EFFECT OF OMEGA-3 FATTY ACID SUPPLEMENTATION AND A CONTROLLED-RELEASE INTRAMUSCULAR THYROXINE INJECTION ON SERUM INSULIN CONCENTRATIONS IN HORSES

by

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ABSTRACT

A completely randomized design experiment was conducted to examine the effects of omega-3 fatty acid and thyroxine supplementation on serum thyroxine and insulin concentrations in horses. Thirty-thee geldings and 11 mares (aged 5 to 27 yr) were stratified by preliminary 4-h postprandial serum insulin concentration. All horses were then randomly assigned to 1 of 3 treatments: control (C), daily oral omega-3 PUFA supplementation (O), or monthly controlled release IM thyroxine microparticle injection (T). The 114-d trial consisted of a d 0 baseline sample, followed by a 30-d and 3 subsequent 28-d periods, named for the month in which the sample was collected – August, September, October, November and December. At the beginning of each period, venous blood was collected to determine serum thyroxine and insulin concentrations. Thyroxine-treated horses had greater ($P < 0.001$) serum thyroxine concentrations compared to both C and O horses. Thyroxine-treated horses also had decreased ($P = 0.001$) serum insulin concentrations compared to both C and O horses.

Results from this experiment indicate that IM thyroxine microparticle injection is an effective method of increasing serum thyroxine concentrations results in decreased serum insulin concentrations compared to untreated animals.

Key words: horse, insulin, omega-3 fatty acid, oral glucose challenge, thyroxine

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CHAPTER I

INTRODUCTION

Metabolic disorders are a growing concern in the modern day equine industry, due to their harmful, and sometimes lethal, secondary complications. Horses are anatomically designed to be roaming, grazing animals; traveling many miles thoughout the day and feasting on fibrous roughage sources as they go. As the horse has evolved from a wild prey animal, to a beast of burden utilized for work and travel, to a companion animal utilized for pleasure; the human viewpoint of the horse has altered inducing many changes in the way we manage them, from both a nutritional and physical standpoint. Many horses are now stalled or kept on small pastures with minimal structured exercise and are fed diets laden with nonstructural carbohydrates, such as sweet feeds and high grain concentrates, which compromise the horse's ability to utilize its hindgut fermentation vat, the cecum, to employ fiber sources for energy production. These alterations in management have resulted in an increase in the prevalence of overweight and obese horses, which increases the animal's risk of developing insulin resistance (IR) and more serious secondary disorders associated with IR. Obesity has been known to cause IR in humans via a process known as lipotoxicity, which occurs as a consequence of high free fatty acid concentration on insulin-sensitive tissues. As the movement

of free fatty acids into tissues increases with excess nutrient availability, skeletal myocytes accumulate diacylglycerols that interfere with insulin signaling and may result in IR (Slawik and Vidal-Puig, 2006). Frank (2009) concluded that weight reduction and management can improve insulin sensitivity in the horse.

Several survey studies have been conducted over the past 20 years assessing the prevalence of obesity among horses and ponies in various countries, due to its role in predisposing horses to serious secondary health issues. In 1998, the USDA National Animal Health Monitoring System (NAHMS) conducted a study estimating that approximately 1.4% of the U.S. horse population was overweight or obese (USDA, 2017). In 2008, another U.S. study was conducted within a subpopulation of horses in Virginia in which a random sample of 300 horses were assessed for various body composition parameters. Of the 300 subjects, 32% were found to be over conditioned and 19% were deemed obese, a marked difference from the projection of the previous study (Thatcher et al., 2008). In the summer of 2011, a UK study that took place at a championship for leisure horses reported 41% of the population of horses to be overweight and 21% to be obese (Harker et al., 2011). More recent studies in 2011 and 2016 in the UK and Australia, respectively, also reported 20% or higher obesity rates among observed populations (Giles et al., 2011; Potter et al., 2016). Collectively, this data indicates that a sizeable number of horses are at risk of developing metabolic disorders, such as IR, which may lead to more harmful health issues such as laminitis (Frank, 2009).

Omega-3 fatty acid supplementation may aid in weight management and reduction in humans (Golub et al., 2011). O'Connor et al. (2004) observed that omega-3

fatty acid supplementation alters glucose metabolism in conditioned horses. Treatment with levothyroxine sodium has been used to aid in the weight management of both humans (American Thyroid Assoc., 2016) and horses (Frank et al., 2006, 2008b). The reduction of body weight, and therefore body fat, has been associated with improved insulin sensitivity in both humans and horses (Slawik and Vidal-Puig, 2006; Frank, 2009). Therefore, the objective of the present study was to assess the effect of omega-3 fatty acid supplementation and a thyroxine microparticle injection on insulin sensitivity in horses.

CHAPTER II

LITERATURE REVIEW

Equine Metabolic Syndrome

Equine metabolic syndrome (EMS) is a condition that has raised concern across the industry for several decades (Johnson, 2002). Affected horses display clinical signs of metabolic efficiency, obesity, and enlarged adipose tissue deposits around the neck and tail head of the animal (Johnson, 2002). The term was coined in 2002 by Johnson to better define a condition that was previously attributed to hypothyroidism, due to the observation that affected animals not only fit the profile of EMS, but in some cases also had low resting total thyroxine (T_4) concentrations (Frank et al., 2002; Johnson et al., 2005). However, the hypothesis that hypothyroidism induced this phenotype was never supported by research studies and thyroidectomized horses failed to develop obesity, abnormal adipose tissue deposits, or laminitis (Frank et al., 2003). Additionally, horses displaying the aforementioned signs responded normally to thyroid challenge tests, indicating that many non-thyroidal conditions are also capable of resulting in reduced resting T_4 concentrations (Breuhaus et al., 2006). Therefore, one author concluded that low resting T_4 concentrations are a consequence, rather than a cause, of EMS in horses and ponies (Frank et al., 2008c).

Clinical signs of EMS include insulin resistance (IR), obesity, regional adiposity, hypertriglyceridemia, and hyperleptinemia (Frank, 2009). This condition has been associated with increased laminitis risk, altered reproductive function, and seasonal alterations in arterial blood pressure (Frank et al., 2006, Treiber et al., 2006, and Bailey et al., 2008). Because of the complications associated with EMS, interest is high in the equine industry to research strategies to both combat and prevent obesity and EMS. However, in order to understand the pathophysiology of IR and its effects, we must first address its various presentations and how equids reach an insulin-resistant state (Frank et al., 2008c).

Insulin and Insulin Resistance

Insulin is a polypeptide consisting of an A and B chain of 21 and 30 amino acids that are linked by a pair of disulfide bonds and is one of a number of hormones required for normal growth and development. Insulin plays a role in metabolism and is the only hormone that directly lowers blood glucose levels (Hadley and Levine, 2006). In response to elevated glucose levels, insulin is released from β-cells within the pancreatic islet. Insulin interacts with receptors on the plasma membrane in many tissues, but most importantly, on hepatic, muscle and adipose tissue cells. In each case, the role of insulin is to enhance the uptake of glucose into cells where it is metabolized and stored as glycogen or used as an energy substrate in the synthesis of proteins or fats (Hadley and Levine, 2006).

Within the liver, insulin activates glycogen synthetase which produces a direct flow of glucose toward glycogen formation (Hadley and Levine, 2006). Glycogen is a polysaccharide of glucose that serves as the storage form of glucose in hepatic and

muscle tissue in animals. Glycogen is readily broken back down to glucose for use as an energy source when needed (Sell et al. 2012).

Insulin stimulates the active transport of glucose and amino acids into muscle cells, and, subsequently, enhances protein synthesis. Glycolysis and oxidative phosphorylation of glucose derivatives provide the energy required for such metabolic activity (Hadley and Levine, 2006).

Lipid synthesis is stimulated by insulin. In adipose tissue, insulin-stimulated glucose uptake results in enhanced catabolism of the sugar to glycerol. Insulin activation of endothelial cell lipoprotein lipase results in the release of free fatty acids (FFA) from chylomicrons. These fatty acids are then transported into fat cells where they combine with glycerol to form triglycerides and are added to the lipid droplets within the fat cells (Hadley and Levine, 2006).

In some individuals, insulin action may be compromised due to obesity or other complications resulting in a need for the body to produce more insulin to compensate for the decreased action (Hadley and Levine, 2006). In such cases, the individual or animal may become IR. Insulin resistance plays a key role in the onset of diabetes mellitus in humans and EMS in horses (Frank et al. 2008c).

Obesity

Obesity has been considered a global epidemic in humans since 1997 (WHO, 2000). Obesity has been observed to cause a variety of serious health consequences, including IR, or type II diabetes mellitus, and metabolic syndrome (Mokdad, 2003). Reduced caloric intake and increased exercise are typically the first and primary recommendations to combat obesity. However, studies show that there is little success in

weight loss of this fashion and that, if there is, it is difficult for patients to maintain the loss (Summerbell et al., 2005; Wing et al., 2006).

Golub et al. (2011) discussed the impact that appetite regulation may have on the induction and maintenance of excess weight and obesity; stating that it is a complex process involving multiple organ systems. When food enters the gastrointestinal tract the vagus nerve relays information concerning pH, gastric stretch and changes in nutrient composition to areas within the brain. These signals are involved in the regulation of feeding. The GIT also secretes hormones that control feeding by acting on the brain. For example, cholecystokinin serves as a satiety signal to the brain and ghelin acts on the hypothalamus to induce feeding. Another key hormone involved in appetite and metabolism is leptin, which is released from adipose tissue. Leptin production is increased in individuals with greater fat mass and decreased in those with lower fat mass. Leptin acts as a satiety signal and stimulates energy expenditure resulting in weight loss. Although leptin production is increased in obese individuals, they may become leptin resistant, due to decreased responsiveness to leptin signaling, a mechanism similar to what occurs in IR, known as hyperleptinemia (Golub et al., 2011).

The endocannabinoid pathway is another key component involved in the regulation of appetite and metabolism (Ahima and Antwi, 2008). Endocannabinoids are lipids derived from arachidonic acid, an omega-6 polyunsaturated fatty acid. Circulating levels of endocannabinoids are regulated by dietary intake of essential fatty acids and the activity of biosynthetic and catabolic enzymes involved in the endocannabinoid pathway (Banni and Di Marzo, 2010). Endocannabinoids activate endogenous cannabinoid CB1 and CB2 receptors in the brain, liver adipose tissue and GIT (Artmann et al., 2008).

Activation of CB1 receptors in the hypothalamus leads to increased appetite and food intake by inhibiting the anorexigenic signals of corticotropin-releasing hormone and cocaine-amphetamine regulated transcript, and activating the orexigenic signal of melanin-concentrating hormone, as well as via other mechanisms (Matias and Di Marzo, 2007). Additionally, research in mice has revealed that endocannabinoids selectively enhance sweet taste, increasing the palatability of foods and increasing feed intake (Yoshida et al., 2010).

In humans and rodents, obesity is associated with elevated free fatty acid concentrations, altered adipokine production by adipose tissues and elevated inflammatory cytokine concentrations within the blood (Ritchie et al., 2004). Obesity and IR are related to one another due to a process called lipotoxicity. This process occurs as a consequence of high FFA concentration on insulin-sensitive tissues. As the movement of FFAs into tissues increases with excess nutrient availability, skeletal myocytes accumulate diacylglycerols that interfere with insulin signaling. Lipotoxicity can result in IR (Slawik and Vidal-Puig, 2006). However, genetics play a role in individual susceptibility to IR, so this process doesn't occur in all cases. This is a possible explanation for the observation that, although IR often accompanies obesity in horses, not all obese horses are IR (Frank, 2009).

Obesity Associated Equine Metabolic Syndrome

Horses with EMS most commonly present as obese with regional adiposity. The major problem in the equine industry that we see today is the interaction between enhanced metabolic efficiency and feeding practices on the farm (Frank, 2009). Horses affected by EMS are able to maintain their body condition with a lower caloric intake and

are often referred to as “easy keepers”. When such horses are restricted to stalls or small pens where their ability to exercise is limited, overfed diets high in nonstructural carbohydrates and insufficiently exercised, their risk of becoming obese is increased. Metabolic efficiency is likely determined, at least in part by genetics, although further research is needed in this area (Frank, 2009). Treiber et al. (2006) conducted one of the few studies addressing this question, in which a hereditary pattern for metabolic status and laminitis predisposition was observed within a closed herd of ponies. According to observations by Frank et al. (2006), EMS is most commonly observed in Morgans, Paso Finos and various pony breeds, but has also been observed in Arabians, Quarter Horses, Saddlebreds, Tennessee Walking Horses, Thoroughbreds, and Warmbloods, indicating that many breed groups are affected.

Frank (2009) describes regional adiposity as the expansion of adipose tissues in certain regions of the body. Analogous to the way that humans accumulate adipose tissue around the waist and hips, horses do so in their neck and tailhead regions, as well as over the ribs and back (Marchesini et al., 2004; Frank et al., 2006). Adipose tissues may also be detected in the prepuce or close to mammary glands in the obese horse and are occasionally seen as randomly distributed subcutaneous masses along the abdomen (Frank, 2009). A scoring system has been developed by Carter et al. (2009) that assesses expansion of adipose tissues around the neck as an indicator of EMS. This physical characteristic is commonly referred to as a cresty neck. An increased neck circumference has been associated with IR in both horses and ponies (Frank et al., 2006; Carter et al., 2009)

Equine Metabolic Syndrome in the Lean Horse

According to Frank (2009), EMS is less commonly observed in leaner horses and it is likely that these animals are affected by a different manifestation of the condition. In the lean animal, the key features of EMS are regional adiposity and increased laminitis risk. These horses typically fall into 1 of 2 categories: (1) horses that were previously obese and are now being maintained in a leaner body condition with effective management, and (2) leaner horses with regional adiposity, IR, and laminitis that do not test positive for pituitary pars intermedia dysfunction (PPID). Middle aged horses (10 to 20 y) or older horses in this category may suffer from PPID that has not progressed to the point that it is detectable by diagnostic tests. Common diagnostic tests for PPID include the dexamethasone suppression test and measurement of adrenocorticotropin hormone in the blood. Unfortunately, these tests likely have limited sensitivity to PPID in its early or mild stages, so it is advisable to consider this possibility when examining middle-aged and older horses. Younger horses that remain lean may also develop EMS, with the horse exhibiting regional adiposity, IR and laminitis while being lean across the ribs and topline. A potential explanation for this presentation is that EMS affected horses have adipose tissues in certain regions of their body that are more metabolically active. In humans, the expansion of adipose tissues in the abdomen is sometimes referred to as visceral or omental adiposity. This condition increases fatty acid uptake into the liver, resulting in hepatic IR (Wasada et al., 2008). The same could be true in these younger, leaner animals suffering from EMS (Frank, 2009).

Pituitary Pars Intermedia Dysfunction

Pituitary pars intermedia dysfunction (PPID), also known as equine Cushing's disease, is a complex progressive disease of the pituitary gland of middle aged and older horses (McCue, 2009). In some cases, horses affected by PPID are also insulin resistant. The pituitary gland is a small structure located at the base of the brain that produces hormones to regulate many physiological functions within the body. Pituitary pars intermedia dysfunction is caused by a decrease in the production of dopamine, an important neurotransmitter that interacts with the pars intermedia of the pituitary gland. Lack of dopamine results in an increase in the size of the pituitary gland, or a pituitary adenoma. This results in an increase in the production of adrenocorticotropic hormone and other hormones produced by the pituitary. Elevated circulating levels of ACTH result in overproduction of cortisol, a hormone associated with stress, from the adrenal gland (McCue, 2009).

Pituitary adenomas are caused by cellular oxidative stress which can be induced by many factors, including metabolic disorders such as IR (McFarlane et al., 2003 and Gospodaryov and Lushchak, 2012). Frank et al. (2008c) discussed the concept of a transition state between a state of IR and the development of PPID. Evidence of a transition state is more recognizable in obese horses as the shift in energy metabolism is more easily detected. An easy keeper will require more calories for weight maintenance and begin to lose weight and muscle mass as pituitary dysfunction progresses. As the obese insulin-resistant horse transitions into a leaner body condition, PPID replaces obesity as the cause of IR (Frank et al., 2008c).

Clinical signs of PPID include long and curly hair coat, increased water consumption and urination, excessive sweating and lactation. Horses with PPID will also

suffer from muscle wasting, particularly over the topline, giving them a sway-backed appearance (McFarlane et al., 2003).

Pancreatic Insufficiency and Diabetes Mellitus

Horses and ponies with chonic IR have elevated blood insulin concentrations, indicating that the pancreas secretes more insulin to compensate for the reduction of insulin function in tissues. However, reduced insulin clearance by the liver likely contributes to hyperinsulinemia in the EMS horse (Frank, 2009). One other author has estimated that 60% of secreted insulin is cleared by the liver on its first pass as portal blood passes though before entering the systemic circulation (Wilcox, 2005). Therefore, hepatic IR may contribute to hyperinsulinemia in affected horses.

Treiber et al. (2005) speculated that pancreatic insulin secretion may decline after the horse has spent many years in a state of compensated IR. This may be referred to as uncompensated IR, pancreatic insufficiency, pancreatic exhaustion, or pancreatic failure (Frank, 2009). In cases such as this, the horse will become hyperglycemic and it is appropriate to refer to this condition as diabetes mellitus, which has been observed by Baker and Ritchie (1974) and Johnson et al. (2005). In the experience of Frank (2009), horses with diabetes mellitus have a leaner body condition and have been suspected or confirmed to have concurrent PPID. According to Jeffrey (1968) and Bakos et al. (2008), diabetes mellitus may also be associated with current or previous pancreatitis.

Hepatic Insulin Resistance

Hepatic IR may occur in either obese or lean animals with EMS if lipid accumulates in the liver as a result of elevated FFA concentrations (Wasada et al., 2008). Frank (2009) stated that only limited evidence of hepatic IR has been presented in horses

and that this data has not been published. In these cases, higher gamma glutamyl transferase activities have been detected in some obese animals with EMS, and mild hepatic lipidosis check spelling has been detected on post-mortem analysis in these cases. Bailey et al. (2008) observed that leaner ponies with EMS have greater insulin responses to fructan when this carbohydrate is provided in a meal than healthy ponies, who fail to respond to the same challenge (Bailey et al., 2008). The liver plays a role in the metabolism of fructose, so the response to fructan may be developed into a diagnostic test for EMS in the future (Frank, 2009).

Laminitis

Increased nonstructural carbohydrate intake exacerbates IR in susceptible animals and may also trigger laminitis by inducing intestinal disturbances (Frank et al., 2008c). Horses are hindgut fermenters who host a microbial population in their cecum and colon. The microbes serve to ferment complex carbohydrates that reach the colon, converting them to volatile fatty acids that are used as an energy source. Microbes are not well acclimated to digestion of nonstructural carbohydrates. When they are exposed to large quantities of nonstructural carbohydrates, they perish due to increased load of lactic acid produced by another subset of microbes. When these microbes die, they release endotoxins and exotoxins into circulation. The toxins can then travel to the hoof where they interfere with endothelial cell function, which results in decreased circulation to the laminae inside the hoof. Decreased lamellar circulation causes stimulation of matrix metalloproteases and separation of the laminae at the dermo-epidermal junction. In the modern equine industry, horses are commonly fed diets high in nonstructural carbohydrates, as corn and other grains are key ingredients in many horse feeds. Horses

may also gain access to nonstructural carbohydrates when the nutrient composition of forages changes or when forage is available in a greater supply than normal (Frank et al., 2008c).

Role of Insulin Resistance in the Induction of Laminitis

Treiber et al. (2006) concluded that IR is the component of EMS that is most likely to predispose horses to laminitis. Insulin resistance is broadly described as the failure of normal concentrations of insulin to induce its expected response in target tissues (Kahn, 1978). The primary function of insulin is to stimulate the uptake of glucose when nutrients are prevalent, for instance, following a feeding. The two major sites of insulin-mediated glucose uptake are skeletal muscle and adipose tissue, but the liver also responds to insulin by increasing glucose uptake from circulating blood. Insulin binds to receptors on the plasma membrane of cells, initiating a signaling cascade that results in the translocation of glucose transporter 4 (GLUT4) proteins to the plasma membrane, which facilitates rapid glucose uptake. A reduction in the density of insulin receptors, malfunction of insulin receptors, defective internal signaling pathways, and interference with the translocation or function of GLUT 4 proteins are some speculated causes of the induction of IR (Kitamura and Accili, 2004).

According to Pass et al. (1998), laminitis predisposition may be determined by the ability of the animal's body to supply glucose to hoof tissues. The hoof is constantly undergoing remodeling processes in order to maintain its dermo-epidermal attachments that are compromised during concussion during movement. Because of this constant remodeling, the hoof likely has a high demand for glucose. Insulin resistance may affect nutrient delivery to hoof tissues by altering vascular tone. When glucose is plentiful,

insulin acts as a slow vasodilator in order to increase blood distribution to muscles to deliver insulin more efficiently (Vincent et al., 2006). This vasodilation is caused by the increased synthesis of nitric oxide from endothelial cells by way of the phosphatidinyllinositol 3-kinase (PI3K) pathway (Muniyappa et al., 2007). However, insulin also promotes vasoconstriction by stimulating endothelin-1 synthesis and activating the sympathetic nervous system via the mitogen-activated protein kinase (MAPK) pathway. Schulman and Zhou (2009) proposed that the PI3K pathway may be compromised in the insulin resistant animal, as this is the pathway that aids in the uptake of glucose. Additionally, in a hyperinsulinemic state, MAPK activity may be increased, contributing to vasoconstriction within the hoof. Detection of increased endothelin-1 concentration in blood from digital veins 12 h after administration of carbohydrate to induce laminitis in healthy horses by Eades et al. (2007) suggests that digital vessels undergo vasoconstriction as a result of carbohydrate overload in horses, which may contribute to the development of laminitis. The MAPK pathway also stimulates the formation of adhesion molecules on the surface of endothelial cells. In another 2007 study, Asplin et al. (2007) observed that laminitis could be experimentally induced in ponies when they are intravenously infused with insulin to create severe hyperinsulinemia, suggesting that hyperinsulinemia can contribute to the onset of laminitis.

Obesity and Laminitis

Obesity may induce a pro-inflammatory state as monocytes move into adipose tissues in response to monocyte chemoattractant protein-1. Adipose tissues secrete more monocyte chemoattractant protein-1 as they reach their storage capacity for lipid and

become stressed (Vick et al., 2007). This increases the number of macrophages residing within the adipose tissue, and these inflammatory cells secrete tumor necrosis factor alpha, a cytokine involved in systemic inflammation, capable of inducing fever, apoptotic cell death, cachexia, and inflammation (Kim et al., 2007). Additionally, excess weight increases the force exerted on the dermo-epidermal attachments within the hoof, further contributing to the risk of laminitis development. Therefore, it can be hypothesized that horses with EMS become more susceptible to laminitis because tumor necrosis factor alpha secretion increases in response to obesity and excess weight exerts greater force on the dermo-epidermal attachments, aiding in lamellar separation within the hoof.

Vick et al. (2007) examined 60 mares of varying body condition and found that obesity was associated with higher blood interleukin-1 and tumor necrosis factor alpha mRNA expression and increased plasma tumor necrosis factor alpha concentrations. Higher plasma tumor necrosis factor alpha concentrations have also been detected in previously laminitic ponies with EMS as compared to ponies with no history of laminitis (Treiber et al., 2008).

Diagnosis of Equine Metabolic Syndrome

Equine metabolic syndrome can be diagnosed by the patient's history, detection of obesity or regional adiposity, and test results consistent with IR. Horses can be screened for IR by collecting a blood sample and submitting plasma for measurement of glucose and insulin concentrations. In the normal animal, serum insulin concentration falls in the range of 2 to 25 µU/mL (BET Labs, 2016) and glucose is below 110 mg/dL (Frank, 2009). Pancreatic insulin secretion increases to compensate for a decrease in tissue effectiveness, so resting serum insulin concentrations are elevated in horses with

moderate or severe IR. Serum thyroxine (T_4) can also be tested, as resting serum thyroxine concentrations are typically reduced in horses with EMS. Walsh et al. (2009) reported a weak negative correlation between insulin and T_4 concentrations in horses with EMS. This finding further reflects that the metabolic status of the animal should be interpreted as a consequence rather than a cause of EMS (Frank et al., 2008c).

Oral and Intravenous Glucose Challenges

Rogers et al. (1988) and Cononie et al. (1994) were notable early users of the glucose challenge as an indicator of glycemic status and insulin sensitivity by measuring serum glucose and insulin concentration over time in response to glucose load. The process requires numerous blood draws over several hours following the oral or intravenous administration of glucose. In each sample, glucose and insulin concentrations are measured to determine the rise and subsequent fall of serum glucose and insulin concentrations in response to the consumption or injection of glucose. Greater sensitivity to insulin results in less secretion being necessary to facilitate glucose uptake by tissues. Therefore, a decreased insulin response to a glucose load is associated with increased insulin sensitivity. Studies have also examined insulin area under the curve data in response to intravenous and oral glucose challenges and have observed reduced insulin AUC flowing insulin-sensitizing interventions, such as exercise and calorie restriction (Rogers et al., 1988; Cononie et al., 1994). Therefore, this technique can also be used as a means of validating changes in insulin sensitivity following an intervention (Rogers et al., 1988 and Cononie et al., 1994).

Impact of Stress and the Fight or Flight Response on Serum Insulin Concentrations

Resting serum glucose and insulin concentrations can be confounded if pain or stress is inflicted on the animal (Frank et al., 2008a). Horses have evolved as prey animals and when exposed to stress or a theat, their instinctual response is to distance themselves from the theat (Evans, 2010). For many horses, having blood collected via jugular venipuncture is a stressful experience. In such a case, the horse may experience a fight or flight response, initiating an endocrine cascade that results in elevated serum glucose concentrations.

Once a stimulus has been perceived as a theat by the autonomic nervous system of the horse, a signal is sent to the adrenal cortex, inducing the release of catecholamines, epinephine and norepinephine. The catecholamines act on various targets thoughout the body, preparing the horse to "make its escape". Most notably, blood flow is increased to muscle tissue, glycogen is mobilized from muscle tissue, and free fatty acids are released from adipose tissue. When glycogen is mobilized from muscle, blood glucose concentrations become elevated resulting in a corresponding insulin response (Hadley and Levine, 2006; Frank et al., 2008a; Bartolome and Cockram, 2016). Therefore, horses that become stressed during blood collection may experience falsely elevated serum insulin concentrations when analyzing for serum insulin concentrations.

Management and Treatment of Equine Metabolic Syndrome

Management of EMS begins with managing body condition of the horse (Frank, 2009). Reduced caloric intake and increased caloric output via exercise can induce weight loss to achieve a lower body condition score in the case of an obese horse. For the

lean horse, it is important to find feed sources that allow the horse to maintain its body condition without exacerbating the effects of IR (Frank, 2009).

In extreme cases, other management practices may include limiting or completely removing pasture access from the horse. Limiting pasture access can be achieved by reducing the number of hours the animal is allowed access to grazing each day, reducing the size of the animal's grazing area, or utilizing a grazing muzzle to reduce the amount of forage the horse is able to ingest (Frank, 2009).

In either case, ensuring that the animal is consuming limited amounts of nonstructural carbohydrate (NSC) is critical. Nonstructural carbohydrates are a component of all plants and include simple sugars, starches and fructans. Horses affected by EMS are generally recommended to be fed a diet consisting only of forage containing less than 10% NSC and little or no grain concentrate, as these feeds contain large volumes of NSC (Frank, 2009). In cases where the forage contains 10 to 12% NSC, the forage can be soaked in cold water for 1 h prior to feeding to reduce the soluble sugar content (Frank, 2009).

If additional energy is needed, feeds that are primarily digested in the large intestine of the horse are recommended in lieu of sweet feeds and other concentrates. Molasses-free beet pulp is highly recommended. Beet pulp may also be soaked prior to feeding to rinse away some of the simple carbohydrates and prevent obstruction of the esophagus. There are also many "low-sugar/low-starch" feeds available on the market that contain beet pulp and are appropriate for most IR horses (Frank, 2009).

Supplementing the diet of an IR horse with fat is controversial. While fats and oils are good sources of energy, increased influx of fatty acids into muscle tissues has been

suspected of inducing IR in humans (Slawik and Vidal-Puig, 2006). In a study by Frank et. al. (2005a), serum nonesterified fatty acid concentrations in healthy mares fed corn or rice bran oil in addition to their diet decreased over a 5-wk period while insulin sensitivity remained unaffected. Therefore, the addition of 0.5 cup (equivalent to 125 mL; contains approximately 100 g fat) rice bran or corn oil is recommended in cases where additional calories are required (Frank, 2009).

Treatment of Equine Metabolic Syndrome Horses with Omega-3 Fatty Acids

Fatty Acid Function

Fatty acids are vital nutritional components in the diet. There are both essential and nonessential fatty acids. Essential fatty acids are those that are not synthesized by the body, and therefore must be acquired via the diet in order to continue proper function of the body (Hall et al., 2004).

Fatty acids are the basic building blocks of fat in the body and are required for brain development and maintenance of proper brain function (Lawrence, 2010). Fatty acids are precursors and antagonists of eicosanoid biosynthesis (Schoene, 1991). Eicosanoids function as chemical messengers that carry information of cell activation from one cell to another (Curtis-Pior, 2004). There are four families of eicosanoids; prostaglandins, leukotrienes, thomboxanes, and prostacyclins. Each family has its own set of physiological and pathophysiological roles, and coordinates events between cells so that proper tissue function occurs. Different eicosanoids are produced from various fatty acids (Curtis-Pior, 2004).

Omega-3 Polyunsaturated Fatty Acids

By definition, omega-3 fatty acids have a double bond on the third carbon from the methyl group. The essential fatty acid for the omega-3 family is alpha-linolenic acid,

which is a precursor for the series of omega-3 fatty acids (Hall et al., 2004). Alpha-linolenic acid undergoes a series of desaturation and elongation processes to produce long-chain derivatives (Dubois et al., 2007). The most important derivatives produced are eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Alpha-linolenic acid is desaturated and elongated into EPA, and can be further elongated into DHA. Eicosapentaenoic acid and DHA are then incorporated into cell membrane phospholipids where they are stored until a chemical or physical insult causes the activation of these fatty acids to mobilize from the cell membrane phospholipids, and metabolize into eicosanoids (Hall et al., 2004). Flaxseed, cranberry seed and walnuts are commonly used sources of ALA (Dubois et al., 2007). Fish oil serves as a common source for both EPA and DHA (Ackman et al., 1989). Omega-3 series fatty acids have general anti-inflammatory, anti-thombotic, anti-hypertensive and anti-arrhythmic effects on the body (Simopoulos, 1999).

Fatty Acids in Forage

Both Dewhurst et al. (2001) and Clapham et al. (2005) reported that ALA is the dominant fatty acid present in all species of fresh forage, comprising an average of 62% of the total fatty acids in forages. In addition, Dewhurst and King (1998) stated that green plants were the primary source of beneficial omega-3 fatty acids and that forage processing decreases overall fatty acid content. After extended periods of wilting, the concentrations of polyunsaturated fatty acids were significantly lower than in corresponding fresh forage. However, forages still contained a higher percentage of ALA than linoleic acid after processing (Dewhurst and King, 1998).

Omega-3 Fatty Acids in Humans

Another series of essential fatty acids are omega-6 fatty acids. Maintenance of the proper balance of omega-6 to omega-3 fatty acids is important in the body (Golub et al., 2011). In humans, the ideal ratio of omega-6 to omega-3 fatty acids is 1:1, though typical western diets have a ratio of 15:1 (Simopoulos and Cleland, 2003). Extensive research has been performed on the benefits of omega-3 fatty acids to humans (Stretenovic et al., 2009). It has been widely reported that populations consuming more fish, which have a high concentration of omega-3 fatty acids, have lower incidence of cardiovascular diseases (Stretenovic et al., 2009). Imbalance of omega-6 to omega-3 fatty acids leads to an overproduction of the pro-inflammatory prostaglandins and cytokines. Experimental studies have provided evidence that incorporation of omega-3 fatty acids into the diet modifies inflammatory and immune reactions (Simopoulos, 2002). Omega-6 to omega-3 ratios of 2:1 to 4:1 were related to reduced mortality caused by cardiovascular diseases, reduced inflammatory process in rheumatoid arthitis patients and reduced risk of breast cancer (Stretenovic et al., 2009).

Omega-3 Fatty Acids in Horses

Hansen et al. (2002) investigated the effects of omega-3 fatty acid supplementation on plasma fatty acid profiles and platelet aggregation in healthy horses. Twelve horses were evenly divided and randomly assigned to either a diet with 10% flaxseed oil added or a control diet for 18 wk. Fasting venous blood samples were collected at week 0, 4, 8, 12, and 16. Horses consuming flaxseed oil had greater plasma omega-6 and omega-3 fatty acid concentrations. Total omega-3 concentrations increased more than omega-6 concentrations in supplemented horses. Eicosapentaenoic acid

concentrations significantly increased in horses fed flaxseed oil. No differences were reported in DHA and arachidonic acid concentrations or platelet aggregation between treatment groups (Hansen et al., 2002).

In 2004, O'Connor et al. studied the effects of fish oil supplementation on the metabolic response to a high intensity exercise test utilizing 10 Thoroughbred and Standardbred horses. Horses were assigned to 1 of 2 treatments; fish oil, containing 10.6% EPA and 8% DHA and control. The fish oil supplement was top dressed daily at 324 mg/kg BW. Horses received their respective diets for 63 d and were exercised 5 d/wk. After d 63, all horses performed a standardized exercise test. Horses supplemented with fish oil had a lower heart rate during exercise and tended to have lower packed cell volume. During recovery, fish oil supplemented animals had lower plasma glucose concentrations. There was also a tendency for serum insulin to be lower and glucose:insulin ratios to be higher in fish oil-treated horses. Serum glycerol and cholesterol concentrations were lower in horses receiving fish oil compared to those consuming the control diet. The authors concluded that fish oil supplementation altered glucose metabolism in conditioned horses (O'Connor et al., 2004).

Hess et al. (2012) conducted a study using 21 maiden mares to determine the effects of different sources of supplemental dietary omega-3 fatty acids on plasma, red blood cell, and skeletal muscle fatty acid profiles. Mares were assigned to one of thee treatments; control, fish oil or flaxseed meal for 90 d. Mares fed fish oil received a combination of 38 g/d of ALA, EPA, and DHA. The flaxseed meal treatment contained 38 g/d of ALA. Blood and muscle samples were taken on d 0, 30, 60, and 90. Plasma linoleic acid and ALA concentrations were significantly lower in mares fed fish oil

compared to mares fed flaxseed meal or control. Eicosapentaenoic acid and DHA were only detected in the plasma and red blood cells of mares fed fish oil and EPA and DHA significantly increased in mares fed fish oil at d 90 as compared to d 30. Plasma ALA was significantly greater in the flaxseed meal group as compared to control at d 30 and also at d 90. The authors reported greater EPA and DHA in skeletal muscle of mares fed fish oil as compared to those fed flaxseed meal and control. The authors stated that omega-3 fatty acid concentrations in the blood and muscle are directly correlated with dietary supplementation (Hess et al., 2012).

Use of Omega-3 Supplementation to Combat Obesity

Animal and human studies have reported that EPA and DHA supplementation may be protective against obesity and may reduce weight gain in already obese animals and humans (Buckley and Howe, 2009). Specifically, studies demonstrated a reduction in visceral (epidydimal and/or retroperitoneal) fat in rats fed high lipid diets that incorporated omega-3 polyunsaturated fatty acids (Parrish et al., 1990; Belzung et al., 1993; Hainault et al., 1993; Baillie et al., 1999; and Ruzickova et al., 2004) and the effect was dose dependent (Belzung et al., 1993). The reduction in visceral fat was associated with a decrease in adipocyte size (Belzung et al., 1993; Parrish et al., 1990) and number of adipocytes (Ruzickova et al., 2004).

A study of obese rats reported that omega-3 PUFA supplementation led to a significant reduction in weight gain compared to controls in the lower and higher dose of omega-3 PUFA (5.9% and 5.1%, respectively), and rats on the higher dose consumed significantly less food compared to control animals (Hassanali et al., 2010). Ruzickova et al. (2004) demonstrated an attenuation of weight gain in mice on a high fat diet

supplemented with omega-3 PUFA and even weight loss in those on the highest concentration of omega-3 PUFA. Similarly, Cha et al. (2001) reported that aged rats on a high omega-3 PUFA diet for 4 months had a significantly lower body weight compared to those on a diet high in omega-6 fatty acids.

There is promising evidence in animal studies that omega-3 PUFA supplementation can modulate fat deposition, food intake, and body weight. However, caution should be used when making inferences to the effects of omega-3 PUFA in humans, because of possible differences in pharmacokinetics of EPA and DHA supplementation between animals and humans, and because doses used in animal studies vary widely and are typically higher than those considered safe in humans (Golub et al., 2011). For example, Perez-Matute et al. (2007) used a dose of 1 g/kg/d EPA in rats while Takahashi and Ide (2000) used 85.2 g/kg/day EPA + DHA in rats. A dosage of 1 g/kg/d in rats corresponds to 9.6 g/9 in a 60 kg person (FDA, 2005). The average intake of omega-3s in the U.S. is approximately 1.6 g/d (approximately 0.7% of energy intake), with 1.4 g of ALA and 0.2 g of EPA/DHA (Kris-Etherton et al., 2000). The Food and Drug Administration (FDA) deems intake of up to 3 g/d of marine omega-3s as "generally recognized as safe" (FDA, 1997).

Fewer studies have examined the association between omega-3 PUFA intake and adiposity in humans (Golub et al., 2011). An observational study of 124 adults reported that obese individuals had significantly omega-3 PUFA concentration compared to healthy weight participants. In obese subjects, there was a significant inverse correlation of -0.4 between plasma omega-3 PUFA and BMI, and correlations of -0.27 and -0.41 for waist and hip circumference, respectively. In addition, there was a significant inverse

relationship between quartiles of plasma omega-3 and BMI, waist, and hip circumference (Micallef et al., 2009). Studies in youth report significantly decreased plasma omega-3 PUFA concentration in overweight youth compared to youth of healthy weights (Klein-Platat, 2005), and in obese youth, plasma omega-3 PUFA is significantly inversely related to BMI z-score quartiles (Scaglioni et al., 2006).

Golub et al. (2011) hypothesized that EPA and DHA act on the human mesocorticolimbic pathway and the human endocannabinoid pathway to decrease the reward associated with food, thereby reducing appetite, food intake, and ultimately reducing excess weight and obesity.

Different organ systems in the body and various pathways are involved in appetite, food intake, and energy homeostasis and the dysregulation of these systems leads to obesity (Golub et al., 2011). These include brain structures, such as the brain stem, hypothalamus and reward pathways, as well as the gastrointestinal tract, adipose tissue and pancreas. Increasing evidence suggests that the omega-3 fatty acids EPA and DHA play a role in these systems, particularly in the central nervous system. Studies in animals and humans have shown promising effects of treatment with EPA/DHA supplemented diets to prevent and reduce obesity. These positive effects have mostly been discussed in the realm of the effect of EPA and DHA on metabolic profiles of subjects, for example, reductions in visceral fat, greater insulin sensitivity, and improved lipid profiles (Golub et al., 2011).

Thyroid Hormones

Thyroid hormones are produced from the thyroid gland, a bilobed glandular organ that develops over a diverticulum of the pharynx. The thyroid gland consists of thyroid

follicles from which thyroid hormones triiodothyronine (T_3) and thyroxine (T_4) are produced and secreted (Hadley and Levine, 2006). Thyroid hormones are unique because they are complexed though covalent bonds to iodine. Terrestrial vertebrates have low iodine availability and therefore have adapted a cellular mechanism known as "iodide trapping" that occurs in the thyroid follicles in order to better utilize and conserve iodine. The sodium-iodide symporter traps iodine at the basolateral membrane of thyroid follicular cells. Iodine is then transported against an electrical gradient across the cell. Once the iodide has reached the apical membrane of the thyroid follicle, porter protein pendrin transports the iodine into the follicular lumen iodine is oxidized and then incorporated into tyrosyl groups of thyroglobulin. Within thyroglobulin, the iodinated tyrosines undergo oxidative coupling, which yields T_4 and smaller amounts of T_3. While T_3 is the biologically active thyroid hormone, the concentration of T_4 in human serum is about 50 times greater than that of T_3. Thyroxine can be converted to T_3 via extrathyroidal monodeiodination of T_4. It is speculated that T_4 has a greater presence in the blood due to the fact that it can be converted to T_3 and to exert a negative feedback on the hypothalamus in order to regulate the secretion of the thyroid hormones. Thyroid hormones play important physiological roles in growth and development and metabolism (Hadley and Levine, 2006).

Thyroid Hormones and Insulin Resistance

Thyroid hormones play an important role in regulating energy balance, metabolism of glucose, and lipids (Chubb et al., 2005). While thyroid hormones oppose the action of insulin and stimulate hepatic gluconeogenesis and glycogenolysis, they upregulate the expression of genes such as GLUT4 and phosphoglycerate kinase,

involved in glucose transport and glycolysis, respectively, thus acting synergistically with insulin facilitated glucose disposal and utilization in peripheral tissues (Raboudi et al., 1989; Weinstein et al., 1994; Clementet al., 2002; and Viguerie et al., 2002). The prevalence of thyroid disease in patients with diabetes is significantly higher than that in the general population (Canaris et al., 2000). Kapadia et al. (2012) concluded that both hypo- and hyperthyroidism may be associated with IR due to various mechanisms such as altered insulin secretion and lipid levels, and that an altered thyroid state can lead to IR.

Chubb et al. (2005) examined the prevalence and progression of subclinical hypothyroidism in women with type 2 diabetes and concluded that subclinical hypothyroidism is a common, but incidental occurrence in female type 2 diabetics, and that routine screening of thyroid function is questionable in such patients.

Hyper- and Hypothyroidism

Hyper- and hypothyroidism are conditions in which either excess or sufficient amounts of thyroid hormones are produced. Symptoms of hyperthyroidism include heat intolerance, muscle wasting, weight loss, increased appetite, increased perspiration, increased body temperature, rapid pulse and elevated basal metabolic rate (BMR). Symptoms of hypothyroidism often reflect the opposite, with cold intolerance, lethargy, weight gain, decreased perspiration, lowered body temperature, slower pulse, and lowered BMR evident (Hadley and Levine, 2006). It is interesting to note that, although hyperthyroid patients have a higher BMR than normal, that they are also capable of gaining excess weight (American Thyroid Association, 2016). This is likely caused by an overconsumption of calories due to their increased appetites (Hadley and Levine, 2006; American Thyroid Association, 2016). Therefore, either form of thyroid disease may

result in excess weight or obesity (Hadley and Levine, 2006; American Thyroid Association, 2016).

Use of Exogenous Thyroxine Supplementation for Weight Loss in Humans

According to the American Thyroid Association (2016), exogenous supplementation of thyroid hormone has been used as a weight loss tool in the past and studies have shown that excess thyroid hormone treatment may help produce more weight loss than can be achieved by dieting alone. However, the excess weight is often regained once the excess thyroid hormone treatment has ceased. Additionally, there are other significant negative consequences that may result from the use of thyroid hormone supplementation for the induction of weight loss, including the loss of muscle protein in addition to the loss of body fat. The American Thyroid Association (2016) concludes that use of exogenous thyroid hormone supplementation is unlikely to significantly change weight and may induce other metabolic problems.

Oral Levothyroxine Sodium Treatment in Horses

In severe and chonic cases of EMS affected animals, in which management strategies such as restricted caloric intake and increased exercise have failed to induce weight loss and/or increase insulin sensitivity of the animal, medical therapies may be implemented to aid in achieving reduced body condition and improved insulin sensitivity. Frank et al. (2006, 2008a, 2008b) have performed several studies on the effects of oral levothyroxine sodium (Thyro L, LLOYD, Inc., Shenandoah, IA) on the horse. In the first of these studies by Frank et al. (2006), levothyroxine sodium was administered to healthy mares at doses ranging from 24 to 96 mg/day over an 8-wk period. Mean body weight decreased and insulin sensitivity increased in treated mares. In 2008 (a, b), Frank et al.

conducted two more studies in which the long-term effects of oral levothyroxine sodium treatment were observed in healthy mares. Levothyroxine sodium was administered at a dosage of 48 mg/day and glucose dynamics were measured at 0, 16, 32, and 48 wk. Concurrently, echocardiographic evaluations, complete blood count, and plasma biochemical analyses were also performed to assess the safety of levothyroxine. A greater than two-fold increase in mean insulin sensitivity was detected as well as reduced mean body weights. No adverse health effects were detected.

According to Frank (2009), measured serum T_4 concentrations are often elevated when levothyroxine is being administered, but concentrations vary considerably within and between horses. Serum T_4 concentrations may range between 40 and 100 ng/mL in treated horses, indicating that levothyroxine is administered at a supraphysiological dosage. However, clinical signs of hyperthyroidism, such as sweating or tachycardia have not been observed in treated horses (Ramirez et al., 1998; Alberts et al., 2000; Frank et al., 2005b; Frank, 2009).

Injectable Controlled Release Thyroxine Treatment

P. J. Burns and R. M. Gilley (BioRelease Technologies LLC, Birmingham, AL, personal communication) evaluated the efficacy of a biodegradable 30 d controlled release T_4 microparticle formulation in healthy mares. Nine mares were used in the study, 5 of which were treated with the thyroxine microparticle injection (BET Pharm, Lexington, KY), containing 100 mg of T_4 microparticles and 4 untreated control mares. Blood samples were collected via venipuncture immediately before administration of treatment and again on d 1, 4, 9, 14, 18, 23, 30, 37, and 42 after treatment. A significant treatment by time interaction for T_4 concentrations was detected between treated and

control mares. Mean T_4 concentrations averaged 20.8 ng/mL on d 0 prior to treatment for all mares. Control mares averaged 22 ng/mL over the 42-d period. In treated mares, a significant increase in serum T_4 concentration was observed, peaking 1 d post administration and averaging 19.1 ng/mL above their pretreatment baseline over the 42-d period (Burns and Gilley, 2004).

Statement of the Problem

Equine metabolic syndrome and EMS associated secondary pathophysiologies have been prevalent in the equine industry for the past several decades. Many authors have suggested that the induction of weight loss may aid in reducing the prevalence of these disorders or alleviate their symptoms by improving insulin sensitivity as a result of weight loss (Johnson, 2002; Treiber et al., 2006; Frank et al., 2008a, 2008b, 2008c, 2009). Although this can be achieved via diet and exercise, sometimes implementation of secondary treatments are necessary to aid in the process (Frank, 2009). Therefore, the objective of the present study was to compare the effects of omega-3 PUFA and a controlled-release thyroxine injection on serum insulin concentrations in horses.

CHAPTER III

MATERIALS AND METHODS

Experimental Design

The present study was conducted at West Texas A&M University (WTAMU) and was approved by the WTAMU Institutional Animal Care and Use committee. Forty-four horses aged 5 to 27 yr (14.8 ± 4.8 yr; 11 mares, 33 geldings) were used to determine the effect of omega-3 polyunsaturated fatty acids and an IM controlled release thyroxine (T_4) injection on resting and post-oral glucose challenge serum insulin concentrations. The 114-d experimental trial consisted of 1 30-d period followed by 3 28-d periods that are identified as "months". Blood samples were collected once per period, and each collection is identified by the month in which it was performed. Prior to the start of the trial, a preliminary test was performed in order to determine the horse's insulin response to a standard meal of hay and concentrate and horses were stratified by serum insulin concentrations and divided into 3 treatment groups control, omega, and thyroxine.

Preliminary Serum Insulin Test

Prior to the start of the trial, 50 horses in the WTAMU horse herd were fed their normal diet, and venous blood was drawn prior to the morning

concentrate feeding, and again 6 h post-feeding to determine serum insulin concentrations in response to their meal. Blood samples were collected in 7 mL vacutainer tubes (Covidien, Mansfield, Ma) and allowed to clot for a minimum of 30 min before centrifugation at 1,250 × g for 20 min at 4°C (Beckman Coulter, Indianapolis, IN). After centrifugation, serum was harvested and stored in triplicate aliquots at -20° C until shipped to BET Laboratories, Inc. (1501 Bull Lea Rd Suite 102A Lexington, KY 40511) for serum T_4 and insulin analysis. Horses with a serum insulin concentration of 20 µU/mL or greater at 6 h post feeding were classified as insulin resistant, according to the parameters set forth by Frank et al. (2008a). All horses were also assessed for body condition according to the scale by Henneke et al. (1983). Horses were then stratified by post-feeding serum insulin concentrations and randomly assigned to treatment groups. To stratify, horses were organized from least to greatest 6 h post-feeding serum insulin concentration and then systematically and randomly assigned to one of the thee treatments going down the list from least to greatest concentration.

Treatments

Horses on the Omega-3 FA treatment (O) were supplemented with 60 g of Equibloom (BET Labs, Lexington, KY) daily for the first 14 d of the trial and supplemented with 30 g for the remainder of the study. This supplementation provided a minimum of 7,120 mg (60 g) and 3,060 mg (30 g) of omega-3 fatty acids, and 1,668 mg (60 g) and 834 mg (30 g) of omega-6 fatty acids, respectively. Horses were individually hand fed the supplement daily. Horses receiving thyroxine (T) were administered thyroxine microparticles (BET Pharm, Lexington, KY), a controlled-release microparticle levothyroxine sodium injection containing 100 mg of T_4. This injection was administered

on each blood collection day following the blood collection period [d 0 (August), 30 (September), 58 (October), 86 (November), and 114 (December)]. The injection was administered IM in the left side of the neck of each animal so that injection sites could be monitored for swelling or abscess; none of which occurred in the present trial. Control horses received no supplemental omega-3 or T_4 during the study.

On each sample collection d (0, 30, 58, 86, and 114), horses were fed their standard diet between 0600 and 0700. At 4 h post-feeding, the time 0 blood sample was collected via jugular venipuncture. Immediately following the time 0 collection, all horses were administered an oral glucose challenge consisting of 75 mL corn syrup (Karo Light Syrup, ACH Food Companies) containing 75 g of carbohydrate administered via oral drench. At 1 and 2 h post oral glucose challenge, venous blood samples were again collected.

All samples were handled in the same manner as in the preliminary blood collection. All serum samples were shipped to BET Labs, Inc. (Lexington, KY) for analysis of T_4 and insulin concentrations.

Herd Care and Management

Horses were housed in large outdoor pens and were fed standard diets of a grain-based concentrate (Hi-Pro WTAMU Pellet, Friona, TX) at a volume capable of achieving or maintaining the horse at a BCS of 5 to 6. Horses also had *ad libitum* access to both blue stem grass hay and water thoughout the duration of the trial. The composition of this diet is displayed in Appendix Table 1. Some pens of horses were allowed access to pasture at night, however, due to the low quality of the pastures, this was not accounted for as an additional source of nutrition. All horses employed in the trial were exercised

regularly (3 to 5 h/wk) as part of the equitation program at WTAMU, with the exception of a few horses who did not exercise for a brief amount of time due to lameness issues.

Laboratory Analysis

Blood was collected, processed and handled in the same fashion as in the preliminary serum insulin test. Serum was analyzed for thyroxine concentration using the radioiummunoassay (RIA) procedure by IVD Technologies (TT4-1000V-100/500). For the analysis of thyroxine, 25 uL of B0, Standards, and sample were placed into designated tubes. Then, 200 uL of ^{125}I-Thyroxine Tracer was placed into each tube. Tubes were covered with paraffin and incubated for 2 h in a shaker at room temperature. All tubes were decanted and 1.0 mL of deionized water was added. Tubes were decanted again and 1.0 mL of deionized water was added and tubes were decanted for the last time. Tubes were then counted with a gamma counter for 2 min. The level of thyroxine in the sample are inversely related to the level of radioactivity that is measured, where:

$$\%B = \frac{CPM\ (X) \times 100}{CPM\ (B0)}$$

Serum insulin concentration was analyzed using Porcine Insulin RIA, MilliporeSigma catalog #PI-12K-Rev. 12-APR-2016.

Statistical Analysis

Body condition score data was reported as mean and median as determined by Microsoft Office 365 Excel (2016). Serum thyroxine and insulin data were analyzed using SAS v. 9.4 (SAS Institute, Inc., Cary, NC) utilizing a 3-way model in the MIXED procedure. The model accommodated repeated measures of both period and time utilizing the direct product compound symmetry covariance structure (UN@CS). Gender was included as a random effect. Means were separated by least significant difference. Data

was analyzed for the effects of treatment, period and time of sample collection within period, as well as the interactions of each independent variable (treatment by period, treatment by time, period by time, and treatment by period by time). Statistical significance was declared at $P \leq 0.05$ and trends were noted at $P \leq 0.1$.

CHAPTER IV

RESULTS AND DISCUSSION

Mean and median body condition scores are represented in Appendix Figures A-1 and A-2. All horses had body condition scores between 4.0 and 7.0 thoughout the duration of the experiment. Serum thyroxine (T_4) and insulin concentrations in control (C), omega-3 (O), and thyroxine (T) horses at times 0, 1, and 2 for each sample collection period are shown in Appendix Tables A-2 and A-9.

Serum thyroxine concentrations in control, omega-3, and thyroxine-treated horses

Least square mean serum T_4 concentrations in C, O, and T treated horses are represented in Appendix Table A-2. There was no effect of the interaction of treatment, period and time ($P = 0.397$) on serum T_4 concentrations (Appendix Table A-2). There was no effect of the interaction of treatment and time ($P = 0.912$) on serum T_4 concentrations (Appendix Table A-3). There was no main effect of time ($P = 0.771$) on serum T_4 concentrations (Appendix Table A-4).

There was an effect of the interaction of period and time ($P = 0.001$) on serum T_4 concentrations (Fig. 1; Appendix Table A-5). There was also an effect of the interaction of treatment and period ($P < 0.001$) on serum T_4 concentrations (Fig. 2; Appendix Table

A-6). There were main effects of both period ($P < 0.001$) and treatment ($P < 0.001$) on serum T_4 concentrations (Fig. 3 and 4; Appendix Tables A-7 and A-8, respectively).

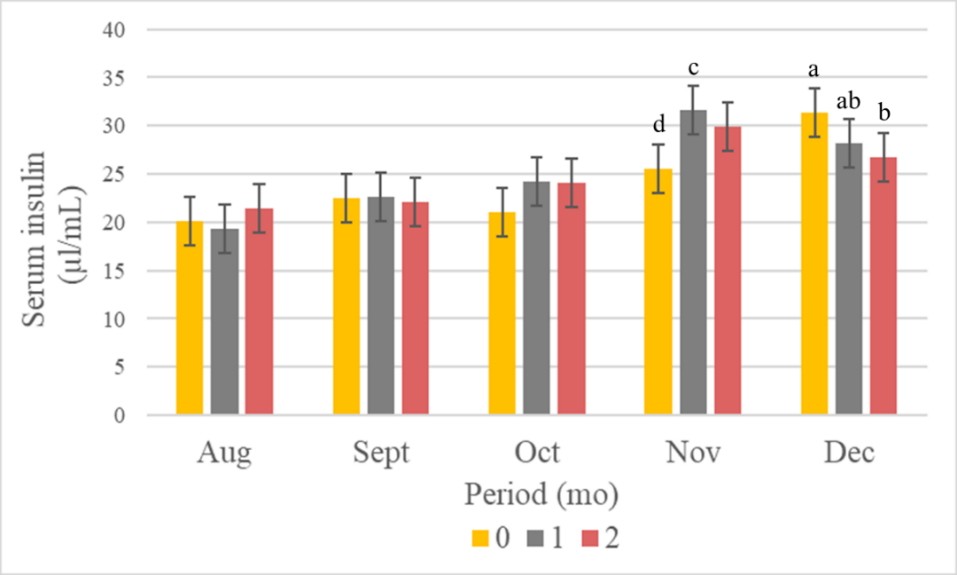

Figure 1. The effect of the interaction of period (mo – Aug, Sept, Oct, Nov, Dec) and time (0, 1, 2) on serum thyroxine concentrations in horses ($P \leq 0.05$)
[a, b] Means for time within period with differing superscripts differ at ($P \leq 0.05$)
[c, d] Means for time within period with differing superscripts tend to differ at ($P \leq 0.1$)

There was an effect of the interaction of period and time on serum T_4 concentrations (Fig. 1). Serum T_4 concentrations were similar across collection times in the months of August, September and October. In the month of November, horses tended to have greater serum T_4 concentrations at 1 h following administration of glucose as compared to time 0. In December, serum T_4 concentrations were greatest at time 0, intermediate at 1 h, and least 2 h following glucose administration. The author has no explanation for these differences in the months of November and December.

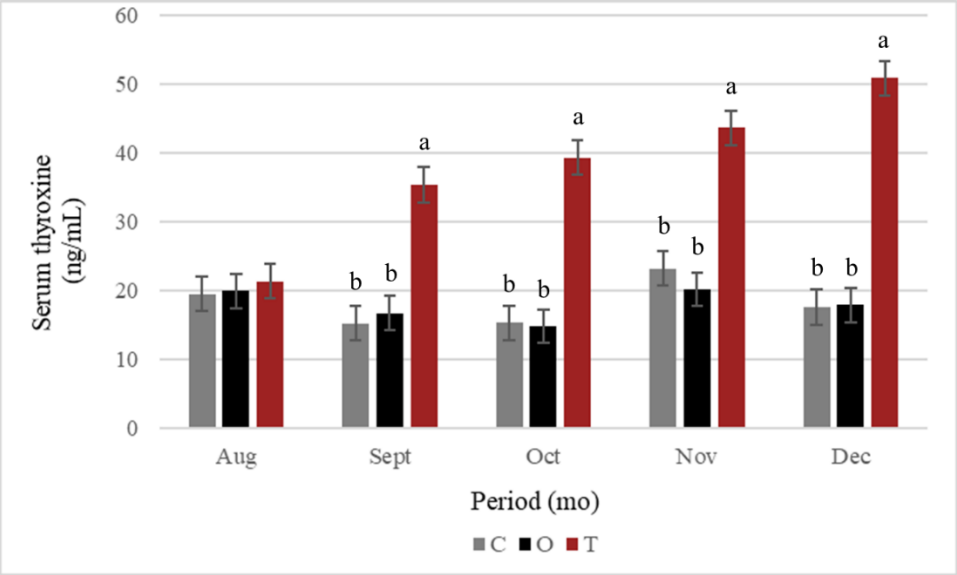

Figure 2. The effect of the interaction of treatment (C, O, T) and period (mo – Aug, Sept, Oct, Nov, Dec) on serum thyroxine concentrations in horses ($P \leq 0.05$)
[a, b] Means for treatment within period with differing superscripts differ at ($P \leq 0.05$)

As expected, there was a significant effect of the interaction of treatment and period on serum T_4 concentrations (Fig. 2). Following the administration of treatments after the August sample collection, T horses had greater serum T_4 concentrations compared to C and O horses in the months of September, October, November and December.

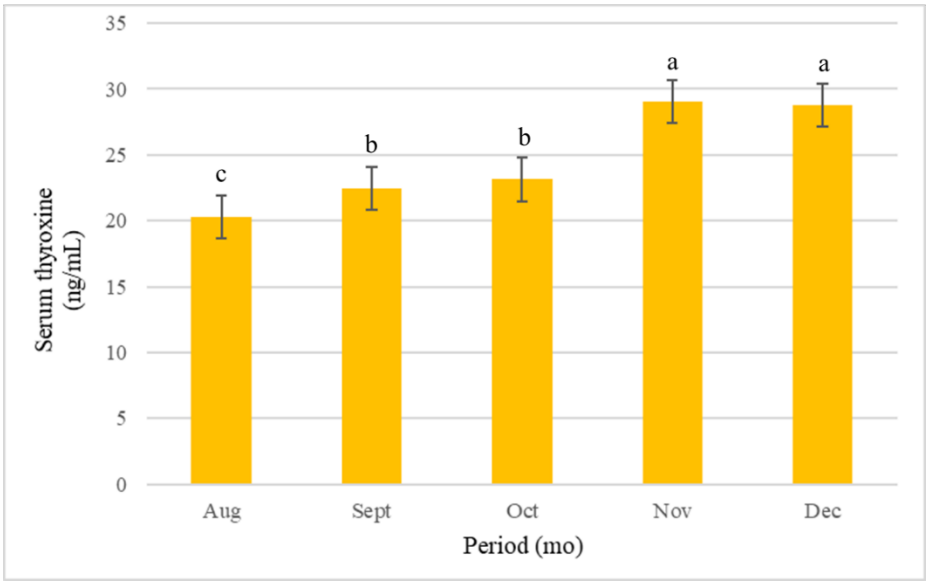

Figure 3. The effect of period (mo – Aug, Sept, Oct, Nov, Dec) on serum thyroxine concentrations in horses ($P \leq 0.05$)
[a, b, c] Means with differing superscripts differ at ($P \leq 0.05$)

There was an effect of period on serum T_4 concentrations (Fig. 3). Serum T_4 concentrations were least in August, increased and were similar in September and October, and then increased and were similar again in November and December.

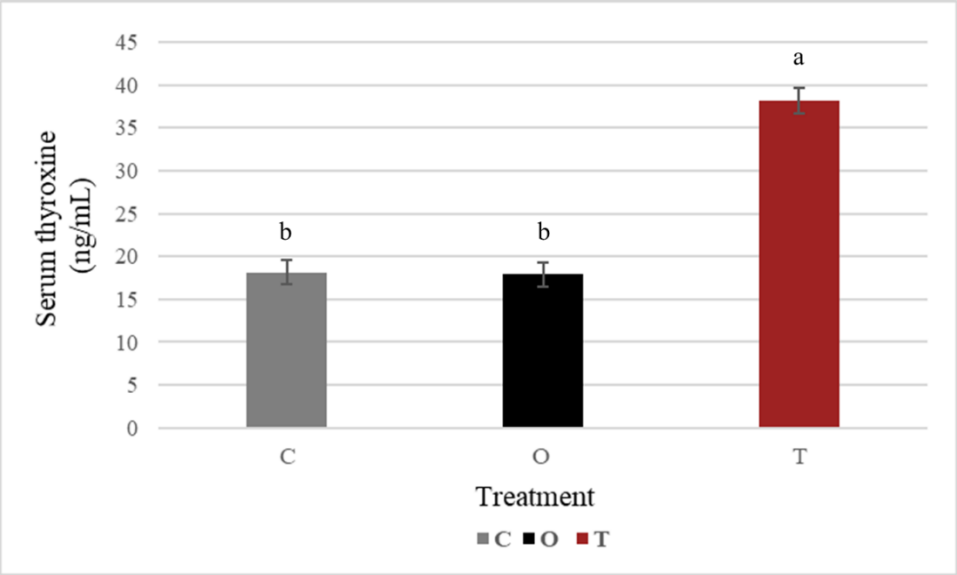

Figure 4. The effect of treatment (C, O, T) on serum thyroxine concentrations in horses ($P \leq 0.05$)
[a, b] Means with differing superscripts differ at ($P \leq 0.05$)

As expected, horses treated with a controlled release T_4 microparticle injection had significantly greater serum T_4 concentrations compared to C and O horses (Fig. 4).

Following the administration of T_4 treatment in August though the remainder of the trial, T horses had greater serum thyroxine concentrations as compared to C and O horses. This agrees with the unpublished work of P. J. Burns and R. M. Gilley (BioRelease Technologies LLC, Birmingham, AL, personal communication). In that study, the authors reported that horses injected with 100 mg T_4 microparticles had greater serum T_4 concentrations at up to 42 d following injection as compared to control horses. In the present trial, serum T_4 concentration appeared to continually increase from September through December, even though the same amount of T_4 was administered at the beginning of each period. The reason for this apparent increase in serum T_4 is unknown. This product was administered every 28 d, while the manufacturer

recommends readministration every 30 d. There is no published data on controlled-release intramuscular T_4 supplementation in horses to which to compare these data.

Serum insulin concentrations in control, omega-3, and thyroxine-treated horses

Least square mean serum insulin concentrations in C, O, and T horses is represented in Appendix Table A-9. There was no effect of the interaction of treatment, period and time ($P = 0.979$) on serum insulin concentrations (Appendix Table A-9). There was no effect of the interaction of treatment and time ($P = 0.867$) on serum insulin concentrations (Appendix Table A-10).

There was a significant effect of the interaction of period and time ($P = 0.001$) and a main effect of time on serum insulin concentrations (Fig. 5 and 6; Appendix Tables A-11 and A-12, respectively). There was an effect of the interaction of treatment and period ($P = 0.012$) on serum insulin concentrations (Fig. 7; Appendix Table A-13). There were also main effects of period ($P < 0.001$) and treatment ($P = 0.001$) on serum insulin concentrations (Figures 8 and 11; Appendix Tables A-14 and A-15, respectively).

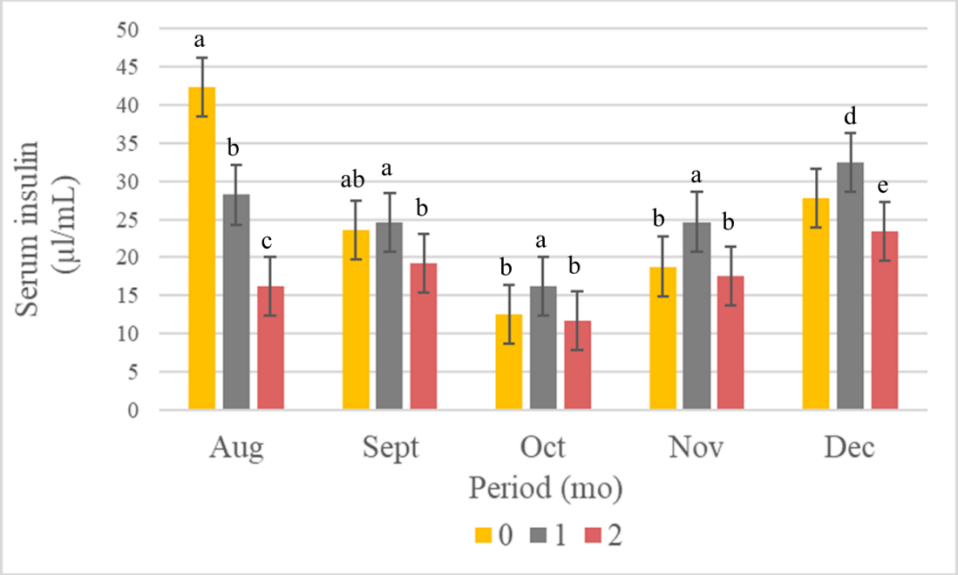

Figure 5. The effect of the interaction of period (mo – Aug, Sept, Oct, Nov, Dec) and time (0, 1, 2) on serum insulin concentrations in horses ($P \leq 0.05$)
[a, b, c] Means for time within period with differing superscripts differ at ($P \leq 0.05$)
[d, e] Means for time within period with differing superscripts tend to differ at ($P \leq 0.1$)

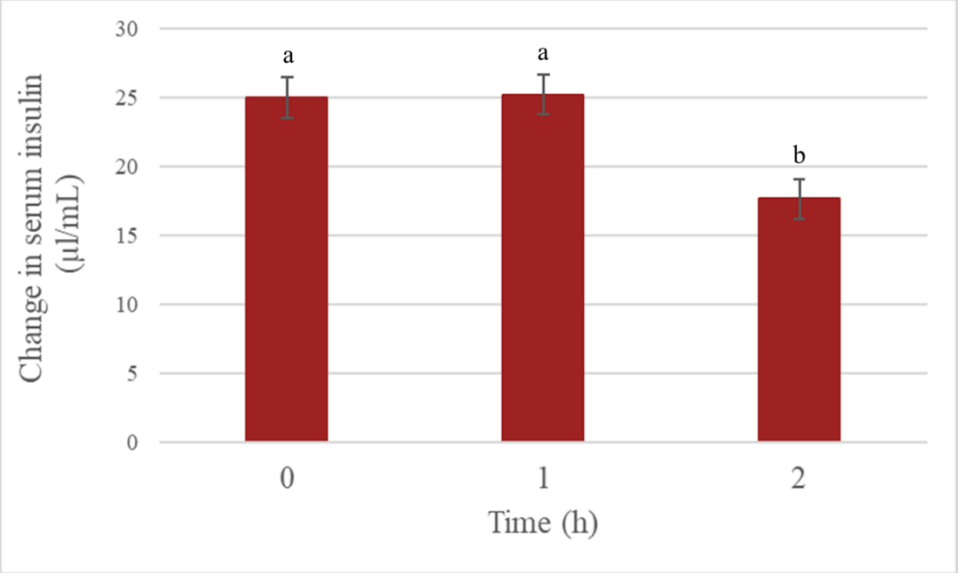

Figure 6. The effect of time (0, 1, 2) on serum insulin concentrations in horses ($P \leq 0.05$)
[a, b] Means with differing superscripts differ at ($P \leq 0.05$)

There was a significant effect of the interaction of period and time on serum insulin concentrations. In the month of August, serum insulin concentrations were greatest at time 0 and significantly decreased at both 1 and 2 h post oral glucose challenge. The response curve for August do not match the curves in any of the subsequent months, or with the work of Richards and Kempton (2016). In the month of September, serum insulin was significantly greater at 1 h as compared to 2 h post glucose challenge, while serum insulin at time 0 was similar to both. In both October and November, serum insulin concentrations were significantly greater at 1 h as compared to both the 0-h and 2-h samples. These responses resemble an expected insulin response to an oral glucose challenge in the horse (Richards and Kempton, 2016). In the month of December, serum insulin concentrations were similar at all 3 collection times, but there was a tendency for horses to exhibit decreased concentrations at 2 h post oral glucose challenge as compared to 1 h post glucose administration. These insulin response curves mostly agree with the expected responses after an oral sugar challenge, except for the initial draw in August. One possible explanation for the greater serum insulin at time 0 in August is a reaction by the horses during the initial blood draw of the trial at time 0, causing an adrenaline secretion resulting in increased blood glucose, and subsequent insulin release.

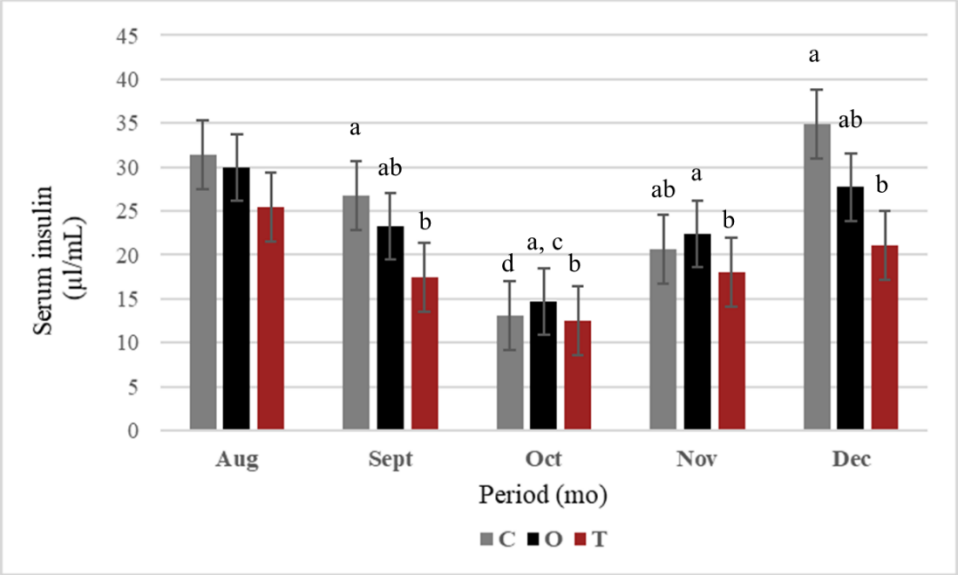

Figure 7. The interaction of treatment (C, O, and T) and period (mo – Aug, Sept, Oct, Nov, and Dec) on serum insulin concentration in horses (P < 0.001)
[a, b] Means for treatment within period with differing superscripts differ within period at ($P \leq 0.05$)
[c,d] Means for treatment within period with differing superscripts tend to differ within period at ($P \leq 0.1$)

There was a significant effect of the interaction of treatment and period on serum insulin concentrations in horses (Figure 7). Horses treated with a controlled release T_4 microparticle injection (T) had significantly decreased serum insulin concentrations as compared to C horses at the September and December sample collection dates. In the month of October, there was no difference in serum insulin concentrations between C and T horses. In the month of November, C horses had similar serum insulin concentrations as compared to both O and T horses, while T horses had significantly lower serum insulin as compared to O horses.

Omega-3 FA supplemented horses had similar serum insulin concentrations as compared to C and T horses in the months of September and December. In the month of October, O horses had significantly greater serum insulin concentrations as compared to

T horses and tended to have greater serum insulin concentrations as compared to C horses.

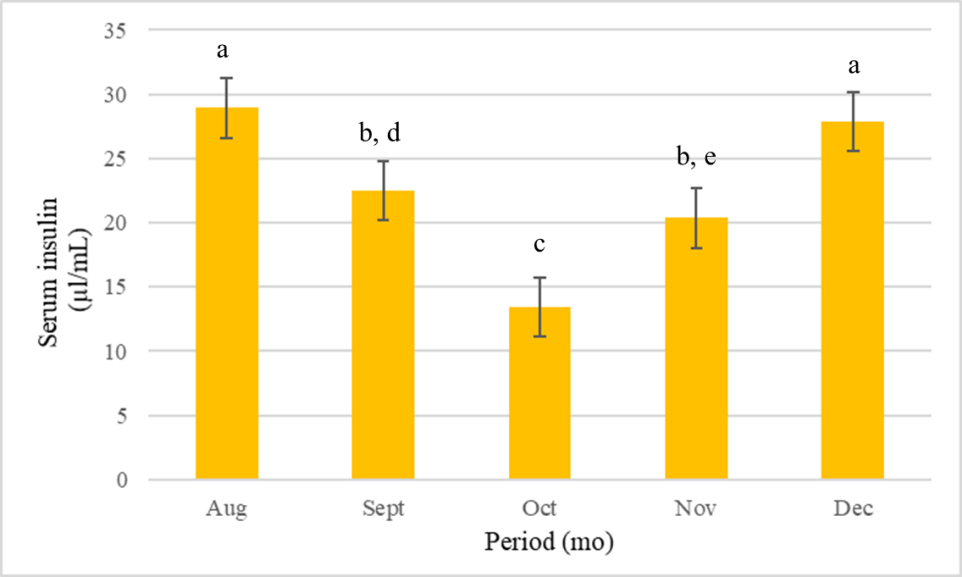

Figure 8. The effect of period (mo – Aug, Sept, Oct, Nov, Dec) on serum insulin concentration in horses (P < 0.001)
[a, b, c] Means with differing superscripts differ at ($P \leq 0.05$)
[d,e] Means with differing superscripts tend to differ at ($P \leq 0.1$)

All horses had decreasing serum insulin concentrations from August to October and increasing concentrations from October to December (Fig. 9). The reason for this pattern is unknown. Speculations have been made regarding the weather and its effect on forage quality and metabolism, however according to weather data on the days blood was drawn, temperatures were well within normal ranges (NOAA, 2018), and no correlation was observed between temperatures and serum insulin. Horse disposition, while not objectively measured, was subjectively assessed on sample collection days, and could be a factor in serum glucose and insulin concentrations. While it is possible that excitement

and anticipation of sample collection could result in higher insulin concentrations (August, December) due to an adrenaline response, the opposite effect could have been observed in October, as the horses appeared relaxed during all 3 collection times. This lack of an adrenaline response could be a factor in the decreased serum insulin observed.

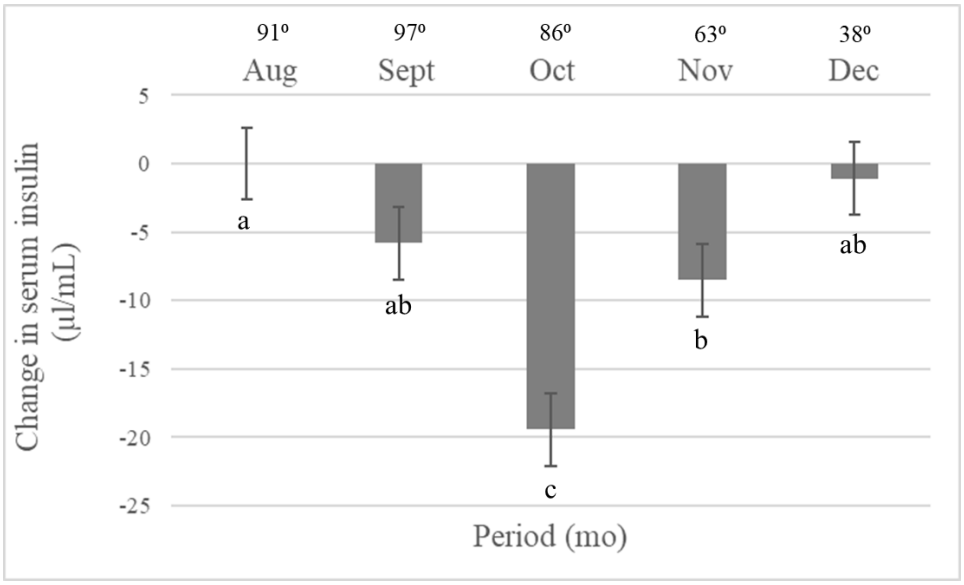

Figure 9. The effect of period (mo – Aug, Sept, Oct, Nov, Dec) on the change in serum insulin concentration from August baseline sample in horses (P ≤ 0.05)
[a, b, c] Means with differing superscripts differ at (*P* ≤ 0.05)

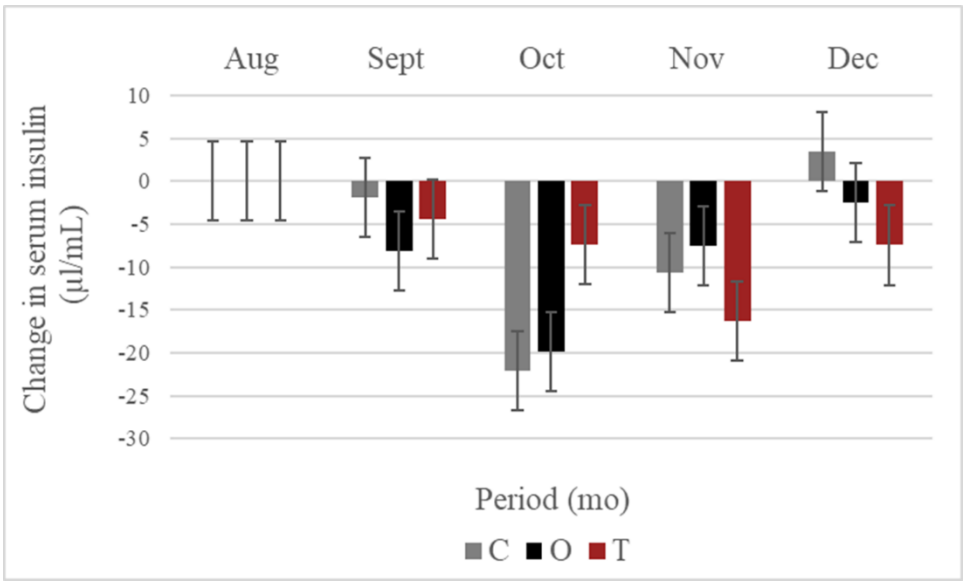

Figure 10. The interaction of treatment (C, O, T) and period (mo – Aug, Sept, Oct, Nov, Dec) on the change in serum insulin concentration from August baseline sample in horses (*P* = 0.8937)

There was an effect of period on change in serum insulin concentration from the baseline draw in August ($P < 0.05$; Figure 9). There was no effect of the interaction of treatment and period on change in serum insulin, however, the graph of this data is displayed for reference as to how these changes varied between treatment (Figure 10).

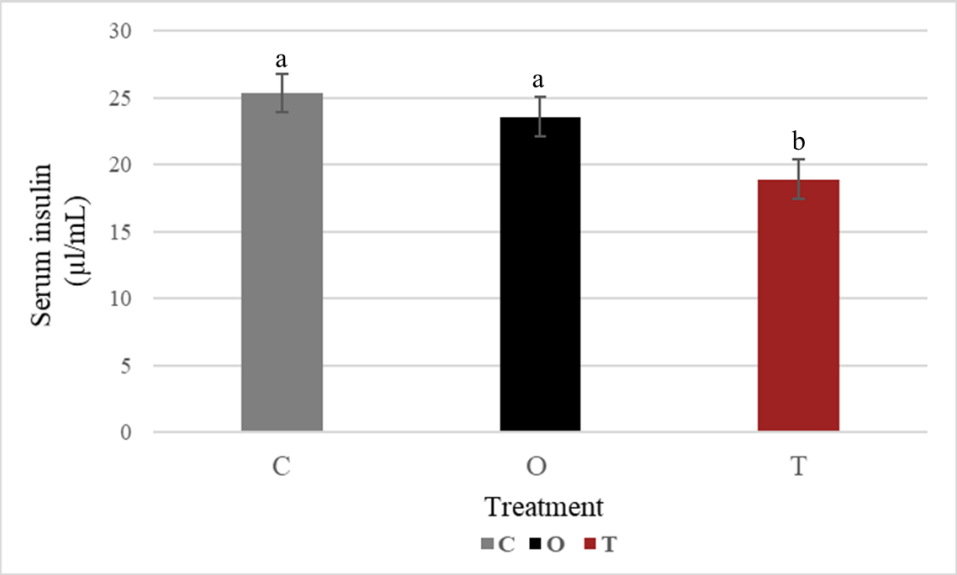

Figure 11. The effect of treatment (C, O, T) on serum insulin concentration in horses ($P < 0.001$)
[a, b] Means with differing superscripts differ at ($P \leq 0.05$)

Horses treated with a controlled release T_4 microparticle injection had significantly decreased serum insulin concentrations thoughout the trial as compared to C and O horses.

These data partially agree with Frank et al. (2006), who reported decreased body weight and increased insulin sensitivity in mares treated with oral T_4 sodium over an 8 wk period. However, that trial differed from the present study in that Frank et al. (2006) administered an incrementally increased dosage of oral levothyroxine sodium that was fed daily. Those horses received 24 g/d of levothyroxine sodium for the first 2 wk of treatment, then 48, 72 and 96 g/d for each following 2-wk period of the 8-wk trial. Horses treated with T_4 in the present trial received 100 mg IM every 28 to 30 d over a 114-d trial. Additionally, Frank et al. (2006) analyzed insulin sensitivity utilizing a combined IV glucose-insulin tolerance test (IVGITT), which differs from the oral glucose challenge

used in the present study. The IVGITT also allowed for the comparison of serum glucose and insulin concentrations. Glucose was not measured in the present study for comparison.

In another trial by Frank et al. (2008b) the long-term effect of oral levothyroxine sodium treatment was examined in healthy mares. Levothyroxine was administered at a dosage of 48 mg/d and glucose dynamics were measured at 0, 16, 32, and 48 wk. A greater than two-fold increase in insulin sensitivity and reduced mean body weights were observed. No long-term studies have been conducted on the use of the T_4 microparticle injection used in the present study.

O'Connor et al. (2004) observed that serum insulin concentrations tended to be lower in and glucose:insulin ratios to be higher in fish oil-treated horses. Fish oil supplies eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids. In contrast, flaxseed oil, the major ingredient in Equibloom (the product used to supply omega-3 PUFA to O horses in the present trial) supplies alpha-linolenic acid (ALA), the precursor for EPA and DHA. This difference in the composition of the omega-3 PUFAs provided maybe the cause for the differences lack of a consistent increase in insulin sensitivity in O horses compared to C. Additionally, parameters were assessed in conjunction with a high-intensity standardized exercise test and routine standardized exercise 5 d/wk. Although horses in the present trial were exercised regularly, the exercise was not standardized across all horses. Exercise may affect insulin sensitivity indirectly by decreasing adipose tissue mass in the horse, allowing for improved insulin sensitivity (Slawik and Vidal-Puig, 2006; Frank, 2009).

This data partially agrees with work completed by Hess et al. (2012), who reported improved insulin sensitivity in mares receiving 38 g/d of omega-3 PUFA of either marine or flaxseed origin for 90 d. Both marine and flaxseed supplemented mares had an overall increase in insulin sensitivity. Although improved insulin sensitivity as a result of omega-3 PUFA supplementation is not reflected in the present trial, Hess et al. (2012) observed similar fluctuations, with acute insulin response decreasing from d 30 to 60 and increasing again at d 90. Disposition index (combined insulin sensitivity and β pancreatic response) increased by 53% in the marine- and 48% in flaxseed-treated horses and did not change with time in control horses (Hess et al., 2012).

Several authors have reported reduced weight gain (Buckley and Howe, 2009) and visceral fat deposition as a result of decreased adipocyte size (Parrish et al., 1990 and Belzung et al., 1993) and number of adipocytes (Ruzickova et al., 2004) in rats as a result of omega-3 PUFA supplementation. Again, these two studies supplemented EPA and DHA sources in the diet in contrast to the present study which provided flaxseed oil as an ALA supplement.

Serum insulin data from this trial also partially agrees with that of O'Connor et al., (2004) and Frank et al. (2006), who both reported reduced serum insulin concentrations in horses treated with omega-3 PUFA or thyroxine.

CHAPTER V

CONCLUSIONS AND IMPLICATIONS

There was an expected effect of the interaction of treatment and period ($P < 0.001$) on serum T_4 concentrations. There were also main effects of both treatment ($P < 0.001$) and period ($P < 0.001$) on mean serum T_4 concentrations. This data shows that the controlled release T_4 injection was effective in increasing serum T_4 concentrations in the horse.

Results from this experiment indicate that horses receiving an IM controlled-release thyroxine (T_4) injection (T) had significantly lower overall serum insulin as compared to control and omega-3 supplemented horses. Further, T horses had significantly lower serum insulin concentrations as compared to C horses at the September (d 30) and December (d 114) collection dates, and had lower serum insulin as compared to O horses at the October (d 58) and November (d 86) collection dates.

The main effect of time and the effect of the interaction of period and time observed on serum insulin concentrations in the present trial indicate that use of an oral glucose challenge may not always provide the desired response to a glucose load. It is important to provide the horse with a standardized quantity of carbohydrates when performing a glucose challenge and in a practical horse environment, it is not always possible.

While data reported from several previous studies indicates that omega-3 PUFA supplementation, particularly in the form of DHA or EPA, may aid in improving insulin sensitivity in the horse, omega-3 PUFA supplementation did not result in significantly decreased serum insulin concentration as compared to C horses in the current study.

Further research needs to be conducted to determine the long-term effectiveness of intramuscular T4 supplementation on reducing serum insulin concentrations and increasing insulin sensitivity in insulin-resistant horses. Reducing the prevalence of insulin resistance in horses could result in a reduction in the prevalence of related metabolic disorders such as pituitary pars intermedia dysfunction and laminitis in horses.

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APPENDIX FIGURES

BODY CONDITION SCORES

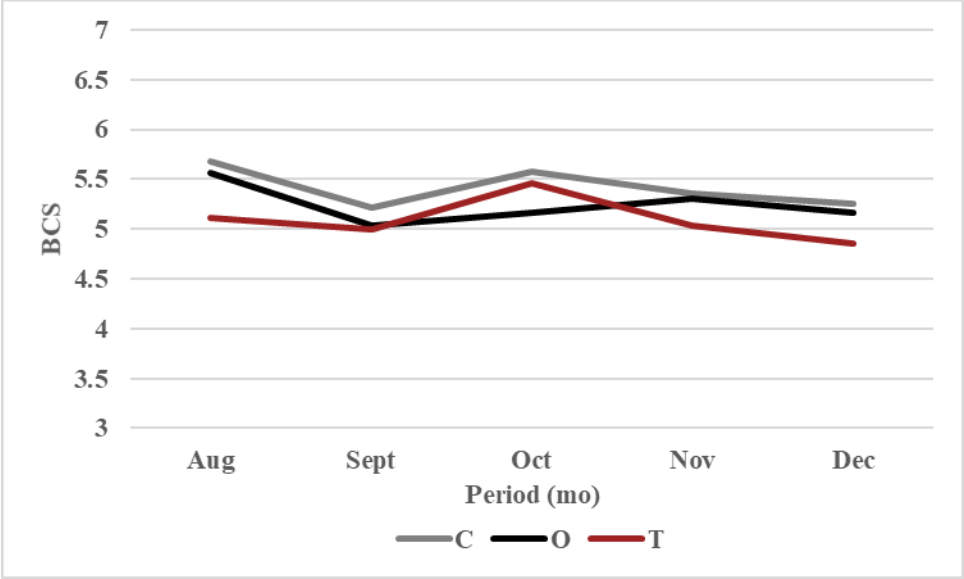

Figure A-1. Mean body condition scores of control (C), omega-3 PUFA (O) and thyroxine (T) treated horses by period

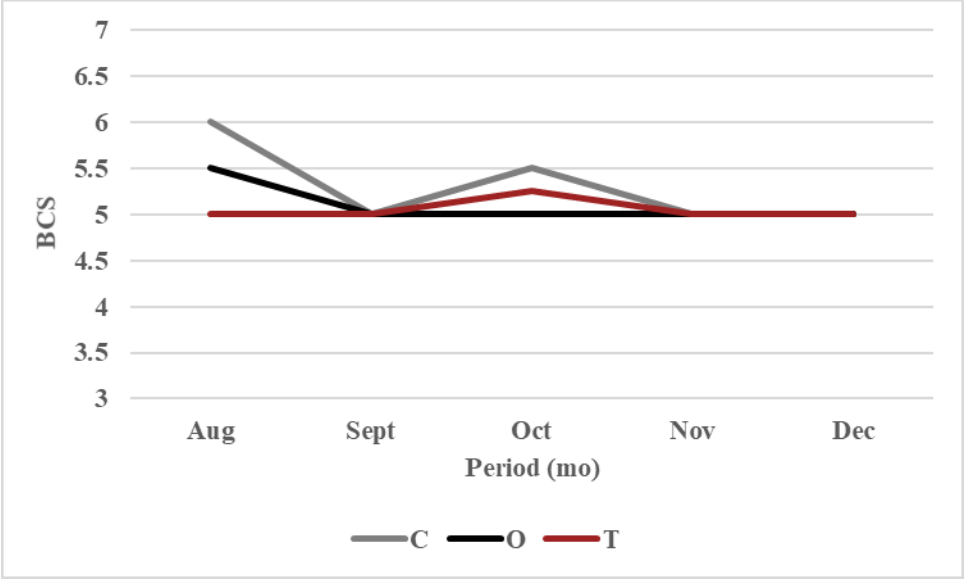

Figure A-2. Median body condition scores of control (C), omega-3 PUFA (O) and thyroxine (T) treated horses by period

APPENDIX TABLES

Table A-1. Analysis of standard diet fed to horses during trial consisting of 2, 4, or 8 lb/d of Hi-Pro WTAMU Pellet (Friona, TX) and ad libitum access to bluestem grass hay

Item	Bluestem Hay	Hi-Pro WTAMU Pellet
Crude Protein, %	4.0	13.6
Crude Fiber, %	-	12.9
ADF, %	51.1	-
NDF, %	73.9	-
Crude Fat, %	-	2.8
Ash, %	-	9.0
TDN, %	45.3	75.8
DE, Mcal/lb	0.91	1.52
NFE, %	-	61.7

Table A-2. Least square mean serum thyroxine concentrations (ng/mL) by treatment (C, O, T), period (mo – August, September, October, November, December), and sample collection time (0, 1, 2) in horses (P = 0.397)

		Treatment		
		C[1]	O[1]	T[1]
Item				
August[2]	0[3]	20.07	18.58	21.65
	1[3]	18.54	18.89	20.36
	2[3]	19.92	22.35	21.98
September[2]	0[3]	14.92	14.93	37.81
	1[3]	15.53	16.04	36.25
	2[3]	15.26	19.09	32.08
October[2]	0[3]	13.62	12.15	37.27
	1[3]	16.29	16.47	40.06
	2[3]	15.87	15.75	40.75
November[2]	0[3]	17.42	18.73	40.58
	1[3]	30.24	21.47	43.18
	2[3]	21.9	20.43	47.31
December[2]	0[3]	20.51	18.88	54.73
	1[3]	15.57	17.12	51.83
	2[3]	16.68	17.58	46.11

[1]C = control; O = Omega-3 supplemented (Equibloom, BET Labs, Lexington, KY); T = thyroxine (T_4) microparticle injection (BET Labs, Lexington, KY)

[2]Blood samples were collected once every 28-30 d, the name of the month corresponds to the name of the period

[3]0 = serum T_4 concentrations prior to oral glucose challenge (OGC); 1 = serum T_4 1 h post OGC; 2 = serum T_4 2 h post OGC

Table A-3. Least square mean serum thyroxine concentrations (ng/mL) by treatment (C, O, T) and sample collection time (0, 1, 2) in horses (P = 0.912)

	Treatment		
	C[1]	O[1]	T[1]
Time			
0[2]	17.31	16.65	38.41
1[2]	19.23	18.00	38.34
2[2]	17.92	19.04	37.64

[1]C = control; O = Omega-3 supplemented (Equibloom, BET Labs, Lexington, KY); T = thyroxine (T_4) microparticle injection (BET Labs, Lexington, KY)

[2]0 = serum T_4 concentrations prior to oral glucose challenge (OGC); 1 = serum T_4 1 h post OGC; 2 = serum T_4 2 h post OGC

Table A-4. Least square mean serum thyroxine concentrations by time (0, 1, 2) in horses (P = 0.771)

Thyroxine (ng/mL)

Time	
0[1]	24.12
1[1]	25.19
2[1]	24.87

[1]0 = serum T_4 concentrations prior to oral glucose challenge (OGC); 1 = serum T_4 1 h post OGC; 2 = serum T_4 2 h post OGC

Table A-5. Least square mean serum thyroxine concentrations (ng/mL) by period (mo - August, September, October, November, December) and sample collection time (0, 1, 2) in horses (*P* = 0.001)

	Time		
	0[1]	1[1]	2[1]
Period			
August[2]	20.1	19.26	21.42

September[2]	22.55	22.61	22.14
October[2]	21.01	24.27	24.12
November[2]	25.58[d]	31.63[c]	29.88
December[2]	31.37[a]	28.17[ab]	26.79[b]

[1]0 = serum T_4 concentrations prior to oral glucose challenge (OGC); 1 = serum T_4 1 h post OGC; 2 = serum T_4 2 h post OGC

[2]Blood samples were collected once every 28-30 d, the name of the month corresponds to the name of the period

Least significant difference was used to determine the probability of time effect within period

[a, b] Means with differing superscripts differ at ($P \leq 0.05$)

[c, d] Means with differing superscripts tend to differ at ($P \leq 0.1$)

Table A-6. Least square mean **s**erum thyroxine concentrations (ng/mL) by period (mo – August, September, October, November, December) and treatment (C, O, T) in horses ($P < 0.001$)

	Treatment		
	C[1]	O[1]	T[1]
Period			
August[2]	19.51	19.94	21.33
September[2]	15.24[b]	16.69[b]	35.38[a]
October[2]	15.26[b]	14.79[b]	39.36[a]
November[2]	23.19[b]	20.21[b]	43.69[a]
December[2]	17.59[b]	17.86[b]	50.89[a]

[1]C = control; O = Omega-3 supplemented (Equibloom, BET Labs, Lexington, KY); T = thyroxine (T_4) microparticle injection (BET Labs, Lexington, KY)

[2]Blood samples were collected once every 28-30 d, the name of the month corresponds to the name of the period

Least significant difference was used to determine the probability of treatment effect within period

[a, b] Means with differing superscripts differ at ($P \leq 0.05$)

Table A-7. Least square mean **s**erum thyroxine concentrations by period (mo – August, September, October, November, December) in horses ($P < 0.001$)

	Thyroxine (ng/mL)
Period	
August[1]	20.26[c]
September[1]	22.43[b]
October[1]	23.14[b]
November[1]	29.03[a]
December[1]	28.78[a]

1Blood samples were collected once every 28-30 d, the name of the month corresponds to the name of the period

Least significant difference was used to determine the probability of period effect

[a, b, c] Means with differing superscripts differ at ($P \leq 0.05$)

Table A-8. Least square mean serum thyroxine concentrations by treatment (C, O, T) in horses ($P < 0.001$)

	Thyroxine (ng/mL)
Treatment	
C[1]	18.16[b]
O[1]	17.90[b]
T[1]	38.13[a]

[1]C = control; O = Omega-3 supplemented (Equibloom, BET Labs, Lexington, KY); T = thyroxine (T_4) microparticle injection (BET Labs, Lexington, KY)

Least significant difference was used to determine the probability of treatment effect

[a, b] Means with differing superscripts differ at ($P \leq 0.05$)

Table A-9. Least square mean serum insulin concentrations (µl/mL) by treatment (C, O, T), period (mo – August, September, October, November, December), and sample collection time (0, 1, 2) in horses (P = 0.979)

		Treatment		
		C[1]	O[1]	T[1]
Item				
August[2]	0[3]	47.94	41.40	37.83
	1[3]	29.47	30.83	24.30
	2[3]	16.71	17.46	14.32
September[2]	0[3]	27.34	24.57	18.78
	1[3]	27.66	27.48	18.63
	2[3]	25.10	17.72	14.98
October[2]	0[3]	12.86	13.06	11.54
	1[3]	15.80	17.42	15.35
	2[3]	10.75	13.72	10.59
November[2]	0[3]	18.91	19.44	18.05
	1[3]	25.12	28.69	20.19
	2[3]	18.02	18.89	15.89
December[2]	0[3]	35.97	26.06	21.27
	1[3]	38.86	33.91	24.51

	2[3]	29.73	23.11	17.53

[1]C = control; O = Omega-3 supplemented (Equibloom, BET Labs, Lexington, KY); T = thyroxine (T_4) microparticle injection (BET Labs, Lexington, KY)

[2]Blood samples were collected once every 28-30 d, the name of the month corresponds to the name of the period

[3]0 = serum insulin concentrations prior to oral glucose challenge (OGC); 1 = serum insulin 1 h post OGC; 2 = serum insulin 2 h post OGC

Table A-10. Least square mean serum insulin concentrations (µl/mL) by treatment (C, O, T) and sample collection time (0, 1, 2) in horses ($P = 0.867$)

	Treatment		
	C[1]	O[1]	T[1]
Time			
0[2]	28.61	24.91	21.49
1[2]	27.38	27.66	20.60
2[2]	20.06	18.18	14.66

[1]C = control; O = Omega-3 supplemented (Equibloom, BET Labs, Lexington, KY); T = thyroxine (T_4) microparticle injection (BET Labs, Lexington, KY)

[2]0 = serum insulin concentrations prior to oral glucose challenge (OGC); 1 = serum insulin 1 h post OGC; 2 = serum insulin 2 h post OGC

Table A-11. Least square mean serum insulin concentrations (µl/mL) by period (mo - August, September, October, November, December) and sample collection time (0, 1, 2) in horses ($P < 0.001$)

	Time		
	0[1]	1[1]	2[1]
Period			
August[2]	42.39[a]	28.20[b]	16.17[c]
September[2]	23.56[ab]	24.59[a]	19.27[b]
October[2]	12.49[b]	16.19[a]	11.69[b]
November[2]	18.80[b]	24.67[a]	17.60[b]
December[2]	27.77	32.43[d]	23.45[c]

[1]0 = serum insulin concentrations prior to oral glucose challenge (OGC); 1 = serum insulin 1 h post OGC; 2 = serum insulin 2 h post OGC

[2]Blood samples were collected once every 28-30 d, the name of the month corresponds to the name of the period

Least significant difference was used to determine the probability of time effect within period

[a, b] Means with differing superscripts differ at ($P \leq 0.05$)

[c, d] Means with differing superscripts tend to differ at ($P \leq 0.1$)

Table A-12. Least square mean serum insulin concentrations by time (0, 1, 2) in horses ($P < 0.001$)

	Insulin (µl/mL)
Time	
0[1]	25.00[a]
1[1]	25.21[a]
2[1]	17.63[b]

[1]0 = serum insulin concentrations prior to oral glucose challenge (OGC); 1 = serum insulin 1 h post OGC; 2 = serum insulin 2 h post OGC

Least significant difference was used to determine the probability of time effect

[a, b] Means with differing superscripts differ at ($P \leq 0.05$)

Table A-13. Least square mean **s**erum insulin concentrations (µl/mL) by period (mo – August, September, October, November, December) and treatment (C, O, T) in horses ($P < 0.001$)

	Treatment		
	C[1]	O[1]	T[1]
Period			
August[2]	31.38	29.90	25.48
September[2]	26.70[a]	23.26[ab]	17.46[b]
October[2]	13.14[d]	14.73[a,c]	12.49[b]
November[2]	20.69[ab]	22.34[a]	18.04[b]
December[2]	34.85[a]	27.69[ab]	21.10[b]

[1]C = control; O = Omega-3 supplemented (Equibloom, BET Labs, Lexington, KY); T = thyroxine (T_4) microparticle injection (BET Labs, Lexington, KY)

[2]Blood samples were collected once every 28-30 d, the name of the month corresponds to the name of the period

Least significant difference was used to determine the probability of treatment effect within period

[a, b] Means with differing superscripts differ at ($P \leq 0.05$)

[c, d] Means with differing superscripts tend to differ at ($P \leq 0.1$)

Table A-14. Least square mean **s**erum insulin concentrations by period (mo – August, September, October, November, December) in horses
($P < 0.001$)

	Insulin (µl/mL)
Period	
August[1]	28.92[a]
September[1]	22.47[b,d]
October[1]	13.45[c]
November[1]	20.36[b,e]
December[1]	27.88[a]

[1]Blood samples were collected once every 28-30 d, the name of the month corresponds to the name of the period

Least significant difference was used to determine the probability of period effect

[a, b, c] Means with differing superscripts differ at ($P \leq 0.05$)

[d,e] Means with differing superscripts tend to differ at ($P \leq 0.1$)

Table A-15. Least square mean serum insulin concentrations by treatment (C, O, T) in horses ($P < 0.001$)

	Insulin (µl/mL)
Treatment	
C[1]	25.35[a]
O[1]	23.58[a]
T[1]	18.92[b]

[1]C = control; O = Omega-3 supplemented (Equibloom, BET Labs, Lexington, KY); T = thyroxine (T_4) microparticle injection (BET Labs, Lexington, KY)

Least significant difference was used to determine the probability of treatment effect

[a, b] Means with differing superscripts differ at ($P \leq 0.05$)