	37.4	2.6	<0.0001	0.002	0.55
	HSO	32.3	38.4	40.5	40.8	37.6	41.8	39.3	42.6	36.6	2.6			

*Fatty acids expressed as a percentage of total fatty acids.

†Treatments with similar letters do not statistically differ from one another.

Table 3.5. Synovial fluid fatty acid profiles in horses supplemented with hempseed oil (HSO) or no supplementation (CON) for 35 days and during the 28-day post-supplementation observation period.

Fatty Acid*	Treatment	Synovial Fluid		SEM	P-values		
		d 35	d 63		Trt	Time	Trt*Time
LA; 18:2n6							
	Control	50.3	50.9	0.84	0.64	0.47	0.96
	HSO	50.7	51.3	0.84			
GLA; 18:3n6							
	Control	nd†	nd	0.03	<0.0001	<0.0001	<0.0001
	HSO	0.270	nd	0.03			
EDA; 20:2n6							
	Control	0.225	0.364	0.074	0.84	0.15	0.72
	HSO	0.237	0.323	0.074			
DGLA; 20:3n6							
	Control	0.262	0.126	0.054	0.04	0.07	0.58
	HSO	0.348	0.272	0.054			
AA; 20:4n6							
	Control	1.25	1.24	0.11	0.49	0.72	0.66
	HSO	1.12	1.21	0.11			
ALA; 18:3n3							
	Control	0.480	0.487	0.094	0.89	0.83	0.78
	HSO	0.494	0.447	0.094			
Total Omega 6							
	Control	52.0	52.6	0.86	0.57	0.53	0.97
	HSO	52.6	53.1	0.86			
Total Omega 3							
	Control	0.474	0.480	0.060	0.33	0.28	0.24
	HSO	0.488	0.344	0.060			
Omega 6:3 ratio							
	Control	113.6	117.8	13.3	0.47	0.57	0.49
	HSO	115.0	102.7	16.3			

*Fatty acids expressed as a percentage of total fatty acids.

†nd= not detected

Table 3.6. Muscle fatty acid profiles in horses supplemented with hempseed oil (HSO) or no supplementation (CON) for 35 days and during the 28-day post-supplementation observation period.

Fatty Acid*	Treatment	Muscle		SEM	P-values		
		d 35	d 63		Trt	Time	Trt*Time
LA; 18:2n6							
	Control	24.5	27.7	1.7	0.81	0.05	0.90
	HSO	24.7	28.3	1.7			
GLA; 18:3n6							
	Control	0.057	0.065	0.019	0.23	0.50	0.27
	HSO	0.101	0.067	0.019			
EDA; 20:2n6							
	Control	0.465	0.513	0.028	0.69	0.04	0.66
	HSO	0.464	0.537	0.028			
DGLA; 20:3n6							
	Control	0.290	.352	0.028	0.10	0.12	0.54
	HSO	0.354	.382	0.028			
AA; 20:4n6							
	Control	1.68	2.22	0.265	0.26	0.24	0.42
	HSO	2.20	2.31	0.265			
ALA; 18:3n3							
	Control	9.27	6.89	0.81	0.09	0.01	0.92
	HSO	7.97	5.44	0.81			
ETA; 20:3n3							
	Control	0.138	0.125	0.004	0.003	0.003	0.79
	HSO	0.125	0.113	0.004			
EPA; 20:5n3							
	Control	0.127	0.126	0.013	0.49	0.58	0.62
	HSO	0.124	0.111	0.013			
DHA; 22:6n3							
	Control	0.139	0.176	0.017	0.40	0.36	0.24
	HSO	0.175	0.170	0.017			
Total Omega 6							
	Control	27.0	30.9	1.8	0.67	0.05	0.91
	HSO	27.8	31.6	1.8			
Total Omega 3							
	Control	1.03	0.92	0.04	0.08	0.005	0.64
	HSO	0.97	0.84	0.04			
Omega 6:3 ratio							
	Control	3.07	4.31	0.62	0.19	0.01	0.70
	HSO	3.50	5.74	0.62			

*Fatty acids expressed as a percentage of total fatty acids.

Chapter 4

Serum and synovial fluid inflammatory biomarkers and stride length in unchallenged, sedentary horses supplemented with hempseed oil

Abstract

Inflammation contributes to reduced comfort levels in horses and elevated disease states which lead to increasing incidence of morbidity and mortality. Polyunsaturated fatty acids (PUFA) are targeted as dietary interventions for their role in inflammation modulation. (γ)-Linolenic acid (GLA), a unique omega 6 fatty acid found in hempseed (*Cannabis sativa L.)* oil (HSO), is a PUFA of particular interest given its potential anti-inflammatory benefits. The goal of this study was to evaluate if supplementing HSO for 35 d would influence inflammatory biomarkers resulting in stride length improvements in horses. Six Thoroughbred geldings (11 ± 1.3 yr) were used in a crossover study with two, 63-d periods. Horses were maintained on 0.4-ha lot with ad libitum access to water and white salt. Treatments included a basal concentrate diet offered at 0.5% BW (CON) or 168 mL HSO delivering 5 g GLA (HSO) top-dressed on the basal diet reduced by the caloric equivalent. All horses were offered 1.3% BW cool season grass hay. Horses were fed their respective diets for the first 35 d of each period and then all horses resumed the CON diet for d 36-63. Blood samples were obtained weekly and synovial fluid was collected on d 0, d 35 and d 63 of each period. Video analysis of horses at both the walk and trot were obtained d -1, d 34 and d 62 of each period for stride length (SL) analysis. Serum was analyzed using ELISA for PGE2, TNFα, IL6, and IL1β, and synovial fluid (SF) was analyzed using ELISA for PGE2 and IL1β. Area under the curve (AUC) was calculated for serum inflammatory biomarkers (IB) using GraphPad (Prism 9.5.1; San Diego, CA). IB and SL data were analyzed using Proc MIXED in SAS Studio (v.15.1 SAS Institute, Inc., Cary, NC) with

repeated measures. Correlations and linear regression analysis between IB and SL were completed using GraphPad (Prism 9.5.1; San Diego, CA).

Treatment did not affect IB AUC, SL, SF PGE2, serum PGE2, TNFα, or IL6. Serum TNFα was greater ($P = 0.04$) for all horses on d 63 compared to d 7 but no treatment effect was observed. HSO increased serum IL1β ($P = 0.01$) compared to CON but there was no treatment x time effect ($P = 0.62$). HSO may contain non-nutritive components that could influence the response of IL1β, but the lack of treatment x day interaction indicates that other factors may have influenced the inflammatory response. Future work is warranted to evaluate the relevance of the observed biological changes. These results indicate that to parse out the effects of HSO on inflammatory parameters in horses may require inclusion of an inflammatory challenge. Along with inflammatory challenge, testing HSO at different dietary inclusion rates may improve understanding of the effects of this supplement on sedentary horses.

Introduction

Chronic inflammation is a contributing factor in numerous disease states (e.g., joint issues, metabolic disease, and gastrointestinal disease, etc.) resulting in increased morbidity and mortality (DeNotta and McFarlane 2023). Inflammation can be managed using traditional medicine (i.e., NSAIDs), but long-term use to mitigate chronic issues can have unintentional and negative consequences such as gastrointestinal ulceration and kidney damage (Flood and Stewart 2022). Feeding polyunsaturated fatty acids (PUFA), most of which has focused on specifically increasing dietary omega 3 FA, is an alternative approach to mitigating inflammation. Although these feed ingredients have been explored extensively for their role in inflammation attenuation, consistent conclusions remain elusive.

PUFA composition in tissues reflects that of the diet; thus, modifying the ratios of FA consumed results in altered membrane composition (Calder 2008). This change comes at the expense of arachidonic acid (AA), which influences substrate availability for eicosanoid synthesis (Gurr et al. 2016). AA is oxidized to produce prostaglandin E2 (PGE2), the most notable and prominent eicosanoid, for its role in the processes that lead to the cardinal signs of inflammation including heat, pain, swelling, and redness (Ricciotti and Fitzgerald 2011). Additional inflammatory molecules of interest are the proinflammatory cytokines: tumor necrosis factor (TNFα), interleukin 1β (IL-1β), and interleukin 6 (IL-6) (Calder and Grimble 2002; Vick et al. 2008; Liburt et al. 2010).

Stride length (SL), which can be influenced by inflammation specifically when associated with joints, is used as an indicator of soundness (Toutain and Cester 2004; Rogers et al. 2005). Inflammatory markers in synovial fluid (SF) provide additional indications of joint health and soundness (Coppelman 2017). The ability of PUFA supplements to mitigate inflammation can

lead to increased SL. Horses supplemented with linseed (*Linum usitatissimum*) oil as a source of omega 3 PUFA had increased trot SL, however cytokines were not measured (Oliveira et al. 2014). SF PGE2 tended to be lower in horses supplemented directly with omega 3 eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) compared to α-linolenic acid (ALA), indicating that directly supplementing these FAs in the diet can reduce inflammatory response in the joint but no effect on gait parameters was analyzed (Ross-Jones et al. 2014).

Increased interest in supplementing industrial hempseed (*Cannabis sativa L*.) oil (HSO) results from the seed's unique PUFA profile (Farinon et al. 2020). Most notable is the presence of γ-linolenic acid (GLA), an omega 6 FA with anti-inflammatory properties (Kapoor and Huang 2006). Supplementing GLA directly in the diet bypasses the need for linoleic acid (LA) conversion. GLA is elongated to dihomo-γ-linolenic acid (DGLA), which can then be oxidated to eicosanoids with anti-inflammatory properties or converted to AA. The fate of DGLA is dependent on enzyme availability in various tissue types (Sergeant et al. 2016).

The effect of GLA on inflammation is poorly characterized in the horse. Craig et al. (1992) reported that 29% of horses with recurrent seasonal pruritus were 'less itchy than in previous years' when supplemented with a GLA/EPA/DHA mixture based on owner observations. However, when replicated in a controlled research study, researchers failed to reproduce similar results (Craig et al. 1997). Neither study evaluated inflammatory cytokines. Another study determined that horses had a lower AA:(EPA+DHA) index, an indicator of inflammatory status (Nelson and Raskin 2019; Tutino et al. 2019; Roškarić et al. 2021), when consuming a GLA supplement (McNiven and Rodriguez 2016), but again no inflammatory cytokines were evaluated. Horses supplemented with GLA/EPA/DHA had lower a lower AA:(GLA+EPA+DHA) ratio compared to horses supplemented with LA/ALA, indicating that

direct supplementation is required to influence inflammatory status (Pagan and Hauss 2023). No studies exist evaluating dietary HSO supplementation to horses, therefore, the objective of this study was to determine if oral supplementation of PUFA, specifically GLA in HSO, would reduce inflammatory biomarkers in plasma and SF and alter stride length in unchallenged, sedentary horses.

Materials and Methods

Use of animals for this experiment was approved by the Virginia Tech Institutional Animal Care and Use Committee. Details for this experiment are previously described at length in Chapter 3. Briefly, six Thoroughbred geldings (11 ± 1.3 yr), with no known history of arthritis, were used in a cross-over design with two consecutive 63-d periods. Treatment diets were fed the first 35 d of each period and included a basal concentrate diet offered at 0.5% BW (CON) or 168 mL HSO delivering 5 g GLA (HSO) top-dressed on the basal diet reduced by the caloric equivalent. HSO was removed and all horses consumed the basal diet from d 36 to 63. Horses were offered 1.3% BW cool season grass hay and had access to a 0.4-ha lot (mowed to 2.54 cm), with shelter and *ad libitum* access to water and white salt. Hooves were trimmed on an 8-wk cycle by a professional farrier.

Sampling. Serum samples were collected weekly. SF was collected on d 0 (baseline), d 35 and d 63 of each period. Walk and trot stride length videos were collected d (-1) (baseline), d 34 and d 62 of each period so as to not interfere with SF sample collection.

Stride Length. Video analysis was captured using a Sony a7iii MILC camera (Sony; Tokyo, Japan) set to capture 60 frames per second. The video camera was placed on a stand 14 m from the center of the recording zone. Each horse was asked to walk and trot in both directions along a concrete path located in front of a barn. The recording zone was set between two

permanent, fixed structures on the barn with a distance of 9 m. Horses were led by the same handler every time and allowed to move freely without lead rope tension. When necessary, a volunteer was asked to drive the horses from behind. Horses were asked to enter the frame in the appropriate gait and to continue steadily until exiting the video frame. Three clean, consecutive strides from the 9 m path were used for frame-by-frame video analysis and results averaged. Video analysis was conducted using Dartfish 360 (DARTFISH, www.dartfish.com; Fribourg, Switzerland). Window height served as a 1.18 m calibration mark for analysis. SL was measured for the left forelimb from the toe during stance phase to the subsequent stance phase.

Inflammatory biomarkers. Inflammatory biomarkers (IB) were analyzed using enzyme linked immunosorbent assays (ELISA). Serum samples were analyzed for PGE2 (PGE2 Multi-Format ELISA Kit, Arbor Assays; Arbor, MI), TNFα (Equine TNFα DuoSet ELISA kit, R&D Systems; Minneapolis, MN), IL6 (Equine IL6 DuoSet ELISA kit, R&D Systems; Minneapolis, MN), and IL1β (Equine IL-1β DuoSet ELISA kit, R&D Systems; Minneapolis, MN). SF was analyzed for PGE2 and IL1β. IL6 ELISA was validated for use in equine serum and PGE2 and IL1β ELISA were validated for use in equine serum and SF prior to analysis.

TNFα. Serum samples for TNFα ELISA were diluted between 1:20-1:80 in reagent diluent. Analysis was performed per manufacturer's instructions with the previously validated modifications (Lavoie-Lamoureux et al. 2010): primary antibody coating was performed in 0.1M Na_2HPO_4 (pH 9.0) overnight at 4^{o}C and the duration of incubations with Streptavidin-HRP and TMB were both increased to 30 min instead of 20 min. Samples were analyzed in duplicate and single absorbance was measured at 450 nm, with wavelength correction set to 540 nm.

IL6. All steps of the IL6 ELISA were performed by the manufacturer's instructions except samples were diluted between 1:20-1:100 in phosphate buffered saline (PBS). Samples

were analyzed in duplicate, and absorbance was measured at 450 nm, with wavelength correction set to 540 nm.

IL1β. IL1β ELISA protocol steps from the manufacturer were followed with the following exceptions: serum samples were diluted 1:40-1:100 in PBS and SF samples were diluted 1:25-1:100 in PBS; Normal Goat Serum was removed from detection antibody incubation step; the recombinant IL1β standard provided was adjusted to make a top standard of 8000 pg/mL and a low standard of 15.6 pg/mL. Serum samples were run in duplicate and SF samples run in triplicate. Absorbance was measured at 450 nm, with wavelength correction set to 540 nm.

PGE2. Serum and SF samples were analyzed following the PGE2 ELISA high-sensitivity format protocol from the manufacturer except serum samples were diluted between 1:30 – 1:60 in PBS and SF samples were diluted 1:5 in PBS. Samples were run in triplicate and absorbance measured at 450 nm.

Statistical analysis. SL and IB data were analyzed using Proc MIXED with repeated measures in SAS Studio (v.15.1 SAS Institute, Inc., Cary, NC). The model included period, treatment, time, treatment x time interaction as fixed effects, period x horse within date as a repeated effect, and d 0 as a covariate. IB data were log transformed for analysis. One horse was unable to complete the walk and trot evaluation on concrete due to consistent foot tenderness, not associated with injury or illness, and was excluded from analysis. Area under the curve (AUC) calculations for serum IB were completed using GraphPad (Prism 9.5.1; San Diego, CA). AUC data analyzed using Proc MIXED in SAS Studio (v.15.1 SAS Institute, Inc., Cary, NC). The model included treatment and period as fixed effects, and horse within treatment as a random effect. PGE2, IL6, and IL1β AUC data were normalized and AUC TNFα data were log transformed prior to analysis. Correlation estimates and linear regression analysis between SL

and IB were completed using GraphPad (Prism 9.5.1; San Diego, CA). Differences were considered significant at $P \leq 0.05$.

Results

Feeding HSO did not affect IB AUC (data not shown). SF PGE2 (Figure 4.1) was not influenced by HSO treatment at either timepoint. SF IL1β (data not shown) was not detected consistently at all timepoints in any of the horses and was therefore not analyzed. Serum PGE2 (Figure 4.2) and IL6 (Figure 4.3) were not influenced by treatment, time, or their interaction. Serum TNFα (Figure 4.4) was not influenced by treatment but was greater ($P = 0.04$) in both HSO and CON horses on d 63 compared to d 7. Serum IL1β (Figure 4.5) was greater ($P = 0.01$) in horses receiving HSO compared to control, but no effect of time or treatment × time was observed.

Feeding HSO did not affect either walk or trot SL (Table 4.1). Horses on both treatments tended ($P = 0.07$) to have a longer walk SL on d 35 compared to d 63. Walk SL was not significantly correlated with any IB measured (Table 4.2). Trot SL had a moderate inverse association ($P \leq 0.01$) with SF PGE2 levels ($r = -0.56$) and was moderately and positively ($P \leq 0.01$) associated with TNFα ($r = 0.58$), IL6 ($r = 0.61$), and IL1β ($r = 0.65$) in serum. Linear regression analysis (data not shown) was run to further investigate the relationship between walk and trot SL and IB. However, no significant relationships were observed.

Discussion

Ability to modulate inflammatory responses in horses using dietary omega 3 PUFA has been evaluated in several studies (Woodward et al. 2007; Manhart et al. 2009; Vineyard et al. 2010; Ross et al. 2012; Ross-Jones et al. 2016). GLA, an omega 6 PUFA, is purported to have

anti-inflammatory benefits, yet few studies have directly investigated its effect on inflammation in horses. Supplementation with *Buglossoides arvensis* oil (a source of GLA and SDA) reduced horse inflammatory status, as measured by AA:(EPA+DHA), but cytokines were not measured (McNiven and Rodriguez 2016). This, however, is the first study to evaluate the inflammatory response in horses supplemented with dietary GLA using HSO.

Serum IL1β was greater in horses receiving HSO compared to CON. This might be explained by the increased consumption of higher fat diets or diets with greater saturated fatty acids such as palmitic acid (PA) and stearic acid (SA). Increased fat intake can increase the expression of IB (Muñoz and Costa 2013; Tan and Norhaizan 2019). Likewise, increased saturated fatty acid intake is associated with increased IB (Huang et al. 2012; Ulven and Holven 2020). Diets were isocaloric, but HSO horses did receive more calories from fat and saturated fatty acids as compared to CON, which may have influenced fat deposition and accumulation. Increasing adipose stores is associated with increased IL1β (Sterling et al. 2022). Horse body condition score (BCS) did not differ between treatment diets or throughout the study, but minor composition differences, not enough to influence BCS, may have been enough to influence the inflammatory response.

Alternatively, hempseed harvest and processing conditions may have indirectly affected the inflammatory response. Hemp is an indeterminate plant, with uneven ripening. To reduce shattering losses, harvest generally occurs before all seeds are ripe (Ely and Fike 2022). However, the presence of immature seeds during harvest can result in increased presence of chlorophyll in the final HSO product which can in turn increase FA oxidation and degradation, resulting in lower quality HSO (Liang et al. 2015). However, we did not measure HSO oxidation. Efforts were made to minimize HSO oxidation through proper storage at 4^{o}C but it is

possible that oxidation still occurred. Overall, the complexity of factors that can influence IL1β response along with the lack of treatment x day interaction suggests that another factor could be contributing to the IL1β response since all horses were consuming the same diet from d 36-63.

The influence of time, but not treatment, on serum TNFα could be attributed to farm activities during the study. Activities included mares foaling and then subsequent weaning in the adjacent pasture to where this study's geldings were housed. Additionally, another study was run concurrently with the beginning of period 1, in which other horses were walked past the geldings on a daily basis contributing to stress and excitement. Stress results in elevated cortisol (not measured in this study), which is a potent inhibitor of TNFα (DeRijk et al. 1997). Therefore, horses may have been stressed resulting in lower TNFα concentrations at earlier vs later timepoints.

The lack of response in SL, SF and serum PGE2, and serum IL6 and TNFα is not unsurprising since the horses in this study were not subjected to an inflammatory challenge and no preexisting inflammatory conditions were identified. Similar findings were observed in horses supplemented with omega 3 in which serum TNFα did not differ in unchallenged horses (Schauermann 2010). Likewise, no effect of EPA and DHA on serum PGE2 or TNFα was observed in exercising horses supplemented with dietary algae (Parsons 2011). Responses to such supplements are not consistent, however.

Fikes et al. (2021) evaluated two supplement rates of bioactive proteins (40 g and 80 g) on SL and inflammatory cytokines in serum. TNFα did not differ between treatments and control, however, IL1β remained unchanged in horses supplemented at 80 g but increased in response to an exercise challenge in both untreated horses and horses supplemented with 40 g. This suggests that the greater supplementation rate was sufficient to attenuate the increase in

IL1β but not TNFα. In another study, plasma PGE2 was decreased in horses, previously diagnosed with arthritis in one or more joints, when supplemented with omega 3 FA (Manhart et al. 2009). Cytokines are short-lived messengers that are rapidly cleared from the body, therefore, the lack of dietary influence is not unsurprising (Zahn and Greischel 1989; Kudo et al. 1990).

The tendency for time but not treatment to influence walk SL could be due to a few different possibilities. Horses used in this study were not regularly worked in hand and were inconsistent in their responses to video data collection data. Additionally, the surface used to walk and trot the horses was not completely smooth and could have influenced horse perception and therefore foot placement. In an attempt to account for this, multiple videos were captured at each gait and the results averaged. The video location was also outside on an active farm and distractions were unavoidable. These distractions increased alertness and disrupted focus in the horses, which could have contributed to the inconsistent response.

While correlation analysis only speaks to an association between two variables, it still provides valuable information regarding future directions. SF PGE2 was inversely associated with trot SL, such that as PGE2 increased trot SL decreased. This is a logical association since elevated PGE2 in SF is a key indicator of joint inflammation (Ross-Jones et al. 2014) which contributes to reduced SL (Toutain and Cester 2004). However, the positive correlations between trot SL and serum TNFα, IL6, and IL1β are opposite of what would be expected. Correlations were only evaluated on d 35 and d 63 therefore data may not have been truly representative. Additionally, cytokines in serum may not be representative of joint inflammation. This work demonstrates the continued need for research evaluating PUFA and inflammation in horses.

Conclusions

Supplementary HSO did not alter SL, SF PGE2, or serum PGE2, TNFα, or IL6 but counter to expectation, it increased IL1β. Lack of treatment x time interaction for cytokines suggests the inflammatory response to dietary PUFA is more complex than a simple dose-response relationship. Further investigation into the effects of dietary PUFA with varying supplemental rates is warranted given the issues of inflammation both for working and sedentary horses.

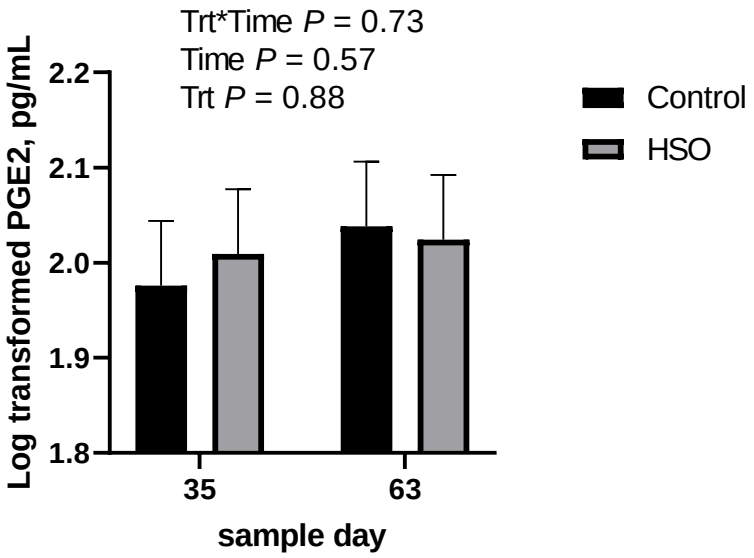

Figure 4.1. Log transformed mean concentration of PGE2 in synovial fluid.

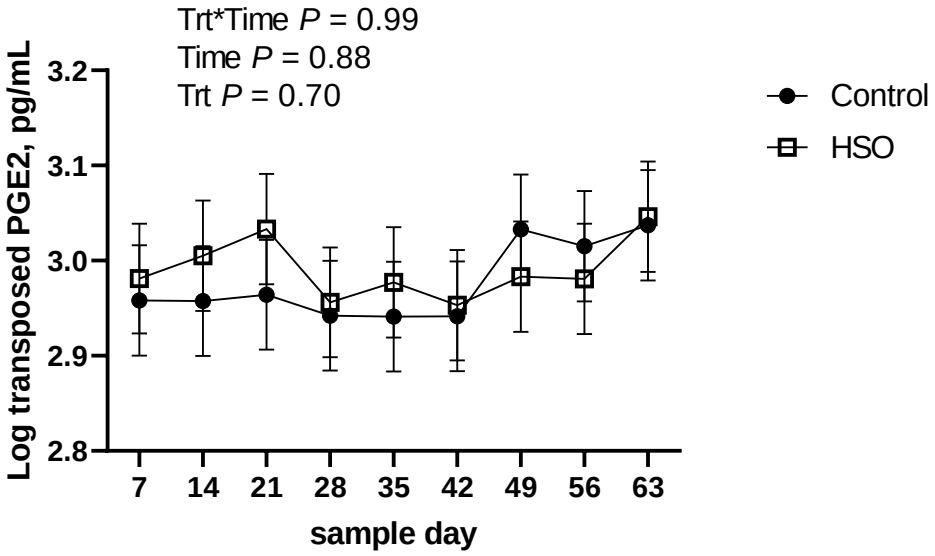

Figure 4.2. Log transformed mean concentrations of PGE2 in serum.

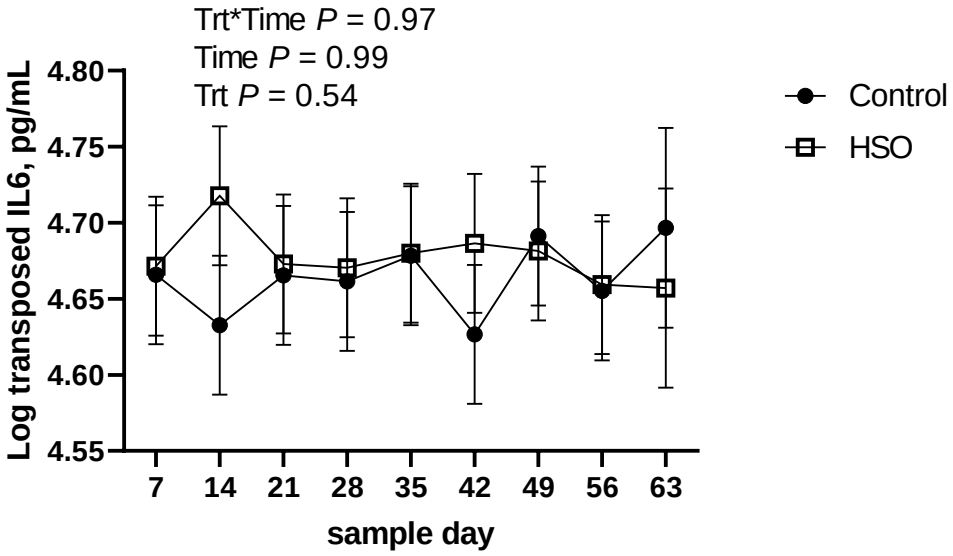

Figure 4.3. Log transformed mean concentration of IL6 in serum.

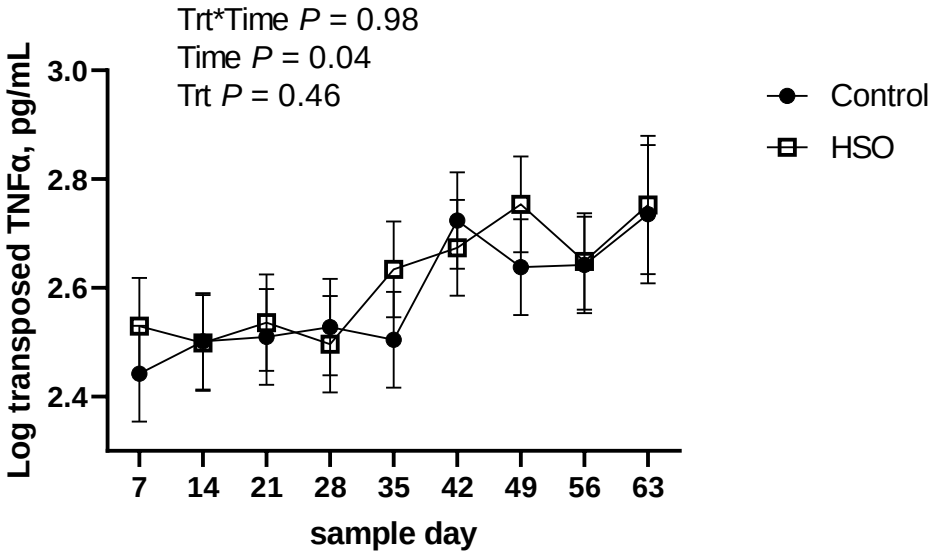

Figure 4.4. Log transformed mean concentration of TNFα in serum.

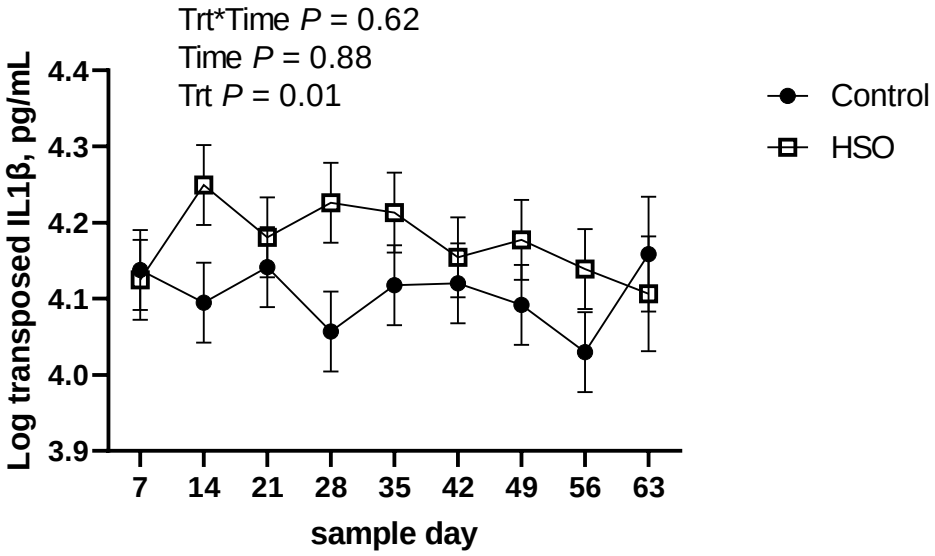

Figure 4.5. Log transformed mean concentration of IL1β in serum.

Table 4.1. Effect of hempseed oil on stride length at both the walk and trot.

Gait	Treatment	Stride length (cm)		SEM	*P*-values		
		d 35	d 63		Trt	Time	Trt*Time
Walk	Control	191.0	184.5	3.1	0.94	0.07	0.92
	HSO	190.4	184.6	3.1			
Trot	Control	228.1	227.0	7.3	0.52	0.64	0.74
	HSO	225.6	219.7	7.3			

Table 4.2. Correlation analysis between walk and trot stride lengths with serum and synovial fluid inflammatory biomarkers.

Stride measurements	Inflammatory biomarkers				
	SF[1] PGE2	Serum PGE2	Serum TNFα	Serum IL6	Serum IL1β
Walk	-0.03	0.03	0.01	-0.01	-0.04
Trot	-0.56*	-0.12	0.58*	0.61*	0.65*

[1]Synovial Fluid
*$P \leq 0.01$

Chapter 5

Cannabinoid concentrations in plasma and synovial fluid following 35 days of hempseed oil supplementation in horses.

Abstract

Interest in industrial hemp (*Cannabis sativa L.*) use in equine diets is based on its favorable nutritional profile, however, concerns over accumulation of cannabinoid contamination limit its acceptance. The goal of this study was to evaluate if residual cannabinoids acquired during processing in hempseed oil accumulate in blood or synovial fluid in horses. Six Thoroughbred geldings (11 ± 1.3 yr) were used in a crossover study with two 63-d periods. Horses were maintained on 0.4-ha lot with restricted access to grass and ad libitum access to water and white salt. Treatments included a basal diet of 0.5% BW concentrate (CON) or 168 mL HSO delivering 5 g GLA (HSO) top-dressed on the basal diet reduced by the caloric equivalent. All horses were offered 1.3% BW grass hay. Horses were fed their respective diets for the first 35 d of each period and then all horses resumed the CON diet for remainder of each period. Blood samples were obtained weekly and synovial fluid was collected on d 0, d 35 and d 63 of each period. Plasma and synovial fluid were analyzed for cannabinoids using high performance liquid chromatography coupled with mass spectrometry with a limit of detection of 50 ppb. Horses consumed 14.0 mg of total cannabinoid content daily. Cannabinoids were not detected in plasma or synovial fluid at any time point. The low intake level of cannabinoids due to residual contamination in the HSO was not enough to cause accrual in plasma and synovial fluid when supplemented for 35 d.

Introduction

Since its legalization in the 2018 Farm Bill, industrial hemp (*Cannabis sativa L.*) has garnered a significant amount of interest in part due to its nutritional profile. Hempseed contains ~30% fat, of which ~80% is polyunsaturated (PUFA) (Farinon et al. 2020). Furthermore, hempseed oil (HSO) has an omega 6:3 ratio of 3:1 and contains unique fatty acids γ-linolenic acid (GLA) and stearidonic acid (SDA). These FA are not found in other commodity seed oils as those species lack the enzymes required to add the appropriate double bonds (Gurr et al. 2016; Farinon et al. 2020). Purported benefits of dietary GLA and SDA include reducing inflammatory mediators by bypassing slow conversion steps of precursor PUFA and increasing the production of anti-inflammatory eicosanoids (Kapoor and Huang 2006).

Interest in HSO use in equine diets is focused on its value as both an energy and PUFA source; however, concerns exist regarding potential accumulation of cannabinoids acquired during processing. Hempseed products currently are not allowed in equine feeds partially based on lack of safety data associated with cannabinoid contamination. Many equine sports authorities (e.g., USEF, FEI, AQHA, etc.) have placed cannabidiol (CBD) and Δ9-tetrahydrocannabinol (THC) on the list of banned medical interventions with a zero-tolerance threshold (AQHA 2019; USEF 2019; FEI 2022). Concerns exist due to the reported anxiolytic effect of cannabinoids and its potential influence on performance (AQHA 2019; USEF 2019).

Most of the recent literature on industrial hemp consumption in livestock has focused on feeding ruminants whole plant silage or plant residues from CBD extraction as the feedstuffs which would have potential for significant cannabinoid intake. However, concern involving cannabinoids (e.g., THC, CBD, etc.) in oilseed-based feed ingredients is largely misplaced since cannabinoids are not directly produced within the hemp achene (more commonly referred to as

seed). Rather, cannabinoids are excreted from trichomes located on leaves and bracts (specialized leaves that contain the seed as it matures; Leme et al., 2020). Trichomes excrete cannabinoids as a sticky, resinous material that can contaminate the achenes and equipment during harvesting and processing. As the cannabinoids primarily adhere to the pericarp, and hempseed oil is commonly extracted by an extrusion process, the presence of cannabinoid contaminants in the end product is typically negligible (Farinon et al. 2020). However, because of the existing federal restrictions, research is needed to determine what, if any, cannabinoids accumulate in tissues when HSO is consumed. To our knowledge, no studies exist in the literature directly evaluating HSO in equine diets. Therefore, the objective of this study was to evaluate if residual cannabinoids acquired during processing are absorbed and transferred to blood or synovial fluid in horses fed HSO for 35 d.

Materials and Methods

Use of animals for this experiment was approved by the Virginia Tech Institutional Animal Care and Use Committee. Details for this experiment are previously described at length in Chapter 3. Briefly, six Thoroughbred geldings (11 ± 1.3 yr; 567 ± 11 kg) were used in a cross-over design with two consecutive 63-d periods. Treatments included a basal diet of 0.5% BW concentrate (CON) or 168 mL hempseed oil delivering 5 g GLA (HSO) top-dressed on the basal diet reduced by the caloric equivalent fed for 35 d. HSO was removed and horses resumed the basal diet from d 36 to 63. All horses were offered 1.3% BW cool-season grass hay throughout the study. Horses were maintained on 0.4 ha lot, mowed weekly to 2.54 cm, with ad libitum access to shelter, water, and white salt.

Sampling. Plasma samples were collected weekly and stored at -80^{o}C until analysis. Synovial fluid was collected on d 0 (baseline), d 35 and d 63 of each period and stored at -80^{o}C

until analysis. HSO was obtained from a single lot for the entirety of the study and kept at 4ºC to prevent spoiling. HSO subsamples were collected throughout the study and composited for cannabinoid analysis.

Cannabinoid analysis. Plasma and synovial fluid samples were analyzed by a commercial laboratory (Sativa Testing Laboratories, Richmond, VA) using high performance liquid chromatography coupled with mass spectrometry. The limit of detection (LOD) for plasma and synovial fluid samples was 50 ppb. Cannabinoids in HSO were analyzed by a separate laboratory (Eurofins Food Chemistry Testing Madison, Inc.; Madison, WI) using ultra-high performance liquid chromatography coupled with mass spectrometry with a LOD of 1 ppm.

Results

Cannabinoids detected in HSO are listed in Table 5.1. Total THC content, calculated as the sum of Δ9-THC and (tetrahydrocannabinolic acid (THCA) x 0.877), equaled 10.2 μg/g HSO. This is far below the required regulatory threshold (≤ 0.3%) that distinguishes industrial hemp from marijuana (Fike et al. 2020). Total cannabinoid content, calculated as: Total THC content + CBD + (cannabidiol acid (CBDA) x 0.877) + (cannabidivarin acid (CBDVA) x 0.877) + (cannabigerol acid (CBGA) x 0.877), equaled 83.2 μg/g HSO. Horses consumed 168 mL of HSO daily which equated to a daily cannabinoid intake of 14.0 mg and an average treatment "dose" of 0.025 mg cannabinoids/kg BW. Supplemental HSO did not lead to detectable cannabinoids in plasma or synovial fluid at any timepoint to a LOD of 50 ppb.

Discussion

Low levels of residual cannabinoid contamination are expected in hempseed and hempseed products, such as HSO, due to current seed processing methods. The commercially available HSO used for this study was representative of products currently available to the

consumer and contained residual cannabinoids albeit at low levels. Cannabinoid accumulation in horses has been evaluated using CBD itself directly supplemented (Draeger 2020; Turner et al. 2022; Yocom et al. 2022; Sánchez de Medina et al. 2023); however, this study is the first to explore the potential to measure cannabinoids in horses following very low-level dietary intake via HSO.

Cannabinoids are highly lipophilic but have poor oral bioavailability, estimated to be as low as 6% in humans (Lucas et al. 2018). Bioavailability of cannabinoid extracts directly supplemented in horses face similar constraints, with values ranging between 7% and 14% (Turner et al. 2022; Sánchez de Medina et al. 2023). Similar to observations of reduced oral therapeutics absorption in horses compared to other species, it is proposed that oral CBD formulations be specifically designed for use in equine to increase efficacy (Rosa 2020).

Residual cannabinoid intake from dietary supplementation of HSO in this study was much lower than the current literature. CBD supplementation rates in the literature range from 0.09 - 10 mg/kg BW in horses (Draeger 2020; Ryan et al. 2021; Turner et al. 2022; Yocom et al. 2022; Leise et al. 2023; Sánchez de Medina et al. 2023). Supplementing 0.12 mg CBD/kg BW for 28 d (in a sesame oil carrier) did not lead to detectable levels of cannabinoids in horses; however, the analysis LOD in this study was not reported (Leise et al. 2023). Cannabinoid concentrations were detected in horses supplemented with 0.35 mg CBD/kg BW but quickly fell below the assay's limit of quantification (LOQ). It should be noted that results for this study also included measurable values for THC. However, THC in the supplement was reported as being below the LOQ and THCA, the active form of THC, was not reported which may have influenced the amount of THC observed in plasma samples (Williams et al. 2022)

Horses supplemented with 50 mg cannabinoids delivered in either a carrier oil or a pelleted supplement resulted in detectable cannabinoid concentrations in serum for both treatments (Draeger 2020). Cannabinoids were detected in horses treated with an average dose of 0.09 mg cannabinoids/kg BW; however, the author also reported detectable cannabinoid concentrations at time 0 prior to treatment administration. Therefore, the presence of false positives cannot be eliminated (Adamchik 2023). Horses dosed with CBD (1 or 3 mg/kg BW in a sunflower oil carrier) demonstrated that more consistent, detectable CBD concentrations in synovial fluid were achieved with the higher dose (Yocom et al. 2022). Turner et al. (2022) indicated that 2 mg CBD/kg BW was selected over 1 mg CBD/kg BW based on better detection at the higher dose concentrations. Guinea pigs and horses, both hindgut fermenters, reach lower CBD plasma concentrations than dogs and humans suggesting it may be necessary to supplement these species at higher doses to reach therapeutic potential (Bartner et al. 2018; Spittler et al. 2021; Yocom et al. 2022; Sánchez de Medina et al. 2023; Schwark and Wakshlag 2023).

Cannabinoid contamination in HSO is a concern because of the potential impacts on health and performance. As mentioned above, no studies have evaluated potential transfer of cannabinoids to horses via dietary HSO, and the possible health consequences; thus, the only comparisons that can be drawn come from studies in which CBD products were fed. Horses administered an oral CBD-infused oil at 1 mg/kg BW for 6 weeks had elevated serum gamma-glutamyl transferase (GGT) concentrations, an indicator of potential liver damage. Yet, a negative control (no CBD) was not included and horses in that same study receiving 3 mg CBD/kg BW did not have elevated serum GGT concentrations (Yocom et al. 2022). However, no behavioral changes were observed at either treatment level. Leise et al., (2023) observed

greater GGT concentrations in horses supplemented with 0.75 mg CBD/kg BW. Yet, no negative effects on GGT were noted in horses dosed with 2 mg CBD/kg BW (Turner et al. 2022).

Horses tolerate dietary fat intakes up to 230 g fat/kg dry matter (DM) before adverse effects on fiber digestion are observed (Kronfeld et al. 2004). However, adding additional dietary fat above 450 mL (~2 cups) of oil daily is typically not recommended to avoid digestive disturbance; though, feeding programs will vary since individual horses may respond differently. This study utilized a treatment of 168 mL HSO (~3/4 cup), well within daily recommendations, which provided a daily intake of 14.0 mg of total cannabinoids. Even if a horse consumed 450 mL HSO, cannabinoid intake would only equal 37.4 mg, which would equate to 0.075 mg CBD/kg BW daily intake for an average 500 kg horse. Such levels are below the amount required to induce detectable CBD concentrations in plasma when supplementing CBD-based products.

Conclusions

The low concentration of residual cannabinoids in HSO from processing were not enough to accumulate detectable levels (50 ppb) in plasma or synovial fluid during a 63-d feeding trial. Cannabinoid consumption can lead to these compounds accruing in blood and synovial fluid, however, the threshold for biological relevance remains to be determined. Additionally, consistent testing methods and acceptable LOD reporting should be developed to accurately compare studies.

Table 5.1. Cannabinoid concentrations in hempseed oil.

	Hempseed Oil (μg/g)
Δ8-tetrahydrocannabinol (Δ8-THC)	<1.00
Δ9-tetrahydrocannabinol (Δ9-THC)	2.62
Tetrahydrocannabinolic acid (THCA)	8.67
Tetrahydrocannabivarin (THCV)	<1.0
Tetrahydrocannabivarin acid (THCVA)	<1.0
Cannabicyclol (CBL)	<1.0
Cannabichromene (CBC)	<1.0
Cannabidiol (CBD)	9.92
Cannabidiol acid (CBDA)	67.7
Cannabidivarin CBDV	<1.0
Cannabidivarin acid (CBDVA)	2.89
Cannabigerol (CBG)	<1.0
Cannabigerol acid (CBGA)	1.34
Cannabinol (CBN)	<1.0
Cannabinol acid (CBNA)	<1.0
Total THC Content[1]	10.2
Total Cannabinoid Content[2]	83.2

[1]sum of THC + (THCA x 0.877)
[2]sum of Total THC + CBD + (CBDA x 0.877) + (CBDVA x 0.877) + (CBGA x 0.877)

Chapter 6

Summary

The purpose of this research was to evaluate the metabolism of the polyunsaturated fatty acids (PUFA) found in hempseed oil (HSO) fed to sedentary horses and their effect on inflammatory parameters. This is the first study to evaluate HSO fed to horses in the literature, therefore, it is also the first to evaluate the relationship between the PUFA profile of dietary HSO and inflammation in the horse. Additionally, this work is the first to determine whether residual cannabinoid contaminants in HSO are measurable in horses fed this supplement.

The first study documented the accumulation, metabolism, and clearance of dietary PUFA supplemented from HSO in serum, synovial fluid, and skeletal muscle over time. Results agreed with the current literature that circulating PUFA reflects dietary intake. Increased intake of γ-linolenic acid (GLA) led to increases in GLA and dihomo-γ-linolenic acid (DGLA) in serum and synovial fluid without further increases to arachidonic acid (AA). Muscle GLA and DGLA were not influenced by treatment, but it is possible the supplementation length and rate were not sufficient to induce alterations. The implication for these results is the potential for reduced inflammatory response. In addition, this study demonstrated that the accumulation and clearance timeframe of PUFA in serum and synovial fluid can be influenced more rapidly than previously thought. PUFA in horses reached a steady state by three weeks of supplementation and returned to baseline by two weeks post-supplementation. The impact of this could reduce required study lengths but it also provides guidance on feeding applications of HSO to see alterations. However, further work is needed to elucidate the biological relevance of the associated changes in fatty acids, as well as identify ideal supplementation rates for cellular incorporation.

The second study evaluated the effects of HSO on inflammatory biomarkers and stride length. In the absence of an inflammatory challenge, an effect was not totally apparent. The only

inflammatory biomarker effected by HSO treatment was Interleukin-1β (IL1β) in which HSO-supplemented horses had greater concentrations than CON. The reason for this is not fully apparent but may be related to greater dietary fat intake or increased saturated fatty acids from dietary fat consumption. Additionally, hempseed harvesting methods may have influenced HSO quality. However, the effect was seen during the post-supplement period as well, when HSO was removed, and all horses were consuming the same diet. This may indicate that another factor was contributing to those results. Even with the lack of treatment effect, correlations between stride length and inflammatory cytokines warrant further evaluation with an inflammatory challenge incorporated.

The third study evaluated the transfer of low-level cannabinoid contaminants from dietary HSO to the horse. Although cannabinoids were measured in the HSO supplement, no cannabinoids were detected in plasma or synovial fluid when tested to a 50-ppb limit of detection. Concerns over cannabinoids are the biggest hinderance for a greater acceptance of hempseed and hempseed products in equine diets. The results of this work provide the preliminary data necessary for establishing acceptable levels of cannabinoid contaminants in hempseed products.

This study raises a number of opportunities for future work in understanding the effects of altering dietary fatty acids. Ideal FA ratios have not been established in the horse and while many claim that diets should reflect FA profiles in forages, few studies demonstrate a benefit. Future work should focus on long term effects of feeding GLA from HSO and at varying supplementation rates in the diet. GLA is not commonly found in the diets of mature horses and the true effect on cellular metabolism is not well characterized. Additionally, HSO should be compared to common FA supplements (e.g., soybean oil, flaxseed oil, ahiflower oil, fish oil, etc.)

to determine how varied dietary FA ratios influence the metabolism of the different FA at a cellular level. Modifying dietary PUFA ratios may influence the inflammatory response but to develop dietary recommendations for horses intended to elicit those changes requires further work.

Ultimately, this research demonstrates the value of HSO as a novel source of PUFA. Additionally, since federal regulations restrict the use of hempseed and HSO in equine feeds, this work provides preliminary evidence for the safe and efficacious use of HSO as a feed supplement for horses and provides an initial basis for federal approval. These results indicate that HSO is a suitable source of GLA, resulting in increased DGLA without further increasing AA in sedentary horses. However, the implications for its effect on inflammation require further evaluation.

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