THESIS

EFFECTS OF TWO DIFFERENT DIETARY SOURCES OF OMEGA-3 HIGHLY UNSATURATED FATTY ACIDS ON INSULIN SENSITIVITY, AND INCORPORATION INTO THE PLASMA, RED BLOOD CELL, AND MUSCLE CELL IN HORSES

Submitted by

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WE HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER OUR SUPERVISION BY JILL KRISTEN REXFORD ENTITLED EFFECTS OF TWO DIFFERENT DIETARY SOURCES OF OMEGA-3 HIGHLY UNSATURATED FATTY ACIDS ON INSULIN SENSITIVITY, AND INCORPORATION INTO THE PLASMA, RED BLOOD CELL, AND MUSCLE CELL IN HORSES BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE.

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ABSTRACT OF THESIS

EFFECTS OF TWO DIFFERENT DIETARY SOURCES OF OMEGA-3 HIGHLY UNSATURATED FATTY ACIDS ON INSULIN SENSITIVITY, AND INCORPORATION INTO THE PLASMA, RED BLOOD CELL, AND MUSCLE CELL IN HORSES

Introduction: Dietary supplementation of n-3 highly unsaturated fatty acids may lead to incorporation into cell membranes throughout the body in humans (Lund et al., 1999) and rats. Incorporation of long chain n-3 fatty acids may increase the fluidity and unsaturation of the membrane, decrease the amount of muscle triglycerides and may improve insulin sensitivity (Oakes et al., 1997; Storlien et al., 1996; Wilkes et al., 1998). The objective of this study was to evaluate the effects of two different dietary sources of n-3 polyunsaturated fatty acid on insulin sensitivity, and the incorporation into the plasma, red blood cell, and muscle cell in horses.

Methods: Twenty one mares were blocked by age, body condition score, and weight and randomly assigned to one of three dietary treatment groups. Treatments consisted of: 1) (FISH) supplemented at 142.4 mg/kg of body weight, where eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) supplied at 58.6 mg/kg of body weight of n-3 fatty acids via a commercial marine source supplement

(Magnitude™; JBS United, Sheridan, IN) plus the basal ration. 2) (FLAX) supplemented at 149.8 mg/kg of body weight of the n-3 fatty acid α-linolenic acid (ALA) via a flaxseed supplement (Nutra-Flax™) plus the basal ration, 3) (CONT) did not receive additional n-3 fatty acid supplementation aside from ALA that was present in the basal ration comprised of hay and barley. Horses were supplemented for 90 days. Horses were weighed and scored for body condition on the week before blood samples were taken. Body weights, body condition scores (BCS), plasma, red blood cells, muscle biopsies, and frequently sampled intravenous glucose tolerance tests (FSIVGTT) were performed on days 0, 30, 60 and 90 of supplementation. Plasma, red blood cells, and muscle cells were analyzed for fatty acid concentrations using a lipid extraction procedure and gas chromatography. Plasma glucose and insulin values from the FSIVGTT were analyzed using enzymatic assay and radioimmunoassay, respectively. Glucose and insulin curves were analyzed using MinMod Millenium software. Statistical differences ($P < 0.05$) were determined with a repeated measures design using the PROC MIXED ANOVA procedure of SAS.

Results: Overall FISH horses had lower plasma linoleic acid (LA) and alpha-linolenic acid (ALA) concentrations versus FLAX and CONT horses ($P < 0.002$). Arachidonic acid (ARA) concentrations in the plasma was higher in the FISH horses versus the FLAX and CONT horses ($P < 0.002$), with time it increased across all treatment groups from day 30 to 60 ($P < 0.007$) and decreased from day 60 to 90 ($P < 0.008$). Eicosapentaenoic acid and DHA were only detectable in the plasma of the FISH horses, where EPA increased from day 30 to 60 ($P < 0.002$) and DHA increased from day 30 to day 90 ($P < 0.005$). Red blood cell LA was lower in the FISH horses compared to

the FLAX and CONT at day 90 ($P < 0.020$). Red blood cell ALA was not significantly different among treatments, but increased from day 30 to 60 ($P < 0.001$) and decreased from day 60 to day 90 ($P < 0.001$). Red blood cell ARA concentrations were higher in the FISH group compared to the FLAX or CONT groups ($P < 0.002$). Red blood cell EPA and DHA were only detected in the FISH horses from day 30 on, where EPA increased from day 30 to 60 ($P < 0.001$), and DHA increased from day 30 to 90 ($P < 0.009$). Muscle LA was lower in the FISH group at day 60 and 90 ($P < 0.040$) and was also lower in the FISH group when compared to the FLAX and CONT across all months ($P < 0.001$). Muscle ALA was lower in the FISH group compared to the FLAX and CONT groups ($P < 0.004$), and the FLAX and CONT groups did not differ ($P = 0.94$). Muscle EPA was significantly higher in the FISH group than the FLAX and CONT at day 60 and 90 ($P < 0.001$). Muscle DHA was significantly higher in the FISH group compared to the FLAX group at day 30, 60 and 90 ($P < 0.020$), and the FLAX and CONT were not different at any of these sampling points ($P < 0.55$). Analysis of the minimal model parameters revealed that the acute insulin response to glucose (AIRg) was not affected by treatment and increased from baseline to day 30, decreased to day 60, and increased to day 90 ($P < 0.020$). Insulin sensitivity (SI) was not different among treatments as well, and increased from day 30 to day 90 ($P < 0.005$). In addition, glucose effectiveness (Sg) was not affected by treatment, and decreased from day 30 to 60 ($P < 0.050$), and increased from day 60 to 90 ($P < 0.020$). Disposition index (DI) did result in a treatment by time interaction ($P < 0.030$), only at day 90 where the FLAX group was greater than the CONT ($P < 0.007$), and the FISH group was not significantly different from the FLAX or

CONT ($P < 0.34$). A negative correlation was found between SI and BCS ($r = -0.381$, $P < 0.003$).

Discussion: Direct supplementation of EPA and DHA significantly increased the equivalent fatty acid concentrations found in the plasma, red blood cell, and muscle cell of equines compared to controls in this study. This study found that insulin sensitivity did not vary between treatments, but its effects could have been confounded with BCS changes, indicating that more closely controlled studies are needed.

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Chapter I

Introduction

There is concern among members of the equine industry with the occurrence of insulin resistance in horses because it has been linked to the development of laminitis, osteochondrosis, and metabolic syndrome (Coffman and Colles, 1983; Ralston, 1996; Treiber et al., 2006b). These diseases can result in a loss of function and performance of the horse. Several factors may predispose a horse to developing a decreased sensitivity to insulin, such as diet, age, breed/genetics and obesity (Jeffcott et al., 1986; Treiber et al., 2006; Vick et al., 2007). Supplementation with certain diets could increase insulin sensitivity in animals that have lower than normal sensitivity or insulin resistance.

The benefit of dietary supplementation with n-3 highly unsaturated fatty acids has been shown to increase insulin sensitivity in previous studies (Behme, 1996; Luo et al., 1996). This may be due in part to an increase in the membrane fluidity from an increase in the unsaturation of the membrane by the incorporation of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) and in consequence improving glucose transport function (Lardinois, 1987; Zhao et al., 2008).

This research focuses on the supplementation of a plant source of highly unsaturated fatty acids containing ALA, and an animal source of highly unsaturated fatty acids containing EPA and DHA, and its effects on the incorporation into the plasma, red blood cell, and muscle cell, and insulin sensitivity. Horses may have a poor conversion efficiency of α-linolenic acid (ALA) to its long-chain derivatives EPA and DHA, so it could be beneficial to directly supplement EPA and DHA through an animal (marine) source. Direct supplementation of EPA and DHA may show an increased incorporation

of those fatty acids into cell membranes, and may increase the animal's insulin sensitivity.

Chapter II

Literature Review

Fatty Acids

Definition and Biological Importance:

A fatty acid is a carboxylic acid with an unbranched, aliphatic tail, which can be divided into one of two classes: saturated or unsaturated. They are used for energy production in cells, and are part of membrane phospholipids. Fatty acids are also grouped as short, medium, or long chain according to the number of carbons in their hydrocarbon chain. A saturated fatty acid contains no double bonds, and as a result is saturated with hydrogen. Unsaturated fatty acids contain one or more double bonds, reducing the number of hydrogen molecules on each carbon. Unsaturated fatty acids with one double bond are referred to as monounsaturated, and fatty acids containing two or more double bonds are referred to as being polyunsaturated.

The parent fatty acids of the n-3 series, α-linolenic acid (ALA), and the n-6 series, linoleic acid (LA), are essential fatty acids, meaning that they are unable to be synthesized de novo by vertebrates (Lands, 1992). Therefore, these fatty acids need to be consumed in the diet. The n-3 and n-6 fatty acid series are essential in growth and development throughout the life cycle, such as normal development of the brain and retina in newborns, and are especially important for premature infants (Simopoulos, 1991). In addition, fatty acids are responsible for the formation of pro-inflammatory eicosanoids and anti-inflammatory resolvins and protectins (Arterburn et al., 2006). Another function of these fatty acids is their role in the structure of the cell phospholipid membrane. Differing levels of incorporation of fatty acids into the membrane have been

shown to change its fluidity (Zhao et al., 2008), improving insulin receptor function, and in turn may increase insulin sensitivity (Lardinois, 1987).

Structure:

The n-3 or n-6 (omega-3 or omega-6) polyunsaturated fatty acid nomenclature refers to the first double bond being three or six carbons in from the terminal methyl carbon counting towards the carboxylic acid end. These polyunsaturated fatty acids (PUFAs) can be converted into long-chain highly unsaturated fatty acids by specific enzymes such as elongase, which adds two carbons to the fatty acid backbone, or desaturase, which inserts double bonds into the molecules. The n-3 series includes the parent fatty acid ALA, and its long chain derivatives EPA and DHA. The n-6 series includes the parent fatty LA, and its most common long chain derivative ARA. The n-3 and n-6 series share the same elongase and desaturase enzymes to convert the parent fatty acids to their long chain derivatives, and these pathways can be seen in Figure 2.1.

Sources of fatty acids in the diet:

A source of α-linolenic acid in the equine diet is hay, or pasture, which is the largest portion of equine rations. Many horses are fed hay or pasture alone, however, performance horses having greater energy needs will require additional energy-dense feeds such as grains or oils. Vegetable oils are also a source of ALA, such as flaxseed and linseed oil, which can be top-dressed onto feed. Linoleic acid is very abundant in cereal grains, such as corn and barley and makes up the majority of the fat in corn oil as well. Algae produce the long-chained n-3 fatty acids EPA and DHA, therefore they are present in fish because algae is an ordinary diet of fish. For that reason, marine foods and fish oil

are a good source of EPA and DHA and can be consumed by humans and other carnivores or omnivores and additionally, have been used as supplements in equine diets.

Digestion and Absorption:

Dietary fat is ingested, and the fat may be slightly hydrolyzed in the stomach, but most of the digestion and absorption occurs in the small intestine. The lipids that exit the stomach enter the upper intestinal tract where secreted bile emulsifies the lipid droplets. The lipid droplets are then available for hydrolysis by pancreatic lipase at the one and three position. The result of this hydrolysis is two free fatty acids and a glycerol backbone with one fatty acid still attached. These free fatty acids and the backbone with one fatty acid form a micelle, which has bile salts surrounding the outer layer. Micelles fit into the intramicrovillus spaces of the intestinal wall, which allows the contents to transfer into the enterocyte. Resterification of the lipid products occurs in the enterocyte, and then they are transported through the lymphatic system into the bloodstream, mostly as chylomicrons or very low density lipoprotein (VLDL). It has been shown in humans that dietary highly unsaturated fatty acids are absorbed from the small intestine and may become incorporated into cell membranes throughout the body (Lund et al., 1999).

Functions of fatty acids:

Fatty acids have several functions throughout the body. One predominant function is the formation of eicosanoids. Eicosanoids are signaling molecules that control body systems, act as messengers in the central nervous system and regulate inflammation (Calder, 2001). Most eicosanoids are derived from arachidonic acid (ARA) and are pro-inflammatory. These include certain prostaglandins (PGE_2), thromboxanes (A_2), and leukotrienes (4-series). Other eicosanoids can be derived from the n-3 long-chain

derivative EPA, and are called resolvins and protectins (Serhan et al., 2008). Resolvins are considered to be anti-inflammatory in action. These include other prostaglandins (PGE_3), thromboxanes (A_3), and leukotrienes (5-series). There is competition between the n-3 series and the n-6 series when ARA competes with EPA as a substrate for cyclooxygenase and lipoxygenase in the eicosanoid pathway (Hansen et al., 2002). These two enzymes convert ARA to prostaglandins, thromboxanes, and leukotrienes, and also convert EPA to resolvins and protectins. It is proposed that when EPA is present in higher amounts than ARA, it will be preferentially directed in to the cell membrane phospholipids, making less ARA available as the substrate in the eicosanoid pathway (Belury et al., 1989; Nieves and Moreno, 2006). The pathway can easily be seen in Figure 2.1.

A cell membrane is composed of a lipid bilayer, which is a thin membrane composed of lipid molecules. The outer layer has a hydrophilic head facing outward and two hydrophobic tails that face inward. The inner layer has its two tails that face the two tails of the outer layer, and then its hydrophilic head faces inward towards the contents of the cell. The head group determines the chemistry of the bilayer, and is mostly composed of phospholipids, while sphingolipids, such as sterols (cholesterol), are a component as well. Lipid bilayers are involved in signal transduction because they house the integral membrane proteins. These proteins are signaling molecules that may be contained in a microdomain of the membrane called a lipid raft, and serve as the organizing center for the assembly of these signaling molecules, influence the trafficking of membrane proteins and membrane fluidity, and regulate receptor trafficking and neurotransmission (Korade and Kenworthy, 2008). Sphingolipids are unable to integrate well into the fluid

phospholipid bilayer, but can exist in the lipid raft of the plasma membrane. The raft's polar lipids contain mostly saturated fatty acyls (Simons and Ikonen, 1997; Stulnig et al., 2001).

Effects of Feeding Fatty Acids

Circulating Triglyceride Levels:

It has been proposed that given the excess accumulation of adipose tissue in rats consuming high n-6 fat diets, addition of n-3 fatty acids may be beneficial (Storlien et al., 1987). One beneficial effect is the decrease in the amount of circulating triglycerides, representing an individual who may not have a decreased sensitivity to insulin (O'Connor et al., 2007). Two studies concluded that supplementation with n-3 highly unsaturated long-chain fatty acids lowered plasma triglyceride levels in rats versus a diet of corn oil or saturated fatty acids (Luo et al., 1996; Storlien et al., 1991).

Interconversion:

One topic of investigation in human research is the possible low conversion efficiency of ALA to its long-chain derivative (Robinson et al., 2007), and it is proposed that horses may have a low conversion efficiency as well. As previously stated, ALA can be converted to its long chain derivative, EPA. For the final conversion to DHA, a β-oxidation reaction occurs after translocation to the peroxisome (Arterburn et al., 2006). The peroxisome is an organelle that houses membrane proteins and plays a role in fatty acid metabolism. Docosahexaenoic acid can also be retroconverted back to EPA in small amounts. In humans, the conversion of dietary ALA to EPA and DHA is estimated to be between approximately 0.3 % to 21 % for EPA, and up to 9 % for DHA (Burdge et al., 2002; Burdge and Wootton, 2002; Hussein et al., 2005). Dietary supplementation of EPA

and DHA has been shown to reduce the conversion of ALA to its long-chain derivatives (Burdge et al., 2003; Vermunt et al., 2000). It has also been reported that diets high in LA reduce the conversion of ALA to its long-chain derivatives in humans (Harper et al., 2006) by 40 % when LA concentrations increased from 15 g/d to 30 g/d over 12 days (Emken et al., 1994). In addition, exchanging LA for ALA in the diet has been shown to increase the conversion to EPA in humans, but these levels are not as high as those provided by direct supplementation of EPA (Goyens et al., 2006).

Incorporation and Influence on Membrane Fluidity and Insulin Sensitivity:

The n-3 and n-6 fatty acid series compete for incorporation into cell membranes. The n-3 fatty acids EPA, DHA, and the n-6 fatty acid ARA compete for the *sn*-2 position on membrane phospholipids (Serhan, 2005). It has also been shown that the cellular membrane composition is determined by the fatty acid content of the diet (Arterburn et al., 2006). The more highly unsaturated long chain fatty acids can have an influence on membrane fluidity by altering the physiochemical properties, which can then affect the activity of transmembrane proteins and increase vascular fluidity. This may in turn have a positive effect on the expression of the receptors of the cell (Ginsberg et al., 1991; Mueller and Talbert, 1988; Rajamoorthi et al., 2005). In horses a study (Portier et al., 2006) examined the effects of supplementation with antioxidants added with EPA and DHA red blood cell membrane fluidity in exercising horses. Increases in RCB EPA and DHA composition were found in treated horses after 3 weeks of supplementation. However, no effects on RCB membrane fluidity were found in supplemented horses compared to control. During an exercise test on the same group study RBC membrane

fluidity was reduced in both treatment groups, but supplemented horses appeared to have a delayed reduction.

Dietary EPA and DHA have been shown to alter lipid rafts and microdomains (Chapkin et al., 2008; Stillwell and Wassall, 2003; Stulnig et al., 2001). Stillwell and Wassall, 2003, noted that select highly unsaturated fatty acids can decrease the dispersing of proteins in cholesterol dependent microdomains, specifically because DHA is incompatible with cholesterol and sphingolipids attached to the outer leaflet raft of the plasma membrane bilayer, indicating that plasma membrane organization of the inner leaflet raft is modified by supplementation with DHA (Stillwell and Wassall, 2003). Another study measured in vitro the effects of EPA on the membrane rafts and found that EPA and DHA were enriched in the rafts when compared to rafts from the control group which were treated with stearic acid (C18:0) (Stulnig et al., 2001). In addition, the amount of unsaturation in the rafts from the EPA treated cells was 42 percent higher than rafts from the control treated cells (Stulnig et al., 2001). It is evident that an increase in the unsaturation of the membrane and the ratio of n-6 to n-3 fatty acids present may increase the fluidity of the membrane (Zhao et al., 2008).

Several studies have shown that dietary supplementation with n-3 fatty acids have elevated the concentrations of these n-3 fatty acids in the composition of different cell membranes in horses. Horses fed three different levels of n-3 fatty acids, which contained n-3 and n-6 fatty acids, specifically EPA and DHA, showed the most incorporation into the plasma when fed 40 g per day, as compared to the control or the 10 or 20 g/d dosages ($P < 0.05$) (King et al., 2008). Another study evaluated the effects of feeding fish oil on the serum fatty acid concentrations (O'Connor et al., 2007). Horses were fed either fish

oil or corn oil top dressed at 324 mg/kg of body weight onto their concentrate ration, with total ration n-3 fatty acids estimated to be 60 mg/kg of body weight. After 63 days horses in the fish oil group had higher levels of n-3 fatty acids ($P < 0.014$), including EPA, in the serum versus the corn oil group which had increased levels of n-6 fatty acids ($P < 0.05$). In addition, serum triglycerides were decreased in the fish oil group compared to the corn oil group ($P < 0.05$) (O'Connor et al., 2007). It has been shown that triglycerides are higher in individuals with a decreased sensitivity to insulin due to the increased concentration of circulating free fatty acids (Pan et al., 1997). An additional study (Hansen et al., 2002) examined the effects of flaxseed oil supplementation for 16 weeks on equine plasma fatty acid concentrations. Horses were fed timothy hay, an alfalfa-based pellet, or an alfalfa-based pellet with 10 % flaxseed oil added. It was found that the horses in the flaxseed oil treatment group had higher levels of n-3 fatty acids, including increased levels of EPA, but not DHA, above the two week acclimation period (Hansen et al., 2002), indicating limited conversion to the long-chain derivative DHA.

Erythrocyte membrane phospholipids have also been studied with respect to the supplementation of different sources of n-3 highly unsaturated fatty acids. One study observed the effects of vitamins supplemented to 99 human subjects, versus vitamins with additional fatty acids, ALA and DHA (Siener et al., 2010). Subjects who received the fatty acids in conjunction with the vitamins had significant increases in erythrocyte EPA concentration ($P < 0.001$). In addition, these same subjects had an increase in ALA and DHA at both weeks eight and 12 when compared to baseline ($P < 0.001$)(Siener et al., 2010). Although EPA was not supplemented in the study, it was presumed that the interconversion was present due to the rise in EPA concentrations of the erythrocytes.

This study supported previous research indicating that dietary intake of ALA in healthy adults increased the concentrations of erythrocyte EPA (Barcelo-Coblijn et al., 2008; Hussein et al., 2005). Another study surveyed the effects of human subjects consuming sunflower oil capsules (control; source of n-6 fatty acids), flax oil capsules, or fish oil capsules (Barcelo-Coblijn et al., 2008). Alpha-linolenic acid was present in all three capsules, and highly abundant in the flax oil capsules. The flax oil capsules were given to three different groups who received doses of 1.2 g, 2.4 g, or 3.6 g/d of flax oil. The fish oil capsules were given to groups in doses of 0.6 g, or 1.2 g, and the control group received 1 g/d of sunflower oil. Eicosapentaenoic acid concentrations in erythrocytes increased in the 2.4 and 3.6 g/d flax oil, and also in both fish oil groups. Both fish oil groups did show a greater increase in EPA concentrations when compared to the flax oil groups, and was also significantly higher in the group receiving the 1.2 g/d of fish oil than the 0.6 g/d group. Docosahexaenoic acid content in the erythrocyte only increased in groups who consumed the fish oil capsules. This lack of elevated DHA concentrations in the flax oil group indicated that the conversion of ALA to DHA and incorporation into red blood cell phospholipids was very low (Barcelo-Coblijn et al., 2008). An additional study conducted on hypercholesterolemic rats looked at the supplementation of EPA or DHA on the fluidity of erythrocytes and the effect on the fatty acid profile (Hashimoto et al., 2006). The results obtained were that the DHA fed rats had a positive correlation with membrane fluidity ($r = 0.39$), and the EPA and DHA fed rats had increased concentrations in the fatty acid profiles of the platelets (482 % and 175 %, respectively). This study concluded that supplementation with both long-chain highly unsaturated fatty acids increased their concentration in the membranes, and DHA transformed membrane

fluidity more effectively than EPA in some specialized dietary regimens such as hypercholesterolemia. Therefore, in that study, DHA was a more potent membrane fluidizer than EPA in rats (Hashimoto et al., 2006). Another study examined the effects of non fried fish or tuna ("fish intake") on the levels of EPA and DHA seen in human erythrocytes. The researchers concluded from the results that EPA and DHA content in red blood cells was reflective of the n-3 fatty acid intake (Sands et al., 2005). Another study was conducted in humans that monitored the effects of short-term or long-term supplementation with different doses of n-3 highly unsaturated fatty acids on the fatty acid composition (Palozza et al., 1996). The doses of n-3 fatty acids were 2.5 g/d, 5.1 g/d, and 7.7 g/d and contained 455 ± 55 mg of EPA and 395 ± 55 mg of DHA. After 180 days of treatment there were significant increases in EPA and DHA concentrations for all three doses in the membrane of erythrocytes, therefore supporting previous research that RBC unsaturation can be increased with fatty acid supplementation (Palozza et al., 1996). In another study (Vineyard et al., 2010) yearling horses with an encapsulated fish oil or ground flaxseed, at a rate of 6g of total n-3 fatty acids per 100 kg of body weight (2,346 g per day) for 70 days. Eicosapentaenoic acid and DHA concentrations in the red blood cell were not detectable before or during the study in the horses receiving the flaxseed or control treatment, and were only found in the horses fed the fish oil. Erythrocyte EPA and DHA increased to day 35 of supplementation, and DHA further increased to day 70 of supplementation ($P < 0.05$) (Vineyard et al., 2010).

Supplementation with EPA and DHA and its direct effects on the fatty acid composition of muscle membrane has not been widely reported. One study did indicate that the fatty acid composition of skeletal muscle phospholipids did reflect the fatty acid

composition of the diet in humans (Andersson et al., 2002), but many studies involving the muscle cell attempt to address the possibility of dietary n-3 supplementation improving insulin sensitivity, rather than just its incorporation into the membrane. It has been shown that an increase in the long-chain fatty acids EPA and DHA leads to an increase in membrane fluidity, the number of insulin receptors, and insulin action. (Lichtenstein and Schwab, 2000; Simopoulos, 1991). Highly unsaturated fats have a favorable effect on increasing insulin sensitivity when compared to saturated fats (Lichtenstein and Schwab, 2000). Other studies also indicate that an increase in the unsaturation of the muscle membrane and a decrease in the content of muscle triglycerides may improve insulin sensitivity in humans and rats (Martín de Santa Olalla et al., 2009; Oakes et al., 1997; Storlien et al., 1996; Wilkes et al., 1998).

Another beneficial effect of dietary supplementation with n-3 highly unsaturated fatty acids is an increase in insulin sensitivity. As discussed above, supplementation with n-3 highly unsaturated fatty acids may increase the vascular fluidity of membranes (Mueller and Talbert, 1988), which may in turn have a positive effect on insulin sensitivity by increasing the function of the receptors (Lardinois, 1987). One proposed action is that the long chain n-3 fatty acids are ligands for the nuclear receptors peroxisome proliferator-activated receptors (PPAR), which is involved in the control of glucose homeostasis because activation of a particular PPAR, PPARγ, results in insulin sensitivity (Picard and Auwerx, 2002). In addition, a study conducted in miniature pigs showed that those fed a diet supplemented with EPA and DHA increased the insulin sensitivity and glucose effectiveness compared to pigs fed a diet supplemented with corn

oil. It is apparent that long-chain highly unsaturated fatty acids have been shown to have an effect on insulin action in different species and should continue to be investigated.

Insulin

Definition, Function, and Biological Importance:

Insulin is an anabolic hormone that is essential to regulating the energy and glucose metabolism in the body. It is secreted by the beta cells of the islets of Langerhans in the pancreas. One of the many actions of insulin in the body is to decrease blood glucose concentrations. After a meal, a glucose bolus injection, or glycogen metabolism, blood glucose concentrations rise, and insulin is released to signal cells to allow glucose to enter and be utilized or stored. The process starts by glucose entering the beta cells in the pancreas through the glucose transporter GLUT-2. Glucose enters glycolysis and the TCA cycle which produces adenosine triphosphate (ATP). Adenosine triphosphate controlled potassium channels close and the cell membrane depolarizes, which opens voltage-gated calcium channels, allowing calcium to enter the cell. This increased level of calcium activates phospholipase C, which cleaves the membrane phospholipid phosphatidyl inositol 4,5-bisphosphate into inositol 1,4,5-triphosphate (IP3) and diacylglycerol. Inositol 1,4,5-triphosphate (IP3) binds to receptor proteins in the endoplasmic reticulum (ER), which allows the release of calcium from the ER through IP3 gated channels, and in turn increases the calcium concentration of the cell even further (Wilcox, 2005). Substantially increased amounts of calcium in the cells causes the release of previously synthesized insulin, which has been stored in secretory vesicles. Insulin is released through circulation to its target organs where it then binds to its receptor on the cell surface and triggers a cascade of events.

After insulin binds to its receptor on the surface of the cell membrane, there is autophosphorylation, and activation of tyrosine kinases, which result in tyrosine phosphorylation of insulin receptor substrates (IRS's)(DeBosch and Muslin, 2008). The binding of IRS's to the regulatory subunit of phosphoinositide 3-kinase (PI3K) results in activation of PI3K, which then phosphorylates membrane phospholipids and phosphatidylinositol 4,5-bisphosphate (PIP2)(DeBosch and Muslin, 2008). This complex then activates the 3-phosphoinositide-dependent protein kinases, PDK-1 and PDK-2, which results in activation of protein kinase B (PKB) and atypical protein kinase C lambda (PKCλ), which is a serine/threonine kinase (Farese et al., 2005). Activated PKB phosphorylates its 160 kDa structure, which stimulates the translocation of insulin-mediated GLUT-4 from intracellular secretory vesicles to the cell membrane (Sano et al., 2003). Once the GLUT-4 transporters are located at the membrane, glucose is able to enter the cell by facilitated diffusion. These events can be seen in Figure 2.2. As soon as the glucose enters the cell, it is phosphorylated into glucose-6-phosphate in order to preserve the concentration gradient so that glucose continues to enter the cell. Glucose-6-phosphate can then continue through the process of glycolysis and produce ATP, or it will be stored as glycogen. When blood glucose levels decrease to the normal physiological values, insulin release from the pancreas slows or stops due to the negative feedback mechanism. It is evident that the activation of PKCλ is involved in the regulation of GLUT-4 translocation in response to insulin (Bandyopadhyay et al., 2002; Farese et al., 2007). The insulin receptor is also dephosphorylated (inactivated) by protein tyrosine phosphatases (PTPs), which make up an extensive family of proteins that have negative effects on insulin action and the metabolism of glucose in the body due to the

regulation of the activation of cytokine receptors or the regulation of cytokine signaling (Bourdeau et al., 2005). Another nuclear receptor that is involved in glucose homeostasis is the nuclear receptor peroxisome proliferator-activated receptor gamma (PPARγ) (Picard and Auwerx, 2002). Peroxisome proliferator-activated receptor gamma one (PPARγ1) is expressed in the skeletal and smooth muscle, liver, and kidney. This receptor's task is to be a transcription factor and regulate metabolism. Peroxisome proliferator-activated receptor gamma activation has been shown to increase insulin receptors and IRS-1 (an insulin receptor substrate) (Iwata et al., 2001).

Insulin also has effects on carbohydrate, protein, and lipid metabolism (Wilcox, 2005). During carbohydrate metabolism, insulin has an effect on the entrance of glucose into the cell, as well as increasing glycogen synthesis by dephosphorylating glycogen synthase. Glycogen breakdown is also decreased, by the dephosphorylation of glycogen phosphorylase kinase. During protein synthesis insulin activates transcription components of specific mRNA, such as the mRNA for glucokinase (the enzyme that facilitates phosphorylation of glucose to glucose-6-phosphate), fatty acid synthase and albumin in the liver. Insulin also decreases mRNA for various liver enzymes. In lipid metabolism, insulin increases fatty acid synthesis by increasing the phosphorylation of acetyl-CoA carboxylase in the adipose tissue and liver, and also increases the formation and storage of triglycerides in these tissues. In addition, when insulin increases, fatty acid oxidation (beta-oxidation) is suppressed by the inhibition of carnitine acyltransferase, which is a necessary enzyme that is required for the transport of fatty acids from the cytosol into the mitochondria. Also, triglyceride synthesis is stimulated by the esterfication of glycerol phosphate, and triglyceride metabolism is suppressed by the

dephosphorylation of hormone sensitive lipase (Wilcox, 2005). Hormone sensitive lipase is inhibited (dephosphorylated)by insulin.

Insulin Resistance:

The most common definition for insulin resistance is when normal concentrations of insulin produce a less than normal biologic response (Kahn, 1978). Therefore, there would not be a normal decrease in blood glucose concentrations after a meal, or when a subject is given a glucose bolus. Insulin resistance suggests insensitivity at the cell's surface, with the receptor, but it can also suggest disorders inside the insulin-sensitive cells, especially in the muscle, adipose, and liver cells, most likely with the signaling cascades (Kronfeld et al., 2005b). Insulin resistance is one of the risk factors for metabolic syndrome and type-2 diabetes mellitus in several different species, such as humans. Tissues that are insulin resistant need greater than normal amounts of insulin to maintain euglycemia. After a progressive resistance of the tissues to insulin there will be an inability to maintain euglycemia, and a reduced function of the beta-cells of the pancreas will occur, which result in a decrease in insulin secretion. This condition is known as type-2 diabetes. (Ferrannini, 1998).

The mechanism of insulin resistance has been proposed to be a problem at the cell surface that could interfere with the cell receptor, which is a glycoprotein associated with receptor tyrosine kinase as previously stated, which is the most common receptor, or a problem with the glucose transporters, or in the translocation of these transporters to the surface of the cell. Although the mechanisms underlying insulin resistance in the muscle are not completely understood, it has been shown in humans and rats that obesity increases the expression or activity of PTPs in muscle and adipose tissue (Ahmad and

Goldstein, 1995). As previously stated, PTPs are protein tyrosine phosphatases that remove phosphate groups on phosphorylated tyrosine residues on proteins, in turn making them responsible for the inactivation of insulin receptors, which can cause the subject to become less sensitive to the secreted insulin. There has also been a strong correlation between total PTP activity and body mass index in adipose tissue and skeletal muscle in obese and lean individuals (Ahmad et al., 1995). Problems are also thought to begin as early in the cascade as the activation of PI3K (Choi and Kim, 2010). In addition, it has been shown that muscle triglyceride levels are inversely related to insulin action (Pan et al., 1997).

Insulin resistance has been a concern as a potential cause in the pathogenesis of some equine diseases, such as laminitis (Coffman and Colles, 1983; Treiber et al., 2006b) and osteochondrosis (Ralston, 1996). When normal insulin secretion does not elicit a decrease in blood glucose concentration and the body secretes more insulin to maintain euglycemia there is a decreased sensitivity to insulin, or insulin resistance. In less severe cases of decreased insulin sensitivity, more insulin can be secreted from the pancreas to decrease blood glucose concentrations. However, in more severe cases, high levels of insulin do not elicit the proper clearance of glucose from the bloodstream. This results in high levels of glucose and insulin in the blood, also known as hyperglycaemia and hyperinsulinaemia, respectively. With respect to glucose homeostasis, there are two primary variables involved: the insulin secretory response of the beta cells to increases in blood glucose concentrations, and the sensitivity of the skeletal muscle and adipose tissues to serum insulin concentrations (Firshman and Valberg, 2007). Insufficiency of the secretion of insulin in response to elevated blood glucose levels, such as what occurs

with Type-I diabetes, is rare in horses (Johnson, 2002; Johnson et al., 2005). Beta-cell failure is proposed to occur in equines as a result of the tissue's decreased sensitivity to the high blood glucose concentrations, and later resulting in pancreatic exhaustion (Kronfeld et al., 2006).

Certain factors may predispose a horse to developing a decreased sensitivity to insulin. The intake of non structural carbohydrates (NSC), such as starch and sugars, found in high concentrations in feedstuffs such as grain and molasses, may reduce sensitivity, while fat supplementation along with fibrous feed may increase sensitivity (Hoffman et al., 2003). In addition, older horses seem to be more prone to developing a decreased insulin sensitivity than younger horses due to an increase in fat accumulation, and a decrease in physical activity (Barbieri et al., 2001). Breed is another factor that may influence insulin sensitivity. It has been shown that ponies have a decreased insulin sensitivity versus standardbred horses (Jeffcott et al., 1986). Studies have identified obesity as a risk factor for laminitis, but it is unclear as to whether obesity directly increased the risk or if it is due to other factors such as insulin resistance and inflammation, which are consequences of obesity (Vick et al., 2007). Exercise has been shown to increase insulin sensitivity with an increased glucose uptake into the muscle cell in rats (Garetto et al., 1984). However, in horses a lack of increase in insulin sensitivity after exercise has been shown in a study (Pratt et al., 2007), which may explain previous observations of a slow rate of post exercise glycogen replenishment (Davie et al., 1994; Davie et al., 1995). However, in other studies, exercise has been shown to improve insulin sensitivity in horses (Powell et al., 2002; Stewart-Hunt et al., 2006). A diet low in non structural carbohydrates (NSC) and high in fat and fiber (262 g

NSC, 212 g fat, 902 g fiber per day) along with exercise three times a week for one hour increased insulin sensitivity in trained Arabian geldings compared to a diet high in NSC and lower in fat and fiber (894 g NSC, 66 g fat, 484 g fiber per day) (Treiber et al., 2006).

Measures to assess insulin sensitivity:

There are several techniques to measure the degree of insulin sensitivity in an individual. These include basal glucose and insulin screenings, oral glucose tolerance tests, clamping techniques, and intravenous glucose tolerance tests.

Basal glucose and insulin screenings:

Testing for basal glucose and insulin levels involves taking a single fasted blood sample to test for concentrations of glucose and insulin. This is common practice in the field of human medicine. The information from these tests has been correlated to more specific results from other insulin sensitivity tests described below. Although single fasting screenings have been used to identify horses with suspected lowered insulin sensitivity (Kronfeld et al., 2005b; Treiber et al., 2006a), the accuracy has been questioned because glucose and insulin concentrations can vary widely in the same individual within a short period of time (Treiber et al., 2006a). The glucose and insulin values are applied to equations and parameters are obtained through the use of these basal proxies. The proxies are the modified insulin-to-glucose ratio (MIRG), measured as [800-$0.30(\text{insulin-}50)^2$]/(glucose-30), which corresponds to the acute insulin response to glucose (AIRg). The second proxy is the reciprocal inverse square of basal insulin (RISQI), which is measured as $\text{insulin}^{-0.5}$, and corresponds to insulin sensitivity (SI). More accurate assessments of insulin sensitivity in the horse have been sought after because it has been proposed that in later stages of insulin resistance, pancreatic

compensation becomes insufficient due to the decreased function of the beta-cells and hyperinsulinaemia may not occur (Kronfeld et al., 2006), however, other studies have shown that basal proxies are good indicators of the horse's acute insulin response to glucose and insulin sensitivity (Treiber et al., 2005b).

Hyperinsulinaemic euglycaemic clamp (HEC) technique:

The hyperinsulinaemic euglycaemic clamp measures the amount of glucose necessary to compensate for an increased insulin level without causing hypoglycemia. The plasma glucose concentration is placed under the control of the researcher and the endogenous glucose-insulin negative feedback loop is broken. Insulin is administered intravenously, and in the other jugular vein glucose is administered. The plasma glucose level is held at a normal fasting concentration. It is known that this test can have some technical difficulties, however it is thought to be one of the most accurate assessments of insulin sensitivity, hyperglycaemia and hyperinsulinaemia. This clamping technique provides greater than maximal steady state insulin concentrations during which the rate of glucose infusion that is required to maintain euglycaemia during the clamp serves as a measure of the insulin sensitivity of muscle and adipose tissues (Defronzo et al., 1979). The rate of glucose infusion during the last 30 minutes of the test determines insulin sensitivity: if high levels are required, the patient is insulin-sensitive. If low levels of glucose are required, this signifies that the body has a decreased sensitivity to insulin. The use of the HEC clamp has been used in the horse (Rijnen and van der Kolk, 2003).

Oral glucose tolerance test (OGTT):

The oral glucose tolerance test measures the intestinal absorption of glucose, hepatic glucose uptake, and the pancreatic endocrine function in the small intestine. This

test requires an overnight fast and blood glucose is then measured at 0, 30, 60, 90, 120, 180, 240, 300, and 360 minutes after administration of a glucose bolus of 1 g/kg of body weight through a nasogastric tube. A peak in blood glucose occurs approximately 90 to 120 minutes after the glucose administration. Blood glucose concentrations should return to normal after four to six hours (Ralston, 2002). A decreased response for glucose to return to normal may indicate an increase in insulin sensitivity, and an increased glucose response may indicated insulin resistance or a decreased insulin sensitivity (Ralston, 2002). This test has been found to be a valuable tool for assessing small intestinal malabsorption in the horse (Mair et al., 1991). The drawbacks of using this test in equines is that blood glucose concentrations may vary due to the stress caused by placing the nasogastric tube in the horse. In addition, other factors stated that may have an affect on the consistency of this test include the rate of administration, the rate of gastric emptying, and differences in intestinal absorption (Kronfeld et al., 2005b).

Intravenous glucose tolerance test (IVGTT):

The IVGTT is a test in which there is an infusion of a glucose bolus via an intravenous catheter. Sampling then occurs at 0, 5, 15, 30, 60, and 90 min and then hourly for 5 to 6 hours post glucose injection. This test can avoid the variability that is associated with gastrointestinal effects of the OGTT. Normal horses should show an immediate rise in blood glucose concentrations which will peak approximately fifteen minutes after the glucose bolus administration. Glucose levels should return to normal within one hour. In addition, the insulin curve should parallel the glucose curve with a peak around thirty minutes post glucose bolus administration (Ralston, 2002). A horse that shows a higher peak of glucose and a delay to return to normal baseline values can

indicate decreased insulin sensitivity. A horse with an impaired beta cell function may show a delayed glucose curve, and the insulin response may be delayed or nonexistent. Insulin resistance at the tissue level is not able to be distinguished by the IVGTT test (Firshman and Valberg, 2007).

Frequently Sampled Intravenous Glucose Tolerance Test (FSIVGTT) and the Minimal Model Analysis:

This test was developed to study glucose and insulin dynamics in a more physiological setting (Firshman and Valberg, 2007). The procedure involves administration of a glucose bolus through a catheter defining minute 0 of the test. Samples are then taken at minutes 1, 2, 3, 4, 6, 8, 10, 12, 14, 16, 19 and at minute 20 insulin is administered. Blood is sampled at minutes 22, 23, 24, 25, 27, 30, 35, 40, 50, 60, 70, 80, 90, 100, 120, 150, and 180 post glucose injection. Glucose and insulin curves are applied to the minimal model using computer software. Parameters are then determined from the software and provide an explicit, quantitative estimate of the insulin sensitivity of the tissues of a given subject. The model simulates plasma glucose dynamics when plasma insulin dynamics are supplied (Kronfeld et al., 2005a). One drawback to this method is that it may be affected by endogenous insulin secretion (Kronfeld et al., 2005a). An advantage of this method is that it has been shown to differentiate between insulin resistance with and without pancreatic compensation (Kronfeld et al., 2005b). Variations have been published, and recent studies show the use of a frequently sampled intravenous glucose tolerance test (FSIVGTT) may include approximately 30 samples over a three hour period of time. Since this test only involves sampling of blood, it is less invasive than the clamp method, and steady-state conditions do not have to be

maintained, however the amount of samples make it almost as labor-intensive as the clamp method (Muniyappa et al., 2008).

Minimal Model parameters

Once glucose and insulin values are obtained curves are then applied and analyzed according to the minimal model of glucose and insulin dynamics (Bergman et al., 1981). The parameters that are obtained are as follows: acute insulin response to glucose (AIRg; (1/mU·L)·min) or the beta cell pancreatic response, which measures the insulin secreted from the pancreas in response to a glucose dose (endogenous insulin). However, AIRg does not distinguish between endogenous and exogenous insulin. Insulin sensitivity (SI; 1/(mU/L·min)), is the tissues' sensitivity to insulin. In other words, SI is the ability of insulin to promote glucose disposal. Glucose effectiveness (Sg; 1/min) is defined as the quantitative enhancement of glucose disappearance due to an increase in the plasma glucose concentration, or the ability of glucose to lower blood glucose concentrations itself. The last parameter, disposition index (DI), is the insulin sensitivity combined with the beta cell pancreatic response. It describes the beta cell responsiveness and accounts for the influence of insulin sensitivity, and the endogenous insulin secretion (Hoffman et al., 2003). Presently, these four minimal model parameters are determined from the glucose and insulin values using MinMod Millennium software.

Effects of Fatty Acid Supplementation on Insulin Sensitivity:

The effects that supplementation of fatty acids have on insulin sensitivity has been a popular research topic in previous years in both humans and animal models. The long-term effects of supplementing with n-3 fatty acids are that the dietary composition would be reflected in the fatty acid profile of cell membranes (Ayre and Hulbert, 1996), and as

described previously it may increase the fluidity of the membrane. Several studies have shown that supplementation of n-3 highly unsaturated fatty acids, especially long-chain EPA and DHA in particular, can increase insulin sensitivity. A study (Ghafoorunissa et al., 2005) conducted using rats fed a diet of fatty acids enriched with either starch or sucrose, where insulin resistance was induced in the sucrose fed rats, revealed that the sucrose fed rats had higher insulin and triglyceride levels than those fed the starch. In addition, increasing the n-3 long-chain highly unsaturated fatty acids fed in the sucrose diet significantly lowered ($P < 0.05$) the plasma insulin response to a glucose load (Ghafoorunissa et al., 2005). A different study (Lombardo et al., 2007) examined the effects of rats fed diets of a purified sucrose-rich diet (SRD), or the same diet replacing the sucrose with starch (control). The SRD was divided into subgroups and one group was supplemented with a mixture of cod liver oil and corn oil (SRD + FO) after an acclimation period on the SRD. The mixture of cod liver oil and corn oil (FO) reduced the triglyceride content that was increased during the acclimation period. In addition, the dietary FO reversed the impaired insulin-stimulated glucose-6-phosphate concentration and glycogen storage during the HEC clamp procedure (Lombardo et al., 2007). Also, the rats fed the SRD diet showed an altered pattern of glucose stimulated insulin secretion from the beta cells, and dietary FO completely normalized the glucose induced insulin secretion in the SRD-fed rats (Lombardo et al., 2007). A study (Behme, 1996) conducted in miniature pigs compared the effects of supplementation with 30 g corn oil (22.7 g/kg n-6 PUFAs) or 30 g Max EPA(6.7 g/kg EPA, 4.8 g/kg DHA, 4.7g/kg n-6 PUFAs). Pigs were supplemented for four to five weeks and submitted to a FSIVGTT and the minimal model was applied. Values obtained for insulin sensitivity and glucose effectiveness were

significantly higher in the pigs fed the MaxEPA ($P < 0.03$) (Behme, 1996). This study showed that insulin sensitivity was enhanced in pigs supplemented with n-3 long-chain fatty acids. Another study (Luo et al., 1996) looked at the effects of feeding rats a diet containing 50 % sucrose for energy, 20 % protein and 30 % fat which only differed in the fat composition: comprised of either a control diet (28 % corn oil, 28 % peanut oil, and 84 % lard) or a diet where the 30 % fat came from fish oil (18 % EPA, 13 % DHA), on insulin sensitivity. Some experimental rats were found to be hyperinsulinaemic, and the fish oil normalized this hyperinsulinaemia ($P < 0.05$). Also, the fish oil fed rats had lower plasma triglycerols than the control fed rats (Luo et al., 1996). This study found that the presence of fish oil in the diet corrected the inhibitory effects of a high sucrose feeding on insulin action. It also found that the fish oil increased the insulin sensitivity of adipocytes in the rats fed that particular diet (Luo et al., 1996). A study (Storlien et al., 1991) conducted that analyzed whether the type of fat fed to rats had an influence on insulin sensitivity looked at the effects of an identical diet, except for the fatty acid composition . Diets fed were saturated fatty acids (136 g tallow and 203 g safflower oil), monounsaturated (339 g olive oil), polyunsaturated (339 g safflower oil), long-chain n-3 polyunsaturated (EPA and DHA; 237 g safflower oil and 102 g fish oil), short-chain n-3 polyunsaturated (ALA; 268 g safflower oil and 71 g linseed oil), or short-chain n-3 fatty acids (268 g tallow and 71 g linseed oil). Results indicated that the n-3 long-chain highly unsaturated fatty acid diet lowered basal plasma triglycerides compared to the group fed saturated fatty acids or monounsaturated fatty acids. In addition, substitution of 11 % of the fatty acids in the polyunsaturated fatty acid diet with n-3 long chain PUFAs from fish oils normalized insulin action. In this study, insulin sensitivity was inversely correlated

with mean muscle triglycerides ($r = 0.95$ and 0.86 for soleus and red quadriceps, respectively; $P < 0.01$). Researchers concluded that n-3 long-chain fatty acids in the phospholipid of muscle may be important for efficient insulin action (Storlien et al., 1991).

Studies have also been conducted which observed the effects of n-3 supplementation on the insulin receptor. One study measured the effects of either a high n-3 fatty acid diet, or a low n-3 fatty acid diet on the insulin binding of skeletal muscle sarcolemma in rats (Liu et al., 1994). The n-3 fatty acids were supplied by fish oil. The rats fed the high n-3 fatty acid diet bound more insulin than the rats fed the low n-3 diet. Also, in the same study two other groups were fed a diet with a highly unsaturated/saturated ratio of one (high P/S), or 0.25 (low P/S). The rats fed the high P/S bound more insulin than the rats fed the low P/S diet. This study demonstrates that dietary supplementation of n-3 long-chain PUFAs increase the amount of insulin bound to its receptor in muscle sarcolemma (Liu et al., 1994). Another study (Taouis et al., 2002) looked at the effects of n-3 fatty acids on insulin receptor signaling in the muscle in rats. Rats were divided into three groups and received either a control diet, a diet high in n-6 highly unsaturated fatty acids, or a diet high in n-3 highly unsaturated fatty acids (19 % fish oil) (Taouis et al., 2002). The most affected enriched tissue in n-3 highly unsaturated fatty acids was the muscle, compared to adipose tissue and liver. In the rats fed the n-3 highly unsaturated fatty acid diet, insulin slightly induced an increased phosphorylation of IRS-1 (one of the IRS's previously described). In the rats fed the n-6 highly unsaturated fatty acids the IRS-1 phosphorylation was actually reduced. The expression of GLUT-4 was also significantly weakened in the n-6 rats (Taouis et al.,

2002). These results indicate that the supplementation of n-3 highly unsaturated fatty acids may increase the ability of insulin to bind to its receptor, in turn increasing insulin sensitivity, and that n-6 may hinder insulin sensitivity.

The effectiveness of α-linolenic acid supplementation compared to the direct supplementation of EPA and DHA and the effects on insulin sensitivity has been studied. Rats were randomly assigned to one of four dietary groups: ALA, EPA, DHA, or EPA/DHA and received 0.5g per kg of body weight per day of each respective fatty acid (Andersen et al., 2008). Compared to the ALA group, the EPA group had an increase in EPA in the liver tissue by 110 %, and an increase in DHA by 10 % ($P < 0.05$). In the DHA group the DHA content of the liver was enhanced by 46 percent compared to the ALA group, and EPA was increased by 50 % due to retro conversion ($P < 0.05$). In the EPA/DHA group, the EPA content was increased by 250 %, and the DHA content was increased by 59 %. In addition, the ALA levels were significantly lower in the EPA, DHA, and EPA/DHA groups (75 %, 76 %, and 62 %, respectively) (Andersen et al., 2008). Fasting plasma glucose and insulin concentrations were significantly lower in the EPA, DHA, and EPA/DHA groups compared to the ALA group ($P < 0.05$), and did not differ from each other. The results in this study clearly showed that ALA supplementation did not represent an alternative to direct supplementation of EPA and DHA in regard to increasing insulin sensitivity.

In humans, a study (McManus et al., 1996) measured the effects of supplementation with 35 mg of fatty acid per kg body weight of linseed oil (LO) or fish oil (FO) for 90 days on fasting glucose and insulin levels, and insulin sensitivity (SI). The design was a randomized double-blind crossover study and treatments were given after

90 days of a placebo treatment of olive oil. Researchers concluded that there were no significant differences between the groups supplemented with linseed oil and fish oil, however, the FO group was associated with a decrease in triglyceride levels and showed a trend towards a decreased insulin sensitivity (McManus et al., 1996), which conflicts with other research. A study in humans (Rasic-Milutinovic et al., 2007) evaluated the effects of n-3 highly unsaturated fatty acid supplementation on insulin sensitivity in patients with chronic renal failure on maintenance hemodialysis, since they presented insulin resistance. Supplementation with a direct source of EPA and DHA at a rate of 2.4 g/d for 60 days resulted in a decrease ($P < 0.001$) in serum insulin values, and also a decrease ($P < 0.001$) in the IR-index IR HOMA values for measuring insulin sensitivity (Rasic-Milutinovic et al., 2007).

Conclusion

It is evident that the supplementation of long-chain highly unsaturated fatty acids has an effect on the incorporation of fatty acids into the cell membranes, and may have a positive effect on insulin sensitivity. With the information available regarding the effects of supplementation with an animal source of n-3 highly unsaturated fatty acids (EPA/DHA) versus a plant source of n-3 highly unsaturated fatty acids (ALA) on insulin sensitivity in other species, but not in horses, it is apparent that this particular matter needs to be researched more thoroughly in the equine species to see if the same beneficial effects apply to equines.

Objective and Hypothesis

This study measured the incorporation of an animal source and a plant source of n-3 highly unsaturated fatty acids into the plasma, red blood cell, and equine muscle cell

and the effects on insulin sensitivity. It is hypothesized that the horses supplemented with the animal source will have greater incorporation of EPA and DHA into the plasma, red blood cell and muscle cell, and have an increase in insulin sensitivity over the horses supplemented with the plant source of n-3 highly unsaturated fatty acids or the control group.

Chapter III

In this study, the experiment was designed to measure the effects of supplying horses with an animal source of n-3 highly unsaturated fatty acids (marine fish oil pellet), which was a direct source of EPA and DHA, or a plant source of n-3 fatty acids, (ground flaxseed) on the incorporation in to the plasma, red blood cell, and muscle cell membranes, and also the effects on insulin sensitivity.

Materials and Methods

Horses:

Twenty one mares of mixed stock horse breeding were maintained on free-choice alfalfa hay for at least one month prior to this study. Horses were maintained according to the policy of Colorado State University's Equine Sciences program, and following the protocol of the Institutional Animal Care and Use Committee. Horses had been dewormed with ivermectin and vaccinated with a five-way vaccine which included tetanus, eastern and western equine encephalomyelitis, influenza, and rhinopneumonitis, in addition to West Nile and Strangles upon arrival at the Equine Reproduction Laboratory before being assigned to this study. They were also dewormed during the study on day 74, May 21, 2009. An initial assessment of body condition score and measurement of weight was made in order to assign treatment groups. The mares, between five and fourteen years of age with an average age of nine, weighed from 525 to 673 kg with an average of 586 kg, and had a body condition scored ranging from five to eight with an average of 6.7. Body condition score was based on a scale of 1 to 9 (Henneke et al., 1983). Final scores were based on the average scores from three trained and unbiased individuals. Horses' weights and body condition scores were assessed on a

monthly basis during the study. Mares were blocked by age, weight and body condition score and randomly assigned into one of three dietary treatment groups which were a control group, a group supplemented with a commercial fish oil pellet, and a group supplemented with a commercial ground flaxseed product. Weights and body condition scores (BCS) were evaluated on a monthly basis three days before days 0, 30, 60 and 90. Age, weight and BCS means and ranges by treatment are given in Table 3.1.

Diets:

Diets included an alfalfa/bromegrass mixed hay, commercial rolled barley, and a fatty acid supplement that varied with treatment. Rolled barley was added to make the diets isocaloric and isonitrogenous, and to ensure the fatty acid supplements would be consumed. The horses underwent a two week adaptation period to the hay fed during the study before baseline samples were taken. All feeds and supplements were analyzed for fatty acid composition. In addition diets were analyzed for nutritional composition by Equi-Analytical Laboratories, shown in Table 3.3, and treatments formulated as seen in Table 3.4. The first group (FISH) received 142.4 mg/kg of body weight of n-3 fatty acids via a commercial fish oil supplement (Magnitude™; JBS United, Sheridan, IN), from which 58.6 mg/kg of body weight was composed of EPA and DHA, plus 1.6 % of body weight in hay and 1.1 g/kg of body weight in supplemental barley daily. The n-6 to n-3 ratio for the entire diet in the FISH group was 0.43:1. The second treatment group (FLAX) received 149.8 mg/kg of body weight of the n-3 fatty acid ALA via a flaxseed supplement (Nutra-Flax™), plus 1.7 % of body weight in hay and 0.51 mg/kg of body weight in supplemental barley daily. The n-6 to n-3 ratio for the entire diet in the FLAX group was 0.44:1. The third treatment group (CONT) did not receive additional n-3 fatty

acid supplementation aside from the n-3 fatty acid ALA that was present in the hay and barley and total dosage was 78.15 mg/kg of body weight of n-3 fatty acids. Group three received 1.6 % of body weight in hay and 1.8g/kg of body weight in supplemental barley daily. The n-6 to n-3 ratio for the entire diet in the CONT group was 0.79:1. The CONT diet contained a similar amount of n-6 fatty acids as the FISH and FLAX groups, but approximately half the amount of n-3 fatty acids. Fatty acid intake for the three treatment groups is shown in Table 3.5. For all groups, hay was fed once daily in a bunk. Barley and the appropriate treatment, if applicable, were fed to horses individually in nose bags once daily. The horses underwent a two week adaptation period to the supplement and barley after baseline samples were taken. Treatments were fed for 90 days. Supplements and barley were mixed together in individual nosebags and fed to the horses. Refusals were weighed on a daily basis and were calculated for each horse and compared to the total amount of that treatment offered throughout the study to develop a percentage of refusal.

Weather:

Average temperature, precipitation and wind speed throughout the study were obtained from the Colorado Climate Center readings from Colorado State University's Foothills Campus.

Sampling:

Weight and BCS:

Weights and body condition scores (BCS) were evaluated on a monthly basis two to six days before sample collection at day 0, 30, 60, and 90.

Fatty Acid Incorporation:

Blood samples and muscle middle gluteal biopsies were taken on day 0, 30, 60, and 90 of supplementation. Blood samples were taken via jugular venipuncture and collected with EDTA vacutainer tubes, centrifuged at 2,700g for seven minutes, plasma was separated and red blood cells were immediately transferred to polypropylene tubes and stored at -80^{o} C until analysis. In preparation for the muscle biopsies the horses were restrained in stocks, hair was clipped in a four inch square over the top of the rump in a line between the tuber coxae and tuber ischii and the area was cleaned with iodine scrub and alcohol. Only a few horses needed further restraint with a twitch. One to three ml of lidocaine was administered subcutaneously and a five to ten mm incision was made into the skin. A Bergstrom muscle biopsy needle was used to sample muscle at a depth of six centimeters from the middle gluteal muscle. Approximately two to three g of wet muscle tissue per horse was extracted, aliquoted, and transferred into polypropylene tubes, and flash frozen in liquid nitrogen until stored at -80^{o} C until analysis.

Insulin Sensitivity:

Insulin sensitivity was measured using the frequently sampled intravenous glucose tolerance test (FSIVGTT). Catheters were placed aseptically into the jugular vein. The FSIVGTT testing was performed on day 0, 30, 60, and 90. Glucose doses were administered using a fifty percent dextrose solution. Insulin used for the test (Humulin N human insulin) was diluted in saline solution at one percent. The FSIVGTT started with two basal samples being taken 20 min after catheters were placed and then 200 mg/kg of body weight of glucose was injected in a bolus and blood samples collected at 1, 2, 3, 4, 5, 6, 8, 10, 12, 14, 16, and 19 minutes after injection. At minute 20, 10 μU/ kg of body weight of insulin solution was administered through the catheter. Blood sampling

continued at 22, 23, 24, 25, 27, 30, 35, 40, 50, 60, 70, 80, 90, 100, 120, 150, 180 minutes post glucose injection. Blood samples were transferred to heparinized vacutainer tubes and centrifuged at 2,700g for seven minutes. Plasma was removed within 15 minutes of collection and frozen at -20˚ C until analysis.

Laboratory Analysis:

Fatty Acid Incorporation:

Fatty acid composition was analyzed using a modified Folch lipid extraction procedure (Folch et al., 1957). Hexane extracts of fatty acid methyl esters were separated by gas chromatography on a 30 m×0.25 mm×0.2 μm film thickness DB-225 column Agilent (Palo Alto, CA) using an Agilent model 6890 GC equipped with autosampler, Chemstation and flame ionization detection.

The procedure was as follows for plasma: 0.5 ml of plasma was added to a labeled screw cap tube, 6 ml of 2:1 $CHCI_3$:MeOH was then added and the mixture vortexed for 60 sec. The sample was filtered through an S&S (#1) filter into a clean labeled screw cap tube. The previous tube and filter were rinsed using one ml $CHCI_3$. The sample was removed from the filter apparatus and two ml of 0.88% potassium chloride (KCl) was added and the sample was vortexed for 30 sec. The sample was then centrifuged at 2500g for 10 minutes. The top layer was aspirated and the lower layer was evaporated ($CHCI_3$) to dryness using light nitrogen flow. Afterward, 0.5 ml of hexane (CH_4) was added and the sample was vortexed for 30 seconds. The entire 0.5 ml sample was plated on a TLC plate. The plate was then placed in the developing chamber filled with 100 ml Hexane:Ethyl Ether:Acetic Acid (70:30: 1) for one hour. The phospholipid band (the band that remains at the origin) was scraped into a screw cap tube. Then 0.5ml of hexane

was added to the tube and vortexed for 30 seconds after which it then underwent methylation. The methylation procedure was as follows: 0.5 ml 0.5N potassium hydroxide (KOH) was added, and the sample was heated in a heating block for 10 min, and then allowed to cool before the cap was removed. Three ml of boron trifluoride (BF_3) was added and the sample was vortexed. The tube was placed on the heat plate for 1.5 hours. The sample was allowed to cool before removing the cap. When the cap was removed, 0.5 ml hexane and 1 ml of high quality water was added and vortexed for 30 seconds. The sample settled and there was an obvious interface between the non polar (upper portion) and polar (lower portion) phase. The non polar phase sample was transferred to the gas chromatography vial.

The procedure for red blood cells was as follows: three ml of methanol (MeOH) was added to a screw cap tube. After the methanol addition, 0.5ml of the red blood cell sample was added drop-wise to MeOH and vortexed for 30 seconds. Three ml of chloroform ($CHC1_3$) was added and the sample was vortexed for 60 seconds. The sample was then filtered through an S&S (#1) filter into a clean screw cap tube. The tube and filter were rinsed using 3ml $CHC1_3$. The sample was removed from the filter apparatus and two ml of 0.88% KCl was added to the sample, vortexed for 30 seconds and then centrifuged at 2500g for 10 minutes. The top layer was aspirated (MeOH and H_2O) and the lower layer was evaporated ($CHC1_3$) to dryness using light nitrogen flow. After drying, 0.5 ml hexane (CH_4) was added and the sample was vortexed for 30 seconds. At this stage the methylation procedure was performed as described above for plasma.

The procedure for the muscle samples was as follows: approximately 30-50mg of tissue was weighed and the tissue was homogenized using a glass-glass homogenizer and

vortexed if needed. One ml of (2:1 $CHCI_3$:MeOH) solution was added to the homogenizer. The sample was homogenized until the entire tissue was in solution. The sample was filtered through an S&S (#1) filter into a clean screw cap tube. The tube and filter was rinsed using one ml of (2:1 $CHCI_3$:MeOH). The sample was removed from the filter apparatus and 0.25 ml of 0.88% KCl was added and the sample was vortexed for 30 seconds, and centrifuged at 2500g for 10 minutes. The top layer (MeOH & H2O) was aspirated and the lower layer was evaporated (CHC13) to dryness using light Nitrogen flow. Afterward, 0.5 ml hexane (CH4) was added and vortexed for 30 seconds. 0.5 ml 0.5N KOH was added and the sample was heated in the heating block for 10 min, and allowed to cool before removing the cap. Three ml BF_3 was added and the sample was vortexed. The tube was placed on the heat plate for 1.5 hours, then the sampled was allowed to cool before removing the cap. Following this 0.5 ml hexane and one ml of high quality water was added and the sample was vortexed for 30 seconds. The sample was allowed to settle and there was an obvious interface between the non polar and the polar phase. Then, .05 ml of the non polar phase of the interface was transferred to the gas chromatography vial and evaporated to dryness using light nitrogen flow. In the final phase, 80 µl of hexane was added, the sample was vortexed and transferred to the gas chromatography vial insert.

All vials were then placed on the gas chromatograph.

Insulin Sensitivity:

Plasma was analyzed for glucose by enzymatic assay using the YSI 2700 SELECT Biochemistry Analyzer with YSI 2365 glucose membranes, YSI 2747 glucose/L-Lactate standard and YSI 2357 buffer concentrate kit. An enzyme for the

specific substrate, glucose, was immobilized between two membrane layers: one polycarbonate and one cellulose acetate. The substrate was oxidized as it entered the enzyme layer, producing hydrogen peroxide, which then passed though the cellulose acetate layer to a platinum electrode where the hydrogen peroxide was oxidized. The current that resulted was proportional to the concentration of glucose in the sample.

Insulin was analyzed using Siemens Coat-A-Count insulin radioimmunoassay kit. Plasma is combined with iodinated insulin in insulin antibody coated tubes. The iodinated insulin competed for a fixed time with insulin in the sample for sites on the insulin specific antibodies. The antibody was then immobilized. The insulin in the sample displaced some of the tagged insulin, and the free tagged insulin was measured with isotope detectors in a gamma counter.

Glucose and insulin curves were applied to the minimal model using MinMod Millennium software to evaluate the following four minimal model parameters: Acute insulin response to glucose (AIRg), or beta cell pancreatic response, which is the animal's insulin response to a glucose dose; Insulin sensitivity (SI), which indicates how sensitive tissue is to insulin; Glucose effectiveness (Sg), which evaluates the glucose mediated glucose disposal; and disposition index (DI), which is the combined insulin sensitivity and AIRg.

Basal proxies were calculated from baseline blood samples as well. Beta-cell responsiveness, MIRG, was measured as $[800\text{-}0.30(\text{insulin}\text{-}50)^2]/(\text{glucose}\text{-}30)$. The reciprocal inverse square of basal insulin, RISQI, is an indication of insulin sensitivity and was measured as $\text{insulin}^{-0.5}$.

Classification of normal or possibly insulin resistant:

After initial analysis of the minimal model parameters, horses were then classified into either a normal group (non-IR), or a possibly insulin resistant group (IR) based on their baseline insulin sensitivity (SI) values. Horses with SI values below 0.78 1/(mU/L·min) were classified into the IR group, based on a previous study's quantification of quintiles which can be seen in Table 3.5 (Treiber et al., 2005b).

Statistical Analysis:

Statistical differences between plasma, red blood cell, and muscle cell fatty acid compositions among treatment groups, along with insulin sensitivity variables at days 0, 30, 60 and 90 were determined by ANOVA using a repeated measures design using the PROC MIXED procedure of SAS. For all analyses, a P-value ≤ 0.05 was accepted as a statistical difference. Significant differences were then further analyzed using Fisher's least square means differences. Results are reported as mean ± sem.

Results

Horses weights and body condition scores:

Weights and body condition scores (BCS) were evaluated on a monthly basis and can be seen in Table 3.6. Since no differences were seen between treatments, results were pooled and averaged across all treatments No differences were found between treatments in body weight and BCS so results were pooled. Weights ranged from 525 kg to 673 kg and decreased ($P < 0.001$) from day 0 to day 60, and then increased ($P < 0.006$) from day 60 to day 90. Body condition scores ranged from five to eight and decreased by 0.36 ($P < 0.021$) from day 0 to day 90. Some mares, particularly two in the FLAX group, exhibited signs of estrus beginning near day 60 of supplementation. Horses remained healthy throughout the study with no adverse effects observed with the different treatments.

Diets:

Refusals were not common among horses, except for the FISH group. Three FISH horses refused almost four percent each of the total barley and fish oil pellet offered in their diet over the four months of the study. The other four FISH horses refused less than two percent each. One FLAX horse refused on multiple occasions totaling 2.0 % of the flaxseed and barley diet over the length of the study.

Weather:

Average temperature, precipitation and wind speed for the months prior to and during sampling can be seen in Table 3.7. Sampling took place during the weeks March 2-6, April 13-17, May 11-15, and June 8-11, 2009.

Treatments, BCS, and weight

There were no differences in body weight variation or body condition score variation (Table 3.6).

Sampling:

Fatty Acid Incorporation:

Plasma fatty acid incorporation is shown in Table 3.8. Linoleic acid (LA; C18:2 n-6) showed a treatment effect ($P < 0.002$), where it was significantly lower in the FISH treated horses compared to the FLAX and CONT. Overall LA decreased with time from 0 to 90 d of supplementation (Day 0 = 37.4 ± 0.52 %, day 30 = 37.5 ± 0.51 %, day 60 = 33.5 ± 0.52 %, and day 90 = 33.5 ± 0.52 %. Additionally, a treatment by time interaction was seen ($P < 0.001$) where LA was significantly lower at day 30, 60 and 90 in the FISH group compared to the FLAX group (refer to Table 3.8). In addition, at day 30 and 60 the FISH group had lower LA plasma concentrations when compared to the

CONT group ($P < 0.002$). At day 90 the FISH and CONT were not significantly different and both lower than the FLAX. Plasma alpha linolenic acid (ALA; C18:3 n-3) was significantly different between treatments ($P < 0.001$). Lowest ALA values were seen in the FISH treated horses, and the highest values in the FLAX treated horses (see Table 3.8). Plasma ALA overall varied with time. It decreased ($P < 0.001$) from day 0 (1.46 ± 0.045 %), to day 30 (1.01 ± 0.045 %), increased ($P < 0.001$) at day 60 (1.48 ± 0.046 %), and further increased at day 90 (1.66 ± 0.045 %). Plasma ALA had a treatment by time interaction ($P < 0.001$) where it was lower at day 30, 60, and 90 in the FISH group compared to the FLAX and CONT. Alpha linolenic acid was higher in the FLAX group compared to the CONT group at day 30 (1.47 % vs. 1.03 %, $P < 0.002$), and also at day 90 (2.22 % vs. 1.82 %, $P < 0.007$), but was not significantly different from each other at day 60 (1.85 % vs. 1.75%, $P < 0.373$). Plasma dihomo-gamma-linolenic acid (DGLA; C20:3 n-6), a derivative of LA and the intermediary before arachidonic acid (ARA; C20:4), concentration was not detected at day 0 or day 90, and was not significantly different among treatment groups or time. Arachidonic acid concentrations were significantly different between treatments at baseline so day 0 was run as a covariate. Arachidonic acid concentrations in the plasma had a treatment effect ($P < 0.002$), as well as a time effect ($P < 0.01$), but no interaction. Arachidonic acid was significantly higher in the FISH (2.32 ± 0.08 %) group compared to the FLAX group (1.87 ± 0.08 %; $P < 0.002$) and also the CONT group (1.95 ± 0.08 %; $P < 0.002$). Arachidonic acid increased in concentration across all treatment groups from day 30 (1.96 ± 0.07 %) to 60 (2.23 ± 0.07 %; $P < 0.007$) and decreased in concentration from day 60 (2.23 ± 0.07 %) to 90 (1.96 ± 0.07 %; $P < 0.008$). Arachidonic acid was not significantly different at day 30

compared to day 90 ($P < 0.99$). At day 0, eicosapentaenoic acid (EPA; C20:5) was below detectable levels in all groups, and was then found in the plasma of the FISH group after thirty days, where it increased ($P < 0.002$) from day 30 (2.17 ± 0.21 %) to day 60 (2.86 ± 0.21 %, and not change ($P = 0.26$) from day 60 (2.86 ± 0.21 %) to day 90 (3.03 ± 0.21 %). In addition to EPA, docosahexaenoic acid (DHA; C22:6) was below detectable levels at day 0, but then was detected in only the FISH group after 30 days. Furthermore, it increased ($P < 0.005$) from day 30 (3.65 ± 0.19 %) to 90 (4.44 ± 0.19 %).

Red blood cell (RBC) fatty acid incorporation is shown in Table 3.9. Linoleic acid was not different between treatments ($P = 0.29$) and overall it varied with time ($P < 0.001$), decreasing progressively ($P < 0.001$) from day 30 (30.7 ± 0.44 %) to day 60 (29.0 ± 0.45 %) and day 90 (27.7 ± 0.45%). A treatment by time interaction effect was seen ($P < 0.030$) where LA was lower in the FISH group compared to the FLAX and CONT ($P < 0.02$) horses, at day 90 (table 3.9). Alpha linolenic acid was different among treatment groups at baseline s day 0 was run as a covariate and overall was not different between treatments ($P = 0.069$) and it increased ($P < 0.001$) overall from day 30 (2.45 %) to 60 (11.0 ± 0.38 %; $P < 0.001$) and decreased from day 60 (11.0 ± 0.38 %) to 90 (3.07± 0.39 %; $P < 0.001$). There was also no treatment by time interaction ($P = 0.47$). Arachidonic acid concentrations in the red blood cell were different between treatments ($P < 0.001$), and also between months ($P < 0.010$). Day zero was run as a covariate due to significant differences between treatments at this time period. The FISH (2.15 ± 0.09 %) group ARA concentrations were higher compared to the FLAX (1.44 ± 0.09 %) and CONT (1.47 % ± 0.09; $P < 0.002$) groups. Overall, ARA concentrations were higher ($P < 0.040$) at day 30 (1.72 ± 0.05%) compared to day 90 (1.57 % ± 0.05). There was no significant treatment

by time interaction (P = 0.056). Eicosapentaenoic acid was undetected at day 0 in the red blood cell of all treatments and reached detectable levels in the FISH group only, after thirty days, where it increased (P < 0.001) from day 30 (1.67 ± 0.16 %) to day 60 (2.34 ± 0.16 %;). In addition, DHA was undetected at day 0, and was detected in the RBC of the FISH group at day 30 (2.04 ± 0.19 %) as well, where it continued to increase significantly at day 60 (2.75 ± 0.19 %) and progressively increased (P < 0.009) to day 90 (3.10 ± 0.19 %).

Muscle fatty acid incorporation can be seen in Table 3.10. There was a treatment effect for LA (P < 0.001). Overall, LA was lower (P < 0.001) in the FISH group (27.8 ± 0.64 %) compared to CONT (33.7 ± 0.64%) and FLAX group (32.7 ± 0.64 %; P < 0.001), but FLAX and CONT were not significantly different from each other (P = 0.31). Day zero was run as a covariate, and there was a time effect (P < 0.001), where it increased from day 30 (28.6 ± 0.54 %) to day 60 (33.03 ± 0.58 %) and did not decrease significantly to day 90 (32.6 ± 0.55 %). A treatment by time interaction was found (P < 0.020). The amount of LA was lower (P < 0.031) in the FISH group compared to the CONT group, but not significantly different from the FLAX group (P = 0.23) at day 30. At day 60 and day 90 the LA concentration of the FISH group was lower (P < 0.040) than the FLAX and CONT groups. Muscle ALA was run with day 0 as a covariate and was different among treatment groups (P < 0.005), in which the FISH group (1.27 ± 0.12%) was lower (P < 0.004) compared to the FLAX (1.87 ± 0.12%) and CONT (1.88 % ± 0.12; group. However, the ALA concentration between the FLAX and CONT groups did not differ from each other (P = 0.94). There was no time effect (P = 0.29) or treatment by time interaction (P = 0.90). Dihomo-gamma-linolenic acid (DGLA; C20:3 n-6) was

found to differ among months, where it increased from day 0 (1.75 % ± 0.12) to day 30 (2.61 % ± 0.11; $P < 0.001$), decreased from day 30 (2.61 % ± 0.11) to day 90 (1.79 ± 0.14%; $P < 0.001$). There was no treatment effect ($P = 0.12$) or treatment by time interaction ($P = 0.65$). Muscle ARA was found to not show a treatment ($P = 0.53$), a time effect ($P = 0.083$), or a treatment by time interaction ($P = 0.15$). Muscle EPA had a treatment effect ($P < 0.001$) and overall was higher in the FISH (2.35 ± 0.15 %) compared to the FLAX (1.58 ± 0.15 %) and CONT (0.99 ± 0.22 %). A treatment by time interaction was found ($P < 0.009$). Muscle EPA was significantly higher in the FISH group (2.82 % ± 0.13) at day 60 and (2.72 % ± 0.11) at day 90 than the FLAX (1.16 % ± 0.13) at day 60 and (1.09 % ± 0.11) at day 90 and the CONT (1.33 % ± 0.13) at day 60 and (1.08 % ± 0.11; $P < 0.001$) at day 90. Muscle EPA also showed a treatment effect ($P < 0.002$) in which the FISH group (2.34 ± 0.13 %) was higher than the FLAX group (1.58 ± 0.18 %). Muscle EPA did not show a significant time effect ($P = 0.14$). Docosapentaenoic acid (DPA; C22:5 n-3) had a treatment effect ($P < 0.047$). It was significantly lower ($P < 0.015$) in the FISH (2.18 ± 0.13%) compared to FLAX (2.69 ± 0.13%), but not different ($P = 0.12$) from CONT (2.49 ± 0.13%). There was no time effect ($P = 0.15$). There was a treatment by time interaction ($P < 0.004$) where muscle DPA was significantly lower ($P < 0.001$) in the FISH group compared to the FLAX and CONT groups at day 90 of treatment (Table 3.10). The FLAX and CONT groups did not differ ($P = 0.37$) at this time point. Muscle DHA had a treatment effect ($P < 0.001$) and overall was higher in the FISH compared to the FLAX and CONT (Table 3.10). Differences between groups were seen at day 0, so month 0 was used a covariate. There was a time effect ($P < 0.018$) where overall DHA increased ($P < 0.028$) from day 0 (4.29

± 0.27 %) to day 30 (5.14 ± 0.26%) and increased (P < 0.008) day 60 (5.18 ± 0.28%) to day 90 (6.29 ± 0.26%). There was a treatment by time interaction (P < 0.050). Muscle DHA was significantly higher in the FISH group compared to the FLAX and CONT groups at day 30, 60, and 90 (Table 3.10).

Insulin Sensitivity:

The parameters that resulted from the minimal model analysis can be seen in Table 3.11. Acute insulin response to glucose (AIRg) was not different between treatments (P = 0.79) and did not have a treatment by time interaction (P = 0.94). It was only different between months (P < 0.001), where it increased (P < 0.008) from day 0 (653 ± 94) to day 30 (794 ± 94), decreased (P < 0.008) from day 30 (794 ± 94) to day 60 (599 ± 95), and increased (P < 0.003) from day 60 to day 90 (812 ± 94). Insulin sensitivity (SI) was not different between treatments (P = 0.85) and did not have a treatment by time interaction (P = 0.65). Insulin sensitivity (SI) was only significantly different between months (P < 0.037) where it increased from day 30 (1.01 ± 0.27) to day 90 (2.08 ± 0.27; P < 0.005). Glucose effectiveness (Sg) was not different between treatments (P = 0.20) and did not have a treatment by time interaction (P = 0.90), but yielded a time effect. Glucose effectiveness (Sg) decreased (P < 0.050) from day 30 (0.018 ± 0.002) to day 60 (0.014 ± 0.002) and increased (P < 0.020) from day 60 (0.014 ± 0.002) to day 90 (0.019 ± 0.002). Disposition index (DI) was not different between treatments (P = 0.41), but did show a time effect (P < 0.002), where it increased significantly from day 60 (801 ± 165) to day 90 (1419 ± 162). showed a treatment by month interaction (P < 0.030). At day 90 the FLAX was greater (P < 0.007) than the

CONT group, and the FISH was not significantly different from the FLAX or CONT ($P < 0.34$) at day 90 (Table 3.11).

Basal proxy results are also shown in Table 3.11. Beta-cell responsiveness, MIRG, measured from basal blood samples as $[800\text{-}0.30(\text{insulin-}50)^2]/(\text{glucose-}30)$, was only different between months ($P < 0.040$), where it increased ($P < 0.020$) from day 60 (6.92 ± 0.48) to day 90 (8.07 ± 0.48). The reciprocal inverse square of basal insulin, RISQI, an indication of insulin sensitivity measured as $\text{insulin}^{-0.5}$, did not show a treatment effect ($P = 0.78$) or a treatment by time interaction ($P = 0.066$). It did result in a significant difference between months ($P < 0.002$). It increased ($P < 0.020$) from day 30 (0.23 ± 0.012) to day 60 (0.25 ± 0.012), and decreased ($P < 0.002$) from day 60 (0.25 ± 0.012) to day 90 (0.21 ± 0.013).

Basal glucose, measured as mg/dL, was analyzed and found to not show any significant treatment by time interaction ($P = 0.32$), treatment ($P < 0.88$) or month effect ($P < 0.10$). Glucose values for the treatment groups are as follows: FISH (100.8 ± 1.73), FLAX (110.6 ± 1.7) and CONT (99.6 ± 1.7). Values for each sampling time across all treatments are as follows: day 0 (101.6 ± 1.6), day 30 (102.8 ± 1.6), day 60 (98.7 ± 1.6), and day 90 (98.3 ± 1.6). Basal insulin values, measured as μIU/mL, were significantly different among months ($P < 0.006$), but did not show a treatment by time interaction ($P = 0.33$) or a treatment effect ($P < 0.79$). Basal insulin increased ($P < 0.062$) from day 0 (18.9 ± 3.2) to day 30 (25.5 ± 3.2), decreased ($P < 0.067$) from day 30 (25.5 ± 3.2) to day 60 (19.0 ± 3.2), and increased ($P < 0.005$) from day 60 to day 90 (29.3 ± 3.2). In addition, day 0 was significantly lower than day 90 ($P < 0.030$).

Horses classified in the IR group were composed of five horses from the FISH group, and three horses from the FLAX and CONT groups each. Average insulin sensitivity for these horses at baseline was 0.38 ± 0.21. The IR group did not show a treatment by time interaction for SI ($P = 0.60$), but did show a main time effect ($P < 0.008$) and a trend towards a treatment effect ($P < 0.090$). Insulin sensitivity (SI) in all these horses increased from day 0 (0.38 ± 0.21) to day 90 (1.47 ± 1.5; $P < 0.002$). The FISH group (1.05 ± 0.16) showed a trend of being higher than the CONT group (0.59 ± 0.16; $P < 0.076$), but was not significantly different from the FLAX group (1.18 ± 0.16; $P < 0.55$). The FLAX (1.18 ± 0.16) and CONT (0.59 ± 0.16; $P < 0.037$) groups were different across the length of the study. Horses classified into the non-IR group did not show a treatment by time interaction or a treatment effect ($P = 0.28$), but did show a time effect ($P < 0.020$). Insulin sensitivity (SI) in these horses was higher at baseline (2.83 ± 0.41) compared to day 30 (1.32 ± 0.41; $P < 0.005$), and then increased to day 60 (2.54 ± 0.41; $P < 0.021$) and continued to increase to day 90 (2.71 ± 0.41) but was not significantly different from day 60 ($P = 0.73$).

Pearson correlation coefficients can be seen in Table 3.12. BCS and weight have a significant positive correlation ($P < 0.01$) with a coefficient of $r = 0.67$. MIRG and AIRg, and RISQI and SI show a significant correlation with a coefficient of $r = 0.60$ ($P < 0.01$) and 0.39 ($P < 0.01$), respectively. BCS and SI showed a significant negative correlation with a coefficient of $r = -0.381$ ($P < 0.003$).

Discussion

Fatty Acid Incorporation:

This is the first study to report the incorporation of supplemental dietary fatty acids into the muscle membrane of horses. In this study the FISH group had lower plasma and red blood cell LA and ALA concentrations than the FLAX or CONT groups, possibly due to higher oxidation or higher conversion to their derivatives. In accordance with plasma and red blood cell LA, muscle LA was significantly lower in the FISH group compared to the FLAX and CONT groups at day 60 and 90. This may be due to a higher oxidation rate of LA or conversion to its long chain derivatives. Muscle ALA was lower in the FISH group compared to the FLAX and CONT. Previous studies have shown that supplementation with 14 g/d of ALA increased the plasma ALA concentration in a dose-dependent manner in humans (Arterburn et al., 2006) In our study we found that horses in the CONT group only had lower ALA compared to FLAX supplemented horses in plasma, but not in muscle or RBC. The lower LA and ALA in the FISH group in this study may indicate that there may be a higher oxidation rate of LA and ALA or a conversion to their respective long-chain derivatives in the FISH group compared to the FLAX and CONT. Arachidonic acid was higher in the plasma and red blood cells of the FISH group indicating higher conversion rate of LA to its long chain derivate. Previous research showed horses supplemented with 6 g of total n-3/100 kg of body weight via fish oil had lower concentrations of LA ($p < 0.05$) and ALA ($P < 0.01$), and higher concentrations of ARA ($P < 0.06$) in the plasma versus horses fed 6 g of total n-3/100 kg via ground flaxseed or those in the control group (Vineyard et al., 2010). However, the researchers did supply one g/100 g of total fatty acids of ARA in the fish oil group, and the current study did not supply ARA to any treatment group. No other similar

supplementation study has shown similar effects indicating conversion of available LA into ARA. However, tracer studies would be necessary to confirm that theory.

In the current study, muscle ARA was not significantly different among treatment groups, indicating that perhaps ARA was being synthesized at the level of blood, but not in the muscle cell. A longer supplementation period may be required to see treatment effects due to the turnover rate of the muscle cells, although ARA was not being supplemented. It is known that the n-6 series competes with the n-3 series for incorporation into cell membranes and also as a substrate in the eicosanoid pathway. As previously mentioned, the n-6 fatty acid series can be converted from the parent fatty acid, LA, to its long-chain derivative, ARA. The n-3 fatty acids series can be converted from the parent fatty acid ALA, to its long-chain derivatives EPA and DHA. These fatty acids compete for conversion enzymes, since they utilize the same elongase and desaturase enzymes. Once the parent fatty acids have been converted to their long-chain derivatives, ARA and EPA continue to compete with one another as a substrate for cyclooxygenase and lipoxygenase to form eicosanoids from ARA and resolvins and protectins from EPA. The higher levels of ARA may also indicate that EPA was the chosen substrate for cyclooxygenase and lipoxygenase in the eicosanoid pathway, as opposed to ARA. However, in a companion study (Schauermann, 2010) done in the same horses, TNF-alpha protein, an inflammatory cytokine, was not different between treatment groups. This does not exclude the chance that cytokine expression might have been different. However, other studies reported a decrease in ARA concentration when evaluating the effects of EPA and DHA supplementation on plasma fatty acid concentrations (Blonk et al., 1990).

Eicosapentaenoic acid and DHA were only detected in the plasma and red blood cells of the FISH group, indicating that in order to observe a substantial increase of these highly unsaturated long chain fatty acids in membranes, direct supplementation is necessary. The lack of EPA and DHA in the FLAX and CONT groups in both the plasma and the red blood cells may have been due to EPA or DHA concentration that was below the detection level of the particular gas chromatograph used for analysis. However, lack of detection of EPA and DHA in horses supplemented with ground flaxseed or a control diet is also supported in previous research conducted by Vineyard et al. (2010) where both EPA and DHA were only incorporated into the plasma and red blood cell membrane of the horses supplemented with marine source of EPA and DHA. Researchers in another study found that horses exhibited an increase in plasma ARA, EPA and DHA in a dose-dependent manner when supplemented with 0g, 10g, 20g, or 40g of encapsulated fish oil (King et al., 2008). The 40g per day dose of fish oil supplied 39.13 g of EPA and DHA. The current study's dose of fish oil supplement was approximately 34 g per day (58.57 mg/kg of body weight), so results in the current study were similar to the highest dose supplied by King et al. (2008).

Previous research has shown that horses receiving flaxseed supplementation (5.6 g/100 kg body weight, equivalent to 56 mg/kg) did not have different plasma ALA concentrations compared to those receiving no supplementation (Siciliano et al., 2003). In the current study supplementation was done at higher doses and showed a dose effect (hay lower than hay plus flax). In contrast, a different study using horses (Hansen et al., 2002) showed that supplementation with 10 % flaxseed oil added to the basal pellet did increase plasma ALA concentrations as well as plasma EPA concentrations, but the rate

of feeding was much greater than the current study, 442 g of ALA per 500 kg horse from flaxseed oil, equal to 1.1g/kg of BW approximately. In addition, in humans it was shown that supplementation with ALA did increase red blood cell EPA concentrations, but not DHA concentrations. Concentrations of DHA only increased in the subjects supplemented with fish oil (Barcelo-Coblijn et al., 2008). Although the EPA concentration was present in the subjects supplemented with both the flax oil capsules and the fish oil capsules, it was the greatest in the highest dose (504 mg/d equal to 5.36 mg/kg of EPA) of the fish oil capsules at 1.66 ± 0.22 mol% of fatty acids (Barcelo-Coblijn et al., 2008).

Overall, in this study the presence of EPA and DHA in the plasma and the red blood cell were both dependent on the presence of those particular long-chain fatty acids in the diet. Even though EPA and DHA were evident in all treatment groups in the muscle membrane, indicating that there is some conversion from the parent fatty acid ALA. The FISH group had extensively higher amounts of EPA and DHA. This supports previous research that has shown supplementation with EPA and DHA has increased tissue concentrations of these fatty acids, respectively (Arterburn et al., 2006; Barcelo-Coblijn et al., 2008). The results of the current study support previous studies that found supplementation with ALA did not result in significantly greater concentrations of the long chain derivatives in plasma in humans (Arterburn et al., 2006), implying that horses may have a poor conversion efficiency as well. In addition, other studies indicate that there may be poor conversion efficiency of ALA in humans (Burdge et al., 2002; Pawlosky et al., 2001).

In the current study, detection of EPA and DHA in the muscle membrane of the FLAX and CONT group may be due to the fact that these fatty acids are synthesized at some extent in the body. At day 0, EPA and DHA were present in the muscle but did increase significantly with direct supplementation in the FISH group. Muscle DPA was lower in the FISH group compared to the FLAX and CONT at day 90. Muscle DPA concentrations have not been reported in horses, and a possible explanation for the lower values in the FISH group is that the competition between the n-3 series and n-6 series for incorporation into the muscle membrane. Eicosapentaenoic acid and DHA may be preferentially incorporated over DPA since they are being directly supplemented and conversion does not have to occur.

Insulin Sensitivity:

Insulin sensitivity, beta pancreatic response, and glucose mediated glucose disposal were not affected by fatty acid supplementation in the current study. Disposition index was higher in the FLAX than CONT group at day 90. Disposition index is a measure that is calculated from insulin sensitivity and beta pancreatic response, so results were not physiologically important because it is a calculated value. In horses classified in the lowest quintile for insulin sensitivity there was a trend for a treatment effect, where FISH and FLAX horses had higher insulin sensitivity compared to the control treatment. In previous studies in rats, insulin sensitivity improved in sucrose induced insulin resistant subjects (Soria et al., 2002; Soria et al., 2001). In healthy humans, supplementation with long chain n-3 highly unsaturated fatty acids improved glucose tolerance (DeLarue et al., 1996). Also, substituting dietary LA with ALA has been shown to improve insulin sensitivity in sucrose fed rats (Ghafoorunissa et al., 2005). It

would be interesting to test supplements in a group of horses that are insulin resistant, to possibly prove that trend.

The beta pancreatic (AIRg) values reported in this study varied with time and are higher than those reported in a study comparing the glucose and insulin dynamics among obese and non-obese horses (Hoffman et al., 2003). Obese horses (BCS of seven to nine) had an AIRg value of greater than 408 ± 49.1 and non-obese horses were much lower at 211 ± 34.7 (Hoffman et al., 2003). The current study's AIRg values averaged across all months is 714.6. However, horses in the current study did have a lower BCS compared to the obese group in Hoffman et al. (2003) indicating that horses in the current study secreted more insulin to maintain euglycemia. This may be due to a breed difference since the current study used a stock horse breed and Hoffman et al. (2003) used Thoroughbreds. The higher insulin secretion in this current study may indicate that horses used in the current study could have been more pre-disposed to insulin resistance.

Insulin sensitivity (SI) did increase significantly over the course of the study, regardless of treatment group and was correlated to a decrease in body condition score. Interestingly, the SI values in this study (1.56) were higher than the obese horses in the study conducted by Hoffman et al. (2003) which revealed the obese horses had a mean SI of 0.370 ± 27, but lower than the non-obese horses, which had a mean of 1.94 ± 0.19. However, in the current study the diet was different from the diet supplied by Hoffman et al. (2003) in which horses were supplemented with concentrates and had access to fructan rich pastures, resulting in a potentially higher consumption of non structural carbohydrates than the current study. The sugar and starch group's NSC content was 46.2 ± 0.7%, the fat and fiber groups NSC content was 14.0 ± 0.8%, the pasture's NSC

content was 8.1 ± 1.0% and the hay's NSC content was 6.9 ± 2.1%. The current study's total dietary intake of NSC was 1,678 g for the FISH group, 1,376 g for the FLAX group, and 1,737 g for the CONT group. A study performed by Treiber et al. (2005a) indicated that a group of thoroughbred weanlings had an increased insulin sensitivity ($P < 0.007$) when fed a diet higher in fat and fiber (SI = 3.59 ± 0.50; 9.7 % fat, 44 % fiber, 12.3 % non structural carbohydrates) compared to a diet high in sugar and starch (SI = 2.28 ± 0.16; 3.0 % fat, 21.3 % fiber, 49.0 % non structural carbohydrates)(Treiber et al., 2005a). The SI values for the thoroughbred weanlings are much higher than the current study's results, indicating that breed and age may have an effect on insulin sensitivity. Results of another study (Durham et al., 2009) confirmed pancreatic beta-cell dysfunction and type 2 diabetes mellitus in three horses using the minimal model analysis (Durham et al., 2009). Those horses showed very low values of AIRg (-0.12 to 21.7), and SI (0.267 to 0.619) and lay within the lowest quintile of normal horses (Durham et al., 2009; Treiber et al., 2005b). This confirmed beta cell dysfunction in these three cases (Durham et al., 2009). In the current study's horses fell into quintiles two and three for insulin sensitivity (average of 1.56 across all months in quintile three), and quintile five (average of 714.6 across all months) for beta pancreatic response and the quintiles can be seen in Table 3.6. A higher value for insulin sensitivity is desired, therefore ideally horses should fall within the higher quintiles to be considered normal in insulin sensitivity. With regard to AIRg, the higher quintiles indicate a decreased sensitivity to insulin, due to the increased amount of insulin secreted for a response to plasma glucose. However, extremely low values of AIRg, indicate beta-cell unresponsiveness or type II diabetes.

The correlation between AIRg and MIRG, and SI and RISQI, show that the basal proxies are significant indicators of their respective minimal model parameters, although other studies have shown higher correlations between the respectable parameters than those obtained in this study. The correlation coefficient of AIRg and MIRG has been reported at r = 0.754 (Treiber et al., 2005b), compared to r = 0.60 in this study. Also, a correlation coefficient of for SI and RISQI of r = 0.774 (Treiber et al., 2005b) is higher than the coefficient acquired in this study (r = 0.394). However, in the study conducted by Treiber et al. (2005b), there was a larger population size compared to this study, which is responsible for the higher correlation coefficient.

The association between BCS and insulin sensitivity throughout the study was found to have a negative correlation coefficient of r = -0.381, so the decrease in BCS could be a possible explanation of the increase in insulin sensitivity among all treatment groups and be partially responsible for and the lack of a treatment effect. In a study conducted by Vick et al. (2007), it was shown that BCS was inversely related to insulin sensitivity with a correlation coefficient of r = - 0.57 ($P < 0.01$), indicating that as horses' BCS decreased, their insulin sensitivity increased.

The mares were beginning seasonal estrous cyclicity near day 60 of sampling, which may explain particular spikes or decreases in the parameters measured. Further research in this area is needed for reference since there are few studies relating stage of the estrous cycle to insulin sensitivity in horses. Insulin sensitivity has been shown to be affected by estrous cycle ($P < 0.001$) and during the luteal phase SI was lower (3.1 ± 0.6) compared to the follicular phase (5.0 ± 0.6) (Cubitt, 2007). However, researchers did not compare insulin sensitivity during estrous and anestrous.

When horses were classified into non-IR or IR groups, the IR horses did show a trend towards a treatment effect. The highest SI values belonged to the horses in the FLAX group. This could be related to the fact that when classified at baseline, the FISH horses in the IR group had a lower mean SI (0.23) than the FLAX group (0.67), yet SI increased the most in the FISH horses by day 90 (FISH 1.72 – 0.23 = 1.49) vs. FLAX (1.92 – 0.67 = 1.25) but was not significant. The average SI totals for the treatments were highest in the FLAX horses (1.18) and then the FISH group (1.05). This could have been avoided by taking into account the SI values of the groups and using that as a block when randomly assigning horses to groups before the study began.

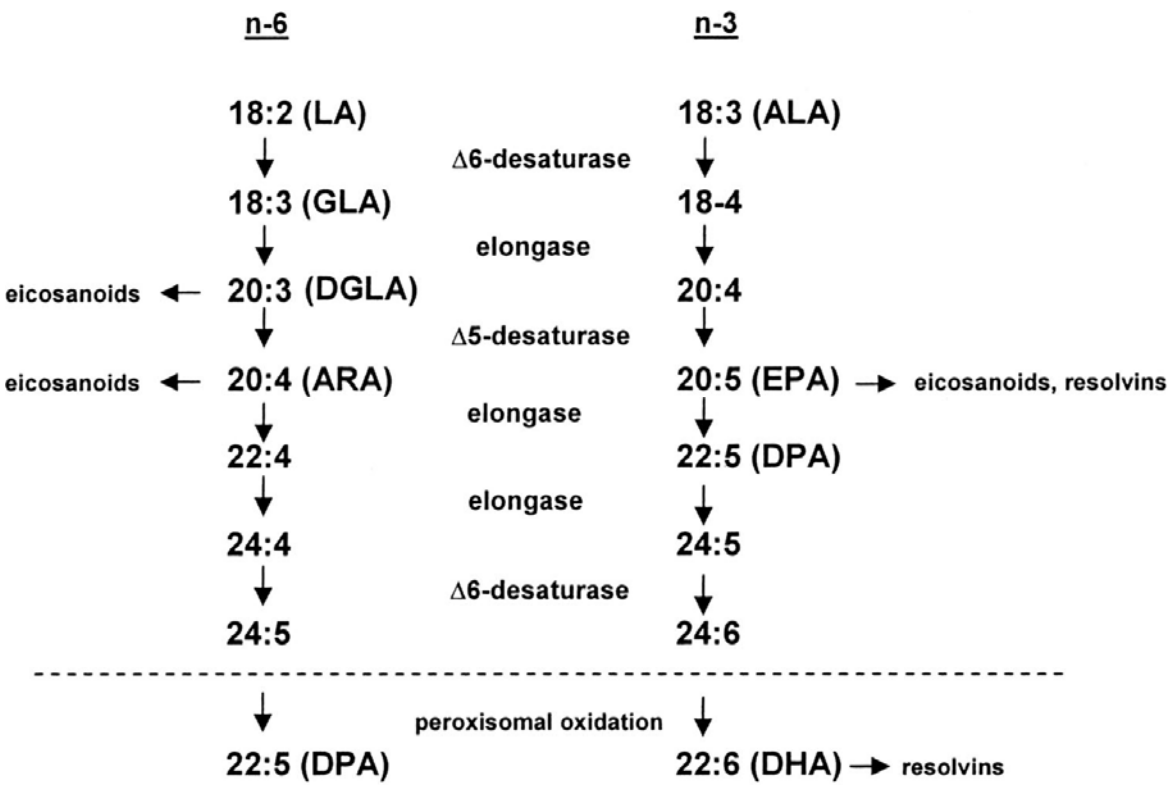
Conclusion and Implications

Direct supplementation of EPA and DHA through a marine source (fish oil) increased the concentrations in the plasma, red blood cell and muscle membranes of equines, and may have a positive effect on insulin sensitivity. A lack of treatment effects for the minimal model parameters may have been influenced by a loss of body condition score, and weight in the horses over the course of the study and perhaps by reproductive cycling,. Future research should attempt to eliminate all possible confounding factors that may influence measured outcomes. Steps to reduce confounding factors, such as a loss of body condition score and weight, in future studies could include the following: more frequent weighing, at least weekly, keeping a 0 kg body weight change by adjusting hay intake based on the weekly weights, providing a longer adaptation period to allow adjustment from prior diets, and also modifying environmental factors (such as providing shelter). In addition, future research should include reproductive records, measure blood

hormone levels, or use geldings so any possible reproductive confounding may be eliminated.

Figures and Tables

Figure 2.1: N-6 and N-3 pathways



(Arterburn et al., 2006)

Figure 2.2 Insulin Signaling Cascade

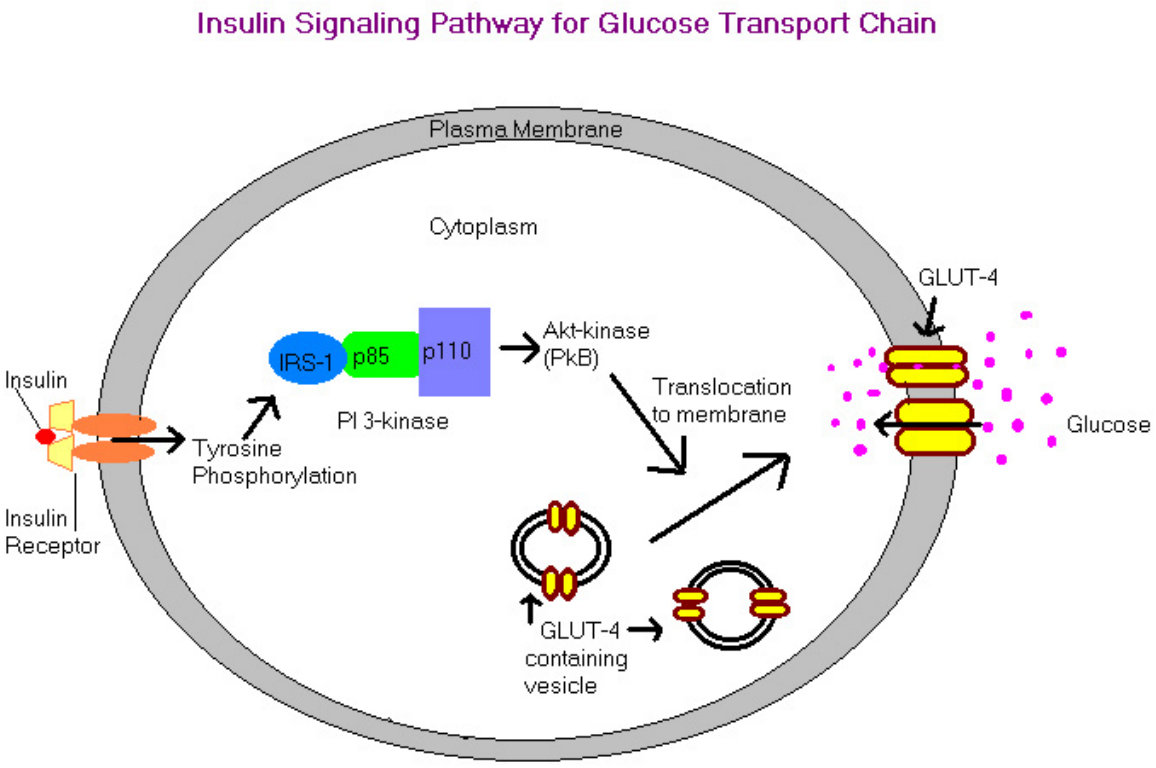


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Table 3.1: Treatment Group Means and Ranges for Age, Body Condition Score (BCS) and Weight at Baseline

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

Table 3.2: Fatty Acid composition of the diet (%)

	Hay	Barley	Magnitude™	Nutra-Flax™
C18:2 % LA	16.98	53.60	5.74	15.69
C18:3 % ALA	35.92	4.25	2.18	56.52
C20:4 % ARA	-	-	-	-
C20:5 % EPA	-	-	7.94	-
C22:5 % DPA	-	-	1.72	-
C22:6 % DHA	-	-	27.64	-

Table 3.3: Nutritional Analysis of the diet

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

Table 3.4: Dietary Intake for Treatment Groups

	FISH	FLAX	CONT
DE (Mcal)	24.71	24.64	24.65
Protein (kg)	1.937	2.075	1.935
Starch (kg)	0.891	0.579	0.941
ADF (kg)	3.010	3.314	3.002
NDF (kg)	3.932	4.290	3.945
Fat (mg/kg BW)	402.9	360.2	256.0
Fatty Acids (mg/kg of BW)			
C18:2 LA	61.84	66.08	61.91
C18:3 ALA	80.97	149.78	78.15
C20:4 ARA	-	-	-
C20:5 EPA	13.06	-	-
C22:6 DHA	45.51	-	-

Table 3.5: Reference Quintiles adapted from Treiber et al., 2005b

Quintile	**1**	**2**	**3**	**4**	**5**
SI X 10^4 (L•min–1•mU–1)	0.14–0.78	0.79–1.50	1.51–2.27	2.28–3.04	3.05–5.94
Sg X 10^2 (min-1)	0.09–0.72	0.73–0.88	0.89–1.28	1.29–1.92	1.92– 2.96
AIRg (mU/L•min–1)	60–148	149–190	191–273	274–337	338–808
DI X 10^4	30–207	208–316	317–427	428–817	818–1,752

Table 3.6: Mean BCS, Weight Across All Treatments

Day	BCS	Weight (kg)
0	6.74 ± 0.85^{a}	585.7 ± 41.51^{a}
30	6.74 ± 0.75^{a}	578.5 ± 36.38^{b}
60	6.57 ± 0.78^{a}	565.0 ± 36.59^{c}
90	6.38 ± 0.82^{b}	576.1 ± 36.81^{b}

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

Table 3.7: Climate Values During the Month of Sampling

Month (Day)	Mean Temperature	Total Precipitation	Average Wind Speed
February	35.7 °F	0.23”	7.1 mph
March (0)	40.4 °F	1.50”	8.0 mph
April (30)	44.9 °F	4.36”	7.8 mph
May (60)	57.3 °F	1.93”	6.3 mph
June (90)	61.5 °F	5.83”	4.9 mph

Table 3.8: Plasma fatty acid incorporation (%)

	TRT				P <		
Item	**FISH**	**FLAX**	**CONT**	**sem**	**TRT**	**TIME**	**TRT*TIME**
C18:2 LA	33.4 [A]	36.9 [B]	36.1 [B]	0.60	0.002	0.001	0.001
d 0 [1]	38.1 [a]	37.1 [a]	36.9 [a]	0.52	-	0.001	0.88
d 30 [1]	34.0 [a]	38.5 [b]	40.0 [b]	0.51	-	0.001	0.23
d 60 [2]	30.1 [a]	36.0 [b]	34.5 [b]	0.52	-	0.001	0.26
d 90 [2]	31.5 [a]	35.9 [b]	33.1 [a]	0.52	-	0.001	0.040
C18:3 ALA	0.94 [A]	1.74 [B]	1.53 [C]	0.055	0.001	0.001	0.001
d 0 [1]	1.44 [a]	1.41 [a]	1.53 [a]	0.045	-	0.001	0.80
d 30 [2]	0.55 [a]	1.47 [b]	1.03 [c]	0.045	-	0.001	0.001
d 60 [1]	0.85 [a]	1.85 [b]	1.75 [b]	0.046	-	0.001	0.37
d 90 [3]	0.94 [a]	2.22 [b]	1.82 [c]	0.045	-	0.001	0.001
C20:3 DGLA	1.41	1.37	1.61	0.16	0.633	0.95	0.15
d 0	-	-	-	-	-	-	-
d 30	1.15	1.49	1.77	0.17	-	0.001	0.61
d 60	1.66	1.25	1.45	0.078	-	0.001	0.37
d 90	-	-	-	-	-	-	-
C20:4 ARA	2.32 [A]	1.87 [B]	1.95 [B]	0.078	0.002	0.010	0.66
d 0	1.53	2.00	1.54	0.068	-	0.001	0.95
d 30 [1]	2.11	1.91	1.84	0.068	-	0.001	0.94
d 60 [2]	2.52	2.13	2.04	0.068	-	0.001	0.97
d 90 [1]	2.23	1.76	1.89	0.068	-	0.001	0.050
C20:5 EPA	2.69	-	-	0.19	-	0.001	-
d 0	-	-	-	-	-	-	-
d 30 [1]	2.17	-	-	0.21	-	0.001	-
d 60 [2]	2.86	-	-	0.21	-	0.001	-
d 90 [2]	3.03	-	-	0.21	-	0.001	-
C22:6 DHA	4.00	-	-	0.26	0.001	0.002	-
d 0	-	-	-	-	-	-	-
d 30 [1]	3.65	-	-	0.19	-	-	-
d 60 [2]	3.91	-	1.28	0.20	-	0.001	-
d 90 [3]	4.44	-	-	0.19	-	-	-

[A, B, C, a, b, c] Values within rows lacking common superscripts differ by $P \leq 0.05$

[1, 2, 3] Values within columns under a certain variable lacking common superscripts differ by $P \leq 0.05$

Table 3.9: Red Blood Cell fatty acid incorporation (%)

	TRT				P <		
Item	**FISH**	**FLAX**	**CONT**	**sem**	**TRT**	**TIME**	**TRT*TIME**
C18:2 LA	28.6	29.6	29.9	0.60	0.300	0.001	0.030
d 0 [12]	30.0 [a]	29.6 [a]	30.4 [a]	0.45	-	0.001	0.73
d 30 [12]	29.9 [a]	30.6 [a]	31.7 [a]	0.44	-	0.001	0.49
d 60 [1]	28.8 [a]	29.4 [a]	28.8 [a]	0.45	-	0.001	0.98
d 90 [3]	25.8 [a]	28.6 [b]	28.7 [b]	0.45	-	0.001	0.94
C18:3 ALA	4.77	5.60	6.14	0.38	0.070	0.001	0.47
d 0	7.48	3.10	3.19	0.64	-	0.001	0.95
d 30 [1]	2.05	2.75	2.71	0.64	-	0.001	0.81
d 60 [2]	9.48	11.3	12.5	0.64	-	0.001	0.16
d 90 [1]	2.28	3.42	3.12	0.64	-	0.001	0.97
C20:4 ARA	2.15 [A]	1.44 [B]	1.47 [B]	0.086	0.001	0.010	0.056
d 0	1.92	2.85	3.07	0.073	-	0.001	0.23
d 30 [1]	1.74	1.58	1.78	0.073	-	-	0.37
d 60 [1]	1.99	1.67	1.65	0.073	-	-	0.41
d 90 [2]	1.78	1.41	1.56	0.073	-	0.001	0.59
C20:5 EPA	2.13	-	-	0.15	0.047	0.001	-
d 0	-	-	-	-	-	-	-
d 30 [1]	1.67	-	-	0.16	-	0.001	-
d 60 [2]	2.34	-	-	0.16	-	0.001	-
d 90 [2]	2.38	-	1.28	0.22	-	0.001	-
C22:6 DHA	2.63	-	-	0.18	-	0.001	-
d 0	-	-	-	-	-	-	-
d 30 [1]	2.04	-	-	0.19	-	-	-
d 60 [2]	2.75	-	-	0.19	-	-	-
d 90 [3]	3.10	-	-	0.19	-	-	-

[A, B, C, a, b, c] Values within rows lacking common superscripts differ by $P \leq 0.05$

[1, 2, 3] Values within columns under a certain variable lacking common superscripts differ by $P \leq 0.05$

Table 3.10: Muscle fatty acid incorporation (%)

	TRT				P <		
Item	**FISH**	**FLAX**	**CONT**	**sem**	**TRT**	**TIME**	**TRT*TIME**
C18:2 LA	27.8 [A]	32.7 [B]	33.7 [B]	0.64	0.001	0.001	0.020
d 0	25.6 [a]	28.4 [a]	32.4 [a]	0.65	-	0.001	0.086
d 30 [1]	26.8 [a]	28.4 [ab]	31.0 [b]	0.65	-	0.001	0.25
d 60 [2]	27.4 [a]	33.8 [b]	37.2 [b]	0.68	-	0.001	0.16
d 90 [2]	28.6 [a]	34.8 [b]	35.1 [b]	0.65	-	0.001	0.80
C18:3 ALA	1.27 [A]	1.87 [B]	1.88 [B]	0.12	0.005	0.294	0.90
d 0	2.30	1.55	1.46	0.13	-	0.001	0.77
d 30	1.16	1.74	1.79	0.14	-	0.001	0.74
d 60	1.49	1.93	2.08	0.15	-	0.001	0.67
d 90	1.18	1.97	1.77	0.13	-	0.001	0.52
C20:3 DGLA	2.26	1.96	1.99	0.11	0.123	0.001	0.65
d 0 [1]	1.82	1.72	1.71	0.12	-	0.001	0.99
d 30 [2]	2.70	2.54	2.59	0.11	-	0.001	0.85
d 60 [1]	2.65	1.82	1.89	0.16	-	0.001	0.87
d 90 [1]	1.86	1.74	1.77	0.14	-	0.001	0.90
C20:4 ARA	6.71	7.11	6.75	0.26	0.530	0.084	0.15
d 0 [12]	6.21	6.66	6.94	0.24	-	0.001	0.61
d 30 [12]	6.94	6.53	6.11	0.24	-	0.001	0.46
d 60 [1]	7.16	7.50	6.90	0.25	-	0.001	0.65
d 90 [12]	6.54	7.73	7.05	0.24	-	0.001	0.35
C20:5 EPA	2.35 [A]	1.58 [B]	-	0.15	0.001	0.135	0.009
d 0	2.23 [a]	2.49 [a]	-	-	-	-	0.62
d 30	1.62 [a]	1.58 [a]	-	-	-	-	0.93
d 60	2.82 [a]	1.16 [b]	1.33 [b]	0.13	-	0.001	0.63
d 90	2.72 [a]	1.09 [b]	1.08 [b]	0.11	-	0.001	0.98
C22:5 DPA	2.18 [A]	2.69 [B]	2.49 [AB]	0.13	0.047	0.154	0.004
d 0	2.71 [a]	2.34 [a]	2.38 [a]	0.14	-	0.001	0.88
d 30	2.58 [a]	2.83 [a]	2.50 [a]	0.10	-	0.001	0.75
d 60	1.90 [a]	2.71 [ab]	2.43 [a]	0.14	-	0.001	0.29
d 90	1.54 [a]	2.89 [b]	2.66 [b]	0.10	-	0.001	0.37
C22:6 DHA	7.96 [A]	4.55 [B]	4.11 [B]	0.27	0.001	0.018	0.050
d 0 [1]	5.44 [a]	4.33 [ab]	3.19 [b]	0.27	-	0.001	0.16
d 30 [2]	6.65 [a]	4.96 [b]	4.00 [b]	0.26	-	0.001	0.23
d 60 [2]	8.62 [a]	4.25 [b]	2.76 [b]	0.28	-	0.001	0.081
d 90 [3]	8.96 [a]	4.62 [b]	4.88 [b]	0.26	-	0.001	0.54

[A, B, C, a, b, c] Values within rows lacking common superscripts differ by $P \leq 0.05$

[1, 2, 3] Values within columns under a certain variable lacking common superscripts differ by $P \leq 0.05$

Table 3.11: Minimal Model parameters and basal proxies

	TRT				P <		
Item	**FISH**	**FLAX**	**CONT**	**sem**	**TRT**	**TIME**	**TRT*TIME**
AIRg 1/(mU/L)·min	632.3	748.0	763.5	147.3	0.79	0.001	0.94
d 0 [12]	563.0	689.7	707.6	163.5	-	0.001	0.94
d 30 [3]	721.7	808.6	851.9	163.5	-	0.001	0.85
d 60 [1]	474.3	645.0	678.8	164.7	-	0.001	0.88
d 90 [23]	770.1	848.8	815.7	163.5	-	0.001	0.89
SI 1/(mU/L·min)	1.62	1.64	1.42	0.30	0.85	0.037	0.65
d 0 [12]	1.47	1.81	1.33	0.47	-	0.001	0.83
d 30 [12]	1.52	0.61	0.91	0.47	-	0.001	0.66
d 60 [12]	1.54	1.82	1.46	0.47	-	0.004	0.89
d 90 [13]	1.96	2.30	1.99	0.47	-	0.001	0.97
Sg 1/min	0.017	0.020	0.014	0.002	0.20	0.041	0.90
d 0 [12]	0.017	0.021	0.012	0.002	-	0.001	0.45
d 30 [12]	0.020	0.020	0.014	0.002	-	0.001	0.98
d 60 [13]	0.014	0.015	0.013	0.002	-	0.001	0.87
d 90 [12]	0.019	0.023	0.017	0.002	-	0.001	0.61
DI AIRg·SI	836.2	1145.6	800.2	195.8	0.41	0.002	0.030
d 0 [1]	600.8 [a]	1172.9 [a]	810.3 [a]	280.5	-	0.001	0.60
d 30 [1]	891.9 [a]	394.5 [a]	597.5 [a]	280.5	-	0.003	0.61
d 60 [1]	549.1 [a]	976.8 [a]	876.0 [a]	285.5	-	0.001	0.80
d 90 [2]	1303.1 [a b]	2038.3 [a]	917.0 [b]	280.5	-	0.001	0.070
MIRG	7.03	7.40	7.69	0.64	0.76	0.040	0.19
d 0 [1]	7.09	6.03	8.01	0.83	-	0.001	0.43
d 30 [1]	7.37	7.29	7.69	0.83	-	0.001	0.95
d 60 [12]	6.32	6.96	7.47	0.83	-	0.001	0.67
d 90 [13]	7.32	9.31	7.59	0.83	-	0.001	0.82
RISQI	0.24	0.24	0.22	0.016	0.78	0.002	0.066
d 0 [1]	0.24	0.28	0.21	0.022	-	0.001	0.44
d 30 [12]	0.23	0.24	0.21	0.022	-	0.001	0.74
d 60 [13]	0.28	0.25	0.23	0.022	-	0.001	0.67
d 90 [12]	0.21 [a]	0.19 [a]	0.24 [a]	0.022	-	0.001	0.48

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

MIRG: $\text{insulin}^{-0.5}$, RISQI: $[800-0.30(\text{insulin}-50)^2]/(\text{glucose}-30)$

Table 3.12: Pearson Correlation Coefficients

Item	BCS	Weight	RISQI	MIRG	AIRg	SI	Sg	DI	Glucose	Insulin	IR
Age	0.338***	0.268**	NS	NS	NS	-0.195*	NS	NS	NS	NS	NS
BCS		0.667***	-0.266**	0.184*	0.313***	-0.381***	NS	-0.271**	0.251**	0.250**	-0.218**
Weight			NS	NS	NS	NS	NS	NS	0.208*	NS	NS
RISQI				-0.838***	-0.477***	**0.394****	NS	NS	-0.344***	-0.873***	0.379***
MIRG					**0.600****	-0.296***	NS	NS	NS	0.650***	-0.289***
AIRg						-0.391***	NS	0.299***	NS	0.336***	-0.295***
SI							NS	0.564***	-0.251**	-0.281***	0.489***
Sg								0.331***	-0.341***	NS	0.200*
DI									-0.248**	NS	0.241**
Insulin									0.357***		

*P < 0.10, **P < 0.05, ***P < 0.01

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